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Carriers for the Targeted Delivery of Aerosolized Macromolecules for Pulmonary Pathologies

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Abstract:

Introduction: Macromolecules with unique effects and potency are increasingly being considered for application in lung pathologies. Numerous delivery strategies for these macromolecules through the lung, have been investigated to improve the targeting and overall efficacy.

Areas covered: Targeting approaches from delivery devices, formulation strategies and specific targets are discussed.

Expert opinion: Although macromolecules are a heterogeneous group of molecules, a number of strategies have been investigated at the macro, micro and nanoscopic scale for the delivery of macromolecules to specific sites and cells of lung tissues. Targeted approaches are already in use at the macroscopic scale through inhalation devices and formulations, but targeting strategies at the micro and nanoscopic scale are still in the laboratory stage. The combination of controlling lung deposition and targeting after deposition, through a combination of targeting strategies could be the future direction for the treatment of lung pathologies through the pulmonary route.

Keywords: Aerosol Devices, Inhaled Macromolecules, Lung Delivery, Microcarrier, Nanocarrier, Nanomedicine, Lipid-based systems, Passive and Active Targeting, Pulmonary Pathologies

Article highlights:

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- Macromolecules for treatment of lung pathologies are heterogenous and can range from proteins such as antiproteases in cystic fibrosis to genetic material such as siRNA in asthma.
- Aerosolization is the main form of delivering materials to the lungs.
- Particle based carriers have been investigated for delivery of macromolecules, as they can be made with specific physicochemical properties to suit a variety of macromolecules and also allows for controlled release and targeting.
- Targeting can be in the form of passive or active targeting, referring to utilizing the unique physiological characteristics of the target environment, or specifically targeting molecules or components of the cell surface, respectively.
- Only a few molecular targets have been investigated for active targeting through the pulmonary route but a considerable number have been identified and could be possible avenues for future investigation.

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LIST OF ABBREVIATIONS

ACE	Aerosol Cloud Enhancer
APC	Antigen-Presenting Cells
ARCs	Archaeosomes
ARDS	Acute Respiratory Distress Syndrome
BCG	Mycobacterium Bovis Bacille Calmette-Guérin
BSA	Bovine Serum Albumin
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
COPD	Chronic Obstructive Pulmonary Disease
CPV	Canine Parvovirus
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
DPIs	Dry Powder Inhalers
dsRNA	Double-Stranded Ribonucleic Acid
EGFR	Epidermal Growth Factor Receptor
FPF	Fine Particle Fractrion
HA	Hyaluronic acid
hpIgR	Human Polymeric Immunoglobulin Receptor
IPF	Idiopathic Pulmonary Fibrosis
LHRH	Luteinizing Hormone Releasing Hormone
lncRNA	Long Non-Coding Ribonucleic Acid
MDIs	Metered Dose Inhalers
miRNA	Micro Ribonucleic Acid
MMP9	Matrix Metallopeptidase 9
MPs	Microparticles

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MW	Molecular weight
NCMPs	Nano-composite Micro Particles
NPs	Nanoparticles
O-SAP	O-Steroyl Amylopectin
PAMAM	Polyamidoamine
PCD	Primary Ciliary Dyskinesia
pDNA	Plasmid Deoxyribonucleic acid
PEG	Polyethylene Glycol
PEI	Polyethylenimine
PGA-co-PDL	Poly (Glyceroladipate-Co-ω-Pentadecalactone)
PLGA	Poly Lactic-Co-Glycolic Acid
PLL	Poly (L-Lysine)
PspA	Pneumococcal Surface Protein A
RNA	Ribonucleic Acid
SFNPs	Silk Fibroin Nanoparticles
siRNA	Small interfering Ribonucleic Acid
SLPI	Secretory Leukoprotease Inhibitor
SLS	Solid lipid nano- and micro-structures
ssRNA	Single-Stranded Ribonucleic Acid
TB	Tuberculosis
Tf-PEI	Transferrin-Polyethylenimine
tgAAVCF	Adeno-Associated Virus Vector Encoding The Human
TPL	Triptolide
VEGF	Vascular Endothelial Growth Factor

1. Introduction

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Pulmonary pathologies are main causes of morbidity and mortality worldwide, such as respiratory infections, chronic obstructive pulmonary disease (COPD), lung cancer, and asthma. Lung delivery of macromolecules through different carrier systems has gained increasing attention for the treatment of respiratory diseases and this review aims to provide a concise, up-to-date overview of recent advances in delivery strategies of macromolecules via inhalation to treat pulmonary pathologies.

1.1. Pulmonary Route for Inhaled Macromolecule Drug Delivery:

The pulmonary route is composed of two structural parts that have different physiological characteristics (Figure 1); the conducing airways and the respiratory airways. The conducting, or, upper airways, extend from the nose, pharynx, trachea and to the terminal bronchioles, branching approximately 17 times until reaching the respiratory bronchioles. The conducting part has pseudo-stratified columnar ciliated epithelium with mucous secreting cells, which are responsible for the mucociliary clearance of the airstream from any exogenous particles, dust, and bacteria. The thickness of the epithelium is approximately 60 μm with a thick mucus layer lined with a lung surfactant layer [1]. The surface area is about 2 m^2. The main functions are to transport the air to the gas-exchange area and to humidify, adjust the temperature, and filter of the incoming air stream.

There are numerous factors that can contribute to the sub-optimal delivery of aerosolized carriers or macromolecules in the conducting airways. One factor is the deposition of aerosolized matter against the walls of the airway. The size, shape and density of particles can influence the deviation of particle flow from the streamlines and result in subsequent impaction away from the main absorptive alveolar surface. Another factor is the humid internal environment (approaches 90%), which can increase of the size of the hygroscopic particles and favors their deposition and early mucociliary clearance [2]. Underneath, the tight junctions between the epithelial cells presents as another barrier for carrier and macromolecule flux. The

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conducting airway is associated with common diseases such as asthma, COPD, CF, and emphysema. The pathophysiology is variable according to the disease conditions leading to bronchoconstriction and increase in the pulmonary tension with resistance to the air flow.

The respiratory airways are distal to the terminal bronchioles (branching around 8 times) ending in alveolar sacs. There is a very thin (0.2-2 μm thickness) epithelium of alveolar cell type I (main cells) and type II secreting lung surfactant layer. The wide surface area (100-140 m^2) is responsible for gas exchange and shows the highest permeability to water and macromolecules making it suitable target for drug delivery. Tight and gap junctions are the main connections between the alveolar cells [3]. A *blood-air* barrier formed from a single fused basal lamina of alveolar cells and endothelium, hosting a plethora of cells like dendritic cells (DCs), mast cells and lymphocytes with secretory immunoglobulin. They not only play a role in antigen recognition and allergic reactions [4, 5], but wandering alveolar macrophages that engulf foreign substances can be another barrier for carrier and macromolecule delivery. The absorption of deposited particles in the alveolar barrier is through either receptor-mediated transcytosis, paracellular passive transport via tight junctions, endocytosis, and engulfed by macrophages [6]. Size and molecular weight (MW) are important factors controlling the macrophage uptake; MW ≤ 25 kDa are rapidly cleared while MW ≥ 40 kDa are slowly cleared, whereas, phagocytosis is optimal for particle size of 1.5-3 μm. To escape the macrophage clearance mechanisms, drug particle and delivery systems must outside of the size range recognized by the macrophages [7]. For macromolecules, e.g., peptides, proteins, siRNA/miRNA, the lung surfactant may induce aggregation, and thus, potentially enhance macrophage clearance. Furthermore, the macrophages secrete peroxidases, inflammatory/immunomodulatory mediators and other host defense molecules that can degrade macromolecules and initiate a local immune response [8, 9]. Disease conditions

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affecting the alveoli have serious percussions to the gas exchange, such as tuberculosis (TB), lung cancer, emphysema, pneumonia, acute respiratory distress syndrome (ARDS), and pulmonary edema. The pathophysiology of the respiratory tract and the severity of the disease are other limiting factors for successful macromolecule delivery.

1.2. Macromolecules and Challenges For Pulmonary Delivery:

The successful production and marketing of the first human recombinant insulin occurred over three decades ago as a protein drug, and subsequent development and production of macromolecule use for theranostic applications has been a rapidly evolving area of pharmaceutical industry [10]. Macromolecules are a heterogeneous group of proteins, including small peptides (20–30 amino acid residues, or called oligopeptides) (cytokines, enzymes, vaccines, monoclonal antibodies and clotting factors), and genetic material (DNA, pDNA, RNA, siRNA, miRNA, ribozymes and aptamers). They are molecules with superior drug-like properties such as potency and specificity, due to highly selective receptor-binding which minimize off-target side effects. However, due to their large size, hydrophilicity, MW, structural instability, and subsequent limited absorption at port of entry, their delivery has predominantly been conducted through injection. Short-circulatory half-life presents with another limitation, requiring frequent injections and consequently lowering the patient adherence to the treatment [11]. Aerosolized macromolecule delivery therefore has promising potentials as an alternative method for their delivery. Such potential benefits include non-invasiveness, possibility for self-administration, bypassing hepatic metabolism and avoidance the harsh proteolytic oral environment. Furthermore, the large surface area (100-140 m^2 in humans) of the lungs with abundantly vascularized thin epithelium, offers an encouraging route for local topical and systemic delivery. Increased bioavailability of the bioactive with a rapid onset of action, requiring smaller doses and reducing the potential unwanted side effects, are among advantages of macromolecule delivery via aerosol inhalation [12].

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However, inhalation of macromolecules includes many challenges in their formulation, storage, and delivery [13]. Manufacturing and purification of macromolecules is a very costly process. Traditional methods of highly purified animal tissue protein extracts are hampered with immunogenicity and lack of specificity [14]. The newer recombinant macromolecule production lines are improving to increase the quantity and the quality of the macromolecules and reducing the cost, which is still very high. Using transfected mammalian cell lines, and recently, human cell lines, are more advantageous than *E. coli* clones with better similarity of macromolecules structure to the natural human proteins, albeit with high cost [14].

Macromolecules have unique structural features and their activity is closely related to their structural integrity, which makes their formulation challenging. Different aerosol formulation approaches (e.g. dry powder, aqueous solution, liquid solution/suspension in a propellant vehicle), are incorporated to preserve their structural integrity. Most carrier based formulations aim to encapsulate the macromolecules in their core to evade exposure to any enzymatic degradation. The carriers/formulations are equipped with different shielding in order to evade macrophages uptake, and targeting moieties can localize the active agent in the required site. Various stabilizers, absorption enhancers, and mucoadhesive adjuvants, such as fatty acids, surfactants and protease inhibitors, have been utilized to promote higher bioavailability [9, 15, 16]. Furthermore, the optimal choice of aerosol delivery device is influenced by the macromolecule type, lung target site and condition.

The safety profiles, including toxicity, local and systemic side effects, and immunogenicity, are important criteria in macromolecules evaluation, in addition to the efficacy and shelf life. In fact, many macromolecules have a major risk of inducing immunogenic reactions [17], with antibody production as the primary immune response. The therapeutic agent, after recognition as ‘foreign’, is internalized, processed, and presented by antigen-presenting cells (APC), resulting in CD4 T-cell responses and the elevation in antibody titer. This immunogenic

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reaction can be used to our advantage and is sometimes targeted as in vaccine prophylaxis and therapeutics [5].

The literature reports that many small RNA, such as siRNA, miRNA and ribozymes could be potential powerful therapeutics [18, 19]. On the other hand, a wide variety of RNA species or analogues have also been reported to be immunogenic including ssRNA and dsRNA [20]. The investigation of the immune-compatibly represents one of the most important features for their translation to the clinic. A wide range of physicochemical properties such as MW, size, surface charge, solubility, and hydrophobicity play a role in macromolecule safety [17]. Hence, the interplay of macromolecule properties, formulation/carrier characteristics, device type, and the targeted lung disease are to be predetermined to achieve successful delivery (Figure 2).

2. Aerosolized Delivery Strategies for Macromolecule Drug Delivery:

Drug delivery to the lungs is achieved via respirable aerosols and can be used for topical as well as systemic delivery because of the advantages offered by the pulmonary route over other systemic routes of administration [9]. The aerosol is a stabilized dispersion of solid or liquid droplets suspended in a gaseous vehicle. The size of these droplets is always referred to as an aerodynamic diameter; the diameter of a sphere of a unit density with the same settling velocity as the particle of interest. The inhalable portion of an aerosol is with an aerodynamic diameter below 10 μm and classified into coarse particles > 2 μm, fine particle fraction (FPF) 0.1-2 μm, ultrafine fraction < 0.1 μm [9]. Most of pharmaceutical aerosols are polydisperse but newer formulations and devices are developing monodisperse systems. It is generally accepted a size range for pharmaceutical applications between 0.5-5 μm will allow deposition in the respirable airways [1]. The aerosol deposits the drug into the airways via different mechanisms mainly inertial impaction, gravitational settling, interception, and Brownian diffusion. The pharmaceutical aerosols which have a positive charge will be exposed to electrostatic precipitation (Figure 3A, B) [21, 22]. A number of factors contribute to the deposition of

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aerosol particles to the airway targets; mainly particle characteristics; size, shape, density and charge, and the pathophysiology of the respiratory system. These factors are not only determinants for the quantity of the deposited particles but also to which regions of the respiratory airways are depositional targets [9]. It has been noted for optimal aerosol delivery, the conditions are aerosol particles with an aerodynamic diameter 0.5-5 μm and lung flow rates of 15-30 liter/minute [21, 23].

2.1. Aerosol Generating Devices:

Three widely known systems for aerosol drug delivery are nebulizers, metered dose inhalers (MDIs), and dry powder inhalers (DPIs). The choice of the device is multifactorial dependent on the active agent, the formulation characteristics, the target site and the pulmonary pathophysiology. The main advantages and disadvantages of each device with recent insights for their development toward macromolecules delivery are represented in Table 1. Successful aerosol delivery to the lung should maintain high efficacy, dose-reproducibility, site-specificity, enhanced aerosol FPF, durability and stability for multiuse, simple handling, environmental-friendly, risk-free, and cost effective treatment [1, 24-29]. Examples of local aerosol macromolecules delivery to the lung are summarized in Table 2.

2.2. Carrier delivery systems:

A carrier delivery system allows an active agent to be stably encapsulated or adsorbed, aiming to improve the pharmaco-kinetics and -dynamics. The carriers can be conveniently used to improve the stability, prevent macromolecule degradation, increase cellular uptake, and confer efficient targeting to the site of action with a homogenous distribution and favorable retention, with a reduced side effect by shielding (Figure 4A) [24]. The carriers are ideally biocompatible, biodegradable, and non-immunogenic, with high stability and scalability [21, 30]. The carrier selection is dependent on interplay of factors mentioned in Figure 2. For

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successful aerosol delivery of macromolecules, preconditioned understanding of macromolecule and aerosol characteristics, target pathophysiological lung condition (inspiratory flow, lung volumes, breath-holding) and the suitability and stability of the macromolecule aerosol generated by nebulization, MDIs, and DPIs is required [1].

2.2.1. Polymeric Particulate Carriers:

Microparticles and Nanoparticles: Although the defined size ranges for microparticles (MPs) and nanoparticles (NPs) can differ based on convention and sources [31], NPs will be referred to particles within the nanometer scale, (1-1000 nm) and MPs as particles in the micrometer scale (1-1000 μm) for the purpose of discussing pharmaceutical carrier formulations in this review, NPs and MPs have a variety of physicochemical properties such as size, surface area, shape, molecular weight, porosity, hydrophobicity and charge, that allow modifications and functionalization to suit a wide range of macromolecules (Figure 4B) [24, 32]. These carriers offer high loading capacity, protection from enzymes resulting in improved macromolecule stability with enhanced lung distribution and retention. Furthermore, they can be actively targeted to the site of action and have mechanisms for controlled release with a net gain of reduced dosing frequency and improved patient compliance. Moreover, they can be easily formulated from a wide variety of natural and polymeric biodegradable and biocompatible materials such as chitosan, alginate, poly Lactic-co-Glycolic acid (PLGA), polyethylenimine (PEI), poly (l-lysine) (PLL) [33]. However, the formulation processes to produce NPs and MPs from the above polymers include harsh steps and solvents that can potentially denature the macromolecule or alter its structure such as emulsification/solvent evaporation, spray dryer, freeze dryer, supercritical fluid technology [34, 35].

Prior optimization steps are required to improve loading and stability. For example, surface coating or functionalization improves the pharmacokinetics; particle shielding with lipids or

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hydrophilic polymers as polyethylene glycol (PEG) lowers the macrophages uptake and recognition, mucoadhesive particles increase the lung retention, i.e., chitosan, coating of carriers with active targeting ligands such as antibodies will be discussed later [32]. A study showed a promising co-block polymer poly(glyceroladipate-co-ω-pentadecalactone), (PGA-co-PDL) MPs with PEG as a suitable carrier for mucolytic enzyme as a protein model; α-chymotrypsin that showed high loading and suitability for DPI [36]. It is also difficult to deliver NPs in their single particulate form due to their high aggregation properties that favor their clearance. Consequently, NPs have been incorporated within microcarrier systems via spraydrying using sugars and amino-acids into NPMPs [37]. Their main advantages are evading the mucociliary and macrophages clearance. Upon their delivery to lower airways, the MPs degrade releasing the NPs that contain the active agent. They show successful DPI formulation with both good flow-ability and dispersibility. Kunda et al., showed successful polymeric cationic NPs formulation loaded with a Bovine Serum Albumin (BSA) as a protein model and was developed into DPI with good biocompatibility against A549 cell line [38]. Furthermore, they successfully developed a NPMPs loaded with pneumococcal surface protein A (PspA) as DPI vaccine against pneumococcal infection that successfully induced antibody response against *in-vitro* DCs line [38]. Recently this work was continued by Rodrigues et al., which demonstrated successful vaccination antibody production through *in-vivo* study via lung instillation in mice compared to subcutaneous injection [39].

Cationic polymers are receiving increasing interest as non-viral vectors for gene transfer such as PLL, PEI, chitosan, dextran, although the transfection rates are still low compared to viral/plasmid vectors [21, 24, 38, 40]. However, they are less immunogenic with anti-degradation shielding. In addition, their cationic charge may play a role in the improved cellular uptake as well as increase the risk of local toxicity due to the interaction with cell membrane, so extensive optimization is prerequisite. A double-blinded clinical trial of PEG-

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PLL NP-mediated gene delivery for CF; where a gene was transfected though nasal mucosa; showed promising results [41].

Dendrimers: Dendrimers are highly branched polymeric carriers that have terminal amine, carboxyl, or hydroxyl groups and their size is within nano-scale (Figure 4C). These groups provide a platform for functionality with different targeting and therapeutic molecules. Their branched structure effectively encloses active (hydrophilic or lipophilic) agents. They have improved physicochemical properties and based on biocompatible and biodegradable polymers. Uniform size with (low polydispersity), stability, versatile ability for modifications, amine group-transfection ability enhances their carrier properties [32, 42]. However, the complex formulations with multifaceted steps to access the core and build branches are limiting factors. Polyamidoamine (PAMAM) dendrimers of different sizes were tested *in-vitro* (Calu-3, primary alveolar cell lines), *ex-vivo* (instillation into perfused rat lungs) to show successful uptake intracellular with no aggregation with the lung fluid. They showed promising results for biocompatible targeting the lung via inhalation [43].

2.2.2. Lipid based Carriers:

Lipid based carriers are efficaciously used in aerosolized macromolecules delivery offering high stability of the formulation and long storage life. They provide protection against enzymatic breakdown and mucociliary or phagocytic clearance with reduced toxicity and immunogenicity. They can provide controlled or sustained release with enhanced cellular uptake with intracellular cargo delivery and suitable for hydrophilic/lipophilic active agents, and can be functionalized with targeting moieties to certain cells [24, 44].

Liposomes: Liposomes are phospholipids assembled vesicles possessing an aqueous core with a size range within the nano to micrometre scale (Figure 4D). They can be formulated from natural or synthetic lipids mostly with neutral or anionic charge such as phosphatidylcholines,

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sphingomyelins, phosphatidylglycerols, and phosphatidylinositols [32]. However, cationic liposomes are produced using lipids with a positive charge and are used for gene delivery due to their capability for ionic interactions with DNA, siRNA/miRNA [24, 32]. They form single bilayer or multilayers that can be used for the controlled release, encapsulate hydrophilic/lipophilic active agents and improve loading capacity, versatile chemical functionalization, and active targeting. Liposomes are extensively investigated carriers for a wide variety of drugs and macromolecules and can be locally delivered via nebulization or DPI [24, 45]. Lung deposition and retention can be targeted via modifications of the physicochemical characters such as size, charge, and formulation composition ratio and lipid type. Liposomes have been used as delivery carriers for vaccines [46], nucleic acids such as siRNA, miRNA, lncRNA, DNA for respiratory conditions as in COPD, CF, lung cancer, although some of these applications are commonly administered via invasive routes, a number of cell and animal studies have proved their suitability for local lung delivery [44, 47, 48].

Solid lipid nano- and micro-structures (SLS): SLS are colloidal carriers having a core of solid lipids and a shell of biocompatible stable emulsifiers as phospholipids, bile salts, poly (vinyl alcohol) (Figure 4E) [49]. They offer improved stability, higher loading, scalability and shielding polymers are used to modify their surface reducing their phagocytic clearance. PulmoShperes™ are porous carriers that can be formulated from lipid-based emulsion spray dried coarsening with a shell of monolayers of phospholipids. They can incorporate the active agent in the core and delivered via inhalation device or instillation. They have been investigated in different clinical trials mainly for delivery of antibiotics but showing promising results for delivery of macromolecules as vaccine, peptides and antibodies [50, 51].

Archaeosomes (ARCs): ARCs are lipid based carriers formulated from a polar type of lipids derived from archae membrane lipid of bacteria; Sulfolobus acidocaldarius. This lipid has di- or tetra-ether groups and has enhanced properties as gene carriers and macromolecules. They

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carriers are biocompatible and biodegradable with high stability cationic lipids with versatile functionality. ARC-DNA complexes were delivered via lung cell line A549 as model therapy for CF [52, 53].

2.2.3. Other carriers for pulmonary delivery:

Viral DNA/iRNA vectors: Viral vectors as gene transfection agent are well renowned such as retroviruses, adeno- and adeno-associated viruses. However, their use and investigation as carriers for pulmonary delivery is limited with majority of administration via parenteral route. Their main limitations are immunogenicity, limited loading, and poor scalability. Human CF transmembrane conductance regulator (CFTR) cDNA that was successfully loaded into adenovirus associate (tgAAVCF) had passed clinical trial phase I via nebulizers in CF patients, however was discontinued after failing to achieve the therapeutic targets in phase II [54]. Another study investigated the delivery of miRNA (Let-7) through lentivirus vectors in mouse lung cancer model resulted in tumor suppression against non-small cell lung cancer [55]. Plasmid-DNA complex have also been investigated using bacteriophages, such as bacteriophage ϕC31 integrase to enhance the efficiency of delivery [54, 56].

Cell-based, biomimetic, and stem cells: Recent advances have enabled using cellular based products for drug delivery purposes, such as using blood cells membrane but at present, no such aerosolized drug is found nonetheless that would be inspirational for future inhaled therapeutics [57].

3 General Approaches for Targeting Pulmonary Delivery of Macromolecules

There are a number of approaches for targeting macromolecules and delivery vehicles to specific locations of lung tissue. Such targeted delivery in the lungs is an important aspect of increasing the concentration of drug to area of interest, which will ideally result in improved efficacy of drugs at the target site, as well as reduced systemic side effects. These methods for

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influencing the destination of active macromolecules can be categorized into passive and active forms of targeting, which refer to utilizing the unique physiological characteristics of the target environment, or specifically targeting molecules or components of the cell surface, respectively.

Passive targeting: The most common form of passive targeting within the lungs is by utilizing the discriminative tendencies of the airway physiology to influence the localization and deposition of the formulation. This bias and inequality in lung localization can arise initially due to how the delivery vehicle transits along the inhalation airflow and through the airways of the lungs. A widely utilized approach of passive targeting in the lungs is through control of particle size in the aerosol, which can be in the form of either suspended liquid or solid, to influence the aerodynamics; dictating the principal mechanisms of impaction. Such methods have been utilized in inhalation therapy for nearly 200 years and can be seen utilized in metered dose inhalers, nebulizers and dry powder inhalers.

It is well established that aerosol particles with aerodynamic diameters in the range of 1 to 5 μm reach the lower parts of the respiratory tract, whereas particles smaller than 1 μm do not deposit, and particles larger than 5 μm accumulate in the conducting airways [58]. Knowing that the deeper alveolar regions of the lungs exhibit greater drug absorption, as well as immune response in the case of immunotherapies, the optimum size range of 1-5 μm is generally the aim for contemporary pulmonary formulations. The particle physicochemical characteristics can be adjusted and utilized to achieve targeting to the desired lung region [59]. The targeted deposition in the lungs is an important mechanism for formulations such as particles containing itraconazole or amphotericin B for lung infections, and has been shown to increase the efficacy of the treatments [60]. Due to the concentration dependent effect of some antibiotics, it is important to have targeted delivery to ensure high concentration only at the affected sites in order to ensure optimum effects while avoiding side effects. Although small

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molecules such as antibiotics and airway therapeutics make up the majority of the delivered material, macromolecules such as siRNA have been heavily considered for pulmonary delivery [61].

Nanoparticulate formulations, such as polymeric NPs or liposomes, can exhibit controlled drug release at the site of delivery, leading to maintenance of therapeutic concentrations in the lungs for a longer duration [62]. Another unique property of NPs is the tendency to be phagocytosed by alveolar macrophages, if they are in the range of 500 to 3000 nm [63, 64]. The simultaneous effects, of NPs uptake and release, were demonstrated simultaneously, when a study of multilamellar liposomes containing rifampicin and isoniazid, exhibited presence in alveolar macrophages and could maintain therapeutic concentrations in the lung tissue for 5 days [65]. Antimicrobial delivery via liposomes to the lungs, has been shown to be beneficial in the case of cystic fibrosis and exhibit a controlled and sustained release and some are currently in phase II clinical trials [66]. Studies involving the delivery of macromolecules such as siRNA have also been conducted using nanocarriers, as summarized more in depth elsewhere [61].

Other than aerosols, liquid micro volumes can also be administered by an airway catheter or bronchoscope to target specific areas of the lungs, but is generally thought to be invasive. Recent development of the delivery of micro-volumes of a liquid plug, by controlling liquid volume and air ventilation, has been hypothesized to be a potential approach for the targeted delivery of precise drug dosages to specific areas in the lung to treat pathologies such as cystic fibrosis and lung cancer [67].

Passive targeting in the lung can be summarized as changing the characteristics of the delivery vehicle to influence localization and uptake based on the physiology of the lung and cellular composition. Optimizing the characteristics of the aerosolized particles comprises a large aspect of this targeting approach as seen in the numerous aerosol delivery devices (Table 1).

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Active targeting: Targeting specific tissue locations, cells, molecules on cells and cell compartments, to increase the concentration of therapeutic macromolecules at the precisely desired location, is the hallmark aim of active targeting. One of the ubiquitous approaches for active targeting generally involves the conjugation of the carrier or macromolecule to a targeting ligand, which is generally in the form of protein and peptides, polysaccharides, glycolipids, glycoproteins or antibodies. Cell surface receptors may be overexpressed on specific cell groups according to the pathologies such as cancer, or expressed on a particular cell group that are useful targets for therapy [68].

For lung cancer, several targets, which can be used to discriminate between healthy and cancerous cells, have been discovered. The epidermal growth factor receptor (EGFR) is known to be highly expressed in non-small cell lung cancer, and studies have been conducted investigating nano-formulations with EGFR targeting ligands for the delivery of cisplatin to the cancer tissue following inhalation [68]. These EGFR targeting particles were able to reduce tumor volume more effectively, as well as increase the bioavailability of cisplatin in the lung compared to the free drug [68]. Another target for lung cancer is carbonic anhydrase IX, which is expressed on tumor cells. Carbonic anhydrase IX antibody was incorporated onto liposome particles with CPP33, which is a cell-penetrating peptide. These liposomes loaded with Triptolide were shown to exhibit anti-tumor effects and reduced systemic side effects when administered endotracheally. Luteinizing hormone releasing hormone (LHRH) receptors are also known to be overexpressed on lung cancer cells and have been successfully investigated for selective targeting [69]. siRNA delivery to cancer cells using nanocarriers targeting LHRH exhibited Although CD44 receptors have been suggested as another potential target, investigation using nanoparticles formulated with hyaluronan in an *in vivo* biodistribution study found that intrapulmonary nebulized administration did not result in increased accumulation of the nanoparticles in the tumors [70].

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In addition to targeting tumor cells and pathogens, there are a number of immune cells which can be targeted for certain lung pathologies. In the case of tuberculosis, alveolar macrophages are recognized as a major part of the pathogenesis and thus a viable target for the delivery of antimicrobial agents. The Mycobacterium tuberculosis resides in the alveolar macrophages and forms the basis for the initiation of inflammation and the formation of granuloma. Macrophages are found in great numbers in the lungs and naturally possess the tendency to phagocytose particles under 5 μm, which can be capitalized for targeting. Formulating antibiotics in nanoparticles has been shown to increase the duration of therapeutic antibiotic concentration in the lungs and studies involving rifampicin, isoniazid and pyrazinamide indicated greater efficacy. Furthermore to phagocytic tendency, alveolar macrophages have also been identified with specific targets, including tetrapeptide tuftsin (Thr-Lys-Pro-Arg),O-steroyl amylopectin (O-SAP) and macrophage scavenger receptors [71], as well as mannose receptors which are also in abundance on DCs.

Activated T cells in the lung are another cell type which can be targeted in the case of asthma. These cells exhibit a Th2 phenotype, which is strongly associated with inducing airway inflammatory responses and chemo-attraction of inflammatory cells. Receptors for transferrin are known to be overexpressed in activated T cells, and in combination with polycations such as low molecular weight polyethylenimine, the conjugated form was investigated for use in asthma. Transferrin-polyethylenimine (Tf-PEI) could be used to selectively deliver small interfering RNA (siRNA) to activated T cells in the lung. SiRNA can be used to silence specific mRNA expression through incorporation into the host cell, and is a promising approach for not only asthma, but also other lung diseases such as cystic fibrosis, chronic obstructive pulmonary disease and cancer. Another cell group associated with asthma is the DCs, which are antigen presenting cells integral to the initiating immune responses. Although no specific study has been conducted targeting DCs by the pulmonary route, DCs exhibit

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tendencies for nanoparticle uptake and several ligands such as DEC-205 have been identified [72]. These targeting possibilities could be useful in applications such as vaccines.

In order to target the general lung epithelium for conditions such as asthma, lung structure cells have been investigated as potential targets, as β2-adrenergic receptors can be commonly found on bronchial smooth muscle cells. siRNA conjugated to salbutamol, which is a commonly used β2-AR agonist for asthma, has shown to exhibit greater gene silencing compared to the non-conjugated siRNA. Another way of targeting the epithelial cells of the lungs is through the human polymeric immunoglobulin receptor (hpIgR) [73]. This approach is postulated to be useful in cystic fibrosis; delivering anti-proteases to the epithelial surface to counter the elastase released from neutrophils, which can otherwise result in unnecessary inflammation and subdued clearance.

An interesting approach for increasing delivery of drugs into the epithelial tissue was tested by the administration of VEGF in an aerosolized form to increase permeability of microvessels in the lungs [74]. It has been hypothesized that the subsequent parenteral administration of magnetized nanoparticles containing the active molecule could potentially accumulate at the bronchial luminal epithelium through increased vascular endothelial permeability. Another example of magnetic targeting is the use of magnetic gradient fields to direct aerosol droplets containing superparamagnetic iron oxide nanoparticles to the desired regions of the lungs in mice. In addition to the delivery of particles to specific areas of the lung, the particles themselves can be used to release the active at specific areas based on physical external stimuli such as light, ultrasound, heat and electric or magnetic fields. An example of targeted release by external stimulus, is the use of ultrasound-sensitive microbubbles for local release of drugs [75] . Microbubbles created from lung surfactants showed a three-fold increase in targeted deposition of the drug compared to common lipid-only microbubbles.

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In addition to the delivery of macromolecules to specific tissue and cells, intracellular organelles such as the mitochondria have also been investigated as pharmacological targets. The mitochondria has been proposed as a target for cancer, as it is integral for cell functioning, and involved in cell proliferation and apoptosis [76]. In order to target the mitochondria, a study investigated the use of histidine to assist with escape from endolysosomes after through the 'proton sponge' effect, which is essentially disruption of the endosomal membrane through osmotic swelling caused by the influx of protons [76]. Another approach for endosome escape was demonstrated by attachment of the active macromolecule to low molecular weight polyethylenimine, which has cellular uptake and endosomal escape properties, leading to relatively high gene transfer efficacy [77].

There are indeed a vast number of identified targets for various cells involved in lung pathologies, but only a limited number have been investigated for efficacy after pulmonary delivery (Table 3). Despite the limited studies specifically for pulmonary delivery, numerous targets on cells of lung tissue and the local immune system have been identified for active targeting, and it may only be a matter of time before these molecular and physiological targets are investigated further for the delivery of therapeutic macromolecules.

4. Conclusion:

This review discusses the approaches for macromolecule delivery in the lungs, and strategies for targeted delivery based on different lung pathologies. The initial deposition of the formulation is mainly dictated by the aerosolization and inhalation, whereas the formulation in the various carriers can further influence the delivery of the macromolecule to specific cells such as immune cells and cancer cells. The targeted delivery of these macromolecules could potentially lead to therapy which has higher efficacy and lower side effects.

5. Expert opinion:

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Targeting the lung via non-invasive aerosolization of therapeutic macromolecules; requires a profound understanding of the macromolecule's physicochemical characteristics, the formulation and carrier methods, the aimed inhaler device, and the pathophysiologic lung conditions.

Macromolecules are a heterogeneous group of high molecular weight molecules (proteins, peptide-based molecules, and genetic material) with superior drug-like properties. The production of macromolecules is still a low yield high cost industry using mammalian cells. This also transfers to their use in developing formulations suitable for pulmonary delivery. Hence, the main challenges in their inhalation formulation development are heterogeneity, loss of their stability, poor loading capacity, susceptible for enzymatic, mucociliary and phagocytic clearances, immunogenicity, and toxicity. Carrier based delivery systems aim to overcome these challenges.

Various carriers systems offer different physicochemical characteristics that might suit one type of macromolecule but not all. The carrier selection process is an active pool of research cross-matching optimization trying to optimize different macromolecules with different carriers under different formulation processes. Inclusive and vigorous characterization for each carrier and macromolecule-carrier combination is important to infer their clinical effectiveness and biocompatibility. It is a very tedious process involving *in-vitro*, and *in-vivo* animal, then human, experiments. Few of them will be clinically translated. The scalability and reproducibility of the formulation is another challenge which needs consideration prior designing carriers and is a major flaw of current methods involving solvent evaporation and ionic gelation. Methods such as microfluidics, which can overcome these issues regarding scaling up, are more frequently being investigated to improve the translation of preclinical formulations to the market [78]. Targeting moieties are important strategies to functionalize the formulation with homing facility that will prevent the non-specific delivery and increase

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the therapeutic outcomes. The availability of biodegradable and biocompatible materials are fueling the development of new carriers which can be used for pulmonary delivery. NPs, MPs, dendrimers, Lipid-based, liposomes, and many other carriers have shown successful intracellular cargo delivery of proteins, peptides and DNA/RNAi. Currently, carriers based on viral or bacterial plasmids are seriously limited by their unfavorable immunogenicity, high costs, and low scalability.

The delivery of the aerosolized formulation via the inhaler device still forms a significant aspect of pulmonary delivery, and the choice of device is based according to the patient condition, as well as the type of formulation. Although nebulizers, pMDI and DPI have many successful, well-established, market products, improvement are still required to use these devices for optimal delivery of macromolecules. It is important to consider approaches which can overcome issues such as low deposition, increasing the FPF, improving stability and shelf-life, operate breath-independent and patient-friendly, and allow for multiuse without limiting their portability.

Lastly, the pathophysiology of lung conditions is variable from one disease to another. Pre-formulation evaluation for the characteristics of the target lung disease and subsequent manipulation of the macromolecule-carrier-device combination is required in order to achieve the desired delivery site, distribution, retention with reduced toxicity and immunogenicity, and better patient compliance. There is currently little known regarding the differences brought by lung diseases affecting the deposition and retention of active agents, which could be an avenue for future research. A need to produce pulmonary delivery systems that can address these limitations for each pulmonary pathology is a future tendency with achieving both site- and cell specific delivery.

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Review Declaration of Interest

A reviewer on this manuscript has disclosed that they are a business associate in a CRO 'Cynbiose Respiratory' specialized field of respiratory medicine.

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Figure 1. The pulmonary barrier structure in the conducting and respiratory airways

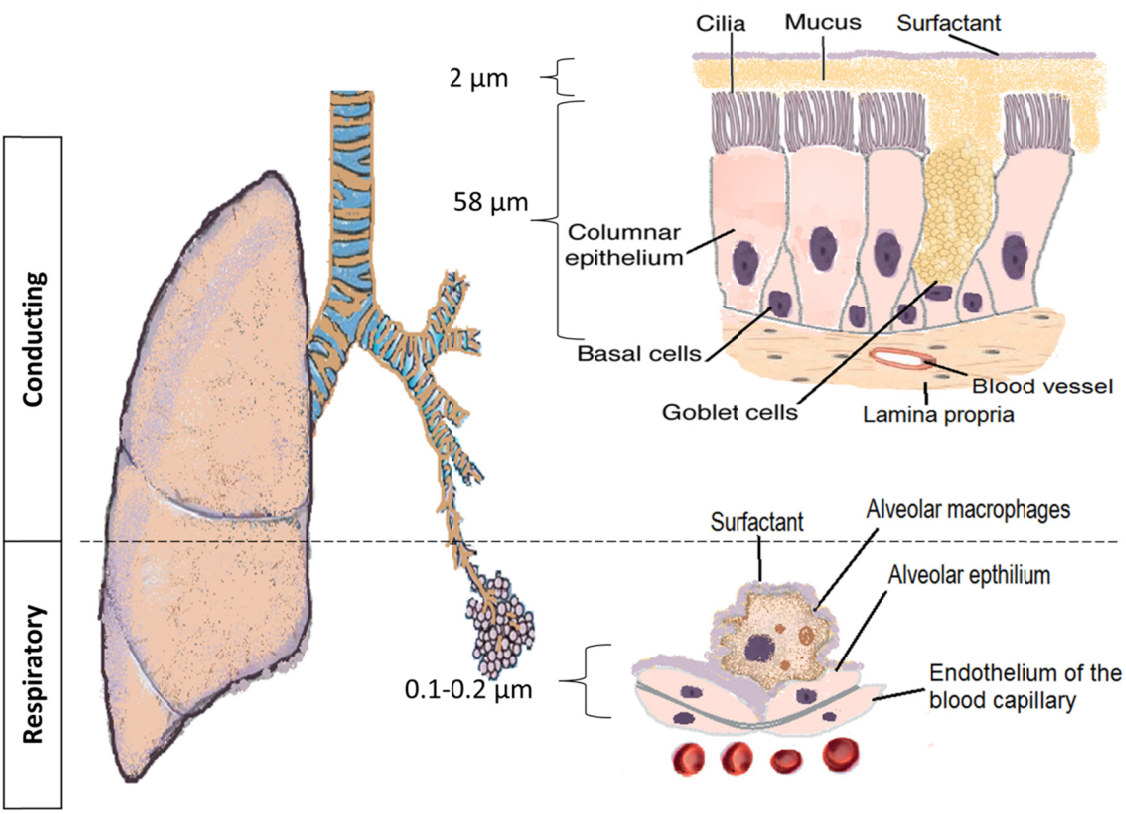

Figure 2. The interplay of factors for successful macromolecule delivery via inhalation

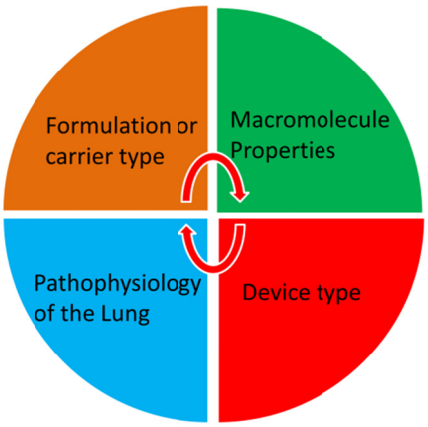

Figure 3A. Particle properties and illustration of their deposition mechanisms

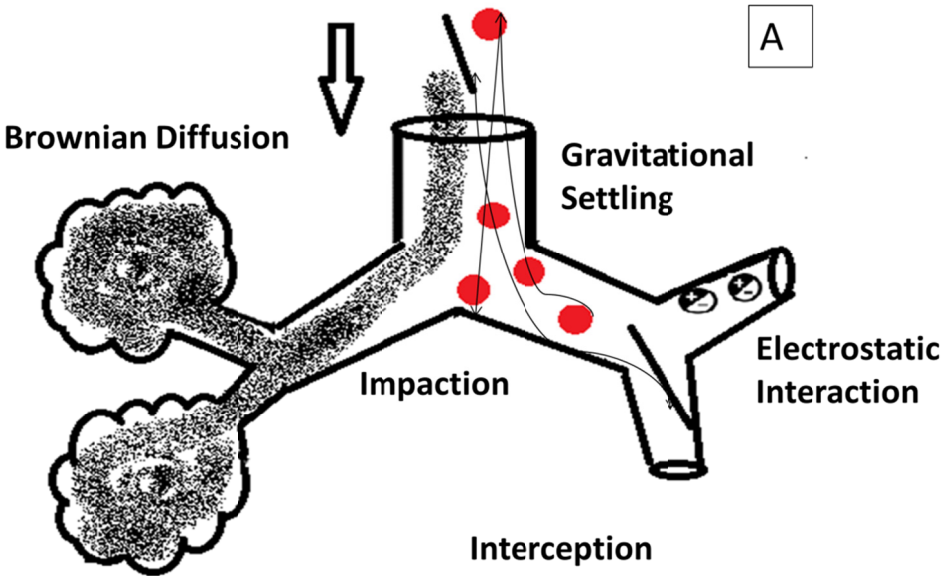

Figure 3B. Particle properties and illustration of their deposition mechanisms

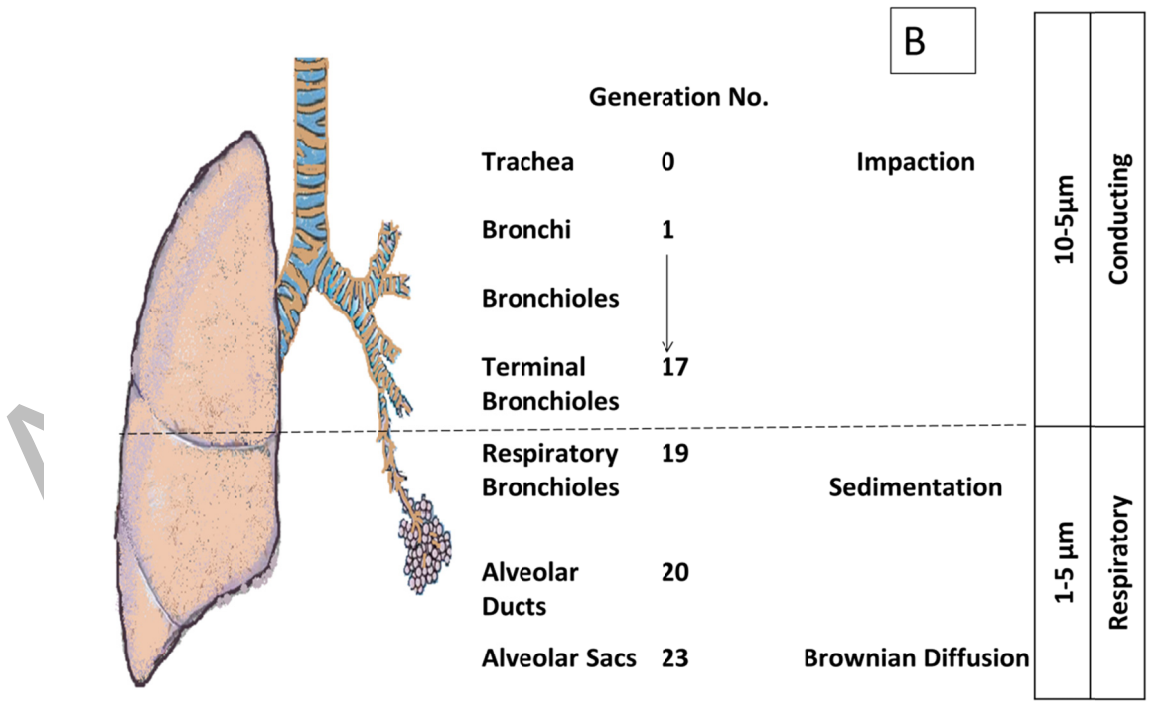

Figure 4A. Ideal Drug Delivery Carrier

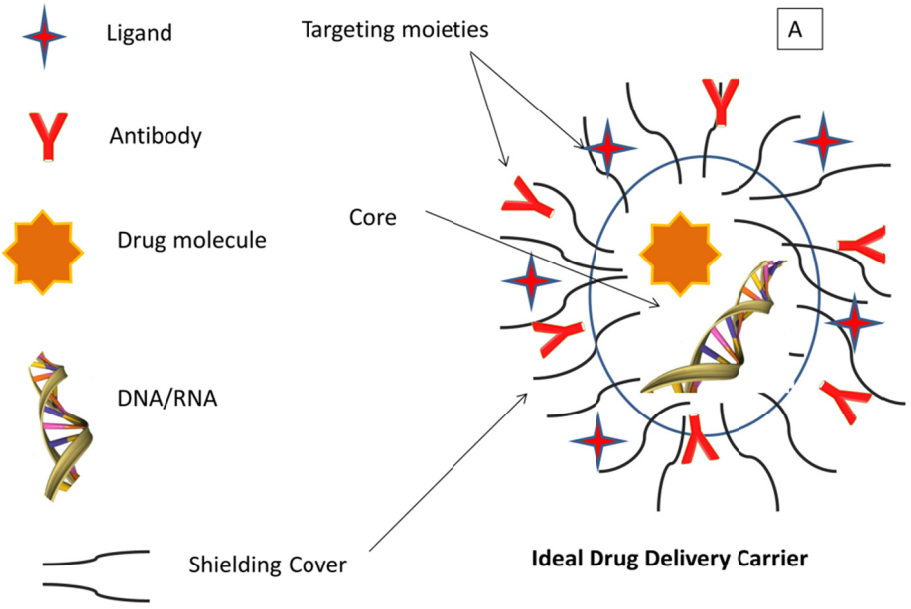

Figure 4 A. Ideal Drug Delivery Carrier.

Figure 4. Different types of macromolecule carriers B: NP/MP, C: Dendrimers, D: Liposomes, E: Solid Lipid Structures

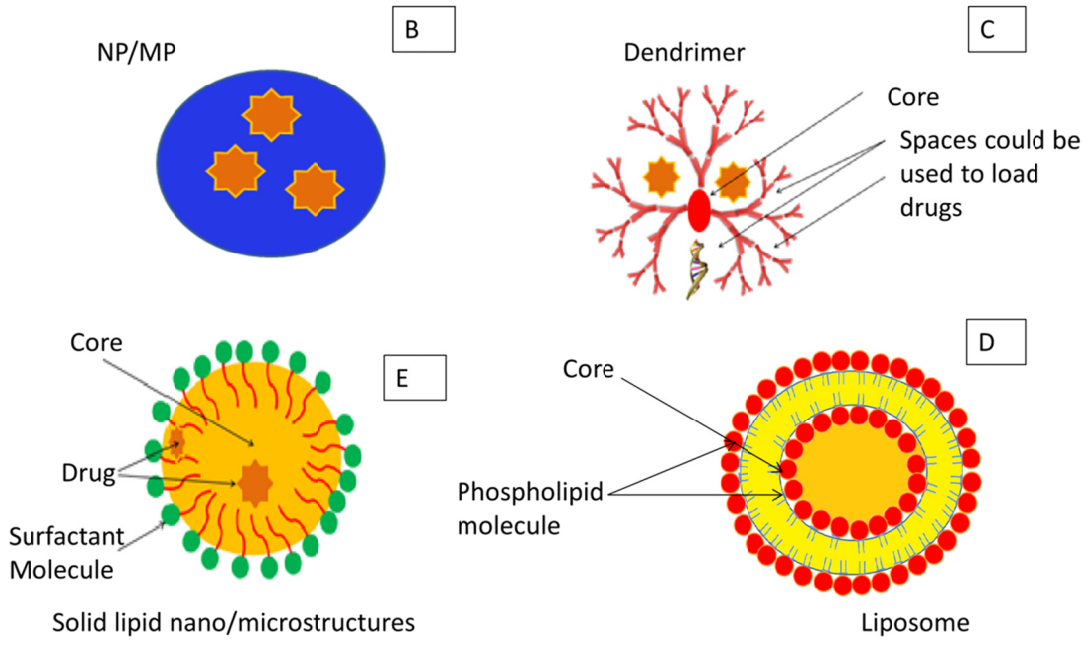

Figure 4. Different types of macromolecule carriers B: NP/MP, C: Dendrimers, D: Liposomes, E: Solid Lipid Structures

Table 1. Aerosol generating devices advantages, disadvantages, and recent developments.

Aerosol Device	Advantages / Disadvantages	Recent development for macromolecules delivery
Nebulizers: Deliver the drug or drug solution mixture.	**Advantages** • No breath-hold • Can be used by young, old or severely ill patients. • Minimal breath training • Drug concentration can be adjusted **Disadvantages** • Expensive and bulky • Nebulizers dose-delivery times are long • Contamination risk • Wet, cold mist	• Newer liquid-based aerosol generators are being developed to improve upon the conventional nebulisation and increase the stability of active agents and the portability of the device. Forexamples: • Respimat® Soft Mist™ Inhaler (Boehringer, Germany): the device generates aerosol upon actuation without a propellant but pressurized by mechanical means. • Surface Acoustic Wave (SAW) nebulizers: new devices that employ certain acoustic frequencies to generate liquid aerosols have proven efficient in macromolecule

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	• Power source needed, electricity or compressed gas • Less portable • Main limitation for macromolecule delivery is unstability of proteins while the nebulization process.	nebulization [79]. • Omron Micro*Air*® VMT (Omron Healthcare, IL, USA): using the vibrating mesh aerosol-generating technology • New systems are able to generate aerosols either under less harsh conditions; mechanically or through vibrating mesh. • They are useful for treatment of asthma, CF, and COPD, for example, rhDNase nebulized enzyme for therapeutic treatment of CF
		• Developments of MDI to improve the patient ease of use,

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MDIs: Deliver the drug as liquid solution or suspension.	**Advantages** • The most widely used oral inhalation device • Mobility/or portability • Short time required for delivery • Deposition of up to 20% of released dose into the airways **Disadvantages** • Hand-breath dependant • Inability to adjust the drug concentration • Failure of dose monitoring • Difficulty to use may aggravate the patient incompliance. • Poor solubility of macromolecule liquid droplets	increase the FPF and lowering the off target deposition, and minimize the drug preparation without limiting their portability, and increasing their cost. • The use of stabilizing or dispersing agents as sugars, surfactants, and ethanol during the formulation process improves the stability of macromolecules dispersed in the propellant and enhances the aerosol characteristics (FPF>50%) and lowers oropharyngeal deposition • Improvements in hand-breath coordination, portability • Accessory devices that can improve the administration process: Bag: InspirEase, Holding chambers: Aerochamber, MediSpacer, ACE (Aerosol Cloud Chamber), Spacer: OptiHaler • DNase has been successfully formulated and delivered through MDIs for CF [87].

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	in a propellant vehicle (currently Hydrofluroalkane). • Propellant allergy • Poor aerosol characteristics and off target deposition in the upper airways. • Expensive with new generations of MDIs	
	Advantages • Small portable devices • No propellant • No hand to mouth coordination • Breath-actuated or energized aerosol generators do not require breath holding • Short dose-delivery time • No cold effect	• DPIs represent the popular choice for delivery of macromolecules through single dose devices [80]. Examples include HandiHaler™ (Boehringer Ingelheim, Germany), and Aerolizer™ (Novartis Pharma, CH). Multidose DPIs, i.e., Diskus™ (GlaxoSmithKline, UK) and Turbuhaler™ (AstraZeneca, Sweden), which are still being improved to enhance FPF of the generated aerosol. • Inefficient breath-actuated DPIs have been improved into

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DPIs: Deliver spray-dried or lyophilized drug as fine dry powders clouds.	• Countable doses and easily monitored • ~12-40% of the emitted dose is deposited into the airways. • Consistent stability with longer shelf life **Disadvantages** • Only available for limited number of drugs, due to production costs associated with challenging formulation of the active agent as a dry powder that exhibits flow-ability, disperse-ability, and stability • Breath-dependant might not have a sufficient inspiratory volume to stimulate the powder dispersion • De-agglomerating challenges to produce inhalable aerosols • Potential for off target deposition in the upper	energized patient-independent devices, such as the Spiros™ (Dura Pharmaceuticals, CA, USA), that don't eliminate the flow-rate need that might affect the drug deposition.

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	airways. • Unit dose might require reloading prior each use • Humidity might increase the size of hygroscopic particles favoring the deposition in upper airways and might affect the stability of macromolecules.	

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Table 2. Examples of aerosolized carriers for macromolecule delivery for lung pathologies.

Indication or Disease	Therapeutic Macromolecule	Carrier/device	Type of study	Outcome/efficacy	References
	• W-1 L19	PLGA NP	Preclinical *in vitro* cell assay	PLGA NPs were successfully formulated and loaded with W-1 L19 oligopeptides prepared from a highly immunogenic part VP2 capsid protein of Canine parvovirus (CPV). These NPs were successfully uptaken on J-774 cell lines with a non-toxic nitric oxide production and high immune response that could be a promising vaccine against CPV	[81] .
	• Ricin Vaccine	Liposome	Preclinical in vivo mouse	A liposomal preparation of a natural toxoid A-chain of Ricin which is	[82]

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Aerosolised Vaccines			assay	natural Lectin from Ricinus communis plant that was sucessfuly delivered by aerosols into mice lung.	
	• Pneumococcal surface protein A (PspA)	NPMP via DPI	Preclinical in vivo mouse assay	NPMPs particles as DPI *and in-vivo* lung instillation in mice induced successful lung immunity.	[39]
	• Tuberculosis (TB) : Anti-TB vaccine Muramyl dipeptide	Instillation	Preclinical *in-vivo* mouse assay	Mycobacterium bovis Bacille Calmette-Guérin (BCG) was successfully intratracheal instillation in mice model of TB and compared to the subcutaneous route to find stronger mucosal lung immunity induced.	[83]
Acute	Surfactant Proteins	DPI,	*in-vivo* animal	Different surfactant proteins were	[84-

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Respiratory Distress Syndrome (ARDS)		nebulization, instillation	study	delivered to the lung via aerosols as dry powders, nebulized or instillations in various *in-vivo* animal models of acute lung injury.	86]
Cystic Fibrosis (CF) Primary Ciliary Dyskinesia (PCD)	A purified solution of recombinant human deoxyribonuclease (rhDNase), a mucolytic agent reducing mucous viscosity and secretions.	Nebulized or MDI	In clinics	Nebulised protein or MDIs [Approved by FDA, Genenzyme NCT01712334]	[87]

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Lung transplant	Cyclosporin A	Nebulized	Phase III clinical trails	Cyclosporin A was delivered via nebulized aerosols that showed high absorption and lung retention lung recipient in a randomized, double-blind, placebo-controlled clinical trial that showed improved the outcomes in chronic rejections.	[88]
Lung Cancer and Metastasis	• Interleukin-2	Nebulized	Clinical trials phase III	Multicentre clinical trial showed high efficient delivery with low toxicity through nebulised aerosols.	[89]
	• IgG1	Nebulized	*In-vitro* study	Nebulised monoclonal antibodies against A431 cells showing promising	[90].

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				results for lung cancer.	
	• Gene delivery: Akt1 siRNA	NP	*In-vivo* animal study	Aerosol Delivery of nanoparticle PEI of Akt1 siRNA Significantly Suppresses Lung Tumorigenesis in K-rasLA1 Mice.	[21]
Alpha-1-antitrypsin deficiency Emphysema CF	Alpha1 proteinase inhibitor (Alpha-1-Antitrypsin)	Nebulized	Clinical trials phase II/III	Nebulized Alpha-1-Antitrypsin was well tolerated in patient with CF in phase II/III clinical trials in Europe. (ClinicalTrials.gov: NCT01684410)	[91]

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Table 3. Summary of targets investigated specifically for formulations administered by the pulmonary route for lung pathologies.

Disease	Target	Effect	Reference
Lung cancer	Carbonic anhydrase IX (CPP33 dual-ligand modified triptolide-loaded liposomes)	Rats that were endotracheally administered triptolide-loaded liposomal formulations exhibited enhanced triptolide-loaded anti-cancer efficacy and reduced concentration of TPL in systemic circulation without apparent systemic toxicity.	[92]
Lung cancer	SP5-52 peptide (conjugated to Gem-loaded SFNPs)	Targeted formulations exhibited higher survival rate, less mortality, and no sign of metastasis in a lung tumor model.	[93]
Lung cancer	Hyaluronic acid (HA)	In vivo biodistribution studies with intrapulmonary nebulized administration of nanoparticle formulated with hyaluronic acid, did not result in increased accumulation in the tumors.	[94]
Lung cancer	High MMP9 concentration	Mesoporous silica-based nanoparticles functionalized with bioresponsive caps exhibited proteasome inhibitor release at high MMP 9	[95]

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		concentrations in a lung cancer cell line transfected with MMP9 cDNA.	
Lung cancer	luteinizing hormone releasing hormone (LHRH) receptors	tumor-targeted local delivery by inhalation of anticancer drugs and mixture of siRNAs was exhibited in a mouse orthotopic model of human lung cancer	[96]
Lung cancer	Integrin avb3	In vitro tumor cell uptake study using RGDfk-histidine-PLGA NPs, suggested that receptor mediated uptake could be taking place.	[97]
Cystic firbrosis	Human polymeric immunoglobulin receptor (hpIgR)	Antiprotease delivery through conjugation to an antibody correlated with areas of hpIgR expression in the respiratory epithelium in an in vivo study.	[73]
Asthma	Beta2-adrenergic receptor (β2-AR	SiRNA conjugated to salbutamol exhibited greater gene silencing compared to the non-conjugated siRNA in an in vivo study.	[98]

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Asthma	Transferrin receptor (TfR)	Biodistribution study of siRNA, transferrin-polyethylenimine polyplexes in an in vivo model confirmed efficient and selective delivery of siRNA to activated T cells.	[77]

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