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REVIEWS

Review

Inhaled RNA Therapy: From Promise to Reality

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RNA-based medicine is receiving growing attention for its diverse roles and potential therapeutic capacity. The largest obstacle in its clinical translation remains identifying a safe and effective delivery system. Studies investigating RNA therapeutics in pulmonary diseases have rapidly expanded and drug administration by inhalation allows the direct delivery of RNA therapeutics to the target site of action while minimizing systemic exposure. In this review, we highlight recent developments in pulmonary RNA delivery systems with the use of nonviral vectors. We also discuss the major knowledge gaps that require thorough investigation and provide insights that will help advance this exciting field towards the bedside.

Potential of RNA Therapy for the Treatment or Prevention of Lung Diseases

The diverse roles of RNA in the body have led to the emergence of different approaches to harnessing RNA for therapeutic use. RNA therapeutics can be broadly divided into three functional classes: (i) inhibition of gene expression [e.g., **small interfering RNA** (siRNA; see Glossary), **microRNA (miRNA)**, and **antisense oligonucleotide (ASO)**]; (ii) protein encoding (e.g., mRNA); and (iii) protein targeting (e.g., **RNA aptamers**) [1]. Despite their diverse mechanisms of action, it is no secret that the biggest barrier to all types of RNA therapeutic is delivery; that is, to bring therapeutic RNA molecules into the target cells effectively in a safe and reproducible manner. With the US Food and Drug Administration (FDA) approval of the first two siRNA therapeutics, patisiran and givosiran, both of which target hepatic disorders, the field of RNA therapy is ready to look for applications beyond the liver [2,3].

There is an increasing number of studies that report the potential of RNA in treating a range of lung diseases including asthma [4], cystic fibrosis (CF) [5], lung cancer [6], and respiratory infections [7]. Inhalation of **aerosol** is an efficient way to deliver RNA to the lung by maximizing local concentration while minimizing systemic exposure. ALN-RSV01, designed to treat respiratory syncytial virus (RSV) infection, was the first siRNA candidate to be delivered through the pulmonary route in clinical trials in 2008 [8,9]. Since then, several clinical trials on inhaled RNA therapy have been initiated (Box 1). However, no inhaled RNA therapeutic has yet been approved for use in clinics.

Naked RNA for Inhalation

RNA is a negatively charged, hydrophilic macromolecule that is incapable of permeating the cell membrane. It is vulnerable to degradation before reaching the target sites due to the abundance of RNase in the body. Therefore, it has to rely on delivery vectors to protect it from premature degradation and facilitate its cell entry. Interestingly, it has been known for over a decade that **naked RNA**, including both siRNA and mRNA, can be transfected in the lung following pulmonary delivery, as shown in many *in vivo* studies [10–24]. Given that the lung comprises various cell types with distinct functions, it is crucial to understand which cell types are susceptible to naked RNA **transfection** for effective clinical translation. To address this issue, Ng *et al.* carried out a comprehensive investigation on the distribution and activity of naked siRNA in the lung of mice following **intratracheal administration** [21]. The silencing activity of naked siRNA was most prominent in lung epithelial cells, dendritic cells, and alveolar macrophages. Similar

Highlights

Inhaled RNA therapy has great potential for treating a range of lung diseases, including lung infections, cystic fibrosis and asthma.

Although naked RNA is able to transfect in the lung following pulmonary administration, its efficiency may be subjected to variation due to the alteration of pulmonary surfactant composition between individuals and disease status, rendering it unfavorable for clinical application.

Many delivery systems have been generated for delivery of RNA therapeutics in the lungs.

Hybrid delivery systems that combine polymers, lipids, or peptides are increasingly popular for RNA delivery to enhance transfection efficiency.

Delivery systems should be tailor-made for different RNA targets and lung diseases to overcome the specific set of delivery barriers associated with the different lung diseases.

Only a few studies have evaluated the aerosol performance, integrity of RNA following aerosolization, dose–response relationship, pharmacokinetic profile, and long-term safety of inhaled RNA therapy formulations. More effort needs to be invested in these research areas for clinical translation.

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Box 1. Inhaled RNA Therapies in Clinical Trials

ALN-RSV01 (Clinical Trial No[i]: NCT00496821, NCT00658086, and NCT01065935)

ALN-RSV01 is a naked siRNA targeting the RSV nucleocapsid protein for the treatment of the associated viral respiratory infection. It was the first siRNA investigated for pulmonary delivery in clinical trials. RSV causes significant illness in immunocompromised patients following lung transplantation, and bronchiolitis obliterans syndrome (BOS) is the major cause of morbidity and mortality in these patients [101]. The Phase I clinical trial (NCT00496821) started in 2007 and demonstrated that ALN-RSV01 was well tolerated following intranasal administration. The Phase IIb clinical trial (NCT01065935) showed that aerosolized ALN-RSV01 was effective in reducing the incidence of new or progressive BOS in lung transplant patients with RSV infection following inhalation. Although ALN-RSV01 failed to progress to a Phase III trial, it marked an important milestone of inhaled RNA therapy [8,101–103].

Excellair

Excellair is an siRNA targeting spleen tyrosine kinase (Syk), which is involved in the inflammatory response in the lung epithelium [104]. It was investigated for the treatment of asthma by inhalation. The Phase I trial began in 2009. There was little information published about the outcome of the study, although it was reported that the drug was well tolerated in patients with asthma. The Phase II trial was discontinued in 2015 [105].

Eluforsen (Previously Known as QR-010) (Clinical Trial No: NCT02532764 and NCT02564354)

Eluforsen is a single-stranded RNA ASO targeting CF transmembrane conductance regulator (CFTR) for inhalation to patients with F508del CF. The Phase I clinical trial (NCT02564354) initiated in 2015 showed that CFTR activity was restored after intranasal administration of eluforsen. The Phase Ib clinical trial (NCT02532764) was completed in 2017 and demonstrated that inhaled eluforsen was safe, well tolerated, and improved respiratory symptoms in patients with F508del CF [106,107]. However, no further clinical development is planned for this candidate.

MRT5005 (Clinical Trial No: NCT03375047)

MRT5005 is the first inhaled mRNA candidate for CF and delivers mRNA encoding fully functional CFTR protein. The Phase I/II clinical trial was initiated in May 2018 to test the safety and tolerability of MRT5005. Patients with CF received mRNA encoding fully function CFTR protein through nebulization. Interim results were encouraging, showing that MRT5005 was well tolerated at low and mid-dose levels (8–16 mg) with no serious adverse events reported at any dose level (up to 24 mg). There was a marked improvement of lung function in patients after single dose of MRT5005 at the mid-dose level[ii]. In early 2020, the FDA granted Fast Track and Rare Pediatric Disease designations for MRT5005 for the treatment of CF[iii,iv].

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observations were reported by other groups in which the activity of naked siRNA was primarily found in lung epithelial cells but not the endothelial cells [23], making it attractive for use in lung conditions affecting these cell types without systemic exposure, for example in RSV infection and CF. Ng *et al.* stressed that **chemical modification** is crucial for naked siRNA to induce effective gene silencing by improving metabolic stability and reducing immunostimulation [21]. Using modified naked mRNA, Tiwari *et al.* successfully developed a mRNA-based approach to express neutralizing antibodies in the lung via intratracheal delivery to prevent RSV infection in mice [19]. The authors also compared naked mRNA with the use of polyethyleneimine (PEI, a synthetic cationic polymer discussed later) derivatives as delivery vectors and noticed that the transfection efficiency of naked mRNA was either better than, or comparable to, these polymers. Despite the promising effect of pulmonary naked RNA delivery, the exact mechanism of how naked RNA crosses the cell membrane barrier in the lung remains unclear, although it has been suggested that the pulmonary surfactant has a significant role in facilitating RNA uptake [25,26]. Some studies also showed that the use of delivery vectors could significantly improve RNA transfection compared with naked RNA in the airways [27–31].

The development of safe and effective inhaled delivery systems in parallel is paramount currently. With the recent success of siRNA in the clinic and the intensive investigation of mRNA in clinical trials, including **mRNA vaccines** against coronavirus disease 2019 (COVID-19) [32,33], we believe that these two RNA candidates are likely to be the first to enter the clinic for treating lung diseases. Therefore, in this review, we focus on the pulmonary delivery of siRNA and mRNA. We

Glossary

Aerosol: suspension of solid particles or liquid droplets in gas. In humans, drug delivery via the pulmonary route has to be administered in the form of an aerosol.

Antisense oligonucleotide (ASO): short single-stranded DNA or RNA (~20 nucleotides in length) that binds to a target mRNA through complementary base pairing, activating RNase H that leads to degradation of mRNA, thereby preventing the translation of mRNA into protein.

Cell-penetrating peptide (CPP): short cationic or amphipathic, natural or synthetic peptide (usually <30 amino acids) that is developed to deliver large cargoes, such as proteins, peptides, and nucleic acids, into cells by promoting cellular uptake.

Chemical modification: strategy to improve the stability and/or reduce immunogenicity of RNA by modifying the structure of RNA while maintaining the biological activity of the molecules (e.g., methylation of the ribose 2'-OH group; alteration of the bases; modification of phosphodiester backbone, etc.).

Intratracheal administration: introduction of substances directly into the trachea, either through the oral cavity via intubation or through a surgical procedure that creates an incision in the trachea (tracheotomy).

MicroRNA (miRNA): a short RNA (~ 21–25 nucleotides in length) that is partially complementary to multiple messenger RNA (mRNA), preventing the translation of mRNA into protein through the RNA interference mechanism.

mRNA vaccine: mRNA that encodes the target antigen to elicit immune responses in the body.

Naked RNA: RNA that is not associated with any delivery vectors or transfection agents, such as polymers and lipids.

Nebulization: conversion of liquid medications into a spray of fine droplets that can be breathed in by the patient.

Nonviral vector: agent or vehicle that transports nucleic acids into the cells without involving the use of viruses.

Parenteral: nonoral route of drug administration; usually refers to injection or infusion of drug directly into the body, bypassing the skin and mucous membranes.

RNA aptamer: singled-stranded RNA oligonucleotide that serves as ligand and binds to specific targets (e.g., proteins

discuss and highlight the recent advances of **nonviral vector**-based pulmonary RNA delivery systems development, gather what we have learnt from these studies, and identify the major gaps of knowledge. With these, we provide insights and direction to move the field forward.

RNA Delivery Vectors for Pulmonary Delivery

Many RNA delivery vectors have been developed for pulmonary delivery and their major functions are to facilitate the uptake of RNA by target cells and to protect RNA from premature degradation. Selected recent studies with different RNA delivery vectors that have demonstrated *in vivo* transfection in animal models are summarized in Table 1. We highlight some important studies in each category and discuss them in more detail herein.

Lipid-Based Delivery Systems

Due to their high transfection efficiency, ease of synthesis, and low batch variability, lipids were popular as transfection agents during the early years of gene therapy studies for various routes of administration [34,35]. The transfection efficiency and toxicity of lipid-based systems are affected by the composition of lipids and the ratio of lipids to RNA. Typically, cationic lipids, such as *N*-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA) and 1,2-dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP), are used to form lipoplexes with, or encapsulate, RNA [36]. The addition of neutral helper lipids, such as dioleoyl phosphatidylethanolamine (DOPE) and cholesterol, in the lipoplexes provide the ability to facilitate RNA complexation, increase stability of the lipoplexes, and reduce toxicity [37,38]. One major problem associated with lipid-based systems for pulmonary delivery is their poor structural stability because they readily fuse with pulmonary surfactants in the airways [39], leading to premature release of RNA before cellular uptake. Lipid nanoparticles (LNPs), which comprise cationic lipids, cholesterol, and polyethylene glycol (PEG), have been developed to improve the structural stability of lipid-based systems [35,40] (Table 1). With proper design and engineering, they can encapsulate RNA efficiently. LNPs are already in use in the clinic for **parenteral** injection of siRNA [41] and also are in an ongoing clinical trial to deliver mRNA (MRT5005) to the lung through **nebulization** for the treatment of CF, with encouraging early results (Box 1).

Polymer-Based Delivery System

There are two main categories of polymers: natural and synthetic polymers. Natural polymers have the advantages of excellent biocompatibility, biodegradability, and safety profiles [42]. Derived from the shells of crustaceans, the natural polymer chitosan is commonly investigated for pulmonary delivery due to its mucoadhesive and mucopermeable properties, enabling it to cross the mucus layer in the airways efficiently [43]. It can be used to form polyplexes with RNA or as a coating layer on the surface of nanoparticles [44,45] (Table 1). However, chitosan is limited by its poor solubility at physiological pH and relatively low transfection efficiency [42]. To overcome these problems, water-soluble chitosan derivatives, such as piperazine-substituted chitosan, were developed and found to be efficient for pulmonary siRNA delivery in healthy mice [46] (Table 1). Furthermore, inhalable chitosan/siRNA dry powder formulations were successfully prepared by supercritical drying [47] and spray–freeze drying [48] (Table 1). Both studies demonstrated a gene-silencing effect of the powder formulations in lung tissues following intratracheal administration in mouse models of lung cancer, taking these delivery systems one step closer to clinical application.

Among the synthetic polymers, PEI is extensively studied due to its high cationic charge density, good aqueous solubility, and wide pH-buffering capacity [49]. Its high versatility allows it to be functionalized to achieve specific targeting. For example, transferrin-PEI was used to target activated T cells in the lung, particularly T helper 2 cells, as potential therapy for asthma by reducing

and nucleotides) with high affinity and specificity.
Small interfering RNA (siRNA): short double-stranded RNA (~21–23 nucleotides in length with two 3'-overhang nucleotides) in which the antisense strand binds to the target mRNA through complementary base pairing, preventing the translation of mRNA into protein via RNAi.
Transfection: a process of introducing nucleic acids into cells artificially.

Table 1. Selected Studies of Pulmonary siRNA and mRNA Delivery in Animals[a]

Delivery vector	RNA type	Disease	Animal model	Refs
Lipid based				
Lipid nanoparticles (LNPs): comprising ionizable cationic lipid, phosphatidylcholine, cholesterol, and PEG	mRNA	Healthy	BALB/c mice	[40]
Polymer based				
Chitosan-coated PLGA nanoparticles: enhancing mucus penetration through a chitosan coating	mRNA	CF	$Cftr^{-/-}$ mice	[45]
Chitosan-derivative: piperazine-substituted chitosans that are water soluble	siRNA	Healthy	Nude mice	[46]
siRNA/Polymer powder: inhalable powder of siRNA complexed with PEI or chitosan prepared by spray–freeze drying and supercritical fluid	siRNA	Lung cancer	C57BL/6, BALB/c mice	[47,48,51]
Tf-PEI: PEI functionalised with transferrin (Tf) for targeting activated T cells	siRNA	Asthma	BALB/c mice	[50]
Hyperbranched poly(beta amino esters) (hPBAEs): polyplexes of biodegradable polymer and mRNA for nebulization; first inhalable mRNA formulation in *in vivo* study	mRNA	Healthy	C57BL/6 mice	[52]
Functional polyesters: synthetic amine A13 modified polyester series is promising for siRNA delivery to lung cancer cells	siRNA	Lung cancer	Nude mice	[27,53]
Peptide based				
Disulfide-constrained cyclic amphipathic peptide: siRNA release and intracellular delivery facilitated by reduction of disulfide bond of peptide in cytosol	siRNA	Healthy	C57BL/6 mice	[28]
siRNA/HMG/OR micelle ternary complexes: comprising oligoarginine (OR) micelles and high-mobility group (HMG) for alveolar macrophages targeting	siRNA	Asthma	BALB/c mice	[30]
PEGylated KL4 peptide: inhalable powder formulation of PEGylated KL4/mRNA complexes prepared by spray–drying and spray–freeze drying; first inhalable powder formulation with *in vivo* bioactivity	mRNA	Healthy	BALB/c mice	[31]
Hybrid based				
Lipid–polymer hybrids				
Lipidoid–polymer hybrid nanoparticles (LPNs): PLGA nanoparticles coated with lipidoid to control siRNA release	siRNA	Healthy	BALB/c mice	[60]
Self-assembled micelle inhibitory RNA (SMAiRNA): comprising hydrophilic polymer/lipid bi-conjugated siRNA	siRNA	Lung fibrosis	C57BL/6J mice	[61]
Lipid–peptide hybrids				
Liposomes-targeting peptide-siRNA (LPR): DOTMA/DOPE liposomes with peptides targeting lung epithelial cells	siRNA	Healthy	C57BL/6 mice	[62,63]
Polymer–peptide hybrids				
Virus-inspired polymer for endosomal release (VIPER): methacrylate-based polymer conjugated with melittin peptide to enhance endosomal escape	siRNA	Healthy	BALB/c mice	[64]
Self-assembled peptide-poloxamine nanoparticles: poloxamine nanoparticles functionalized with multiple moieties	mRNA	CF	B6CF mice	[65]

Table 1. (continued)

Delivery vector	RNA type	Disease	Animal model	Refs
Functionalized nanoparticles				
Gold nanoparticles: functionalized gold nanoparticles with targeting peptides	siRNA	Lung cancer	B6 albino, nude mice	[67,74]
Surfactant-coated nanogel system: enhances intracellular delivery by coating dextran nanogel with surfactant protein B	siRNA	Healthy, ALI	BALB/c mice	[29,69]
Exosomes				
Exosomes: serum-derived exosomes targeting lung macrophages	siRNA	ALI	C57BL/6J mice	[73]

[a]Abbreviations: ALI, acute lung injury; CFTR, CF transmembrane conductance regulator; DOTMA, N-[1-(2,3-dioleyloxy)propyl]-n,n, n-trimethylammonium chloride; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane chloride salt; PEG, polyethylene glycol; PLGA, poly(lactic-co-glycolic acid).

airway inflammation [50] (Table 1). Inhalable powder formulations have also been investigated for PEI delivery system. Okuda *et al.* used a spray–freeze drying technique to produce inhalable PEI/ siRNA dry powder with good pulmonary gene-silencing activity in mice with lung metastasis [51] (Table 1). However, the relatively high toxicity of this nonbiodegradable polymer remains a considerable concern, even with a low-molecular-weight PEI, rendering it difficult to be translated for clinical application. Thus, biodegradable synthetic polymers were developed to address this issue. For example, in a recent study, hyperbranched poly(beta amino esters) (hPBAEs) [52] were used to deliver mRNA through nebulization, with promising gene expression observed in the lung epithelium of mice without local or systemic toxicity after repeated dosing (Table 1). To enhance RNA delivery efficiency for lung cancer therapy, Yan *et al.* used a combinatorial library of functional polyester and high-throughput screening to identify matching cancer cells for specific targeting [27,53] (Table 1). After screening a library of >500 polyester candidates, synthetic amine A13 modified polyester PE4K-A13 was found to be potent for siRNA delivery to mice with lung tumors [27] (Table 1).

Peptide-Based Delivery Systems

Cell-penetrating peptides (CPPs) have attracted increasing attention for RNA delivery due to their versatility and cell entry ability [54]. The design of peptide sequence can be inspired by natural peptides, proteins with known functions, or by computational simulation [55,56]. The sequence of amino acids determines the properties of peptides, such as structure, charges, solubility, and polarity, which further affect the interaction with RNA, cellular uptake, toxicity, and transfection efficiency. One of the drawbacks of CPPs is the lack of cell specificity, which can be addressed by introducing cell-targeting sequences [57]. Furthermore, natural L- amino acids are susceptible to protease degradation; although strategies such as replacing L- amino acids with D- analogs has been proposed, this approach may lower the efficiency of the peptides [58]. Although many CPPs appeared to be promising candidates for delivering RNA to the lung, only a few have shown *in vivo* transfection. Welch *et al.* developed disulfide-constrained cyclic amphipathic peptides that form complexes with siRNA with good transfection efficiency because the disulfide bond reduction in the cytosol facilitated the release of the cargo as well as proteolytic clearance. Efficient gene knockdown was also observed in lung tissues of healthy mice following pulmonary delivery [28] (Table 1). Choi *et al.* developed a peptide-based vector that comprises micelles of oligoarginine peptides as the siRNA carrier and high mobility group (HMG) peptide ligand that targets the activated alveolar macrophages, with promising biological effects in an asthma model [30] (Table 1). Recently, Qiu *et al.* developed KL4 peptide, a surfactant protein B mimic, for delivering both siRNA and mRNA to the lung with good transfection efficiency

[25,31]. The PEGylated KL4 peptide was also used to formulate mRNA as inhalable dry powder by spray–drying and spray–freeze drying techniques, with efficient gene expression observed in the lung of healthy mice [31] (Table 1).

Hybrid Delivery Systems

To overcome the limitations of a single class of delivery vector, hybrid delivery systems, which are defined as the combination of two or more delivery vectors into a single entity, have been investigated and developed. This formulation strategy aims to increase the strengths of these delivery vectors while decreasing their disadvantages, with lower toxicity compared with their precursors [59].

Lipid–Polymer Hybrids

The combination of lipids with polymers intends to address the problems of poor structural stability associated with lipids and the low biocompatibility of polymers. Thanki *et al.* developed lipid–polymer nanoparticles (LPNs) comprising a poly(lactic-co-glycolic acid) (PLGA) matrix core coated with lipidoid, with siRNA localized in both the core and the shell [60]. This design compensated the low siRNA-loading capacity of PLGA by introducing cationic lipidoids while controlling the rate of siRNA release through degradation of the polymer. Enhanced lung retention upon pulmonary administration of the siRNA-loaded LPNs was observed in animal studies, suggesting their potential for controlled siRNA delivery in the lung (Table 1). Yoon *et al.* developed self-assembled micelle inhibitory RNA (SMAiRNA) nanoparticles that comprised bioconjugated siRNAs with a hydrophilic polymer on one end and a lipid on the other end [61] (Table 1). This hybrid system demonstrated effective gene silencing in animal models of pulmonary fibrosis with good stability and low toxicity.

Lipid–Peptide Hybrids

Another common hybrid strategy is to combine lipids with peptides, with the latter serve as a hydrophilic group and facilitate cellular uptake and transportation. For example, cationic liposomes comprising DOTMA/DOPE were blended with epithelial-targeting peptides to deliver siRNA to the lung of healthy mice, leading to successful silencing of the epithelial sodium channel at the airway epithelium [62,63] (Table 1). The results demonstrated the potential application of this hybrid system for CF therapy.

Polymer–Peptide Hybrids

Similar to lipid–peptide hybrids, the major aim of combining polymers with peptides is to enhance the cellular transport efficiency. Polymers are usually conjugated covalently with the peptides. Feldmann *et al.* developed a polymer system called VIPER (virus-mediated polymer for endosomal escape), which comprised a cationic methacrylated-based copolymer for siRNA binding and a membrane lytic peptide melittin, to facilitate endosomal escape [64] (Table 1). The hybrid system demonstrated more effective gene-silencing effects in the lung of healthy mice compared with the unmodified polymer system. Guan *et al.* developed a multimodular synthetic peptide with anchor, cationic, and targeting moieties that can form complexes with biocompatible poloxamine-based copolymers and mRNA via self-assembly [65] (Table 1). These ternary complexes showed excellent mRNA expression in the lungs of mice with CF with negligible toxicity, making them an attractive gene delivery system for CF and other lung diseases.

Functionalized Nanoparticles

In addition to the aforementioned conventional delivery vectors, inorganic metal-based nanoparticles have also been evaluated for the pulmonary delivery of RNA. However, these nanoparticles cannot act as transfection vectors by themselves due to their lack of RNA-binding ability.

Therefore, they are usually functionalized on the surface to enhance the transfection efficiency. For example, gold nanoparticles are attractive delivery vectors because of their ease of synthesis and conjugation as well as their superior stability [66]. Gold nanoparticles were conjugated with siRNA through gold–thiol bonds and functionalized with M2pep, a peptide that selectively targets tumor associated macrophages [67] (Table 1). Specific gene-silencing effects were observed in targeted macrophages following intratracheal administration in a mouse lung tumor model. It has been suggested that pulmonary surfactant facilitates the delivery of polymer-based delivery systems in the lung [68]. To take advantage of this phenomenon, pulmonary surfactant and surfactant protein B-coated dextran-based nanoparticles were developed for siRNA delivery, with successful gene-silencing effects observed in healthy mice and in a model of acute lung injury (ALI), respectively, following pulmonary administration [29,69] (Table 1).

Exosomes

Exosomes are extracellular vesicles secreted in many cell types. They are involved in communication through the transfer of substances, such as lipids, proteins, and nucleic acids, between cells [70,71]. They have many desirable features for RNA delivery, including high biocompatibility with low inherent toxicity and immunogenicity [72]. Zhang *et al.* presented a new approach to delivering small RNA to the lung with the use of exosomes. siRNA was loaded into serum-derived exosomes via calcium-mediated transfection. Following intratracheal administration, the siRNA-loaded exosomes were successfully taken up by lung macrophages to achieve specific gene silencing in a mouse model of ALI [73] (Table 1).

Lessons Learnt from these Studies

We carried out a survey of studies published between 2015 and early 2020 that reported siRNA or mRNA transfection following pulmonary delivery in animals (Figure 1). A PubMed search was performed using the search terms 'siRNA' or 'mRNA', 'pulmonary delivery', 'intratracheal', 'inhalation', 'nebulization', and with the filters 'last 5 years' (publication date) and 'other animals' (species) . In total, 53 articles were included in the survey [10–23,27–31,36,38,40,45–48,50–53,60–65,67,69,73–87]. Each article was categorized manually according to the type of RNA delivery vector used or naked RNA; the animal model (disease or healthy); the method of administration to animal; and the type of RNA (siRNA or mRNA). While polymer was the most commonly used RNA delivery vector due to its high versatility and ease of preparation, the hybrid delivery system is gaining popularity, many of which include a targeting peptide to improve specificity. With the successful transfection of naked RNA in the lung and the technological advances in chemical modifications that greatly enhance the stability and reduce the immune activation of RNA [88,89], many researchers opt for the use of naked RNA to transfect lung tissues due to its simplicity (Figure 1A). This approach can also eliminate the risk of toxicity and immunogenicity associated with the delivery vectors. It is particularly popular when researchers are interested in examining the biological function of a particular protein rather than its therapeutic potential, such as the role of Yes-associated protein (YAP), ribosomal protein S3 (RPS3), and PI3K/SGK1 pathway in ALI [13,14,18].

Nevertheless, the successful use of naked RNA should not lead us to discard the use of delivery vectors for clinical applications. The transfection efficiency of naked RNA may not be robust enough for therapeutic use. Also, as mentioned earlier, the cellular uptake mechanism of naked RNA in the airways is still unclear. It has been speculated that lung lining fluid and pulmonary surfactants have a critical role in facilitating the transportation of RNA into cells [68]. The composition of lining fluids could vary substantially among patients and pathological conditions. Indeed, it has already been observed that pulmonary surfactant protein and lipid composition change significantly as a result of aging [90]. Lung diseases, such as CF, idiopathic pulmonary fibrosis, and chronic

(A) Delivery vector

Hybrid 23%
Naked 26%
Exosome 2%
Nanoparticles 7%
Peptide 6%
Lipid 6%
Polymer 30%

(B) Disease model

Inflammation 23%
Healthy 32%
Asthma 9%
Lung infection 2%
Fibrosis 8%
LIRI 2%
Lung cancer 24%

(C) Administration method

Powder aerosol 9%
Nebulisation 11%
Liquid
instillation
55%
Liquid aerosol 25%

(D) RNA type

mRNA 15%
siRNA 85%
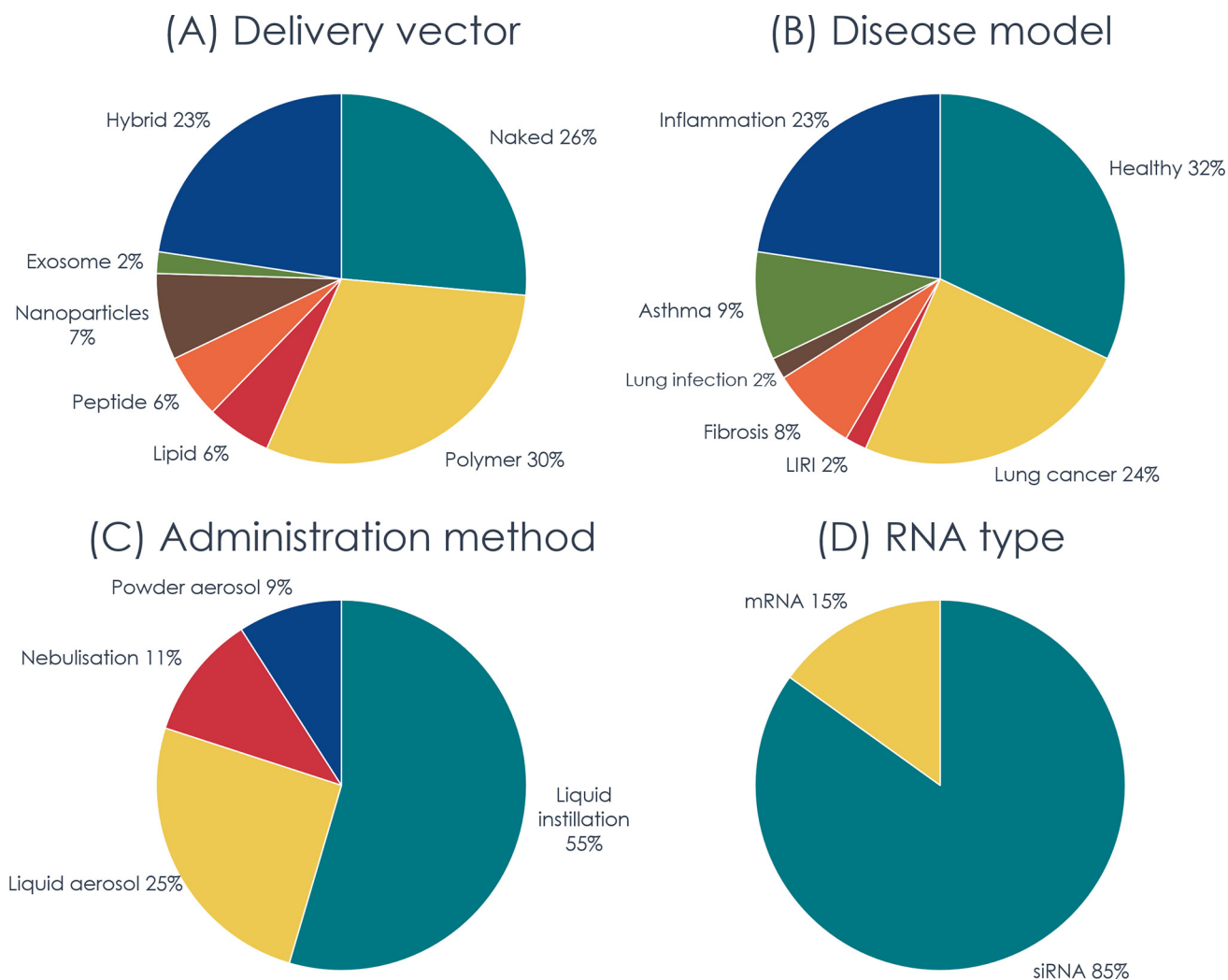
Trends in Pharmacological Sciences

Figure 1. Survey of Recently Published Studies on Pulmonary Small Interfering RNA (siRNA) or mRNA Delivery in Animals. Studies published between 2015 and early 2020 that reported siRNA or mRNA transfection following pulmonary delivery in animals were surveyed using an article search on PubMed with the search terms 'siRNA' or 'mRNA', 'pulmonary delivery', 'intratracheal', 'inhalation', 'nebulization', and with the filters 'last 5 years' (publication date) and 'other animals' (species) applied (N = 53). The studies are classified by (A) type of delivery vector; (B) disease model used; (C) administration method used in animal studies; and (D) RNA type. The classification was done independently by Y.Q. and M.C. and was cross-validated by J.K.W.L. Abbreviation: LIRI, lung ischemia–reperfusion injury.

obstructive pulmonary disease (COPD), are also associated with surfactant deficiency and altered lipid compositions [91–93]. Consequently, the transfection efficiency of naked RNA could be easily influenced by the age and disease status of the patients, leading to problems of reproducibility. Moreover, naked RNA lacks active targeting ability. Given that the lung tissues contain a variety of cell types, it would be desirable to use a delivery vector with a targeting moiety to enhance specificity while reducing transfection in unintended cell types.

The potential of RNA therapeutics to treat a range of lung diseases prompts us to examine the possibility of developing a universal delivery platform that can be adopted by different RNA therapeutics for different conditions. While such a system would accelerate the clinical translation of

inhaled RNA therapy, delivery barriers could be unique to a specific physiological condition. In that case, it would be more effective to have a carefully designed system to overcome these specific sets of barriers. For example, patients with CF have thick and excessive mucus in the airway; hence, a delivery vector with excellent mucus penetration ability would be favorable. Moreover, in preclinical studies, healthy animals are sometimes used to evaluate the efficiency of a delivery vector, with the use of reporter or housekeeping genes as the RNA target (Figure 1B). This approach may run into a similar problem in that a delivery vector may perform differently in a healthy animal versus a disease model due to the different delivery barriers in the airways. Furthermore, a non-disease RNA target (which is generally used to evaluate the efficiency of a delivery vector) does not provide any information regarding the pharmacodynamics properties of the proposed therapy. Even if a disease model is used, there is still the challenge of the clinical translation because many lung disease models have limitations that prevent their direct translation to human disease [94–97]. For instance, asthma is a complex condition that is observed exclusively in humans. The most common model used in preclinical studies is murine allergic airway inflammation. However, the distribution of lung inflammation in mice is different from human asthma, and the animals develop tolerance after repeated allergen exposure [97]. Therefore, a collaborative approach between formulation scientists, pharmacologists and clinicians will facilitate the development of a clinically relevant delivery system for inhaled RNA therapy.

Crucial Steps Involved in the Development of Inhaled RNA Delivery Systems

Many studies have focused on the development of RNA delivery vectors, but the translation of these vectors into suitable dosage forms for clinical application is currently lacking. Here, we summarize the critical steps for the development of successful pulmonary RNA delivery system (Figure 2, Key Figure) and discuss the areas/factors that should be focused on.

In general, it is desirable to use delivery vectors that exhibit cell-targeting properties with the ability to overcome the specific barriers associated with the disease concerned. Delivery vectors that are nonbiodegradable or with a low RNA-loading capability would be less attractive. A thorough understanding of the excretion pathway is required to ensure that the delivery vectors would not accumulate in the body, especially when synthetic or nonbiodegradable materials are used. Vectors with high loading capacity could avoid the use of excessive excipients, thereby minimizing the risk of toxicity. Moreover, to evaluate the *in vivo* efficacy of the delivery system, RNA is often administered intratracheally to the lung of animals either as large droplets instilled by pipette or as a fine spray aerosolized by a microsprayer or similar device (Figure 1C). Clearly, these administration methods are impractical for human use. For clinical practice, RNA drugs can be delivered either as liquid aerosols through nebulization or as dry powder for inhalation (Figure 2). While the former can deliver high doses of liquid formulations over a period of time (typically ~10–20 min, depending on the dose), the latter is more portable and convenient to use. However, there are stringent requirements for particles to be suitable for inhalation in clinical settings and animal studies cannot reflect the 'inhalability' of a formulation. Surprisingly, only a few studies have evaluated the aerosol performance of RNA formulations designed for inhalation [31,48,51] (Box 2). A good understanding of the aerosol properties of the formulation could boost the chance of successful clinical translation (Figure 2). Moreover, nebulizers or dry powder inhalers are needed to generate aerosol for inhalation. Given that both devices could damage the fragile RNA molecules (especially single-stranded mRNA) due to the high shear stress during aerosolization, it is essential to examine the RNA integrity, in terms of physical structure and biological activity, in the aerosolized particles (Figure 2).

It is unclear whether the RNA dose is optimized in *in vivo* preclinical studies because the dose–response relationship of RNA following pulmonary delivery is often not reported. Dose

Key Figure

Summary of Crucial Steps Involved in the Development of Inhaled RNA Delivery Systems
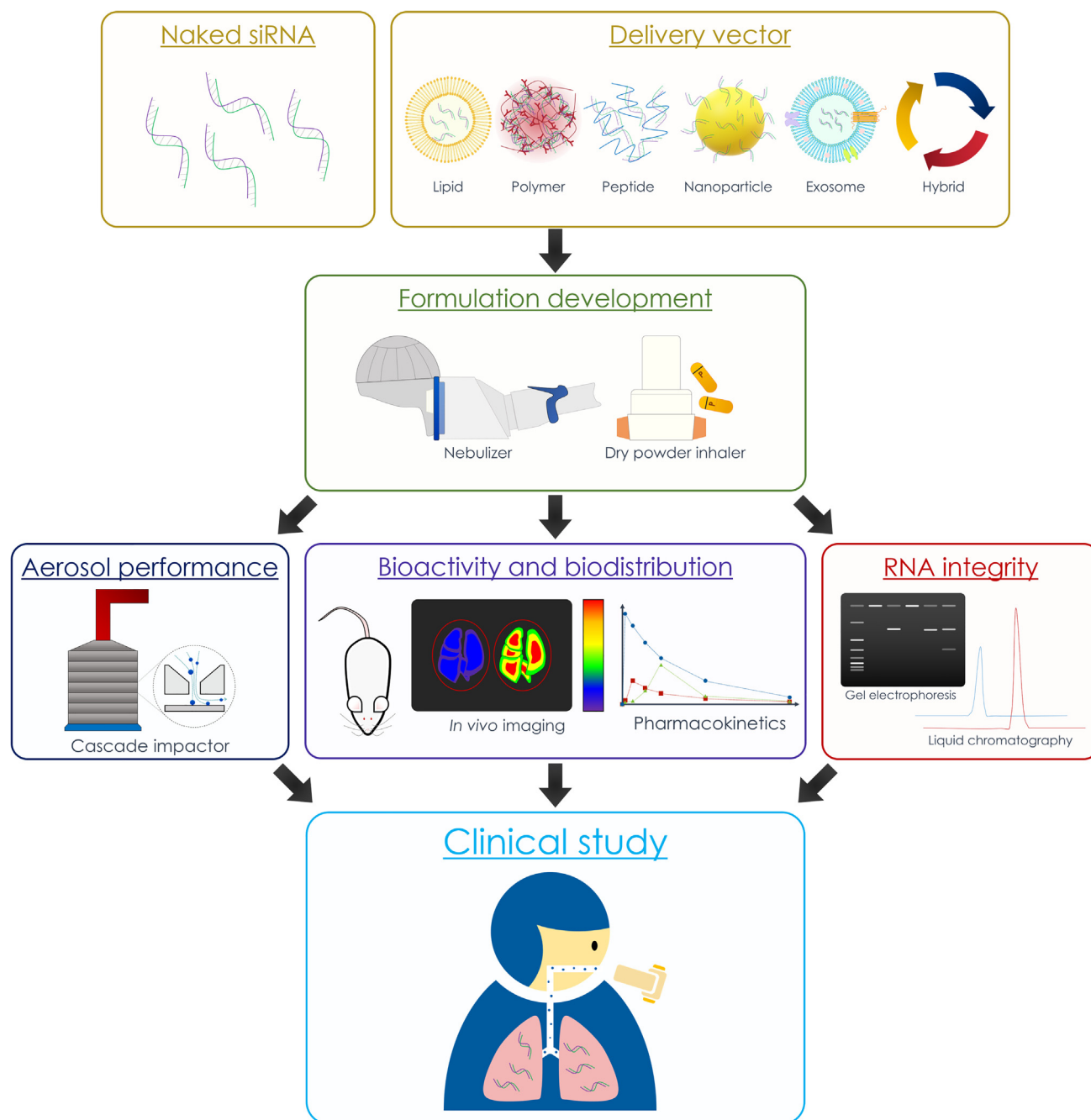
Figure 2. Firstly, either naked RNA is used or a delivery vector is designed to facilitate the uptake of RNA therapeutics to the targeted cells. A formulation (liquid or powder aerosol) is then developed with the identification of a suitable inhalation device for clinical use. The formulation is thoroughly characterized and evaluated for its aerosol performance, RNA integrity after aerosolization, and the biological activity and pharmacokinetic profile of the inhaled formulation in suitable animal models. Lastly, the potential candidates are identified for clinical study.

optimization is critical for clinical translation not only for maximizing the therapeutic efficacy, but also for the practicality of administration. The amount of excipient (which may be included in the formulation to improve stability or to enhance aerosol performance) needs to be considered carefully because this can also affect the final liquid volume or powder mass to be administered. When RNA is delivered through nebulization, a high dose would increase the volume and, hence, the administration time, leading to the increased risk of RNA degradation due to prolonged exertion of shear stress on the RNA molecules. For dry powder formulations, there is a constraint on the amount of powder that could be inhaled by a patient each time. Currently, TOBI® Podhaler® (tobramycin inhalation powder) approved for treating *Pseudomonas aeruginosa* infection in patients with CF, has the highest inhaled dose of ~30 mg per actuation [98]. The RNA dose has to be taken into consideration during the development of an effective delivery system that is fit for purpose.

Another area that requires attention during development of a pulmonary RNA delivery system is understanding the biodistribution and pharmacokinetic profile of the inhaled RNA formulation (Figure 2). Many studies focus on the evaluation of gene expression in the lung tissues as a whole. While naked RNA is shown to manipulate gene expression primarily in the lung epithelial cells and macrophages [21], the sites where nanoparticulate RNA delivery vectors exert their biological activity are less clear. For delivery vectors that rely on nonspecific cellular uptake mechanisms, RNA could be effectively taken up by various cell types and absorbed into the systemic circulation. Therefore, it is critical that biodistribution as well as pharmacokinetic profiles are thoroughly investigated. Furthermore, the long-term toxicity of the delivery system also needs to be carefully evaluated.

Concluding Remarks and Future Perspectives

RNA therapeutics have great potential in lung diseases. Here, we have discussed nonviral delivery systems developed for siRNA and mRNA therapeutics in the lung and briefly summarized the

current preclinical and clinical state of the field. Currently, it appears that the development of mRNA therapeutics is lagging behind that of siRNA therapeutics (Figure 1D), possibly because of the relatively poor stability of the long single-stranded RNA molecule. With technological advances in RNA modification that improve the stability, specificity, and safety of RNA therapeutics [88,89] and the recent success of siRNA therapeutics, we believe that both siRNA and mRNA will enter the clinic for the treatment of respiratory diseases in the near future. However, some crucial questions remain to be addressed before a successful RNA inhalation delivery system can be realized (see Outstanding Questions).

In light of the current COVID-19 pandemic, development of an inhaled version of mRNA vaccine is an area that deserves more attention. Currently, there are several clinical studies (Clinical Trial No[i].- NCT03164772; NCT03908671; NCT02662634; NCT03076385; and NCT03345043) that demonstrate the safety and efficacy of mRNA vaccines for the treatment of and protection against lung cancer and influenza, respectively [99,100]. mRNA vaccines for COVID-19 are being explored in several different clinical trials (Clinical Trial No.: NCT04283461; NCT04470427; NCT04405076; NCT04449276; NCT04480957; NCT04380701; and NCT04368728). These mRNA vaccine candidates are designed to be administered through parenteral injection. If any of these are successful, we believe that an inhaled version of the successful mRNA vaccine will be an area to explore because it will provide a non-invasive route of administration with the possibility of self-administration, especially dry powder formulations, which show superior stability. The challenges of manufacture and scale-up, including the production of RNA, delivery vectors, and the loading of RNA into the vectors without losing their physicochemical properties and biological activities, also need to be overcome. Overall, a safe and efficient RNA delivery system to the lung remains the key to successful clinical translation.

Resources

[i] www.clinicaltrials.gov

[ii] https://investors.translate.bio/news-releases/news-release-details/translate-bio-announces-interim-results-phase-12-clinical-trial

[iii] https://investors.translate.bio/news-releases/news-release-details/translate-bio-receives-fda-fast-track-designation-mrt5005

[iv] https://investors.translate.bio/news-releases/news-release-details/translate-bio-provides-mrt5005-program-updates

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