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Review

Pulmonary Delivery for miRs: Present and Future Potential

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Abstract: Administration through the respiratory tract can be advantageous, with high drug bioavailability, limited enzymatic activity, reduced dose requirements compared to oral, and potentially diminished side effects. Among the different types of drugs studied for pulmonary delivery, genetic material delivery has gained favorable scientific interest, using polymer-, lipid-, inorganic-, or vector-based nanocarriers. As pulmonary drug delivery has been associated with challenges, including physiological barriers and lung metabolism, the delivery of sensitive molecules such as nucleic acids can exacerbate these challenges. While short-interfering RNAs (siRNAs) have been extensively reported as suitable ribonucleic acid interference (RNAi) candidates for pulmonary delivery, discussion on micro-RNA (miR) pulmonary delivery is limited despite their significant therapeutic potential. Recently, these non-coding RNAs have been explored in targeted or non-targeted pulmonary administration against various diseases. This review addresses the information gap on miR-pulmonary delivery with updated and concentrated literature. We briefly discuss the barriers to lung administration, describe different functional nanocarriers for miR delivery, and provide an extensive literature update on the different miRs and their targeted diseases currently being studied.

Keywords: pulmonary delivery; RNA interference; micro-RNAs; nanocarriers; liposomes; naked miRs

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1. Introduction

The pulmonary route for the delivery of active pharmaceutical ingredients has attracted significant interest due to the advantages associated with the direct and localized action this route provides, which include: (a) rapid drug accumulation in the lung that provides a large surface area for the absorption of compounds; (b) ease of administration; and (c) relatively decreased enzymatic activity [1]. Not surprisingly, several products are currently available utilizing this route, which include dry powder inhalers, metered dose inhalers, and nebulizers [2]. The pulmonary route presents versatility as it has been used to administer compounds for systemic and localized action. At the same time, challenges associated with efficient delivery to the lungs, especially when higher dosing is required, can hinder the development of novel pulmonary systems [1].

Similarly, nucleic acid-based therapeutics have recently received an accelerated interest, predominately stemming from the development of the mRNA-based COVID-19 vaccines, their rapid translation and associated benefits to patients [3]. In fact, the successful translation of the nucleic acid-based vaccines propelled a large number of research endeavors and clinical trials that expanded to different types of nucleic acids and diseases, spanning from short interfering RNAs (siRNAs), micro-RNAs (miRs), to message RNAs (mRNAs), and diseases such as different types of cancer, infections, and genetic disorders [4–6]. The benefits associated with nucleic acid-based therapeutics emerge from their capacity to simulate natural endogenous molecules (such as miRs or mRNAs) or take advantage of natural cell mechanisms towards therapeutic outcomes (such as protein translation and RNA interference mechanisms). This has the potential for personalized

therapeutics, as diseases are frequently associated with gene dysregulation by the cells, potentially leading to improved outcomes [6]. Similarly, short nucleic acids, such as miRs and siRNAs, have been established as promising gene regulators against a plethora of diseases [7–9]. In fact, their capacity to utilize the cells' RNAi mechanism makes them unique and effective post-transcriptional gene regulators. Not surprisingly, both miRs and siRNAs are the focus of clinical trials, with siRNAs [10] arguably having a relatively stronger presence compared to miRs [6,11] in the clinical landscape at the moment, with siRNA products already approved for patient treatment [12]. Nonetheless, miRs are promising endogenous products that have demonstrated significant potential for regulating a plethora of pathways and diseases, resulting in their extensive evaluation in recent years.

miRs are a group of highly conserved non-coding RNAs with 19–22 nucleotides that show partial base pair complementarity with their respective targeting messenger RNA (mRNA) [13,14]. In mammalian cells, the miRs bind to either the 3′ or the 5′ untranslated region (UTR) of their mRNA targets, whereby, via the activity of the Argonaute protein (AGO2), the formation of the RNA-induced silencing complex (RISC) is initiated. Through this miR-initiated RISC or miRISC, the targeted mRNA translation is suppressed, causing dysregulation of respective genes [15]. As natural endogenous transcriptional products, miRs present the unique potential of using existing miR dysregulation profiles generated during different disease states for treating these diseases, including lung diseases. Thus, pulmonary delivery of miRs becomes an interesting application for treating lung diseases. Local delivery of miRs to the lungs can be simpler than other routes of administration (i.e., intravenous), minimize drug degradation due to the lung's suggested low nuclease activity, minimize non-tissue-specific delivery and side effects, and allow the administration of reduced dosing due to increased accumulation in the targeted tissue [16].

Although there is strong literature reviewing the pulmonary delivery of siRNAs, similar content on the potential of miRNAs is limited. In this review paper, we aim to describe the potential limitations of pulmonary delivery in general and in specific for nucleic acids, as well as summarize and provide updates on current methodologies used for the pulmonary delivery of miRs, the miRs that have been studied, and the diseases that have been targeted using these miRs. Our interest is to identify the most common approaches for delivering miRs, focusing on non-viral delivery systems and the routes of administration, as well as potential delivery approaches that may indicate potential benefits towards the translation of pulmonary miR-based treatments into patient care.

2. Lung Structure and the Barriers of the Pulmonary Route of Administration

Pulmonary drug delivery systems are recognized for their targeted drug delivery capabilities due to their capacity to induce high bioavailability and site-targeting in the lungs. The respiratory system is an attractive route of administration for drug delivery and absorption due to its large surface area of approximately 100 square meters, which provides access to various tissues along its path [17]. Furthermore, drug dosing via the pulmonary route can be less effective compared to traditional delivery routes (i.e., oral) to achieve comparable or higher localized levels in the lungs, as it by-passes the first-pass metabolism, and a reduced enzymatic activity in the lungs has been reported [1,18–20]. Anatomically, the respiratory system is divided into the upper and lower respiratory tracts, also referred to as extra-thoracic and intra-thoracic regions. A total of 23 generations of divisions are present in the respiratory system, 16 of which are part of the conducting zone, and the remaining fall under the transitory zone (Figure 1) [21].

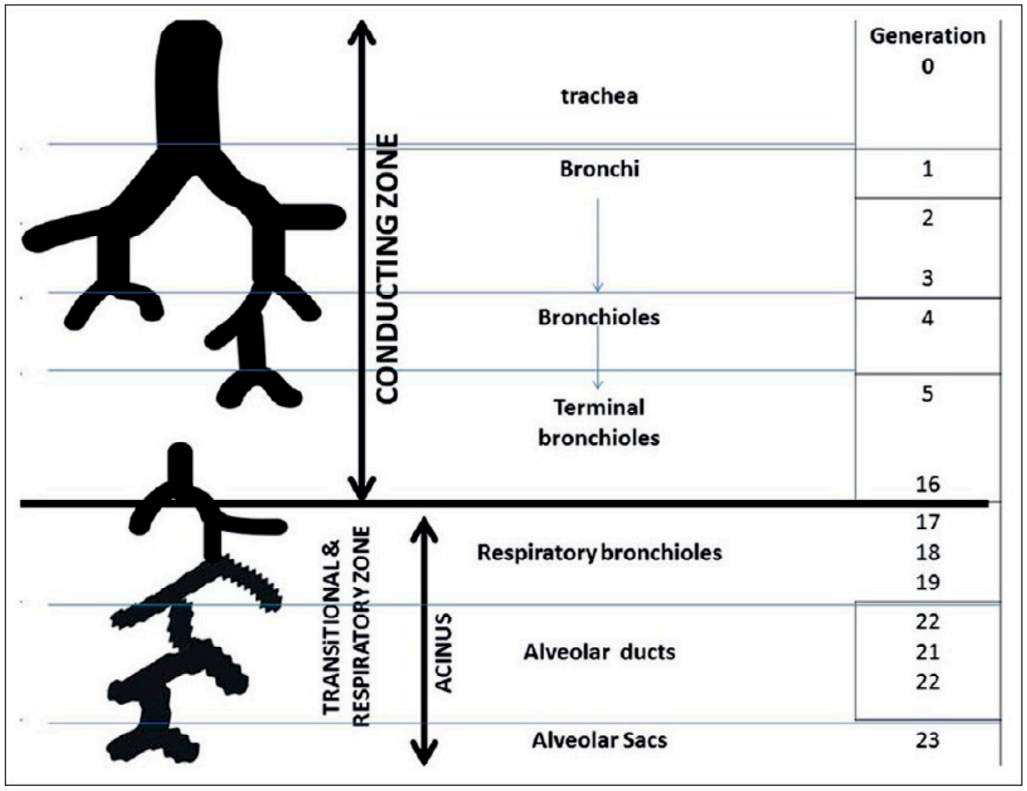

Figure 1. The tracheobronchial tree and the ductal branching generations produced. The image is a reprint from [22].

The extra-thoracic region consists of the oral and nasal cavities, the pharynx, and the larynx. The intrathoracic region is further subdivided into the tracheobronchial and alveolar, or acinar, regions. Trachea, main bronchi, and bronchioles are parts of the tracheobronchial regions, whereas respiratory bronchioles, terminal bronchioles, alveoli, and alveolar ducts are part of the acinar regions [23].

Despite the associated benefits of pulmonary drug delivery, such as localized action and diminished enzymatic activity compared to other administration routes, certain challenges exist for successful drug delivery. A list of barriers is presented here, which we further analyze below. These include (Figure 2):

(a) Physiological barriers, such as:
    i. Mucosa
        a. Composition of the mucociliary membrane
        b. Mucosal clearance
        c. The binding mechanism in the mucosal layer
    ii. Pulmonary Surfactants
(b) Particle size of the drug formulation
(c) Lung metabolism.

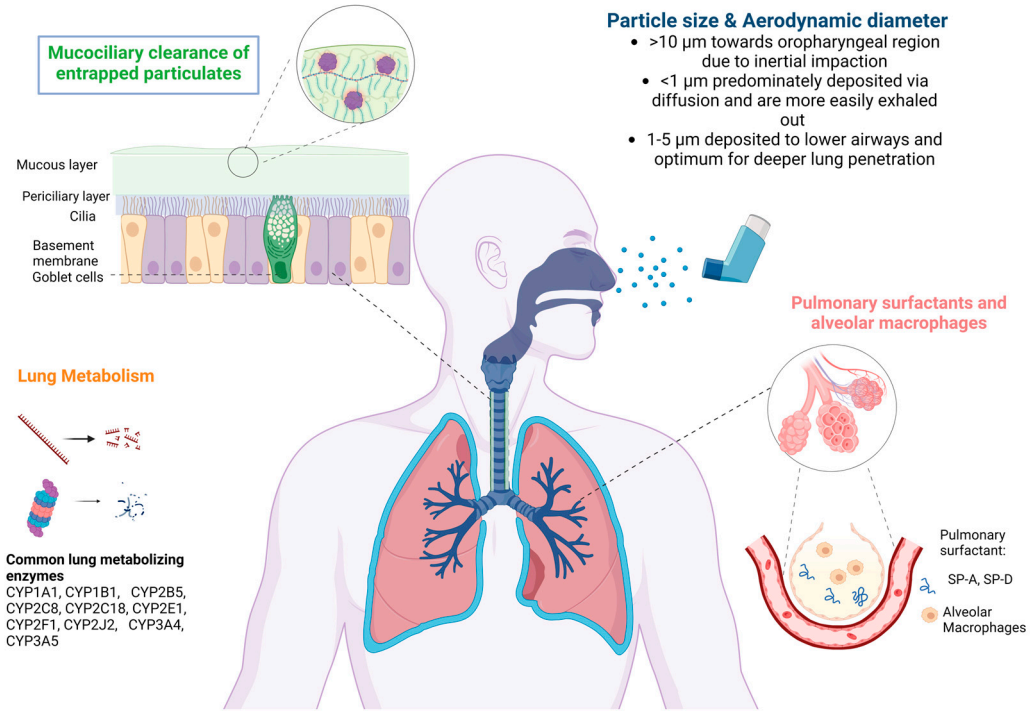

Figure 2. Barriers commonly present during pulmonary administration of active pharmaceutical ingredients.

2.1. Mucosa as a Barrier

As a part of the pulmonary defense mechanism, a protective mucus layer, lining the pulmonary epithelium, exists along the respiratory tract, and it constitutes a major hurdle for the entry of organic, inorganic, and gaseous materials [24]. The defensive mucus barrier in the respiratory system is formed by a network of dense and elastic, high molecular weight (MW) mucin fibers forming a strong fibrous mesh connected via peptidoglycan chains [18]. The mucus is secreted through the surface secretory cells, i.e., goblet cells, and the secretory cells of submucosal glands known as serous cells [24]. As is the nature of most physiological membranes, the mucosal layer is anionic due to its high sulfate and sialic acid contents. The mucosa also contains lipids, sugars and salts, nucleic acids, and various antibodies and immunoglobulins, such as IgA, while membrane-attached mucins, such as MUC1, MUC4, and MUC 16, are involved mainly in signal transduction, cell differentiation regulation, proliferation, and generating immune responses against insults [19,25,26]. The viscosity is the product of the mucin network, which is interconnected to one another through disulfide chains [18]. In normal physiological conditions, mucus comprises 97% water and only 3% solids, made of mucins, lipids, salt proteins, and cellular debris [27]. However, in respiratory pathophysiology, the concentration of solids increases up to 15%, which increases the mucus' viscosity and elasticity [27].

Not surprisingly, as the mucus layer covers most of the surface area along the respiratory tract, gases and solid materials need to efficiently transverse the mucus to reach the living tissue. Thus, the superficial mucosal layer entraps inhaled particles, pathogens, and therapeutic particles in its viscous gel layer, whereas the periciliary liquid layer beneath the mucosa lubricates the cilia for continuous movement.

Depending on the capacity of particles to penetrate and transverse the mucus, they can become eliminated from the respiratory system through the ciliary beating, either towards the outside of the airways by coughing or towards the esophagus and the stomach's acidic environment by swallowing [28]. Similarly, from a drug delivery perspective, therapeutic particles or drugs trapped in the mucus layer before traversing to the living tissue will be cleared away/removed from the lungs. As the mucus' clearance and structure depend on the pathological condition in the lungs, drug presence and duration in the lungs can vary.

For instance, the secreted mucus is highly viscous and elastic in cystic fibrosis, asthma, and airway obstructive diseases [29]. This causes increased airway obstruction due to thick mucus deposition, resulting in the mucus and its adhesive properties being a significant diffusion barrier for inhaled drug molecules [29–31].

As mucins form an intensive network of cross-linkage through strong disulfide bonds, the mucus layer forms a mesh-like network of sizes ranging from 0.1 to 2 microns, varying by location in the body [31]. In addition, mucin fibers are formed with both positively and negatively charged amino acids at the physiological pH and a negatively charged glycan coating, thus being regarded as having a net negative charge [18,32,33].

The mucus layer's complexity and its constant removal suggest that drugs and/or drug carriers face a significant challenge in traversing the mucosa successfully, and their success depends on the molecule size and charge in the case of free drugs or the surface chemistry, charge, and particle size in the case of drug carriers. The negatively charged nature of the mucus layer can also result in a specific disadvantage for gene delivery vectors, which tend to be formed by cationic lipids and polymers, resulting in their high adherence to the mucin layer and their removal along with it [18].

2.2. Pulmonary Surfactants

The pulmonary surfactants (PS) are lipoprotein complexes, predominately produced by Type II alveolar epithelial cells, and are composed of an amalgamation of lipids and proteins. The surfactants in the pulmonary system protect against alveolar collapse in extreme conditions due to a decrease in surface tension in the alveolar regions [34]. A deficiency of these surfactants in the peripheral respiratory system can cause several respiratory distress symptoms. However, the pulmonary surfactants can also significantly impact nanocarrier pulmonary delivery.

Nguyen et al. [35] exhibited the effect of a bovine-derived lung surfactant, Alveofact™, in aerosol gene therapy, where nanocomposites with PS displayed enhanced intracellular uptake. It was found that the in vitro transfection efficiency of nanocomposites coated with PS in A549 cells was 30-fold higher compared to plasmid DNA (pDNA) encapsulated in polyethyleneimine (PEI) and consistently remained as such even after nebulization. Similarly, Kukowska et al. [36] demonstrated that the use of a synthetic surfactant, Exosurf™, increased the transfection of cationic dendritic polymers in different eukaryotic cells, which suggested that surfactants facilitate cellular and nuclear membrane permeability by disrupting their lipid bilayer. Furthermore, incubation of gene-expressing adeno-associated viruses (AAV) with surfactants promoted transgene expression in the peripheral lungs of rabbits upon instillation [37], as well as augmented the transfection efficiency of AAV gene transfer to the A549 cells [38], indicating potential benefits for pulmonary delivery. In contrast, adding Alveofact to the PEI/DNA complexes followed by intratracheal administration to mice resulted in reduced luciferase gene expression in the mouse lungs compared to PEI/DNA complexes without the natural PS [39]. This finding led Rudolph et al. [39] to conclude that the natural surfactant did not increase gene expression in the mice's lungs. Alveofact also decreased lipofectamine-induced transfection efficiency due to the disruption of the lipid-DNA lipoplexes. However, PEI polyplexes were not affected upon incubation with Alveofact [40]. Therefore, pulmonary surfactants can either augment or curtail gene delivery to the lungs based on the choice of a delivery vehicle/gene vector. This presents pulmonary surfactants as a crucial factor to consider while designing a delivery vehicle.

2.3. Particle Size

Aerodynamic particle size is critical in predicting particle deposition in different respiratory tract regions. Particle sizes ranging from 0.5 μm to 5 μm are optimal for proper lung deposition. More so, smaller particles are taken up by more peripheral areas of the lungs, such as alveolar sacs, while the larger ones preferentially accumulate towards the central part, the conducting airways. It has also been emphasized that particles sized less

than 0.1 μm depend entirely on Brownian diffusional transport, whereas for particles sized above 1 μm, deposition by diffusion becomes negligible [41,42]. The deposition of particles in different respiratory regions depends on their different physical parameters (i.e., size and density), their structure, and air velocity. Of note, in the first ten respiratory tract generations, particle deposition is dominated by inertial impaction due to the high airway velocity in these regions for larger particles. Similarly, gravitational sedimentation would be the primary reason for particle deposition at the last six airway generations (towards the central airways) due to low air velocity characterized by a lesser cross-sectional area in this region [43]. For instance, particles with an aerodynamic diameter of >10 μm are commonly deposited in the oropharyngeal region due to inertial impaction. In contrast, particles with an aerodynamic diameter of less than 1 μm are predominately deposited via diffusion while suspended in the airstream. Due to the tidal air movement, they are more easily exhaled [44]. Additionally, for particles with sizes in between, a combination of diffusion, sedimentation, or inertial impaction takes place [44]. Therefore, particle size consideration is necessary for proper deposition during pulmonary delivery, as this can affect the deposition location and efficient administration with minimal losses. Yet, the method of administration through the pulmonary route impacts this discussion, as the dimensions reported above refer mainly to the inhalation method of particles. The significance of the pulmonary delivery method in lung deposition has been thoroughly addressed in the comprehensive review by Youngren-Ortiz et al. [45]. It has been noted that while intratracheal instillation can circumvent the oropharyngeal drug deposition, it neither accounts for nor allows the prediction of the effect of aerodynamic particle size on lung deposition. This can also be substantiated by the study conducted by Osier and Oberdorster [46], where they demonstrated how particle deposition is affected by intratracheal inhalation or instillation. Their findings suggested that when animals were subjected to titanium dioxide particles (~250 nm and ~21 nm sizes) via inhalation (ventilated) or instillation, the instilled group of animals showed increased responses as detected by bronchioalveolar lavage fluid (BAL) analyses. This took place despite inhalation presenting a more homogeneous particle distribution pattern, which, unlike instillation, does not create areas of high and low particle burden within the lungs. In the context of pulmonary miR delivery via intratracheal instillation, Niemiec et al. [47] developed cerium oxide nanoparticles for miR-146a pulmonary delivery with a 190 nm average particle size, while Zhang et al. [48] used exosomes with encapsulated miRs, which presented an average diameter of approximately 86 nm. On the other hand, for intranasal miR delivery, Osorio et al. [49] utilized miR-219a-encapsulated liposomes with sizes that varied between 160 nm and 230 nm, and Liu et al. [50] formulated gold nanoparticles encapsulating miRs with a hydrodynamic diameter of ~160 nm. Although all of the above studies focused on nanoparticles, their administration took place in the form of a liquid suspension (i.e., instillations) or ventilated suspended droplets. The deposition of nanoparticles through inhalation, as individual air-suspended nanoparticles, would have been limited due to their lack of gravitational deposition or impaction. Therefore, droplet-forming instillation or inhalation (spray) does not necessitate the particles in suspension inside the spray droplets to be of the suggested microsized dimensions. For example, Xu et al. [51] reported on the pulmonary delivery of nanoparticles carrying a drug and a nucleic acid. Their approach involved spraying the suspension into the lungs for nanocarrier delivery.

Thus, although nanoparticles for their pulmonary delivery may present advantages for mucus penetration or cell uptake, among others, a droplet of the suspension would be required for inhalation [44,45], or else instillation must occur [46]. Taking together how microsized particle diameter affects lung deposition, droplet size should be of the proper μm dimensions for successful inhalation.

2.4. Lung Metabolism/Enzymes of Different Parts of The Respiratory Tract

Unlike oral administration, the metabolism during the pulmonary drug delivery route is based on xenobiotic-metabolizing enzymes that influence the pharmacokinetics of

inhaled compounds. The lungs have comparable types of metabolic enzymes to the liver, though the overall metabolic activity in the lungs is lower than that of the liver [52–55]. The region expressing xenobiotic enzymes is distinguishable based on the expression of certain metabolizing enzymes. For instance, the CYP enzymes responsible for phase I metabolism are abundantly present in the bronchiolar epithelium, mainly in the club cells, the ciliated columnar epithelial cells, the alveolar cells, and macrophages [54]. For perspective, the alveolar surface area in the lungs constitutes approximately 100 square meters of surface area, out of which the alveolar type (AT)-I pneumocytes cover the majority of the area, which makes it the most likely area for the deposition of therapeutic agents [56–58].

Several articles demonstrated the presence of metabolic enzymes in the lung. Willey et al. [52] performed reverse transcriptase polymerase chain reaction (RT-PCR) and identified that cytochromes P450 (CYP), types 1A1, 1B1, 2B7, 2E1, 4B1, and 2F1, were expressed by human bronchial epithelial cells, which indicates that the types of enzymes present among different parts of the respiratory tract are likely to vary from the cellular components within the tract. In another study, CYP3A was detected in human lungs, with the authors concluding that the most frequently observed CYP3A expression is of CYP3A5, localized on the bronchial wall and glands, type I and II alveolar epithelium, vascular endothelium, and alveolar macrophages, though a variation in expression among individuals most likely takes place [53]. Similarly, different types of NADPH oxidases (NOX) in the lungs are responsible for innate immune responses [59].

With the focus on miR pulmonary administration, nuclease activity in the lungs has been reported to be diminished, which is attributed to the successful administration of unmodified nucleic acids to the lungs [20]. Further analysis of the nuclease activity in the lungs may be necessary to establish the decreased levels confidently.

3. miRs Local Delivery to the Lungs—A Brief Overview of Their Application and Their Delivery Approaches for Local Treatment

Pulmonary delivery of nucleic acids has the same advantages as described above for any other drug type, including reduced non-specific tissue distribution and increased local nucleic acid concentration in the lungs. Furthermore, as nucleic acids can degrade in circulation, avoiding systemic administration can minimize this effect [7,8]. As inhalation is the most prominent method for pulmonary delivery in humans, intranasal and intratracheal administration are the most frequently used in animal models due to their simplicity [60]. Thus, miR therapeutics have been studied for pulmonary delivery primarily using these two routes of administration.

miRs, short non-coding RNAs of ~22 bases, are steadily recognized for their significance in diseases. miRs, being natural endogenous products of the cells, regulate gene expression through the cell's RNAi mechanism, similar to siRNAs. miRs target mRNAs with which they present partial complementarity, causing translational repression. The ability of the miRs to have partial complementarity with targeted mRNAs allows them to affect the expression of numerous genes and regulate multiple pathways [7,8]. This is particularly important as miR dysregulation occurs in diseases, so they are being used as predicting biomarkers or potential targets/tools for treatment [61–63]. For example, miR dysregulation occurs in cancer, with one of the best-studied miRs being miR-34a. This miR has been identified as dysregulated in numerous cancers, has been characterized as a tumor suppressor, and has been used for the treatment of colon [64], lung [65], breast [66], and prostate [67] cancers, among others [68]. The extensive literature on miR-34a led to its eventual clinical evaluation [6,69].

Similarly, miR dysregulation takes place in lung diseases. For example, miR dysregulation has been identified in lung cancer [70,71], chronic obstructive pulmonary disease [72], lung injury [73], pulmonary hypertension [74], and asthma [75], among others. With nucleic acids becoming powerful tools for regulating gene expression and pathway activity, delivery of the nucleic acids in vivo presented significant challenges associated with their stability and capacity to enter the cells [7,8]. Nanotechnology-based methodologies for

drug delivery have rapidly proliferated in the last few decades, with novel systems demonstrating unique and promising capabilities [76]. There have been a growing number of clinical trials and publications utilizing nano-based carriers to deliver a plethora of drugs, including nucleic acids [77,78]. Nanocarrier systems have evolved to accommodate the sensitive nucleic acids, protect them from degradation, and deliver them in vivo to the cells.

In the following sections, we briefly describe representative examples of nanotechnology-based systems used for the delivery of nucleic acids, with a focus on pulmonary administration, utilizing inhalation, intratracheal, and intranasal administrations. Subsequently, we assess what methodologies have been evaluated for the delivery of miRs and their purpose for treating lung diseases. Our analysis gives an overview of four main categories of delivery systems: polymer-based, lipid-based, inorganic nanoparticles, and naked nucleic acids.

3.1. Polymer-Based Nanoparticles

Polymer-based nanocarriers have proliferated in recent years due to their safety, versatility, and capacity to carry various drugs with diverging physicochemical properties [76]. Natural and synthetic polymers are utilized for encapsulating active pharmaceutical compounds for the treatment of cancer [79], inflammation [80], diabetes [81], infections [82], Alzheimer's [83], and osteoarthritis [84], to name a few, among others. As nano-delivery approaches have demonstrated the capacity to be introduced into or researched for compound-based therapeutics of various characteristics and diagnostic methodologies, polymer-based systems have presented a reliable approach. Similarly, polymer-based systems have been utilized to deliver nucleic acids, including siRNAs, miRs, shRNAs, plasmids, mRNAs, and others [7,9].

Polymers have versatile synthetic processes and preparations of their respective nanocarriers, which permit researchers to modify their properties accordingly for each application. The most commonly used nanocarriers for nucleic acid delivery rely on positively charged materials due to their capacity to complex with the negatively charged nucleic acids, such as poly-amines, like polyethyleneimine (PEI) [85], polyamidoamines [86], polylysine [87], protamine [88], or natural polymers, such as chitosan [89] and gelatin [90] (Figure 3).

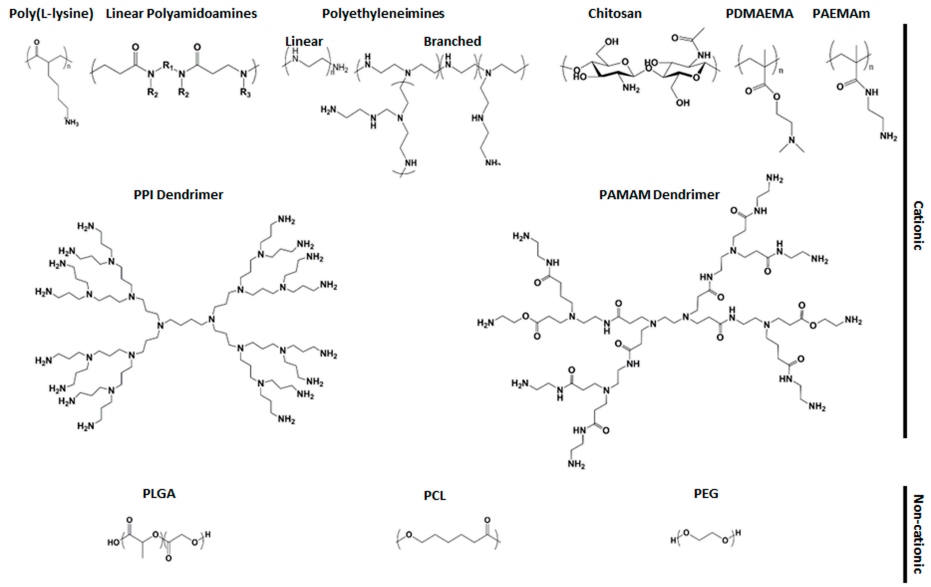

Figure 3. Representative structures of polymers used for nucleic acid delivery. PDMAEMA: Poly(2-(dimethylamino)ethyl methacrylate); PAEMAm: poly(2-aminoethyl methacrylamide); PPI: Poly(propylene imine); PAMAM: Polyamidoamine; PLGA: Poly-lactide-co-Glycolide; PCL: Poly-ε-caprolactone; PEG: Poly-ethylene glycol.

Furthermore, it has been suggested that polymer-based nanocarriers may present decreased immunogenic responses from the host, though this may vary depending on the polymer [91]. Similarly, the biocompatibility and, more importantly, the inherent cytotoxicity of the cationic polymers may significantly differ among the different materials [92].

Polyethyleneimine is a synthetic polymer that has been extensively studied, due to its capacity to complex with nucleic acids such as siRNA [93], DNA [94], miR [95], and mRNA [96]. In fact, PEI is frequently considered a go-to material for exploratory transfection studies due to its capacity to efficiently transfect cells, although its biodegradability has been a concern for in vivo applications [97,98]. One example of PEI's application for pulmonary delivery was the development of spray-freeze-dried siRNA/PEI powder [99]. The authors prepared siRNA against luciferase and complexed it with branched PEI (MW: 70 kDa), along with or without additional materials, such as L-leucine and D-mannitol, in water. The samples were spray-freeze-dried to produce fine powders of ~ 10 µm diameter, capable of being aerosolized for lung delivery. The powder induced strong gene silencing via intratracheal administration in mice.

In contrast, non-cationic polymers have also been used to entrap nucleic acids for delivery. This includes poly (lactide-co-glycolide) (PLGA) polymers, which are synthetic, FDA-approved, biocompatible, and biodegradable polymers that have been traditionally used for drug delivery applications [100] and the formulation of nanocarriers [101]. The polymers have been used directly to encapsulate nucleic acids [101,102] with endosomal escape promoted by a selective reversal of the nanocarrier's surface charge [102]. As these polymers form hydrophobic cores, and to further enhance the endosomal escape, the encapsulation of the hydrophilic nucleic acids inside the nanoparticles can be assisted by other materials, such as PEI [103,104]. One example would be from Frede et al. [105], where the authors developed nanoparticles that consisted of siRNAs coated on a calcium phosphate core, which was encapsulated inside PLGA nanoparticles. Finally, the authors coated the nanoparticles with PEI, and the final constructs were administered intranasally to mice with pulmonary inflammatory diseases using siRNAs against pro-inflammatory mediators, with therapeutic benefits against the respective diseases [105].

3.2. Lipid-Based Carriers

Liposomes are among the most commonly known and used nanocarriers, as evidenced by the fact that liposomal doxorubicin, Doxil, was the first cancer-treating nanocarrier approved by the FDA [106]. Liposomes can have different classifications, including multilamellar or unilamellar vesicles, long-circulating, flexible, immunoliposomes, and others, depending on their structure, composition, size, and surface modifications [107].

Two major types of lipid-based nanocarriers have attracted attention for the delivery of nucleic acids: (a) cationic liposomes and (b) lipid nanoparticles. Cationic liposomes are similar in structural characteristics to traditional liposomes, having a hydrophilic core and a lipid bilayer, and can be prepared through thin-film and extrusion methodologies, with the negatively charged nucleic acids complexing with the lipids during or after the carrier formulation, forming lipoplexes [108,109]. Furthermore, cationic liposomes (Figure 4) usually have a positive surface charge, and the cationic lipids have been associated with non-specific binding and potential toxicity, though this depends on the overall composition of the lipids and surface modification of the carriers [110,111]. It has been reported that plasma proteins bind onto the cationic liposome's surface due to their positive charge, which promotes their clearance in circulation by the reticuloendothelial system (RES) and alters their in vivo behavior [109]. Nonetheless, numerous clinical trials with cationic liposomal systems are undertaken to deliver nucleic acids [109]. In one example of cationic liposomes used for pulmonary delivery, cationic liposomes using DOTAP and the thin-film hydration/extrusion method were prepared with siRNA. The liposomes were intratracheally administered to animals and presented prolonged lung retention compared to intravenous administration.

In contrast, lipid nanoparticles (LNPs; Figure 4) commonly form a solid, micelle-like structure at their center instead of the familiar single, aqueous core of liposomes [5,112]. Lipid nanoparticles are usually formulated using an ionizable lipid, such as SM-102 or DLin-MC3-MDA, which is ionized at low pH values and neutral at physiological pH values. This allows for the complexation of the nucleic acids with the lipids during the preparation in an acidic environment, and an eventual neutral to slightly negative carrier form after buffer exchange. Furthermore, lipid nanoparticles utilize helper lipids, such as DSPC and DOPC, and cholesterol to build the outer layer of the nanoparticle, which contributes to the production of a neutral to negative surface charge and the uptake/transfection of the LNPs [5]. An example of LNPs used for pulmonary administration is from Zhang et al. [113], where the authors evaluated the formulation parameters of LNPs to deliver mRNAs. Subsequently, the authors administered the formulations intratracheally and evaluated the expression of luciferase in vivo following intraperitoneal injection of luciferin. The authors identified how the lipid composition could impact LNP stability for aerosolization and presented that intratracheal administration can induce gene expression with this approach.

Finally, lipid nanoparticles have become prominent in recent years due to their clinical translation to patient treatment. For example, Onpattro/Patisiran is the first RNAi-based therapy using LNPs [78], and the COVID-19 vaccine using LNPs was recently developed [114]. Several other products are also evaluated or approved for patient treatment [115]. Another example of a clinical trial using LNPs through inhalation is MRT5005, a LNP formulation of mRNA encoding cystic fibrosis transmembrane conductance regulator for treating cystic fibrosis (NCT03375047) [116].

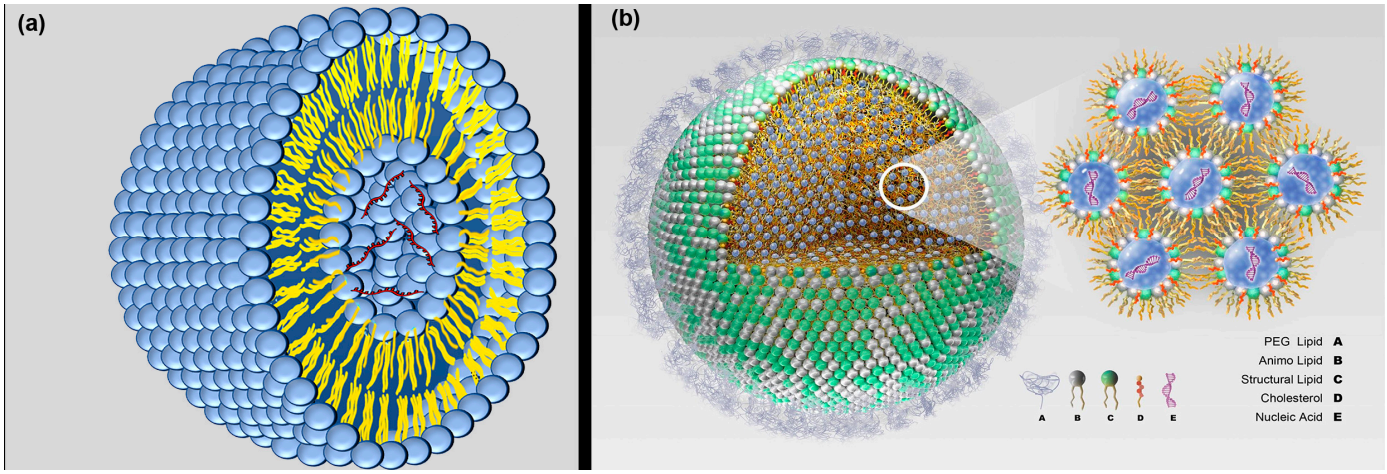

Figure 4. Illustration of (**a**) liposome and (**b**) lipid-nanoparticle structures (image is a reprint with permissions from [117]).

3.3. Inorganic Nanoparticle-Based Delivery

Inorganic nanoparticles (NPs) have been extensively explored in small molecule delivery in the past decade due to their significant advantages, such as chemically versatile formulations, uniform nanosizes suitable to evade biological barriers, traceability of inorganic NPs by various non-invasive imaging techniques, and overall less immunogenicity facilitated by decorating the core surfaces with suitable ligands [118].

Many studies have suggested the potential role of gold nanoparticles (AuNPs) for successfully delivering therapeutic molecules to the lungs [119–127]. AuNPs are used to incorporate drug molecules due to their notable inert characteristics, low cytotoxicity, photothermal attributes, and their ability to move around at a frequency as a function of their size and shape [128,129]. They exhibit versatile physical characteristics, as they can take various forms, including nanospheres [130–132], nanorods [133], nanostars [50,134,135], nanoshells [136–139], and nanocages [140,141]. Additionally, AuNPs are convenient to

functionalize through surface conjugation, granting them additional properties and delivery capabilities, with prolonged circulation and active targeting being the most commonly evaluated [128].

In a very interesting study conducted by Sukumar and colleagues [142], gold iron oxide nanoparticles (GIONs) coated with β-cyclodextrin-chitosan(CD-CS) hybrid polymer co-loaded with miR-100 and anti-miR-21 and decorated with PEG and glioblastoma (GBM) cell targeting T7 peptide were synthesized for intranasal delivery in a U87-MG-cell-derived orthotopic xenograft mouse model. The study aimed to sensitize the GBM cells with the miRs prior to systemic administration of a chemotherapeutic drug, temozolomide (TMZ), to increase the drug's efficacy. Furthermore, the potently cationic CD-CS hybrid polymers successfully encapsulated the negatively charged miRs via electrostatic interaction, followed by an electrostatic coating of miR-loaded CD-CS complexes with a net positive charge on the surface of the GION nanostars. Their data showed that the intranasal delivery of their formulation displayed efficient accumulation of Cy5-miRs in mice treated with the T7-targeting poly-GIONs, as the nanoparticles showed strong brain retention compared to only poly-GIONs. They also validated the synergistic roles of miR-100 and anti-miR-21, showing the decline of cell viability post-48 h by approximately 1.5-fold compared to only individual miR delivery. Furthermore, the authors showed that the co-delivery of the two miRs followed by TMZ administration at 100 μm is suitable for the optimal decline in cell viability. Overall, there was a significant increase in survival of mice co-treated with T7-poly-GIONs loaded with miR-100/anti-miR-21 plus systemic TMZ compared to the untreated control group or the animals receiving non-targeted poly-GIONs-miR-100/anti-miR-21 or TMZ alone [142]. Using the same design concept in a different report by the same group [134], they designed an intranasal drug delivery vehicle using gold nanoparticle chitosan (AuNS-chitosan) against SARS-CoV-2 (SC2) using DNA coding for S protein as antigen. Though the study was focused on the intranasal delivery of SC2-DNA loading against respiratory disease, it could be extended to the intranasal delivery of miR against lung-specific diseases. Furthermore, Liu et al. [50] formulated AuNPs coated with CD-CS that were further functionalized with a urokinase plasminogen activator (uPA) peptide to encapsulate a novel triple suicide gene (thymidine kinase-p53-nitroreductase: TK-p53-NTR) along with therapeutic miRs (anti-miR-21, anti-miR-10b, and miR-100) as a therapeutic intervention for lung metastases in mouse models via intranasal delivery. Initially, they employed a microfluidic-based coating process to optimize different forms of AuNPs: CD-CS-coated Au-nanostars at 20 and 50 nm, and Au-nanodots at 20 nm. After successive experiments for evaluating the NP concentration post-CD-CS coating, gene loading capacity and transfection efficiencies using Fluc-eGFP-pcDNA of all three formulations in HEK-293T and 4T1 cells were performed. The results for Au-nanostars sized at 50 nm (later named pAuNS) showed the highest transfection efficiency and were used for further experiments. Subsequently, the researchers assessed the TK-p53-NTR pDNA and miRs loading efficiency for pAuNS, which showed that the co-loading capacity of the suicide gene and miRs was 1 μg TK-p53-NTR pDNA and 200 pmol miRs for 0.06 μL of pAuNs. By further coating the pAuNs with a uPA peptide, uPA receptor-mediated endocytosis in 4T1 cells elevated the TK-p53-NTR pDNA and miRs gene loading and cell transfection efficiency by up to 4 folds. After establishing the optimized formulation and doses of the genes, they chose four formulations to administer to the mouse model in a 20 μL dose (5 μL/drop into the nares of the mouse). Their findings indicated that the TK-p53-NTR genes were significantly present throughout the lungs as opposed to other vital organs. Even though the TK-P53-NPR groups demonstrated around 39% tumor growth inhibition compared to only around 20% in the miRs co-delivered groups, they emphasized that the miR co-delivery improved the overall survival rates relative to the control mice groups. Therefore, these studies show that not only does the AuNP demonstrate their suitability in lung delivery, but also, through miRs co-delivery with other therapeutic agents or small biomolecules, favorable therapeutic effects can be augmented.

3.4. Naked Nucleic Acids

Naked nucleic acids refer to the administration of nucleic acids without any transfecting agent or carrier. Briefly, nucleic acids are directly administered in an aqueous solution, such as phosphate buffered saline (PBS), saline, or pure water. Systemically, the administration of unmodified nucleic acids results in limited transfection efficiency due to their rapid elimination through degradation by nucleases, among other barriers [143,144]. To overcome this limitation, modified nucleic acids have been developed and used, where chemical modifications to the structure of the nucleic acids enhance their stability against nuclease degradation, improve uptake from cells, and can diminish immunogenic responses [145]. These chemical modifications (Figure 5) include phosphodiester linkage, 2′-oMe, 2′-MOE, 2′-F, locked nucleic acids (LNA), cholesterol modification, or the introduction of other conjugations for increased cell type selectivity [146–148].

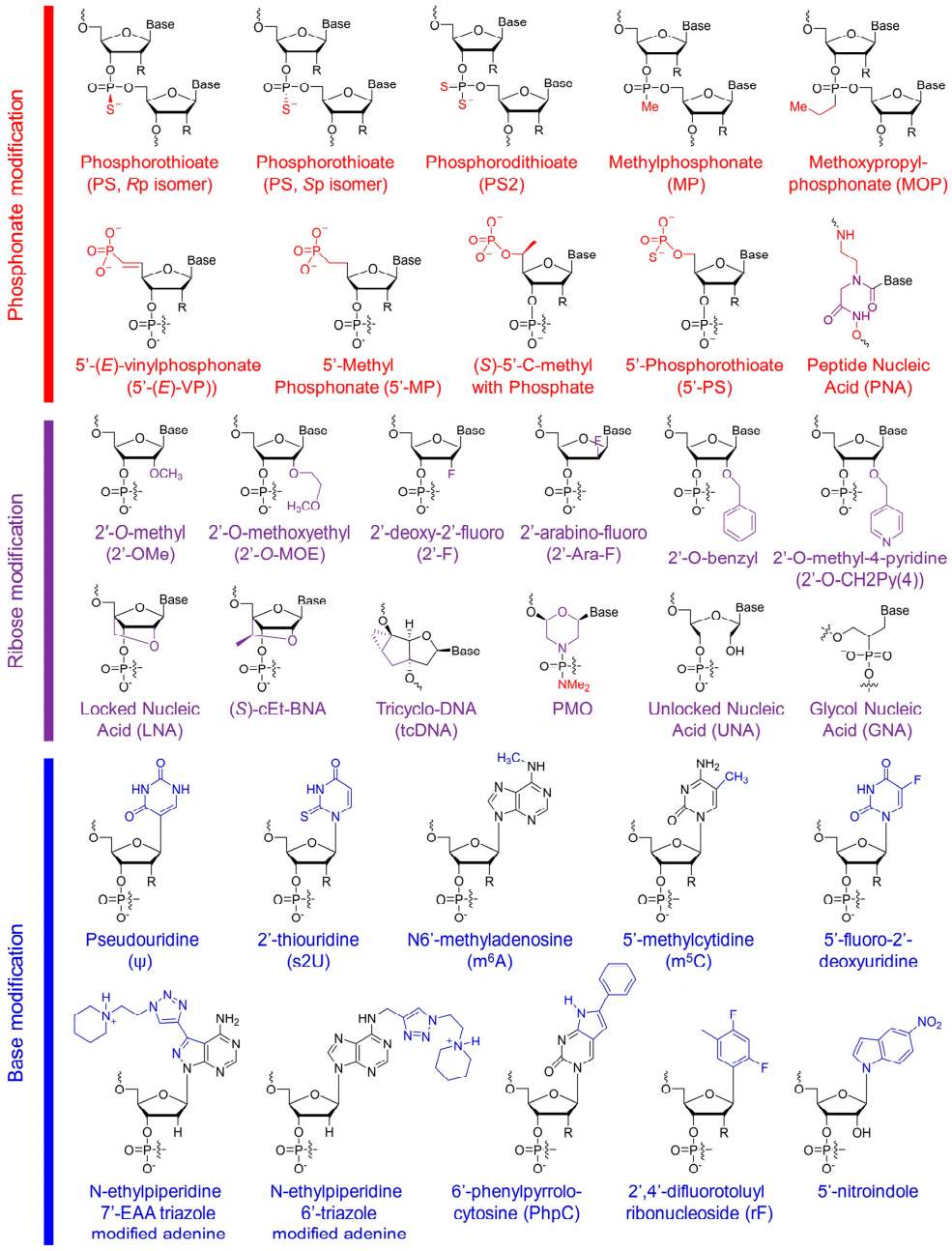

Figure 5. Representative chemical modifications for improving short nucleic acid stability and/or delivery. The image is a reprint from [148].

In fact, several products have received FDA approval for patient treatment and rely on modified nucleic acid technology [115,149]. Interestingly, a decreased nuclease activity in the lungs has been reported [20], which also correlates to a significant number of studies that evaluate naked nucleic acid administration to the lungs with promising results and demonstrate that in vivo nucleic acid application to mucosal surfaces does not necessitate a transfection vehicle [150]. One example is Fulton et al.'s [151] work, where the authors administered siRNAs intranasally with and without a transfecting agent (i.e., lipofectamine). The authors concluded that the naked siRNAs successfully induced gene downregulation without the need for the transfecting agent [151], though the utilization of a transfection agent has been reported that can provide advantages in terms of stronger transfection or active targeting [91].

Nonetheless, the advantages of nucleic acid administration without needing a transfecting agent, which adds complexity to the system and has potential side effects from any cationic components, were evaluated in clinical trials. ALN-RSV01 is a naked siRNA that was evaluated in clinical trials (NCT00496821, NCT00658086, and NCT01065935) against viral respiratory infection [116], and it was reported that the treatment was well tolerated in vivo and potentially beneficial for patients [148].

3.5. Pegylation and Lung Mucosa

As described above, one of the significant barriers to pulmonary delivery through inhalation is the mucus layer on the lung airways. The viscoelastic characteristics of mucus result from mucin fibers creating an interconnected network between the molecules and can greatly affect the penetration of nanocarriers and compounds through the mucosa [152]. With the properties of any materials being classified as more or less mucoadhesive, their residence time in the respiratory tract can greatly be affected, recognizing that the mucus layer is in motion and can be cleared through mucociliary clearance at a rate of 1–10 mm/min, though this clearance can be affected by diseases. This is a natural defense mechanism of our bodies to protect from undesired particles, clearing the luminal gel layer every ~10–20 min [24,153].

Pegylation, i.e., the attachment of a hydrophilic, neutral polyethylene glycol polymer chain to the surface of a nanocarrier or compound [154,155], has extensively been applied for delivery purposes due to the methodology's fundamental advantages. For example, pegylation of carriers or compounds has been reported to: (a) improve the pharmacokinetic profile (i.e., increase half-life); (b) decrease opsonization and immunogenicity; (c) improve aqueous solubility; (d) decrease toxicity; (e) increase carrier stabilization; and (f) decrease compound degradation [154–156]. Several FDA-approved products currently exist for patient treatment that incorporate pegylation [157]. One example is also Doxil, the pegylated liposomal doxorubicin used for cancer treatment.

With pegylation being commonly incorporated into drug delivery carriers, the effect of pegylation on penetration through the mucus layer is a subject of interest. Pegylation can have a complex impact associated with its transportation through the mucus layer. It is generally understood that a decrease in PEG coverage of nanoparticles reduces particle penetration through a mucus layer [158].

In an article from Conte et al. [159], the authors evaluated how pegylation affects the penetration of hybrid lipid/polymer nanoparticles in the lung mucosa for inhaled siRNA therapy. The authors prepared two nanoparticle formulations, which consisted of a PLGA core and a lipid shell with a siRNA against nuclear factor-κB, with one formulation being coated with PEGs (using DSPE-PEG) and the other without PEGs (using DPPC). The nanoparticle formulations were evaluated in vitro using artificial cystic fibrosis mucus, mucus from cystic fibrosis cells, and sputum samples from patients with cystic fibrosis. The authors reported that pegylation could inhibit nanoparticle penetration and cell uptake. For example, mucus-lined Calu-3 cells demonstrated that non-pegylated nanoparticles efficiently crossed the mucus and cellular barriers. This led to the conclusion that non-pegylated nanoparticles may be advantageous for the pulmonary delivery of nucleic

acids, especially under pathological conditions and in the presence of gel-forming mucins MUC5AC and MUC5B, compared to their pegylated counterparts [159].

Contrastingly, Schuster et al. [160] evaluated nanoparticle diffusion in human respiratory mucus from healthy lungs. The authors prepared polystyrene nanoparticles of different diameters with and without a coating of 5 kDa methoxy-PEG. They identified that 100- and 200-nm particles coated with PEG rapidly penetrated the mucus 15 and 35 times faster than the uncoated nanoparticles of the same dimensions, respectively. In contrast, >500 nm nanoparticles coated with PEG did not transverse the mucus layer. The authors reported that the nanoparticles demonstrated different penetration capacities with mucus from different organ origins. Similarly, Suk et al. [161] prepared DNA nanoparticles using polyethyleneimine and poly-L-lysine with a dense 5 kDa PEG coating and compared them to uncoated nanoparticles. The authors determined that nanoparticles with smaller sizes and PEG coatings efficiently transverse mucus from cystic fibrosis patients compared to larger or uncoated carriers. This was a follow-up study the group had prepared, concluding that insufficient PEG coverage on the nanoparticle's surface can limit sputum penetration of the carriers [162].

These results demonstrate the significance of PEG coating in mucus penetration and how PEG can be beneficial or detrimental to mucosal penetration. This was also elaborated in an earlier paper by Wang et al. [158], where the authors presented PEG coating's contradicting behavior. The authors evaluated the different parameters, such as PEG coverage percent on the surface of polystyrene nanoparticles and the MW of PEG. They concluded that PEG coating of nanoparticles with a dense low-MW PEG layer diminishes the particles' hydrophobic interactions with mucus, promoting their penetration [158].

Thus, despite the challenging effects of nanocarrier pegylation for mucus penetration, it would appear that the overall studies lean towards the fact that pegylation assists mucus penetration, recognizing that certain conditions, such as PEG density on the carrier surface and PEG MW, need to be taken into consideration during carrier development [32].

3.6. miR delivery to the Lungs: Current Application and Updates

With miRs and siRNAs having similar structures, pulmonary delivery approaches developed for siRNAs should easily translate to the delivery of miRs. As several publications review siRNA delivery to the lungs (indicatively: [60,163]), here we focus on miR delivery research, what has taken place, the methodologies used for their pulmonary administration, and the diseases currently targeted for treatment.

Inhalation has been a traditional approach for delivering active compounds for treatment of lung diseases in humans due to the local action of compounds, the reduced side effects, and the simplistic and non-invasive nature of the method. Inhalation requires the nebulization of any solution to be inhaled, which makes dosing challenging to control as it relies on aerosolization efficiency, the volume of the tubing/aerosolization chamber/device, and the rate of breathing (tidal effects) [164]. In contrast, intratracheal administration has been extensively used in animal studies and involves the instillation of a solution into the trachea for evaluating the effects of compounds or viruses. This takes place in a more controlled and simplified manner, where dosing can be more accurate, even though it does not mimic the inhalation of compounds [165]. Finally, intranasal administration has also been frequently studied to substitute inhalation in animal studies for pulmonary administration. The application relies on applying a small volume of the tested solution to the animal's nasal area, allowing the tidal forces of the animal's breathing to carry it to the lungs [166]. An unintended consequence of intranasal administration is that the drug may also be deposited/absorbed in the nasal cavity or upper respiratory tract, not just in the lungs [166]. Interestingly, this also allows for bypassing the blood–brain barrier. Thus, intranasal administration has been attempted to target central nervous system diseases [167].

An interesting analysis of the different administration approaches for miRs was performed by Schlosser et al. [168]. The authors evaluated the optimal delivery method for

cel-miR-39 in rats using intratracheal liquid instillation or aerosolization and intranasal liquid instillation or aerosolization and compared these to intravenous, intraperitoneal, and subcutaneous deliveries. The miR mimic was administered using Invivofectamine 3.0 as a transfecting agent. It was determined that all lung-targeting delivery approaches achieved lung-selective miR presence when compared to intravenous, subcutaneous, and intraperitoneal administrations, signifying the importance of local delivery for lung diseases. Furthermore, the authors concluded that intratracheal administration of a liquid produced the optimal results, significantly achieving the highest lung levels among all other administration methodologies, whether they were lung-targeting or not [168].

3.6.1. Intratracheal Administration of miRs

As intratracheal administration presents significant advantages in precise dosing and simplicity, not surprisingly, intratracheal instillation has been one of the major approaches for miR delivery. Liu et al. [169] evaluated the intratracheal administration of anti-miR-21a for the treatment of fibrotic lung disease. The authors administered intratracheally a LNA-modified miR-21 inhibitor to an animal model of pulmonary fibrosis and identified a diminished severity of lung fibrosis in mice, which the authors attributed to a TGF-β1-mediated decreased pro-fibrogenic activity in fibroblasts. Using miR-26a to treat pulmonary fibrosis, Liang et al. [170] developed a cholesterol-conjugated miR-26a mimic and administered the product through intratracheal injection to animals that had induced pulmonary fibrosis. It was concluded that miR-26a attenuated fibrogenesis in vivo, and the authors identified a positive feedback loop between p-SMAD3 and miR-26a, where p-SMAD3 is involved in the progression of idiopathic pulmonary fibrosis. In an attempt to inhibit pulmonary fibrosis using miR-200c, Yang et al. [171] introduced the miR mimic dissolved in saline through intratracheal instillation without using a transfecting agent. The treatment ameliorated pulmonary fibrosis in mice, and the authors presented the importance of the miR-200 family in the disease's progression.

Zhuang et al. [172] recently evaluated the intratracheal administration of a miR-92a inhibitor to treat acute lung injury. The anti-miR-92a (AMO92a) oligonucleotide was administered with an RP1-linked R3V6 peptide, which also has anti-inflammatory effects in the lungs as RP1 is an advanced glycation end-products receptor antagonist. The administration of the peptide-oligonucleotide complexes inhibited miR-92a levels more efficiently than R3V6/AMO92a and PEI/AMO92a complexes. Furthermore, the complexes decreased the TNF-α and IL-1β levels, indicating a decrease in lung damage in an animal model of acute lung injury. In another study, Bobba et al. [173] recently formulated miR-146a in LNPs. They identified that increasing miR-146a during mechanical ventilation can mitigate lung injury, and a nanocarrier system is an effective approach for delivering the pre-miR-146a. To prepare the nanoparticles, empty LNPs were prepared using DOPE, DOTAP, linolic acid, and TPGS, and pre-miR-146a was mixed with PEI (2k) at an N/P ratio of 10 to form complexes. The resulting polyplexes were mixed with the empty LNPs at a lipid-to-nucleic acid mass ratio of 10 to form the miR-146a-loaded LNPs before being intratracheally instilled in the mice. In a similar approach, Niemiec et al. [47] developed cerium oxide nanoparticles (nanoceria) for the delivery of miR-146a for the treatment of acute lung injury, also using intratracheal administration. According to the paper, the nanoparticles were prepared using a precipitation method, and the miR-146a was conjugated to the particles using a 1,1-carbonyldiimidazole (CDI) coupling method between the nucleic acid's amino group and the nanoparticle. The final products were diluted in PBS prior to intratracheal administration to the animals, preventing acute lung injury. In an earlier study, naked miR-146a (i.e., without using a nanocarrier) was also administered intratracheally to ameliorate lung injury [174]. In another study for the treatment of lung injury, miR-802 was administered intratracheally to animals with the assistance of Invivofectamine 3.0. The authors identified that intratracheal administration of the miR had a protective role against acute lung injury through Peli2 mediation [175].

Using the same transfecting agent, Courboulin et al. [176] evaluated the role of miR-204 in the treatment of pulmonary arterial hypertension. The authors intratracheally nebulized miR-204 mimic along with Invivofectamine for administration to rats, and the treatment decreased disease severity in male rats with induced pulmonary arterial hypertension. In a recent study on pulmonary arterial hypertension, Ma et al. [177] evaluated the inhibition of miR-30a for treating the disease. The authors used miR-30a inhibitors and mimics to assess the activity of the miR in vitro using lipofectamine 3000, while they used naked miR mimics and inhibitors in PBS for in vivo administration through intratracheal instillation. They concluded that miR-30a inhibition could inhibit pulmonary arterial hypertension in mice by mediating the p53 signaling pathway.

Exosomes are extracellular vesicles secreted from cells and carry molecules from the original cells, such as proteins, RNA, receptors, and others. They are part of the cell-to-cell communication system and can contribute to or regulate diseases. As exosomes are small vesicles in the submicron dimension and carry receptors that allow them to target specific cell sub-types, they were identified as potential carriers for drugs, including nucleic acids [178]. In two interesting studies from the same group, the authors utilized exosomes for the intratracheal administration of miRs. The approach relied on the isolation of exosomes from either serum or murine macrophages and the post-loading of short nucleic acids, such as a miR-15a mimic or inhibitor. This was achieved using a modified calcium-mediated transfection or electroporation directly on the exosomal samples. Subsequently, the modified exosomes were administered intratracheally to animals. The authors concluded that intratracheal administration of exosomes with enriched miR content could be a viable approach for administering miRs to the lungs [48,179].

3.6.2. Intranasal Administration

As an alternative to intratracheal administration, intranasal administration presents similar advantages due to its simplicity and capacity to deliver efficient compounds, as well as the potential administration to the central nervous and upper respiratory systems [167,180]. Intranasal administration has traditionally been used in humans to absorb compounds in the nasal cavity, whether for treating local symptoms in the upper respiratory tract, systemic administration of compounds, or brain disorders [167]. In contrast, in rodents, intranasal administration can be used to deliver substances to the upper and lower respiratory tracts [166,181].

For example, Olave et al. [182] identified that miR-489 is involved in hyperoxia-induced abnormal lung development. Using mice exposed to various oxygen environments along with the intranasal administration of a LNA-miR-489 inhibitor suspension in water, the authors determined that inhibition of miR-489 in the lungs can potentially assist in alveolar septation. In a similar study with detailed experimental procedures for intranasal administration, Deng et al. [183] evaluated the inhibition of miR-143/145 expression to prevent chronic hypoxia-induced pulmonary hypertension in mice using intranasal administration. The nucleic acids were dissolved in PBS before administration, and the intranasal dosing of anti-miR-145 successfully decreased the expression of miR-145 in the lungs.

Targeting inflammation in the respiratory tract, Song et al. [184] identified that miR-218 was significantly downregulated in chronic obstructive pulmonary disease patients. To evaluate the role of miR-218, the authors used a miR-218 inhibitor in water administered to mice through intranasal administration, and they concluded that miR-218 has a protective role in cigarette smoke-induced inflammation and chronic obstructive pulmonary disease. In another study, Mattes et al. [185] evaluated miR-126 for allergic airway disease. Intranasal administration of a modified miR-126 inhibitor in saline suppressed the asthmatic phenotype and inflammation in mice. The same group later reported a similar approach for nucleic acid delivery, where they evaluated the intranasal administration of a modified miR-155 antagomir. The authors evaluated whether intranasal administration of the antagomir could downregulate miR-155 expression to alter the disease phenotype in

an ovalbumin-induced allergic airway disease model. Although the phenotype was not altered, miR-155 expression was reduced in the lung immune cells [186].

As the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) developed into a worldwide pandemic, the COVID-19 disease emerged as a significant health risk of particular immediate interest. With the disease being transmissible through inhalation and having a negative impact on the lungs and their function, a connection between lung miR dysregulation and the disease was evaluated [187]. McDonald et al. [187] reported the importance of miR-2392 and SARS-CoV-2. The authors identified that circulating miR-2392 correlated with downstream suppression of gene expression at mitochondria, increased inflammation, hypoxia, and glycolysis, in addition to other symptoms associated with COVID-19. To this end, the authors developed a miR-2392 inhibitor as an antiviral therapeutic, administered intranasally or intraperitoneally, and determined that the treatment significantly reduced viral viability in hamsters.

As we mentioned above, intranasal delivery allows for the administration of drugs to the central nervous system and brain, bypassing the BBB. To this end, miRs have been evaluated for brain delivery using intranasal administration. For example, Su et al. [188] reported on developing targeted PLA-PEG nanoparticles decorated with wheat germ agglutinin, which has been reported to bind to oligosaccharides on nasal epithelial cells, promoting delivery from the nose to the brain. The authors entrapped miR-132 in the nanoparticles, with the miR being initially complexed with spermidine, and intranasally treated animals after cerebral ischemia. The nanoparticles demonstrated accumulation in the brains of rats, increasing the levels of miR-132 in the temporal cortex, and it was concluded that the approach could be used to cross the BBB and treat brain diseases.

These examples demonstrate that the intratracheal and intranasal routes of administration for miRs have been a reliable approach for treating a variety of lung diseases (and beyond), while the carriers used for the administration have not been the particular focus for many of these research papers. In fact, the use of LNA, chemically modified nucleic acids, or naked nucleic acids has been extensively utilized with promising results, at least for evaluating the activity of the respective nucleic acids. The use of a drug carrier can potentially provide benefits (i.e., improved transfection or active targeting), but the simplicity of successful pulmonary delivery and transfection without a transfecting agent can be valuable for the evaluation of miR activity. In Table 1, we summarize research on intratracheal and intranasal administrations of miRs we identified during the preparation of this article for a direct comparison of the two routes of administration and the potential carrier systems used.

Table 1. Intratracheal, intranasal and inhalation administration of miRs.

miR (Or Target)	Disease or Targeted Organ	Carrier	Ref.
	Intranasal		
miR-132	Brain (Neurodegenerative Diseases)	PLA-PEG nanoparticles with wheat germ agglutinin decoration. Nucleic acids were premixed with spermidine	[188]
Anti-miR-21 and miR-100	Glioblastoma	T7 peptide decorated Gold-Iron oxide nanoparticles	[142]
	Glioblastoma	Microfluidically processed extracellular vesicles	[189]
Anti-miR-21, miR-100 and anti-miR-10b	Triple Negative Breast Cancer induced lung metastases	Microfluidics optimized CS-CD coated Au-nanostars decorated with uPA peptide via host-guest chemistry	[50]
Anti-miR-146a	Alzheimer's	Chemically modified nucleic acids in water	[190]
Anti-miR-155-5p	Hippocampal Inflammation	Chemically modified nucleic acids in water	[191]
Let-7	Lung cancer	Adenovirus	[192]

Table 1. *Cont.*

miR (Or Target)	Disease or Targeted Organ	Carrier	Ref.
miR-155 agomir and antagomir	Allergic rhinitis	Saline	[193]
miR-127	Lung Inflammation	Chemically modified nucleic acids and LNA in PBS	[194]
miR-135a	Allergic rhinitis	Chemically modified nucleic acids in saline	[195]
	Allergic rhinitis	Lentiviruses	[196]
miR-410	Airway inflammation	Chemically modified nucleic acids in water	[197]
miR-223-3p	Allergic rhinitis	Saline	[198]
Let-7i	Traumatic Brain Injury	Water	[199]
miR-203 antagomir	Chronic epilepsy	Chemically modified nucleic acids in PBS	[200]
Anti-miR-134	Epilepsy seizures	LNA and chemically modified nucleic acids in water	[201]
miR-124	Ischemic brain injury	RVG29-modified PLGA-PEG nanoparticles. Nucleic acids were premixed with spermidine	[202]
Anti-miR-21	Glioblastoma	Self-assembled nanoparticles of RAGE-antagonist peptide and nucleic acids	[203]
miR-219a-5p	Multiple sclerosis	DSPC liposomes, PLGA nanoparticles, and extracellular vesicles comparison	[49]
Anti-miR-210	Hypoxic-ischemic brain injury	LNA in saline	[204]
Anti-miR-143/145	Pulmonary Hypertension	LNA in PBS	[183]
Anti-miR-223-3p	SARS-CoV-2/lungs	LNA	[205]
miR-29	Allergic rhinitis	Saline	[206]
miR-146a	Temporal lobe epilepsy	Water	[207]
Anti-miR-155	Allergic airways disease	Chemically modified nucleic acids	[186]
Anti-miR-489	Bronchopulmonary dysplasia	LNA in water	[182]
Anti-miR-126	Allergic airways disease	Chemically modified nucleic acids in saline	[185]
Anti-miR-218-5p	Chronic obstructive pulmonary disease	PBS	[184]
miR-2392	SARS-CoV-2/lungs	Peptide nucleic acid in nanoparticles (nanoligomer SBCoV207)	[187]
miR-101	Pulmonary fibrosis	Adenoviruses	[208]
	Intratracheal		
Anti-miR-92a	Acute Lung Injury Model	RP1-linked R3V6 peptide complexed with nucleic acids	[172]
Anti-miR-21	Lung fibrosis	PBS	[169]
miR-146a	Lung injury during mechanical ventilation	Lipid nanoparticles	[173]
	Acute lung injury	Cerium oxide nanoparticle	[47]
	Acute Lung Injury	Saline	[174]
	Lung injury during mechanical ventilation	Lipid nanoparticles	[173]
miR-802	Acute lung injury	Invivofectamine	[175]
miR-204	Pulmonary arterial hypertension	Invivofectamine	[176]
Anti-miR-26a	Idiopathic Pulmonary Fibrosis	Chemically modified nucleic acids	[170]

Table 1. *Cont.*

miR (Or Target)	Disease or Targeted Organ	Carrier	Ref.
Anti-miR-30a	Pulmonary arterial hypertension	PBS	[177]
miR-15a agomir and antagomir	Lung	Exosomes	[48]
miR-200c	Pulmonary fibrosis	Saline	[171]
Let-7	NSCLC	Lentivirus	[209]
miR-663	Pulmonary arterial hypertension	Adenovirus	[210]
	Inhalation		
Let-7b	Lung cancer	Aerosolization using a custom-made collision-type atomizer	[211]
miR-17	Bronchial epithelial cells	Nebulized lipid–polymer hybrid nanoparticles	[212]

4. Conclusions and Future Perspectives

Targeted delivery of small biomolecules has received favorable attention and can be considered promising for their direct role in gene regulation. It is evident that with the advent of the newer generation of drug carriers, the innate negatively charged nature of the small RNAs such as miR, siRNA, shRNA, circRNA, etc. can be exploited for successful delivery into the cells. Among the small RNAs, while siRNAs have become a major focus of interest in targeted delivery, including pulmonary delivery, and have been extensively reported in the literature, limited literature exists on other types of nucleic acids. Given the similar mode of action of siRNAs and miRs in the cells' RNAi mechanism, both of these small molecules often have large similarities [213]. However, unlike siRNAs, miRs are endogenously transcribed and regulate more than one target mRNA. Given the complexity of gene deregulation in diseases such as cancer, cystic fibrosis, and others, miRs can be used to regulate multiple critical pathways associated with each disease. Thus, miRs can be employed in combination therapies to supplement existing therapeutics and upregulate downregulated miRs, or they can be targeted in antisense miR applications for their respective downregulation if upregulated. Despite the extensive literature presence of siRNA-focused reviews, limited literature reviews exist on miR-based pulmonary. Here, we have accumulated impactful research that demonstrates the promising future of miRs using pulmonary delivery.

As miR-based therapeutics are promising therapeutic tools for treating multiple diseases, including lung diseases, pulmonary delivery methodologies have expanded for evaluating these novel therapeutic tools for directly targeting the lungs. In animal models, intratracheal and intranasal administration of the nucleic acids appear to dominate the delivery methodologies for the administration of miRs. At the same time, using nanocarrier systems is not necessarily the main focus of current research. Instead, the activity of the miRs appears to be the main focus, and consequently, naked miRs provide sufficient effect to evaluate their activity. Despite that, carrier systems may present benefits for the delivery of nucleic acids, even through the use of the pulmonary airways, as they may assist in cell targeting, nucleic acid stability, penetration through the mucosa, and overall transfection. With naked miRs (chemically modified or not) currently presenting a significant portion of the research on pulmonary delivery of miRs, the research on carrier systems should proportionately increase in the coming years, following any proof-of-principle for miR activity in the different lung diseases. Nonetheless, miR pulmonary delivery has presented a significant increase in recent years, and it should be anticipated that its benefits will be further investigated with the goal of patient care translation, despite any current limitations.

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