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Pulmonary siRNA delivery for lung disease: Review of recent progress and challenges

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ABSTRACT

Lung diseases are a leading cause of mortality worldwide and there exists urgent need for new therapies. Approval of the first siRNA treatments in humans has opened the door for further exploration of this therapeutic strategy for other disease states. Pulmonary delivery of siRNA-based biopharmaceuticals offers the potential to address multiple unmet medical needs in lung-related diseases because of the specific physiology of the lung and characteristic properties of siRNA. Inhalation-based siRNA delivery designed for efficient, targeted delivery to specific cells within the lung holds great promise. Efficient delivery of siRNA directly to the lung, however, is relatively complex. This review focuses on the barriers that impact pulmonary siRNA delivery and successful recent approaches to advance this field forward. We focus on the pulmonary barriers that affect siRNA delivery, the disease-dependent pathological changes and their role in pulmonary disease and impact on siRNA delivery, as well as the recent development on the pulmonary siRNA delivery systems.

1. Introduction

Lung diseases are a significant global public health problem creating a large economic burden [1]. Because the lung directly interfaces with the external environment, inhaled drug delivery to therapeutically address pulmonary diseases has been a longstanding goal. In addition to endogenous stimuli, the lungs are susceptible to inhalation injury and multiple related diseases as an organ exposed directly to harmful substances [2–4]. Pulmonary delivery offers the potential to address unmet medical needs in lung-related disease including allergy [5], asthma [6], idiopathic pulmonary fibrosis (IPF) [7], cystic fibrosis (CF) [8], both viral and bacterial infections [9], acute lung injury (ALI) [10], chronic obstructive lung disease (COPD) [11], and lung cancer [12].

Inhalation-based delivery of small interfering RNA (siRNA) designed for efficient, targeted delivery to specific cells within the lungs holds great promise but remains yet to be realized. In general, inhalation drug delivery systems, if designed effectively, reduce the overall dose required to treat pulmonary disorders in comparison to oral or parenteral delivery systems. They also avoid first-pass metabolism, reducing dose and risk of toxicity from metabolic byproducts. They provide a reproducible and possibly more economical platform that can be provided to diverse patient populations within a variety of settings and on a daily basis if necessary. The now commonly used dry powder inhalation (DPI) devices used to treat asthma or COPD serve as excellent examples [13].

Active targeting of the lung using siRNA has been a postulated approach for a variety of lung diseases. siRNAs induce gene silencing by a sequence-specific posttranscriptional process known as RNA interference (RNAi) [14]. With the first siRNA medication (Patisiran) approved in the USA and EU [15], the field of siRNA delivery received a strong stimulus to expand the scope of RNAi therapeutics to other diseases [16]. Patisiran is a nanoparticle formulation containing a chemically modified siRNA encapsulated with lipid excipients for delivery to hepatocytes. The lipid nanoparticles are composed of ionizable cationic lipids (DLin-MC3-DMA), phospholipid (DSPC), cholesterol, and polyethylene glycol modified lipids (PEG2000-C-DMG), that are combined via rapid mixing under acidic conditions. The siRNA is modified with eleven 2'-methoxy-modified sugar residues and four 2'-deoxythymidine residues to improve stability and to avoid off-target effects [17]. Another RNAi drug GIVLAARI™ (givosiran) was approved in November 2019 for

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the treatment of acute hepatic porphyria (AHP) [18]. The treatments based on siRNA provide potential benefits compared to traditional drugs, including target specificity and ability to inhibit the expression of mutant proteins without affecting wild type ones [16]. For RNAi-based therapies, a suitable delivery strategy is critical for optimum therapeutic effect. In many diseases like cancer, amyloidosis, viral infections, hypercholesterolemia, and acute kidney injury (AKI), systemic delivery is necessary for siRNA to reach a specific target. More than 25 systemically administered siRNA drug candidates are under clinical trials [19,20]. However, systemic delivery of therapeutic siRNA targeting tissues other than the liver has proven challenging. Local delivery of siRNA provides potentially better gene silencing at the target site and circumvents the first-pass effect thus resulting in lower doses and reduced off-target effects [21]. Thirteen clinical trials investigate local siRNA delivery as of the fall of 2020, and two of them are focused on lung delivery: ALS-RSV01 and Excellair^{TM} [20]. Direct delivery of siRNA into the lungs is non-invasive and allows for self-medication [22]. Addressing pulmonary delivery barriers and intracellular delivery of siRNA is critical for success of pulmonary siRNA delivery. The use of nanoparticles to deliver siRNA can help to overcome anatomical barriers, mucociliary clearance (MCC), intracellular siRNA uptake limitations, and macrophage alveolar clearance. For optimum siRNA pulmonary delivery, siRNAs need to be deposited to the target region of the lungs and released at the target cells. An ideal siRNA delivery system should: (1) condense siRNA into a stable particle, (2) protect siRNA from nuclease degradation, (3) improve cellular uptake and promote endosomal escape to release siRNA to the cytoplasm of target cells, and (4) specifically silence the target gene with low off-target effects and toxicity [3]. In this review, we highlight the progress of pulmonary siRNA delivery over the past decade.

2. Pulmonary barriers that affect siRNA delivery

Efficient delivery of siRNA directly to the lungs with the intent to treat lung diseases is complex despite the convenience of established inhalation delivery strategies. Depending on the desired location of delivery for a given disease state (upper airway, lower airway, alveolar region, systemic absorption) a number of considerations that include packaging, particle size, morphology, geometry, surface properties, cell target, and host defense mechanisms must be taken into consideration. There are three mechanisms of drug deposition in the respiratory system: impaction, sedimentation, and diffusion. From a particle point of view, if the intended location of drug deposition is the alveolar region, it will require navigation past 23 branching segments [23]. Therefore, the formulation must be designed with an optimal aerodynamic particle size that can be maintained and afford deep penetration into the lung. In particular, large particles with aerodynamic diameters greater than 6 μm deposit on the back of the larynx in the upper airways, never reaching the lower airway. If the intent is to deliver siRNA to the bronchiolar or alveolar region then smaller particles are required. Likewise, if particles are designed with an aerodynamic diameter less than 1 μm they are predominantly exhaled due to Brownian motion, never achieving deposition [24,25]. Accordingly, the optimal aerodynamic diameter for efficient lung deposition typically is in a range between 1-5 μm.

Considering that innate host barrier defense mechanisms evolved to keep foreign particles out of the lungs, careful consideration of these natural defense mechanisms is necessary when formulating pulmonary siRNA therapeutics. Perhaps the biggest obstacle to particulate drug delivery is innate lung clearance, including mucociliary transport and phagocytosis by macrophages. A variety of epithelial cell subtypes forms a continuous barrier, sealed by tight junctions, that secrete mucus and other host defense factors into the lumen that work in concert with oscillating ciliary projections particularly in the upper airway and bronchiolar regions [26]. Together this system works to capture foreign particles and escalate them up and out of the airway. Further, excessive mucus production is a characteristic encountered in many inflammatory-based lung diseases including, but not limited to, CF, chronic bronchitis and asthma thereby potentially making it more difficult for a particle to avoid capture or to traverse this barrier in order to reach intended target cells [27]. The deeper a particle penetrates into the lung, the more frequently it will encounter alveolar macrophages that are designed to phagocytose and destroy foreign particles, as this too may be modified further in certain disease states (discussed in more detail later). Finally, the epithelial barrier is bound together via multiple tight junctions designed to maintain a patent airway. The epithelium is in a polarized state such that the expression of surface bound proteins may be restricted to or predominantly expressed on the apical or basolateral surface, thus making it more or less accessible for receptor-targeted approaches [28].

The pulmonary surfactant (PS) and macrophages in the airspace are another obstacle to affect the pulmonary siRNA delivery. The alveolar macrophages are phagocytes residing in the airspace that play a critical role in homeostasis, host defense and tissue remodeling. In the following section, we will focus on the details of the most important pulmonary barriers to siRNA delivery and on how to strategically overcome the barriers in order to improve siRNA delivery efficiency.

2.1. Pulmonary surfactant (PS)

PS is a surface-active material covering the entire alveolar surface, which is secreted by specialized alveolar type II epithelial cells. The main physiological role of PS is to maintain low surface tension upon expiration to prevent alveolar collapse. PS has been extensively studied because of the functional role in mammalian breathing [29,30]. Inhaled siRNA delivery vectors that make it to the alveoli must retain their stability and function in the presence of PS, which are abundant in the airspace. Natural human PS has a complex composition of ~8% of surfactant proteins and ~92% of lipids by mass. The protein part consists of four surfactant proteins which can be structurally divided into two parts: the larger hydrophilic SP-A and SP-D, and the smaller hydrophobic SP-B and SP-C [31]. The hydrophilic surfactant proteins, also known as collectins, participate in the opsonization process to promote pathogen and particle uptake by phagocytic cells [32]. The hydrophobic surfactant proteins regulate the interfacial surfactant absorption dynamics, which improve the lipid transfer and membrane fusion process. The lipid fraction mainly contains zwitterionic phosphatidylcholine (~60-70 wt%), anionic phosphatidylglycerol (~10 wt%), and neutral lipids, of which cholesterol is the most abundant (~8-10 wt%) [29,30]. From an inhalation therapy perspective, PS is primarily considered as one of the extracellular barriers in the deep lung which needs to be overcome to gain access to the underlying target cells [33,34]. Poractant alfa, a modified PS from swine, and beractant, bovine PS, are commercially available for treating neonatal respiratory distress syndrome [35].

2.2. Alveolar macrophages

Alveolar macrophages are phagocytes that reside in the airspace and play a critical role in homeostasis, host defense, and tissue remodeling. There can be several macrophages in each alveolus though numbers may vary in pathological states, such as observed increases in smokers [36]. Following inhalation, particles can be rapidly cleared by alveolar macrophages via opsonin-independent scavenger receptors or by opsonin-dependent mechanisms, which trigger the release of pro-inflammatory cytokines TNF-α and IL-1β and initiate the production of further inflammatory mediators, consequently leading to acute lung toxicity [37,38]. In alveoli, phagocytosis by macrophages and migration towards the mucociliary escalator contribute to overall elimination. Although macrophages are less efficient in the phagocytosis of nanoparticles than microparticles [39,40], siRNA-containing nanoparticles may aggregate in the presence of PS, which increases their susceptibility to macrophage clearance. In the case of polymeric nanoparticles, studies have shown

that particles with hydrophobic surfaces are more susceptible to phagocytosis by macrophages than those with hydrophilic surfaces [41].

The alveolar macrophages in the bronchoalveolar lumen also play a critical role in lung inflammatory pathologies but also constitute an attractive target for siRNA therapeutics. However, delivery of siRNA to macrophages is challenging, because differentiated alveolar macrophages are difficult to transfect. Several strategies have been devised to overcome this challenge. PLGA microparticles with encapsulated siRNA were successfully aerosolized and resulted in efficient macrophage transfection [42]. Macrophage uptake of anionic PLGA-lipid hybrid microparticles was greater than uptake of positively and negatively charged precursor PLGA particles [43]. Lynn *et al.* developed a hybrid core-shell nanoparticle that mediated efficient *in vivo* delivery of siRNA to alveolar macrophages [44]. Duo *et al.* evaluated inhaled exosomes to deliver siRNA to modulate the alveolar macrophages immune responses demonstrating efficacy *in vivo* [45]. Despite these promising results, a better understanding of how environmental damage affects alveolar macrophage function is necessary for the development of macrophage-targeted treatment strategies.

2.3. Mucociliary clearance (MCC) and periciliary layer (PCL)

MCC by ciliated epithelial cells serves as a barrier to pulmonary siRNA delivery. In the upper and central airways, epithelial cell mucus is a defense barrier against irritants such as bacteria, allergens, and other inhaled particles (Fig. 1). Airway mucus is produced by secretory cells including goblet cells and club cells and forms an adhesive and hyperviscoelastic barrier to impede particle deposition in the deeper lung [46,47]. Airway mucus is composed of mucins, water, and other gel-like constituents [46]. Mucins are glycoproteins with densely glycosylated and negatively charged regions. The mucus covering the airway epithelium has been recognized as one of the greatest obstacles to siRNA delivery. Electrostatic interactions with positively charged siRNA particles may trap the particles in the mucus layer resulting in increased MCC and decreased delivery efficacy [48]. Demeester *et al.* showed that cationic DOTAP/DOPE lipoplexes were stable when incubated with diluted CF sputum obtained from patients but binding of mucins decreased the transfection efficacy. Mucins, however, did not cause aggregation or dissociation of the lipoplexes, indicating that mucins alter (intra)cellular steps in the transfection process [49]. Nanoparticles trapped in the mucus layer of the airways are cleared by MCC or cough-driven clearance. However, particles that rapidly penetrate through the mucus layer and into the PCL may be retained significantly longer in the lungs. The PCL is believed to be nearly stationary and presents a significantly steric barrier to particle penetration. In CF lungs, dehydration of PCL mediated by dysregulation of epithelial sodium channels in the airway epithelium can cause osmotically driven collapse of the PCL, which makes the PCL mesh tighter and thus increases further the barrier to particle transport. Overall, particles that can penetrate the mucus layer but not the PCL would also be cleared via MCC or by macrophages [39].

Multiple strategies have been studied for effective mucus layer penetration and MCC avoidance, including mucus-penetrating particles (MPPs). Unlike mucoadhesive particles, which are trapped in the luminal mucus layer and eliminated by the MCC, MPPs diffuse through mucus and avoid MCC. Surface properties of the MPPs are crucial in improving mucus penetration [38,46,48,50,51]. To favor penetration, MPPs should be small enough to avoid entrapment in the mucin mesh and have hydrophilic and neutral surface charge to avoid interactions with the hydrophobic and negatively charged groups of mucins. However, siRNA delivery often relies on lipophilic and positively charged carriers [46]. Therefore, careful surface engineering strategies are needed. Among those, dense surface coating with polyethylene glycol (PEG) has been the most successful approach. Surface PEG affects lung distribution, mucus penetration, and lung-residence time of the siRNA particles [38,50,52]. Densely PEGylated DNA MPPs showed greatly enhanced particle distribution, lung retention time, and gene transfer following intranasal administration compared to similarly sized mucoadhesive nanoparticles [52]. A recently reported alternative to PEG to facilitate mucus penetration relies on a unique combination of lipophobic and hydrophobic properties of perfluoroalkyl polymers [51].

The use of adjuvants which can modulate biological barriers is another suitable approach to overcome such barriers. Adjustment of biological barriers with properly selected adjuvants is one possible way to enhance the efficiency of pulmonary siRNA delivery. However, it is important to ensure that any adjuvant approach does not cause significant toxicity or disrupt normal lung function. The most widely explored method in this context has been the use of mucolytic agents that degrade or loosen primary macromolecular components of airway mucus, such as recombinant human DNase (rhDNase, Pulmozyme®) and N-acetyl cysteine (NAC, Mucomyst®) [53], which can significantly reduce the viscoelasticity of airway mucus. Both are clinically used in helping CF patient clear accumulated mucus from the airways. It has been reported that pre-treatment with NAC increases mucus mesh spacing which leads to rapid diffusion and improved penetration through airway mucus [54]. Improving airway surface hydration with osmotically active inhaled hypertonic saline provides another effective way of mucus clearance [55,56]. Inhaled mannitol creates an osmotic drive for water to move into the airway lumen. The subsequent increased hydration of the airway surface decreases the adherence of mucus to the epithelium and facilitates the coupling of mucus and cilia, thereby increasing mucus clearance. Inhaled dry powder mannitol (Bronchitol®) is promising

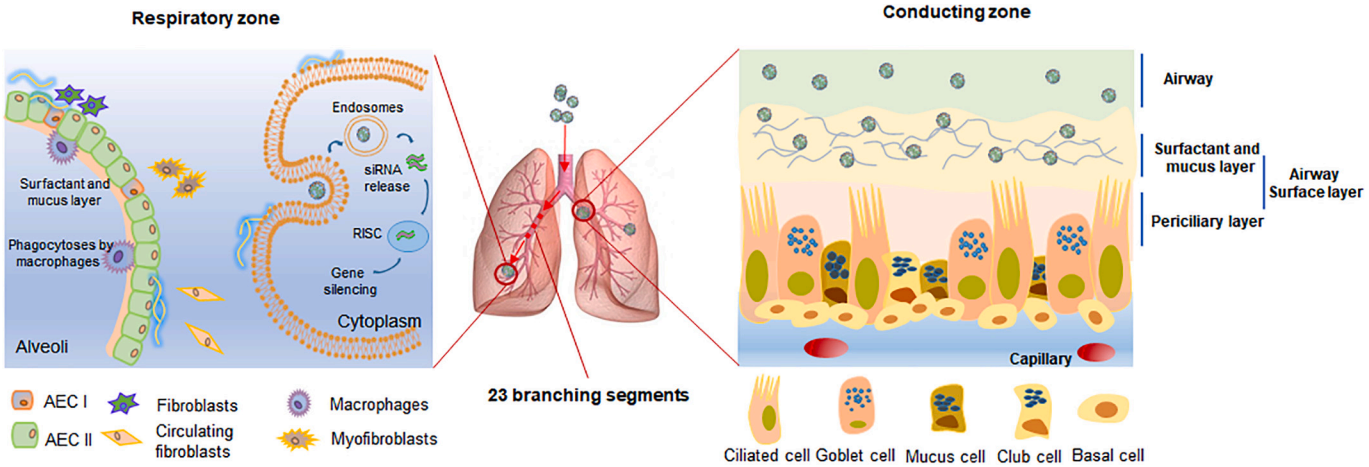

Fig. 1. Schematic illustration of the pulmonary barriers that affect siRNA delivery.

treatment for the clearance of retained airway secretions in the patients with CF and thereby may also be useful as an adjuvant to inhaled siRNA therapy [57]. Combination treatment with inhaled corticosteroids and bronchodilators may also be considered to optimize siRNA lung deposition. Combination therapy using intranasal delivery of dexamethasone-conjugated PEI and siRNA showed improved efficiency in treating asthma [58]. The use of inhalable β2-adrenoceptor ligand-directed chitosan carriers also improved the efficacy of pulmonary siRNA delivery [59].

2.4. Cellular and intracellular barriers

To exert its therapeutic effect, siRNA must be delivered to the cytoplasm of the target cells. The cell uptake pathway associated with the siRNA delivery by non-viral vectors is endocytosis. There are four main mechanisms of endocytosis. First, clathrin-mediated endocytosis, which is the most widely studied mechanism and is involved in receptor-mediated uptake of nanoparticles. For example, transferrin receptor, low density lipoprotein receptor, and epidermal growth factor receptors have been explored to mediate endocytic uptake. Second, caveolin-dependent endocytosis, which generates cytosolic caveolar vesicles following nanoparticle binding to the cell membrane. Third, macropinocytosis, which generates large macropinosomes containing extracellular fluid and soluble proteins. Fourth, other endocytic mechanisms that rely on actin-driven membrane protrusions, which subsequently fuse with and separate from the plasma membrane to generate macropinosomes [60]. For most obstructive lung diseases, such as COPD and CF, airway epithelial cells are the primary target for treatment. Unfortunately, these cells display low endocytic activity on the apical side [39]. Agents such as the natural airway surfactant lysophosphatidylcholine (LPC) or the calcium chelator EGTA were employed to transiently disrupt epithelial tight junctions and improve vesicular stomatitis virus (VSVG)-HIV entry *in vivo* [61]. Particles smaller than 150 nm allow for endocytosis and also limit macrophage uptake, both of which help to avoid lung clearance [62]. Upon endosomal cellular entry, siRNA must escape from the endosomes into the cytoplasm. Multiple strategies have been studied to overcome and promote the endosomal escape of siRNA that are directly applicable to pulmonary delivery [63–68]. Active targeting using ligand moieties that bind to over-expressed receptors on the surface of target cells is a widely explored strategy to enhance cell uptake and to improve therapeutic efficiency [69]. For example, T-cell targeted pulmonary siRNA delivery using transferrin and melittin-PEI conjugate shows efficient uptake in active T cells and endosomal escape for the treatment of asthma [70]. As cholesterol is an essential component of the cell membrane and can be metabolized, it is often used as the lipid anchor for improving stability and cellular uptake as well as decreasing the cytotoxicity of the carriers [71]. Once taken up by the target cells, siRNA particles must overcome several intracellular barriers, including but not limited to the endosomes and lysosomes. These barriers are shared in numerous organs and tissues in addition to the lungs and have been extensively reviewed elsewhere [72].

3. Disease-dependent pathological changes and their role in pulmonary disease and impact on siRNA delivery

When considering whether a patient with a given lung disease would benefit from inhalation of siRNA there are a number of important host factors that should first be determined. Pulmonary mechanics play a vital role in deciding whether medication can be inhaled deep into the lung, retained, and made available to target cells. In the majority of asthma or COPD patients, the day-to-day fluctuation in respiration is suitable for metered-dose inhalers (MDI), DPI, or nebulizers [13]. In extreme settings of lung diseases, such as stage IV COPD with forced expiratory volume (FEV_1) < 50%, severe asthma (FEV_1 < 50%), or end-stage IPF with diffusion capacity of the lung for carbon monoxide (CO) (D_{LCO}) < 50%, respiration may be severely obstructed or restricted to the extent that inhalation is ineffective because therapeutic deposition of particles cannot be achieved [73]. This would be most apparent during acute disease exacerbations.

Tissue composition must also be considered. In the case of obstructive lung diseases, such as asthma or chronic bronchitis, airway tissue composition changes significantly with increases in mucus production, hypertrophy of the basement epithelial membrane and surrounding smooth muscle, activated immune cells, and the many inflammatory factors that they produce [74]. This also leads to changes in the composition of the aqueous and mucous layers that line the airway thereby in most instances decreasing fluidity and increasing viscosity [74]. Whether siRNA particles can traverse through this complex admixture *en route* to target cells that lie underneath remains a major challenge in this field.

In CF, the levels of endogenous DNA and actin filaments released from necrotic neutrophils are elevated which further contributes to the dense mesh structure of the airway [75]. The elevation of oxidative stress in CF increases disulfide cross-links between mucin fibers which unfavorably changes mucus transport properties for particles [76]. The size of the CF mucus mesh ranges from 60 to 300 nm [77]. Nucleic acid delivery vectors that have been used in CF clinical trials have been shown to be incapable of efficiently penetrating CF mucus, most likely due to the positively charged surface of the formulations that interact with negatively charged mucus.

COPD patients experience increased airway inflammation, dynamic lung hyperinflation, elevated bacterial colonization in the lower airways, and increased susceptibility to viral airway infections [78]. Significant efforts have been made to develop siRNA therapeutics targeting mRNAs involved in the pathogenesis of COPD, including RIP2, RPS3, MAP3K19, and CHST3 [7,79]. Clinical trials using either viral or non-viral delivery vectors have failed to show clinical benefits because of inefficient transfection of target cells [39]. Poor ability of the particles to penetrate the mucus layer and aggregation caused by PS are likely other reasons for the lack of success in clinical testing. COPD and CF show activation of the alveolar macrophages, which provides additional challenge in siRNA delivery [80]. In asthma, the barrier function of the airway epithelium is impaired through defective tight junction formation. The tight junction proliferation results in increased epithelial resistance. However, the pro-inflammatory cytokines and bacterial toxins reduce the permeability of tight junctions, which means that the barrier properties of the airway epithelium may vary with disease state [81].

Respiratory infections can be caused by a wide range of microorganisms in airways, including bacteria and viruses. Respiratory infections are among the most common reasons for hospitalization, partly because many infectious diseases have become difficult to treat due to the rise of antimicrobial resistance. The respiratory syncytial virus (RSV) replicates in the superficial layer of the respiratory epithelium, and thus local delivery of siRNA to the lungs is a rational approach to treat RSV infection [82]. There has already been a clinical trial of an siRNA formulation, ALN-RSV01, which was directed against mRNA that exhibits specific anti-RSV activity [82]. Tuberculosis (TB) is a bacterial lung infection caused by *Mycobacterium tuberculosis*. RNAi approaches against MTb typically aim to modulate the host gene expression or host immune response instead of targeting the microorganism directly due to the lack of the requisite machinery for RNAi [83,84]. The 2020 pandemic of the newly discovered coronavirus (SARS-CoV-2, COVID-19) spurred interest in siRNA as a possible treatment [85]. In principle, multiple proteins encoded in the viral genome can be targeted by siRNA. Zheng *et al.* designed 48 siRNA sequences that potentially target the entire SARS-CoV genome, including open reading frames (ORFs) needed for the translation of key proteins [86]. Li *et al.* developed an siRNA which improved several symptoms of SARS-CoV, like fever, viral load and acute alveolar damage [87]. He *et al.* demonstrated synergistic antiviral effects through the use of siRNA pool targeting various

structural genes of the virus [88]. Since coronaviruses use ORF1a and ORF1b replicases [89], siRNA could be an efficient approach to control the virus by silencing the viral mRNA at particle stage. Future studies are called for to evaluate their potential efficiency and safety.

Finally, a significant limitation for the success of inhalation therapies is in the development of validated animal models of different lung disease states [2,90]. Approaches to enhance RNA stability, tissue targeting, cell penetration and intracellular endosomal escape are critical to realize the full potentials of RNAi drugs. This is important in preclinical development to demonstrate efficacy, toxicity profiles, and ultimately the establishment of appropriate human dosing regimens and how *in vitro* models can enrich pulmonary drug delivery research allowing for faster and more reliable clinical translation [91][90].

4. Recent developments in the pulmonary siRNA delivery systems

Pulmonary siRNA delivery requires the use of suitable vectors that can safely circumnavigate the unique environment and barriers in the lungs [2,21,22,92]. In addition to the delivery barriers discussed above, the stability of delivery systems is critical for efficient pulmonary siRNA delivery. For the systemic siRNA delivery, the siRNA is rapidly degraded with poor stability. Even though the nuclease levels are low in the lungs, the particle stability in the presence of mucus and PS poses an obstacle. It is critical to evaluate the impact of mucus and PS on the delivery systems. Cationic lipid-based siRNA nanocarriers (Lipofectamine™ and RNAiMAX) were incompatible with PS, however, the gene silencing potential of siRNA-loaded dextran nanogels (DEX-NGs) was maintained in the presence of PS and the intracellular siRNA delivery by DEX-NGs was enhanced [93]. Moreover, BALF contains inhibitory components for non-viral gene transfer, and a study showed that mucins adsorbed more to lipoplexes than to polyplexes. However, the specific inhibitory components have not been identified. The inhibition was most likely due to the charge in the surface charge of the gene vectors [94]. The shielding of siRNA particles to circumvent interaction with the airway surface layer (ASL) environment should be a focus for pulmonary administration.

In the following sections, we will review safety considerations and focus on the main types of vectors employed in pulmonary siRNA delivery (Table 1). Because each type of delivery vector offers a unique set of advantages and disadvantages, there is a great need for direct head-to-head comparisons of the different delivery systems to provide evidence for their relative delivery performance. A good example of such a study is the evaluation by Garbuzenko *et al.* of micelles, liposomes, mesoporous silica nanoparticles, cationic dendrimers, quantum dots, and PEG in their ability to accumulate in the lungs [95].

4.1. Safety of pulmonary delivery systems

Careful safety evaluation of siRNA delivery systems is critical for their use because of the finely-tuned and sensitive immune response of the respiratory system to foreign particles. Evidence shows that the observed toxic effects related to particulate delivery systems are primarily mediated by inflammatory responses that often occur after particle-induced oxidative stress. The extent of the response is sensitive to the physicochemical properties of the particles, including their size, chemical composition, surface properties, charge, and shape [96]. Intracellular oxidative stress can regulate the expression of endothelial cell adhesion molecules (CAMs) by transcription-dependent mechanisms involving redox-sensitive transcription factors and resulting in the activation of the MAPK and NF-κB pathways leading to the release of the inflammatory cytokines [97]. Multiple studies show that most positively charged particles induce lung inflammation following pulmonary delivery. For example, positively charged chitosan microparticles and gold and iron oxide nanoparticles caused inflammatory lesions in rodents [98]. Rats given positively charged graphene nanoplates exhibited greater pulmonary inflammation than when given negatively charged counterparts [99]. Mice administered with cationic liposomes and cationic NLCs developed pulmonary inflammation, while neutral and anionic liposomes and anionic NLCs exhibited normal lung histology [99,100]. For some particles, however, negative surface charge can elicit stronger release of proinflammatory cytokines than positively charged particles [101]. For example, in RAW264.7 macrophages, negatively charged silica nanoparticles induced the highest secretion of proinflammatory TNF-α compared to neutral and positively charged silica NPs [102]. Negative surface charge on quantum dots enhanced the mRNA levels of TNF-α in A549 cells, whereas IL-1β expression was enhanced by all quantum dots regardless of their surface charge [103]. Lipid-based delivery vectors induced toxicity and non-specific activation of inflammatory cytokines and interferon responses [104], while alveolar macrophages were not activated by exposure to polymeric microspheres [105].

To reduce non-specific toxicity to normal lungs following pulmonary delivery, nanoparticles with site-specific targeting and triggered release characteristics have been evaluated. PS-based pH-sensitive nanoparticles were cytotoxic to lung tumor cells but proved safe to healthy lung cells, indicating that safety and selective toxicity may be achieved [106]. Biodegradable PLGA nanoparticles did not induce apoptosis, oxidative stress or cell cycle arrest compared with nonbiodegradable forms [107]. A block copolymer composed of PEG and PAsp(DET) achieved safe gene transfection without inducing severe lung inflammation [108]. Covalent siRNA conjugates are among the most promising delivery approaches with a usually favorable toxicity profile [109]. Further, exosomes as a new class of delivery systems did not trigger the lung immune response and were less likely to aggregate [45].

4.2. Lipid-based carriers for pulmonary siRNA delivery

A majority of commercial siRNA transfection agents are based on cationic lipids and some of them have been used in pulmonary delivery [110]. Lipid-based formulations used in pulmonary siRNA delivery include liposomes, lipoplexes, and nanostructured lipid carriers (NLC) [111]. Despite their outstanding transfection efficacy, the presence of cationic lipids in many of the formulations raises toxicity concerns associated with non-specific activation of inflammatory cytokines and interferon responses [112]. The inflammation response can be minimized by incorporating PEG lipids into the formulations [113]. However, a balance must be found between the positive surface charge and PEG surface density. The exclusive use of neutral lipids such as cholesterol in the form of covalent siRNA-lipid conjugates may reduce the toxicity and inflammation associated with cationic lipids [114].

Incorporation of specific ligands to achieve receptor-targeted siRNA delivery has been explored in various delivery strategies, including pulmonary delivery. Tumor-specific receptor luteinizing hormone-releasing hormone (LHRH) has been used in NLC formulations of siRNA and paclitaxel (PTX) in pulmonary treatment of lung cancer [115]. Inhalation delivery of the targeted formulation showed limited adverse effects and clearly outperformed non-targeted formulation as well as intravenously administered control. Oligolysine epithelial-targeting peptide have been used as part of DOTMA/DOPE formulations to deliver siRNA to mediate silencing of airway epithelial ENaC [116]. siRNA encapsulated in a vitamin A-coupled liposome efficiently suppressed HSP47 expression and induced apoptosis of myofibroblasts in the IPF model [117]. When Genzyme GL-67 cationic formulation composed of DOPE and DMPE-PEG, was clinically tested for the delivery of DNA for cystic fibrosis treatment [118], chloride abnormalities were improved and bacterial adherence in patients' lungs was reduced.

Lipids have also been widely used as components of other of siRNA delivery systems. For example, hybrid lipid-polymer nanoparticles containing poly(lactic-co-glycolic) acid (PLGA) and dipalmitoyl phosphatidylcholine (DPPC) have shown great potential for pulmonary siRNA delivery [119]. Similar hybrid nanoparticles have been optimized

Table 1
Recent developments in the pulmonary siRNA delivery.

Delivery system	siRNA target	Disease	Delivery route	Key observations	Ref
Nano-embedded porous microparticles co-loaded with DOX and siRNA	MRP1	Lung cancer	Dry powder inhalation	Excellent aerodynamic performance and sustained drug release. Anticancer efficacy in chemoresistant lung cancer.	Xu PY, *et al.* 2018. [136]
Transferrin (Tf)-PEI	Fluorescently labeled siRNA	Asthma	Intratracheal	Optimal physicochemical properties and selective siRNA delivery to activated T cells.	Xie Y *et al.* 2016. [127]
Noncovalently PEGylated ternary complex	CTGF	IPF	Intratracheal	Significant reduction in target gene expression, collagen deposition, inflammatory cytokines production, attenuation of pulmonary fibrosis, increased survival rate.	Sung DK *et al.* 2013. [190]
Micelles	Amphiregulin and CTGF	PF	Intratracheal and intravenous delivery	Collagen accumulation in the lung of animal was effectively inhibited both in the intratracheal or intravenous delivery.	Yoon PO *et al.* 2016. [191]
Exosomes	ICAM-1	Human primary pulmonary microvascular endothelial cells (HMEVCs)	In vitro	The Exo/siRNA compound efficiently delivered the target siRNA into HMVECs causing selective gene silencing, inhibiting the ICAM-1 protein expression, and PMN-EC adhesion induced by lipopolysaccharide (LPS).	Ju Z *et al.* 2017. [192]
Exosomes	Myd88	ALI	Intratracheal	No lung immune response to exosomes. Delivery of siRNA into lung macrophages in vivo.	Zhang D *et al.* 2018. [45]
LHRH-NLC	EGFR	Lung cancer	Inhalation	Enhanced efficiency of lung cancer therapy.	Garbuzenko OB *et al.* 2019. [115]
Liposomes	Mcl1	Lung cancer	Intratracheal	High delivery efficiency and reduced formation of melanoma metastasis.	Shim G *et al.* 2013. [193]
Liposomes	αENaC	CF	Oropharyngeal	Rapid translocation across mucus. Corrected aspects of the mucociliary defect in human CF cells. Effective delivery and silencing in vivo.	Tagalakis AD *et al.* 2018. [116]
Targeted liposomes	αENaC	CF	Oropharyngeal	Nebulized formulations retained biophysical properties and transfection activity. In vivo silencing of the α-ENaC subunit gene expression.	Manunta, M. D. I *et al.* 2017. [194]
Targeted liposomes	αENaC	CF	Oropharyngeal	Rapid translocation across mucus. Transfections of primary CF epithelial cells.	Tagalakis AD *et al.* 2018. [116]
Hybrid lipid-polymer nanoparticles	αENaC	CF	Inhalation	Delivery to airway epithelial cells. No acute proinflammatory effect.	d'Angelo I *et al.* 2018. [119]
Targeted gold nanoparticles	c-myc	Lung cancer	Intratracheal	Tumor cell proliferation, tumor growth inhibition, prolonged survival.	Conde J *et al.* 2013. [159]
Surfactant-coated nanogels	CD45	Healthy mice	Intratracheal aerosolization	Safe and effective siRNA delivery to alveolar macrophages.	De Backer L *et al.* 2015. [44]
PEG-PEI polyplexes	EGFP	actin-EGFP mice	Intratracheal	Efficient in vivo gene silencing.	Merkel OM *et al.* 2009. [128]
PEI-cyclam polyplexes	PAI-1	IPF	Intratracheal	Decreased collagen deposition in the lungs.	Ding L *et al.* 2018. [131]
PEI polyplexes	Bcl2	Lung cancer	Intratracheal aerosolization	Co-delivery of siRNA and DOX improved the antitumor effect with low side effects on the normal tissues.	Xu C *et al.* 2015. [132]
Chitosan polyplexes	EGFP	Lung cancer	Intratracheal aerosolization	Deposition throughout the lungs. Silenced the EGFP expression in lung tumors.	Capel V *et al.* 2018. [133]
G4-NH4 dendriplexes	EGFP	In vitro	pMDI	High respirable fractions (up to 77%) and fine particle fractions (~50%). Preserved biological activity of the siRNA after exposure to the pMDI propellant.	Conti DS *et al.* 2014. [139]
Dendrimer polyplexes	EGFP	In vitro	pMDI and DPI	Effective in producing aerosols suitable for deep lung deposition for both pMDI and DPI with no impact on the in vitro gene knockdown efficiency of the siRNA.	Bielski E *et al.* 2017. [140]
Dendrimer polyplexes	Fluc-TYE563	In vitro	Inhalation	Retained siRNA integrity and bioactivity after processing into dry powders. A binary mixture of trehalose and inulin showed optimal stabilization, enhanced cellular uptake and gene silencing efficiency.	Agnoletti M *et al.* 2017. [141]
VIPER polyplexes	GAPDH	Healthy mice	Intratracheal	Robust gene silencing (>75% knockdown) within the lungs.	Feldmann DP *et al.* 2018. [142]
Fluorinated polypeptide polyplexes	TNF-α	ALI	Intratracheal	siRNA delivery into macrophages. Fluorination enhanced the mucus permeation.	Ge C *et al.* 2020. [195]
Hybrid nanoparticles coated with SP-B	TNF-α	ALI	Tracheal aspiration	PS enhanced the siRNA delivery.	Merckx P *et al.* 2018. [30]
Hybrid fluorinated polymers	PDL-1	Lung cancer	Intratracheal	Decreased tumor fibrosis, increased T cell infiltration and relieved immunosuppression.	Li Z *et al.* 2020. [145]
PFC emulsion polyplexes	PAI-1	IPF and ALI	Intratracheal	Prolonged lung retention and widespread lung distribution. Promising therapeutic efficacy in ALI and in early fibrinogenic stage of IPF. Increased survival in IPF.	Wang Y *et al.* 2019. [150]
PFC emulsions stabilized with polycations	STAT3	Lung metastasis	Intratracheal	Improved anticancer effect.	Li Z *et al.* 2019. [149]

(*continued on next page*)

Table 1 (*continued*)

Delivery system	siRNA target	Disease	Delivery route	Key observations	Ref
Targeted mesoporous silica nanoparticles	MRP1 and BCL2	Lung cancer	Inhalation	Enhanced cytotoxicity of anticancer drugs. Prevented systemic off-target organ exposure.	Taratula O *et al.* 2011. [157]
HMGB1A/R3V6 ternary complexes	S1PLyase	ALI	Intratracheal	Significantly reduced inflammatory response. Reduced S1PLyase expression in ALI model.	Oh B *et al.* 2014. [196]
Multi-shell nanoparticles of CaP and PLGA with PEI	Mixture against pro-inflammatory mediators	Lung inflammation	Nasal	Decreased lung inflammation.	Frede A *et al.* 2017. [158]
Naked siRNA	TGF-β1	TB	Intratracheal	Increased expression of antimicrobial mediators (NO and iNOS). Reduced bacterial load in the lungs.	Rosas-Taraco AG *et al.* 2011. [84]
Naked siRNA	Interleukin 10	In vitro	Dry powder	First use of naked siRNA as inhalable dry powder using spray drying technology with integrity of siRNA retained.	Chow MYT *et al.* 2017. [165]
Naked siRNA	TGF-β1	IPF	Intratracheal	Inhibited pulmonary fibrosis, improved lung function, and prolonged survival.	D'Alessandro-Gabazza CN *et al.* 2012. [160]
Naked siRNA	TGF-β1 and miR-326	IPF	Intranasal	Attenuated fibrotic lung response.	Das S *et al.* 2014. [197]
Naked siRNA	VEGF	Lung cancer	Dry powder	Dry powder siRNA inhibited lung metastasis.	Miwata K *et al.* 2018. [134]
Naked siRNA	$SOCS_3$	Asthma	Intranasal	Decreased lung eosinophilia, improved mucus secretion, reduced lung collagen content.	Zafra MP *et al.* 2014. [161]
Naked siRNA	IL-4 and anti-RSV	Asthma	Intranasal	Reduced total cell count and eosinophilia in bronchoalveolar lavage fluid.	Khaitov MR *et al.* 2014. [162]
Naked siRNA	Rip2	Asthma. COPD	Intratracheal	OVA-induced cytokine release, inflammatory cell infiltration and mucus hypersecretion inhibited in experimental allergic airway inflammation [163]. Rip2 siRNA suppressed CS-induced inflammatory and oxidative damage markers [198].	Goh FY *et al.* 2013. [163] Dong J *et al.* 2019. [198]
Naked siRNA	CD86	Asthma	Intratracheal	Inhibited OVA-induced airway eosinophilia and airway hyperresponsiveness.	Asai-Tajiri Y *et al.* 2014. [199]
Naked siRNA	c-kit	Asthma	Intranasal	Inhibited expression of c-kit, reduced airway mucus secretion and infiltration of eosinophils in bronchoalveolar lavage fluid. Reduced production of SCF, IL-4, and IL-5.	Wu W *et al.* 2104. [200]

for scale-up, spray-drying production to fabricate physiochemically stable, biologically functional powders with properties optimal for pulmonary siRNA delivery [120].

4.3. PS for pulmonary siRNA delivery

Recent studies have shown that PS can be used to prepare nanoparticles with control over their biological fate, toxicity, pulmonary distribution, cell targeting, and intracellular delivery [44]. In the context of pulmonary siRNA delivery, PS can be used as carriers in the design of unique and bio-inspired systems to improve the siRNA distribution at the alveolar interface in the deep lung. The PS-based delivery strategy was first reported in 1994 in a study that used poly(lysine) conjugated to SP-B to deliver DNA into airway cells *in vitro* [121]. Because SP-B is positively charged, it is one of the most investigated PS for pulmonary delivery [33,34,122,123].

Amphiphilic cationic KL4 peptide was developed and tested as a synthetic SP-B mimic [124]. KL4 is one of the active compounds in Surfaxin, an FDA-approved intratracheal PS suspension for the prevention of RDS [124]. The KL4 peptide formed complexes with siRNA and helped in delivery in lung epithelial cells. Compared with conventional lipoplexes, the KL4/siRNA complexes remained stable and mediated efficient siRNA transfection in the presence of PS [125].

Systematic studies have been conducted to delineate how lipid composition affects pulmonary siRNA delivery with SP [29,30,33,34,44,93]. Hybrid nanoparticles consisting of siRNA-loaded dextran hydrogel core were coated with Curosurf® (a clinically used porcine PS) as an outer shell. The studies demonstrated that the outer layer of PS enhanced the intracellular siRNA delivery [34,44]. SP-B was identified as the key component responsible for the enhanced siRNA delivery following pulmonary administration [30]. SP-B can also promote siRNA delivery to other cell types, suggesting a more universal carrier potential [29].

4.4. Polycation-based systems for pulmonary siRNA delivery

The utility of polycations as siRNA delivery systems called polyplexes has been explored for several decades. A plethora of synthetic and natural polycations have been pursued for pulmonary delivery of siRNA, including poly(ethylenimine) (PEI) and chitosan [62].

PEI has become one of the most widely studied siRNA delivery vectors because of its highly modifiable amine-rich structure that facilitates effective cellular internalization and endosomal escape [126–129]. Clinical application of PEI or its conjugates has been so far limited by toxicity concerns [127]. Modification of PEI with neutral or anionic moieties, like PEG, perfluoroalkyls, hyaluronic acid, and attachment of targeting moieties has been shown to reduce toxicity [127–130]. Transferrin-PEI was designed to selectively deliver siRNA to activate T cells in the lung to avoid potential systemic effects [127]. An investigation by Merkel *et al.* showed that polyplexes formulated with PEG-PEI demonstrate better targeted delivery of siEGFP compared with PEI polyplexes *via* intratracheal administration [128]. In our previous study, we designed polyplexes based on CXCR4-inhibiting PEI derivative (PEI-C) for pulmonary delivery of siRNA as a combination treatment of IPF [131]. Similarly, pulmonary co-delivery of chemotherapy and siRNA can improve therapeutic efficacy in lung cancer [132]. Doxorubicin was conjugated to PEI using a pH-sensitive cis-aconityl linker and the polymer-drug conjugate was then used to condense siBcl-2 into polyplexes [132]. The combined polyplexes exhibited enhanced antitumor efficacy compared with either monotherapies.

Chitosan is another polycation that has been evaluated for pulmonary siRNA delivery [133]. Findings showed that chitosan dry powder prepared by spray freeze drying delivered siRNA against VEGF decreased the number of metastatic lesions in the lungs [134]. A limitation of chitosan is its poor solubility at physiological pH [133,135]. Various chemical modifications of chitosan have been used, including PEG conjugation. Piperazine substitution of chitosan increased aqueous

solubility at physiological pH, lowered cytotoxicity, and increased gene silencing efficacy. The system was used for inhalation delivery with good tolerability [133]. Xu *et al.* developed inhalable chitosan particles for co-delivery of doxorubicin and siRNA. The nanoparticles, which were embedded in poly-L-lactide (PLA) were highly stable and achieved deep lung deposition with excellent aerodynamic performance and sustained release of doxorubicin [136].

Cationic dendrimers have well-defined hyperbranched 3D structure with spherical shape [137]. The high density of surface groups allows for specific ligand modification for improved siRNA delivery [138]. Generation 4 PAMAM dendrimer was used for siRNA delivery using pressurized metered-dose inhaler (pMDI) [139]. The dendrimer/siRNA polyplexes were stabilized with mannitol and given with hydrofluoroalkane propellant to achieve siRNA delivery to alveolar epithelial cells. Triphenyl phosphonium modification of PAMAM dendrimers achieved successful aerosol delivery to the lungs using portable inhalers [140]. Dendrimer/siRNA polyplexes prepared using microfluidic assembly were successfully evaluated in spray-dried microparticle form [141].

Virus-inspired polymer for endosomal release (VIPER) was developed based on a polycation block to electrostatically condense siRNA into polyplexes [142]. Pulmonary administration of VIPER polyplexes in mice improved accumulation of the particles in both the bronchial and alveolar epithelium.

Compared with simple polyplexes, core-shell polymeric NPs can encapsulate siRNA in a polymer matrix to improve siRNA stability in the formulation [143]. Receptor-targeted nanoparticles comprising multifunctional mixtures of cationic segments (oligolysine epithelial-targeting peptide and the liposome DOTMA/DOPE) demonstrated effective mucus penetration and siRNA delivery to airway epithelium with low cytotoxicity, high transfection efficiency, and increased gene expression [116].

Fluorinated polycations emerged as attractive materials for nucleic acid delivery due to their unique serum resistance associated with the lipophobic and hydrophobic features of fluorocarbons [144]. Fluorination as an effective strategy for transmucosal nucleic acid delivery using guanidinated and fluorinated bifunctional helical polypeptides was reported recently [51]. The authors found that fluorination prevented undesired dissociation and decreased the mucin aggregation, which improved mucus penetration. This was due to reduced interactions between the polyplexes and the mucins. We have recently taken advantage of the fluorinated polycations in a pulmonary delivery strategy to successfully modulate the immune response and enhance anti-PD-L1 immunotherapy in lung cancer [145].

4.5. Peptide-based systems for pulmonary siRNA delivery

Bioactive cationic peptides represent an interesting class of siRNA delivery systems due to the possibility to take advantage of their inherent biological activity to improve the efficacy of the delivery process. Cell penetrating peptides (CPPs) used alone or covalently conjugated to polymers are a common example. CPPs consist of short amino acid chains and can interact with the plasma membrane to allow for cellular uptake during siRNA delivery [146]. A recent study shows that the silencing of chitinase-3-like-1 (Chi3l1) expression in the lung using CPPs-siRNA complexes (dNP2-siChi3l1) inhibits lung metastasis with enhanced Th1 and cytotoxic T-lymphocyte responses [147]. However, when compared to naked siRNA, conjugation of siRNA to transactivator of transcription (TAT) and penetrating CPPs failed to increase siRNA-mediated gene knockdown in healthy mouse lung [148].

4.6. Emulsion-based systems for pulmonary siRNA delivery

Direct formulation delivery to a disease site provides an attractive approach to treating lung disease. In our previous work, we reported that combining CXCR4 inhibition with plasminogen activator inhibitor-1 (PAI-1) silencing could serve as a promising strategy for treating IPF [131]. However, simple cationic polyplexes poorly penetrated the lung mucus layer [49]. Perfluorocarbons (PFCs) are biocompatible materials widely used as ultrasound contrast agents, in treating lung diseases, and in organ transplantation because of their high oxygen-dissolving capacity [149–151]. We have reported that PFC emulsions could improve cellular siRNA delivery to attenuate lung cancer metastasis, IPF, and ALI [149,150]. The PFC emulsion polyplexes formulated for pulmonary siRNA delivery improved cellular internalization and endosomal escape when compared with polyplexes. Furthermore, PFC in PLGA-PEG emulsions can serve as an agent for highly efficient organ reoxygenation [152]. A reverse water-in-PFC emulsion has been evaluated as a potential drug delivery system for pulmonary administration using pMDIs [153–155], but has not yet been used for siRNA delivery.

4.7. Inorganic nanoparticles for pulmonary siRNA delivery

In recent decades, various inorganic materials have been developed for therapeutic and diagnostic applications [156]. Among them, mesoporous silica nanoparticles (MSN) and hybrid lipid-calcium phosphate nanoparticles have shown promise in pulmonary siRNA delivery. MSN are well-suited for co-delivery of small molecule drugs and siRNA due to large porous surface area. LHRH-targeted MSN were tested in delivery of anticancer drugs and siRNA to treat lung cancer [157]. When compared with intravenous injection, where only 5% of the injected dose accumulated in the lung, pulmonary delivery of the MSN increased lung accumulation to 73% and prevented the absorption into the systemic circulation. Hybrid lipid-calcium phosphate nanoparticles were developed for pulmonary delivery of siRNA to abate lung inflammation [158]. The nanoparticles consisted of a calcium phosphate core coated with siRNA directed against pro-inflammatory mediators, encapsulated in PLGA, and finally coated with the outer layer of PEI. Nasal instillation of the particles led to a significant reduction of target gene expression and modulation of the inflammation response [158]. Conde *et al.* developed functionalized gold nanoparticles for targeted delivery of siRNA to lung cancer cells toward effective silencing of the specific target oncogene [159].

4.8. Naked siRNA for pulmonary delivery

The term “naked siRNA” refers to the delivery of siRNAs without any delivery vectors. Multiple studies showed that systemic vascular delivery of naked siRNA is compromised because of the instability and poor pharmacokinetic performance. However, for local pulmonary delivery, naked siRNA has shown promising results in the treatment of lung infections [84,134,160–163]. To improve siRNA stability and efficacy without using delivery systems, the siRNA was either chemically modified or conjugated to other biomolecules. Zhang *et al.* demonstrated that pulmonary delivery of siRNA can be achieved by intranasal administration without any vectors [164], although many of the mechanistic considerations, such as cellular uptake, are not clear. Nevertheless, the delivery of naked siRNA has been extended to clinical trials. A modified form of siRNA (ALN-RSV01) was administered using nasal spray and the treatment reduced RSV infection. In addition to liquid formulations, dry powder forms of naked siRNA also showed gene silencing effects [165,166]. Kohie *et al.* showed that naked siRNA was not affected by nebulization when processing using ultrasonic, air-jet, and vibrating-mesh nebulizers [167], providing important evidence of siRNA stability.

4.9. Microparticles for pulmonary siRNA delivery

Aerosol particles with an aerodynamic diameter 1-5 μm are optimal for penetration into the deep lungs and lower airways. Despite their large size (5-30 μm), large porous hollow particles (LPHPs) can achieve the desired aerodynamic size range via enhanced porosity within the

particles, which aerodynamically balances the large size [168]. A main advantage of this approach is that the actual geometric size is too large for phagocytosis by alveolar macrophages, which permits therapeutic retention for longer periods of time. LPHPs show other beneficial features, including high dispersibility from an inhaler and enhanced deposition in the lungs upon inhalation. The large particle size reduces the fractional surface area of particle-particle contact in a dry powder or liquid suspension and leads to decreased tendency to aggregate. A dry powder inhalable PLGA LPHP containing insulin showed prolonged drug release and decreased macrophage uptake and MCC *in vivo* compared with nonporous particles [169]. LPHP of PEG-PLGA containing heparin reduced the uptake by isolated rat alveolar macrophages *in vitro* more than small nonporous particles [170,171]. A 3D-printed micromixer was used for preparation of siRNA-dendrimer nanocomplexes, which were then processed into nano-embedded microparticles shown to retain siRNA integrity and bioactivity [141]. siRNA can be encapsulated in PLGA microparticles optimized using a double emulsion technique, and the results showed significant downregulation of the target gene expression compared to negligible knockdown using commercial transfection reagents [42,172]. Xu *et al.* developed PLA porous microparticles which contained siRNA-loaded chitosan and doxorubicin by the supercritical anti-solvent process. The particles exhibited a favorable aerodynamic performance and sustained drug release that led to higher anticancer efficiency [136]. A porous silicon micro/nano composite was reported to deliver siRNA to the lungs with melanoma metastasis [173].

4.10. Exosomes for pulmonary siRNA delivery

Exosomes are a type of extracellular nanovesicles released from living cells. Exosomes were once thought to be a mechanism for removing unwanted proteins, but we now know that they are also involved in intercellular communication. The fact that exosomes can bind a wide range of surface receptors makes them an interesting option for therapeutic siRNA delivery. Recent studies suggest that exosomes regulate the development of lung inflammation in response to diverse stimuli, potentially providing novel therapeutic and diagnostic targets for ALI/ARDS [174][175]. Inhaled exosomes have been developed for efficient delivery to inhibit or activate the alveolar macrophages and also generate pulmonary immune responses [45]. Importantly, these serum-derived exosomes themselves did not trigger the lung immune response. Intratracheal siRNA delivery using host serum-derived exosomes attenuated LPS-induced inflammation in alveolar macrophages [45]. Intratracheal instillation deposited exosomes in alveolar regions rather than in bronchioles and macrophages were the main recipient cells. The presence of surface proteins in exosomes may facilitate uptake by specific cell types in the lungs and avoid uptake by macrophages. Exosomes from human induced pluripotent stem cells were used as siRNA delivery vectors to silence ICAM-1 expression as a potential treatment of ALI. Recent study showed the potential of exosomes in delivering siRNA in an *in vitro* airway model [176].

5. Pulmonary siRNA inhalation delivery: Promise of dry powder inhalers

Therapeutic delivery via inhalation provides direct access to target cells for siRNA therapy in a relatively non-invasive manner. Early developmental and preclinical studies of pulmonary siRNA delivery typically use intratracheal and intranasal administration for their simplicity. However, these administration routes are not usually directly translatable to clinical use due to considerable risks and discomfort for patients. Hence, the development of inhalation aerosol systems is critical for the practical translation of experimental siRNA delivery systems from the laboratory to clinical use [177]. The three most common used commercial aerosol inhalation systems are nebulizers, pressurized metered-dose inhalers (pMDIs), and dry powder inhalers (DPIs). Nebulizers generate inhalable micron-sized liquid droplets that can carry large amounts of siRNA delivery vectors per droplet and reach virtually all areas of the lungs. Some of them have already been successfully used in clinical pulmonary nucleic acid delivery. A nebulized cationic lipid/DNA formulation has been tested in phase I clinical trial in CF [118]. More recently, two additional inhalable RNAi-based products have been tested in clinical trials: ALS-RSV01 [82,178] and Excellair™ [179]. ALS-RSV01 uses cholesterol-siRNA conjugate to treat RSV infection in lung transplant patients [178]. Excellair™ (whose phase II trial was discontinued in 2015) was aimed at treating asthma through silencing Syk [179].

Dry powder siRNA formulations have several advantages over liquid aerosol formulations, including better stability, ease of handling, and lower cost of storage and transportation [180]. Nevertheless, the development of DPIs for pulmonary siRNA delivery remains limited due to unresolved issues related to destabilization of the siRNA and the carrier system caused by the stress of heating, freezing, or spraying [181]. Spray drying, spray freeze drying (SFD), and supercritical fluid drying are the three major dry powder techniques applicable for the use in siRNA formulations [181]. Even naked siRNA can be formulated into an inhalable dry powder using co-spray-drying with mannitol and L-leucine (a dispersion enhancer), while maintaining the integrity of siRNA [165]. An early example of siRNA formulation in the form of inhalable dry powder using spray drying technology dates back to 2010 [182]. The initial formulation was improved by incorporating DOTAP into the original PLGA matrix to improve encapsulation and transfection activity of the siRNA in the spray-dried formulations [183]. Inhalable dry powder formulations of pH-responsive peptide/siRNA complexes was successfully produced by spray drying without compromising the physical integrity and biological activity of siRNA [184]. SFD is a multistep process including a spray-freezing step and a freeze-drying step (Fig. 2). The powder production by the SFD maintained the physicochemical properties of polyplexes without negating transfection efficiency [185]. SFD can provide high recovery of the powders even when the formulation amount is small, which makes SFD well-suited for the task due to the relative expense of siRNA [186]. SFD process parameters and choice of excipients could be optimized to maintain integrity and biological activity of siRNA, for example by co-spraying with carbohydrates and using low inlet temperatures [183]. Similarly, co-spray drying with mannitol and L-leucine as excipients also successfully preserved siRNA integrity even in the absence of any delivery system [165]. The presence of siRNA also alters the molecular arrangement and solid-state composition of carbohydrate excipients during the spray drying process [187].

Among the polycation-based siRNA delivery systems, PEI and chitosan have been the most often used vectors for the preparation of dry powders. SFD was used to prepare chitosan formulation with siVEGF, which was successfully used for pulmonary delivery to treat metastatic lung cancer [134]. PEI/siRNA powder with a spherical and highly porous structure was also prepared by SFD with high aerosol and lung delivery performance [188]. Merkel *et al.* demonstrated successful spray drying of PEI polyplexes in the form of nano-embedded microparticle powder [126]. The authors optimized the spray-drying parameters to generate powder with appropriate aerodynamic properties suitable for deep lung deposition.

In several published studies, the inhalable siRNA dry powder formulations were limited by the siRNA content, which was too low to be moved to clinical study. Inhalable spray-dried powder formulation with high siRNA loading (>6% w/w) was developed using human serum albumin as a dispersion enhancer to improve aerosol performance [189]. As a dispersion enhancer, albumin favorably modifies the surface properties of the spray-dried powder leading to the fine particle fraction consistently over 50% [189]. L-leucine is often used as the dispersion enhancer, but due to its small size compared to macromolecular siRNA, the benefits of L-leucine in siRNA formulation were modest [165].

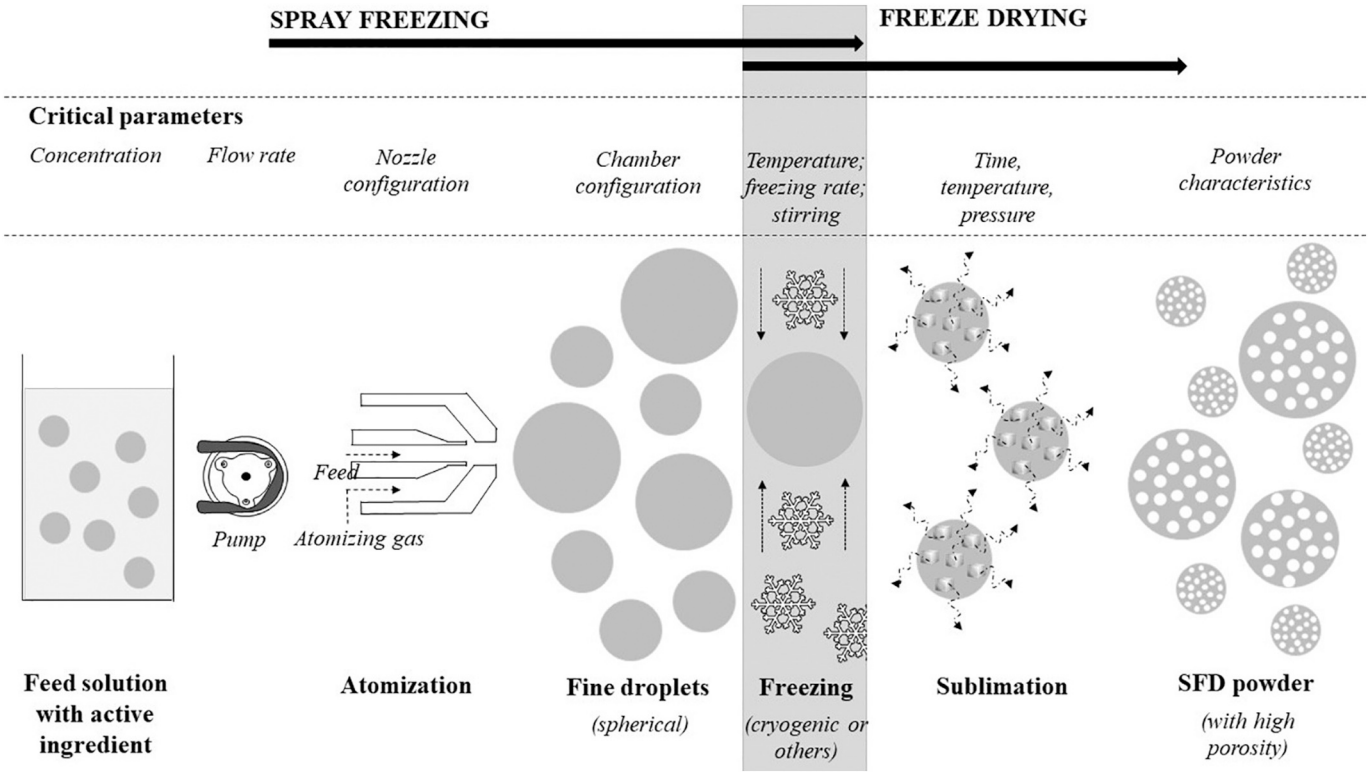

Fig. 2. Process of spray freeze drying (with permission [186]).

6. Conclusion

In this review, siRNA delivery barriers and recent approaches to pulmonary delivery were described. Pulmonary delivery of siRNA has gained increased attention due to the specific physiology of the lungs and characteristic properties of siRNA. However, how to translate laboratory studies to clinical practice and the difficulties that exist in evaluating this route of administration still remain a significant challenge. Even though the DPIs seem a strong choice for pulmonary siRNA delivery, more studies are required in regard to dose, siRNA loading, optimal excipients, and inhaler device for maintenance of siRNA stability and biological function.

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