BIO-INSPIRED MATERIALS IN DRUG DELIVERY: EXPLORING THE ROLE OF PULMONARY SURFACTANT IN SIRNA INHALATION THERAPY

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Abstract

Many pathologies of the respiratory tract are inadequately treated with existing small molecule-based therapies. The emergence of RNA interference (RNAi) enables the post-transcriptional silencing of key molecular disease factors that cannot readily be targeted with conventional small molecule drugs. Pulmonary administration of RNAi effectors, such as small interfering RNA (siRNA), enables direct delivery into the lung tissue, hence reducing systemic exposure. Unfortunately, the clinical translation of RNAi is severely hampered by inefficient delivery of siRNA therapeutics toward the cytoplasm of the target cells. In order to have a better control of the siRNA delivery process, both extra- and intracellular, siRNAs are typically formulated in nanosized delivery vehicles (nanoparticles, NPs). In the lower airways, which is the targeted site of action for multiple pulmonary disorders, these siRNA-loaded NPs will encounter the pulmonary surfactant (PS) layer, covering the entire alveolar surface. The interaction between the instilled siRNA-loaded NPs and the PS at this nano-bio interface results in the adsorption of PS components onto the surface of the NPs. The formation of this so-called biomolecular corona conceals the original NP surface and will therefore profoundly determine the biological efficacy of the NP. Though this interplay has initially been regarded as a barrier towards efficient siRNA delivery to the respiratory target cell, recent reports have illustrated that the interaction with PS might also be beneficial for local pulmonary siRNA delivery.

Keywords: Lung, nano-bio interface, nanoparticle, pulmonary delivery, pulmonary surfactant, siRNA

1. The lung is an attractive target organ for nanoparticle-mediated siRNA delivery

According to the latest update by the World Health Organization (WHO), chronic obstructive pulmonary disease (COPD), lower respiratory infections and lung cancer are respectively the third, fourth and fifth cause of death worldwide [1], illustrating the limited available therapeutic possibilities. In addition, numerous other respiratory disorders are characterized by an urgent and unmet therapeutic need. Since the pathophysiology of many of these incurable pulmonary diseases can be linked to genetic aberrations, they represent potential candidates for treatment by genetic interference [2]. Over the last two decades, extensive research in the field of RNA interference (RNAi) has created new therapeutic opportunities, as virtually all genes are susceptible to targeting by RNAi effectors such as small interfering RNA (siRNA) duplexes. Introduction of siRNAs into the cytoplasm of the diseased cells triggers the RNAi machinery, resulting in the sequence-specific post-transcriptional silencing of a target gene [3-5].

Targeting the respiratory tract for siRNA delivery offers the possibility for local siRNA applications via the intranasal, intratracheal or inhalation route. The direct pulmonary delivery of siRNA is considered beneficial since (i) the siRNA dose that needs to be administered is lower when compared to systemic administration, (ii) the risk of systemic side effects is reduced, and (iii) it provides a noninvasive route of administration and the possibility for self-administration. Most importantly, pulmonary delivery of siRNA mediates direct access to respiratory target cells. Depending on the type of airway disease, the target cells vary from epithelial cells, alveolar macrophages, interstitial dendritic cells to T lymphocytes. While epithelial cells are key players in e.g. cystic fibrosis [6] or different types of lung cancer [7], alveolar macrophages, dendritic cells and T lymphocytes are strongly involved in the pathogenesis of inflammatory diseases like asthma or COPD [8, 9].

To date, the potential of siRNA-based therapeutics has been evaluated in more than 20 clinical trials [10-12], of which only two have addressed the clinical potential of siRNA *via* inhalation. ALN-RSV01 (Alnylam® Pharmaceuticals) is a siRNA designed to inhibit the replication of respiratory syncytial virus (RSV) by interrupting the synthesis of the viral nucleocapsid [13].

The Phase IIb trial was initiated in 2010, but unfortunately the primary endpoint was not achieved and no further developments in the study program are described [14]. Another siRNA that was delivered *via* direct administration to the lung (Excellair™-ZaBeCor Pharmaceuticals), targeting spleen tyrosine kinase (Syk), is reported to improve the asthmatic symptoms in a Phase I trial. However, details on the clinical outcome of the Phase II trial that started in 2009 are still not available.

It is important to note that these studies evaluated the clinical potential of naked siRNA. The major advantage of this approach is its simplicity, thereby avoiding possible inflammatory responses associated with certain delivery vectors. However, it is anticipated that the local therapeutic response can be markedly enhanced through the use of nanoparticles (NPs), since they display the possibility to target specific pulmonary cell types and afford a better control of the drug delivery process, both extra- and intracellular [15, 16]. Novel nanoformulations are needed that show high drug loading, protect the siRNA, improve its pulmonary distribution and enhance its cellular internalization in specific lung cell types.

In this review, an overview of the different extra- and intracellular barriers that siRNA delivery platforms encounter following pulmonary delivery is provided, with a special emphasis on the pulmonary surfactant (PS) layer. Upon deep lung deposition, the PS layer is one of the first interfaces that siRNA-loaded NPs encounter, as it covers the alveolar surface of the respiratory tract. At the interface between NPs and biological systems (i.e. nano-bio interface), NPs progressively and selectively associate with a range of biomolecules, forming a so-called biomolecular corona [17]. Also in the respiratory tract, pulmonary administration of NPs typically results in the adsorption of PS components onto their surface. The latter interaction might alter the PS function and lead to NP-induced toxicities. On the other hand, it can also confer a new biological identity to the inhaled NPs, which profoundly determines their biological efficacy [18], a feature that might be exploited for the benefit of pulmonary drug delivery using nanomedicines.

2. The major barriers in nanoparticle-mediated siRNA delivery to the lung

The local delivery of siRNA towards the respiratory tract *in vivo* in pre-clinical models has been mediated by numerous types of nanosized delivery systems, including lipid-, peptide-, or polymer-based NPs [19]. Since recent review papers comprehensively describe the plethora of available NPs for pulmonary siRNA delivery, it will not be covered here [20, 21]. Instead we will focus on the obstacles that these siRNA-loaded NPs encounter in the respiratory tract, as the efficacy of the siRNA delivery system greatly depends on the successful delivery to its targeted site of action.

Despite the advantages of the local route of administration, the lung imposes intrinsic anatomic, physical, immunological, and metabolic barriers to efficient siRNA delivery (**Figure 1**) [22].

The highly branched structure of the lungs presents an early barrier to the deposition of instilled NPs into the lower respiratory tract (**Figure 1 (1)**). Here, the most important factor that determines the regional deposition of particles in the lungs, is the aerodynamic diameter (d_{aer}) of the liquid or dry powder aerosol. Particles with a d_{aer} between 1 and 5 µm are predominantly deposited in the lower airways, with a maximum alveolar deposition reached around 3 µm. Also aerosols characterized by particles with a d_{aer} reduced to the nanoscale mainly result in alveolar deposition [23]. Of note, besides the d_{aer} of the inhaled particles, also the breathing pattern of the patients determines the deposition in the respiratory tract. Lowering the respiratory rate (*e.g.* by breath-holding) for instance prevents the direct particle exhalation, thereby promoting deep lung deposition [24].

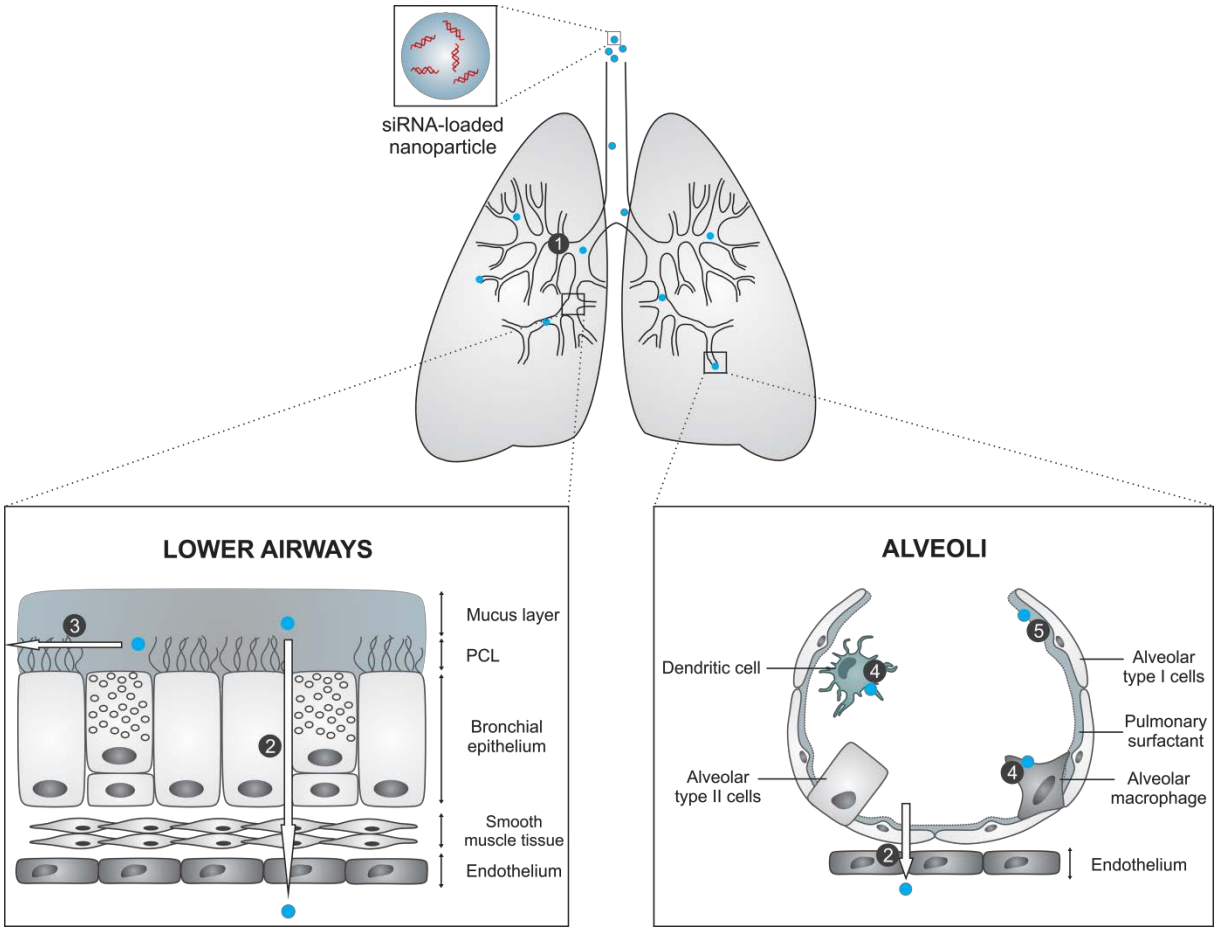

Figure 1. The predominant extracellular biological barriers encountered upon pulmonary application of siRNA-loaded nanoparticles.

(**1**) The highly branched airway structure can present an early barrier to inhaled nanoparticles (NPs). For local therapeutic effects, NPs must avoid rapid clearance from the lung *via* (**2**) translocation across the pulmonary epithelium or (**3**) the mucociliary clearance following entrapment in the respiratory mucus. Furthermore, the interaction with (**4**) the respiratory cellular defense mechanism or (**5**) the pulmonary surfactant layer must be considered.

PCL: periciliary layer

Considering local pulmonary genetic interference, the translocation of siRNA-loaded NPs to extra-pulmonary tissues will also compose an important barrier, as it will decrease the siRNA dose present in the respiratory tract (**Figure 1 (2)**). It has already been demonstrated that the pulmonary administration of small molecule drugs leads to efficient and rapid drug transport across the air-blood barrier, owing to the high vascularization, large surface area and ultra-thin

epithelium of the alveoli (*i.e.* 0.1-0.4 µm) [25, 26]. Interestingly, also the topical application of naked siRNA has shown a rapid systemic distribution [27]. In stark contrast, the translocation and accumulation in extra-pulmonary organs of NPs is very limited, again illustrating the need of a delivery vehicle to achieve local therapeutic effects and avoid systemic exposure. In addition, the majority of the NPs that do translocate, is directed towards the regional lymph nodes, a process largely governed by dendritic cells, without further distribution throughout the body [28-30].

Still, the most important elimination route in the airways is the mucociliary clearance (**Figure 1 (3)**). The respiratory mucus is primarily composed of a three-dimensional network of cross-linked and entangled mucin fibers, lying on top of a periciliary layer [31]. The latter is also grafted with mucins and proteoglycans that are tethered to the cilia and the epithelial surface with increasing density from the top of the layer to its bottom [32]. For local NP-mediated drug delivery to mucosal surfaces (*e.g.* gastrointestinal or vaginal surfaces), different types of mucoadhesive NPs were developed in an attempt to increase the local bioavailability [33]. However, a fundamental limitation of mucoadhesive NPs in the lungs is the physiological turnover of respiratory mucus, which is dramatically elevated by the so-called mucociliary clearance [34]. This clearance mechanism is based on the propulsive movement of 10^9 cilia per cm^2 in the periciliary layer, resulting in a continuous transport of the mucus layer from the upper airways to the esophagus [35]. As a result, mucoadhesive systems are especially unsuitable for topical delivery to the lung. In order to avoid the rapid mucociliary clearance, siRNA-loaded NPs deposited in the upper airways will have to efficiently penetrate the respiratory mucus layer [36, 37], which can be impeded by steric hindrance or adhesive interactions.

The alveolar epithelium is not covered by respiratory mucus, but by a thin layer of PS (**Figure 1 (5)**), which will be described in more detail in section 3. An important NP clearance mechanism in the alveoli is the phagocytosis of the NPs by alveolar macrophages (AM) (**Figure 1 (4)**). AMs comprise more than 90 % of the pulmonary immune cell population. The air-side surface of each alveolus in the human respiratory tract is covered by 12 to 14 AMs

[38]. The AMs ingest foreign insoluble particles deposited in the lower airways by phagocytosis, which plays a prominent role in the first line defense against inhaled pathogens. Next, the particles will be either degraded or the particle-carrying macrophages will migrate to the ciliated airways, followed by mucociliary transport to the larynx. Importantly, although it was previously assumed that NPs are able to bypass the phagocytic uptake by AM [39], recent findings illustrated the opposite namely that AM preferably capture particles with a hydrodynamic diameter in the nanometer scale [29, 40, 41]. Of note, in the context of pulmonary pathologies with an underlying inflammation, the resident AM might also constitute important target cells for siRNA delivery.

Once the siRNA-loaded NPs have successfully conquered the different extracellular barriers, they reach the surface of their target cell in the respiratory tract. At this stage, the NPs have to bypass several intracellular barriers prior to reaching its site of action, *i.e.* the cytoplasm of the target cell. Considering the non-drug like properties of siRNA, its high negative charge and high molecular weight, this can be a dramatic limiting step in the clinical translation of siRNA therapeutics. Therefore, as mentioned before, much effort has been undertaken to incorporate siRNA in NPs optimized to circumvent (most of) the intracellular barriers. **Figure 2** represent the major intracellular barriers, such as cell binding [42], cellular internalization [43, 44], and cytoplasmic release of intact siRNA [45, 46]. As illustrated in Figure 2, a transport mechanism is required to transfer the NPs across the plasma membrane of the target cell. Generally, non-viral NPs are internalized via endocytosis after immobilization on the cell surface [43]. Briefly, the plasma membrane invaginates locally, thereby surrounding and enclosing the NPs. After membrane fission, the NPs are located inside the lumen of the newly formed intracellular vesicles, generally referred to as endosomes. Upon maturation, these endosomes will intracellularly fuse with lysosomes that represent a hostile environment for the siRNA-loaded NPs, given the low pH (± 4.5) and the presence of various degradative enzymes. It is imperative for siRNA-loaded NPs to escape from these detrimental compartments in order to transfer the siRNA payload into the cell cytoplasm [47].

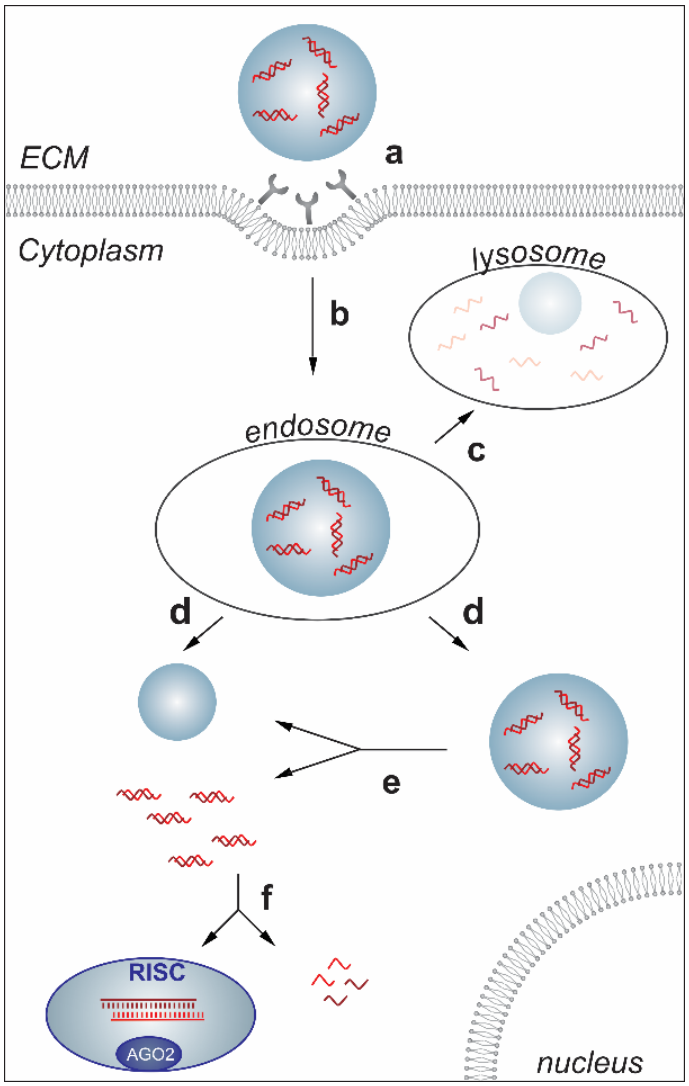

Figure 2. The predominant intracellular barriers encountered once the siRNA-loaded nanoparticles reach the respiratory target cell.

(**a**) Cellular attachment by *e.g.* specific recognition by membrane proteins. (**b**) Endocytic uptake of the nanoparticles (NPs) across the plasma membrane. The NPs inside the endosomes can either (**c**) be degraded upon fusion of maturated endosomes with lysosomes or (**d**) escape from the endosomes and (**e**) release the siRNA. (**f**) The cytosolic siRNA can either be enzymatically degraded or incorporated in the RNA induced silencing complex (RISC).

AGO2: Argonaute 2; ECM: extracellular matrix.

3. Pulmonary surfactant

3.1. Composition and biophysical activity

Pulmonary surfactant (PS) is synthesized and secreted from the respiratory epithelium by alveolar type II cells onto the air-liquid interface, where it reduces surface tension. PS is a complex mixture of lipids and proteins, of which the lipid fraction accounts for more than 90 wt% (**Figure 3**). The lipid fraction mainly contains phosphatidylcholine (PC) (around 60-70%) and phosphatidylglycerol (PG) (approximately 10%) species. Among PC molecular species, dipalmitoylphosphatidylcholine (DPPC) represents 41 % of the total surfactant mass. DPPC is essential to sustain the extremely low surface tensions observed during expiration as its saturated acyl chains can adopt a highly lateral packed state occupying smaller area per molecule compared to unsaturated phospholipids [48, 49]. Other minor phospholipids such as phosphatidylethanolamine, phosphatidylinositol, sphingomyelin, as well as lysophosphatidylcholine comprise the remainder of the phospholipid pool. Additionally, neutral lipids are also present in PS membranes and account for approximately 13% by mass, with cholesterol being the most abundant (around 8 % by mass).

On the other hand, approximately 10% of surfactant in mass is constituted by proteins, including specific surfactant proteins, which can be classified with respect to their water affinity: hydrophilic proteins (SP-A and SP-D) and hydrophobic proteins (SP-B and SP-C). SP-A, SP-B and SP-C are membrane-associated proteins and are present in surfactant in small amounts (3-5% by mass in the case of SP-A, 0.5-1% by mass for SP-B and SP-C). SP-D is not usually associated with surfactant membranes and it constitutes less than 0.5 % by mass [50]. SP-A and SP-D belong to the collectin protein family and they are involved in the innate immune response and inflammatory responses in the lung as well as in the maintenance of alveolar homeostasis, whereas SP-B and SP-C are crucial for the biophysical function of surfactant [51, 52].

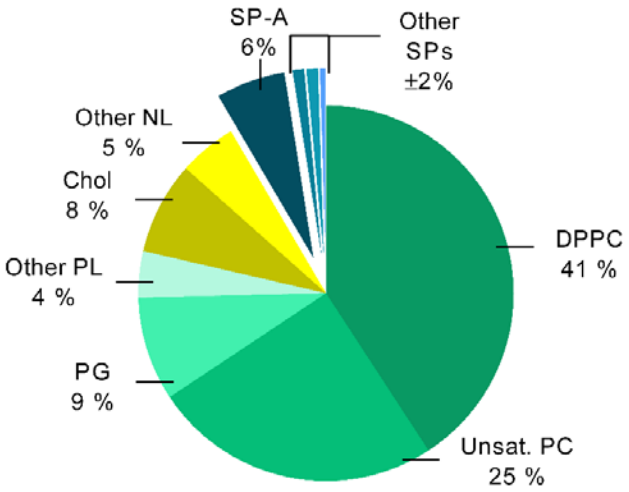

Figure 3. Composition of human pulmonary surfactant.

Pie chart representing average weight percentage of each component with respect to the total mass of pulmonary surfactant. This figure was adapted from Parra *et al.* [53].

(DP)PC: (dipalmitoyl)phosphatidylcholine. PG: phosphatidylglycerol. PL: phospholipids. Chol: cholesterol. NL: neutral lipids. SP: surfactant protein.

The major function of PS is to reduce the surface tension at the air-liquid interface of the lower airways, thereby preventing collapse of the lung on expiration and stabilizing the respiratory surface along compression-expansion breathing cycles [54]. Despite the fact that DPPC and PG are the main surface active agents, the presence of the small hydrophobic surfactant proteins SP-B and SP-C is strictly required to facilitate phospholipid dynamics during surface film formation and stabilization.

PS is also involved in lung host defense against inflammation and infection [51, 55]. In general terms, the hydrophilic section of surfactant proteins is specialized in the clearance of pathogens from the alveolar spaces by binding and agglutinating a variety of bacterial, fungal or viral pathogens and molecules such as allergens or environmental inorganic particles as well as in the modulation of the inflammatory cellular response in the lung. Thus, these proteins are in charge of recognition and opsonization of pathogens, presenting them to immune cells such as alveolar macrophages and monocytes [56]. However, also in the absence of immune cells, they still exhibit intrinsic antimicrobial activity by increasing the permeability of the microbial cell membrane [57].

Of note, several pathologic processes may modify the composition, structure and/or function of PS. For example, inflammatory pulmonary disorders are often associated with vascular injuries, causing an influx of *e.g.* serum proteins, cholesterol, and free fatty acids into the airways. At the air-liquid interface, these molecules will partly inhibit the function of the PS layer, resulting in higher surface tensions [58]. In addition, modifications in the composition of PS have been reported in several pulmonary disorders. For example, different studies have indicated that altered levels of cytokines in the alveolar space can affect the surfactant biosynthesis, thereby causing aberrant PS lipid and/or surfactant protein levels [59].

3.2. Therapeutic use of pulmonary surfactant

The integrated regulation of surfactant synthesis, secretion and metabolism is vital for air breathing and ultimately, survival. Indeed, deficiency or inactivation of surfactant affects lung compliance and gas exchange and contributes to the development and outcome of severe respiratory pathologies, such as neonatal respiratory distress syndrome (NRDS). Preterm babies with still immature lungs lack fully differentiated alveolar type II cells, therefore resulting in insufficient PS production, which contributes to the pathogenesis of NRDS [60]. Current medical practice guidelines recommend respiratory support (mechanical ventilation and nasal continuous positive airway pressure) combined with surfactant treatment [61, 62], which substantially ameliorates the signs of NRDS and reduces mortality and respiratory morbidity in premature babies [63].

Initial surfactant preparations consisted of synthetic surfactants that contained only phospholipids, and lacked surfactant proteins [64]. However, once the presence of the hydrophobic surfactant proteins was proven to be essential for the optimal surface activity, surfactant preparations extracted from lungs of mammals were developed [65]. These natural surfactants, derived from either bovine or porcine animal sources, are extracted from lung lavages or minced lungs. Although these natural surfactants have been clinically approved for the treatment of NRDS, they also present some drawbacks, such as batch-to-batch variations,

high costs per dose, and the potential risk of transmission of pathogens [66]. To avoid these possible side effects, several synthetic lung surfactant preparations, consisting of surfactant lipids and synthetic surfactant protein analogs, are currently tested in (pre)-clinical trials (Venticute®, CHF5633®). The synthetic surfactant Surfaxin® has even recently been approved by the Food and Drug Administration for the treatment of NRDS.

To date, the instillation of exogenous PS is solely used in the treatment of NRDS [63]. However, lack or deficiencies of the surfactant system are also associated with other respiratory pathologies [60], *e.g.* acute respiratory distress syndrome (ADRS) or Meconium Aspiration Syndrome (MAS). ARDS is characterized by respiratory failure with many different origins, manifested in severe pulmonary inflammation, diffuse alveolar damage and alveolar capillary leakage [67]. Impaired vascular permeability and pulmonary edema evoke the infiltration of surface-active molecules, such as serum and plasma proteins, into the alveolar air-liquid interface from the blood capillaries, exerting an inhibitory action on surfactant, generating abnormally high surface tensions [68, 69]. MAS results from incorporation of cholesterol into surfactant membranes and films when a newborn inhales his first stool before or during delivery due to prenatal stress [70]. In this case, PS is inactivated due to the membrane-perturbing effect of meconium and particularly by an excess of cholesterol, which alters the structure and dynamics of surfactant membranes and films. As the inactivation of PS somehow contributes to the development and outcome of these pulmonary disorders, research is currently ongoing in order to understand how PS replacement could benefit these lung diseases.

Importantly, besides the therapeutic use of PS in surfactant replacement therapy, there is an increasing interest in the use of PS for the benefit of pulmonary drug delivery. Due to its unique biophysical properties mentioned above, PS provides several advantages in drug delivery such as (i) the solubilization of poorly-water soluble drugs, (ii) the efficient transport along the entire respiratory surface based on its spreading capabilities, and (iii) the protection from other extracellular barriers [58]. A recent review has comprehensively described the current knowledge on the potential use of PS as a drug delivery vehicle for existing small molecule drugs, which will not be covered here [71]. Instead, the major aim in this work is to discuss a

number of inspiring reports that evaluated the interaction between NPs and PS components, and how this could impact on NP-mediated siRNA delivery to the respiratory tract.

4. The interplay between pulmonary surfactant and nanoparticles

4.1. Biomolecular corona formation: evaluating the adhesion of endogenous pulmonary surfactant components to engineered nanoparticles

The majority of publications on the adhesion of endogenous PS components onto the NP's surface have focused on the hydrophilic surfactant proteins, SP-A and SP-D. This can be attributed to their molecular structure and their function in the innate immune response. As outlined above, these proteins function as opsonins to enhance the uptake of inhaled particulate matter, such as microorganisms, by antigen presenting cells [72]. It is therefore conceivable that these proteins can also adsorb onto the surface of inhaled NPs, thereby playing a key role in the formation of a biomolecular corona on inhaled NPs.

Being members of the collectin family, both hydrophilic surfactant proteins enclose a carbohydrate recognition domain (CRD). This domain allows them to associate with carbohydrates at the surface of various pathogens, necessary to fulfill their role in the first line defense of the respiratory tract. Despite this structural resemblance, SP-A and SP-D showed a divergence in their interactions with other biological structures. For example, SP-A has a general preference for lipophilic patterns and a high affinity for phospholipids, which allows the protein to associate with PS membranes [73]. In contrast, SP-D preferably interacts with strong hydrophilic structures, owing to the presence of a large positively charged area near its CRD site. For a more detailed insight into the structural determinants of this biomolecular recognition, the reader is referred to recent comprehensive reviews available in the literature [72, 74].

Recent reports have illustrated that these variations in pattern recognition also exists for the interaction of SP-A and SP-D with deposited NPs. While isolated human SP-A preferentially interacted with hydrophobic NPs, a pronounced binding of recombinant SP-D to the hydrophilic NPs was observed [75].

Nevertheless, this work on isolated surfactant proteins did not consider the impact of other biomolecules in the lung lining fluid on the propensity for SP-A and SP-D to bind NPs. In an

attempt to better represent the *in vivo* situation, Marchetti and coworkers recently collected lung lining fluid from human lung tissue and isolated the soluble proteins. This fraction, including SP-D and other non-surfactant related proteins originating from a broad range of sources (*e.g.* pulmonary cell products, serum, etc.), allowed the authors to evaluate the impact of these atypical proteins on the SP-D surface association. Apparently, these proteins did not significantly alter the binding affinity of SP-D, nor its preference for hydrophilic surface modifications [76].

However, the latter samples were still depleted of surfactant lipids and surfactant proteins other than SP-D. Given the illustrated impact of phospholipids on protein adsorption patterns, it is of particular interest to evaluate protein surface association in more complex media [77]. To this end, the surfactant protein surface association has been evaluated upon incubation of different types of the NPs with bronchoalveolar lavage fluid (BALF) [78-80]. These results indicated that the presence of other proteins or phospholipids did not inhibit the association of SP-A and SP-D on different types of NPs, including carbon nanotubes, metal oxide NPs, or polystyrene NPs. Importantly, a recent publication provided the first evidence on the formation of a biomolecular corona onto NP's surface *in vivo* [81]. In this study, mice were treated with single walled carbon nanotubes (SWCNT) *via* pharyngeal aspiration. Upon different incubation periods, the lungs were lavaged and the NPs were recovered from the murine BALF. Next, the adsorbed lipids and proteins were identified and quantified *via* mass spectrometry. Not surprisingly, these data revealed that both surfactant lipids and surfactant proteins interact with the SWCNT. The predominant components in the biomolecular corona were the phospholipids phosphatidylcholine (PC) and phosphatidylglycerol (PG). Computer modeling indicated that an uninterrupted coating of phospholipids was formed, where the hydrophobic alkyl chains of the phospholipids are oriented toward the hydrophobic NP surface while the hydrophilic headgroups are exposed toward the aqueous phase. The surfactant proteins that interacted with the SWCNTs were SP-A, SP-D and SP-B. The absence of SP-C was related to the lowered SP-C expression level in the pro-inflammatory environment that was induced by the SWCNT exposure and to its physicochemical characteristics, making it more difficult to

visualize *via* gel electrophoresis. The computer modeling indicated that SP-D can interact with the hydrophilic headgroups of the phospholipid-coated SWCNT. However, the complete 3D structure of SP-A and SP-B are not available, implying that details on the surface binding of these proteins could not be obtained *via* computer modeling assessments. Unfortunately, such detailed information on the formation of a biomolecular corona on other types of (drug-loaded) NPs *in vivo* is still lacking.

Of note, to date no information is available in the literature on the interaction between NPs and PS in relevant disease models. As mentioned in section 3.1., it is known that the structure and composition of PS can be significantly altered in airway disease. On the other hand, pulmonary surfactant dysfunction and the concomitant destabilization of the respiratory surface is usually associated with reduced compliance and partial alveolar collapse, compromising proper aireation and preventing homogeneous distribution of NPs. It is therefore conceivable that all these changes can also modulate how inhaled NPs interact with surfactant and vice versa and to what extent NPs effectively reach deep regions of the lung . It will be important in future research to consider the impact of these alterations on NP toxicity and drug delivery performance.

4.2. The impact of pulmonary surfactant on nanoparticle-mediated drug delivery: exploring the potential role in pulmonary siRNA delivery

The interaction with biomolecules can obscure the original NP surface. Consequently, it confers a new biological ‘identity’ to the NPs characterized by both an altered size and surface charge as well as the surface presentation of adsorbed biomolecules which all are critical determinants for the toxicological profile and the biological activity of NPs [18].

Most studies have focused on the toxicological consequences, which can be manifested on two different levels. For one, as the deposited biomolecules conceal the original NP surface, the interaction with the target cells will be significantly altered [18]. Consequently, the internalized fraction of siRNA-loaded NPs will be modified, which will have a definite impact on

the induced cellular toxicity [82]. Besides the cytotoxic consequences, the adhesion of biomolecules to the surface of NPs can have an important impact on the normal biophysical function of PS. Depending on the amount of adsorbed biomolecules, the associated depletion of surfactant lipids and proteins from the air-liquid interface will affect the molecular arrangement of surfactant molecules in the PS film [83]. For example, the adhesion of surfactant proteins onto the surface of NPs inevitably results in a perturbation of PS function. As outlined above, the hydrophilic surfactant proteins SP-A and SP-D contribute to the first line host defense in the respiratory tract. As a result, it is conceivable that the sequestration of these proteins augment the susceptibility to microbial infections [72, 84]. On the other hand, as the hydrophobic proteins SP-B and SP-C are strictly required for the formation and stability of the PS film, the squeeze-out of these proteins will have a direct effect on the biophysical function of PS [85]. The clarification of the extent and mechanisms involved in this PS inhibition by inhaled NPs may guide future development of safer nanomedicines, and has therefore been a major topic of investigation [83, 85, 86].

Besides its impact on nanotoxicity and PS function, the interaction of PS and NPs might also affect the biological activity of the latter. As SP-A and SP-D function as opsonins to enhance the uptake of inhaled particles, the association of SP-A and SP-D onto the surface of various NPs will probably exert a triggering effect on the uptake of NPs by alveolar macrophages. This has been observed by Ruge *et al.*, where the surface association of isolated native human SP-A onto inorganic NPs significantly enhanced their internalization by cultured murine AM [87]. In a follow-up publication, these findings were extrapolated to the interaction with isolated native human SP-D [75]. The enhancement in phagocytosis by AM upon SP-D functionalization has also been illustrated by another research group. Here, a significant higher internalization of amine-modified polystyrene NPs (A-PS) by primary AM isolated from wild-type mice was observed compared with the internalization by primary AM isolated from SP-D deficient mice. In addition, it was illustrated that upon interaction with isolated SP-D, the high uptake of A-PS by AM isolated from SP-D deficient mice could again be recovered [88]. A recent publication investigating the impact of SP-A has highlighted a different outcome. Here,

the internalization levels of A-PS were comparable in AM isolated from wild-type mice or from SP-A deficient mice. Also the application of exogenous SP-A could not alter the uptake. Comparing the adhesive interaction of SP-D and SP-A to A-PS revealed for SP-A only a weak surface adhesion, which implies that the observed cellular effects could not be directly related to protein association [89].

Overall, these studies indicate that certain drug-loaded NPs might be cleared from the lung to a greater extent upon interaction with the lung-specific collectins. At the same time, these data also demonstrate that including decoration with SP-A or SP-D in NP design could improve their uptake by AM, suggesting that it might be beneficial for NP-mediated drug delivery in the context of inflammatory disorders.

So far, no data on the impact of isolated hydrophobic surfactant proteins, i.e. SP-B and SP-C, on the internalization of NPs is available. These proteins are structurally different from SP-A and SP-D and will therefore likely not induce a similar interaction with AM as illustrated above for the pulmonary collectins.

While the impact of isolated surfactant proteins on pulmonary drug delivery efficacy has not yet been explored, synthetic or animal-derived surfactant preparations have recently been used to modulate the therapeutic efficacy of nucleic acid loaded NPs. In fact, already in the late 1990s, PS preparations have been combined with lipid- and polymer-based delivery vehicles. The primary goal of these studies was to understand the barrier properties of PS towards the respiratory delivery of therapeutic nucleic acids. To this end, different types of NPs were mixed with synthetic or animal-derived surfactant preparations [90-93]. Collectively, these reports outlined the incompatibility of cationic lipid-based NPs with PS *in vitro* [90-92]. It has been suggested that the observed inhibitory effect results from disintegration of the lipid NPs by the negatively charged lipids present in the PS preparations, resulting in premature release of its nucleic acid payload prior to reaching the target cell. This hypothesis was supported in recent work by De Backer *et al*.. Here, experimental evidence on a quantitative release of siRNA complexed with Lipofectamine^{TM} (i.e. a commercially available lipid-based transfection reagent) in the presence of animal-derived surfactant preparations was reported [94].

Interestingly, polymer-based NPs appeared to be more resistant to PS preparations compared with lipid-based NPs [92, 93]. Therefore, PS was represented as a critical barrier mainly for lipid-based NPs. However, as opposed to the surfactant preparations, bronchoalveolar lavage fluid (BALF) inhibited both poly- and lipoplex – mediated gene transfer *in vitro* [95]. This was also confirmed in more recent publications, where transfection efficiencies of lipid-based and polymer-based delivery vehicles were compared in cultured lung epithelial cells. Surprisingly, the DNA [96] or siRNA [97] transfer mediated by the lipid-NP was less compromised in the presence of BALF compared to the polymeric NP.

Recently, the approach to combine polymeric NPs and PS in a single siRNA delivery vehicle has been explored. A single delivery vehicle consisting of siRNA loaded NPs and animal-derived PS preparations, was evaluated by the group of Kissel [98]. It was reported that the siRNA transfer activity of biodegradable NPs, fabricated from diamine modified poly (vinyl alcohol) grafted with PLGA, was significantly improved upon the use of Alveofact® during the preparation procedure. The production protocol involved a single-step solvent displacement technique, applying PS as a surface altering component. Here, the improved siRNA transfer activity was attributed to a facilitated cellular uptake [98].

Recent findings by De Backer *et al*. advocate that the high transfection levels cannot always be correlated with the alterations in cellular uptake. In the latter report, colloidally stable PS-coated and siRNA-loaded dextran nanogels were constructed. The high siRNA delivery efficiency of the nanogels had previously been established in various cell lines *in vitro* [99-102]. Although the decoration with PS substantially inhibited cellular uptake of the nanogels in lung epithelial cells and alveolar macrophages, their gene silencing potential in both cell types was maintained or even improved (**Figure 4**). These intriguing data suggest that PS may enhance the fraction of internalized siRNA that is ultimately delivered into the cytosol, possibly mediated by the altered interactions with biological membranes, i.e. the cell membrane and/or endosomal membrane, in the presence of PS. For instance, the presence of PS could influence the predominant cellular uptake mechanism and subsequent intracellular processing, both of which could impact on the siRNA delivery efficiency [94, 103].

Important to note, the decoration of siNGs, with a PS shell could additionally enhance their colloidal stability and largely prevented release of its siRNA payload in the presence of competing polyanions (e.g. mucins) [103]. Because of its promising features, this NP platform was subsequently evaluated *in vivo* for siRNA delivery to murine resident alveolar macrophages (rAM) [104]. Of note, already more than fifteen years ago, a first *in vivo* experiment on the co-delivery of animal-derived PS and DNA-loaded polymeric NPs was conducted [105]. Here, no significant improvement in DNA transfer activity was observed in the presence of PS. This is in stark contrast to the *in vivo* performance of the PS-coated siRNA-loaded nanogels (siNGs). Both the uncoated and PS-coated siNGs achieved high levels of siRNA uptake in rAM, yet only the PS-coated formulation could significantly reduce gene expression on the protein level. PS-coated nanogels induced a profound downregulation of target mRNA levels, reaching 70 % knockdown upon a single local application (with ~1 mg kg^{-1} siRNA dose) in notoriously hard-to-transfect primary rAM [104].

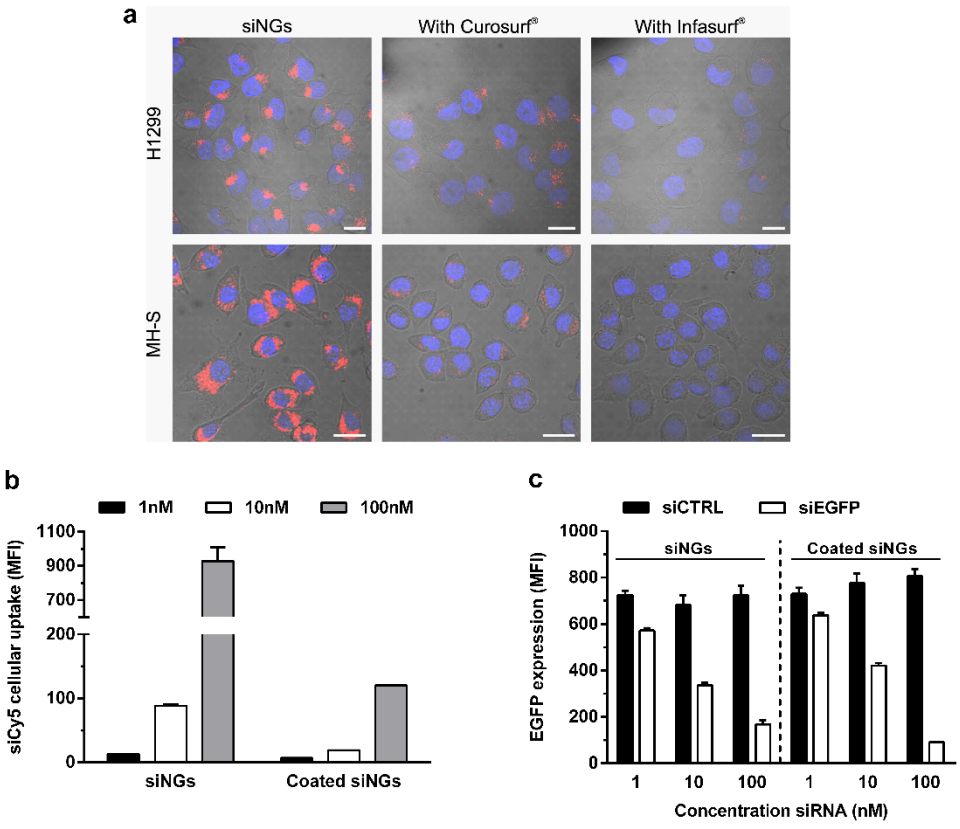

Figure 4. Impact of pulmonary surfactant on the cellular uptake and gene silencing potential of siRNA-loaded nanogels (siNGs)

(**a**) Commercially available pulmonary surfactant (PS) preparations (Curosurf®, Infasurf®) significantly inhibit the cellular internalization of siNGs in both lung epithelial cells (human H1299) and alveolar macrophages (murine MH-S). Scale bars correspond with 20 µm. Reproduced with permission from [94]. Copyright 2013. Elsevier. (**b**) Cellular uptake by H1299 cells of siNGs or siNGs coated with PS (coated siNGs) as a function of siRNA concentration. (**c**) Evaluation of enhanced green fluorescent protein (EGFP) silencing by uncoated siNGs or coated siNGs. To evaluate the uptake, the NGs were loaded with Cy5-labeled siRNA (siCy5). To assess the gene silencing potential, they were loaded with control siRNA (siCTRL) or active siRNA (siEGFP). (*n*=3). Reproduced with permission from [103]. Copyright 2015. Elsevier.

5. Conclusion and future outlook

Inhalation therapy of siRNA is a valuable strategy to silence key molecular targets involved in respiratory pathologies. However, formulation of siRNA therapeutics in safe and efficient nanosized delivery systems (nanoparticles, NPs) is imperative for clinical translation. Upon pulmonary administration, siRNA-loaded NPs have to overcome many extra-and intracellular barriers. Many groups have investigated the interaction of (drug-loaded) NPs with pulmonary surfactant (PS), *i.e.* a complex mixture of lipids and proteins, and stated deleterious effects on both PS and NP function. In contrast, recent reports described that combining PS and NPs into a single polymeric delivery systems might improve siRNA delivery, both *in vitro* and *in vivo*. Therefore, as synthetic nanomedicines often fail to surmount the numerous biological barriers en route to their intracellular target, a growing interest exists in the implementation of bio-inspired materials, such as PS, to advance the field of macromolecular drug delivery.

In addition, despite the fact that the instillation of exogenous PS is solely used as standard therapeutic intervention for NRDS, research is currently ongoing in order to understand how PS replacement could benefit a wider range of lung diseases. As revie123.wed above, it was already indicated that PS dysfunction contributes to the development and outcome of several respiratory diseases. This implies that the combination of a siRNA-loaded NP and exogenous PS may provide new and synergistic therapeutic opportunities for the treatment of lung diseases with underlying PS dysfunction.

In summary, future research addressing the promising features of the co-administration of siRNA-loaded NPs and PS may strongly contribute to the design of tomorrow's effective siRNA delivery systems for the local treatment of respiratory pathologies.

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