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The Lord of the Lungs: The essential role of pulmonary surfactant upon inhalation of nanoparticles

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ABSTRACT

The rapid development of nanotechnology is opening a huge world of promising possibilities in healthcare, but this is also increasing the necessity to study the potential risk of nanoparticles on public health and the environment. Since the main route for airborne particles to enter into our organism is through the lungs, it has become essential to prove that the nanoparticles generated by human activities do not compromise the respiratory function. This review explains the key role of pulmonary surfactant to sustain the normal function of breathing, as well as the stability and immunity of lungs. Particular emphasis is made on the importance of analysing the features of nanoparticles, defining their interactions with surfactant and unravelling the mutual effects. The implication of the nanoparticle-surfactant interaction on the function and fate of both structures is described, as well as the main *in vitro* methodologies used to evaluate this interaction. Finally, the incorporation of pulmonary surfactant in appropriate *in vitro* models is used in order to obtain an extensive understanding of how nanoparticles may act in the context of the lung. The main goal of this review is to offer a general view on inhaled nanoparticles and their effects on the structure and function of lungs derived from their interaction with the pulmonary surfactant system.

1. Introduction

In recent years, nanotechnology has become one of the most promising fields of new technologies worldwide. This is visible in the diverse applications and rapid increase of the global nanotechnology market value, which has reached $50 billion in 2018 and it is expected to exceed $132 billion by 2023, according to the latest report from Industry ARC [1]. Nanotechnologies include manufacturing methodologies, nanomaterials, nanoparticles (NPs) as well as the production of nano-scale devices with ever-growing potential in energy, electronics, food, textiles and medicine, providing new advances to optimize many industrial and medical applications. Particularly in medicine, nanotechnology is improving the development of personalized and targeted methods of diagnosis, monitoring and treatments [2]. The production and usage of NPs have a great range of potential applications, principally due to the wide range of possibilities due to the modification and the modulation of their capabilities to interact and cross biological barriers. This has put them at the centre of cutting-edge drug-delivery strategies for more precise treatments, controlling drug release and reducing side effects [3,4]. However, they have a reverse side that should be analysed carefully to optimize their use while reducing their potential toxicity. Since the early 2000s, the global concern about the impact of NPs on the environment and human health has risen significantly. The necessity of further information about the risks associated with the production and use of NPs as well as their adequate regulation has led to a sustained increase in research and investment leading to the creation of different scientific disciplines, such as nanotoxicology, as well as to governmental programs, such as the National Nanotechnology Initiative (NNI) of the USA [3].

Most NPs, produced *ad hoc* or derived from industry, combustion of fossil fuels or even from natural processes, are released into the environment, increasing the opportunities of contact with different organisms including human beings. The main route of entrance for these airborne NPs into the body is through the lungs, which are highly specialised organs in charge of the gas exchange between the atmosphere and the bloodstream. The gas exchange surface is mostly formed by epithelial cells that are constantly exposed to the external environment and, therefore, to potential harmful substances (e.g., viruses, bacteria, airborne pollutants, etc.). In contrast to the skin (the other epithelial surface in continuous contact with the atmosphere), lungs surface cannot be increased in thickness or in the number of its protective layers because the gas exchange requires a thin but resistant,

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elastic and gas-permeable barrier. Therefore, evolution has developed other strategies to protect the body from the entrance of external particles through what is the large respiratory surface of more than 100 m^2 in adult human lungs [5]. In mammals, these barriers involve a branched structure (23 tubular bifurcations), fluid counter-flows generated by ciliated cells, secretion of renewable and transportable protective materials (mucus and pulmonary surfactant) and the presence of immune cells (e.g., interstitial and alveolar macrophages). Even so, any detrimental compound which manages to circumvent these barriers and enters the body without control, might trigger a whole series of responses including oxidative stress, inflammation and genotoxicity [6]. For instance, the exposure to silica particles has been associated with the development of silicosis, lung injury, interstitial fibrosis, industrial bronchitis and emphysema [7,8]. Therefore, taking into account the rising levels of airborne NPs, it becomes extremely important to recognize the potential dangers derived from their impact on the respiratory system in order to optimise their benefits and minimise the deposition, interaction with the barriers and entrance into the body.

One of the challenges to evaluate the effects of NPs on the airways and to investigate the mechanisms behind the crossing of these barriers is to simulate the complexity of the *in vivo* situation using *in vitro* approaches. Most of the respiratory surface models developed so far try to replicate the air-blood barrier culturing pulmonary cell lines in monoculture or co-culture systems. In the last few years considerable efforts have been made to improve these models and many sophisticated models such as the "breathing" *lung-on-chips* [9,10] or the inclusion of 3D bio-printing technology [11,12] are creating unprecedented models mimicking with increasing detail the *in vivo* structure, function and dynamics. However, pulmonary surfactant (PS), one of the most relevant substances in the alveolar context is not yet systematically included in these cellular *in vitro* models [13,14]. It literally enables the process of breathing [15], contributing to the maintenance and stability of the alveolar fluid lining preventing the ultra-filtration of fluid coming from the blood stream [16]. PS is considered the first protective layer in contact with airborne particles in the deep lung, conditioning the formation of *coronas* and the fate of NPs [4,17]. Therefore, this review focuses on the mechanisms and implications of the interaction of NPs with PS with the hope to further encourage scientists working in this field to integrate PS in the *in vitro* systems and to lead to improved understanding of the *in vivo* situation. This in turn should lead to advances in studies in toxicology in addition to improved drug delivery systems and the design of smart biomedical applications.

2. Nanoparticles as a double-edge sword

The continuous development of nanotechnology has extended the use of NPs with a consequent increase in their release into the environment and, therefore, in the exposure to humans. Despite the growth in new fields of application (e.g., in biomedicine, agriculture, food, renewable energy, electronics, cosmetics, etc.), NPs may also bring detrimental effects to the natural environment and human health. This is particularly true of the respiratory system, which may lead to a need to considerably limit their use. The attractiveness of using NPs is mainly based on their versatility (i.e., customizable surface, core, shape and size) and large surface/volume ratio, all of which enhance their physicochemical properties, reactivity and distribution when compared to larger particles. Particularly in drug delivery, they are good candidates mainly because of their solubility and stability in aqueous media, ability to adsorb and transport different molecules of interest, crossing biological barriers and targeting them to desired locations [5]. Nevertheless, all of these potential advantages may act as a double-edge sword with reverse effects that need to be carefully analysed on a case by case basis, especially when the entrance into the body occurs without control. The interaction with the environment and the facility of different molecules to adsorb on their surface could drastically change the targeted destination, retention times and cargo release properties of NPs, as well as promoting the formation of aggregates. The aggregation phenomena reduce their solubility and stability in biological media, which may contribute to increased toxicity and a possible inflammatory response, hindering their detection and analysis in the *in vivo* context [18–20]. For instance, titanium dioxide (TiO_2) NPs are widely used for daily products such as paint pigments, sunscreen lotions, plastics and food colouring, but they may also induce cytotoxicity, inflammation, generation of Reactive Oxygen Species (ROS) and structural damage of the lung tissue [21]. Therefore, any kind of NP that potentially ends up in the atmosphere and, consequently, in humans, animals or plants, must be carefully designed and the possible impact on health related to their physicochemical properties must be investigated to prevent any undesirable effect. This is especially important in biomedicine, where the investigation and use of NPs for diagnosis, imaging and drug delivery has risen considerably in the last few years [5]. In this sense, it is important to investigate and develop the production of biodegradable and biocompatible NPs that balance low cytotoxicity and inflammation potential while carrying molecules of interest to desired locations. The best candidate materials proposed for imaging and drug delivery are lipid-based NPs (e.g., liposomes, micelles, nanostructured lipid carriers or solid lipid nanoparticles), polymeric NPs (e.g., polysaccharides: chitosan or hyaluronic acid; polyesters: polylactic co-glycolic acid -PLGA- or polycaprolactone; and amino acid-based polymers) or protein and metallic NPs [22]. In this regard, there is much interest to develop both adequate experimental *in vitro* models mimicking physicochemical and cellular respiratory barriers to determine the impact of NPs on these specific barriers (i.e., pulmonary surfactant, mucus or cell cultures) as well as *in vivo* experiments to determine the impact of NPs on the organism as a whole.

3. When not only size matters

The study of NPs as well as the regulation of their production and usage should start by unifying the different definitions to facilitate communication and understanding between researchers and institutions. NPs have received numerous definitions in the past few years, with size being the most remarkable characteristic. However, these definitions remain ambiguous and incomplete. The International Organization for Standardization (ISO) has published their ISO 2015 definitions in the field of nanotechnology, where a nanoparticle is defined as a "nano-object with all external dimensions in the nanoscale (range from 1 to 100 nm) where the lengths of the longest and the shortest axes of the nano-object do not differ significantly" [23]. Nevertheless, the European Union adopted a wide-ranging definition of a nanomaterial in 2011 as "a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm-100 nm" [24]. Considering the importance of origin and main morphological, structural and compositional features of NPs in both the production and effectiveness, they will be briefly described in this section.

3.1. Origin

NPs can be classified as natural, incidental or manufactured, as referred in the EU Recommendation 2011 [24]. Natural NPs are released into the environment as a consequence of natural processes without human intervention (e.g., volcanic ash, soil erosion, clay minerals and forest fires). Incidental and manufactured NPs comes from anthropogenic activities. The former refers to NPs resulting from a process in which the production is a consequence but not the objective of the process (i.e., fuel burning, demolition and industrial processes). These NPs play an important part in atmospheric pollution and constitute the major group of toxic NPs. Some industrial procedures and work sites constitute a huge source of exposure to incidental airborne NPs with damaging health impacts on the lungs, including silica particles or asbestos, that are associated with occupational diseases, including silicosis and industrial bronchitis [7]. For example, long-term effects including chronic inflammation, pleuritis, vasculitis, lung cancers, asbestosis and mesotheliomas have been demonstrated as a result of exposure to asbestos. This effect is apparently due to ROS generation and asbestos-cell interactions that activate some intracellular signalling pathways which up or down-regulate the transcription of genes involved in inflammation and fibrosis [25,26]. In contrast, manufactured NPs are made with a specific objective and are designed with desired characteristics hopefully avoiding possible negative effects [19,27]. Any of these NPs may be present in the atmosphere as airborne particles and subsequently affect both the environmental and public health. Consequently, it is important to understand the origin and toxicity of NPs in relation to their source.

3.2. Size and size distribution

The definitions mentioned above are based on NP size, considering them as objects with a size or median-particle size in the threshold of between 1 and 100 nm [18,27]. In the context of lungs, different studies have shown that interactions between NPs and PS, as well as coating by PS components, is dependent on size [28,29]. The smaller sizes, with a higher surface/volume ratio, have an enhanced tendency to form agglomerates/aggregates [18]. NP aggregation affects deposition in the lung and subsequent interaction with PS and clearance mechanisms [30]. It has been demonstrated that size affects the interaction with PS components, but since it is not the sole factor involved in the interaction, it should be considered in combination with other properties.

3.3. Composition

Composition is normally used as a key feature to classify NPs. NPs can be classified as carbon-based, metal-based, organic polymeric or lipid-based. All four groups of NPs are found in the atmosphere. Carbon- and metal-based NPs are widely used in the fields of science and engineering because of their remarkable strength, reactivity, electrical and optical properties. Magnetic NPs, normally metal- or iron oxide-NPs, have been widely used in the last few years to develop biomedical applications. Polymeric NPs are an excellent option for biomedical applications given that they are based on organic molecules that are biodegradable and biocompatible [19,20,31,32]. Dendrimers constitute a good example of this group which allow for the generation of different environments to encapsulate both hydrophobic and hydrophilic active pharmaceutical ingredients (APIs) at the same time. Lipid-based NPs contain lipid sections and are also widely used in biomedicine. For example, anionic liposomes are used for targeting macrophages for the treatment of Chronic Obstructive Pulmonary Disease (COPD) [33]. However, they are not the best nanocarriers to combine with PS as they easily fuse with surfactant membranes releasing the cargo before it can be vehiculized. Nevertheless, the composition of a NP is not the sole characteristic that determines its effect. A good example of this is the difference in toxicity between mesoporous silica NPs (MSNPs) and non-porous silica NPs, associated with the development of silicosis and other lung diseases. The SiOH and SiO^- groups of the surface can form hydrogen bonds and electrostatic interactions, respectively, with the cell membrane which may lead to membranolysis. The porosity of the MSNP surface reduces the extent of these groups, in turn reducing the interactions with the cell membrane and the reaction of radicals present on the surface generating ROS, being much less toxic than silica NPs. The high drug loading capacity of MSNPs helps to reduce the amount administered while maintaining the needed drug doses and, hence, decreases the potential toxicity [34]. This example highlights the importance of considering the composition when designing NPs and predicting their effects *in vivo*.

3.4. Charge and hydrophobicity

Both the surface charge and hydrophobicity, defined by NP composition, strongly affect how a NP interacts with the environment. Depending on these two characteristics, NPs will interact more strongly with some proteins or lipids in the context of PS. This determines the formation of the biological corona and the fate of NPs. Consequently, charge and hydrophobicity have a different impact on the biophysical function and metabolism of PS and, ultimately, on the associated toxicity [4,30,31,35].

4. From nose to alveoli: an arduous trip through the lungs

When foreign bodies, NPs in this case, get into respiratory tract, they encounter numerous and diverse obstacles hindering their transit into the deeper regions in the lungs. These obstacles include physical, biochemical and cellular barriers that prevent unhindered passage through the airways and absorption into the body.

4.1. Physical barriers

The first difficulty that NPs encounter is the characteristic branched architecture of the lungs; 23 tubular bifurcations from nose to the final destination of the airways, the alveoli, obstruct the advance of NPs. Propelled by the air currents produced by breathing (i.e., breath pattern, flow rates and tidal volume), NPs will be forced to deposit onto a tubular respiratory surface that progressively subdivides and reduces its diameter (from 1.5 cm in trachea to ≈200 μm in alveoli). Mainly depending on their aerodynamic diameters (i.e., size, density and shape of NPs) and the special architecture of the airways, this deposition will be governed firstly by *inertial impaction* in the first 10 bronchial generations where high air speed and turbulent air flows occurs (NPs $>$ 10 μm). After this, *gravitational sedimentation* occurs mostly in the central bronchial bifurcations in the conductive zone, where the air speed is slower and the residence time of NPs longer (1 μm $<$ NPs $<$ 10 μm). Finally, deposition is caused by *Brownian diffusion* and *electrostatic attraction* in the alveolar region, where the air velocity is close to zero (NPs $<$ 0.5 μm) [36,37]. Taken all together, and especially in the case of difficulty in the control of breathing patterns common to neonates, children and adults with respiratory disorders (e.g. COPD, cystic fibrosis or alveolar proteinosis), this makes the delivery of drugs via the pulmonary pathway particularly challenging. Therefore, in order to control the specific place of deposition and enhance drug delivery, efforts need to be mainly focused on a correct design in the morphology of therapeutic NPs (i.e., size, shape, density, porosity and surface charge). NPs that are small enough to avoid deposition in the upper and conductive airways but too large to be exhaled should reach deeper areas in the lungs [5,38]. According to the predictions of Byron [39], the optimal aerodynamic diameter for a particle to reach the alveolar region is 1–3 μm, but a slow and deep inhalation is necessary. Fig. 1 summarizes these concepts.

4.2. Renewable biochemical barriers

In the case that NPs are deposited on the respiratory surface, they encounter a second battery of barriers that are continuously being cleared away and renewed. This is the *mucus* in the upper and conductive airways, and *pulmonary surfactant* in the alveoli. The former is a complex viscous solution principally composed by water and mucins structured in two layers with different viscosity (for a more thorough explanation of composition, structure, barrier function and role in drug delivery, it is suggested that the reader consults the recently published review by Murgia *et al.* [40]). A low-viscosity periciliary layer surrounds the ciliated cells with a thicker layer above [41–43]. Mucins (MUC5AC and MUC5B in the airways) generate a complex network where NPs can be filtered by size (physical filtering barrier) or interact

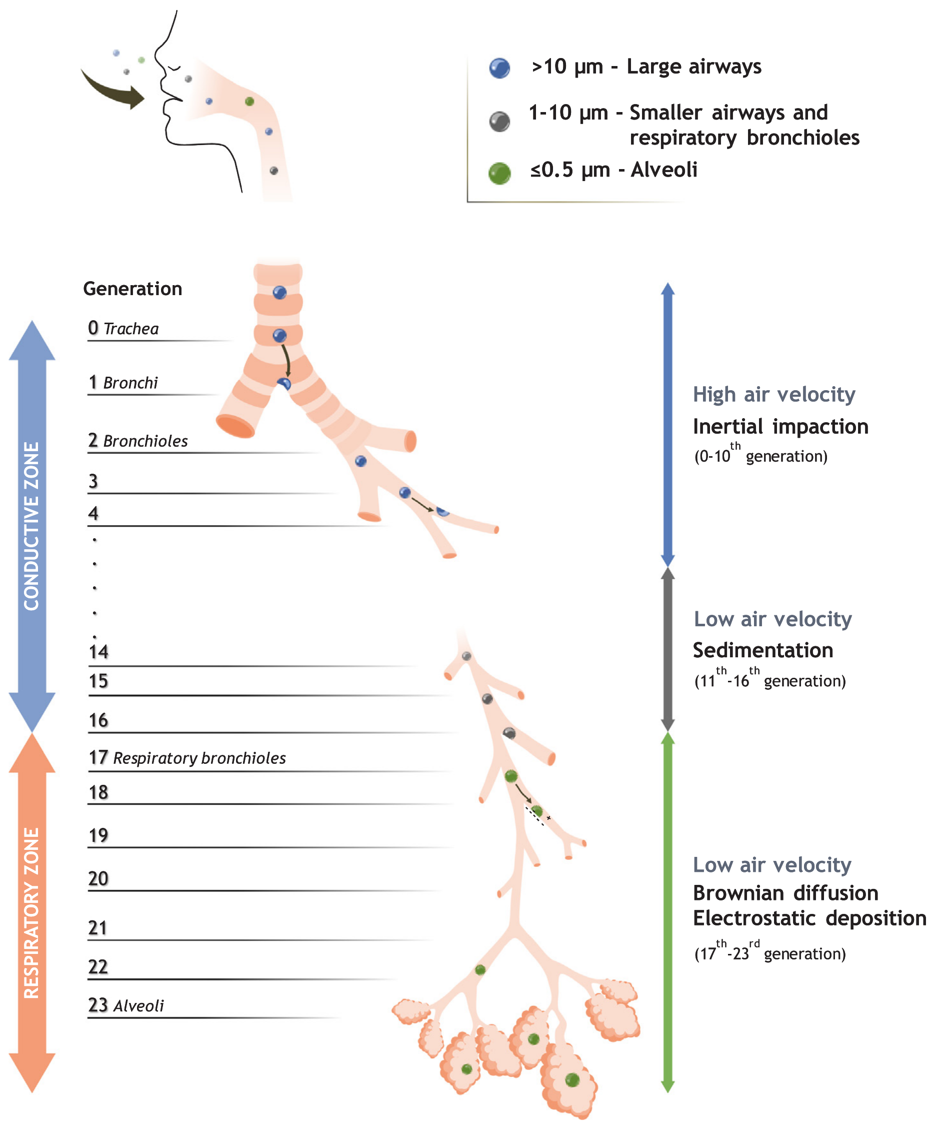

Fig. 1. Architecture of the lung and the main forces that govern diffusion and deposition of nanoparticles. The inertial impaction mainly occurs in the upper airways through the nasopharynx and large airway bifurcations, especially if the particles possess an aerodynamic diameter greater than 10 µm. Particles with an aerodynamic diameter from 1 to 10 µm are deposited by gravitational sedimentation on smaller airways and in the respiratory zone. Brownian diffusion is the main process that leads to deposition of particles equal or smaller than 0.5 µm on the alveoli.

with a variety of functional groups present in the mucus that check penetration and diffusion [40]. In any case, those NPs retained in the mucus will be transported up to the glottis via the mucociliary escalator, where they will be coughed away or swallowed to the gastrointestinal tract [5,36]. When the inhaled NP interacts with the mucus layer, some of the mucus components might adsorb onto the surface forming a corona that will affect the subsequent interactions and internalization. Modifying the surface of NPs should affect these interactions (e.g., the negative charged mucins will not interact with neutrally charged NPs [41]) and the formation of the corona which will ultimately determine the fate of the NP. The modifications made to prevent NPs from interacting with mucus and therefore to escape the mucociliary escalator include the slight adjustment of the mucus pore size by mucolysis and the careful selection of the NP size to fit the pore or the surface to prevent interactions [36,41]. One of the most used strategies consists of coating NPs with polyethylene glycol (PEG), a hydrophilic, non-charged, biocompatible polymer that reduces adhesion promoting the diffusion through the mucus [40,42–44]. Additionally, PS, whose barrier function will be explained later in this section, could also be used to overcome the mucus barrier. Coating NPs with PS could impede the adhesion to the mucus layer [41] facilitating interfacial spreading [45,46].

If inhaled NPs have managed to penetrate the upper or conductive airways, escaping deposition, they will finally reach the alveolar regions where they will encounter the PS. Here, regardless of the composition and surface of NPs, they will interact with surfactant components. Some surfactant components will be adsorbed onto the surface of NPs contributing to the corona formation and, consequently, their fate. In the case of hydrophilic NPs, it is proposed that hydrophilic surfactant proteins, mostly the collectin SP-A that facilitates the recognition, binding, and clearance of foreign entities from the lung [47], firstly binds to the NP surface and subsequently the remaining surfactant components (mostly lipids together with the hydrophobic surfactant proteins, SP-B and SP-C). On the other hand, hydrophobic NPs are thought to bind surfactant components the reverse order [17]. Therefore, all NPs reaching the alveolar spaces will be embedded into the surfactant membranes and exposed to the highly dynamic environment. During the process of breathing, alveoli are continuously dilating and contracting, which forces PS to constantly be compressed and expanded [48]. As a result, all the NPs that integrate into the surfactant film will similarly be subjected to these compression/expansion dynamics. These breathing dynamics lead to the folding and exclusion of different PS structures from the interface ensuring an ongoing removal of the less surface-active materials [49,50]. In a similar manner, it could also enhance the release of the NPs interacting with PS towards the alveolar spaces, making them free to interact, internalise and eventually, cross the last line of defence, the cellular barrier [4,36].

4.3. Cellular barriers

In the event that NPs succeed in overcoming the former barriers, the epithelial and immunological cells will constitute a last line of defence. The characteristics of the epithelial barrier change progressively through the whole respiratory tract in terms of both morphology and cell types. It progressively reduces in thickness from 3 to 5 mm in bronchi, 0.5–1 mm in bronchioles and 0.1–0.4 µm in alveoli [51,52]. The epithelium in the conductive areas is mainly constituted of Club cells and ciliated cells, that respectively synthesize and secret the mucus, moving it up to the glottis. In the alveolar region, a monolayer of type I and II (ATI and ATII) pneumocytes constitutes the thinnest and largest epithelial barrier of the body, evolved to optimize gas exchange between the atmosphere and the bloodstream. ATII also synthesize and secrete PS into the alveolar spaces. The distance from the air space to the blood stream in the alveolus (less than 400 nm), in addition to the large surface area, enable the inhaled particles to enter the body [5,52].

Nevertheless, a heterogeneous group of macrophages are present to protect the whole respiratory surface. They are mainly in charge of maintaining a low inflammatory context, ensuring the correct function of the tissue, performing non-immune, tissue-specific and homeostatic functions [53]. The microenvironment that macrophages encounter in the lungs is markedly fluctuant and peculiar. The continuous exposure to environmental antigens and particles, hypoxia, oxidation, microflora, airway mucus, surfactant and epithelial cells that constantly change this microenvironment [54], forces pulmonary macrophages (PMϕ) to adapt to this very variable environment. Under homeostatic situations, PMϕ have specific roles depending on whether the location is in bronchiolar submucosa, alveolar interstitium, vascular adventitia or luminal side of alveoli [55]. The latter location in particular harbours the so-called alveolar macrophages (AMϕ), while the other PMϕ form a larger and more heterogenous group referred to as the interstitial macrophages (IMϕ). Apart from being guardians of the alveolar surface, AMϕ are the cells mainly responsible for PS clearance [15,56,57], although a small part of the 'used' surfactant is also degraded by ATII pneumocytes. The NPs that are excluded from the interface during the process of breathing are thought to be either phagocyted by IMϕ and AMϕ or endocyted by non-phagocytic cells like ATII via clatrhin or caveolae pathways [36,58–60]. The phagocytosis by macrophages also depends on the particle size, composition and surface [5,61]. Particles smaller than 0.26 µm [62] are more likely to escape from phagocytosis than microparticles (> 500 nm) [5].

PMϕ play a crucial role in several respiratory disorders, such as asthma, pulmonary fibrosis and tuberculosis and, as in the case of AMϕ, are specialised in PS clearance. Therefore, using PS as a carrier of NP could be a good strategy to target PMϕ and treat these diseases [33,36,63]. Ruge *et al.* [64] demonstrated that both surfactant lipids and the hydrophilic proteins coating NPs modulate the uptake by AMϕ. Therefore, this effect could be used positively to trigger the interaction of NPs with these cells. Additionally, the design of NPs with the potential to cross the epithelium and endothelium and reach the bloodstream has been proposed as a potential way of entry to treat peripheral diseases. Nemmar *et al.* [65] have demonstrated that inhaled carbon NPs diffused into the systemic circulation of hamsters through phagocytosis via macrophages and endocytosis via epithelial and endothelial cells. ATI and ATII pneumocytes have shown selective incorporation of lecithin-coated and albumin-coated NPs, and their subsequent transport to the circulatory system by transcytosis [5]. These findings indicate the importance of studying the properties of NPs and the mechanisms that lead to particle clearance so that they can be avoided when the intention is to reach the deep lung. Investigation of the interaction between PS and NPs in this region should help in the development of new biomedical applications.

5. Pulmonary surfactant: the guardian of the door

Deep within the lung, PS is the first barrier of the respiratory surface to make contact with external elements. Therefore, it is important to study how it interacts with inhaled particles, and in this sense, PS needs to be considered as a having a fundamental role when simulating the *in vivo* situation. Accordingly, a brief overview of the composition, structure and function of PS is presented. For more details about PS function, structure and interfacial activity, we recommend consulting other reviews in the literature [15,48,50,66,67].

A thin aqueous layer coats the pulmonary epithelium and therefore, the lung must overcome the surface tension at the air-liquid interface during inhalation and exhalation. To achieve this, ATII pneumocytes synthetize, assemble and secrete into the alveolar space a surface-active material which referred to as the pulmonary surfactant [48,66]. PS is a complex mixture of lipids and proteins. Lipids comprise more than 90% of the mass and the most abundant species is the saturated lipid dipalmitoylphosphatidylcholine (DPPC) representing around 40% by mass [48,50]. It enables PS to reduce surface tension to extremely low values [4]. Fig. 2 summarizes the main fractions of lipid and protein composition of PS. Proteins, including four specific surfactant proteins, constitute the remaining 8–10% of surfactant by mass and they can be classified into two families. The hydrophilic proteins, SP-A (6%) and SP-D (< 0.5%) are involved in innate host defence mechanisms and are responsible for pathogen recognition, binding and clearance from the alveolar space, while the hydrophobic proteins, SP-B (1%) and SP-C (1%), contribute to the formation and maintenance of stable films at the air–liquid interface [15,68,69].

Lipid and protein components are synthesized and assembled by ATII pneumocytes and stored as tightly packed multi-lamellar structures, called lamellar bodies (LB), before being secreted into the alveolar spaces [50,70]. When secreted, intermediate structures are formed by changes in pH, hydration and calcium concentration [15,70]. The main structures formed are tubular myelin (TM),

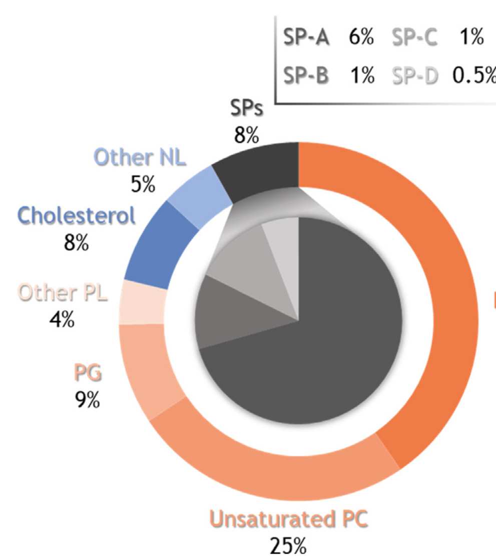

Fig. 2. Lipid and protein composition of pulmonary surfactant. The representation of the main fractions of lipid and protein components in pulmonary surfactant is based on [48]. Dipalmitoylphosphatidylcholine (DPPC); phosphatidylcholine (PC), phosphatidylglycerol (PG); phospholipids (PL); neutral lipids (NL); surfactant proteins (SPs).

predominantly stabilised by SP-A, and large membrane layers that have the potential to transfer large amounts of material into the interface. To maintain the functionality of the PS, it has to be continuously recycled and replenished [4,15].

Alveoli are very dynamic structures, continuously subjected to cycles of compression and expansion, forcing PS to continuously adapt to an ever-changing environment. PS can spontaneously form films at the air-liquid interface, excluding water molecules from the interface and so reducing surface tension to values below 2 mN/m after exhalation [48]. First, PS rapidly adsorb into the air-liquid interface reducing the surface tension and stabilizing the respiratory surface. To do this, large amounts of phospholipids (PL) are transferred by surfactant aggregates, a process that is facilitated by calcium and SP-A and when this material is near to the interface, the proteins SP-B and SP-C transfer the PL into the interface forming a dense film. The density of this film depends on the surface concentration and membrane composition, and is facilitated by unsaturated PL. Then, during expiration, the alveolar volume is reduced to a minimum and PS should reduce the surface tension to very low values by the lateral compression of the interfacial film leading to the formation of associated three-dimensional structures. This is facilitated by the presence of DPPC, which supports extreme surface packing, and the exclusion of structures rich in unsaturated PL by the so-called "squeeze-out" process. SP-B and SP-C play a crucial role orchestrating the lateral sorting and the three-dimensional organization of the surface film that leads to extremely low surface tensions of < 2 mN/m which is then maintained during moderately long periods of time. When the lung subsequently expands on inspiration, the condensed film has to rapidly and efficiently re-spread across the interface. This is accomplished by the transference of material from the interconnected reservoirs in the subphase, again, facilitated by the two hydrophobic proteins [66,69,71].

The correct function of PS is essential to allow the opening and stabilisation of alveoli during breathing. PL, especially DPPC, are responsible for the extreme reduction in the surface tension due to its amphipathic character, thus allowing them to spontaneously form interfacial films at the air-liquid interface that prevents the water molecules from being exposed to the air. The role of SP-B and SP-C is essential in order to reach the equilibrium surface tension very rapidly, reducing it to minimum values during compression and favouring the re-extension of the film during expansion. The recruitment of some of these essential components by inhaled NPs may compromise the function of PS. Thus, due to the key function of PS in the normal function of the lung, it is important to analyse the interaction of NPs from a toxicological point of view, to reduce the impact on PS function.

6. When nanoparticles and pulmonary surfactant meet

Each time we breathe, external NPs enter our organism. Most of them cause insignificant effects, but others may cause serious harm. The interaction of NPs with PS can lead to changes in both NP properties and PS functions. The inhalation of NPs has been related with the generation of ROS, an effect that leads to damage in the epithelium and directly affects ATI and ATII pneumocytes. Epithelium damage and inflammation further affect PS in various aspects (synthesis, composition, structure, metabolism and function) [72]. Depending on the physicochemical properties of the NP, different PS components will participate in the interactions, which could equally interfere with the intended biomedical application of the particles as well as affecting the normal functioning of the PS system subsequently determining the biocompatibility or toxicity of NPs. Thus, it is essential to understand these interactions and their consequences on the performance of PS to improve the design of novel nanomaterials. Having described the composition, structure and function of PS as well as the main important features of NPs, the interaction between these two entities and how this may affect the normal function of PS will now be described in this section.

6.1. Formation of the nanoparticle corona

When a NP contacts with any biological fluid, some of its components will adsorb onto the NP surface forming a "biological corona". Vroman described in 1962 [73] the adsorption of plasma proteins onto the surface of hydrophobic or hydrophilic solids, but it was not until 2007 that Cedervall *et al.* [74] introduced the term "corona". The formation of the corona is a dynamic and competitive process governed by colloidal forces, the nature of the environment and the NP, and will also depend on the time of exposure [75,76]. Several molecules form transient complexes with NPs, following a kinetics of adsorption and desorption defined by the "Vroman effect". This is described as a time-dependent process, in which molecules with low affinity but highly abundant will adsorb first but which are then subsequently replaced by less abundant molecules with higher affinity [77,78]. While the NP moves from one biological environment to another, the corona is continuously changing, with some molecules of the first scenario being replaced by others from the new one. This has been described as the "fingerprint" of the NP, indicating the pathway it has followed through the organism, which can be used to understand the biological fate of the NPs [76,79,80]. The adsorption of these molecules onto the NP strongly depends on their affinity as well as on biomolecule-biomolecule interactions. The former governs the formation of the called "hard corona", the first layer of the corona composed by molecules tightly adsorbed with high affinity to the NP. The biomolecule-biomolecule interactions is the driving force that mainly determines the formation of the outer layers of the corona, the "soft corona", consisting of molecules bounded transiently to the hard corona by weaker interactions [75,80]. The hard corona is believed to be more important than the soft corona, since it is responsible for the aforementioned "fingerprint" and determines the interactions with cells, affecting the translocation through the body and tissue and, hence ultimately, the NP function. The composition of the hard corona has been widely studied, in contrast to the soft corona, which is basically unknown due to the difficulties to isolate it [75]. Apart from the corona formation, the high ionic strength and protein adsorption could destabilize the NP surface and cause the formation of NP aggregates [81,82]. This destabilization depends on both the protein concentration and the physico-chemical properties of NPs [82]. When proteins adsorb to the NP surface, they tend to unfold and interact with other proteins in the media, leading coated NPs to aggregate and form larger complexes [83,84]. This alters the behaviour, properties and biodistribution of NPs *in vitro* and *in vivo* (e.g., sedimentation, uptake and cytotoxicity) [82,85]. Aggregation of AuNPs, for example, has been connected with a reduction of 25% in their uptake by A549 cells in comparison with single NPs [81]. Other studies reported that the adsorbed unfolded proteins expose certain epitopes that may help the NP uptake by interacting with cell surface receptors [84,86]. Therefore, it is important to study and understand the NP-protein interactions and the process of formation and composition of the biological corona under physiological conditions. Improving the design of drug delivery systems and the processes by which NPs may accidentally be released should contribute to controlling the bioavailability and biodistribution of NPs entering the organism, both in terms of toxicological effects and desired action [80,87].

The first biological fluid to make contact with inhaled NPs in the deep lung is the PS. The content of lipids in this region is much higher than in other biological fluids, such as plasma, and this leads to the formation of a corona with significantly different composition compared to the extensively investigated plasma corona [17]. The structure of this lipid-protein corona depends on key NP features (specially its composition, hydrophobicity and surface charge) [88]. Some studies have shown that the lipid composition of the PS corona formed on different inhaled NPs presents no significant differences in terms of lipid species, although the content of lipids surrounding hydrophilic NPs is lower [17,87,88]. In the case of lipid-based NPs, the adsorption and exchange of lipids between the NP and PS depends on the lipid composition of the NP [89]. It has been demonstrated that different surface functionalization of silica oxide (SiO_2), or zirconium oxide (ZrO_2) NPs do not interact directly with pure lipid samples, which indicates that NP-lipid interactions in PS may be mediated by the proteins [90]. The adsorption of some proteins to NPs, such as SP-D or the antimicrobial peptide cathelicidin, triggers the adsorption of lipids [17,90]. Regarding protein adsorption, increases with the concentration and surface area of the NPs have been described [35]. The hydrophilic surfactant protein SP-A directly interacts with lipid-based (amphiphilic) NPs as well as with purely hydrophobic NPs, in contrast with SP-D that preferably associates with hydrophilic NPs. The hydrophobic surfactant proteins selectively adsorb onto anionic NPs although, interestingly, SP-B but not SP-C adsorbs to anionic hydrophilic NPs, and even to cationic or neutral NPs, due to its higher positive charge density and significantly less hydrophobicity in comparison with SP-C [35,88]. It is clear that, depending on the characteristics of the NP and the PS components adsorbed, the performance of PS and subsequently, lung function might be somehow affected. In the following section, the characteristics that matters and how they affect the PS system will be extensively described.

The corona has been shown to play an important role in the internalization of NPs intended for drug delivery to the lungs. Kato *et al.* [91] administered intratracheally lecithin-coated and uncoated polystyrene beads to rats. Both beads were taken up by AMϕ, but ATI and ATII pneumocytesincorporated only the lecithin-coated beads. PS, besides its unique capability to act as a shuttle for NP delivery into the deep lung, determines the coating of the NPs and its ability to promote internalization by alveolar cells. De Backer *et al.* [46,92] observed that dextran nanogels coated by PS were more efficient than uncoated nanogels in the delivery of siRNA to different cell types both *in vitro* and *in vivo*. Although the PS-coated nanogels exhibited lower cellular uptake, they were able to significantly reduce the expression of a gene at the protein level. This indicates that PS components somehow facilitate intracellular translocation of NPs into the cytosol, where siRNA produce silencing. This group also reported that protein SP-B could be the key element in PS responsible for the efficiency of the siRNA delivery. In fact, the mere incorporation of SP-B into a PL mixture coating, significantly improved siRNA delivery from loaded siRNA nanogels into lung epithelial cells *in vitro* as well as in AMϕ *in vivo* [93]. This is an illustrative example of how a smart designing of the corona can enhance the internalization of NPs to target cells and the potential use in biomedical applications.

6.2. Key nanoparticle features determine their interaction and impact on pulmonary surfactant

Once NPs reach the alveoli, their characteristics govern the interaction with PS and its consequences. Additionally, an important factor determining the effect of NPs on PS performance is the amount of inhaled NPs that ultimately make contact with the system. In most cases, the derived toxicity is concentration dependent and increases considerably when high doses are applied [94–98]. The toxicity of NPs on the PS is manifested at two levels affecting the biological function and the metabolism of PS. In this section, the effect of NPs on the PS system regarding these two aspects will be described as defined by physiochemical properties of the NPs (Fig. 3).

6.2.1. Effects on the biophysical function of pulmonary surfactant

The normal breathing process of the lungs requires the correct biophysical functioning of the PS. As mentioned above, a good operative PS should rapidly form an interfacial film to cover the air-liquid interface and reduce the surface tension to equilibrium values. It also needs to present low compressibility to further reduce surface tension to extremely low values during expiration, and finally to efficiently re-spread during inspiration [67]. In these processes, the synergistic action of the lipids and proteins plays a key role. Therefore, if one or more constituent of PS is removed or damaged, the biophysical function of PS can be severely compromised. Depending on the properties of a NP, it may either interact selectively with certain components of PS, or with the environment, leading to the formation of ions that could affect PS function. Surfactant dysfunction due to the inhalation of NPs is believed to occur when there is direct interaction with individual PS components. When key PS elements such as proteins SP-B and/or SP-C are adsorbed onto the surface of a NP they are squeezed-out and removed from the PS film, leading to inhibition of surfactant activity. A reduction in the concentration of hydrophobic proteins below certain levels leads to a loss in PS adsorption to the air-liquid interface resulting in limiting its ability to reduce the surface tension as well as its compressibility. In contrast, the formation of NP/PS aggregates is thought to act as a protective action of PS reducing the diffusion of NPs into the sub-phase and the internalization by AMϕ and pneumocytes [99].

There are many studies using various biophysical models focusing on the effect of NPs, with different composition and from different sources (air pollutants or biomedical NPs). These will be described in the next section. It has been shown using the constrained drop surfactometer that the addition of engineered mesoporous carbon NPs (MCNPs) or multi-walled carbon nanotubes (MWCNTs) to Infasurf®, a clinical PS, even at low concentrations, increases the minimum surface tension and the compressibility of PS [96,100]. Langmuir-Blodgett and Wilhelmy balances were also used to show that the presence of carbon-based NPs in native surfactant (obtained from porcine bronchoalveolar lavage fluids) leads to significant increases in the minimum surface tension and negatively affects the typical lateral phase transitions exhibited by PS films under compression. This may be due to the adsorption of PS components to the NPs [98]. Some *in vivo* studies have demonstrated the adsorption of PS lipids and proteins onto single-walled carbon nanotubes (SWCNTs) in animal models. After inhalation, SWCNTs are coated by phosphatidylcholine (PC) and phosphatidylglycerol (PG) lipids, and proteins SP-A, SP-B and SP-D, which strongly affects the normal function of PS [101]. It has been shown that SP-A and SP-D bind to carbon nanotubes, while SP-A adsorbs to metal oxide NPs but SP-D prefers gold NPs (AuNPs) [102]. The total or partial absence of these two proteins is also associated with impairment of the innate immunological defence of the lungs [66]. In the case of AuNPs, a model for metal air pollutant, they have been described as getting coated by PL at the contact with a model lung surfactant (DPPC/palmitoyloleoyl phosphatidylglycerol (POPG)/SP-B; 70:30:1 w/w), adsorbing to the air-liquid interface and inhibiting the adsorption of free PL. This increases the minimum surface tension and reduces the capability of PS to correctly re-spread during inhalation [103]. Polymeric NPs have shown the mildest effects on the biophysical function of PS and could represent the best option as a basis for the design of drug delivery NPs for inhalation therapies [104,105]. The interaction of lipid-based NPs with PS has been also studied. Wan *et al.* [89] showed that the interaction between both structures leads to the exchange of lipids, which changes the liquid-condensed domains of PS films. However, even though the composition of a NP determines some characteristics, it does not fully define all the effects and it is necessary to further evaluate other physicochemical aspects such as size, charge and hydrophobicity to obtain a more complete understanding of the effects.

NP size could determine the fate of NP *in vivo* or the degree to which they are toxic or not. Schleh and Hohlfeld [30] studied the effect of TiO_2 micro- and nano- particles on the clinical surfactant Curosurf® using the pulsating bubble surfactometer. TiO_2 microparticles slightly increased the minimum surface tension, in contrast with TiO_2 NPs, which significantly increased the minimum surface tension, severely inhibiting the PS function in a concentration-dependent manner. Some *in vitro* and *in vivo* studies performed with silver NPs (AgNPs) of different sizes included in consumer products demonstrated that the smaller NPs (20 nm) present higher toxicity due to their larger surface/area ratio and high dissolution rates (Ag^+ ions induce acute inflammatory effects), compared with larger NPs (110 nm) [106]. In

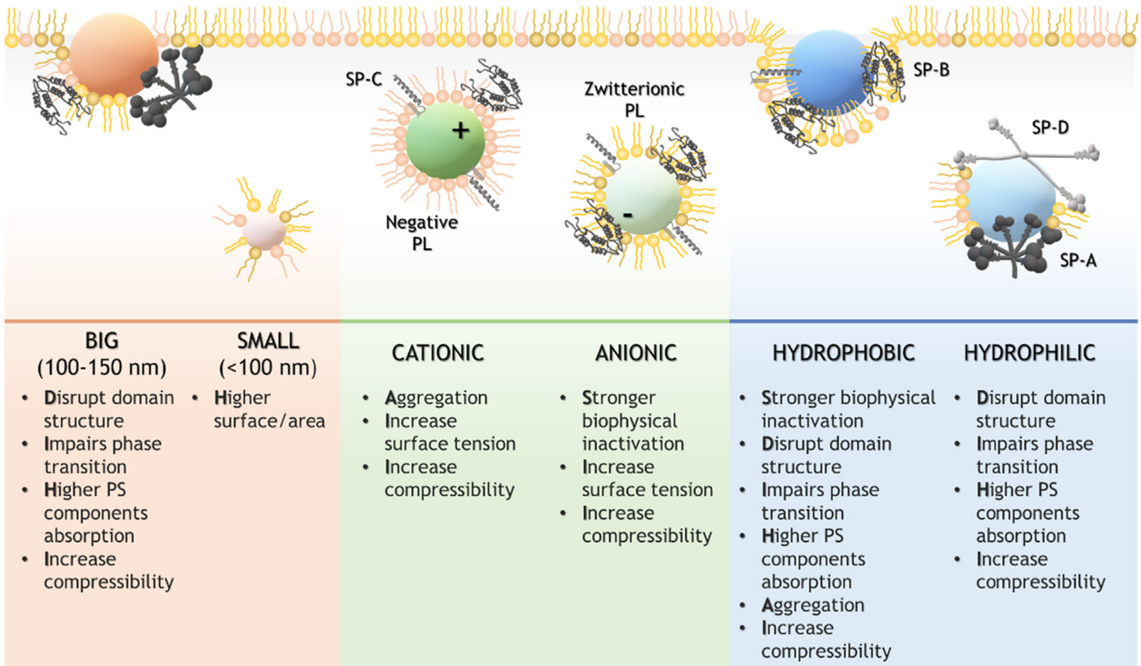

Fig. 3. Principal effects and interactions of nanoparticles upon contact with pulmonary surfactant. The main physicochemical properties of NPs that affect their interactions with surfactant are listed and represented in the scheme.

contrast to these studies, Dwivedi *et al.* [28] reported different observations with reference to the NP size. They investigated the effect of two hydrophobic polymeric NPs with different diameters (12 and 136 nm) on DPPC monolayers and PS model films (DPPC/dipalmitoyl phosphatidylglycerol (DPPG)/SP-C; 80:20:04 mol%). In both models, the small NPs had no significant effect until high concentrations were applied. In contrast, atomic force microscopy (AFM) and Langmuir-Blodgett balance studies showed that the large NPs disrupt the domain structure of surfactant films by the formation of multilayer protrusions during compression, impairing compression-driven phase transitions and increasing the compressibility of the films, even at low concentrations. Chen *et al.* [100] also showed that larger MCNPs induced more pronounced cytotoxic effects than small MCNPs. These studies suggest that NPs could present stronger inhibitory effects than microparticles due to their higher surface/area ratio, which enhances other physicochemical properties and pronounces their effects, i.e., by increasing the adsorption of PS components or promoting the formation of NP aggregates [35]. However, while NPs seem to be more toxic than microparticles, when the dose of delivered particles to the lungs is normalized to the surface/area ratio the difference between both sizes disappear [76]. It can be concluded that the size of NPs is not a determining feature that should be taken separately from surface charge and hydrophobicity when interpreting toxicological studies.

Surface charge and hydrophobicity have been largely proposed as key parameters defining the interactions of NPs with the PS components and, hence, their effects. The surface charge is a determining characteristic in the interaction with PS and the formation of NP/PS aggregates. The aggregates are formed due to electrostatic interactions, the strength of which has been shown to be higher in positively-charged than in negatively-charged NPs [99]. Both negatively- and positively-charged polystyrene NPs cause biophysical inactivation of PS, but this was stronger for the anionic NPs [94]. The inhibition of PS function produced by inhaled NPs is believed to occur by its interaction with PS components and its depletion. Following this hypothesis, the adsorption of SP-B and SP-C to negatively-charged NPs is thought to be one of the mechanisms for NP-promoted loss of function in PS. Protein SP-B exhibits stronger affinity for anionic NPs than SP-C, owing to the difference in charge density [88,94]. On the other hand, positively-charged NPs present strong affinity for anionic PL, with which SP-B interacts. This leads to the depletion of SP-B from the surfactant films and leads to the formation of elongated strand-like structures contributing to the development of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) [103].

The core composition and the coating of the NP define the surface hydrophobicity, which modulate the interaction with the molecules within the biological fluids. The surface hydrophobicity of NPs seems to play the most decisive role defining the effect of NPs on the biophysical function of PS. However, to simplify, we refer to the surface hydrophobicity as hydrophobic or hydrophilic. Studies performed in Langmuir-Blodgett balances, AFM and constrained drop surfactometer revealed that polymeric NPs with different hydrophobicity present different inhibitory effects on Infasurf®, which increases with the hydrophobicity of the NPs [107]. Hydrophobic NP could remain longer at the air-liquid interface, promoting the interaction with the lipids and the hydrophobic proteins of PS, having an inhibitory effect on PS function. Different hydrophobic NPs have shown similar effects on DPPC monolayers. All of them were able to adsorb onto the air-liquid interface, interact with the lipid tails and present higher retention times in comparison with hydrophilic NPs [88,95,107,108]. Hydrophobic NPs are normally segregated into liquid-expanded regions of the films at low surface pressures, while at higher surface pressures they interact with structures outside of but associated with the interface [95]. During compression of the films, the hydrophobic NPs aggregate and form protrusions that locate at the phase boundaries [35]. From there, they negatively affect the phase transition and the compressibility of the film, making it more fluid by transforming the large condensed microdomains into small ones [95,97,109]. Since hydrophobic NPs are retained by the PS film, they are wrapped by PS as they are excluded from the interface during the compression/expansion cycles [110]. In contrast, even though hydrophilic NPs could interact with the head-group of the lipids [108], they are normally translocated to the subphase where they interact with the hydrophilic proteins. While hydrophilic NPs cross the monolayer, they can also form PS/NP complexes and remove lipids, which increase the minimum surface tension [109,110]. In summary, both hydrophobic and hydrophilic NPs have demonstrated inhibitory effects on the biophysical function of PS (increasing the minimum surface tension, reducing the LE-LC plateau, impairing the phase transition), but the effects are typically more pronounced in the case of hydrophobic NPs.

6.2.2. Influence on the pulmonary surfactant metabolism

Breathing dynamics and the highly oxidative environment lead to a progressive loss of PS functionality, so the PS pool must be continuously replenished, through the exchange of material between the interface and the subphase [15]. Apart from the direct effects on PS function, NPs could have an impact on surfactant metabolism. Impairment of PS catabolism and recycling has been related to some lung injuries, such as pulmonary alveolar proteinosis (PAP), a disease associated with the accumulation of used and non-functional PS in the alveoli [67]. The PS metabolism could be affected by the inhalation of NPs mainly at two levels; firstly, in the production of new PS to replace the spent material, and secondly in the removal and recycling of spent surfactant from the interfacial structures. Referring to the former, the exposure to AgNPs produced an alteration in the surfactant composition and triggered the production of lipids by ATII pneumocytes [111]. Wang and Petersen showed that lipid-coated AuNPs were internalized by A549 human lung cancer cells, widely used as an *in vitro* model of ATII pneumocytes [112]. Lipid-coated AuNPs were endocytosed by the cells during the recycling process, but the amount of the NPs decreased over time, while the number of LB increased dramatically. Since the LBs are the structures that serve to secret surfactant lipids, they proposed that A549 cells increase the number of LBs in the presence of these NPs as a protective mechanism to remove them from the cell. Later studies confirmed that ATII pneumocytes increase the amount of LB and the PC synthesis in response to the exposure to silica-coated superparamagnetic iron oxide NPs (SiO_2-SPIONs). SiO_2-SPIONs interfered with the normal packaging of PS into LBs, which lead to a sequestration into autophagic vacuoles and subsequent elimination [113]. LBs are also damaged after the intratracheal administration of TiO_2 NPs in mice [114].

With respect to the removal of spent PS components, the PS complexes adsorbed at the interface are released during breathing dynamics in the form of small aggregates with less interfacial activity, and subsequently cleared by AMϕ or recycled by ATII cells [57]. This conversion of active large PL structures into smaller ones has been shown to increase upon inhalation of hydroxyapatite NPs, even at low concentrations. This is due to the adsorption of surfactant proteins to the NP surface [115]. Other studies have focused on the vesicle insertion process, which occurs in two steps: initial adsorption of vesicles to the interfacial film and posterior fusion and integration, a process that is Ca^{2+}-dependent and protein-mediated. It has been proposed that the fluid regions of the film are the areas where the vesicles insert. As NPs normally localize in this type of regions and recruit surfactant proteins that mediate the fusion of vesicles, they could affect the PS recycling process [95]. Polyorganosiloxane NPs and polymeric NPs inhibit the vesicle insertion process by adsorbing at the air-liquid interface and recruiting surfactant proteins [28,95].

An efficient strategy to mitigate the negative effect of NPs on both the biophysical function and the metabolism of PS is to coat the surface of NPs, reducing the bioreactivity. AgNPs coated with DPPC presents more stability and less aggregation than the uncoated counterparts, thus decreasing toxicity [116]. Similar observations were made later by coating zinc oxide nanowires or AgNPs with Curosurf®, so that the contact with the aqueous environment is blocked and the release of Zn^{2+} or Ag^{+} ions delayed [117,118]. This is a potential aspect to explore in benefit of the delivery of NPs to the lungs combined with PS. Beck-Broichsitte [105] modified the surface of polymeric NPs with polyethylene glycol (PEGylation), reducing the negative effect of the NPs in the interfacial activity of PS. Plain negatively charged NPs adsorb SP-B and SP-C, essential for the biophysical function of the PS, but PEGylation of polymeric NPs lead to a protein repellent surface and a reduction in the impact on the performance of PS. The coating of AuNPs with PEG has also shown a significant decrease in the toxicity of these NPs towards ATII cells, which reduces the possible negative effects on the metabolism of PS carried out by these cells [119]. Again, this is an example of how an appropriate design of the NP coating is important to produce more stable and less toxic NPs intended for delivery to the lungs.

7. Importance of including PS in cellular and tissue *in vitro* models of the lung epithelium

Besides the effect of NPs on the biophysical properties of PS, it is also relevant to analyse the potential toxicity and interactions of NPs with the different respiratory barriers and their capability to cross them. In this sense, cellular and tissue *in vitro* models of the air-blood barrier are novel approaches useful to perform safe and reproducible assessments of NPs. Standardization of *in vitro* systems that can be used to predict the *in vivo* situation, at least partially, is an important issue in pharmaceutical research with important advantages in reducing the costs and time of *in vivo* studies, minimising animal testing, allowing for the modelling of different respiratory diseases as well as conferring more significance to the human context since primary human cells can be used [11,13]. In this section, the most important advances in respiratory surface models will be summarized outlining the importance of including PS.

7.1. An overview of the current cellular and tissue in vitro models

Although current models cannot yet cover all the conditions and complexity of the *in vivo* scenario, there are sophisticated respiratory surface models that are starting to mimic some of the most representative and relevant characteristics (i.e., physicochemical, structural, dynamical and functional properties) to test the applicability and toxicity of new strategies in pulmonary drug delivery. The simplest models consist of monocultures of the most characteristic cells in the respiratory milieu (e.g., epithelial cells, AMϕ or endothelial cells), either as cultured cell lines (e.g., human Alveolar Epithelial Lentivirus immortalized (hAELVi) [120]) or primary cultures of isolated cells. An exclusive setup described by Schürch *et al.*, which introduced ATII cells in a captive bubble surfactometer (CBS), was used to include breathing-like dynamism into cell culture models to evaluate the internalization of AuNPs under conditions of breathing [121]. Co-cultures of these cell types on both sides of permeable membranes have increased the complexity of the system modelling the most relevant structural characteristic in the air-blood barrier context (i.e., epithelial and endothelial cell layers separated by the basement membrane) [122–124]. Additionally, these models could be cultivated both in media-submerged or under air-liquid interface conditions. The former is easier to handle, but the latter is closer to the *in vivo* scenario [125]. The combination of these cell cultures and microfluidics has led to the development of lung-on-a-chip models, which considerably improves the structural, functional and mechanical properties of the *in vitro* air-blood barrier models [10,126–128]. The microfluidic chips also provide a precise control of the cell environment and allow to produce long-term cultures [125]. Some of these devices also allow to recreate physiological breathing conditions, stretching the flexible membrane and cells by applying a vacuum to the side chambers of the membrane [126] or to the adjacent microcavities [10]. These breathing lung-on-a-chip models have been used to test the exposure of NPs to alveolar-like structures and have demonstrated the importance of breathing dynamics on the nanotoxicological analysis (Fig. 4) [126]. Additionally, the recent inclusion of 3D bioprinting technology, which aims to design and produce biocompatible structures closer to *in vivo* architectures, offers more versatility, reproducibility and accuracy than the manual seeding method, where erratic and undesirable structures may be formed [11]. The first bioprinted air-blood barrier was developed by Horváth *et al.* in 2015 [11], in which ATII pneumocytes and endothelial cells where printed separately in a thin basal membrane-like layer. Recently in 2019,

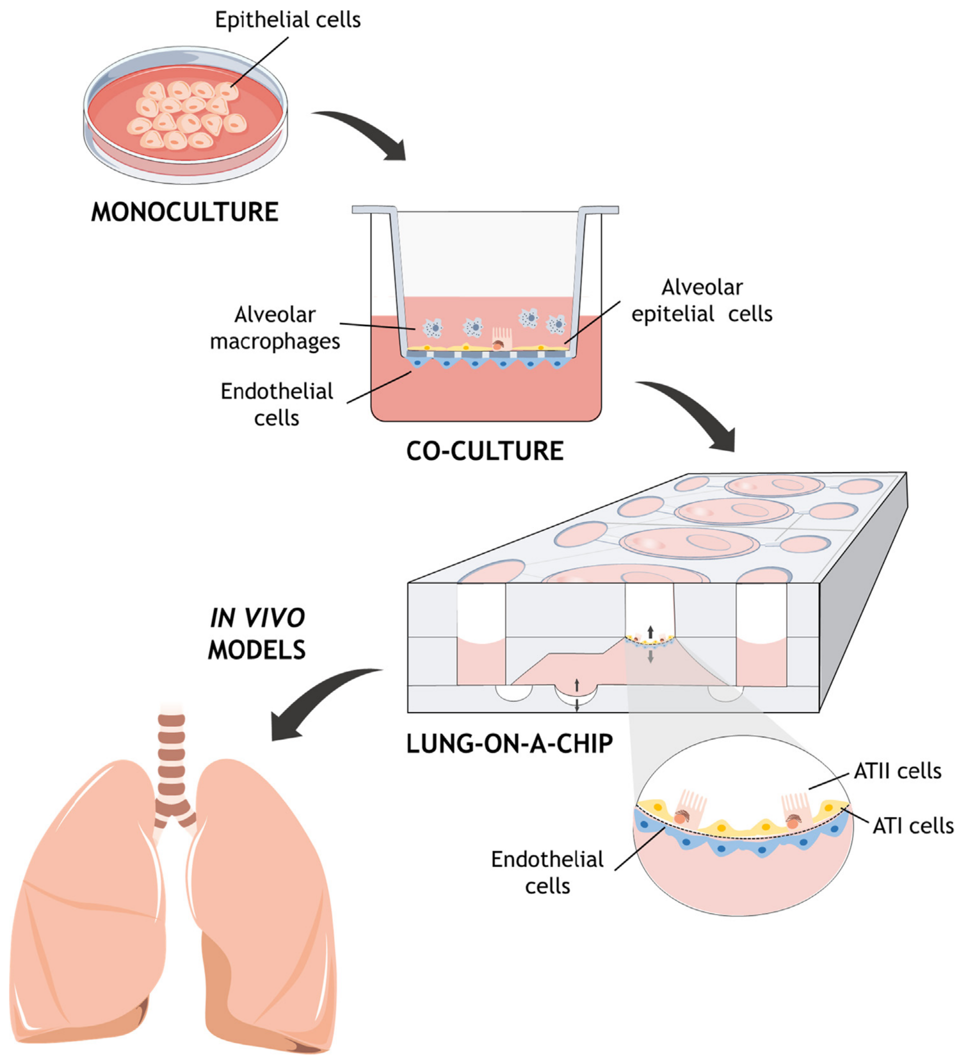

Fig. 4. Models of the respiratory air-blood barrier. The complexity of *in vitro* models increases from the simple monocultures to co-cultures of the most representative cells in the lung (i.e., epithelial and endothelial cells and macrophages). The latter combines different cell types on both sides of permeable membranes to better mimic the lung. The most complex model is the breathing lung-on-chip devices [10], with the aim to reproduce as close as possible the *in vivo* conditions.

Grigoryan *et al.* have also developed a hydrogel-based structure mimicking a breathing air sac with vascular networks using 3D bioprinters [12]. This hydrogel-based model allows for the investigation of the oxygenation and flow of human red blood cells during breathing dynamics simulations. But most importantly perhaps, it could also serve as the scaffold for respiratory cells to build a complete and totally functional alveolus, offering new possibilities in tissue engineering, regenerative medicine and pharmaceutical research.

7.2. Why we should include pulmonary surfactant

Throughout this review, the importance of PS in the alveolar context has been described as well as how the presence of PS could affect the behaviour of inhaled NPs. Nevertheless and to some extent surprisingly, PS is not yet systematically included in the aforementioned models. As explained before (see the section *Pulmonary surfactant: the guardian of the door*), PS is present across the whole respiratory interface to avoid pulmonary collapse and to minimize the entry of foreign entities through the air-blood barrier. In this barrier, the reduction of surface tension by PS also prevents the oedema due to extravasation of plasma components into the alveolus, by reducing the hydrostatic pressure gradient between the circulatory system and the alveolus [129,130]. The protective role of PS also includes the protection of the alveolar microenvironment from oxidative damage, carried out by the surfactant proteins SP-A and SP-D. The antioxidant activity of these two proteins is based on the interference with the formation and propagation of free radicals in the extracellular spaces or on the plasma membrane [131]. These hydrophilic proteins are also critical components of the innate host defence of the lung. In addition to the well-known function of SP-A and SP-D in the recognition, binding and elimination of infectious particles from the lung, they bind to the surface receptors of immune cells to modulate their activity by the production of inflammatory components [132–134]. In fact, SP-D has been closely related with the regulation of the phenotype and function of AMϕ [135,136]. Besides the proteins, the PS lipids have also shown an immune modulatory role in the local defence of the respiratory epithelium [133]. Several surfactant PL have been identified as anti-inflammatory and immunosuppressive components that acts on multiple receptors on the AMϕ surface. The unsaturated lipids and cholesterol could also act as biosensors of oxidative stress, thanks to the unsaturated bonds that confers them the flexibility to transform into effectors to regulate the host response to environmental damage [137]. It was shown that AMϕ need the presence of sufficient PS to ingest bacteria, revealing an essential role in the clearing of foreign material by macrophages that should be considered *in vitro* [16]. Some studies have shown an increase in the cytotoxicity of NPs when they interact with PS. On the one hand, MWCNTs coated with Curosurf® lead to an increase in the generation of ROS, the release of cytokines and apoptosis in alveolar cells when compared to uncoated MWCNTs [138]. On the other hand, silica NPs present an enhanced cytotoxicity when the PS was added to an *in vitro* model of the air-blood barrier [139]. These effects demonstrate once again the importance of considering PS in the *in vitro* models due to its capability to modify the impact and interactions of inhaled NPs.

Most components of PS exhibit an essential role in alveolar homeostasis. Thus, it is crucial to systematically include and characterize all of this material in the studies involving respiratory surface models, especially for those that explore therapeutic and toxicological effects of new pulmonary drug delivery strategies. However, it is even more important to select the proper PS material, depending on which role has to be modelled; as an endogenous protective system, as an exogenous surfactant with intrinsic therapeutic purposes, or as an "excipient" to improve the distribution of drugs and NPs through the lungs, enhancing internalization into cells. Commercially available lung surfactants derived from animal sources (porcine and bovine) only contain the hydrophobic components (i.e., they lack the hydrophilic proteins SP-A and SP-D), and typically do not contain cholesterol. Since those components play a critical role in alveolar homeostasis, inflammatory responses, recycling and clearance of surfactant as well as trapping external substances, surfactants that maintain the native composition and structures are desirable to mimic the endogenous surfactant. However, a caveat in the use of native surfactants comes from the potential induction of inflammatory processes by materials derived from animal sources. Thus, exhaustive characterization of the materials is crucial to prevent the introduction of spurious pro-inflammatory factors into cell models such as endotoxin. In the case of clinical surfactants, inflammation/allergy-derived problems are mitigated and they are good candidates for use as carriers in drug delivery. Nonetheless, these animal-derived surfactant preparations are relatively susceptible to inactivation (by agents that compete for the interface, such as albumin, or that degrade its components such as phospholipases [72,140]) and may present substantial batch-to-batch variations. They are also expensive to produce and supplies are limited. An additional major problem is the non-null possibility of animal-to-human transmission of potential pathogenic agents. Hence, other non-animal preparations that could allow producing surfactant in large quantities at a reasonable cost while controlling composition and reducing the inflammation and infective potential are preferred. In this sense, entirely synthetic surfactants could be a potential choice in pharmaceutical and toxicological research.

8. Conclusions

Nanoparticles present extraordinary properties, from which numerous applications can be derived. The high surface/volume ratio and their great versatility make them perfect candidates to develop more efficient materials and products in various fields, including promising characteristics to overcome emerging problems in biomedicine, improving diagnosis and drug delivery techniques. Nevertheless, these unique NP properties could also present undesirable effects, resulting in toxicity to humans. This double-edge potential of opportunities and threats increases the necessity to carefully analyse the characteristics of each NP preparation as well as their interaction with biological systems. Given the high amount of NPs from various different sources that are released into the atmosphere, it has become essential to study their possible effects on the lungs as their main route of entry. The most damaging effects of inhaled NPs could occur when they reach the deep lung, where they interact with PS, alveolar cells and even diffuse into the circulatory system. Since the first barrier encountered by NPs when they reach the alveolus is PS, it is crucial to study the reciprocal interaction between both structures. On the one hand, the interaction with PS forms a surfactant corona around the NPs that determines the further interaction with alveolar cells and modulates the internalization of delivered NPs to the target cells in the case of drug administration. This corona involves the recruitment of certain components of the PS, depending on the characteristics of NPs, which will affect its functions and metabolism. The hydrophobicity of NPs seems to be the most determining feature that affects the biophysical function of PS, and hydrophobic NPs show the highest inhibitory activity towards surfactant performance. Permanence in the interface, interaction with PS elements and interference with the cells related with PS metabolism lead to failure in the degradation and recycling and ultimately affect the function of PS. These effects may contribute to the development of respiratory diseases, demonstrating the importance of testing the NP/PS interaction. Evaluation of the bioavailability or the toxicity of inhalable NPs using *in vitro* techniques is important in order to reduce the use of animal testing and to model and standardize the analysis. As has been described throughout this review, PS plays a crucial role in lung homeostasis as well as in the toxicity and fate of inhaled NPs, so to include it in these models is essential if an accurate prediction of their effect on the lung is to be made, defining the NP-PS interaction. NPs have the potential to become important tools with a diversity of applications but, also, due to their possible impact on the respiratory system, an analysis of their characteristics and how they interact with all the elements constituting the lung epithelium should be made as the first step towards an optimization of their properties and biocompatibility.

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