Overcoming the pulmonary barrier: new insights to improve the efficiency of inhaled therapeutics

Abstract: The pulmonary route offers an exceptional, non-invasive administration site for drug delivery. The principal characteristics that make the lungs an appealing route for drug administration include a large surface for drug dispersion (approximately 100 m²), a low content of drug-metabolizing enzymes, and a high vascularization for systemic drug delivery. Recent advances in this field such as the development of modern inhalation devices, novel inhalation-adapted formulations, and innovative drug carriers have contributed to a significant improvement in the low level of lung aerosol deposition achieved in the past, and have allowed for an enhancement in aerosol penetration into the lungs. Less focus however has been placed on the fate of inhaled particles after they deposit onto lung surfaces. After first contact with a pulmonary surface therapeutic particles are exposed to complex microenvironments and biological barriers (both cellular and non-cellular) that may vary widely in composition depending on the region of the lung in which the particles deposit. Most of the current inhaled therapies aim to achieve deep lung deposition at the alveolar air-blood barrier. In this particular region, the epithelium is coated with the pulmonary surfactant, a thin liquid layer composed of lipids and proteins that reduces surface tension in the alveoli, but which also interacts with and may influence the fate of inhaled therapeutics within the alveolar region. In addition, alveolar macrophages efficiently engulf inhaled particulates in the 1–5 μm size range; these therefore also pose a significant barrier to the effective delivery of therapeutic micro- and nanoparticles (NPs). Furthermore, the tightly-joined epithelium of the airways is coated with a dynamic viscous mucus layer which forms the mucociliary escalator, an efficiently coordinated piece of machinery that entraps inhaled particulates including pollutants, pathogens and, eventually, therapeutic NPs, and removes them from the lungs. A better understanding of the complex processes to which inhaled particles are subjected within distinct regions of the lungs may allow for the design of innovative therapeutics, including biocompatible polymeric NPs, aimed to efficiently overcome the complex pulmonary barriers and thus enhance the therapeutic efficiency of NP-associated actives. The current review therefore discusses the structure of the pulmonary barriers, as well as some of most innovative strategies to overcome them in order to facilitate an enhanced delivery of inhaled therapeutics.

Keywords: mucus; nanoparticles; pulmonary barriers; respiratory epithelium; surfactant.

Introduction

The use of aerosols with therapeutic intention was made possible in the late 1950s, with the advent of the first efficient jet nebulizers (1). In the following decades the use of inhaled therapies has significantly increased, powered by intensive multidisciplinary research which has led to the development of modern inhalation devices, novel inhalation-adapted formulations and innovative drug carriers, including therapeutic nanoparticles (NPs) (2, 3).

The rationale for the use of the pulmonary route for drug delivery is based on several aspects. Firstly, this route is readily accessible (via oral or nasal access), and constitutes a non-invasive approach for drug delivery. The pulmonary route also presents an extensive surface area for drug dispersion, of approximately 100 m² (4). In addition, drug metabolizing enzymes are in lower concentration within the lungs in comparison to other organs, which may a priori enhance drug bioavailability.
after inhalation, compared for instance to oral administration (5). In the case of pulmonary diseases (e.g., lung cancer or cystic fibrosis, CF) aerosolized therapeutics enable localized treatment of the pathology, thereby avoiding systemic exposure and its associated potential side effects (2, 6).

The pulmonary route may however also be used for systemic drug delivery (7). Besides a large alveolar surface, the lungs are also highly vascularized, allowing for a rapid absorption of many small drug molecules directly into the left heart (8). This feature is of special interest in relation to drugs which show a poor oral bioavailability due to first pass metabolism, as well as therapeutics for which a rapid onset of action is desired (9). In this regard, insulin is probably the most studied inhaled drug for systemic delivery, and has been proven to be effective in clinical trials when delivered via the pulmonary route (10, 11).

Extensive basic and clinical research on aerosol delivery of therapeutics, especially for the treatment of pulmonary diseases of high prevalence such as chronic obstructive pulmonary disease (COPD), asthma and CF, has resulted in a better understanding of inhalation technology. Traditionally, research efforts into pulmonary aerosol delivery have mainly aimed to improve lung deposition of inhaled drug particles. In this regard, the aerodynamic diameter of therapeutic particles has received great consideration as a predictor of deposition within different anatomical regions of the lung, resulting in the designation of an acceptable aerodynamic diameter range (1–5 μm) by the pharmaceutical industry (6). In current research, the effect of a great number of other parameters that significantly impact on particle size and lung deposition, including temperature, humidity, driving gas flow, drug viscosity, surface tension, and patient characteristics and breathing technique (6), is additionally considered during the design and development of efficient aerosol delivery devices. As a result of such additional research considerations, lung deposition levels as high as 70% of the device-filling dose can be currently achieved in patients with a good inhalation technique (12), in comparison to the relatively low lung deposition percentages (10–20%) achieved previously (2, 13).

Interestingly however, much less research focus has been placed on the fate of inhaled particles once they have been successfully deposited onto lung surfaces (14). After deposition a particle is exposed to a complex microenvironment, the exact composition of which varies greatly depending on the anatomical lung region in which the particle deposits (i.e., conducting airways or alveolar region). Moreover, the deposited particle encounters a number of biological barriers that might favor or preclude interaction with its therapeutic target. For instance, if a particle deposits in the upper airway, it may be trapped within the mucus layer and cleared by the mucociliary escalator before it reaches the underlying epithelium (15). After alveolar deposition, a particle will interact with the alveolar surfactant layer and may potentially be taken up by a macrophage before it can be absorbed through the thin alveolar epithelium (16). Thus, it cannot be assumed that all deposited drug particles will ultimately facilitate a therapeutic effect. Gaining understanding of the complex interactions that occur between therapeutic particles and pulmonary elements within the lungs can give rise to an improvement in design of novel pulmonary carriers, such as therapeutic NPs, for enhanced drug bioavailability.

The use of NPs may offer significant advantages for pulmonary administration of drugs, including facilitation of sustained or controlled release, deep tissue penetration, protection of the therapeutic cargo, and enhanced cellular uptake and subcellular trafficking (17, 18). In order to achieve these goals, therapeutic NPs can be engineered from a wide range of biocompatible materials (3) and further modified through changes in shape or surface chemistry (19, 20). The use of biocompatible NPs therefore holds great potential as a method to enhance the efficiency of pulmonary drug delivery, not only due to their intrinsic advantages but also as a result of the possibility to adapt their design in terms of size, shape and surface chemistry to the demanding conditions imposed by pulmonary (both cellular and non-cellular) barriers. In this regard, recent advances in drug delivery confirm the viability of delivering aerosolized NPs by means of a vibrating mesh nebulizer for targeted drug delivery of poorly soluble compounds (21).

The present review will discuss the histological organization of the lungs and the structure of the pulmonary barriers, as well as the most innovative strategies to overcome these barriers for enhanced delivery of inhaled therapeutics.

**Histological organization of the lungs**

The respiratory epithelium represents the body’s largest exchange surface with the surrounding environment. The main function of the respiratory system is to deliver air to and excrete air from the alveoli, while simultaneously offering protection from exogenous threats contained within the inhaled air, such as pathogens and pollutants.
Accordingly, the structure of the respiratory system has evolved to efficiently carry out this function. The lungs resemble an inverted tree in which the conduction airways branch systematically over more than 20 generations before the alveoli are reached (22). As the air flows down through the conducting airways the turbulent nature of the inhaled air diminishes and it is warmed and humidified. This particular design allows for filtering of a significant amount of exogenous, inhaled particles at upper airway level, preventing them from reaching the alveoli, where gas exchange occurs through the large surface of the extremely thin air-blood barrier. More than 40 cell types contribute to ensure the proper functioning of the respiratory system (23), creating two well-differentiated histological regions along the respiratory epithelium: the gas exchange area or alveolar region and the conducting airways.

The trachea, bronchi and bronchioles form the conducting airways of the lungs. The conducting airway epithelium is pseudo-stratified and columnar in nature, and is formed by ciliated, basal and secretory cells (24). Ciliated cells (20–60 μm tall) make up approximately half of the epithelial surface and each one is coated with approximately 250 cilia (25). A vital function of the ciliated cells is to propel the protective mucus layer on top of the airway epithelium towards the proximal airways, for gastric clearance and metabolism. Basal cells are situated near the basal membrane and therefore do not contribute to the luminal surface of the epithelium. However, a role as progenitor cells of ciliated and Clara cells have been proposed for basal cells (26). Clara cells, together with mucous, goblet and serous cells constitute the secretory cell set of the airways; these cells secrete a wide variety of molecules that are further incorporated into the mucus layer.

In contrast to the aforementioned pseudo-stratified columnar epithelium in the proximal airways, the structure of the airway epithelium in the distal bronchioles is more cuboidal and non-ciliated in nature (27). The protective mucus layer that covers the epithelium also progressively reduces in thickness as the alveolar region is approached, decreasing from a thickness of 10–30 μm at tracheal level, to 2–5 μm in the smaller bronchi (28, 29) (Figure 1).

The alveolar epithelium is dominated by two cell types: type I and type II pneumocytes. Type I pneumocytes show a flattened shape with a variable thickness of 2–3 μm around the perinuclear region and just 0.2 μm in the cell periphery (25). This particular shape allows them to cover more than 90% of the alveolar surface, although their contribution in terms of cell number accounts for only approximately 10% of all alveolar cells (23, 24, 30). The thin squamous alveolar epithelium provides an excellent platform for gas exchange between the alveolar lumen and the underlying blood capillaries. Type II pneumocytes, on the other hand, cover just 5% of the total alveolar surface even though they are significantly greater in number in comparison to type I pneumocytes (31). Their shape is rather cuboidal and they play a secretory role within the alveolar region. Indeed, pulmonary surfactant, a complex mixture of lipids and proteins that lines the alveolar surface, is synthesized, stored and secreted by type II pneumocytes (32, 33). The main function of this surfactant layer is to avoid alveolar collapse by reducing surface tension within the alveoli, while the hydrophilic surfactant-associated proteins (SPs) A and D also play a role in the innate immune defense of the lungs (34, 35). Alveolar macrophages are additionally involved in the immune response of the lungs; they are derived...
from bone marrow monocytes and are transported to the alveolar space via the capillaries, where they patrol the alveolar air-spaces and have the ability to phagocytize a number of elements including pathogens, less active surfactant components and, ultimately, microparticles and NPs (36–38).

The large alveolar surface is mirrored, to some extent, by a large capillary endothelial surface, which accounts for approximately 40% of the total cellular content of the lungs. The thin capillary barrier may vary in thickness from 200 nm to as little as 35 nm and, even though it is readily permeable to respiratory gases, it may pose a barrier to inhaled particles (24). Other cellular components of the lung epithelium include cartilage, smooth muscle cells, and the cells of the interstitium, encompassing the elastin- and collagen-producing fibroblasts along with inflammatory (dendritic and mast) cells, neural elements and other additional cell types (22).

Biological barriers of the alveolar region

The alveolar region represents a capital target site for drugs delivered via the pulmonary route. Inhaled therapeutics reaching the deep lung may have a local therapeutic function or may be intended for systemic drug absorption. In the case of inhaled β-2 agonists and inhaled corticosteroids, indicated for the management of mild to severe asthma or COPD, both local and systemic actions account for therapeutic effect. On the one hand, these drugs are directly delivered to the local site of action within the lungs and their action may result in the majority of inhaled particles being cleared and degraded before they carry out their therapeutic effect (unless the alveolar macrophages themselves represent the therapeutic target; e.g., in tuberculosis).

As mentioned, uptake of particles by alveolar macrophages has been found to be size dependent. Particles in excess of 5 μm are apparently taken up to a much smaller extent, and NPs with a diameter below 240 nm are also minimally taken up by macrophages (46). The particle size for optimal macrophage uptake however remains controversial (47), and in addition to size, particle uptake may vary according to particle shape and surface chemistry (36). Additionally, alveolar macrophages play a significant role in the modulation of the immune response triggered by pathogens, inflammatory cytokines, and other factors that contribute to the pathogenesis of pulmonary diseases.

Alveolar epithelium

The alveolar epithelium is designed to serve as an efficient structure for gas exchange. For this purpose a vast portion of the epithelial surface (>90%) is covered by the flattened type I pneumocytes, which form a thin layer (0.1–0.3 μm) (23, 25) and allow for a fast diffusion of oxygen into the blood stream, as well as an evacuation of carbon dioxide to the alveolar air-space prior to its complete removal from the organism. In contrast, type II pneumocytes play a role in the regulation of surfactant metabolism, ion transport, and alveolar repair in response to injury (41). In the healthy lung, type I pneumocytes form tight junctions with other type I pneumocytes as well as with type II pneumocytes, resulting in the formation of a tight alveolar epithelial barrier that displays a high transepithelial electrical resistance (5, 42). Particles and molecules below 100 nm in diameter are considered to efficiently penetrate this barrier (43), whereas larger particles may cross the air-blood barrier chiefly by endocytic pathways (Figure 2). The specific mechanisms of particle uptake by alveolar epithelial cells remains an area of active research, and seems to be highly dependent on the interaction of inhaled particles with the pulmonary surfactant layer. For instance, particle-surfactant interactions may lead to translocation of particles across epithelial barriers via receptor-mediated recycling of pulmonary surfactant components by type II pneumocytes (44); particles may also/alternatively be cleared from the alveolar space by SP-A- and SP-D-mediated macrophage phagocytosis (16, 45).

Alveolar macrophages patrol the air spaces in the deep lung and have the ability to efficiently clear inhaled particles in the 1–5 μm size range (44), paradoxically the same particle size range expected to reach the alveolar region after aerosol therapy (6, 24). Thus, alveolar macrophages pose a significant barrier to therapeutic particles reaching the alveolar space as their presence and action may result in the majority of inhaled particles being cleared and degraded before they carry out their therapeutic effect (unless the alveolar macrophages themselves represent the therapeutic target; e.g., in tuberculosis).
by inhaled NPs by releasing inflammatory (TNFα, IL-1α and IL-1β) or anti-inflammatory (IL-10) mediators, or by interacting with dendritic cells acting as antigen presenting cells (48).

Figure 2  Schematic diagram of the air-blood barrier at the alveolar region. The air-blood barrier consist primarily of a layer of pulmonary surfactant, composed of phospholipids (PL) and surfactant proteins (SP) A, B, C and D, followed by the tightly joined alveolar epithelium built up by type I (gray) and the surfactant-producing type II pneumocytes (lighter gray). Underneath the endothelial cells (brown) represent the last cellular barrier before the bloodstream is reached. In this context, the fate of a nanoparticle (NP) might significantly be influenced after a first contact with the surfactant layer; PLs and hydrophilic SPs (SP-A and SP-D) might absorb to the surface of the NP facilitating its clearance by alveolar macrophages (yellow) (1). On the other hand, NPs can be immediately displaced to the hypophase and put in close contact with the alveolar epithelium (2), or might be internalized by type II pneumocytes via receptor mediated recycling of pulmonary surfactant components (3). Eventually, NPs internalized by epithelial cells by means of endocytic routes might penetrate into the capillaries and be suitable for therapeutic systemic delivery (4).

The barrier properties of the alveolar epithelium are difficult to mimic in vitro. To date significant efforts in the development of stable in vitro cell lines (tumor-derived or immortalized cell lines) intended to model the alveolar region have not yet resulted in a cell line which is able to form tight monolayers of polarized cells (as assessed by measurement of transepithelial electrical resistance), an important characteristic of the epithelium in vivo. Therefore, primary cultures remain the option of choice for transport studies. Primary cells however have a number of limitations including their high cost, a limited life span, and a high level of variability between donors, passages, and experiments (49).

Animal- and human-derived type II pneumocytes differentiate into type I pneumocytes after 5–10 days in culture (24, 41). Under in vitro culture conditions type II pneumocytes lose their cuboidal appearance, and become flattened; also lamellar bodies, characteristic of type II pneumocytes, decrease in size and in number progressively (41). As a result, a tightly joined type I pneumocyte-like cell layer with a high transepithelial electrical resistance is achieved.

Cell lines that model the alveolar region have been frequently used for metabolic and toxicity studies (25), with A549 being the prevalent cell line used for these purposes. A549 cells exhibit a phenotype similar to that of type II pneumocytes due to the presence of lamellar bodies and SPs. Indeed, A549 has been widely used as a system to study the regulation of pulmonary surfactant synthesis (24). Moreover, A549 cells have also been incorporated into sophisticated triple co-cultures, together with macrophages and dendritic cells, to produce tools for the investigation of immunological crosstalk between cell-types after exposure to NPs (23). Nevertheless, the development of an alveolar cell line model that further displays significant epithelial barrier properties would be highly desirable. Recently, Salomon et al. have reported the potential of the NCI-H441 cell line as a model of the distal lung for transport studies (30). NCI-H441 cells express markers typical of human type II pneumocytes, including SPs. In the study by Salomon et al., high transepithelial electrical resistance values and evidence of the presence of ZO-1 and E-cadherin, both markers of tight junctions, were observed under liquid culture conditions. The barrier properties, however, were significantly lowered under air-liquid culture conditions, a culture condition that represents more accurately the in vivo situation.
Pulmonary surfactant

Pulmonary surfactant is a complex mixture of lipids and proteins that lines the inner epithelial surface of the alveoli. The pulmonary surfactant enables efficient gas exchange by reducing the surface tension that originates at the air-liquid interface at the end of expiration (50). It is composed of more than 50 lipid species, with PLs representing approximately 80% of the total surfactant mass (32, 51). Dipalmitoylphosphatidylcholine (DPPC) is the most important lipid for the surface tension reduction function of pulmonary surfactant (52). However, at physiological temperature the dynamic properties of DPPC are significantly limited, while its actual function is dramatically enhanced in vivo by the hydrophobic SPs, SP-B and SP-C (53). Four different surfactant-associated proteins have been described to date, named SP-A, SP-B, SP-C and SP-D as previously mentioned, following the order in which they were discovered (54). SP-A and SP-D are hydrophilic proteins with a minor function in surface tension reduction, but with an important role in the host defense of the lung (35, 55). On the contrary, SP-B and SP-C are small hydrophobic proteins which are involved, together with PLs, in the surface tension reduction at alveolar level (56).

Although pulmonary surfactant may impose a barrier to pathogens and certain inhaled particles, the barrier properties of the alveolar lining fluid cannot be compared to those of the pulmonary lining fluid of the airways (see Section Respiratory mucus). Pulmonary surfactant might in fact enhance the bioavailability of inhaled therapeutics, by increasing their solubility through complex drug-surfactant interactions (57). Particle wetting with surfactant PLs may induce their immediate displacement into the alveolar lining fluid, provided that the size of the particle does not exceed the thickness of the surfactant layer (58, 59). Current structural models for pulmonary surfactant propose the existence of several PL layers that act as a reservoir for those PLs exposed to the air-liquid interface. According to this model, these PL layers are connected by SPs which would further form porous structures that facilitate the diffusion of small polar molecules such as ions, defense proteins and peptides through surfactant membranes (34).

In the case of larger structures like NPs- and microparticles, the barrier properties of pulmonary surfactant rely on the complex interplay between the hydrophilic surfactant proteins, SP-A and SP-D, epithelial cells, and alveolar macrophages. In this regard, Ruge et al. have recently demonstrated that both SP-A and SP-D have the ability to bind magnetic NPs and to promote their clearance by alveolar macrophages in vitro (16, 45). Interestingly, these studies have shown that the adsorption of SP-A or SP-D to NPs is largely influenced by the coating material of the magnetic NPs, with SP-A showing a higher preference for those particles with a PL coating and SP-D adsorbing to a greater extent to starch-coated magnetic NPs (45). These findings strengthen the theory of the formation of a protein corona around inhaled NPs that will further determine their fate within the alveolar environment (60). In the case of the alveolar region in vivo, one could expect the resulting corona around the NPs to be composed not only of SPs but also of many of the different lipid species contained within pulmonary surfactant, which have the ability to modulate the adsorption patterns of the hydrophilic SPs (45). Therefore, both lipid and protein components should be considered when investigating NP-surfactant interactions.

The interaction between inhaled NPs and the hydrophobic SPs, SP-B and SP-C, has not been widely investigated, although this potential interaction has been pointed out as a possible mechanism of surfactant dysfunction as provoked by NPs (61). From a therapy safety perspective it is thus of critical importance that NPs have minimal or negligible effects on the surface tension reduction function of pulmonary surfactant. Furthermore, they should not induce lung inflammation and/or alveolar permeability, since many of the components of the inflammatory cascade and some of the proteins contained in the edema fluid may lead to pulmonary surfactant inactivation (34). The effects on surfactant function of therapeutic NPs should therefore be properly assessed in vitro prior to their in vivo use (61).

Biological barriers of the airways

Although alveolar deposition is the aim of many inhaled therapies, the airways remain a major site for drug deposition as a result of the use of currently-available aerosol delivery devices (62). Therefore, the therapeutic efficacy of such inhaled therapies will be highly dependent on the ability of deposited particles to overcome biological barriers present in this particular anatomical region. As mentioned, in the conducting airways the mucociliary escalator and the underlying epithelia represent the major barriers for inhaled therapeutics.

Mucociliary clearance is probably the most important defense mechanism of the lungs. This mechanism works as a coordinated system of epithelial water and ion transport, mucin secretion and cilia action, resulting in the entrapment and clearance of distinct particulate matter in the
nano and micro size range (63). Such a size range includes pathogens, harmful particulates and, ultimately, therapeutic NPs. The exogenous particulates trapped in the viscous mucus blanket are continuously propelled towards the upper airways for gastric elimination. In the case of therapeutic NPs intended for intracellular or systemic delivery, even if they manage to traverse the mucus barrier (17), they will still have to cross the epithelial barrier. Here the tight junctions between neighboring cells provide a significant barrier property to the epithelium (64, 65) and the fate of the particles (cellular uptake, or not) will principally depend on their size, shape and surface chemistry (19).

Airway epithelium

The epithelium of the conducting airways is a tightly organized layer that poses a significant barrier to those therapeutic particles aimed for intracellular or systemic delivery. The apical membranes of both bronchial and alveolar epithelial cells are joined by tight junctions dividing the cell membranes into functionally distinct apical and basolateral domains (24, 64). Interestingly, apical-to-basal electrical resistance across the epithelium (a measure of the tightness of cell junctions) seems to decrease from a maximum in the trachea to a minimum in the distal airways before returning to a high value in the alveoli (5). Since the mucus layer also decreases in thickness in the more distal airways, the terminal section of the conducting airways might represent an appealing target site for pulmonary drug delivery.

Particles are internalized (or not) by airway epithelial cells depending on a number of factors such as their size, shape, charge, and surface functionalization (19). The uptake can happen through different mechanisms: phagocytosis, macropinocytosis, clathrin-dependent endocytosis, caveolae-mediated endocytosis or clathrin- and caveolae-independent endocytosis (Figure 3). Small molecules can be transported by passive diffusion through the plasma membrane, absorbed as a result of the action of specific transporters, or translocated via tight junctions (paracellular transport), whilst larger particles may require vesicular transport systems for their translocation through the epithelium (5, 19, 25). In this regard, among the different endocytic routes available for particle translocation, some require receptor-mediated activation, whereas others such as macropinocytosis and phagocytosis (which use membrane folds to encircle and take up particles) are considered as nonspecific internalization modalities (19).

In vitro cell culture models represent an excellent tool to investigate particle trafficking through the airway epithelium. Ideally, a relevant in vitro model of the airway epithelium should accurately represent the barrier properties of the bronchial epithelium in vivo. Primary cells, those

![Figure 3](Image)

**Figure 3** Possible uptake mechanism for cellular entry of particles and their subsequent trafficking. Small molecules may be transported passively via diffusion through the plasma membrane (A). Particles may be also internalized via clathrin-dependent endocytosis (B), caveolin-dependent endocytosis (C), clathrin- and caveolae-independent endocytosis (D), macropinocytosis (E) and phagocytosis (F). Endocytic stages (B-F) proceeds from the plasma membrane and involves engulfment of particles into intracellular vesicles, which usually are carried to early endosomes (EE), late endosomes (LE) and lysosomes in order to be digested. Sometimes particles might be also exocytosed (G).
obtained directly from human or animal lung tissue, are considered the gold standard model to study the barrier properties of the airway epithelium. On the other hand, stable cell lines, represented by tumor or immortalized cells, are readily accessible at a significantly lower cost. Their biological relevance has however been frequently brought into question due to their genetic alterations, and because important physiological characteristics may be lost when such cells are grown in vitro (25).

Calu-3 is one of the most used cell lines for the study of the airway epithelium and has been used for transport and metabolic studies (66, 67). When grown on collagen-coated permeable filters, Calu-3 cells are able to form tight junctions as shown by the expression of occludin, claudin-1, connexin 43 and E-cadherin (24, 25). Calu-3 cells can be cultured in liquid culture conditions as well as with an air-liquid interface (27). Air-liquid culture conditions better resemble the in vivo situation of the airways; in such circumstances Calu-3 cells form a pseudo-stratified layer of columnar cells, display enhanced ciliogenesis, secrete a mucus gel layer on the cell surface, and possess barrier integrity typical of the upper airway epithelium (62). Other utilized cell lines of the airway epithelium include 16HBE14o- and BEAS-2B (68, 69). Nevertheless, neither of these models is able to secrete mucus and the latter cannot form tight junctions (25), which presents a serious drawback when the barrier function of the epithelium is considered.

**Respiratory mucus**

Upon deposition within the airways inhaled particulates make contact with one of the most important defense mechanisms of the lungs, the respiratory mucus. Human mucus can be defined as a protective semipermeable layer that coats several mucosal epithelia throughout the body, including the gastrointestinal tract, the female cervicovaginal tract, the ocular surface epithelium, and the pulmonary airways (70). Some of the secreted elements are common to many mucosal tissues (71), however, the specific composition and the physicochemical characteristics of mucus may significantly vary between different anatomical locations in the human body (17, 72).

In the conducting airways of the lung, the mucus barrier possesses gel-sol characteristics and is organized in two differentiated phases (24). The upper layer of the mucus, that which is exposed to the airway lumen, possesses a high viscosity (gel) and acts as a potent sticky filter for inhaled particulate matter. On the other hand, the lower layer, also termed the periciliary fluid (73), is considered a watery (sol) layer. This layer allows the cilia to beat and recover, so that the rather thick mucus blanket can be propelled towards the proximal airways (24, 25).

Mucus is primarily composed of water (95%), glycoproteins (mucins, 2–5%), salts, non-mucin proteins, lipids, DNA, enzymes, cells and bacteria, and most likely of a layer of pulmonary surfactant (24, 72–74). Mucins are glycoproteins that are made up of a polypeptide backbone containing high levels of O-glycosylated tandem repeats (75). The glycans account for 40–80% of the weight of the mucins and confer upon them a negative charge at physiological pH (17, 75). Five major mucins are expressed in the airways: MUC1, MUC4, MUC5AC, MUC5B, and MUC16 (76). Of these, MUC1, MUC4 and MUC16 are cell membrane-tethered mucins with a single-pass intracellular domain and an extracellular domain that may extend 500–1500 nm into the extracellular space (15, 76, 77). Some of the proposed roles for membrane-associated mucins include intracellular signal transduction pathways, control of inflammation and immune responses, and regulation of cell differentiation and proliferation (76). On the other hand, MUC5AC and MUC5B are the most important secreted mucins of the airways (77). MUC5AC is produced and secreted by goblet cells (78), whereas MUC5B is mainly produced by submucosal glands (76). The gel-forming MUC5AC and MUC5B are the major macromolecules of mucus and are primarily responsible for the viscous nature of the luminal mucus mesh. This complex mesh is formed due to the ability of single mucin units to interact with other mucin molecules by means of intermolecular disulfide bonds (75, 79), yielding large molecular structures which may be several micrometers in length. For instance, in a 2–5% mucus gel, each mucin may crosslink up to 100 other mucins (44). This systematic overlapping of mucin macromolecules leads to the formation of a tight mesh with a highly heterogeneous pore size, and a bulk viscosity at physiological shear stress levels up to 10,000 times higher than that of water (72, 80).

The mucus gel pore size (size filtering), on the one hand, and polyvalent particle-mucus interactions (electrostatic and hydrophobic filtering) on the other, represent the governing mechanisms that enable exogenous particulate material to be trapped and immobilized in the viscous mucus layer (81). With regard to the pore size of pulmonary mucus the average mesh spacing remains a point of discussion, in part because the sample preparation protocols for the imaging techniques used to estimate the pore size (e.g., scanning electron microscopy, SEM) alter the native structure of the gel layer during the dehydration and fixation processes (72). Mucus mesh pore sizes for cervicovaginal mucus has been suggested to be
around 350 nm, whereas human CF mucus and rhinosinusitis mucus have been estimated to form a mesh with an average spacing of approximately 150 nm (44). Recent data from Kirch et al. using cryogenic SEM (cryo-SEM), which allows for unbiased determination of the native mucus structure, has revealed a highly heterogeneous pore size for respiratory mucus ranging from pores of approximately 100 nm to voids of several micrometers in diameter (Figure 4). In this study, the authors highlighted the significant thickness and rigidity of the polymer scaffold that resulted in the entrapment of all types of studied nanoparticles (size range 170–500 nm) (80).

In addition to size filtering, polyvalent low-affinity interactions are of paramount importance in order to entrap and remove foreign particles smaller in size than the mesh pore of mucus. Besides the negative charge provided by the glycan side chains (82), the mucins also possess non-glycosylated regions with a high capacity for hydrophobic interactions (17). Hence, one would expect that positively charged particles (i.e., chitosan-coated NPs) as well as hydrophobic NPs will interact with mucins by electrostatic and hydrophobic interactions, respectively, even when their diameter is lower than the mesh pore size (72). Thus, the interplay between stearic forces and polyvalent low-affinity interactions provide the mucus with an exceptional barrier property for any inhaled particulate matter, including those microparticles and NPs intended for therapeutic purposes.

In order to improve the therapeutic delivery of NPs to mucosal tissues, several delivery strategies are currently being explored. The use of mucoadhesive substances has been proposed as a method to prolong the residence time of the particles in mucosal tissues (83, 84). One of the mechanisms of mucoadhesion in pulmonary delivery would be the alteration of mucus rheology (increased viscosity), which would lead to a decrease in the rate of mucociliary clearance and would therefore prolong the residence time of the therapeutic NPs in the respiratory tract. However, while this strategy might be of interest for oral delivery of therapeutics (adhesion to gastrointestinal mucus), in the case of pulmonary drug delivery it can be expected that mucoadhesive particles will most likely remain trapped in the luminal gel mucus layer, even if the overall mucus clearance rate could be diminished. Moreover, in the course of several pulmonary diseases in which mucus rheology is already altered (i.e., CF) an increase of the bulk viscosity of mucus should be rather avoided than promoted.

In many lung disease states mucolytics can be used as an adjuvant therapy for the delivery of inhaled therapeutics. Mucolytics can alter the mucus mesh structure by degrading or interacting with some of the components of the mucus mesh. For instance, N-acetylcysteine reduces the bulk viscosity of mucus by interfering with the disulfide bonds that hold mucin molecules together (79), while Pulmozyme (DNase) is useful for degrading the DNA of pulmonary purulent secretions which accumulate in the mucus layer during several pulmonary conditions and lead to increased mucus viscosity (85). Nevertheless, even if the use of mucolytics might be of advantage in certain cases, it should be noted that the use of these substances disrupts the natural barrier property of the mucus layer. The use of mucolytics might therefore result in undesired particulate matter traversing the mucus barrier together with therapeutic NPs.

The most innovative and promising method to overcome the mucus layer for therapeutic purposes is the development of mucus-penetrating NPs (17, 70, 86). The rationale for the design of such NPs is derived from the observation that some viruses can cross the mucus gel

---

**Figure 4** Representative cryo-SEM images of pulmonary mucus. The cryo-SEM images of mucus show the strongly heterogeneous nature of mucus polymer mesh (left image). Large as well as very small pores can be observed (left image). Furthermore, the enlarged view of the mucus (right image, representing the area on the left image indicated by a square) shows a thick wall of the polymer scaffold (Scale bar: left 10 μm; right 500 nm.). From reference (79), reprinted with permission from PNAS.
layer at similar rates as they move in water (81). In fact, if the pores of the mucus mesh are filled with a fluid with a similar viscosity to that of water, then particles with a diameter below the average mesh spacing would theoretically penetrate the mucus gel layer, provided they do not interact with airway mucins. In the case of viruses, a muco-inert armor is achieved by the presence of alternating surface charges (positive and negative) that make the virus into an hydrophilic “particle” with overall neutral charge (87). The complex natural surface of these viruses would be difficult to mimic in the design of therapeutic NPs and thus feasible approaches have been investigated in the development of mucus-penetrating NPs (20, 86).

In general, uncoated polymeric NPs possess hydrophobic surfaces that adhere to the hydrophobic domains of mucins (72). Adding a cationic coating, as achieved through the use of chitosan, also leads to mucus adhesion of particles due to electrostatic interactions (83). In contrast, mucus-penetrating NPs are hydrophilic and generally possess a neutral or slightly negative surface charge (20, 70). Kai et al. demonstrated that covalently-modified polystyrene NPs coated with high density, low molecular weight polyethylene glycol (PEG) of 200 or 500 nm in diameter could penetrate fresh undiluted human cervical mucus, contradicting the previously reported mesh pore size for this particular mucus layer (70). Moreover, in a further study using PEG-coated NPs exposed to human airway mucus collected from the endotracheal tubes of patients undergoing non-thoracic surgery, the authors were able to demonstrate that 100 and 200 nm diameter PEG-coated NPs were small enough to diffuse through the fluid-filled pores of the mucus network (72). In this second study, 500 nm PEG-coated particles were immobilized by respiratory mucins; this highlights the difference in structure of cervicovaginal and respiratory mucus, despite the presence of similar rheological properties. In a similar fashion, Mura et al. have recently shown that PLGA NPs coated with a low molecular weight and negatively-charged molecule (Pluronic F68) could diffuse unimpeded through the mucus layer and were highly internalized in an in vitro model (Calu-3) of the airways (20).

Paradoxically, Ungaro et al. have reported that 10% of chitosan coated (positive) and about 7% of poly-vinyl alcohol (negative) coated PLGA NPs can penetrate a synthetic mucus layer after 24 h of exposure (88). This apparent discrepancy in the results might be partly explained by the use of diverse NPs, different mucus types (native human-derived, calu-3-derived, or synthetic mucus) which might display different characteristics, but also by the time-scale of the NP penetration measurements (24 h in the Mura and Ungaro studies) that might in some cases be significantly higher in comparison to the clearance rate of the mucociliary escalator. Therefore, in vivo, mucus penetrating NPs should penetrate the mucus layer in a relatively short time period and in a significant amount, in order to achieve a proper therapeutic effect. A key factor for the efficient mucopenetration of these particles seems to be appropriate coating (high density) with neutral or slightly negatively charged low molecular weight molecules. This further minimizes the electrostatic interactions of the particles with mucins, while conferring on them a hydrophilic nature by efficiently hiding the hydrophobic, surface-exposed areas.

Conclusion

Significant technological advances over the last decades have dramatically improved the lung deposition of aerosolized therapeutics, achieving deposition percentages as high as 70% with the most innovative devices. Nevertheless, increasing interest in particle-lung interactions has identified a number of pulmonary barriers that may hinder the therapeutic effect of the deposited particles. The lung epithelium at both alveolar and airway level, together with present macrophages, represent the main cellular components of the pulmonary barrier. The pulmonary surfactant in the alveolar region and the mucus layer in the conducting airways constitute the pulmonary lining liquid, which can be considered as the major non-cellular pulmonary barrier to inhaled therapeutics. Recent investigations of the pulmonary barrier have identified a number of mechanisms by which particles can be entrapped and cleared from the lungs. For example, inhaled therapeutics may be entrapped in the mucus layer by polyvalent interactions or stearic forces, whilst in the alveolar region NPs may be cleared from the alveolar space via SP-mediated macrophage uptake.

Nanotechnology has the potential to revolutionize pulmonary drug delivery by utilizing the scientific knowledge of pulmonary barriers for the design and development of more efficient inhaled (nano)therapeutics. Present developments in this field include mucus-penetrating NPs, or NPs with different shapes that can evade clearance by alveolar macrophages. Further possibilities of nanotechnology in the field of lung delivery however remain extensive, and are expected to significantly improve the efficacy of many pulmonary therapies in the coming years. On the other hand, the main challenge of delivering nanoparticulates by inhalation remains on the ability to couple the new developments in the field of nanotechnology to efficient aerosol delivery devices.
Acknowledgments: This work was supported by the Marie Curie Initial Training Network PathChooser (PITNGA-2013-608373). The authors gratefully acknowledge the critical review of the manuscript performed by Dr. Sarah Gordon.

References


Xabi Murgia graduated in biochemistry at the University of the Basque Country and achieved a Master's degree in Neuroscience in 2010. From 2009 to 2013 he worked at Cruces University Hospital, in Bilbao, where he investigated the delivery of surfactant and perfluorocarbon aerosols as a treatment for experimental Respiratory Distress Syndrome. As a result of this work he was awarded a doctoral degree in 2014. Since January 2014, he is part of the Department of Drug Delivery at the Helmholtz Institute for Pharmaceutical Research of the Saarland (HIPS) as a Marie Curie fellow within the framework of the PATHCHOOSER initial training network (www.pathchooser.eu). Currently he focuses his investigations on the cellular and non-cellular barriers of the lung.

Cristiane S. Carvalho studied Biology at Universidade Estadual do Norte Fluminense (UENF), Rio de Janeiro state, Brazil. She studied the effect of Thiosemicarbazones in the development of intracellular *Toxoplasma gondii*. During her PhD, in the same university, she spent 2 years at the Federal University (UNIFESP), in Sao Paulo, Brazil, and 3 months in Germany (Braunschweig), at Helmholtz Centre for Infection Research (HZI), with a DAAD fellowship. At this time she started to work with *Mycobacterium spp.* infection in epithelial cells. She graduated in 2009 at the UENF and worked for 2 years as a Postdoctoral fellow at the UNIFESP, mainly focused on the characterization of the *M. abscessus* intracellular survival. In 2011 she continued the studies with *Mycobacteria* infection in human lymphatic primary cells at HZI-Braunschweig. Since March 2013, she is working as a postdoctoral fellow at the Helmholtz-Institute for Pharmaceutical Research of the Saarland (HIPS) in projects concerning pulmonary drug delivery.

Claus-Michael Lehr is Professor at Saarland University, and also cofounder and head of the department “Drug Delivery” at the Helmholtz-Institute for Pharmaceutical Research Saarland (HIPS). HIPS belongs to the Helmholtz Centre for Infection Research (www.helmholtz-hzi.de), Braunschweig, and is the first public research institute in Germany explicitly dedicated to the Pharmaceutical Sciences. The main focus of research of Prof. Lehr’s team (currently 8 senior and 30 junior researchers/PhD-students) over the past 15 years has been on the one hand exploring the biological barriers, in particular the gastro-intestinal tract, the skin and the lungs, and on the other hand developing the appropriate carriers capable of crossing these epithelial barriers and deliver the active molecule to the target. Prof. Lehr is (co)author of more than 250 publications and Fellow of both the American Association of Pharmaceutical Scientists (AAPSS) and the Controlled Release Society (CRS). His team has won several international and national research awards in the areas of Drug Delivery and of Alternative Methods to Animal Testing. Prof. Lehr has been coordinator of the Marie Curie ITN “Galenos EuroPhD in Advanced Drug Delivery” from 2004 to 2008. He is Co-editor of the European Journal of Pharmaceutics and Biopharmaceutics. Moreover, he is cofounder of two companies, Across Barriers GmbH and PharmBioTec GmbH.