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Interactions between inhaled nanomaterials and biomolecules in the lung

– a study of the risks of systemic effects

REPORT 14/16

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KEMI
Swedish Chemicals Agency

The Swedish Chemicals Agency is supervisory authority under the Government. We work in Sweden, the EU and internationally to develop legislation and other incentives to promote good health and improved environment. We monitor compliance of applicable rules on chemical products, pesticides and substances in articles and carry out inspections. We also provide guidance regarding enforcement and inspections to municipalities and county administrative boards. We review and authorise pesticides before they can be used. Our environmental quality objective is A Non-toxic Environment.

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Preface

The Swedish Chemicals Agency has been assigned by the Swedish Government to produce a national action plan for a toxic-free everyday environment: Action plan for a toxic-free everyday environment 2011 – 2014 – protect the children better. The work on the action plan has been extended until 2020.

Efforts are now going on in several areas, both in Sweden, within the EU and internationally and often in cooperation with other authorities. Reducing chemical risks in the everyday environment is one step towards attaining the Swedish Parliament´s environment quality objective A Non-Toxic Environment, which is the objective that we are responsible for.

Within the framework of the action plan, the Swedish Chemicals Agency compiles knowledge in our report and PM series elaborated by experienced colleagues, researchers or consultants. In this way, we present new and essential knowledge in publications which can be downloaded from the website www.kemikalieinspektionen.se

This report/PM has been produced within the framework of the government assignment to carry out the strategy on a non-toxic everyday environment and reaching the environmental quality objective A Non-Toxic Environment 2015–2017.

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Contents

Summary

Inhaled Nanoparticles (NPs) that are deposited in the airways, will immediately and spontaneously bind to endogenous biomolecules present in the liquid film covering the epithelium of the lungs. This formation of a new surface, a so-called biomolecule corona, can lead to completely new surface properties of the NPs that can affect the particles' bioavailability and interactions with biological tissue.

The general knowledge of NPs interactions with biomolecules is mainly based on interactions with proteins in the blood, and several studies have pointed out that such interactions can lead to systemic effects, for example, impact on the immune system and blood circulation.

The upper airway is constructed to clean the inhaled air on its way down to the alveoli where the gas exchange takes place. This is made possible by the cells of the upper respiratory tract that secretes mucus having a function to capture inhaled particles and microbes. The interaction between the mucus and the inhaled NPs may vary depending on the particles physical and chemical properties. It is shown that electrically charged NPs or fat-soluble (lipophilic) NPs are trapped in the mucus and transported back to the oral cavity by the cilia on the lung epithelial cells. NPs with a water soluble (hydrophilic) and/or neutral charge can more easily penetrate the mucus and reach the underlying cells. The size and form of the particles are important for their penetration through the mucus. Particles with a size below 500 nm have a possibility to penetrate the mucus, and the smaller particles penetrate more easily. Materials with an elongated shape, like carbon nanotubes, have not been shown to penetrate the mucus. Nanoparticles that are transported down to the alveoli will first encounter a thin liquid amphipathic (both lipophilic and hydrophilic) film termed surfactant. The function of surfactants is to reduce the surface tension and thereby prevent the alveoli from collapsing during expiration. This is accomplished due to the amphiphilic nature of surfactant molecules, which are composed of lipids (90 %) and proteins (10 %). Studies have shown that the NP with a lipophilic surface to a larger extent remains in the surfactant film, which may affect the surfactants' ability to reduce the surface tension. Independent of the property of the NPs surface, it has been shown that both fatty acids and proteins bind to the surface of the NP. This corona of biomolecules covering the surface of the NPs promote the uptake of phagocytic white blood cells having the function to "clean up" particles and microbes in the inhaled air.

To date, there are only few studies that have shown an association between systemic effects and the biomolecules that covers the surface of the NP. In several of these studies, the NPs have been administered in a solution which does not reflect a realistic situation for NP inhalation. These results suggest, however, that small neutral, negatively charged or polar NP (<6 nm) can cross the air-blood barrier, and enter the blood and hence reach other organs. Systemic uptake of NPs may lead to systemic effects both in blood circulation and in tissues. The fraction of NPs that cross the air-blood barrier has been found to be very low. However, there are studies showing that NPs with properties like; neutral, negatively charged or polar and with a size that is less than 34 nm can reach the blood circulation via lymph nodes draining the lungs. Neutral NPs that is less than 6 nm has been shown to be excreted by the kidneys. These studies

suggest that NPs that are larger than 6 nm and less than 34 nm pose the greatest risk to be distributed systemically to the blood circulation and tissues.

Studies of NPs in blood have shown that the NPs can affect the biological mechanisms associated with autoimmune diseases, cardiovascular diseases and cancer. For example, some studies have shown that NPs are able to induce blood coagulation and secretion of mediators for immune activation and inflammation.

In conclusion, there are suspected relationships between biomolecules that bind to their surface of NPs and adverse systemic effects. However, the origin mechanisms to these health effects is not clear and needs further investigation.

Sammanfattning

Nanopartiklar (NP) som inhaleras och deponeras i luftvägarna binder spontant till kroppsegna biomolekyler som finns i den vätskefilm som täcker cellerna i lungorna. Denna beklädnad kan leda till helt nya ytegenskaper som potentiellt kan påverka partiklarnas biotillgänglighet och interaktioner med biologisk vävnad. Under senare år har kunskapen om NPs interaktioner med biomolekyler i blodet ökat snabbt och flera studier har pekat på att sådana interaktioner skulle kunna leda till systemiska effekter på exempelvis immunsystemet och blodcirkulationen.

De övre luftvägarna har som uppgift att rena inandningsluften på sin väg ner till lungblåsorna där själva gasutbytet äger rum. Detta möjliggörs genom att cellerna i övre luftvägarna är täckta av en slemhinna (mukus) som har till funktion att fånga upp så mycket partiklar och mikrober som möjligt. Interaktionen mellan slemhinnan och inhalerade partiklar kan variera beroende på partiklarnas fysikaliska och kemiska egenskaper. Det är visat att elektriskt laddade NP eller fettlösliga (lipofila) NP fastnar i mukus och därmed effektivt transporteras tillbaka till munhålan med hjälp av flimmerhåren på lungepitel cellerna. NP med en vattenlöslig (hydrofil) och/eller neutral laddning har lättare att ta sig igenom mukus och därmed nå underliggande celler. Penetrationen av partiklar genom mukus är också storleks- och formberoende. Partiklar under 500 nm har möjlighet att ta sig igenom, ju mindre desto lättare. Långa material såsom kolnanorör har svårt att penetrera mukus.

Nanopartiklar som tar sig ner till lungblåsorna kommer först i kontakt med en tunn amfifil (både lipofil och hydrofil) vätskefilm (surfaktant), vilken har som funktion att minska ytspänningen vid in- och utandning. Surfaktanter består till 90 % av fettsyror och till 10 procent av proteiner. Studier har visat att NP med en lipofil yta i större utsträckning stannar kvar i surfaktant-filmen, vilket kan påverka surfaktantens förmåga att minska ytspänningen. Oavsett ytegenskap har det visats att både fettsyror och proteiner binder till ytan av NP. Denna nya beklädning av biomolekyler främjar upptaget i fagocyterande vita blodkroppar, vars uppgift är att ”städa upp” partiklar och mikrober som kommer med inandningsluften.

Hittills har endast ett fåtal studier visat på systemiska effekter som är relaterade till biomolekyler som täcker ytan på NP när de kommer i kontakt med mukus och surfaktant. I flera av dessa studier har man emellertid administrerat NP i lösning vilket inte återspeglar en realistisk situation för inandning av NP. Dessa resultat antyder dock att små neutrala, negativt laddade eller polära NP (< 6 nm) kan ta sig över luft-blod barriären och nå blodbanan, och därmed också andra organ. Detta kan leda till systemiska effekter i såväl blodcirkulationen som i de organ där partiklar deponeras,. Andelen NP som tar sig över luft-blod barriären har emellertid visat sig vara liten. Däremot finns det resultat som pekar på att NP som är neutrala, negativt laddade eller polära, samt mindre än 34 nm, kan ta sig ut i blodet via de lymkörtlar som dränerar lungorna. Neutrala NP som är mindre än 6 nm utsöndras via njurarna. Det antyder att NP med en storlek större än 6 nm och mindre än 34 nm utgöra den största risken för att spridas systemiskt till andra organ.

Studier gjorda på blod har visat att NP kan påverka biologiska mekanismer som har associerats med autoimmuna sjukdomar, hjärt-kärlsjukdomar och cancer. Som exempel kan nämnas studier som visat att NP kan påverka vita blodkroppar att skicka signaler som aktiverar immunsystemet och starta inflammationsreaktioner.

Sammanfattningsvis finns misstänkta samband mellan negativa systemiska effekter av NP och de biomolekyler som binder till dess yta när de interagerar med mukus och surfaktant. Mekanismerna för uppkomsten av sådan hälsoeffekter är emellertid inte klarlagda och behöver utredas vidare.

1 Background

Nanotechnology is an important and promising field that is contributing to social and economic development, as well as to the improvement of environmental and human health [1, 2]. The production and use of new products based on nanomaterials (NMs) are increasing. When a material is reduced to the nanoscale, unique physicochemical properties appear, which enables new technical applications and functions. Due to these unique physicochemical properties, such as size, surface area, and quantum phenomena, NMs interact with ther environment differently than the same material in bulk [3-5]. Nanosafety and nanorisk analyses have evolved as a result of the increased use and production of NMs and the health effects of NM exposure have been evaluated. The highest risk of being unintentionally exposed to synthetically manufactured NMs is through inhalation in occupational settings related to NM production. Moreover, people may come in contact with synthetically manufactured NMs via inhalation when using sprays containing NMs, via gastrointestinal exposure from food, via the airways by swallowing the mucus from mucuciliary clearance, or via dermal exposure when using skin products containing NMs. Dermal uptake is not considered a significant route of entry, unless there are open wounds or the skin is otherwise defective, e.g. due to disease. Inhalation exposure is generally considered the easiest route for NMs to enter the circulatory system. The lung has the largest surface area being in direct contact with the surrounding environment, and with the short distance across the air-blood-barrier, there is a high theoretical risk of NMs migrating from the inhaled air to the blood circulation [6, 7]. Several factors affect whether NMs enter the circulation system via inhalation: i) particle deposition in the airways, ii) clearance of NMs in the airways, iii) solubility of the particle, and iv) physical and chemical properties of the NMs. The NMs bioavailability in the body depends on their possibility to translocate through the air-blood-barrier [8]. Knowing what physical and chemical properties of the NMs that determine particle deposition and which initial interactions take place between the surface and biomolecules in the fluids facilitates predicting the particle clearance and translocation in biological systems [9-12].

2 Objectives and methods

This study aims to compile current knowledge on how the physical and chemical properties of inhaled NMs determine the kind of NM-biomolecule surface complexes, or biocoronas, that are formed when NMs encounter the respiratory lining fluid (RLF). Additionally, this article summarizes data on how the properties the NM-biomolecule biocorona determine the fate of NMs related to cellular uptake and clearance from the airways. From a nanosafety perspective it is essential to understand the properties of both the NM in its initial state and the NM-biomolecule complex that is formed in the RLF. This study is based on our own current research in the field, as well as on literature available in online databases (Web of Science, Scopus, PubMed). Articles related to nanomedicine have not been included in the literature survey. Similarly, *in vitro* studies on lung epithelial cells performed in cell media are not included, except in areas where no other information is available.

3 Characteristics of manufactured nanomaterials

Nanomaterial refers to a general term for nano-objects with different shapes and forms. A NM is defined 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”. According to the EU commissions´s recommended definition [13].

NMs can be manufactured in many different forms, such as spheres, wires, sheets, tubes, rods, fibers, etc. Thin films of materials, for example, usually manufactured in large quantities in semiconducting and engineering industries, are classified as nanomaterials. This is a cause of concern from a nanosafety perspective due to the potential release of NPs as a result of these nm-thick films being decomposed. The morphology, structure, and surface of NPs are important properties that affect the fate of the particles and their potential to induce toxicity within the body. The Organization for Economic Co-operation and Development (OECD) has produced guidelines for relevant NM metrics that should be used when evaluating nanosafety. The guidelines state that “characteristics requiring determination might include (but are not limited to): particle size, size distribution, aggregation, agglomeration state, shape, chemical composition, surface area, surface chemistry, dissociation constant, crystal structure, surface charge, zeta potential, Hamaker constant, interfacial tension, and porosity” [14]; this list needs to be continuously scrutinized and updated due to the rapid development of the field. The novelty of NMs is that they exhibit physical and chemical properties that are different to those of the very same material in bulk, a difference that sometimes cannot be predicted based on their known macroscopic properties. Properties of NMs should be measured by methods validated for NMs.

4 The respiratory tract –adapted to its challenges

The respiratory tract can be divided into the upper respiratory region (nasal airway, pharynx and larynx), the lower respiratory region (trachea and bronchi) and the alveolar region (Figure 1). Inhalation of dust and microbes occurs with every breath and thus humans have a sophisticated host defense mechanisms that has developed through evolution. The RLF plays an important role in the host defense system; the RLF is composed of two structures in the airways, with mucus in the proximal part of the airways, and surfactants in the distal part of the airways [15]. The mucus and the surfactants are composed of different biomolecules, specifically adapted for their respective functions. When NMs encounter the RLF they immediately interact with these endogenous biomolecules to form a new surface, a so-called biomolecule corona [16-20]. The following sections give an overview of the physiology of the respiratory tract and describe NM properties that affect their fate of the particles inside the respiratory tract.

5 Deposition of NMs in the lung and its relation the NMs properties

Upon inhalation, particle deposition in the lung may occur through five different mechanisms: sedimentation (gravity), inertial impaction, interception (NM-surface contact), electrostatic deposition, and diffusion. These mechanisms generally occur in different regions of the respiratory tract [21, 22]. The extent of NM deposition in the lung is determined by the physicochemical properties of the NMs, such as size, shape, density, and surface chemistry [23]. Breathing conditions, like ventilation rate, mouth or nose breathing, and airway geometry are other factors that affect NM deposition [24]. The transportation of NMs into the lung can be explained by their aerodynamic diameter [25]; Materials with an aerodynamic diameter below 5 μm are predominantly deposited in the alveolar regions of the airways [26].

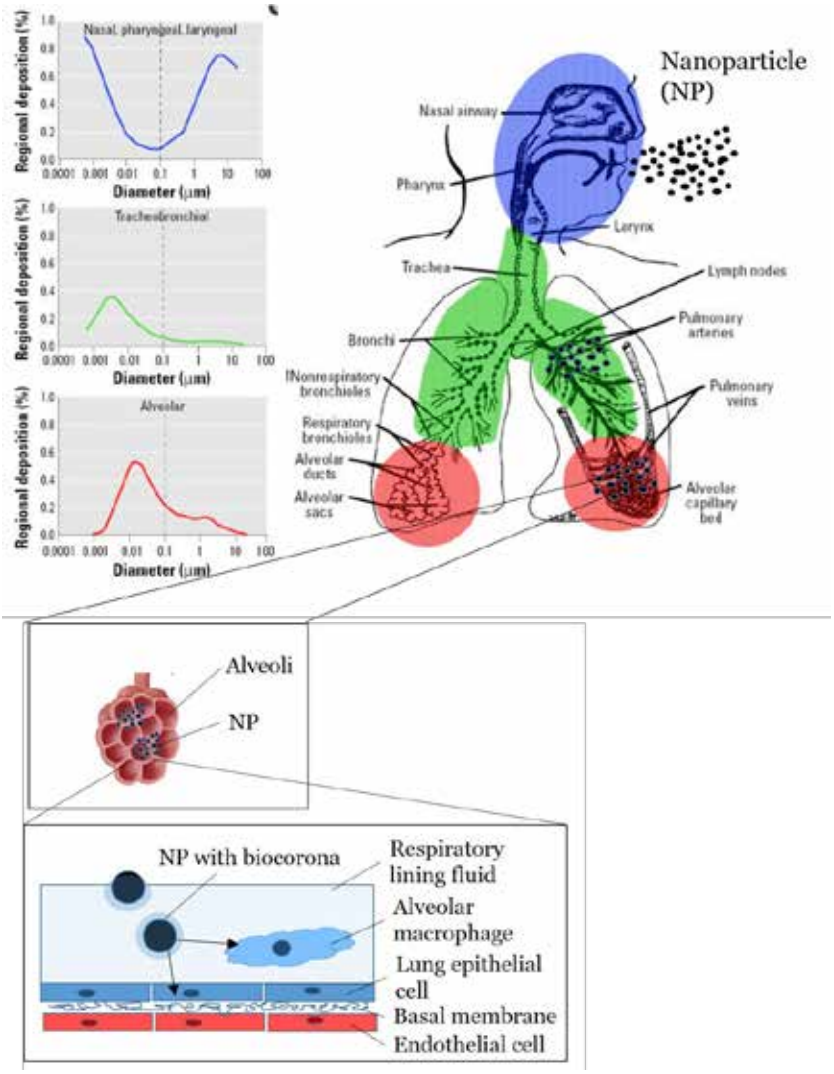

Figure 1. *A schematic representation of nanoparticle deposition and interaction with the respiratory lining fluid. The image is reproduced version from Oberdörster et. al., 2005. [26].*

NMs primarily deposit in the peripheral airways within the alveolar region [26, 27] (figure 1). The airflow in the alveoli is minimal and therefore the major deposition of NMs with a particle size of 10-100 nm predominantly occurs by diffusion and is more efficient compared to larger particles with a particle size between 0.1 and 1 μm [23]. It is rare for NMs to exist as single, isolated entities. The primary particles are attracted to each other due to strong electrostatic forces, as well as weaker van der Waals forces, which causes them to form larger agglomerates. The strength of the forces that keep an agglomarate together can be classified with the agglomeration parameter, *A*, which is found through $A = D/d$, where *D* and *d* are the agglomerate and the primary particle diameter, respectively [28]. The agglomeration parameter has been shown to influence the NMs bioavailability [29]. Hard agglomerates, attracted with strong forces, are called aggregates but the literature is often not consistent with the nomenclature and the term aggregate is sometimes also used for soft agglomerates (weak forces). In this article the term agglomerate is used for any NP agglomerate where there are no data placing it in either the hard or soft agglomerate category. The propensity for NM agglomeration in air or in non-biological fluids is affected by the NM's shape, structure, surface charge, acid-base properties, and also by the molecules adsorbed onto its surfaces. Upon inhalation, the agglomeration state and the specific physicochemical properties of the NMs determine the deposition pattern within the respiratory tract [23, 27].

The shape of the NMs also affects the aerodynamic actions, and thus the lung deposition. For long fibers and wires the aerodynamic size may be many times their diameter. Long fibers and wires predominantly deposit in the upper airways due to interception with the surface of the airways [23].

6 Properties of nanomaterials affecting particle interaction

The first medium that NMs encounter following inhalation is the RLF. NM characteristics that determine the interaction with the biological components in the RLF are: size, shape, and chemical properties on the surface [8]. NPs have a large surface-to-volume ratio compared to larger, micrometer-sized particles. For example, in a 1 cm^3 cube only about 10^{-5} % of the cube's atoms are on the surface, compared to 10% when dividing the 1 cm^3 cube into many 10 nm^3 cubes [30]. Due to high free energy at the surface of NMs and because of the lower atomic coordination compared to their larger counterparts, there is incomplete chemical interactions at the surface. In order to reduce the free energy at the surface, the NM adsorbs biomolecules present in the biologic milieu and thus increases the dispersion of the nanomaterial in the milieu [10, 30]. When a NM adsorbs biomolecules a biomolecule corona is formed and this biomolecule corona modifies the properties of the NM in the biological fluid and affects its agglomeration properties. Surface properties, such as hydrophobicity/hydrophilicity, surface charge, acid-base properties, and particle size, all influence the formation of a corona of biomolecules covering the NM's surface. The composition of the hard corona is independent of the NM, however, the biologically active proteins that form the corona may change their properties depending on the surface characteristics of the NMs, which may result in altered biological impact (figure 2) [31].

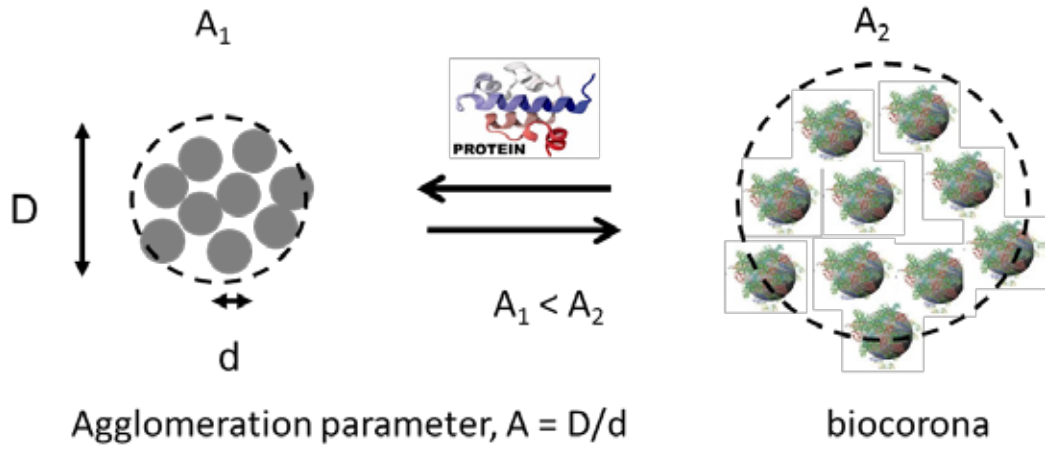

Figure. 2. *The agglomeration of nanoparticles is described by the agglomeration parameter, which distinguishes soft from hard agglomerates. Adsorption of biomolecules in the respiratory lining fluid can affect the inter-particle interaction by e.g. changing the nanoparticle surface charge and screening van der Waals bonding that weakens their interaction.*

7 Properties of nanomaterials affecting cellular uptake, particle clearance, and translocation

The modified physical and chemical properties that result from the interaction between the NP and the biomolecule determine the biological fate of the NM-biomolecule complex. As discussed previously, the primary particle size, shape, and agglomeration state all influence the fate of the NM; they affect the efficiency of particle clearance, the agglomerate dissociation, and the ability for cellular uptake of the NM and its translocation [9, 31]. The translocation of particles depends strongly on the agglomeration process and how it is affected by the NM's interaction with the lung fluids. Small differences in the surface properties of NMs may completely alter rheological properties to either promote or reduce agglomeration [9], and this may affect the capacity for clearance of particles by macrophages. For instance, it has previously been shown that NPs in the human lung are less efficiently cleared by phagocytosis than larger micron-sized particles [9, 32], which could enhance their bioavailability [9]. Another important factor is the NM concentration. Because agglomeration rates increase with increasing NM concentration (by Ostwald ripening), and if agglomeration promotes accelerated biological clearance, it is not certain that high concentrations of NMs are more toxic than low concentrations [9].

Clearance mechanisms in the airways include dissolution, mucociliary transport, and phagocytosis. The composition of the NM plays a decisive role in whether the material is able to dissolve in the biological fluids or not. When particles containing metals dissolve in the airways, the free ions could exert classic metal toxicity. Iron oxides, for example, have been shown to dissolve and release iron ions in the acid milieu found within the lysosomes in cells [10, 15]. Iron is essential for oxygen transportation in the blood but an excess of iron can lead to increased free radical production and cytotoxicity [16]. In contrast, TiO_2 is an ionic oxide and does not dissolve at the pH levels found in realistic biological environments; therefore it remains a stable particle in

the airways over a long period of time [17]. Particles with low solubility, such as TiO_2, are primarily removed from the airways by phagocytosis and mucociliary clearance. The agglomeration properties affect phagocytosis and thus particle clearance [27]. For instance, *in vitro* studies have demonstrated that the different crystal structures of TiO_2, namely anatase and rutile, affect the agglomeration parameter and determine their ability to be internalized into the cells [29]. In the anatase phase, TiO_2 NPs form soft agglomerates which easily dissociate and consequently are more easily internalized in cells, whereas rutile TiO_2 NPs form hard agglomerates which are not internalized to the same extent. The shape of particles also affects their degradation following internalization of macrophages. It has been shown that NMs with fiber-like structures are difficult for the macrophages to internalize, which results in a frustrated phagocytosis and a release of toxic mediators, inducing cellular stress to the surrounding environment [9, 23]. NMs shaped like sheets or platelets have also been shown to induce toxicity due to the incapacity of macrophages to internalize them [33].

8 Airways and mucus: functions and components

8.1 Surface epithelium in the trachea bronchiolar region

Gas exchange results in the inhalation of about 8500 L of air per person and per day during normal ventilation [34]. In addition to oxygen, inhaled air also contains environmental particles and microbes. The respiratory system has evolved excellent barriers to resist and fight undesirable intruders, making the air in the alveolar region almost sterile [35]. The upper and lower respiratory tracts consist of secretory and ciliated epithelial cells mixed in a mosaic pattern [36]. The secretory cells are further subdivided into Clara, goblet, and serous cells. These cells are regionally distributed in the airways, with the goblet and serous cells present in the upper respiratory tract, and the Clara cells present in the lower respiratory tract [37]. The secretory cells secrete mucins and several biomolecules with antimicrobial functions (e.g., β-defensins, lysozyme, IgA, lactoferrin); these biomolecules are part of the mucus covering the apical side of the epithelial cells [35, 36]. The ciliated epithelial cells clear the particles and pathogens that have been trapped in the mucus [36]. In the epithelium there are about 10^9 cilia per cm^2; they work in a synchronized manner as they beat 12-15 times per second, causing an upward movement of the mucus towards the pharynx (mucociliary clearance) where it is swallowed [35-37]. About 10 ml of mucus is estimated to reach the mouth each day in a healthy adult [37].

8.2 Mucus

In order to understand what makes certain NMs able to penetrate the mucus barrier we need to understand the physical and chemical properties of the mucus. The mucus is the first line of defense and functions as a robust barrier against foreign intruders, preventing them from reaching the underlying epithelial cells [38]. The mucus gel possesses physical viscoelastic properties which enable both flow (viscosity) and deformation (elasticity) [36, 39]. In a normal lung, 90-98% of the mucus consists of water and the remaining fraction consists of solids, such as glycoproteins (mucins), proteins, lipids, and inorganic salts [36, 40]. The thickness of the airway mucus varies

from 5 to 55 µm, with the thinnest areas in the bronchial region and increasing thickness higher up in the airways [40]. The mucus is divided in two layers: the upper and the underlying layer. The upper layer, closest to the lumen, is highly viscoelastic which facilitates its ability to stick to and trap inhaled foreign intruders, whereas the underlying layer, known as the periciliary liquid, is less viscoelastic which facilitates the movement of cilia in the mucus [38].

The viscoelastic features of mucus depend on the biomolecule composition in mucus, in particular the composition of mucins. Mucins are produced in the secretory cells and are stored in granules in dehydrated conditions. During the secretion the mucins swell one hundred times as a result of hydration and ionic strength [36, 39]. The mucins are composed of long glycoprotein chains, which are densely glycosylated with carbohydrate chains (1-20 sugars) [36, 38, 39]. The glycoproteins form long fibers (1-10 µm long, 3-10 nm in diameter) that are bundled and entangled with reversible linkages of both low affinity non-covalent interactions and stronger disulphide interactions (Fig. 3). The reversible linkages contribute to the viscoelastic properties of the mucus [39]. The mesh of mucin fibers forms pores up to 500 nm wide [36]. The chemical composition of mucins causes a net negative charge due to sialic acids and sulfate end groups of the carbohydrate chains. The negative net charge gives rise to a repulsive force between the carbohydrate chains which increases the stiffness of mucus (Figure 3) [39]. The central parts of the glycoproteins hold both hydrophobic and hydrophilic areas, where the protein core is hydrophobic and the glycosylated carbohydrates are highly hydrophilic [38]. Lipids present in the mucus will adsorb to hydrophobic sites on the mucins which further increases the viscoelastic properties of the mucus [38].

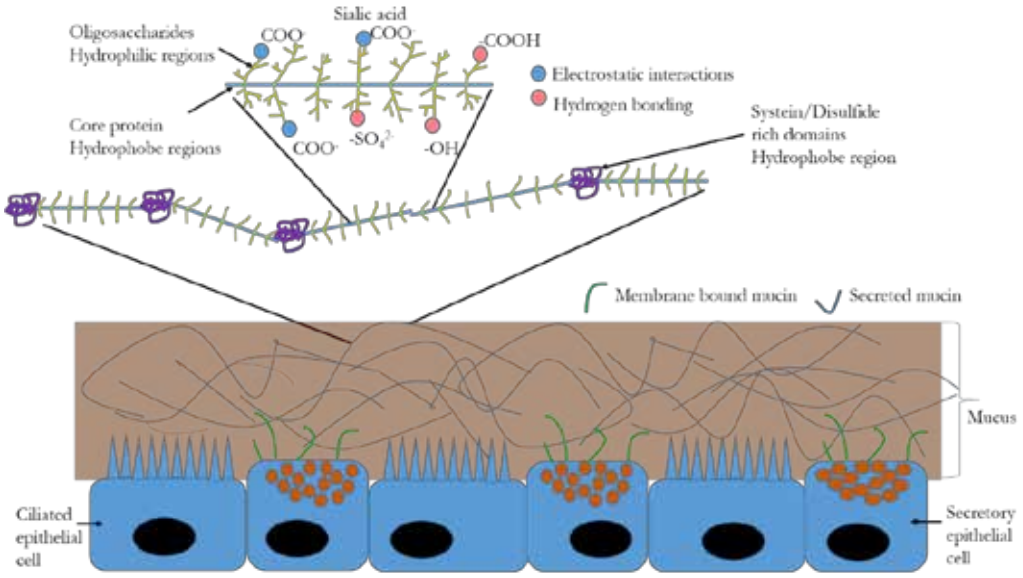

Figure. 3. *A schematic picture of lung epithelial cells, mucus, and the structure and physicochemical properties of glycoproteins.*

8.3 Nanoparticle interactions with mucus

Based on studies on exposure to cigarette smoke and exposure to air pollution it is known that particle exposure affects the function of mucus. Upon exposure to the particles in the irritant smoke the viscoelasticity of the mucus decrease, which in turn increases the mucociliary clearance due to increased secretion of mucin. On the other hand, a prolonged exposure to particles, as is also the case for cigarette smokers, the function of mucociliary clearance deteriorates [39].

To come in contact with the epithelial cells the NMs need to penetrate the mucus, meaning that the NMs must be able to move in the opposite direction of the flow of the mucus: the mucus is secreted by the epithelial cells and moves outwards to the lumen [38]. To penetrate the mucus the NMs must be small enough and have surface properties which allow them to escape steric hindrance and adhesion to the mucin mesh. Furthermore, they must penetrate the mucus fast enough in order to overcome the velocity of the mucus barrier, i.e. the forward-moving force must be larger than that exerted by the mucus barrier [40]. The physical and chemical properties of mucin allow for a variety of possible interactions with various inhaled intruders. This flexibility enables multiple weak interactions between mucins and many diverse surfaces. The alternating hydrophilic and hydrophobic regions in the mucins facilitate weak interactions at the surface of incoming particles [38]. However, since more than 90% of the mucus consists of water the penetration capability for particles with hydrophilic properties is enhanced [40]. It has, for example, been shown that a virus with a hydrophilic capsid easily penetrates the mucin mesh [36]. These weak interactions keep the NMs trapped in the mucus, furthermore, the weak interactions between mucin fibers enable the fibers to reorganize themselves and recoil, forming an elastic gel that enhances their capacity to interact with different surfaces of particles [38]. The weak interactions retain the NMs in the mucus while being moved up to the pharynx by the stirring cilia on the epithelial cells.

For soluble proteins to penetrate the mucus the surface needs to be densely coated with positive and negative charges at equal density, separated by as little as 0.5 nm [38]. These proteins with a neutral surface are neither attracted to, nor repelled by the negative charge in the oligosaccharide domain and penetrate the mucus [38]. NPs with a neutral surface also penetrate the mucus. The net negative charge of mucins allows only a selection of particles to penetrate the mucus. Particles with a net negative surface charge are not likely to penetrate the mucus due to the repulsion from the negative sites at the end of the oligosaccharide chains. Particles with a net positive charge will be attracted to the negatively charged sites on the glycoproteins and become trapped in mucus [38]. Both positively and negatively charged particles will be cleared by the mucociliary escalator (figure 4), thus, in order to efficiently penetrate the mucus, the surface of NMs should have a net neutral charge [38]. (Figure 4).

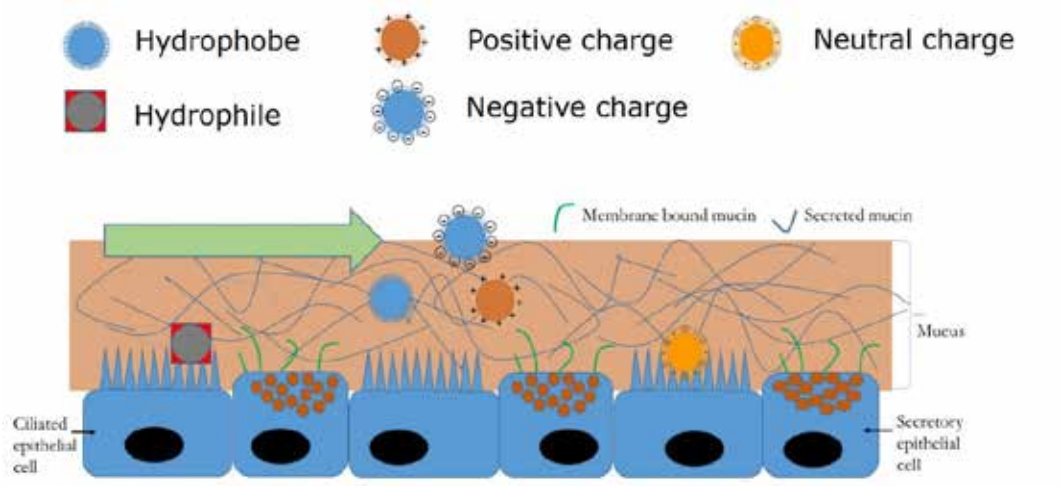

Figure 4. *A schematic presentation of how hydrophobicity/ hydrophilicity and surface charge affect particles' fate in the mucus of the airways. Hydrophilic particles with a net neutral surface charge will reach the lung epithelial cells to a larger extent than hydrophobic particles with a net negative or net positive surface charge, which to a larger extent will be trapped in mucus.*

Similar results have been reported in a study by Schuster et al. (2013), in which the movement in mucus was compared between two polystyrene (PS) particles: PS particles densely coated with polyethylene glycol (PS-PEG), and PS particles densely coated with carboxylate (PS-COOH). The PS-PEG particles have a neutral net charge while the PS-COOH particles are negatively charged. By measuring their motion in mucus they found that neutral PS-PEG, both at 100 nm and 200 nm, moved easily in the mucus, whereas the negative PS-COOH, also 100 nm and 200 nm in size, were observed to move less in the mucus. At a particle size of 500 nm neither PS-PEG nor PS-COOH moved in the mucus. The results from this study indicate that something prevents movement of the NPs The authors relate this inhibition of NPs movement to the mucin mesh, which functions as a net capturing inhaled particles dependent on their size [41].

Table 1 *Nanoparticle trajectories in the airway mucus, illustrated by their random movement within a circle during 1 sec. The picture is a reproduced version from Schuster et al. Biomaterials, 2013.* ***34****(13): p. 3439-46 [41].*

Particle (Size/charge)	Random movement within a circle in airway mucus during 1 sec. — 1 µm
PS-COOH 100 nm (negative)	
PS-PEG 100 nm (neutral)	
PS-COOH 200 nm (negative)	
PS-PEG 200 nm (neutral)	
PS-COOH 500 nm (negative)	
PS-PEG 500 nm (neutral)	

As described above, the mucus prevents penetration by foreign particles, both by steric hindrance due to the dense mucin mesh, and by direct interactions with mucus components. In a study by Jachak and colleagues the penetration of mucus by carbon nanotubes and three different metal oxides were analysed; the mucus was human cervical vaginal mucus (CVM), (table 2).

Table 2. *The physical dimension and zeta potential ( ) of some metal oxides and single-walled carbon nano tubes (SWCNT) used in the mucus penetration experiment by Jachak et. al., Nanotoxicology, 2012.* ***6****(6): p. 614-22 [42].*

Particle	Dimension measured	Vendor-lis diameter (	Dimension, suspended in PBS* (nm)	ζ-potential (r
Cerium (IV) oxide (C	Diameter	< 25	~ 495	- 16
Zink oxide (ZnO)	Diameter	< 50	~ 114	- 9
Zirconium (IV) dioxid (ZrO2)	Diameter	< 100	~ 505	- 23
Single-walled carbon nanotubes (SWCNT)	Length		~ 76	- 9
Single-walled carbon nanotubes (SWCNT)	Length		~ 210	- 10

* Phosphate-buffered saline

The study showed that size, shape, and surface charge affect mucus penetration. The NP with the smallest size and lowest surface charge in PBS, ZnO, was most efficient at penetrating the mucus, although only in low quantities. It was concluded that the tested NMs probably adhered to the mucin by electrostatic attraction, as well as by hydrogen and hydrophobic interactions, rather than being trapped by steric hindrance. However, the low zeta-potential of the NMs suggests that they were all agglomerated, forming much larger particles, thus, further studies are required to evaluate the size dependency in this study. The authors also performed theoretical calculations and found that very low amounts of ZnO (~ 2%) would penetrate 10 µm thick mucus during one hour. Negligible fractions of CeO_2 and ZrO_2, and virtually no SWCNT would penetrate the same mucus according to the calculations [42]. Carbon-based NMs have a hydrophobic surface, thus the SWCNT is expected to interact with the hydrophobic entities of the mucin [43].

9 Alveolous and surfactants: functions and components

9.1 Surface epithelium in the alveolar region

The airways end in the large distal alveolar region responsible for the vital gas exchange of oxygen and carbon dioxide [44]. The fine-tuned communication between the alveoli and the dense net of capillaries that wrap around them is essential for ventilation. The surface of the alveoli are primarily (95 %) composed of the thin type I alveolar epithelial cells, which are specialized for gas exchange. The epithelial type II alveolar cells are large cuboidal cells that produce and secrete surfactants that cover the surface of the alveoli. As a part of the host defense system antigenic-presenting cells, such as alveolar macrophages and dendritic cells, are present at the surface of the alveolar epithelial cells with around 12-14 alveolar macrophages per alveolus [45-47]. The

alveolar macrophages clear inhaled particles either by destroying them intracellularly or by migrating to ciliated airways where the macrophages are cleared by mucociliary clearance [47].

9.2 Surfactants in the alveolar region

Surfactants form a liquid film that covers the surface of alveolar epithelial cells. This liquid film consists of an aqueous hypophase and a surface-active lipid-protein mixture [48]. The function of surfactants is to reduce the surface tension and thereby prevent the alveoli from collapsing during expiration [49], and this is accomplished due to molecules with amphipathic properties [44, 50]. The surfactant molecules are composed of lipids (90 %) and proteins (10 %) [44, 51]. The most commonly found surfactant lipids are: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG), sphingomyelin (SM), and cholesterol (CL), (figure 5) [7].

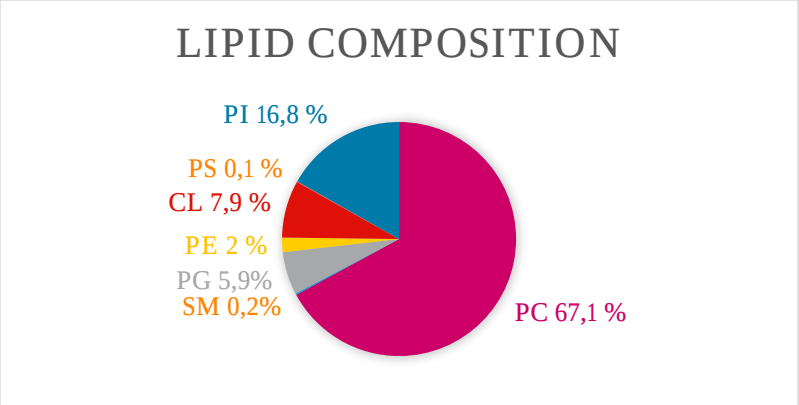

Figure 5. *Lipid composition of lung surfactant: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) phosphatidylglycerol (PG), sphingomyelin (SM) and cholesterol (CL), [52]*

About half of the proteins in surfactants are made up of the four surfactant proteins: SP-A, SP-B, SP-C and SP-D [52]. The proteins SP-A and SP-D are hydrophilic while SP-B and SP-C are hydrophobic. The most abundant phospholipid in surfactants is the dipalmitoylphosphatidylcholine (DPPC), which is a zwitterionic PC, and the second most abundant phospholipid is the negatively charged PG [53]; overall the phospholipid membrane is negatively charged [54]. DPPC has properties which cause lowered surface tension and it can withstand high surface pressure, however its capacity for re-spreading is low [53, 55]. The re-spreading of a surface film is enhanced and promoted by the presence of the negatively charged PG, unsaturated phospholipids, and the hydrophobic surfactant proteins SP-B and SP-C; together they form a mosaic film with lipid-protein components that can manage the biophysical forces that occur during the compression and expansion cycles of ventilation [15, 27, 48, 53]. A study of proteins in porcine lung lavage has determined what proteins, other than the surfactant proteins, that are the most abundant in the porcine lung (table 3). The proteins were analyzed with label-free shotgun proteomics. The surfactant protein SP-C could not be detected using this approach, indicating that this analysis not detect all proteins [52].

***Table 3**. A list of the most abundant proteins, other than SP A-D, that have been found in porcine lung surfactant, using a proteomic approach.*

Native proteins found in porcine lung surfactant
serum albumin
sodium-dependent phosphate transport protein 2B
tubulin alpha-4A chain
fibronectin
myosin-9
deleted in malignant brain tumors 1 protein
complement C5
actin, cytoplasmic 1
complement C3
Ig alpha-1 chain C region
haemoglobin subunit beta
I-xylulose reductase
tubulin beta-4B chain
tubulin alpha-1A chain
calcium-activated chloride channel regulator 1
polymeric immunoglobulin receptor
AP-2 complex subunit beta
serotransferrin

Four of the proteins that were found in the porcine lung surfactant were also found in the porcine plasma: serum albumin, complement C3, Ig alpha-1 chain C region, and serotransferrin. Their presence in the lung surfactant may be explained by small ruptures of the alveoli during the lung lavage [52]. In addition to identifying the proteins in the porcine lung surfactant, the authors classified them according to their biological functions. Among other functions they found proteins associated with binding and catalytic activity; which implies that the proteome of lung surfactant is interacting with cells (e.g. macrophages) and intruding pathogens. The proteome profile found in the porcine serum was mostly associated with antioxidant and enzyme regulator activity [52].

The morphological structure of surfactants is divided into two subfractions: the surface-active fraction which consists of tubular myelin and lamellar bodies (the hydrophobic lipid layer), and the underlining layer which consists of unilamellar vesicles [27]. There are only three cell layers that separate the air from the vascular bed in the alveoli: the lung epithelial type 1 cell, the basal membrane, and the endothelial cell, which together measure 0.1 μm [56]. A schematic presentation of the air-blood barrier is presented in figure 6.

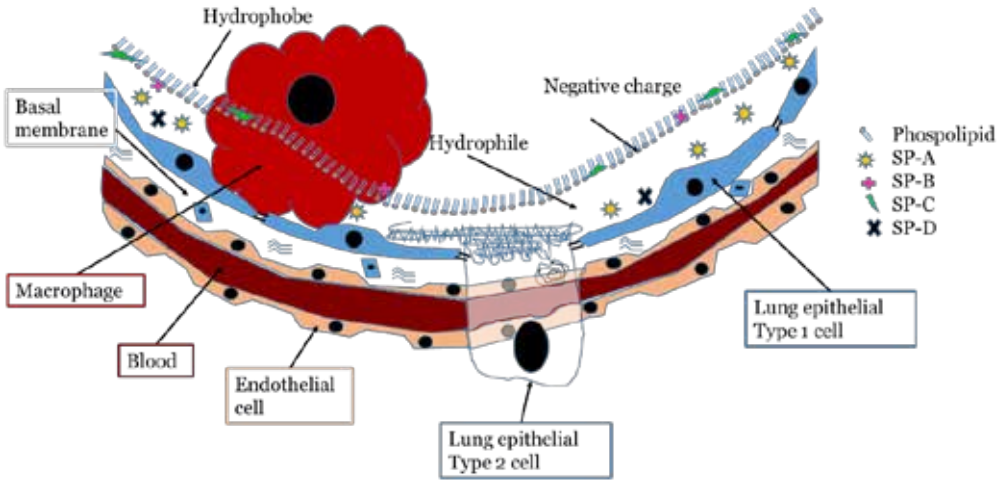

Figure 6*. A schematic presentation of the air-blood barrier in the alveolar region of the lung. The ratio between the components is not to scale. The image is reproduced from Murgia et al. 2014, Eur. J. Nanomed. 2014; 6(3): 157–169.*

SP-A and SP-D belong to the collectin protein family and they are part of the innate immune system; they bind to the surface of inhaled intruders (opsonization) and thus facilitate the recognition of the intruders by alveolar macrophages [47-49]. Both SP-A and SP-D contain a carbohydrate recognition domain (CRD), which enables them to associate with various pathogens with carbohydrate surfaces, however, the proteins differ in their interactions with biological structures. SP-A has a high affinity for phospholipids and lipophilic patterns, whereas SP-D has a high affinity for strong hydrophilic structures.

9.3 Nanoparticle interactions with alveolar surfactant

Interactions between solid surfaces, such as NPs and surfactant, are generally complex. As a NP encounters a surfactant the nature of the interaction depends on the properties of the solid NP and the surfactant. The interactions between NPs and surfactants may occur through van der Waals interactions, electrostatic forces, and dispersion interaction [57].

In order for NPs to come in contact with the cell membrane in the epithelium they first need to diffuse through the lipids at the air-liquid interface of the surfactant and thereafter move through the aquatic phase between the lipids and the lung epithelial cells. The interaction between NPs and lipids and the penetration through the lipid layer depend on the physicochemical properties of the NPs, which modify the surface tension of the lipids [57]. The NP biocorona may further increase the interaction with the liquid, promoting the transport of the NPs to the cell membrane [52].

9.3.1 Nanoparticle morphology: size and shape

NPs may affect the air ventilation by disturbing the re-spreading and the adsorption of surfactants (biophysical function) at the air-liquid interface. *In vitro* studies have demonstrated that particles' impact on pulmonary surfactant function depends on particle size. Schleh and colleagues used pulmonary surfactant (Curosurf, natural porcine surfactant) *in vitro* and showed that nanosized TiO_2 induced biophysical

function, as well as structural alterations of the surfactant layer, to a larger extent than micron-sized TiO_2 particles [58]. The mono lipid membrane of surfactant re-spreads through hydrophobic interactions affecting the surface tension and curvature energy of the particle surface. In an *in vitro* model where they examined the interactions between NPs and lipid membranes, silica NPs were used together with the lipid membrane 1.2-Dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPC), a phospholipid similar to those in cell membranes and vesicles. The study showed that silica NPs with a dimension greater than 22 nm were mostly covered by a lipid bilayer, unless the surface was very rough and irregular. The smaller silica NPs (1.2 nm and 22 nm) remained almost uncovered by lipids and passed through the lipid membrane through pores. The study showed that the dimension and shape of a NP are decisive in how much the lipid membrane will spread over the NP surface [59].

When using an artificial surfactant, composed of DPPC, palmitoyl-oleoyl-phosphatidylglycerol (POPG), and SP-B (70:30:1), Bakshi and colleges showed that gold NPs, with a primary particle size of 15 nm, were coated with the surfactant film and that it resulted in increased surface tension, measured at ~23 mN/m. Increased surface tension may result in diminished pulmonary function [60]. Braydich-Stolle and colleagues studied the effects of silver NPs (25 nm) coated with either hydrocarbon (HC) or polysaccharide (PS) within the alveolar and lysosomal fluid. Both the HC and the PS silver NPs formed extensive agglomerates in the alveolar fluid as well as in the lysosomal fluid. The PS-coated silver NPs demonstrated a loss of coating following dispersion in the alveolar and lysosomal fluid. Regardless of coating, the aggregation pattern and the morphology were altered [61].

With a computer simulation model it has been shown that the penetration of NPs through a DPPC monolayer varies during pulmonary ventilation and that it is dependent on both the NP shape and the hydrophobicity/hydrophilicity of the particle [62]. NPs with three different shapes (rod, barrel, and disk-shape) were analyzed with either a hydrophilic or a hydrophobic surface. During inspiration (surfactant film expansion) the hydrophobic NPs immerse in the hydrophobic tails of the DPPC layer, whereas the hydrophilic NPs immerse in the hydrophilic head groups of DPPC. At inspiration there was no observed structural disruption, regardless of the shape or hydrophobicity/hydrophilicity of the NP. In contrast, during the expiration (surfactant film compression), the DPPC monolayer became very dense and no hydrophobic NPs, regardless of shape, could penetrate the DPPC layer. The model showed that the shape of the particles is of importance for the structural disruption of the DPPC monolayer, with different penetration abilities for different NP shapes. The hydrophilic NPs with a rod-like shape demonstrated the largest penetration ability and caused the least damage to the DPPC monolayer. The authors concluded that the rod shape has the smallest surface area and thus the least contact with the DPPC layer. Among the hydrophilic NPs, the disk-shaped NPs showed the lowest penetration ability whereas the hydrophobic NPs did not penetrate the DPPC monolayer independent of their shape [62]. The authors concluded that the particle shape affects the capillary forces that act on the particle at the air-liquid interface as well as its rotation and orientation abilities [62]. Additionally, the shape of the material is a decisive factor for accumulation at a low floatability. Angular particles promote thinning and rupturing of the air-liquid

interface more than round particles, and therefore angular particles accumulate more easily.

Different shapes of carbon NMs affect the surfactant film differently. The carbon NPs (fullerenes, C60), carbon nanotubes, and nano sheets (graphene) all have a hydrophobic surface and therefore interact with the lipid monolayer and affect the biophysical function of ventilation [63], [64, 65]. Notably, the graphene has been found to induce pores in the surfactant film, resulting in an increased compressibility of the surfactant film, which inhibits the functions of the surfactant [64].

9.3.2 Nanoparticle surface

Chemical properties of the NP surface are of great importance for the biophysical functions in the lung; this has been demonstrated in artificial systems where both surface charge (ζ-potential) and hydrophobicity were shown to inhibit the lung surfactant's ability to decrease the surface tension during the cycle of compression and expansion [66]. Surface properties of NPs, such as hydrophilicity/hydrophobicity and surface charge, are important factors for biomolecule adsorption and particle translocation across the RLF [43].

In an *in vitro* system set up with Infasurf (a modified surfactant prepared from newborn calves) it has been shown that the biophysical function of the surfactant was affected both by hydrophobic polystyrene NPs and by hydrophilic hydroxyapatite NPs; these NPs are both negatively charged at neutral pH and of similar size (~ 90 nm). The hydrophobic particles induced a much faster inhibition of the biophysical function, which indicates that there are different mechanisms of interaction between the surfactant and the surface of hydrophobic particles and between the surfactant and the surface of the hydrophilic particles. Hydrophilic NPs seem to quickly penetrate the monolayer whereas the hydrophobic polystyrene NPs are trapped within the monolayer of Infasurf [43]. In an *in vitro* study, performed with spherical hydroxyapatite NPs (~90 nm) in Infasurf, it was shown that NPs inhibited the biophysical function of ventilation at low NP concentration. The authors found that this biophysical deterioration was due to adsorption of SP-B and SP-C onto the surface of the NPs, rather than the NPs' interference with the surfactant film at the air-liquid interface. SP-B and SP-C are important for the re-spreading of phospholipids, and a depletion of these proteins results in transforming large phospholipid vesicles to small vesicles, which inhibits the surface activity and hence the biophysical function [67]. A computer-simulated model has shown that SP-B but not SP-C adsorbs onto negative NPs, furthermore, negative NPs with a hydrophilic surface pull the adsorbed SP-B out of the interfacial surfactant monolayer and down to the aqueous sub phase [43]. Such a reduction of SP-B at the air-liquid interface may have an impact on the respiratory function. In mice a 25 % reduction of SP-B in the lung surfactant induces fatal respiratory failure [68]. In contrast to the negative and hydrophilic NPs, negative NPs with a hydrophobic surface are trapped in the phospholipid monolayer and do not pull SP-B out of the interfacial surfactant layer; SP-B is however in close contact with the negatively charged NPs. With positive or neutral NPs SP-B remains within the phospholipid monolayer [43]. Studies performed in artificial RLF (Infasurf) indicate that hydrophobic NPs are

retained in the phospholipid monolayer and therefore inhibit the biophysical function of the surfactant [66].

Ruge and colleagues performed an *in vitro* study of the interactions between NPs and surfactant proteins SP-A and SP-D. Negatively charged magnetite particles, with either starch (ST-mNP / hydrophilic /~150 nm) or phosphatidylcholine (PL-mNP / hydrophobic /~120 nm), were studied and it was observed that SP-A binds to the hydrophobic PL-mNP, whereas SP-D to a larger extent adsorbs onto the hydrophilic ST-mNP [48]. SP-A is more hydrophobic than SP-D and therefore it binds more easily to lipophilic compounds, whereas SP-D rather binds to polar substrates [69]. The adsorption of SP-A and SP-D depends on the hydrophilic and lipophilic properties of the NP surface. Because of the diverse adsorption properties of SP-A and SP-D, the possibility of binding to different biological structures increases. The binding of SP-A or SP-D to NPs enhances endocytic ingestion by alveolar macrophages. In the study of SP-A and SP-D an elevated internalization of PL-mNP by macrophages was observed, indicating that the components in surfactant coats the surface and provide a more rapid clearance of NPs [48]. In a study by Harishchandra and colleagues an artificial RLF with lipids and SP-C was used to study the interactions of the lung lining fluid with amorphous silica NPs (AmorSil20); the AmorSil20 NPs are hydrophobic and 22 nm in diameter. They found that the NPs interact with the artificial lung lining fluid at the air-liquid interface. In addition, they found that the NPs disturb the surface structure as well as penetrate the artificial lung lining fluid, reaching the underlying alveolar epithelial cells [55].

Carbon-based nanomaterials are hydrophobic and may be trapped in the phospholipid monolayer and retained there over a long period of time, which may affect biophysical function [43]. In a study performed by Kapralov and colleagues it was shown that SP-A, SP-B, SP-D, phospholipids phosphatidylcholine (PC), and phosphatidylglycerol (PG) all had adsorbed onto the surface of single-walled carbon nanotubes (SWCNTs) which were administrated to the airways of mice. These interactions between the lipids and the SWCNTs were further investigated by computer modeling, which indicates that the hydrophobic alkyl chains of the phospholipids are adsorbed onto the surface of SWCNT, while the hydrophilic polar head groups of phospholipids are pointed into the aqueous phase. The same authors also presented data on *in vitro* tests where they show that SWCNTs, coated with biomolecules from surfactants, exhibit enhanced internalization by macrophages compared to uncoated SWCNTs [70]. Raesch et al. conducted an extensive investigation to determine the lipid and protein composition of coronas on NPs with a magnetite core [52]; the results were evaluated for three different NP coatings: phosphatidylcholine coating (lipid-NP), PEG5000 coating (PEG-NP), and poly(lactic-co-glycolicacid) coating (PLGA-NP). The study was performed using native porcine pulmonary surfactant from lung-lavaged pigs. Raesch and colleagues found that the amount of lipids per surface area significantly differed between the NPs with different coatings. The smallest amount of lipids (0.12 mg/m^2) was adsorbed onto the hydrophobic PEG-NPs and the lipid-NPs adsorbed the highest amount of lipids (0.61 mg/m^2); the PLGA-NPs had a surface coating of 0.38 mg/m^2. The relative distribution of lipids in the corona of the NPs in this investigation is presented in figure 7. All NP coronas preferably bound to lipids with a short carbon chain length (30 and 32 carbon

chain length); this is potentially explained by the higher fluidity of the shorter carbon chain lipids, making the interaction with the NP surface more dynamic. Although the amount of lipids on the NP surface differed between the hydrophobic PEG-NPs and the lipid-NPs, their surface coating was composed of the same types of lipid. As much as 90 % of the lipids at the NP surface is made up of the same ten lipids. These data indicate that the lipids mask the surface independent of the surface hydrophobicity/hydrophilicity of the NP; these results indicate that the surface corona might be of importance for further interactions within the lung [52]. The authors assume that the strength of the interactions between the lipids and the PEG-NPs, PLGA-NPs, and lipid-NPs must be different and possibly associated with the proteins present in the surfactant.

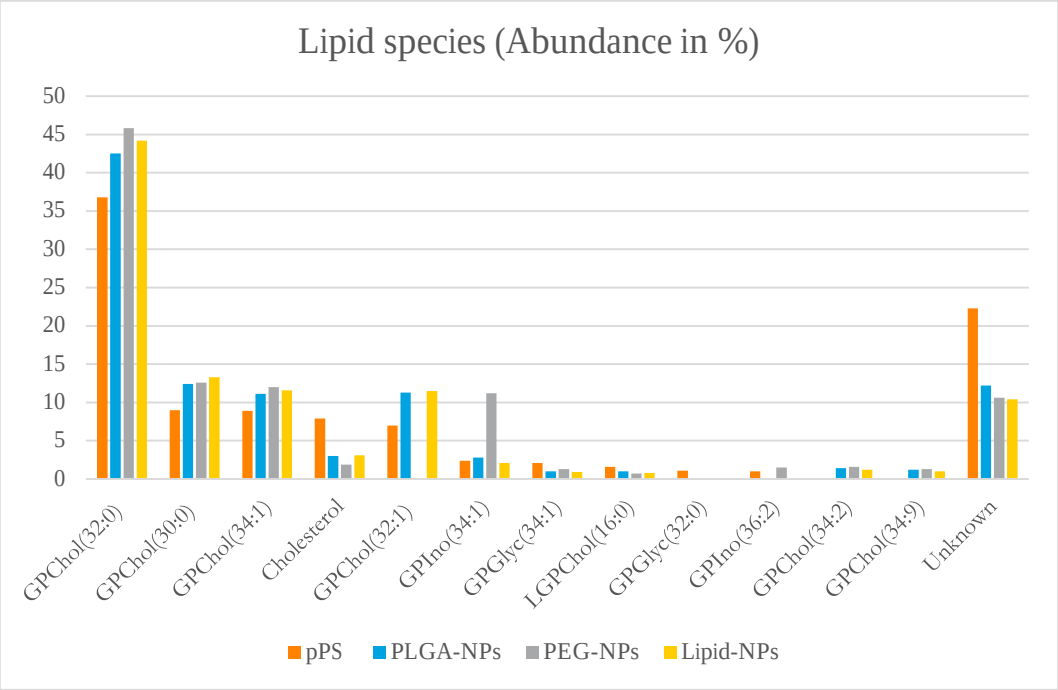

Figure 7. *A graph of the abundance (%) of lipid species on the surface of different NPs with different surface chemistry: pPS (pristine NPs), PLGA NPs (poly(lactic-co-glycolicacid)), PEG NP (PEG5000-coating), lipid NP (phosphatidylcholine-coating), GPChol (phosphatidylcholine), GPIno (phosphatidylinositol), and GPGlyc (phosphatidylglycerol). After each NP name in the graph there are two numbers, the first of which refers to the number or carbons in the chain and the second of which refers to the number of double bonds of the phospholipids.*

Raesch and colleagues performed a comprehensive investigation of the proteins present on the surface of a few NPs: PEG NPs, PLGA NPs, and lipid NPs. The ratio of proteins and lipids was approximately 1:10 on the tested NPs. Altogether, 413 proteins were identified on the PLGA-NPs, 376 proteins on the PEG-NPs, and 417 proteins on the lipid-NPs. The authors followed up with a study of those proteins that had a high binding affinity for the NP surfaces and those proteins that were present in high concentrations (table 4).

***Table 4**. A list of the adsorption pattern of proteins to the different types of NPs studied: PEG NPs, PLGA NPs, and lipid NPs. +++ = highest relative corona concentration, + = lowest relative corona concentration.*

Protein	PLGA	PEG	lipid
Surfactant protein A	++	+	+++
Surfactant protein D	+	+++	++
Cathelicidin antimicrobial peptide (CAMP)	++	+	+++
Myisin-3	+++	+	++
Apolipoprotein A-I (APOA-1)	+++	++	+
Sodium-dependent phosphate transport protein 2B	+++[a]	+	+++[a]
Deleted in malignant brain tumors 1 protein (DMB	++	+	+++
BPI fold-containing family B member 1	++	+	+++
Ficolin 1/2	+++	+	++

[a] No statistical difference

The proteins with high binding affinity and those present in high concentrations were all associated with host defense and interaction with lipid membranes. SP-A and SP-D are both involved in host defense and they bind to carbohydrate epitopes. In the adsorption study, SP-D adsorbed in the highest concentration on PEG NPs, whereas the highest concentration of SP-A was found on lipid NPs [52]. These results support previous knowledge that SP-A is more hydrophobic than SP-D [69]. The Cathelicidin antimicrobial peptide (CAMP) is an antibacterial peptide involved in host defense. CAMP interacts with lipid membranes and in the study CAMP was mostly found on lipid NPs and on PLGA NPs. The Apolipoprotein A-I (APOA-1) is a lipophilic protein that appeared on the NPs in the following concentration order: PLGA NPs > PEG-NPs > Lipid NPs; APOA-1 is involved in phospholipid and cholesterol binding. The sodium phosphate transporter protein 2B is a membrane-associated protein that is involved in the synthesis of surfactants; it appeared in highest concentration on the lipid NPs and the PLGA NPs. The deleted in malignant brain tumors 1 protein (DMBT1) is a protein involved in host defense; it binds to bacteria and interacts with SP-D. DMBT1 was present on the NPs in the following order of concentration: lipid NPs> PLGA NPs > PEG NPs. The fold-containing family B member 1 (BPI) and the Ficolin ½ are both proteins associated with binding to pathogens as well as with interactions with lipid membranes [52]. Both proteins were most abundant on the lipid NPs. Some limitations of this study are the use of magnetic NPs and magnetic separation, which might be selective for some of the proteins, and also that the surface charge of the NPs was not addressed.

The phospholipid bilayer of cell membranes is negatively charged and therefore attracts NPs with a positive surface. This attraction results in enhanced internalization of positive NPs compared to neutral and negative NPs [31, 71]. Leonenko et al. have studied the influence of surface charge on NPs in RLF. The initial surface charge of the NPs (ζ –potential) ranged from -60 to +30 mV, and upon being transferred to RLF (lavaged RLF from rats washed with PBS) the ζ –potential changed to -27 to -33 mV for all NPs. The ability of biomolecules within the RLF to mask their surface charge by

changing ζ –potential suppresses the particles' capability to induce toxicity. This study was performed with polystyrene NPs that had been functionalized with different charged molecules. The authors found that the major molecules on the surface of the NPs were proteins. They also identified the same type of proteins present in the NPs' coronas, regardless of surface charge, indicating that proteins in surfactant have high affinity for NP surfaces regardless of surfaces [72]. The authors inferred that the adhesion of biomolecules onto the surface of NPs is due to strong hydrophobic and/or π-π interactions (non-covalent interactions) [72].

Kasper et al., compared amorphous silica NPs (aSNP) that were either unmodified or that had -COOH groups (negatively charged) or $–NH_2$ groups (positively charged) attached to the surface. The different aSNPs were exposed to cell cultures *in vitro,* with and without the presence of a lung surfactant (Alveofact, bovine alveolar lavage). The results indicate that the presence of the surfactant increases the cytotoxicity of the unmodified aSNPs as well as for the aSNPs with $–NH_2$ groups. The increased cytotoxicity is believed to be due to reactive silanol groups (Si-O-H), formed in the presence of lung surfactant [73]. These results by Kasper et al. are in contrast to the previously described results from Leonenko et al, in which proteins from the lung surfactant masked the positive surface charge of the NPs and made them less toxic. This contrasting results may be due to different methodological setups. Fröhlich et al., concluded from their experiments that the chemical composition of NPs is less important than their surface charge regarding making them cytotoxic. They studied several NPs (silica, ZnO, hollow silica-titania, and gold NP) and found that NPs with a positive surface charge induce a higher cytotoxicity than the negatively charged or neutral NPs [74].

It has previously been shown that positively charged NPs have the potential to induce membranolytic and hemolytic activity. However, following a protein corona formation on polymeric and metal oxide NPs, the surface charge changed to negative which resulted in complete loss of hemolytic activity [72, 75]. The adsorption of biomolecules to the surface of NPs may mask the surface charge and mediate properties for aggregation [72]. This increased property for aggregation is further known to enhance the recognition of alveolar macrophages [9].

10 Clearance of nanomaterials

Following deposition in the RLF, and formation of a biomolecule corona, the NMs will in most cases interact with cells, especially in the alveolar region. The formation of a biomolecule corona will take place instantly following deposition in the RLF, therefore it is likely that interactions with the cell surface occurs with the NP–biomolecule complex [73]. NMs are internalized in cells by active and energy-dependent mechanisms, compared to small molecules which diffuse by passive processes according to the equilibrium principles [12]. The formation of a biomolecule corona is of great importance for particle internalization since some proteins induce protein-mediated binding that might be very specific, such as a receptor-ligand interaction; other interactions between the NP and cell are non-specific and generic [76]. The different mechanisms for particle uptake into cells are size-dependent and can be mediated by either phagocytosis, macro-pinocytosis, clathrine-mediated endocytosis,

caveolin-mediated endocytosis, or clathrine/ caveolin -independent endocytosis [31, 77]. The binding of SP-A or SP-D to the surface of NPs has been shown to enhance an endocytic ingestion by alveolar macrophages [48]. Zhu and colleagues report that opsonization of NPs with SP-A and SP-D results in internalization by phagocytosis [31]. It has been shown that phosphatidylcholine-coated NPs are internalized by macrophages to a larger extent than uncoated NPs, indicating that the surface coating with surfactant lipids provides a more rapid clearance of NP [48].

The clearance of NMs can occur by active migration of cells or by translocation (Figure 8):

i) Macrophage migration towards the mucociliary escalator and movement up to the larynx,
ii) Macrophage migration towards the interstitial tissue,
iii) Dendritic migration towards the lymphatic vessels and draining lymph nodes,
iv) intracellular destruction of NMs within macrophages, and
v) translocation to secondary organs

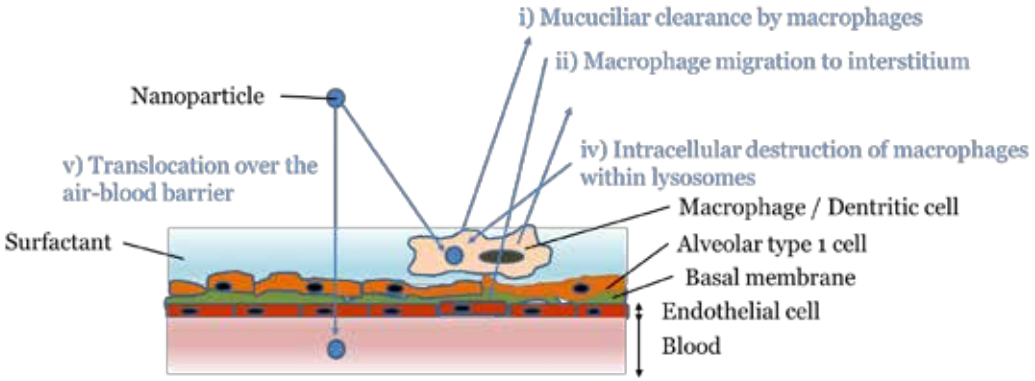

Figure 8. *Illustration of different mechanisms for clearance of NMs following deposition in the alveolar region of the lung.*

The mucociliary escalator is generally a very efficient clearance pathway for particles [25, 78]. However, the retention time is longer for NMs, compared to larger particles, due to increased pathway up to the mucociliary escalator, as well as lower mucus transport velocity in the small airways [79]. When comparing retention times in relation to particle size, Kreyling and colleagues, showed that gold NPs with a core diameter of either 5 nm, 18 nm, 80 nm, or 200 nm had longer retention times than the NPs with core size 1.4 nm and 2.8 nm. The authors show that the two smallest particles more easily cross the air-blood barrier, hence their short retention time [80].

Macrophage migration to interstitial tissues has been studied by Kreyling et al. NPs of TiO_2, iridium, and elemental carbon, were relocated to interstitial tissues to a greater extent than agglomerates of TiO_2 particles with a primary size of 200-300 nm. It was also shown that NPs may re-appear on the epithelial surface, followed by macrophage migration toward the ciliated airways [79]; the re-appearance is mediated by macrophages [25]. Further studies are needed to determine if these findings apply to human epithelium.

The translocation of NPs to lung draining lymph nodes has been shown in many studies [81-85]. Recently it was shown that the translocation of 50 nm polystyrene NPs to lung draining lymph nodes was due to active transport of dendritic cells and not macrophages. This particle translocation was also shown to be size dependent, with particles of 500 nm being found to a lesser extent than 50 nm in the lymph node [86]. Translocation of particles by simple drainage has only been shown for very small NPs. In a study by Choi et al., both organic and inorganic NPs below 34 nm were rapidly translocated (within 30 minutes) after an intratracheal instillation, and NPs with a size below 6 nm were translocated within 3 minutes. The NP surface properties were of importance for this translocation, with zwitterionic, polar, and negatively charged NPs being translocated but not positively charged NPs [87].

The intracellular degradation of foreign substances takes place in the lysosomal compartment of macrophages, which is an acidic milieu with digesting enzymes. In a study by Cho et al., the fate of different metals and metal oxides were studied following internalization into macrophages and lysosomes. They found that the conditions in the acidic lysosome (pH 5.6) affect the different metals and metal oxides. A comparison of 15 different metal and metal oxide NPs revealed that two important properties determine their potential for cellular toxicity (Table 5), one of which is the NP surface charge in acid conditions and the other being the NPs' property to be dissolved to toxic ion species at pH 5.6. They found that the hemolytic properties are enhanced with increased positive surface charge of the NPs. They also found that some of the metal oxides have high solubility in acid conditions (CuO NPs, MgO NPs, and ZnO NPs) [75].

Table 5. *Physicochemical properties and sizes of metals and metal oxides and theirhemolytic properties and solobility in acid (pH 5.6) condition. NP_a and NP_b refers to different sizes of the metal oxides. Adapted from Ref. [75], (Cho et al. 2012 Toxicol Sci, 2012.* ***126****(2): p. 469-77).*

Particles	Primary size	Hydrodynamic size	Z-potential (pH 5.5)	Hemolytic prop	Solubility (%)
Ag-NP	91.9 ± 4.7	81 ± 12	-	No	0.0 ± 0.0
Al_2O_3-NP	6.3 ± 0.2	65 ± 18	+>15	Yes (~20%)	5.5 ± 0.1
CeO_2-NP_a	9.7 ± 0.4	88 ± 12	+>15	Yes (>30%)	0.3 ± 0.0
CeO_2-NP_b	4.4 ± 0.2	132 ± 11	+<15	No	0.2 ± 0.0
Co_3O_4-NP	18.4 ± 0.8	185 ± 12	+>15	Yes (~10%)	1.6 ± 0.0
Cr_2O_3-NP	205 ± 20.7	173 ± 17	+<15	No	1.5 ± 0.0
CuO-NP_a	23.1 ± 1.0	112 ± 10	+<15	Yes (30%)	97.3 ± 1.4
CuO-NP_b	14.2 ± 1.2	99 ± 9	+<15	No	89.5 ± 0.3
MgO-NP	15.0 ± 1.8	116 ± 26	-	No	100 ± 0.0
NiO-NP	5.3 ± 0.4	92 ± 2	+ >15	Yes (>60%)	8.9 ± 0.0
SiO_2-NP	6.2 ± 0.4	378 ± 70	-	No	5.0 ± 0.0
TiO_2-NP_a	5.6 ± 0.2	105 ± 26	+<15	No	0.0 ± 0.0
TiO_2-NP_b	30.5 ± 1.8	119 ± 16	+<15	No	0.0 ± 0.0
ZnO-NP_a	10.7 ± 0.7	306 ± 42	+<15	No	100 ± 0.0
ZnO-NP_b	137 ± 9.2	282 ± 62	-	No	100 ± 0.0

Similar results were found by Braydich-Stolle and colleagues when they studied the induction of cytotoxicity, inflammation and phagocytosis of different coated silver NPs in different physiological fluids: interstitial, alveolar, and lysosomal. Both the interstitial and alveolar fluids have a pH of 7.4, whereas the lysosomal fluid has a pH of 4.5. Polysaccharide-coated silver NPs in alveolar and lysosomal fluid show dispersion and loss of coating. Due to the low pH in the lysosomal fluid, the particles showed some agglomeration, and significant dissolution of silver into silver ions, which resulted in increased phagocytosis, cytotoxicity and inflammation. The authors summarize that the NM properties may be altered depending on the surrounding environment and with that also the toxicological effects [61]. If the ions are toxic, as is the case for Zn^{2+} and Cu^{2+}, they may induce cytotoxic effects and trigger inflammation [75]. It has previously been shown that positively charged NPs have the potential to induce membranolytic and hemolytic activity of cells. However, following a protein corona formation on polymeric and metal oxide NPs the surface charge turns negative, which results in complete loss of hemolytic activity. However, the hemolytic potential can be recreated when the metal oxide NPs are introduced to an acid environment, similar to the lysosomal environment (pH 5.6); the protein corona is digested by enzymes and the positively charged surface is regained [72, 75].

The air-blood barrier separating the external air in the alveolus from the systemic blood circulation is only 0.1-0.2 µm thick. Consequently, there is only a short distance for particles to translocate into the vascular system. At present there are still many unknown factors involved in this translocation into the blood and the secondary organs [88]. A translocation of a NM means crossing the membranes with either para-cellular or trans-cellular mechanisms; the trans-cellular mechanism includes a particle release (exocytosis) into the blood [80]. The translocation of NPs has been shown to be size dependent and previous studies have shown that NPs with very small sizes below (~6 nm) are more able to translocate [80, 87]. Studies on the sizes 18 nm [89], 24 nm, 110 nm, and 190 nm showed minimal or no translocation into the vascular system [90]. The translocation of small NPs seems to be independent of material, however, the surface properties of NPs are of importance. It has been shown that both organic and inorganic NPs with zwitterionic, polar surfaces and a net negative charge translocate over the air-blood barrier [87]. In another study performed by Kreyling and colleagues gold NPs with a size of 2.8 nm and with a negative surface charge were translocated from the lung to a higher extent (3.4 %) than gold NPs with a positive surface charge (1.2%) [80]. Both these studies were performed using intratracheal instillation [80, 87]. In another distribution study by Kreyling and colleges they used iridium NPs with a count mean diameter (CMD) of 20 nm (Ir NP 20 nm) and 80 nm (Ir NP 80 nm) as well as a composite iridium-carbon NP with a CMD of 25 nm (Ir-carbon NP 25 nm). All particles were aggregated and agglomerated to a chain-like structure built from the primary particle size of 2-4 nm for Ir and 5-10 nm for Ir-carbon. It was shown that the Ir NP 20 nm fraction was bigger than that of Ir NP 80 nm and Ir-carbon NP 25 nm in all of the studied organs: liver, spleen, kidneys, heart, brain, blood, and “remainder”. In this study the authors stated that both the size and the material characteristics of the NPs are determinants for translocation and accumulation in organs [91]. One study exposed mice and rats to a dry aerosol of carbon fullerens (C_{60}; 50 nm) and then analyze the concentration of C60 in different organs: lungs, blood, liver, spleen, kidney, bronchial

lymph nodes, and brain. Low levels of C60 were detected in the bronchial lymph nodes, the liver and the spleen, in addition to the lung. The authors state that this biodistribution maybe a result of gastrointestinal exposure following clearance by the mucocilary escalator [85]. Kreyling and colleagues also found that depending on the route of administration (intravenous injection or intratracheal instillation) the relative amount of gold NPs in secondary organs was very different. The authors suggest that this is related to the formation of different types of protein coronas following the different routes of administration [80]. In a comprehensive review by Kermanizadeh and colleagues they discuss that some literature show translocation over the air-blood barrier, although to a very low extent. They conclude that the size and surface charge are important factors in translocation; the solubility is another important determinant, where soluble particles show a larger percentage of translocation compared to insoluble NPs [92].

11 Systemic effects

Particulate matter in air pollution may induce systemic effects that result in impaired health, such as increased respiratory and cardiovascular morbidity and mortality [93, 94]. While this is general knowledge, the mechanisms by which the particles induce these effects are still under investigation. In the health context, there is a concern about the ability of small NPs to cross the air-blood barrier and thereby have a direct impact on cells and molecules in the blood circulation and in other organs. Choi and colleagues found that NPs with a size of 34 nm and with a negative surface charge were translocated into the blood, but not through the air-blood barrier. This translocation is possible through the lung-draining lymph nodes. They also found that zwitterionic NPs with a size of 6 nm were efficiently cleared by renal filtration into the urine, whereas the larger NPs 34 nm were not excreted by the urine. Based on this, the authors state that the NPs with zwitterionic or negative surface charge and with a size larger than 6 nm and below 34 nm are the most dangerous NPs since they can translocate into the bloodstream but are not excreted by the urine [87].

Several studies have addressed systemic affects following inhalation exposures to NPs. Although the mechanisms of the induction of systemic effects are unknown, they are in many cases thought to be due to secondary effects and not directly due to the presence of NPs. For example, it is known that inhalation of NPs (of different kinds) induce airway inflammation following the secretion of inflammatory mediators (cytokines and chemokines) from the lung residential cells and the recruitment of cells from the circulatory system into the airways [82-84, 95-102]. It is also known that inhalation of NPs induces an activation of the immune system. The mechanisms for this immune induction is still unknown, although it has been suggested that the antigen is a configured endogenous biomolecule covering the surface of the NP rather than the NP itself [103]. Exposure to NPs is also known to induce oxidative stress within the lung, which may be a co-actor in systemic effects [104]. Only a few studies focus on the markers that reflect an effect on the cardiovascular system following NP exposure. Nurkweiz and colleagues show a dysfunction in the microvascular system that they relate to an activation of systemic inflammation and/or an activation of neurogenic mechanisms [105].

To our knowledge no study has been able to link the formation of biocoronas on the surface of NMs following encounter the RLF to systemic effects. The reported systemic effects following inhalation of NPs originate mostly from studies performed with NP aerosol inhalation, intratracheal instillation, and oropharyngeal-/laryngeal aspiration, and not dry-powder inhalation that mimics the real scenario where dry NPs encounter the RLF.

12 Conclusions

This study summarizes the current knowledge on how physical and chemical properties of inhaled NMs determine the NMs' fate upon being deposited in the Respiratory Lining Fluid (RLF) and also the toxicological response. NMs deposited in the respiratory tract are efficiently removed by the mucus and the mucuciliary escalator. The different properties of mucus enable various interactions between the mucus and inhaled intruders, such as NMs. Negatively and positively charged particles are efficiently trapped within the mucus and consequently removed. In contrast, NMs with neutral or hydrophilic surfaces appear to more easily penetrate the mucus and reach the lung epithelial cells present underneath the mucus.

NMs deposited in the alveolar region first encounter the surfactant film, which consists of lipids and proteins and covers the lung epithelial cells. Both *in vitro* studies and computer modelling reveal that the surface charge and hydrophobicity of NMs are of major importance for the formation of a biomolecule corona and for the translocation of NMs across the surfactant film. Independent of the NMs' surface properties, both the lipids and the proteins in the surfactant interact with the NMs and mask their surface, indicating that the biomolecule corona is of importance for further interactions within the lung. Most proteins that adsorb onto the NMs have functions associated with host defense; they interact with lipid membranes and enhance the uptake by macrophages. Following internalization by macrophages, the NMs can be removed by several clearance mechanisms such as: mucuciliary clearance, translocation to interstitial tissues within the lung, and intracellular destruction within the lysosomal compartment. Only some NMs are toxic due to their composition, as in the case with classic metal toxicity following the release of metal ions. NM toxicity is also related to the shape of the NM. The internalization by macrophages of NMs structured like fibers and sheets are difficult, which leads to a frustrated phagocytosis and a release of toxic mediators inducing cellular stress to the surrounding environment. Given the small extent of translocation of NPs across the air-blood barrier into secondary organs, there is evidence that systemic response is a consequence of secondary effects. For instance, a systemic release of mediators, such as cytokines and chemokines, has been observed following NP exposure to the airways; activation of the adaptive immune system has also been observed. It is not known whether it is the NP itself that is the antigen or if it is the configured endogenous biomolecules coating the NP surface.

Interactions between NMs and surfactants also affect and disturb the biophysical function of ventilation. Studies have indicated that hydrophobic NMs are retained in the surfactant film and therefore alter the surface tension of the surfactant, which affects the biophysical function of ventilation.

This study shows that there is a lack of knowledge regarding the consequences of the formation of biomolecule coronas on NPs and their influence on systemic effects. Further investigation is needed to determine whether systemic effects are a result of the NPs themselves or whether they are secondary effects from inflammatory mediators, neural activation, extracellular vesicles, etc.

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