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Review article

Nebulised delivery of RNA formulations to the lungs: From aerosol to cytosol

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ABSTRACT

In the past decade RNA-based therapies such as small interfering RNA (siRNA) and messenger RNA (mRNA) have emerged as new and ground-breaking therapeutic agents for the treatment and prevention of many conditions from viral infection to cancer. Most clinically approved RNA therapies are parenterally administered which impacts patient compliance and adds to healthcare costs. Pulmonary administration via inhalation is a non-invasive means to deliver RNA and offers an attractive alternative to injection. Nebulisation is a particularly appealing method due to the capacity to deliver large RNA doses during tidal breathing. In this review, we discuss the unique physiological barriers presented by the lung to efficient nebulised RNA delivery and approaches adopted to circumvent this problem. Additionally, the different types of nebulisers are evaluated from the perspective of their suitability for RNA delivery. Furthermore, we discuss recent preclinical studies involving nebulisation of RNA and analysis in *in vitro* and *in vivo* settings. Several studies have also demonstrated the importance of an effective delivery vector in RNA nebulisation therefore we assess the variety of lipid, polymeric and hybrid-based delivery systems utilised to date. We also consider the outlook for nebulised RNA medicinal products and the hurdles which must be overcome for successful clinical translation. In summary, nebulised RNA delivery has demonstrated promising potential for the treatment of several lung-related conditions such as asthma, COPD and cystic fibrosis, to which the mode of delivery is of crucial importance for clinical success.

1. Introduction

In recent decades the potential of exogenous RNA has emerged for the treatment and prevention of many diseases previously thought to be "undruggable". The profound impact of RNA as a therapeutic agent has received unprecedented global attention in the past two years with the approval of mRNA-based vaccines targeting the SARS-CoV-2 virus which have been a game changer in the world's response to the COVID-19 pandemic [1]. Other conditions which can be successfully targeted by clinically approved RNA-based therapies include hypercholesterolemia [2], muscular dystrophy [3] and acute hepatic porphyria [4]. RNA therapeutics can be divided into several categories which differ in terms of structure and mechanism of action, including antisense oligonucleotides (ASOs) [5], small interfering RNA (siRNA) [6], and most notably, messenger RNA (mRNA) [7]. Other forms of RNA therapies currently in various preapproval stages of development include microRNA (miRNA) [8] and short hairpin (shRNA) [9]. Exogenous miRNA is delivered as two short strands of RNA arranged in a duplex structure of 19–25 nucleotides in length [10]. shRNA is a single-stranded RNA molecule that forms a stem-loop structure consisting of a 19-22 nucleotide base-pair region of double stranded RNA linked by a short loop of 4–11 nucleotides akin to a hairpin in appearance [11]. Upon intracellular delivery, exogenous RNA can either inhibit or enhance protein expression depending on the type of RNA. The short non-coding RNAs including ASOs, siRNA, miRNA and shRNA bind endogenous target mRNA resulting in the inhibition of protein translation while the delivery of mRNA allows for the transient upregulation of expression of the encoded protein. The use of therapeutic mRNA is an effective approach for the treatment of genetic diseases caused by a defective protein-encoding gene, and for vaccination against infectious diseases (as well as cancer immunotherapy) through

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the synthesis of pathogen (or tumour)-specific antigens [7].

In August 2018, the launch of Onpattro® (Patisiran, Alnylam Pharmaceuticals, Cambridge, MA, USA), indicated for the treatment of hereditary transthyretin-mediated amyloidosis (hATTR), heralded a new era in RNA therapeutics as the world's first clinically approved siRNA therapy [12]. siRNA consists of two RNA strands, arranged in a duplex structure, both of which are 19–25 base pairs in length each with a characteristic overhang of two nucleotides at their 3' end [13]. Inhibition of gene expression i.e., gene silencing can be achieved through a phenomenon known as RNA interference (RNAi) whereby the translation of mRNA into protein is inhibited by sequence-directed cleavage of target mRNA (Fig. 1)[14]. As part of the RNAi mechanism, only the strand of siRNA complementary to the sequence of the target mRNA, known as the guide strand, is incorporated into the multinuclease, RNA-Induced Silencing Complex (RISC). The remaining strand, referred to as the passenger strand is degraded by the Argonaute protein, a component of RISC [15]. Following association of the guide strand with the target mRNA, the Argonaute protein catalyses mRNA cleavage which results in specific silencing of the target gene [16].

To date, the majority of licensed RNA drugs are indicated for systemic administration [17]. Notably, all 5 currently approved siRNA therapies target liver related conditions [12,18–21]. Systemic administration presents numerous barriers to naked RNA delivery. These include a short circulatory half-life due to rapid nuclease degradation [22] and potential renal clearance in the kidneys owing to its small size [23]. Short molecules of RNA such as siRNA and ASOs can be chemically modified in several ways along their sugar phosphate backbone to improve nuclease resistance and extend half-life, a common approach being replacement of the phosphodiester bond with a phosphorothioate (PS) backbone [24]. Other examples of chemical modifications to siRNA are detailed comprehensively by Wan et al. [25]. Despite an improved pharmacokinetic profile however, the introduction of modifications to the RNA molecule is often limited due to a decrease in binding affinity towards its endogenous mRNA target [26].

The potential therapeutic applications of mRNA extend well beyond vaccines for infectious diseases to include protein replacement therapy, gene therapy and cancer immunotherapy. mRNA therefore provides novel opportunities for the treatment and prevention of a wide range of respiratory diseases, including genetic disorders e.g., cystic fibrosis (CF) [27], inflammatory diseases e.g., asthma [28] and lung infections [29]. However, the therapeutic use of mRNA molecules poses considerable challenges related to its physicochemical properties; (i) large molecular weight (10^5-10^6 Da), (ii) high negative charge density and (iii) intrinsic instability and susceptibility to degradation by exo- and endonucleases, as opposed to shorter RNAs [30]. To mitigate these challenges, delivery systems are imperative to achieve intracellular delivery of mRNA and protect from RNase-mediated degradation. The replacement of uridine with pseudouridine in mRNA also improves the thermodynamic stability of the mRNA molecule, thereby mitigating the increased susceptibility of uridine linkages to hydrolysis [31,32]. Differences in the physicochemical properties and stability of mRNA and small oligonucleotides should be considered in the development of RNA formulations. As previously mentioned, a wide range of chemical modifications can be applied to small oligonucleotides to increase their stability [33]. For example, the presence of the 2'-hydroxyl of the ribose is responsible for the hydrolytic degradation of RNAs (absent in DNA) and is often methylated in siRNA products to improve the storage stability [34]. In contrast, modified nucleotides play significant roles in the effectiveness of mRNA translation, but can only tolerate modifications that do not interfere with ribosome binding [35]. As a result, mRNA products have a shorter shelf-life and have more stringent storage requirements as compared to small oligonucleotide formulations [34]. The mRNA molecule is increasingly fragile dependent on (1) the length of the mRNA chain, (2) the secondary/tertiary structure of the mRNA and (3) selection of nucleotides, which may pose additional challenges in resisting shear stress effects, such as those associated with nebulisation [34,36]. Other factors that can impact the stability of mRNA-based drugs include the composition of the formulation (e.g., lipid type, excipient type, solution pH) and the presence of impurities, which can induce mRNA degradation, as reviewed extensively elsewhere [34].

In general, biological drugs are traditionally administered systemically via intravenous injection for treating diseases including those associated

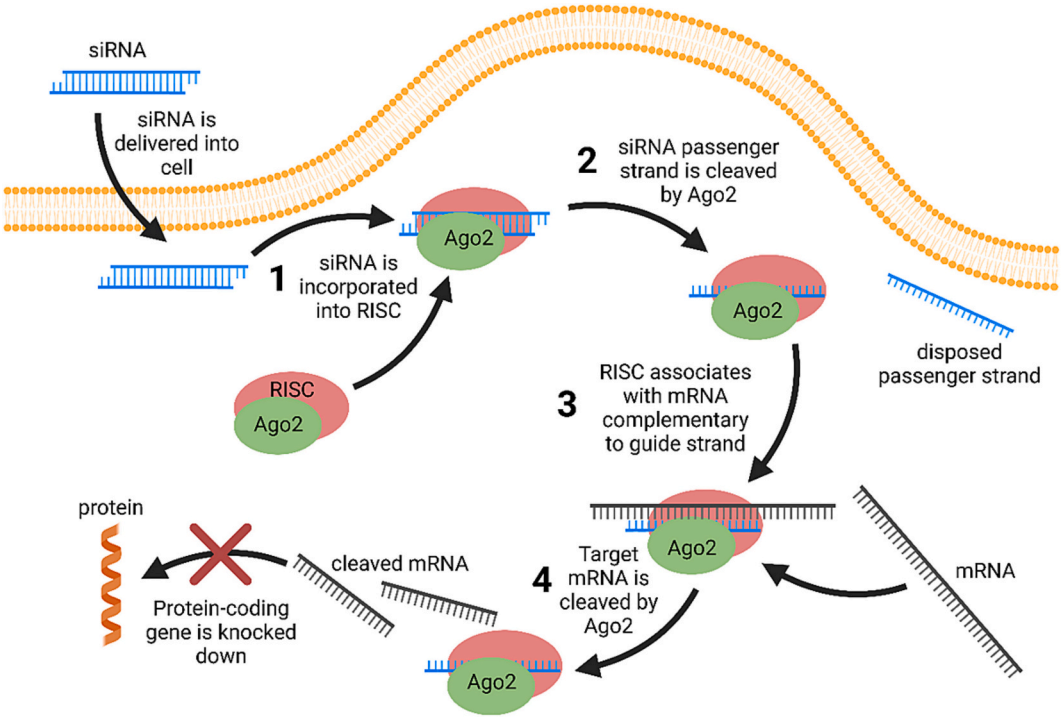

Fig. 1. Mechanism of siRNA-based RNA interference (RNAi): 1) Exogenous siRNA delivered into the cell is incorporated into the RNA-Induced Silencing Complex (RISC), a multiprotein complex. 2) The argonaute-2 protein (Ago2) of RISC cleaves the siRNA passenger siRNA strand. 3) The remaining guide strand complexes with the target mRNA. 4) Ago2 cleaves the mRNA inhibiting its translation into protein. Created with BioRender.com

with the lung [37]. To prevent its degradation in the circulation and achieve sustained plasma levels, RNA is often encapsulated in a delivery system such as lipid nanoparticles (LNPs) [38–40]. However, the intravenous application of commonly used conventional LNP formulations as RNA delivery systems is hampered by an often-unfavourable biodistribution profile skewed towards preferential uptake into the liver [41,42]. Achieving adequate RNA delivery to the lungs following systemic administration can thus be challenging. That said, considerable work has been done in recent years in adjusting mRNA-LNP formulations to successfully achieve targeted delivery to organs beyond the liver including the lungs [43]. For example, using selective organ targeting (SORT) LNPs containing a permanent cationic lipid, exclusive delivery of mRNA to mouse lungs was obtained following IV administration [43]. In other studies, the helper lipid in the LNPs was replaced with a cationic lipid causing increased protein expression in the lungs [44,45]. The use of charged lipids is a promising method for achieving mRNA lung delivery via systemic injection. However, the testing performed thus far was carried out in mice only therefore, it remains to be seen if such organ specific delivery as a function of particle charge can be achieved in larger species including humans [46]. Further, the addition of a fifth lipid component, as is the case for SORT LNPs, also further complicates already complex LNP formulations in terms of potential manufacturing challenges and regulatory hurdles [43].

Given that the development of LNP formulations containing charged lipids for extrahepatic RNA delivery is currently in its infancy, administration via the pulmonary route offers an attractive alternative for delivering RNA to the lungs. Pulmonary administration is advantageous as it provides a direct pathway to the lungs [47], and potentially circumvents challenges associated with systemic delivery including the tendency of traditional LNP formulations to skew towards the liver [43]. Pulmonary delivery can also overcome many of the clinical disadvantages of systemic administration as it is painless, carries less risk of infection and injury and allows for self-administration [48]. Additionally, there are a plethora of lung-related conditions which can be targeted by local administration of RNA such as CF, asthma and infectious diseases for example tuberculosis (TB) [49–51]. Delivery of RNA vaccines via inhalation presents a more patient-acceptable, needle-free alternative to parenteral administration which has been the mainstay for vaccination programmes so far [52]. Inhaled vaccines also initiate a mucosal immune response thus providing mucosal protection which is generally not obtained with injection administration [53,54].

Although numerous strategies exist for the pulmonary administration of RNA therapeutics, their delivery via nebulisation will be the primary focus of this review. In recent years, several preclinical studies have been published investigating nebulised RNA for lung delivery with promising results noted [55–59]. Hence, there is a need to critique these studies to gauge the prospect of future progression into clinical trials. This review will assess the various types of nebulisers, their application and general suitability for RNA delivery. RNA faces a unique set of anatomical, physiological and delivery barriers to successful pulmonary administration. This review will also discuss these different barriers to RNA delivery and the methods by which they can be circumvented. The studies performed thus far in this area indicate that a delivery vector is of high importance for the enhanced stability of nebulised RNA. Lastly, this review will therefore aim to assess the different polymer, lipid and lipid-polymer hybrid-based delivery systems utilised in nebulised RNA formulations, with reference to specific examples present throughout the literature.

2. Pulmonary delivery of RNA

The pulmonary route holds numerous advantages for the local treatment of respiratory diseases for example, asthma [60]. High concentrations of drug can be delivered directly to the site of action resulting in a rapid clinical response with minimal risk of systemic side effects [61]. By avoiding first pass metabolism, an inhaled dose may therefore achieve the same or greater therapeutic effect when given as a fraction of the systemic dose [47]. In the case of systemic diseases, the pulmonary route offers the advantages of being non-invasive, and suitable for delivery of a wide range of molecules including large macromolecules such as proteins [62]. The lungs have a large absorption surface area (approximately 100 m^2), are well vascularised and have low enzymatic activity [63]. In addition, the alveolar epithelium is extremely thin with a thickness of approximately 0.1–0.5 μm which facilitates rapid drug absorption [64]. However, treatment of systemic diseases using therapeutic RNA administration via the pulmonary route has yet to be sufficiently demonstrated as studies published thus far have predominantly focused on targeting local lung conditions [56,65,66]. The contrasting approaches taken depend on whether local or systemic administration is required. For instance, local treatment aims to promote retention of the therapeutic agent in the lungs as opposed to its absorption from the alveolar region which is desirable from a systemic delivery standpoint [67]. Given the progress made using nebulised RNA for local conditions, the central focus of this review will be its use for lung-related conditions rather than treatment of systemic diseases.

2.1. Respiratory system: anatomy and aerosol deposition

The respiratory system can be divided into two anatomical regions; the upper respiratory tract encompassing the nose, nasal cavity, mouth, pharynx and larynx, and the lower respiratory tract which extends from the trachea down to the terminal alveoli [68]. The lower respiratory tract undergoes extensive dichotomous branching, beginning with the bifurcation of the trachea into two bronchi, which branch into bronchioles. These bronchioles further split into alveolar ducts which are connected to alveolar sacs [69]. Each successive branching point corresponds with increasingly smaller diameter, with 23 generations in total as per the Weibel model [70,71]. The lower respiratory tract can also be divided into two zones in accordance with functionality. The first zone known as the conducting zone, spans from the upper airways to the terminal bronchioles and conducts air into the second zone known as the respiratory zone [71]. The respiratory bronchioles, alveolar ducts, and alveolar sacs comprise the respiratory zone, which facilitates gaseous exchange [72].

Aerosols can deposit in the lungs, depending on a variety of physicochemical parameters, via several mechanisms including inertial impaction, sedimentation, and diffusion. The location and deposition mechanism are strongly influenced by the particle's aerodynamic diameter, which is a function of its size and density [73,74]. Particles with an aerodynamic diameter greater than approximately 5 μm will typically deposit by inertial impaction in the oropharyngeal region and are swallowed into the GI tract or else deposit in the large conducting airways [75,76]. Particles with an aerodynamic diameter of approximately 1–5 μm on the other hand, deposit in the bronchioles and alveoli through sedimentation [74]. To ensure deposition in the alveolar regions an aerodynamic diameter of approximately <2 μm is desirable [77]. Particles less than approximately 0.5 μm deposit in the alveoli via the mechanism of diffusion. Nevertheless, most particles <0.5 μm in size do not ultimately reach the alveoli. Instead, they are removed from the respiratory tract during exhalation due to their smaller size [78,79]. The numerous techniques used for aerodynamic analysis of nebulised aerosol droplets are summarised in Table 1.

2.2. Barriers to RNA delivery by the pulmonary route

2.2.1. Intracellular barriers

For therapeutic RNA to exert effects *in vivo*, it must be able to migrate to its intracellular site of action within the cell. In general, there are two ways by which drugs may enter a cell: endocytosis and directly crossing the cell membrane [88]. RNA is a large hydrophilic molecule with a polyanionic charge attributed to the phosphate groups present in its nucleotide structure [89]. Due to its physicochemical properties therefore, RNA is generally unable to cross the phospholipid bilayer of the cell membrane by simple diffusion [90]. Non-viral delivery vectors e.g., in nanoparticle (NP) form are often employed to facilitate RNA entry into

Table 1
Techniques used to analyse the aerodynamic properties of nebulised aerosol droplets.

Technique	Technique Principle	Advantages	Disadvantages	Apparatus	Measurement outputs
Cascade impaction	The aerosol dose is separated as it is drawn through a series of stages. Each stage contains a defined number of nozzles, the diameter of which decrease from one stage to the next. Particles are separated into size fractions which are recovered and quantified by mass, usually with High Performance Liquid Chromatography (HPLC) [80].	Considered a gold standard and is recommended by the USP and Eur Ph. API mass per size fraction can be measured (pharmacopeial requirement). A direct link can be established therefore between aerodynamic size and API mass. Direct measurement of aerodynamic diameter [80].	Labour intensive and time consuming. Size distribution changes as a function of distance travelled and droplet evaporation cannot be studied. Size distribution results can be highly dependent on the flow rate applied [81].	Next Generation Impactor (NGI) (seven stages) Andersen Cascade Impactor (ACI) (eight stages) Multi-Stage Liquid Impinger (MSLI) (four stages) Twin-Stage Impinger (TSI) (two stages)[a]	Mass Median Aerodynamic Diameter (MMAD) The diameter which divides the mass of the aerosol in half i.e., the diameter at which 50% of particles are smaller and 50% are larger [82]. Fine Particle Fraction (FPF) The Fine Particle Dose (FPD) expressed as a percentage of the total emitted dose [83]. Geometric Standard Deviation (GSD) Indicates the variability in particle diameter within an aerosol. In a cumulative distribution curve, GSD is calculated from the ratio of the particle diameter at the 84.1% point to the median diameter [47].
Laser Diffraction	Light from a laser beam is diffracted by particles onto a photodetector array. Large particles scatter light energy at smaller angles compared to smaller particles thus the light diffraction pattern can be used to deduce particle size distribution [84].	Rapid real-time analysis. Non invasive. Wide dynamic size range. Measurement is not dependent on flow rate [81].	API is not quantified. No direct measurement of aerodynamic diameter. Calculated volume distribution curves assume that particles are spherical. Droplet evaporation during measurement results in a bias to finer sizes [84].	Malvern Spraytec® Sympatec® laser particle sizer	Volume Median Diameter (VMD) This diameter divides the aerosol volume in half i.e., 50% of the aerosol volume is contained within droplets smaller than the VMD and 50% is contained within droplets larger than the VMD [85].
Time-of-flight (TOF) Analysis	The time taken for accelerated aerosol particles to transit between two beams of light i.e., Time-of-flight (TOF) is accurately measured. Particle size can then be determined as TOF is a function of aerodynamic size [86].	Real-time analysis. Non invasive. Direct measurement of aerodynamic diameter [84].	API is not quantified. Acceleration in the measurement zone can distort droplet shape making aerodynamic size appear smaller than reality [84].	Aerodynamic Particle Sizer (APS®) Aerosizer®	MMAD FPF GSD
Phase Doppler Particle Analysis (PDPA)	Aerosol droplets pass through two intersecting laser beams and light is scattered onto multiple detectors. The particle diameter is proportional to the phase shift between the Doppler burst signals from different detectors [87].	Wide dynamic size range. Non invasive. Size can be measured at the immediate exit of the device prior to droplet evaporation [84].	API is not quantified. No direct measurement of aerodynamic diameter. Droplet sphericity is assumed [84].	Phase Doppler Particle Analyzer (PDPA)	VMD

[a] TSI is used to measure FPF rather than MMAD and GSD.

the cell, which is frequently accomplished by endocytosis [91]. Non-viral vector-mediated cellular uptake of RNA can be further improved using a targeting ligand which binds to specific receptors on the cell surface inducing receptor-mediated endocytosis [92]. For example, multidrug resistance protein (MRP-1) siRNA has been delivered using folic acid as a targeting ligand for the folate receptor, which is often overexpressed on the surface of cancerous cells [93]. Alternatively, uptake through the cellular membrane can be enhanced using cell-penetrating peptides (CPPs) [94].

Therapeutic RNA faces other numerous challenges in addition to poor cellular uptake. Following endocytosis, exogenous material such as an RNA drug is transported by endocytic vesicles to the endosome. From here, the internalized RNA may be transported to the lysosome and be subject to degradation [95] in the acidic endosomal environment, which is maintained by ATP-dependent proton pumps found on the membrane surface [96]. Numerous studies have shown that endosomal escape of siRNA into the cytosol may be achieved by taking advantage of proton influx into the endosome [97–99]. Non-viral vectors with a high buffering capacity such as polyethyleneimine (PEI) have been employed to release the RNA cargo before it is trafficked to the lysosome. The polymer acts as a "proton sponge", whereby protons accumulate into the endosome resulting in increased osmotic pressure [100]. Eventually, endosome rupture occurs with release of the delivery vector and its RNA cargo into the cytosol [97]. While in the endosome, RNA can also stimulate the innate immune system through activation of toll-like receptors (TLRs) [101]. TLR activation in turn, can activate the protein complex, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) which stimulates the production of several pro-inflammatory cytokines such as interleukin 6 (IL-6) [102]. Numerous studies have shown that certain chemical modifications applied to RNA can help to curtail this initiation of an innate immune response [103].

2.2.2. *Extracellular barriers*

Several extracellular barriers exist which can impede the delivery of

RNA by the pulmonary route, Fig. 2. Mucociliary clearance is a protective mechanism by which inhaled particles are removed from the lungs [104]. Viscoelastic mucus, produced by Goblet cells in the lung epithelium acts as a physical barrier and can entrap inhaled particles [105]. Mucins, large molecular weight glycosylated proteins, present in mucus can bind drugs and their delivery systems. Mucin-binding can occur through electrostatic, hydrophobic and H-bonding interactions and prevent penetration of the mucus [106,107]. Ciliated epithelial cells also beat in a coordinated manner to sweep the mucus containing entrapped materials upwards towards the nasopharynx so that it can be swallowed into the GI tract [108]. Clearance of this mucus by the ciliated cells is a rapid process [109]. Consequently, the overall time available to inhaled RNA for absorption is restricted [110].

Airway mucus has been shown to trap and promote the removal of RNA delivery vectors to great effect, therefore presenting a formidable barrier to inhaled RNA. Moreover, in lung disease the mucus barrier is often more formidable compared to the healthy state. For example, in CF and chronic obstructive pulmonary disease (COPD) mucus viscosity and elasticity is greater making RNA penetration harder [111]. Indeed, mucus accumulation may obstruct the airways causing impaired lung function [112]. Airway dehydration can also occur leading to concentration of the mucus gel [113]. Furthermore, airway inflammation, often seen in lung diseases can cause mucin hypersecretion leading to a greater number of mucins available to potentially bind RNA delivery vectors and impede their delivery [114]. One strategy to overcome challenges posed by mucus is to employ a delivery vector for RNA such as chitosan NPs, which have mucoadhesive properties [115,116]. Chitosan's mucoadhesive nature can be attributed to its cationic charge which facilitates interaction with anionic mucins. The contact time between chitosan NPs and the mucosal surface is thus prolonged and enhances the absorption of its drug cargo [117]. Furthermore, chitosan can disrupt intercellular tight junctions present in the lung epithelium which potentially allows for increased uptake [118].

Another delivery strategy proven effective in traversing the lung mucus barrier is to densely graft poly(ethylene glycol) (PEG) onto the surface of NPs. Indeed, PEGylation has been widely investigated to overcome the mucus barrier for several routes of delivery including vaginal [119], oral [120], ocular [121] and nasal [122]. Coating with PEG, a hydrophilic and neutrally charged polymer has been shown to effectively enable NPs, which would have otherwise been immobilised, to diffuse rapidly through lung mucus [111], and its success is well-documented in the literature over the past decade or so [123,124]. For example, a study by Hanes' group demonstrated that PEGylated polystyrene NPs covalently modified with a dense PEG coating could diffuse through samples of fresh human respiratory mucus at a rate of up to 35-fold greater than uncoated control NPs, as shown by multiple-particle tracking (MPT) analysis. This study also highlighted the important role played by particle size in penetrating mucus as the PEGylated particles $\leq$200 nm in size displayed rapid penetration in contrast to those $\geq$500 nm, which were sterically hindered by the mucus mesh [125]. This observation regarding particle size influence on mucus penetration is supported by another study by Hanes' group carried out with samples of CF sputum. PEGylated polystyrene NPs 500 nm in size were immobilised whereas NPs $\leq$ 200 nm in size could diffuse in the sputum [126]. These studies suggest that particle size is another important consideration when tackling the mucus barrier with NPs 200 nm or less being most appropriate for delivering therapeutics such as RNA into lung cells. In another study by the same group, PEGylated mucus-penetrating NPs (MPPs) based on the polymer poly (β-amino ester) (PBAE-MPPs) were used to successfully deliver plasmid DNA encoding green fluorescent protein (GFP) in BALB/c mice [127]. A microsprayer apparatus was used to intratracheally administer 50 μL of NPs at a concentration of 0.5 mg/mL plasmid DNA. Following treatment, PBAE-MPPs achieved uniform GFP transgene expression in mouse lungs in contrast to several other DNA NP formulations for example, non-PEGylated PBAE NPs [127].

The advantageous nature of PEG's mucopenetrating properties has also been utilised in the field of RNA lung delivery for the treatment of lung cancer [128]. NPs comprised of modified PEG-co-poly(lactic-co-glycolic acid) (PLGA) block copolymers and oligo (β-aminoesters)

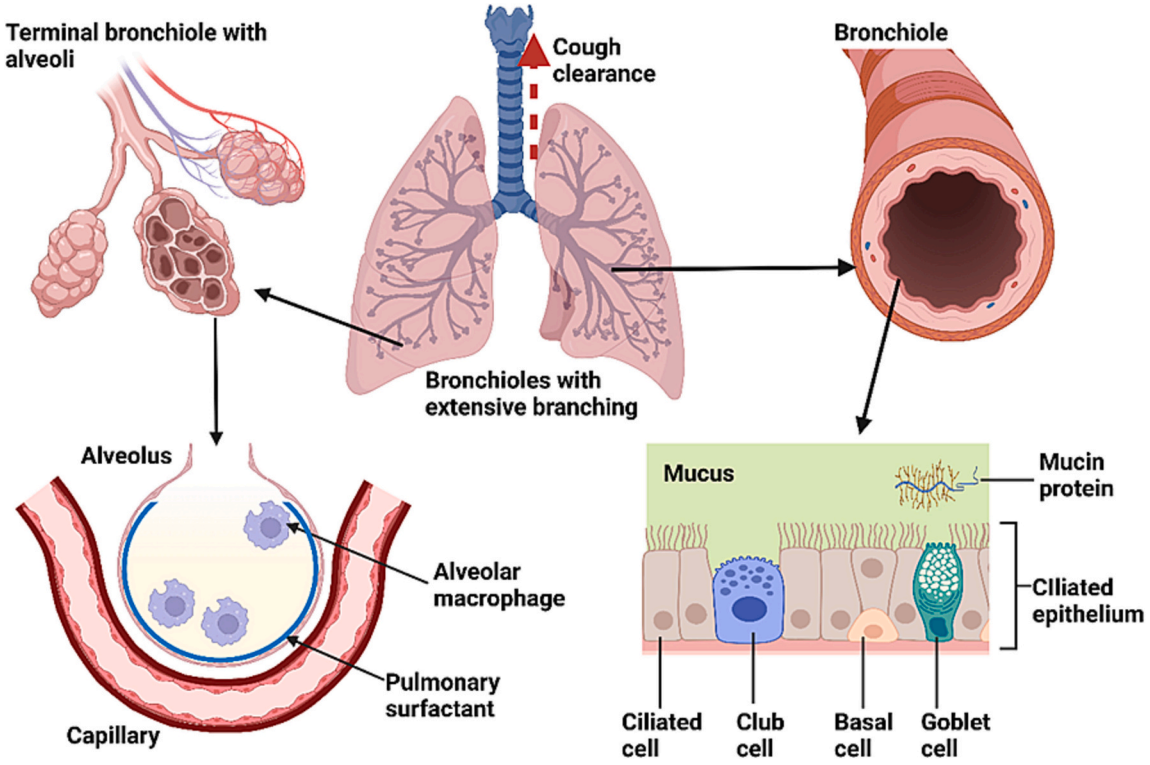

Fig. 2. Schematic diagrams of the barriers associated with pulmonary drug delivery; cough clearance, pulmonary surfactant, alveolar macrophages, mucociliary clearance and mucin proteins. Created with BioRender.com

(OBAE) containing luciferase targeting siRNA (antiluc-siRNA) were formulated and administered intratracheally to mice previously injected with bioluminescent A549-luc cancerous cells. These PEG-coated anti-luc-siRNA NPs subsequently yielded a strong decrease in bioluminescence 72 h post treatment compared to the scrambled siRNA control NPs. In another study, lipoplexes containing siRNA were modified with PEG as well as with hyaluronic acid (HA) to target the CD44 receptors expressed by tumour cells [129]. These fluorescently labelled lipoplexes were administered intratracheally in mice and exhibited uniform distribution throughout the lung. The modified lipoplexes also exhibited diffusion in a sample of fresh human mucus, contrasting with an unmodified control which was almost completely immobilised in the mucus sample. Interestingly, it was found that non-PEGylated lipoplexes modified with HA only also had enhanced mucus diffusion suggesting that HA itself has mucopenetrating properties [129]. Further studies are warranted however, to optimise the role of HA as a potential enhancer of lung mucus penetration for RNA NPs.

Pulmonary surfactant, a mixture of phospholipids and proteins, is secreted by Type II alveolar cells to lower alveolar surface tension [130]. While pulmonary surfactant is essential for pulmonary function, it may also represent a challenge for delivery to the alveolar region [107]. Cationic lipid carriers can interact with pulmonary surfactant resulting in a loss of transfection capability. Regarding cationic polymers, however, the presence of pulmonary surfactant has been shown not to impact their stability [131]. In a previous study, the low molecular weight, cationic PEI was used to form polyplexes with radio-labelled siRNA. Intratracheal delivery in mice led to a wide distribution of the polyplexes within the lung including the deep alveolar regions thus demonstrating the potential of PEI as an siRNA carrier in preventing lung clearance [132]. Other studies found pulmonary surfactant to be beneficial in facilitating cellular uptake of RNA in the respiratory tract [133] and to improve the transfection efficiency of polymer-based delivery vectors of RNA [134]. For example, Curosurf®, a commercial pulmonary surfactant has been used to coat cationic dextran-based hydrogel NPs containing siRNA targeting enhanced green fluorescent protein (eGFP). The Curosurf®-coated NPs, which had also been lyophilised and subsequently reconstituted, were nebulised using an Aeroneb® Pro vibrating mesh nebuliser and administered to eGFP-expressing H1299 lung epithelial cells. Subsequent flow cytometry analysis demonstrated successful siRNA uptake and eGFP down-regulation in the cells [135].

Alveolar macrophages pose another barrier to effective pulmonary delivery of RNA. These cells operate as part of the immune system to protect the body by engulfing and degrading inhaled foreign macromolecules through phagocytosis [136]. This can result in premature removal and loss of biological response. Several measures focusing on controlling particle size and shape have been investigated to evade macrophages in the alveoli. Large porous particles with a physical diameter > 10 μm that are too big for phagocytosis but have a smaller aerodynamic diameter due to their lower density, represent one option to evade macrophages in the deep alveolar regions [137,138]. Alternatively, NPs < 200 nm in size renders them too small for macrophage phagocytosis [139–141].

The lungs consist of different cell types each being potential targets for RNA transfection depending on the lung-related condition of interest. Consequently, the target cells must be transfected with enough specificity and in sufficiently high numbers to enable a therapeutic effect, which can be challenging. The ciliated cells of the pseudostratified columnar epithelia have been previously targeted for the delivery of nebulised mRNA encoding DNAI1. This gene is frequently mutated in primary ciliary dyskinesia (PCD), a disease characterised by impaired mucociliary clearance due to ciliary dysfunction [142]. Goblet cells have also been successfully transfected with both siRNA and shRNA targeting the vesicle-associated membrane protein 8 (VAMP8), which regulates mucin granule exocytosis thus bringing about an overall reduction in mucin secretion [143]. In addition to being a potential delivery barrier, alveolar macrophages can also be a target of pulmonary administered RNA [144,145]. In one study siRNA targeting methyl-CpG-binding protein 2 (MECP2), a protein abnormally expressed in idiopathic pulmonary fibrosis, could specifically target macrophages in mouse alveoli following intratracheal administration and was shown to reverse the established disease [145].

RNA administered via the pulmonary route has been explored for the treatment of a wide range of lung related conditions including lung cancer [146], CF [49], COPD [134], asthma [50] and viral infections [57]. During treatment, it is crucial to note that the physiology of the lung can oftentimes be altered by disease and may therefore have an impact on the effectiveness of RNA treatment. For example, in both COPD and asthma, obstruction in the airways can cause a decline in lung function meaning a patient's ability to inspire an RNA dose may be reduced [147]. As previously mentioned, these conditions are also often associated with excessive mucus secretion which can further complicate effective dose administration [148]. Testing the therapeutic efficacy of RNA delivery in healthy subjects may therefore not fully recapitulate the response in a diseased state. Furthermore, most *in vivo* studies performed thus far on RNA studies have only been carried out in mouse models [59,129,146,149]. Mouse lungs differ considerably in anatomy and physiology compared to human lungs including a greatly reduced size, increased respiratory rate and reduced vascular penetration of the intraparenchymal airways, which can limit the translation of findings to human patients [150–152].

2.3. Routes of pulmonary RNA delivery

Inhalation is by far the most efficient and patient acceptable means for local delivery of therapeutics to the lung [153]. However, to date, no inhalation products have been licensed for RNA delivery to the lung. Other approaches to target the lungs include the intratracheal and intranasal routes which have been commonly employed in pre-clinical animal studies. However, the invasive nature and inefficiency of these administration routes limit their clinical translation into human patients [154]. The intratracheal route has been widely used in preclinical animal studies for the delivery of RNA to the lungs as it enables direct administration of the RNA formulation into the animal's lungs thereby minimising loss in the oropharyngeal and upper airway regions [115,155]. Accuracy of dosing can therefore be achieved [156].

Intratracheal administration can involve surgical incision into the trachea followed by insertion of tubing providing a pathway directly to the trachea. Alternatively, the animal is intubated endotracheally whereby the trachea is accessed through the mouth [65]. Dry powder formulations can be given by loading in a disposable syringe tip and dispersing the formulation with compressed air or in the case of liquid formulations, a microsprayer can be used [157,158]. Previously the intratracheal route was used to deliver siRNA complexed with thiolated PEI chemisorbed onto gold NPs in BALB/c mice. A microsprayer apparatus was used to administer the NPs intratracheally and 2D epifluorescence imaging confirmed deposition in the lungs [159]. Intratracheal administration is a variable technique as the site of deposition in the lung can differ significantly from one intratracheal method to another. Since the formulation is administered directly into the trachea, the influence of aerodynamic diameter is also difficult to assess. Consequently, given the anatomical differences previously discussed, it is difficult to extrapolate results from intratracheal mice studies to humans [150,160]. As outlined, the intratracheal route is an invasive technique which causes considerable distress and discomfort which thus hinders its translation from animal models to humans. Endotracheally intubation is employed in human patients, but it is normally restricted to emergency situations in a healthcare setting and undertaken by skilled personnel [161].

The intranasal route has also been utilised in preclinical studies for RNA lung deposition in mice [49,162,163]. For intranasal administration, the mouse is under general anaesthesia and positioned vertically to

allow for drug administration to the nostril using a micropipette. Bohr et al. used the intranasal route to deliver tumour necrosis factor alpha (TNFα) siRNA complexed to phosphorus-based dendrimers in a lung inflammation mouse model [162]. The siRNA mediated inhibition of TNFα led to a strong anti-inflammatory effect demonstrating potential in treating lung inflammation [162]. Although, the intranasal route is more straightforward and less invasive compared to intratracheal administration, it is not as effective in achieving deep lung deposition, since most drug deposits into the upper airways [160]. The different physiological traits of the lungs of mice and that of humans are also an important consideration as mice, like all rodents, are obligatory nose breathers [160]. Intranasal administration can also result in the dose being swallowed into the GI tract. Furthermore, intranasal administration can result in an unevenly distributed dose within the lungs as shown in a previous study utilising a mucosal atomization device (MAD) for intranasal drug delivery in pigs [164].

3. Nebuliser devices

In the past decade, several papers have emerged detailing the use of nebulisers to deliver RNA, some of which have examined *in vivo* delivery [57,59,149]. Nebuliser devices aerosolize an aqueous-based formulation to produce respirable droplets that are inspired under normal tidal breathing [165,166]. Most nebulised aqueous formulation are in the form of a solution however aqueous suspensions can also be nebulised. The nature of the aerosolization mechanism within the nebuliser varies depending on the type of nebuliser device [167]. Significant advantages of nebuliser devices include delivery during a patient's normal tidal breathing, thereby overcoming actuation and coordination difficulties (compared to metered-dose inhalers (MDIs), and the modality is suitable for patients with compromised inspiratory effort which can be a limitation with dry powder inhaler (DPI) devices. Nebulisers can also deliver much larger drug doses compared to DPIs and MDIs [168]. There are three main types of nebuliser devices; jet nebulisers, vibrating mesh nebulisers (VMNs) and ultrasonic nebulisers, Fig. 3 [169]. Of the studies published involving the nebulisation of RNA, the majority have utilised vibrating mesh technology [115,135,139]. Jet nebulisers have also been used for RNA delivery, albeit to a lesser extent [116,170,171]. A summary of relevant studies is provided in Table 2. A recently published study details the effective use of a novel nebulisation method termed the nanotech membrane (NM) method for the delivery of SARS-CoV-2 mRNA vaccines [172]. This new design is characterised by a lower energy input compared to the more established nebuliser designs. Briefly, fluid is pushed through the porous NM at a low pressure creating equally sized jets, which, then break up into droplets thus forming an aerosol. Two COVID-19 mRNA vaccines, BNT162b and mRNA-1273, both encapsulated within LNPs were nebulised using the NM method as well as with commercial VMN and colliding jet nebulisers. There was no noticeable change in LNP sizes post NM nebulisation, in contrast to the other nebulisers which caused aggregation of LNPs. Nebulised samples from each nebuliser type were administered to HEK293T cells and expression levels of SARS-CoV-2 spike protein were measured. It was found that both vaccines produced significantly higher expression levels of spike protein when nebulised with the NM compared to other nebuliser designs [172]. This study is significant as it demonstrates that low energy nebulisation may be preferable for better maintaining the integrity and efficacy of mRNA-LNPs. However, it must also be noted that biological activity was not measured in a lung-relevant cell line nor in any *in vivo* subjects. In addition, the vaccines including their LNPs formulations are indicated for intramuscular injection and not for inhalation. Future experimentation is therefore warranted.

MRT5005 is a candidate mRNA treatment for CF (Translate Bio Inc., Lexington, MA, USA) and is the first mRNA therapeutic indicated for nebulised administration to undergo clinical trials. The treatment entered Phase I/II clinical trials in May 2018 and has been administered to adult participants with CF across a range of doses from 4 mg (low dose) to the highest dose of 24 mg. (ClinicalTrials.gov Identifier: NCT03375047) [187]. To the best of our knowledge, it is the only nebulised RNA candidate which has been recently investigated in a clinical setting. MRT5005 contains mRNA encoding a fully functional CFTR protein encapsulated within a LNP formulation intended for delivery to lung epithelial cells. Initial results were promising as repeated

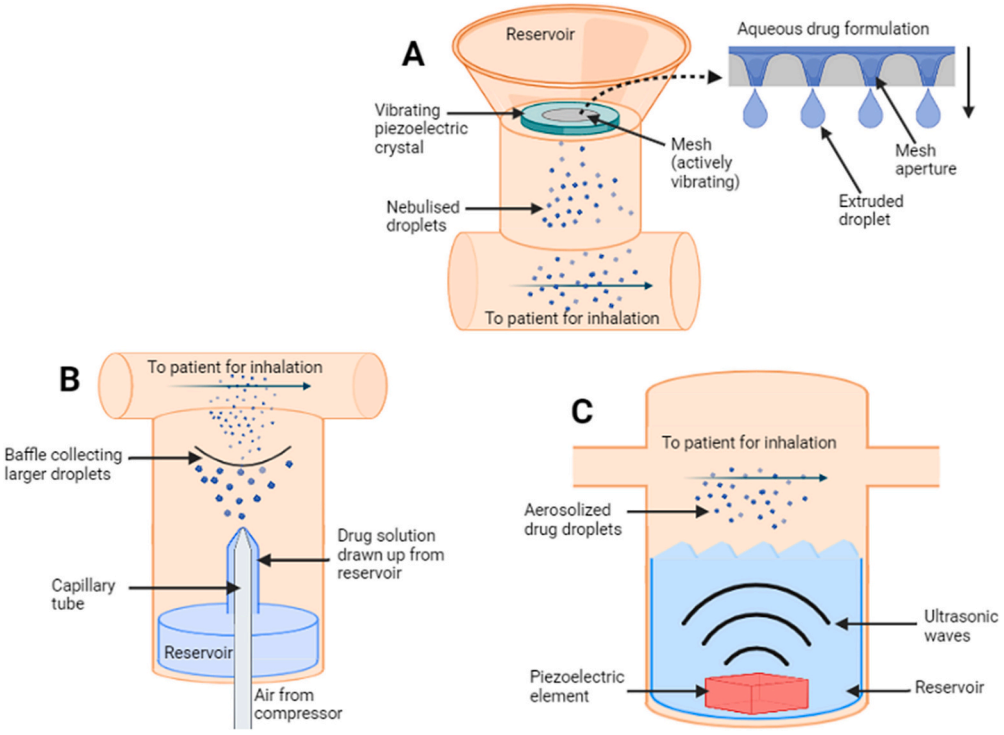

Fig. 3. Schematic representation of main nebuliser designs: (A) vibrating mesh nebuliser (actively vibrating), (B) jet nebuliser and (C) ultrasonic nebuliser. Created with BioRender.com

Table 2
Summary of studies performed involving nebulisation of RNA using *in vitro/in vivo* models related to the lung. Type of nebuliser, delivery vector and RNA target included.

Nebuliser Type	Nebuliser Model	RNA	Target/encoded gene	*In vitro/in vivo* model	Delivery vector	Reference
Vibrating Mesh	Aerogen® Pro	siRNA	CXCL-1 IL-8	Calu-3 Sprague Dawley rats	PEI-LPEG polyplexes	[65]
		siRNA	Luciferase	Calu-3	SC12CDClickpropylamine (novel cyclodextrin)	[173]
		siRNA	GFP	HEK293, A549, 16HBE C57BL/6-Tg mice	Salbutamol-coupled guanidinylated chitosan NPs	[115][a]
		siRNA	ENaC	Triple cell co-culture: 16HBEs, MDM & MDDC	DPPC/PLGA based lipid polymer NPs	[56][a]
		siRNA	GFP	H1299	Cationic dextran nanogels	[135][a]
	Aeroneb® Lab	mRNA	Luciferase	A549, HeLa BALB/c mice	LNPs	[174]
		siRNA	Luciferase	H1299	DEAPA-PVA – PLGA NPs	[139]
		siRNA	Luciferase	H1299	OEI-HD polyplexes	[175]
		mRNA	GFP Luciferase Cre recombinase CFTR	HeLa, A549, 16HBE, CFBE41o- BALB/c mice, Ai9 mice, CFKO mice	LNPs	[55]
		siRNA miRNA	cel-miR-39 MYD88	BALB/c mice, C57BL/6 mice	Small extracellular vesicles	[176]
	Aerogen® Solo	mRNA	Luciferase eGFP	HEK-293, NuLi-1 BALB/c mice	LNPs	[149]
		miRNA	IL-8	NuLi-1	PLGA/DOTAP based lipid polymer NPs	[58]
		mRNA	TdTomato	CFBE BALB/c mice	LNPs	[177]
		mRNA	GFP IκBα-SR SOD3	BEAS-2B, A549, SAEC Sprague Dawley rats	Lipofectamine™ 3000 *in vivo*-jetPEI®	[178]
		LNA	miR-101-3p miR-145-5p miR-223-3p miR-494-3p miR-509-3p	16HBE, NuLi-1, CFBE41o-, CuFi-1	PLGA NPs	[179]
		mRNA	DNAI1	HEK293 CD1 mice, CreER KO mice	LNPs Lipofectamine™ 2000	[142]
		mRNA	Luciferase Cas13a dCas9-VPR IgG	BALB/c mice DBA/2 mice LVG Golden Syrian Hamsters Fitch ferrets Rhesus macaques Holstein calf	PBAE NPs	[180]
	Aeroneb®[b]	mRNA	Luciferase	A549 C57BL/6 mice	hPBAE polyplexes	[181]
		mRNA	Luciferase aFI6	BALB/c mice	LNPs	[59]
		crRNA	Cas13a	A549	PBAE NPs	[57]
		mRNA	Luciferase	BALB/c mice LVG Golden Syrian Hamsters		
		mRNA	Luciferase Cre recombinase	A549, primary large airway cells, primary small airway cells C57BL/6 J mice, Ai14 mice, C57BL/6 N Scnn1b-Tg mice	LNPs hPBAE polymer NPs	[182]
		siRNA	IL-11	A549, mouse lung fibroblasts (MLFs) C57BL/6 mice	PPGC NPs based on cationic lipid-like molecule G0-C14 and PLGA-PEG copolymer	[183]
		mRNA	Luciferase			
Jet	Omron NE-C801	siRNA	Survivin	A549	PBAE/GOCMCS NPs	[171]
		siRNA	Survivin	A549	GOCMCS/N-2-HACC NPs	[184]
	One-jet Collison nebuliser	siRNA ASO	MRP1	Nude nu/nu mice	DOTAP liposomes	[146]
	PARI Boy®	mRNA	Luciferase GFP	16HBE	Lipofectamine™ 2000 DMRIE C PEI polyplexes	[170]
		mRNA	A1AT	16HBE	Lipofectamine™ 2000	[66]

(*continued on next page*)

Table 2 (*continued*)

Nebuliser Type	Nebuliser Model	RNA	Target/encoded gene	*In vitro/in vivo* model	Delivery vector	Reference
	PARI LC Sprint®	siRNA	N/A (non-targeting)	H-292	Crosslinked chitosan NPs	[116]
	AeroEclipse® II BAN™	siRNA	α-ENaC	16HBE, 1HAE, A549, H441, NHBE C57BL/6 mice	Cationic Lipid-Peptide-RNA NPs	[185]
Surface Acoustic Wave nebulisation	Respite™	siRNA	Luciferase	A549	PEI INTERFERin	[186]

A1AT: alpha-1-antitrypsin, BAN: breath actuated nebuliser, CFTR: cystic fibrosis transmembrane conductance regulator, DEAPA: Diethyaminopropylamine, DMRIE C: 1,2-dimyristyloxy-propyl-3-dimethyl-hydroxy ethyl ammonium bromide and cholesterol, DNAI1: dynein axonemal intermediate chain 1, DOTAP: 1,2-dioleoyl-3-trimethylammonium-propane, DPPC: dipalmitoylphosphatidylcholine, ENaC: sodium transepithelial channel, GFP: green fluorescent protein, GOCMCS: guanidinylated O-carboxymethylchitosan, hPBAE: hyperbranched poly (β-amino ester), IgG: Immunoglobulin G, IκBα-SR: nuclear factor-κB(NF-κB) inhibitor super-repressor, IL: interleukin, LNA: locked nucleic acid, LPEG: linear poly(ethylene glycol), MDM: monocyte-derived macrophages, MDDC: monocyte-derived dendritic cells, MRP1: multidrug resistance-associated protein 1, N2-HACC: N-2-hydroxypropyltimehyl ammonium chloride chitosan, NPs: nanoparticles, OEI-HD: Hexanediol diacrylate cross-linked oligoethylenimine, PEG: poly(ethylene glycol), PEI: polyethyleneimine, PLGA: poly(lactic-co-glycolic acid), PBAE: Poly (β-amino ester), PVA: polyvinyl alcohol, SOD3: superoxide dismutase 3.

[a] This reference refers to the Aerogen® Pro as Aeroneb® Pro as per its former brand name.

[b] Exact Aeroneb® model was unspecified in these studies.

doses were well-tolerated and appeared to indicate an increase in the forced expiratory volume in one second (FEV1) in subjects [188,189]. However, follow-up studies failed to show any meaningful trends in FEV1 across any dose group indicating a lack of therapeutic efficacy. The investigators reported that sufficient expression was likely not obtained in the differentiated bronchial epithelium following administration hence a lack of consistent improvements in FEV1 [187].

3.1. Vibrating mesh nebulisation

Vibrating mesh nebulisers (VMNs) have come to market more recently compared to the more established jet and ultrasonic nebuliser technology. VMNs contain a perforated mesh plate which vibrates resulting in aerosol generation (Fig. 4) [190]. VMNs may be categorised as either active or passive devices. In active VMNs, for example the Aerogen® Solo device and the PARI eFlow® nebuliser, the mesh is typically vibrated directly by a piezoelectric crystal resulting in aerosolization of the aqueous fluid containing drug by extrusion through the mesh apertures [191]. In passive VMNs, for example, the Omron MicroAir®, vibrations from the piezoelectric crystal are transmitted to a transducer horn adjacent to the drug formulation in the nebuliser reservoir and resulting waves pass through the formulation [192]. "Passive" vibrations of the perforated mesh are then induced, and aerosol is generated [193]. In general, passive VMNs are no longer widely available, with some exceptions such as some Omron® nebulisers. Their lack of availability stems from some of their technical limitations, such as incompatibility with suspension-based formulations, which consequently limits their usefulness. Active VMN have been shown to deliver significantly more drug to the human lung than jet nebulisers, and thus allow for greater flexibility in formulation design and dosing strategies [194]. Active VMNs are more efficient than jet and ultrasonic nebulisers in that they can produce fine, respirable droplets, have a lower residual drug volume, and have been shown to deliver more drug to the patient lung [194,195]. The efficient nature of active VMNs is a particularly important attribute when nebulising RNA given its high cost. VMNs are suitable for delivery of heat-labile cargoes such as RNA because they do not produce significant temperature changes during nebulisation [196]. VMNs are also faster, more discreet, and user-friendly compared to older generation nebulisers [195]. Additionally, in combination with filters, VMN have been shown to not emit any fugitive medical aerosols or potentially infectious patient-derived bioaerosols, thus mitigating the risk of bystander exposure. Consequently, VMN have been identified as the preferred nebuliser type by several guidance and consensus documents globally [197–199].

The Aeroneb® Lab VMN was used in a recent study to nebulise several luciferase mRNA containing LNP formulations, each containing a different naturally occurring lipid in place of the common structural lipid, distearoylphosphatidylcholine (DSPC) [174]. The hydrodynamic size and PDI of two LNP formulations of particular interest, one containing 1,2-dipalmitoyl-sn-glycero-3-O-4'-(*N*,*N*,*N*-trimethyl)-homoserine (DGTS) and the second containing the standard, DSPC were found to increase after nebulisation. This demonstrated that the changes in physical properties of the LNPs post aerosolization did not depend on the structural lipid type, however, the transfection efficiency of the DPSC LNPs was significantly greater than the DGTS LNPs when administered to A549 lung cells post nebulisation. The results demonstrate the effectiveness of the Aeroneb® Lab for aerosolising mRNA LNPs. However, the choice of LNP formulation is an important consideration,

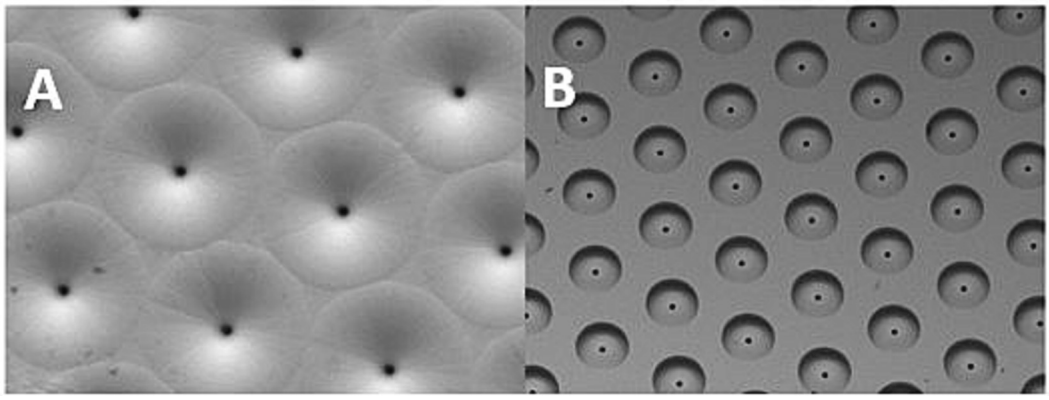

Fig. 4. Perforated meshes (A) (reservoir side) of the Aerogen® Solo and (B) Omron MicroAir U100 vibrating mesh nebulisers. Images captured using a scanning electron microscope at Aerogen Ltd., Galway, Ireland.

particularly when striving for efficient transfection [174].

The Aeroneb® VMN was also used to nebulise PBAE polymer-based particles containing mRNA encoding the CRISPR-associated nuclease Cas13a, as a potential treatment for influenza virus and SARS-CoV-2 infection [57]. Firstly, glycosylphosphatidylinositol (GP–) - anchored nanoluciferase (aNLuc) mRNA was used to optimise the final mRNA concentration in the formulation and administered, using a nose-only exposure system fitted with 3D printed nose cones, to mice previously infected with influenza A/WSN/33 at a dose of 100 μg per animal (Fig. 5). The Cas13a mRNA significantly reduced influenza viral RNA by 89.1% compared to a non-targeting control and also reduced both the rate of viral replication and symptom severity in hamsters infected with SARS-CoV-2. These results are highly encouraging from a VMN perspective as they indicate effective delivery of RNA *in vivo* to the lungs without any adverse impact on its stability or efficacy [57].

In another study involving VMN-mediated RNA delivery, an Aeroneb® Pro was used to nebulise guanidinylated chitosan (GCS) NPs carrying GFP siRNA [115]. The GCS NPs were also coupled to salbutamol acting as a targeting ligand to increase uptake in cells expressing β2-adrenergic receptors in the airways and reduce potential unwanted side effects. The salbutamol-coupled NPs were nebulised and then administered endotracheally to eGFP transgenic mice at a dose of 5 μg siRNA once daily over three consecutive days. Significant downregulation of GFP expression in the murine bronchial epithelial cells was observed and was also significantly greater in the salbutamol-coupled NPs compared to those without salbutamol. This study indicated the important role that may be played by targeting ligands when nebulising RNA for pulmonary delivery [115]. In the decade or so since this study was published, however, there has a been a lack of follow up studies utilising targeting ligands for nebulised RNA.

The Aerogen® Solo VMN also features in numerous studies for example, for the delivery of mRNA into a rat model of *E. coli* pneumonia as an investigated treatment for acute respiratory distress syndrome (ARDS) [178]. mRNA molecules encoding two proteins known for their role in reducing ARDS severity, nuclear factor-κB(NF-κB) inhibitor super-repressor (IκBα-SR) and extracellular superoxide dismutase 3 (SOD3) were complexed with the transfection reagent *in vivo*-jetPEI® and nebulised into ventilated adult Sprague Dawley rats inoculated with *E coli*. In the IκBα-SR mRNA treated group, there was a significant drop in arterial carbon dioxide (pCO2) concentration. In SOD3 treated rats, both alveolar-arterial oxygen gradient (AaDO2) and bronchoalveolar lavage (BAL) bacteria load dropped from 364 to 263 mmHg and from 5000 to 2000 CFU/mL, respectively. Both mRNAs also led to a reduction in BAL levels of total white cell infiltration and in the pro-inflammatory mediators, cytokine-induced neutrophil chemoattractant-1 (CINC-1) and IL-6. The research signified the potential of nebulised RNA as an anti-inflammatory agent, which is particularly pertinent given that ARDS is a serious complication of COVID-19 infection [178]. It is likely further studies targeting genes with nebulised RNA to reduce airway inflammation will be published in the years ahead. The studies performed thus far have shown the potential of VMNs as a suitable device to aerosolize RNA containing formulations and produce a size range appropriate for deposition into the lower respiratory tract. This has been observed across a range of different RNA vectors including lipid-based, polymer-based and hybrid carriers. Critical product attributes and RNA pharmacodynamic properties can also be maintained throughout the nebulisation process.

3.2. Jet nebulisation

Jet nebulisers utilise the energy from compressed gas as the driving force for droplet aerosolization. This gas may be air or oxygen, which is usually supplied from a tank or portable compressor [200]. The compressed gas is pumped through a narrow nozzle generating a region of low pressure at its orifice, which causes the bulk drug solution to be drawn up from the reservoir by capillary action in response to this low pressure. The drug solution is then sheared by the high velocity airstream into aerosol droplets which range in particle size. Larger droplets are removed by a baffle through inertial impaction and consequently only droplets of an appropriately small size can remain in the airstream and leave the nebuliser cup, Fig. 3B. Larger droplets trapped by baffles will ultimately re-enter circulation for further nebulisation [201]. Aerosol performance varies across jet nebuliser types [202]. More efficient jet-nebuliser designs include breath-enhanced and breath-actuated devices [203]. The PARI LC Star®, for example, a breath-enhanced nebuliser contains an inspiratory valve allowing inspired air to entrain into the nebulising chamber resulting in more droplets being carried into the patient's airstream. It also contains an expiratory valve on the mouthpiece preventing entry of exhaled air [204]. Breath-actuated nebulisers on the other hand, produce aerosol only during inhalation. For example, in the AeroEclipse® an opening valve is triggered only in response to a patient's inspiration and aerosol is thus generated [205].

Breath-enhanced and breath-actuated devices are advantageous as less aerosol is lost to the external environment, which reduces expensive drug loss, drug wastage and environmental risks. In addition, they are quieter and user-friendly [195]. Jet nebulisers do, however, produce less aerosol in the fine particle range compared to the newer VMN meaning loss of drug in the upper airways. Also, as larger droplets are recirculated within the jet nebuliser, the drug solution is subjected to the shear forces of the nebulisation cycle on a repetitive basis [206]. This recirculation feature of jet nebulisers is potentially disadvantageous in the case of RNA formulations as these repeated shear forces could damage the fragile RNA molecule. Encapsulation of RNA within a protective

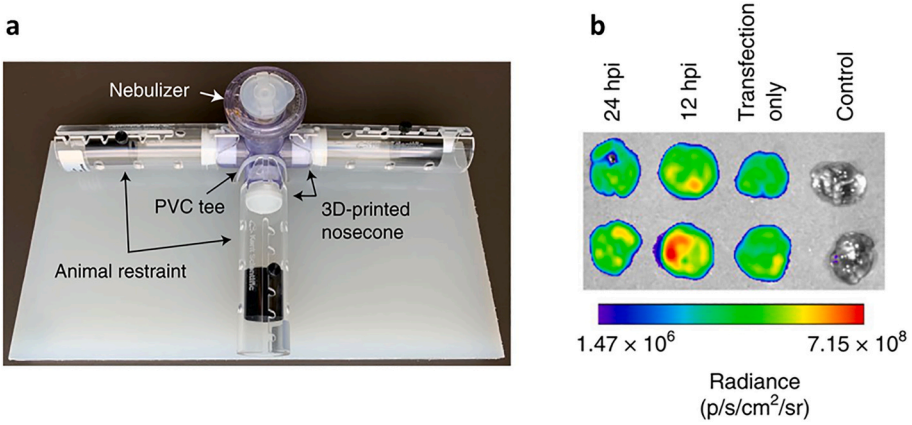

Fig. 5. Nebulisation of poly (β-amino ester) (PBAE) polymer-based particles containing mRNA encoding for Cas13a including (a) Nose–cone active vibrating mesh nebuliser (Aeroneb®) apparatus. (b) Luminescence of mice lungs post treatment with 100 μg of aNLuc mRNA at 12 and 24 hpi. hpi = hours post infection (with influenza A/WSN/33). Adapted from [57] with permission from Springer Nature.

delivery vector such as a LNP could therefore prove essential for the viable use of jet nebulisers for pulmonary delivery of RNA. This presumption is supported by a study in which survivin siRNA containing NPs consisting of the polymer PBAE and guanidinylated O-carboxymethylchitosan (PBAE/GOCMCS) and a sample of naked siRNA were both nebulised using an Omron NE-C801 jet nebuliser [171]. Gel electrophoresis analysis showed that the integrity of the siRNA within the NPs was preserved while the naked siRNA's integrity was decreased due to the shear forces encountered during nebulisation. Additionally, the nebulised PBAE/GOCMCS formulation had a FPF (<6.4 μm) of approximately 58% indicating a large proportion of the aerosolized dose was within the respirable range [171]. This study is noteworthy as it demonstrates the damaging impact of jet nebulisation's repeated shear forces on naked RNA and the necessity for a delivery vector such as PBAE/GOCMCS NPs to preserve RNA integrity.

The choice of delivery vector is an important consideration to overcome the shearing forces generated during RNA nebulisation [170]. Using the PARI Boy® jet nebuliser, one study nebulised the lipid-based commercial transfection reagents, Lipofectamine™ 2000 and DMRIE C (a 1:1 mixture of DMRIE(1,2-dimyristyloxy-propyl-3-dimethyl-hydroxy ethyl ammonium bromide) and cholesterol) as well as PEI-based polymer complexes. A PARI Boy® jet nebuliser was used to aerosolize these vectors complexed with GFP mRNA for transfection in human bronchial epithelial cells (16HBEs). In the case of Lipofectamine™, the number of GFP transfected cells dropped from 50.5% ±3% pre nebulisation to 38% ±4% post nebulisation indicating a reduction in transfection efficiency. In contrast, the number of cells transfected by branched PEI did not change after nebulisation. It must be noted however, that the transfection efficiency of branched PEI was far less with only 2.8% ± 1% cells transfected. The authors were able to increase the resistance of Lipofectamine™ formulations to the effects of nebulisation shear forces by increasing the amount of Lipofectamine™ to prepare the mRNA lipoplexes. Despite this, increasing the amount of lipids was not successful for DMRIE C as a reduction in transfection efficiency continued to be seen post nebulisation [170]. Indeed, further studies could investigate the same formulations using a VMN and/or ultrasonic nebuliser to investigate if the observation regarding delivery vector are consistent across device type.

In another study, a PARI LC Sprint® jet nebuliser was used to aerosolize crosslinked chitosan NPs encapsulating siRNA. The chitosan NPs did not exhibit any evident cytotoxicity in H-292 muco-epithelial lung cells and viability remained high at >85% even at the highest chitosan concentration tested of 83 μg/mL. Gel electrophoresis analysis also confirmed that post nebulisation the integrity of the siRNA was not affected, and it remained bound to the chitosan NPs [116]. However, the overall study outcome is also limited as transfection efficiency of the chitosan NPs was not evaluated and therefore must certainly be accounted for in any future experimental work. The one jet Collison nebuliser is documented in a study for the delivery of DOTAP liposomes co-loaded with the anticancer agent, doxorubicin and ASO or siRNA targeting MRP-1 into mice [146]. Over a 25-day period, the co-loaded liposomes were administered to lung tumour bearing mice housed within a five-port nose-only exposure chamber with each inhalation session lasting for 10 min. Nebulised treatment resulted in a reduction in tumour volume of over 90% compared to a reduction of about 40% only in a separate treatment group receiving IV injection of doxorubicin liposomes. Size analysis also showed that the liposome integrity was retained over the 60 min of aerosolization [146]. This study was one of the first published detailing the use of nebulised RNA *in vivo* hence its significance should be acknowledged. Additionally, it highlighted the potential of nebulised RNA in conjunction with chemotherapy agents as a viable alternative to IV administration.

3.3. Ultrasonic nebulisation

Ultrasonic nebulisers generate aerosol via high frequency ultrasonic waves produced from a piezoelectric transducer. Ultrasonic waves pass through the drug containing solution resulting in aerosol generation at the surface, Fig. 3C [207]. The drug solution may be positioned directly over the transducer or in other cases, there is a water couplant chamber present between the transducer and the drug reservoir [208]. Aerosolization occurs at the liquid surface by capillary wave formation and/or cavitation. For the most part, ultrasonic nebulisers have the advantage of being compact, energy efficient and silent [201]. However, they often have a large residual drug volume [209]. Importantly, ultrasonic nebulisers usually generate heat during operation [193]. This could be particularly problematic in the case of heat sensitive biomolecules or indeed thermolabile RNA formulations. This drawback has hindered their usage in the field of inhaled RNA. This is clearly reflected in Table 2 with a notable lack of studies performed utilising ultrasonic nebulisers in comparison to VMNs and jet nebulisers. Due to these limitations, it is likely that the preference for other nebuliser types over ultrasonic nebulisers will continue in the coming years.

A study carried out in 2016 investigated the Omron® NE-U07 ultrasonic nebuliser for the delivery of naked siRNA solutions [210]. A CompAir™ NE-C28 jet nebuliser and MicroAIR™ NE-U22 VMN (both manufactured by Omron®) were also tested for comparison. Gel electrophoresis showed that nebulisation did not degrade the naked siRNA solution in any of the three nebuliser types used. Aerodynamic performance was also assessed using an aerodynamic particle sizer (APS®) and the Andersen cascade impactor (ACI). Interestingly, the VMN produced the largest MMAD, and this decreased with increasing siRNA solution concentration, perhaps due to changes in surface tension [210]. This study contrasts with numerous others which have shown reduced naked siRNA integrity post nebulisation [116,171,211]. Furthermore, there are several other factors which were not considered. The transfection efficiency was not tested in an *in vitro* or *in vivo* setting and the numerous physiological barriers presented by the respiratory system detailed in Section 2.2.2 must be recognised. It is also worth noting that naked mRNA was not investigated in this study and due to its far longer nucleotide sequence, it is potentially much more susceptible to the nebulisation shear forces than siRNA [14].

3.4. Surface acoustic wave nebulisation

Recently another type of nebuliser has emerged in the literature for the nebulisation of RNA. The Respite™ System, is an aerosol technology which employs surface acoustic waves (SAW) as the driving force behind nebulisation [186]. Like traditional ultrasonic nebulisers, SAW technology utilises the propagation of waves for aerosol generation. In the case of SAW however, nanometre amplitude Rayleigh waves are propagated as opposed to the millimetre-order wavelengths in ultrasonic nebulisers. SAW based nebulisers are thus considered a new investigational type of nebulisers separate to established conventional ultrasonic nebulisers [192]. The SAW device operates at a far higher frequency of ≥10 MHz at which large biomolecules are less prone to cavitation and shear damage [212]. In addition, it requires a lower power of 0.5–3 W compared to the 10 W or above consumed by traditional ultrasonic nebulisers [213]. Within Respite™ an oscillating electrical signal is supplied to a single crystal piezoelectric substrate such as lithium niobate which generates and propagates SAW. Capillary waves are then formed on the surface of the solution in contact with the substrate resulting in its nebulisation (Fig. 6) [186].

SAW technology has been used in one study to nebulise a solution containing siRNA targeting luciferase. Laser diffraction analysis showed a mean aerodynamic diameter of 2.7 μm, demonstrating its potential applicability for inhalation. Subsequent gel electrophoresis analysis of the nebulised RNA revealed that it was minimally damaged by SAW mediated nebulisation. siRNA complexes were also formed with both PEI and INTERFERin, a lipid-based transfection reagent, nebulised and administered to A549 cells expressing firefly luciferase (A549-luc-C8 cells). Up to 47% luciferase knockdown was obtained, which

demonstrated that some of the siRNA's therapeutic efficacy was retained throughout the SAW nebulisation process [186]. SAW technology holds promise for RNA nebulisation based on these results however, to the best of our knowledge, this is the only published study utilising this platform for RNA nebulisation [186]. Further evaluation and progression towards *in vivo* studies is warranted to gauge feasibility of clinical translation more accurately. In addition, the nebulisation process which used a Falcon tube (Fig. 6b) would require modification to allow efficient aerosol inhalation by *in vivo* subjects.

3.5. Pertinent factors influencing nebuliser device choice

Several key considerations affect the choice of nebuliser technology. Foremost amongst these include whether an aerosol can be successfully generated. The aerosol generated must be sufficient to deliver the entire dose in a reasonable timeframe and the integrity of the cargo should be maintained throughout the process of nebulisation. Device selection is also informed by the likely use scenario for the product, and indeed, the formulation design, with dose volume being an important consideration. The volume added to the reservoir will be dictated by the concentration and minimum effective dose, but nevertheless, it is unlikely that standard volumes of 2.5 mL or 5 mL of medication will be used, for reasons of cost per dose, or indeed usability. Further, the large residual volume reported to remain in jet nebulisers suggests they are unsuitable for small dose volumes <1 mL [208]. Limitations also exist in the case of certain formulations when used with some nebuliser devices, e.g., jet nebulisers can preferentially nebulise the buffer in suspension formulations, resulting in a greater mass per unit volume remaining in the medication cup, and passive mesh nebulisers can become clogged when used in combination with suspension formulations. Further, jet, and ultrasonic nebulisers have been shown to be associated with solvent evaporation [214] and an increase in drug solution concentration during the nebulisation period [215]. Other considerations include the physicochemical characteristics of the bulk formulations themselves. Surface tension, viscosity and tonicity can all potentially influence aerosol generation using nebulisers [191]. Increasing the viscosity has been shown to produce smaller droplet sizes, however the output rate was compromised in passive mesh devices [192]. While the lower the surface tension of the formulation the more difficult it is to nebulise using vibrating mesh (active and passive) devices. The higher the osmolality, the higher the output rate with active VMNs, but no change for jet or ultrasonic.

Beyond this ability to generate a bulk aerosol, is the primary consideration as to whether the cargo survives the process. Stresses owing to temperature changes and shear forces exerted on the cargo during nebulisation can cause degradation of labile cargoes [216]. To date there has been no definitive determination of the impact of shear and more systematic investigation is warranted. As discussed above and further in Section 4.1, the choice of delivery system is an important consideration in helping to mitigate against any potential damage induced by shear forces [217,218]. Nebulisation is known to induce changes in drug formulation, in a device specific manner, as discussed above. This is particularly relevant in the context of delivering thermolabile therapeutics as they may be adversely affected by temperature increases and decreases. Active VMNs have been shown to produce small changes in temperature, again in a device dependent manner. The Aeroneb® Pro, vibrating mesh device, was reported to produce a small increase of 3 °C over a 5-min nebulisation window [219]. While the volume of formulation added to the reservoir of a Pari eFlow® vibrating mesh device was shown to influence the extent of the temperature increase [220]. Further, the authors demonstrated that active cooling strategies using a micro-Peltier attached to the reservoir were beneficial in preserving the activity and stability of thermolabile proteins during nebulisation. It is well established that jet nebulisers tend to produce a drop in temperature, due to the latent heat of evaporation of the nebuliser solution [221]. Temperature decreases of between 4.2 and 7.9 °C were seen in one study [219], while another reported an approximate temperature decrease of 7 °C during the first 2 min in the reservoir [221]. As discussed above ultrasonic devices are renowned for generating heat during nebulisation due to the conversion of excess energy from the piezoelectric crystal to heat [192]. Temperature changes between 8.9 and 10.1 °C were observed [219] although potential increases in drug solution temperature by up to 20 °C above ambient temperature have also been reported in the literature [222]. Consequently, it is not surprising that the use of ultrasonic devices is not recommended in the delivery of some protein molecules. Ultimately, device selection for a particular formulation, or range of formulations, should be made following a robust screening process, cognisant of the ability to generate a viable, active aerosol with cargo, suitable for use in the intended patient population and delivery setting.

4. RNA delivery vectors

Administration of RNA in combination with a delivery vector is established as an effective way to overcome physiological, anatomical and stability challenges. Previous studies have shown the potential of viral vectors for RNA delivery [223–225]. However, the safety and production limitations associated with viral vector-mediated delivery are well documented [226–228]. Consequently, much attention has been dedicated to non-viral delivery systems for nebulised RNA (Fig. 7). These include lipid-based delivery systems such as liposomes and LNPs [146,149] polymer-based systems e.g., PEI [65], PBAE [181] and chitosan [115] as well as lipid-polymer hybrid systems [58].

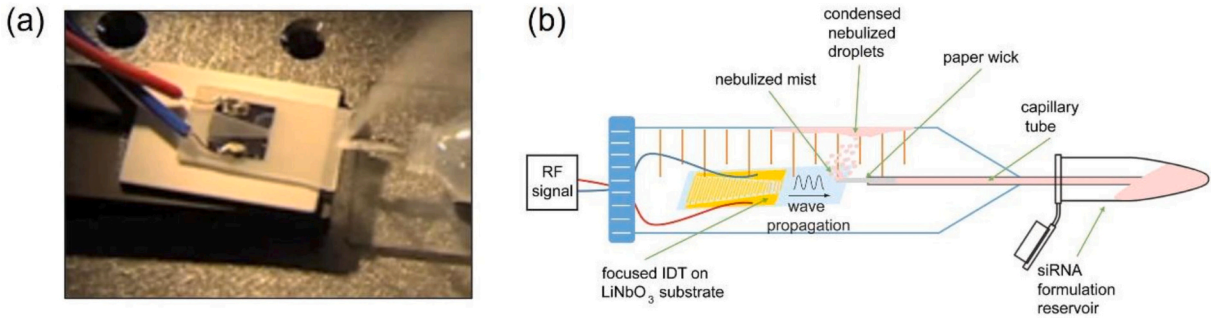

Fig. 6. (a) SAW device used by Cortez-Jugo et al for nebulising siRNA formulations. (b) Nebulisation process by SAW in a 50 mL Falcon tube; a paper wick is placed in contact with the single crystal piezoelectric lithium niobate ($LiNbO_3$) substrate through which the siRNA formulation is drawn via a capillary tube from a reservoir vial. Power is applied via interdigital transducer (IDT) electrodes and the nebulised mist is collected as condensed droplets on the inner wall. Adapted from [186] with permission from Elsevier.

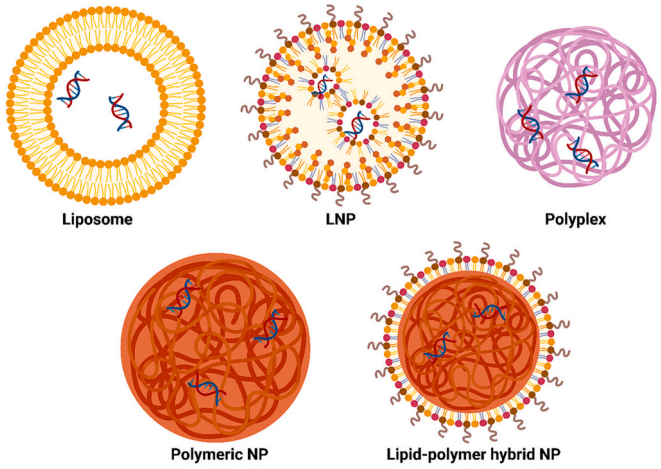

Fig. 7. Non-viral vectors used for RNA delivery: liposomes consist of lipid bilayer(s) with an aqueous core are less commonly used than lipoplexes and LNPs. A diverse array of LNPs exist - a four lipid component system including cholesterol is depicted; polyplexes composed of polycations interacting with RNA; polymeric nanoparticles can involve RNA dispersed within a polymeric matrix and lipid-polymer hybrid nanoparticles can include a polymeric core surrounded by a lipid shell. Created with BioRender.com

4.1. Lipid based delivery vectors

Lipid based delivery systems have proven their value for delivery of RNA molecules, most notably in Patisiran (Onpattro®), the first clinically approved siRNA therapy [12]. Lipidic delivery systems possess numerous characteristics which make them attractive vectors for RNA delivery. They have an inherent propensity for favourable interaction with the cell membrane lipid constituents facilitating RNA transfection into the cell. Additionally, many are biodegradable, biocompatible, and readily available [229]. Liposomes are typically spherically shaped vesicles and may be composed of one or more phospholipid bilayers [230]. Liposomes which possess a neutral charge, including those composed of the neutral lipid, 2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) have shown their potential to encapsulate and deliver siRNA into cells both in *in vitro* and *in vivo* studies [231,232]. Neutral liposomes however do not undergo significant interaction with the cell membrane which can hinder intracellular delivery of their cargo [233]. Cationic liposomes on the other hand, have a much stronger interaction with the negatively charged cell membrane surface. Cationic lipids such as N-[1-(2,3-dioleyloxy)propyl]-n,n,*n*-trimethylammonium chloride (DOTMA) and 1,2-dioleoyl-3-trimethylammonium-propane chloride (DOTAP) are often introduced into the liposomal formulation therefore to enhance cell uptake [234]. Moreover, due to their positive charge, these cationic liposomes can interact with negatively charged RNA through electrostatic attraction and form complexes termed lipoplexes [235].

Cationic liposomes have the potential to cause inflammation and toxicity in the lungs [236,237] and can undergo structural changes when aerosolized leading to premature RNA release [133]. Considering these issues, RNA liposomes intended for treating lung related conditions have predominantly been administered via a parenteral route [238–241]. Some studies have been carried out however, investigating nebulisation of cationic liposomes for delivery of RNA with positive outcomes [66,146,170]. Lipofectamine™ 2000, is a commercially available liposomal formulation, widely used as a transfection agent for nucleic acids and contains the cationic lipid, 2,3-dioleoyloxy-N- [2 (sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-1-propaniminium (DOSPA) and the neutral lipid, dioleoylphosphatidylethanolamine (DOPE) in a 3:1 weight ratio [242].

A recent study documented the use of Lipofectamine™ 2000 for the delivery of mRNA encoding alpha-1-antitrypsin (A1AT) via nebulisation [66]. Due to its function of inhibiting the serine protease, elastase, deficiency in A1AT can result in elastase hyperactivity leading to destruction of alveoli and reduced elastic recoil of the lungs. The A1AT liposomal complexes were aerosolised using a PARI Boy® jet nebuliser for five minutes prior to addition to 16HBE cells. Secreted A1AT protein was detected confirming successful transfection of mRNA while an anti-elastase assay also demonstrated functional activity of the secreted protein. Overall, nebulised mRNA encoding A1AT has shown promise as a therapeutic in this study [66]. The use of Lipofectamine™ 2000 however may be restricted to *in vitro* experimentation due to its toxicity meaning future *in vivo* studies will necessitate an alternative delivery vector.

Lipid nanoparticles (LNPs) are another type of lipid-based delivery system which have been effectively utilised for nebulised RNA [59,149,174]. LNPs typically contain an ionizable lipid, an important component for RNA encapsulation and its cytosolic release [243]. Ionizable lipids used in LNPs have a pKa value of less than pH 7 allowing for a neutral surface charge at physiological pH 7.4 [244]. Utilising ionizable lipids, LNPs can therefore mitigate against the biocompatibility concerns associated with charged lipids [245]. In a low pH environment, such as the endosome, ionizable lipids become protonated due to the presence of an amine headgroup in the chemical structure. Consequently, the acquisition of a positive charge allows for endosome destabilisation and efficient release of RNA into the cytoplasm thus preventing its degradation in the lysosome [246]. Several different ionizable lipids have been used for LNP production such as dilinoleylmethyl-4-dimethylaminobutyrate (DLin-MC3-DMA) [247], 1,2-dioleoyl-3-dimethylammonium-propane (DODAP) [43] 1,2-Bis (linoleoyloxy)-3-(dimethylamino)propane (DLinDAP) [248] and *N*,*N*-dimethyl-2,2-di-(9Z,12Z)-9,12-octadecadien-1-yl-1,3-dioxolane-4-ethanamine (DLin-KC2-DMA) [249]. DLin-MC3-DMA is considered to have optimal pKa properties for *in vivo* performance [250]. The physical structure of RNA containing LNPs consists of an electron dense core in which entrapped RNA material is surrounded by inverted micelles of the ionizable lipid [251]. LNPs therefore differ structurally to liposomes which are composed of a lipid bilayer containing an aqueous core [252]. In addition to an ionizable lipid, LNPs also typically contain cholesterol to enhance membrane fusion and support particle stability, a helper lipid, such as DSPC or DOPE, which encourages fusion with the cell and endosomal membrane and a PEG lipid [253,254]. The incorporation of a PEG lipid for example, 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DMPE-PEG2000) into the LNP structure aids in preventing aggregation of particles, reducing particle size, and also increasing systemic circulation time by acting as a steric shield against clearance by the mononuclear phagocyte system [255,256]. However, the repeated administration of PEG *in vivo* has the potential to elicit immunogenicity [257,258] and must be considered, especially where long-term use is indicated.

The impact of the molar percentage of PEG lipids on LNP performance has been comprehensively reported in a 2021 study investigating a series of different LNP formulations for optimum delivery of luciferase mRNA to mouse lung using an Aeroneb® VMN [59]. Formulations containing a PEG lipid generated stable and monodisperse LNPs ~200 nm or less. In the case of LNPs containing a cationic lipid, increasing the molar PEG percentage led to an increased efficiency in mRNA delivery. Conversely, LNPs containing a neutral phospholipid achieved more potent mRNA transfection efficiency when the percentage of PEG in the formulation was lowered. A lead LNP formulation termed NLD1, carrying mRNA encoding the antibody, membrane anchored FI6 (aFI6), known for neutralising influenza A virus subtype H1N1 was nebulised and administered to mice subsequently inoculated with influenza A/Puerto Rico/8/1934 (PR8). Mice were given 50 μg of nebulised mRNA per dose at three days and two days before infection. All six mice in the group treated with aFI6 mRNA later survived, in contrast to the control group where five out of six subjects succumbed to influenza and died

[59]. This study thus demonstrated the effectiveness of nebulised mRNA-LNPs for combating influenza A *in vivo* and how the LNP formulation parameters may be adjusted for optimal performance.

Another recent study also investigated adjustment of formulation parameters in the LNP-mediated delivery of nebulised RNA [177]. Four novel cationic lipids, achiral functional analogues of DOTAP, were each incorporated into a LNP formulation along with a proprietary ionizable lipid, DSPC, cholesterol and PEG2000-DMG for encapsulation of mRNA encoding for the fluorescent tdTomato protein. Post nebulisation with an Aerogen® Solo VMN, all four had efficient encapsulation efficiencies ≥97% and mRNA purity was ≥82% when characterised by parallel capillary electrophoresis. Immunohistochemistry analysis on the lungs of BALB/c mice 24 h post intratracheal administration of the mRNA-LNPs revealed that two of the four novel cationic lipid LNPs (L2 and L4) had a similar delivery profile and TdTomato protein expression levels in the airways compared to the control DOTAP LNP formulation. The DOTAP analogues' potential for efficient LNP-mediated RNA delivery is clear from this study however, further validation testing is required including more extensive *in vivo* testing [177].

It is previously documented that the shear forces of nebulisation can destabilise LNPs culminating in loss of RNA cargo and functionality [55]. It is evident that LNP formulations require careful optimisation to improve resistance to shear stress encountered during nebulisation [59,149,174]. In a recent study LNPs comprised of the same lipid components as those found in the Onpattro® and containing luciferase mRNA were nebulised, which resulted in aggregation and instability [55]. By increasing the percentage of PEG in their LNP formulation the authors noted an improvement in particle stability during nebulisation. Using an excessively large amount of PEG however, also caused LNP destabilisation and reduced mRNA encapsulation efficiency. They hypothesized that the decrease in encapsulation post nebulisation is due to mRNA leaking to the LNP's exterior as a result of its structure rearranging during aerosolization [55]. mRNA-LNP destabilisation during nebulisation was also observed in another study wherein 18 different LNP formulations were loaded with eGFP mRNA, nebulised and used to transfect HEK-293 and NuLi-1 cells [149]. All 18 nebulised formulations had significantly decreased GFP fluorescence intensity compared to their readout pre-nebulisation. Indeed, in several cases, GFP expression in cells was negligible post nebulisation. Four lead formulations out of the original 18 were chosen to deliver Luciferase (FLuc) mRNA into BALB/c mice at a dose of 1.5 μg and interestingly, the luminescence intensities of the nebulised samples were comparable to those pre nebulisation [149]. Given their encouraging results, it would be interesting to see these LNPs progress into further studies with a specific lung-related gene target. This study demonstrated how a Design of Experiments approach can be invaluable for selecting specific LNP formulations which are more resistant to the shear forces of nebulisation. It should also be noted however that reduced RNA delivery vector stability and potency post nebulisation is not exclusive to LNP based formulations. For example, in one study utilising polymeric nanoparticles composed of tertiary-amine-modified polyvinyl alcohol (PVA) and poly (lactic-co-glycolic acid) (PLGA) to deliver luciferase siRNA, there was decreased transfection after nebulisation across multiple siRNA doses at one particular N/P ratio. The authors hypothesized that the VMN's vibrational forces may have affected the NPs' integrity resulting in reduced siRNA encapsulation and therefore amount of siRNA delivered [139].

4.2. Polymer based delivery vectors

Polymers, both synthetic and naturally derived are a popular drug delivery platform as they are widely available, versatile and may be modified chemically and structurally in numerous ways to improve drug delivery performance [259,260]. RNA delivery is no exception and polymer-based systems have been researched extensively in recent years for RNA delivery [57,261–263]. There are generally two types of polymer systems used for RNA delivery: polycation materials and polymeric NPs. Polycations, for example PEI, interact electrostatically with RNA due to their cationic charge to form polyplexes. Polymeric NPs on the other hand, usually consist of a polymer like PLGA in which RNA is encapsulated within its core or else is dispersed within the polymer matrix [138]. Due to its hydrophobic nature, efficient encapsulation of hydrophilic agents such as RNA in PLGA can prove challenging [264]. One method to improve RNA encapsulation within PLGA NPs is to incorporate a second positively charged polymer into the NP formulation which complexes with RNA thereby improving its uptake. Examples of additional charged polymers co-formulated within PLGA nanoparticles include PEI [98] and poly(L-lysine) (PLL) [265]. PEI has been extensively investigated to deliver RNA as polyplexes in the form of both a linear and branched polymer and at various molecular weights [266]. PEI is advantageous due to its availability and low cost [267]. As mentioned in Section 2.2.1, its high buffering capacity enables it to act as a "proton sponge" promoting the influx of protons into the endosome [100] culminating in endosome rupture and release of the delivery vector and its RNA cargo into the cytosol [97].

Nanoparticles composed of PEI and PEI in combination with linear poly (ethylene glycol) (PEI-LPEG) have been formulated as part of a study for the nebulised delivery of siRNA targeting Interleukin 8 (IL-8), a proinflammatory cytokine. siRNA NPs were nebulised into a glass twin stage impinger (TSI) and directed onto a transwell insert containing a monolayer of Calu 3 cells placed in Stage B (lower stage). Only the nebulised PEI-LPEG NPs were successful in significantly reducing IL-8 expression *in vitro*. The NPs containing siRNA targeting CXCL-1, a homologue of IL-8, were administered intratracheally at a dose of 75 μg into rats which were subsequently lipopolysaccharide (LPS) stimulated. The PEI NPs resulted in a 10-fold decrease in average CXCL-1 gene expression compared to those in the PBS-LPS and non-targeting (NT) siRNA control groups. Unexpectedly, there was no significant difference between the *in vivo* results for PEI-LPEG siRNA and the NT siRNA control which suggests the reduction of CXCL-1 gene expression in the case of pegylated PEI-siRNA was not exclusive to siRNA mediated inhibition [65]. This study is significant for highlighting the therapeutic potential of nebulised siRNA in airway inflammation, however the need for reliable disease models both *in vitro* and *in vivo* is paramount.

The viability of PEI as a clinically translatable vector is hindered by its potential toxic effects, which are particularly evident at high molecular weights [268], as well as its lack of biodegradability [269]. In contrast to PEI, PLGA has shown to be biodegradable and possess a desirable safety profile for RNA delivery [270]. However, due to its negative charge PLGA cannot form polyplexes with RNA and consequently it is formulated as polymeric NPs. The negative charge of PLGA can also adversely affect *in vivo* performance due to opsonisation and rapid clearance from the bloodstream in addition to hindering cell uptake [271]. To improve its general viability as an RNA carrier, PLGA often undergoes modification to enhance performance for example, pegylation [272], incorporation of lipids [273] or cationic polymers such as PEI into the PLGA matrix [98] or coating with the polysaccharide, chitosan [274].

Poly (β-amino ester)s (PBAEs) are another polymeric class which have been used effectively for polyplex delivery of nebulised RNA [57,171,181]. PBAEs are advantageous due to their biodegradability and low cytotoxicity [275]. They are available as a diverse range of distinctive compounds synthesized through conjugation of amine monomers to diacrylates [276]. Protonation of amine groups on PBAEs in the acidic endosome is also thought to facilitate the endosomal escape of their RNA cargo into the cytosol [277]. As previously discussed in Section 4.1, the adoption of a delivery vector which is sufficiently physically robust to withstand the shearing forces of nebulisation is paramount for the effective delivery of nebulised RNA. A 2019 study introduced hyperbranching into PBAE polymers as a means of achieving the physical properties necessary for nebulisation of their RNA payload [181]. Upon aerosolization with an Aeroneb® VMN, the hyperbranched

PBAEs (hPBAEs) complexed with mRNA remained stable with particle sizes below 200 nm, whereas linear PBAE polyplexes aggregated into large, unstable NPs. The hPBAEs were then used to aerosolize luciferase mRNA at a concentration of 0.5 mg.mL^{-1} to C57BL/6 mice housed within a whole-body nebulisation chamber connected to the nebuliser via a silica-containing spacer device (Fig. 8). Luciferase mRNA expression was found to be maximal at 24 h post nebulisation and decreased significantly by 48 h after administration. Luminescence was also uniform across all five lung lobes [181]. The whole-body chamber in this study is an effective, non-invasive means to administer nebulised RNA *in vivo* with minimal subject handling. However, the practicality of such apparatus for larger and more complex animal species must be considered.

Natural polymers can offer attractive properties supporting their use in pulmonary RNA delivery, as they are generally biocompatible, biodegradable, non-toxic and can have good mucoadhesive properties [278]. Chitosan, a derivative of chitin, is a biodegradable natural polysaccharide which has shown to be advantageous as a drug carrier due to low toxicity and immunogenicity. It is also capable of easily complexing with RNA due to electrostatic interaction owing to its positive charge [279,280]. Conversely, chitosan's development as an RNA carrier is hindered by its low buffering capacity and resultant poor endosomal escape. Chitosan, therefore, is oftentimes modified to improve its effectiveness as a carrier of RNA [115,184,281]. From a nebulisation perspective, there are examples of chitosan being used for RNA delivery with some of these progressing to *in vivo* studies [115,282].

In one study, pH-responsive NPs comprised of two chitosan derivatives; guanidinylated O-carboxymethyl chitosan (GOCMCS) and N-2-hydroxypropyltimehyl ammonium chloride chitosan (N-2-HACC) were developed to carry siRNA targeting survivin, an inhibitor of apoptosis [184]. The NPs were administered to A549 cells *in vitro* for 72 h and resulted in a downregulation of the survivin gene to 6.9% compared to the control group treated with empty nanoparticles. Upon aerosolization with an Omron NE-C801 jet nebuliser a FPF of >60% was produced indicating the potential for lower lung region deposition while gel electrophoresis analysis also confirmed that the siRNA integrity was maintained during nebulisation [184]. To conclude, based on their *in vitro* performance, chitosan derivatives appear promising carriers for RNA via nebulisation. Future animal model studies are needed however to more accurately evaluate their clinical potential given the poor *in vitro - in vivo* correlation often observed with preclinical RNA nebulisation studies.

4.3. Lipid polymer hybrid vectors

Lipid polymer hybrids have been explored as a formulation for delivering RNA cargoes and can overcome some of the disadvantages associated with carriers which are purely lipid or polymer based. These include poor RNA encapsulation rates in the case of polymeric nanoparticles and instability during storage associated with lipid-based systems [283]. Lipid polymer hybrid nanoparticles (LPNs) usually consist of a polymeric core that is surrounded by a lipid shell which may also be pegylated. Combining the favourable traits of both polymers and lipids as drug carriers, LPNs, can provide an RNA delivery vector which is both highly stable and biocompatible [284].

LPNs containing PLGA and DOTAP have been previously assessed for the delivery of miR-17, microRNA targeting the proinflammatory cytokine, IL-8. The LPNs were lyophilised, reconstituted, and nebulised using an Aerogen® Solo VMN. The nebulised LPNs were approximately 232 nm in size with a PDI of 0.172, produced an aerosol MMAD of around 4.2 μm and FPF of approximately 89.8% therefore exhibiting satisfactory physicochemical and aerodynamic properties. *In vitro* administration of the nebulised LPNs to NuLi-1 bronchial epithelial cells (BECs) downregulated IL-8 secretion by over 40% [58]. Further optimisation may be warranted however with the aim of increasing the current gene knockdown levels perhaps by substituting DOTAP in the LPN formulation with a different lipid component. The effective use of lyophilisation in this study to prolong the stability of the LPNs is also noteworthy.

LPNs have also been nebulised to deliver siRNA targeting the α and β subunits of the sodium transepithelial channel (ENaC) protein [56]. Dipalmitoylphosphatidylcholine (DPPC), a key component in the lung lining fluid was incorporated into hybrid nanoparticles (hNPs) containing both PLGA and PLGA/PEI. The hNPs had a triphasic release profile over around five days and were both stable in and capable of penetrating samples of artificial mucus. They could also penetrate a 3D triple cell co-culture model (TCCC) consisting of HBE cells, human blood monocyte-derived macrophages and dendritic cells used to mimic the human epithelial airway barrier. The nebulised hNPs elicited a 60% and 40% reduction in A549 cell ENaC protein expression for the α and β subunits, respectively. Additionally, incorporation of PEI into the hNP formulation caused a further 10% decrease in the protein expression for both subunits [56]. As a whole, this study demonstrated the effective use

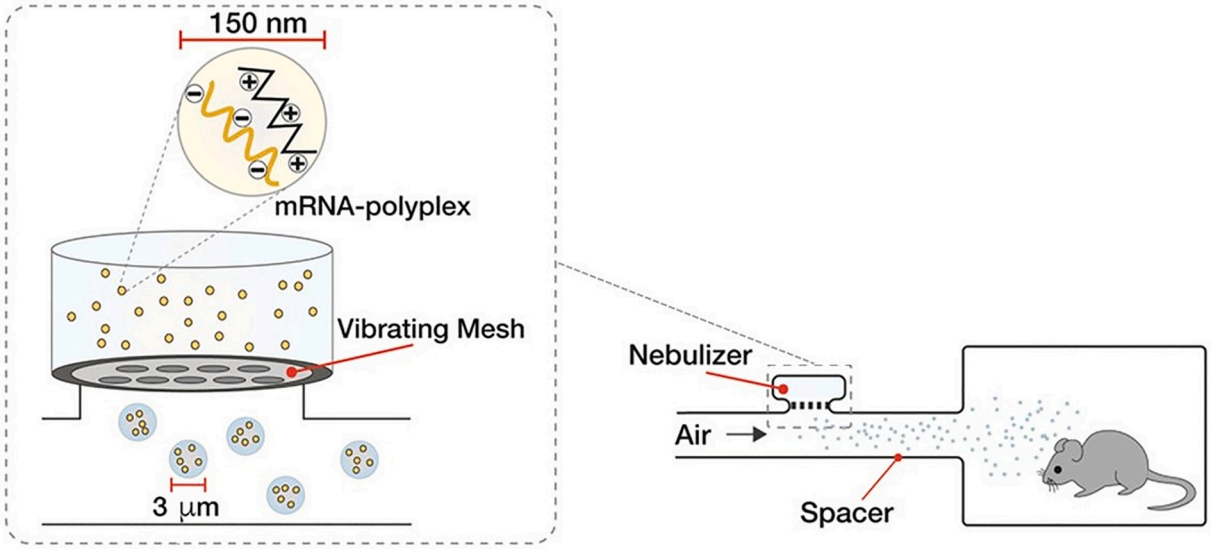

Fig. 8. Whole-body chamber with VMN used to deliver hPBAE polyplexes containing mRNA encoding for firefly luciferase to mice. Adapted from [181] with permission from Wiley.

of an *in vitro* model to mimic the epithelial airway barrier and how LPNs may be utilised for RNA delivery [56]. Further work is required however to assess if the nebulised LPNs' effectiveness can be replicated in an *in vivo* environment.

In another study lipopolyplexes were formed by mixing liposomes comprised of the neutral phospholipid, DPPC and DPPC in combination with one of the two co-lipids, 1,2-dipalmitoyl-sn-glycero-3-phospho-(10-rac-glycerol) (DPPG) and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanol-amine (DPPE) with PEI-based polyplexes [285]. The lipopolyplexes containing luciferase siRNA were nebulised using an Aeroneb® Solo VMN and produced a significant level of luciferase knockdown in SKOV-3 cells thus maintaining the functionality of their siRNA cargo after nebulisation. It should be noted however that testing was performed only in ovarian carcinomic SKOV-3 cells and there is an obvious need to also evaluate transfection efficiency in a cell line relevant to lungs [285]. In summary, lipid-polymer hybrids, as a delivery vector are an attractive alternative to those which are comprised entirely of lipids or polymers and offer potential for the delivery of nebulised RNA.

5. Conclusion and future outlook

There have been enormous strides in the development of RNA therapeutics in the last decade culminating in the approval of several treatments, with more expected to follow from the clinical pipeline. The RNA treatments approved to date are intended for delivery by the parenteral route, despite its drawbacks. Pulmonary inhalation is an attractive means of drug administration due to its non-invasiveness and the plethora of lung-related conditions which can be effectively targeted by exogenous RNA. However, there are currently no clinically approved RNA treatments indicated for pulmonary administration and there are only a few products in clinical trials. Although the pulmonary route is attractive, several anatomical and physiological barriers need to be overcome to achieve effective local delivery of the RNA and systemic targeting via inhalation. A cost-effective, efficient method of delivery that preserves the integrity of the labile RNA cargoes is necessary to ensure the viability of pulmonary delivery. Nebulisation is a particularly enticing method due to its capability to deliver large RNA doses during normal tidal breathing. The preclinical studies performed involving RNA nebulisation are wide-ranging targeting conditions such as cystic fibrosis, cancer, and asthma. The results of these studies are largely positive indicating the potential for further development and progression into clinical testing. Yet, a sizeable percentage of nebulised RNA treatments are only in their early preclinical phase, emphasising the efforts still required to realise the potential of pulmonary delivery. Many have only advanced as far as a "proof of concept" stage wherein the RNA's ability to remain stable, produce an aerosol in the respirable range and transfect cells *in vitro* has been evaluated. In several studies aerodynamic testing was only progressed to analysis with the twin stage impinger, a long-established yet rudimentary apparatus with a large fine particle fraction (FPF) cut off of 6.4 μm, which overestimates the percentage deposited in the lower lungs. Moreover, numerous papers have reported mediocre FPF values of close to 50% suggesting sizeable loss of dose in the upper airways which is particularly burdensome given RNA's expensive cost. There is a need to progress further towards *in vivo* studies to better gauge the behaviour of these RNA treatments in human subjects. That said, the vast anatomical and physiological differences between human lungs and that of mice, the most widely applied animal model, indicate that preclinical *in vivo* studies may only give a limited insight into clinical effectiveness. Furthermore, in most mice studies the RNA is administered via the intratracheal route, an invasive method impractical for routine human application except in emergency situations. It is also worth noting that in studies which have performed both *in vitro* and *in vivo testing*, poor correlation is often seen between the two particularly in relation to transfection efficiency.

Regarding nebuliser designs, the vibrating mesh nebuliser has been the preferred type for researchers in the last decade or so due to its low residual volume, compact size and production of fine aerosol droplets, and this trend is expected to continue. Numerous research papers have detailed the fragility of naked RNA molecules due to the shearing forces of nebulisation, and this may be especially challenging for mRNA given the molecular length. A robust and efficient delivery vector, one which can maintain the stability of RNA from nebulisation to cell entry will therefore be of paramount importance for efficacious nebulisation of RNA. Studies so far broadly indicate that lipid-based delivery vectors have superior RNA transfection efficiency. However, polymeric vectors may possess greater stability in an *in vivo* lung environment. Thus far, polymeric, and lipid-polymer hybrid vectors have generally shown greater robustness in resisting nebulisation shear stress and to maintain better stability during aerosolized delivery. That said, through optimisation by substitution or changing the molar percentage ratios of individual components, the stability of lipid vectors during nebulisation can also be enhanced. Formulating lipid-polymer hybrids is a favourable strategy of combining the advantageous attributes of both vector types for RNA nebulisation. Any delivery system adopted will need to be carefully tailored to tackle both the challenges inherently associated with RNA cargoes and the *in vivo* barriers. Indeed, studies have shown that painstaking optimisation and subtle adjustments in formulation parameters through design of experiments, notably for LNP-based systems are necessary in achieving the maximum amount of RNA at its target site. Specialised modification of delivery vectors such as incorporation of mucus penetrating agents and the use of targeting ligands represent other effective means of increasing the efficiency of nebulised RNA.

Looking beyond the current stage of development towards a future world in which nebulised RNA clinically approved for patient use is a reality, there are several questions that need to be addressed. Exogenous RNA as a treatment will not be a once off "silver bullet" which cures or permanently reverses a patient's disease. Like most pharmacological treatments, nebulised RNA has a transient therapeutic effect which diminishes upon the interruption or cessation of therapy. Consequently, this necessitates repeated administration at defined intervals over time and this frequency of administration will need to be established for each treatment. One must therefore consider the biocompatibility of nebulised RNA formulations repeatedly given over extended periods of time and the impact of accumulated levels of exogenous RNA and its delivery vector in the body. There are several reports for example, suggesting that poly (ethylene glycol), a component within LNPs elicits immunogenicity following repeat administration. Preclinical animal studies are usually restricted to treatment over a period of weeks. This means the impact of adverse effects due to prolonged administration may only come to the fore during clinical trials by which point considerable money and time have already been expended.

In several conditions such as cystic fibrosis and COPD, patients already spend lengthy periods of their daily life adhering to complex medication regimens. Any advancement in RNA therapeutics should aim to simplify this rather than complicate further. Another noteworthy consideration is that in many respiratory conditions lung physiology is altered compared to healthy individuals, e.g., increased mucus production in cystic fibrosis patients and reduced inspiratory flow rate and capacity. Nebulised RNA treatments may therefore need to be adapted to deal with such changes in physiology, which are poorly mimicked in animal models. Finally, it is important to acknowledge the stringent storage needs for RNA medicinal products, which oftentimes require storage at as low as -80°C post manufacture, which present additional challenges relating to shipping and overall cost. To preserve stability, RNA products are often supplied as a lyophilised powder which is reconstituted prior to administration. This presents additional challenges. For example, patients and/or healthcare workers must be adequately trained on how to accurately reconstitute RNA and the reconstitution itself is a further step in the nebulisation process prior to filling the nebuliser reservoir. For patients in advanced stages of

debilitating conditions such as lung cancer, this may require input from healthcare professionals. The challenges facing nebulised RNA are no doubt, abundant at each development stage from *in vitro* testing to clinical trials as discussed. However, the progress made so far is encouraging. A nebulised RNA treatment may therefore in the future be within grasp, a viable alternative to parenteral administration, delivering RNA from an aerosol to the cytosol.

CRediT authorship contribution statement

Michael T. Neary: Writing – original draft, Visualization, Conceptualization. **Lianne M. Mulder:** Writing – review & editing, Writing – original draft. **Piotr S. Kowalski:** Writing – review & editing, Writing – original draft, Supervision. **Ronan MacLoughlin:** Writing – review & editing, Writing – original draft. **Abina M. Crean:** Writing – review & editing. **Katie B. Ryan:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition.

Declaration of Competing Interest

Ronan MacLoughlin is an employee of Aerogen Limited. The remaining authors declare no competing interests.

Data availability

No data was used for the research described in the article.

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