Interaction of nanomaterials at the air-liquid interface of the lung

Nanomaterial interactions within the respiratory unit

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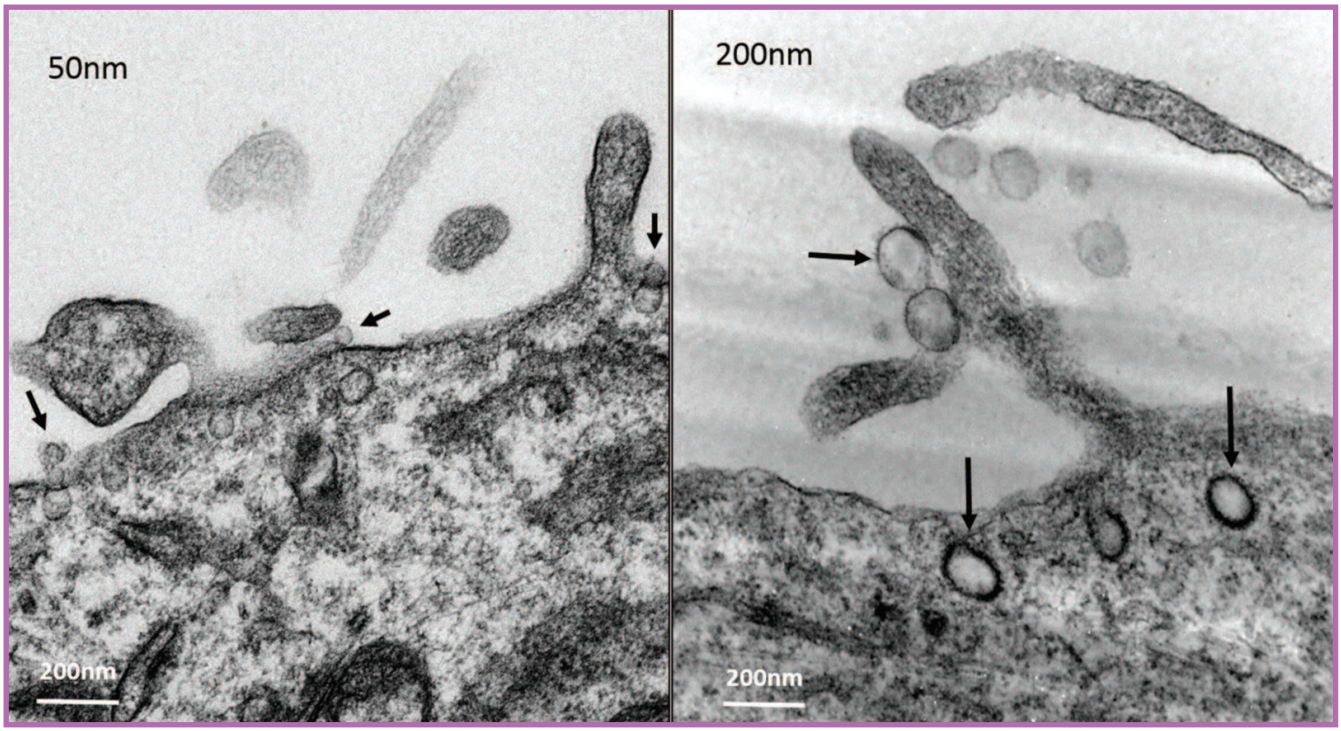

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Transmission electron micrographs showing the interaction of 50 nm and 200 nm polystyrene spheres with human lung alveolar epithelial type 1 cell membranes, illustrating macropinocytosis and endosomal uptake.

Nanotechnology

Nanotechnology involves the use of materials at the nanoscale, defined as having at least one dimension of less than 100 nm. However, there is still considerable debate over the definition and terminology of these nanomaterials.[1,2] The field of nanotechnology has expanded rapidly since the early 1990s, with a wide range of applications in many diverse areas of everyday life. This is reflected in the marked increase in publications in this area. For example, a search of PubMed for the word "nanotechnology" revealed an increase in publications from 1,196 in 2003 to 5,817 in 2013, while a search for the word "nanomedicine" for the same years showed a rise from 5 to 1,927 publications. Although a relatively new discipline, the potential number of formulations and applications is enormous, including medicine, engineering, cosmetics, textiles, sporting goods and many other purposes. The market for final products incorporating nanotechnology rose from less than $100 billion in the year 2000 to $254 billion in 2010, and is predicted to rise to $3 trillion in 2020.[3]

Nanosized objects and the lung

As a consequence of rapid market expansion, release of nanoparticles into the air during synthesis, use and disposal of nanomaterials is a concern. It is predicted that when nanoparticulate material is inhaled, a high percentage (~50%) will access the gas exchanging zone, where it may have unwanted effects.[4] For example, compilation of the results from a number of rat and mouse studies showed that inhalation of titanium dioxide (20 nm and 250 nm) by rodents caused pulmonary neutrophilic inflammation, which was directly related to increased surface area and therefore nanosize.[5] On the other hand, in the realms of nanomedicine and drug delivery to the lung for local and/or systemic treatment, some of the new biopersistent nanomaterials offer alternative approaches to conventional biodegradable products.[6,7] For example, nanogold is relatively non-toxic, readily taken up by cells, is easy to functionalize, to track and, once delivered to target cells, can also be heated to either kill targeted cells or to release conjugated ligands.[6] Carbon nanotubes,

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which can pierce cells as well as being endocytozed, can easily be functionalized, while drugs can be loaded into the lumen for slow release within the target tissues.[7]

Inhalation of the fine (<2.5 μm aerodynamic diameter) and nanoparticulate fractions (less than 100 nm aerodynamic diameter) of ambient air pollution has adverse health effects, including exacerbation of COPD, increased asthma attacks and lung cancer, as well as increased stroke, heart attacks, atherosclerosis and other cardiovascular conditions.[8, 9] However, it is important to appreciate that there are distinct differences between the nanoparticles in engineered nanomaterials and those in ambient air. For example, a significant component of nanoparticles in the fine component of particulate matter (PM less than 2.5 μm aerodynamic diameter) in ambient air is derived from diesel exhaust, which consists of a carbon core coated by organic compounds, including polycyclic aromatic hydrocarbons (PAH), and small amounts of sulfate, nitrate, metals and other trace elements. Such chemicals may irritate asthma (sulphate) or contribute to the development of cancer (PAH).[10] In contrast, engineered nanomaterials are essentially designer products, where the chemical composition, shape, size, dimensions and surface functionalization have been selected for specific applications.[9] They are synthesized using many different chemicals, may contain more than one chemical, may be functionalized in a variety of ways and can have different morphology. Nanoplates have one nano dimension, nanorods have two nano dimensions while spheres have three.[2] Materials on the nanoscale exhibit unique properties compared to the same material in bulk form. The smaller the product, per unit mass there are millions more particles, which have a greater surface area compared to the bulk material. This increase in surface area provides a greater number of chemically-active surface molecules, which confers bioreactivity and also provides opportunities to modify the surface of the material for specific purposes. A wide range of materials are being used, including metals, metal oxides, carbon-based materials and quantum dots. The non-carbon-based materials include silver, gold, silicon dioxide, iron oxides, titanium dioxide, zinc oxide and cerium oxide. Carbon-based materials include carbon nanotubes, nanowires, fullerenes and graphene. In order to appreciate processes that might follow deposition of a relatively low mass but potentially high distribution of inhaled nanomaterials deep in the lung, it is important to understand and anticipate adverse events that are likely to occur. This will help to circumvent toxic effects and optimize processes at the nano-bio interface of the lung, particularly in designing safe nanoproducts. This article briefly describes a series of *in vitro* studies, and supporting *in vivo* studies, of nanoparticle interactions with the first targets of inhaled nanoparticles, affecting particle translocation and interaction in the alveolar region of the lung: the alveolar lining liquid, alveolar macrophages and the alveolar epithelium.

Alveolar lung lining liquid—the first target

The ultimate fate and bioreactivity of inhaled nanosized materials will depend on a myriad of factors, including the physicochemistry of the materials, inhaled dose, and target cells and fluids. Here the focus is the alveolar respiratory unit, as opposed to the terminal respiratory unit which includes the respiratory bronchioles. The first target biomembrane in the alveolar respiratory unit, consisting of the alveoli and associated ducts distal to the respiratory bronchiole, is the lung lining liquid, a major component of which is pulmonary surfactant[7, 11] secreted by alveolar epithelial type 2 cells. It is enriched with phospholipids (~80%),[11] particularly saturated dipalmitoylphosphatidylcholine (DPPC), which maintains reduced surface tension to prevent alveolar collapse. This is aided by surfactant-associated proteins (5-10%), SP- A, SP-B, and SP-C. SP-B and SP-C are hydrophobic and bind to the lipid component to increase stability and structure of surfactant. SP-A and another surfactant protein, SP-D, are complex hydrophilic molecules, termed collectins. They contribute to host defense by binding microbes and other foreign material, facilitating clearance by macrophage phagocytosis.[12] They also contribute to immune defense.

Alveolar lining liquid also contains serum-derived and locally-produced antioxidants, antiproteases, albumin and other bioreactive molecules. It is suggested that particulate material penetrates the thin surfactant layer (<200 nm deep),[13] forcing the particles to interact with the underlying cells, where mechanotransduction and mechanosensing pathways can stimulate cellular activity.[14, 15] The exact processes are not known but the size and surface charge of the nanomaterials are crucial to this stimulation. For example, low concentrations (10-100 μg/ml) of hydrophobic 12 nm and 20 nm polyorganosiloxane (AmorSil) did not disrupt the surface-tension-reducing capacity of DPPC surfactant films on a Langmuir-Blodgett trough, while slowly crossing them. However, the same concentrations of 136 nm hydrophobic nanoparticles caused significant disruption of the DPPC film.[16, 17] The surface charge of particulate material also affects the activity of surfactant. For example, 100 nm negatively-charged polystyrene nanoparticles were more disruptive of surfactant films, measured using a pulsating bubble surfactometer, compared to 100 nm positively-charged polystyrene nanoparticles. This study also indicated that whole surfactant (e.g., rabbit lung surfactant isolated from bronchoalveolar lavage) was more resistant to nanoparticle disruption compared to synthetic mixtures of component parts of surfactant (e.g., DPPC

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and SP-B).[18] During submersion, nanomaterials can interact with and adsorb components of the lung lining liquid layer which will alter their behavior at the gas-liquid interface, and possibly affect dispersion, clearance and translocation of the nanaomaterials. It has long been known that airborne particulate materials such as mineral particles,[19, 20] adsorb proteins and other biological molecules, although the term "corona"[21] has only recently been used to describe this process. Early studies *in vitro* indicated a corona of lung surfactant mitigated mineral particle induced cytotoxicity, suggesting the protective potential of the local extracellular milieu of the alveolar units[22] to modify the behavior of inhaled (nano)particles delivered to the lung. Surfactant-associated proteins SP-A and SP-D both bind to nanomaterials, likely reflecting the mechanism by which they facilitate microbial clearance *in vivo.*[12] SP-A and SP-D differentially bind to carbon nanotubes, depending on their purity and the chemical variation between the nanotubes.[23] The ability of nanomaterials to adsorb specific proteins, including SP-D, from human lung lining liquid, can be studied by incubating the nanomaterials and acellular human bronchoalveolar lavage fluid for one hour at 37°C, followed by protein separation and immunoblotting. Immunoblotting using antibodies to SP-D demonstrated that 20 μg/ml acid-oxidized carbon nanotubes (700 nm long, 20 nm diameter) adsorbed three times as much SP-D as the equivalent poly(4-vinyl pyridine)-modified carbon nanotubes. This likely reflects the uneven topography and therefore increased surface area due to acid treatment of the acid-oxidized carbon nanotubes. Using the same methods, negative-surface-charged 100 nm polystyrene nanoparticles (20 μg/ml) adsorbed two and three times more SP-D, respectively, than equivalent neutral-surface-charged and positive-surface-charged 50 nm polystyrene nanoparticles.[24]

The degree of surfactant protein binding varies among nanomaterials, even those of the same class (e.g., metal oxides) and the equivalent bulk materials.[25] Neither SP-A nor SP-D seem to de-agglomerate nanoparticles.[25-27] Indeed, it has been suggested that increased agglomeration/aggregation and macrophage phagocytosis due to SP-A/D binding might be an important mechanism of nanoparticle clearance from the alveolar region.[26, 28] DPPC also bound to, and caused agglomeration of, carbon black nanoparticles,[29] although binding of DPPC to silver spheres resulted in improved dispersal and delayed nanosilver dissolution.[30] Stimulation of macrophage clearance of magnetite nanoparticles by SP-A and SP-D depended on whether the nanoparticles were hydrophobic (SP-A enhanced; 5-20 μg/ml, $p<0.05$) or hydrophilic (SP-D enhanced; 20 μg/ml, $p<0.05$). However, addition of natural surfactant isolated from pig bronchoalveolar lavage or a synthetic surfactant made from relevant concentrations of phospholipids (66% DPPC), supplemented with SP-D to mimic *in vivo* composition, modified these effects so that nanoparticle uptake by macrophages was much the same, regardless of addition of natural surfactant (containing SP-A) or synthetic surfactant (containing SP-D).[28]

These studies indicate that inhaled nanoparticles that deposit onto the lung lining liquid will adsorb components of lung surfactant, possibly a mixture of proteins and lipids, particularly since these molecules are abundant at this interface. This process is complex and could have a number of consequences. Coating nanomaterials with natural components from the local milieu may enhance phagocytosis, endocytosis and other cellular clearance mechanisms. This may neutralize toxicity to underlying epithelial cells or enhance uptake by the epithelium, a desirable process in nanodrug delivery to the lung. However, sequestration of components of lung lining liquid, especially surfactant, and the consequent depletion of components essential to the lung's immune defense system and to the normal surface-tension-reducing function would not be ideal. This is particularly the case in the absence of sufficient regeneration of lung surfactant. The effects of loss of surfactant on lung function in humans are well documented,[31] for example, in acute respiratory distress syndrome and interstitial pulmonary fibrosis.[31] Animal models deficient in SP-D exhibit pulmonary emphysema, and deficiency in SP-A and SP-D affects antimicrobial defense.[12, 31] Alternatively, it is clear that the degree of surfactant adsorption by nanomaterials depends on many factors, including surface chemistry and functionalization, size and shape, as well as conditions in the surrounding environment. For example, low pH enhances silver nanoparticulate dissolution,[30] while calcium aids SP-A and SP-D binding to carbon nanotubes *in vitro.*[23] It is a complex bio-interface and much still needs to be established. Importantly, the diversity of nanomaterials, and complexity of the molecular interactions with lung surface components, will be critical in subsequent interactions at the gas-liquid interface.

Nanoparticle clearance by alveolar macrophages

Alveolar macrophages are responsible for clearance of organic and inorganic particulate material from the alveoli. They readily phagocytoze material in the micrometer range. However if particles are too long, for example, with a length greater than the diameter of a macrophage (~20 μm), there could be ineffective phagocytosis and increased activation of macrophages. This could lead to undesirable consequences, for example, as observed in asbestos-induced mesothelioma. This is a possibility with tubular structures that have a high aspect ratio, such as carbon nanotubes.[32] Both *in vitro* and *in vivo* studies have indicated that inhalation of long carbon

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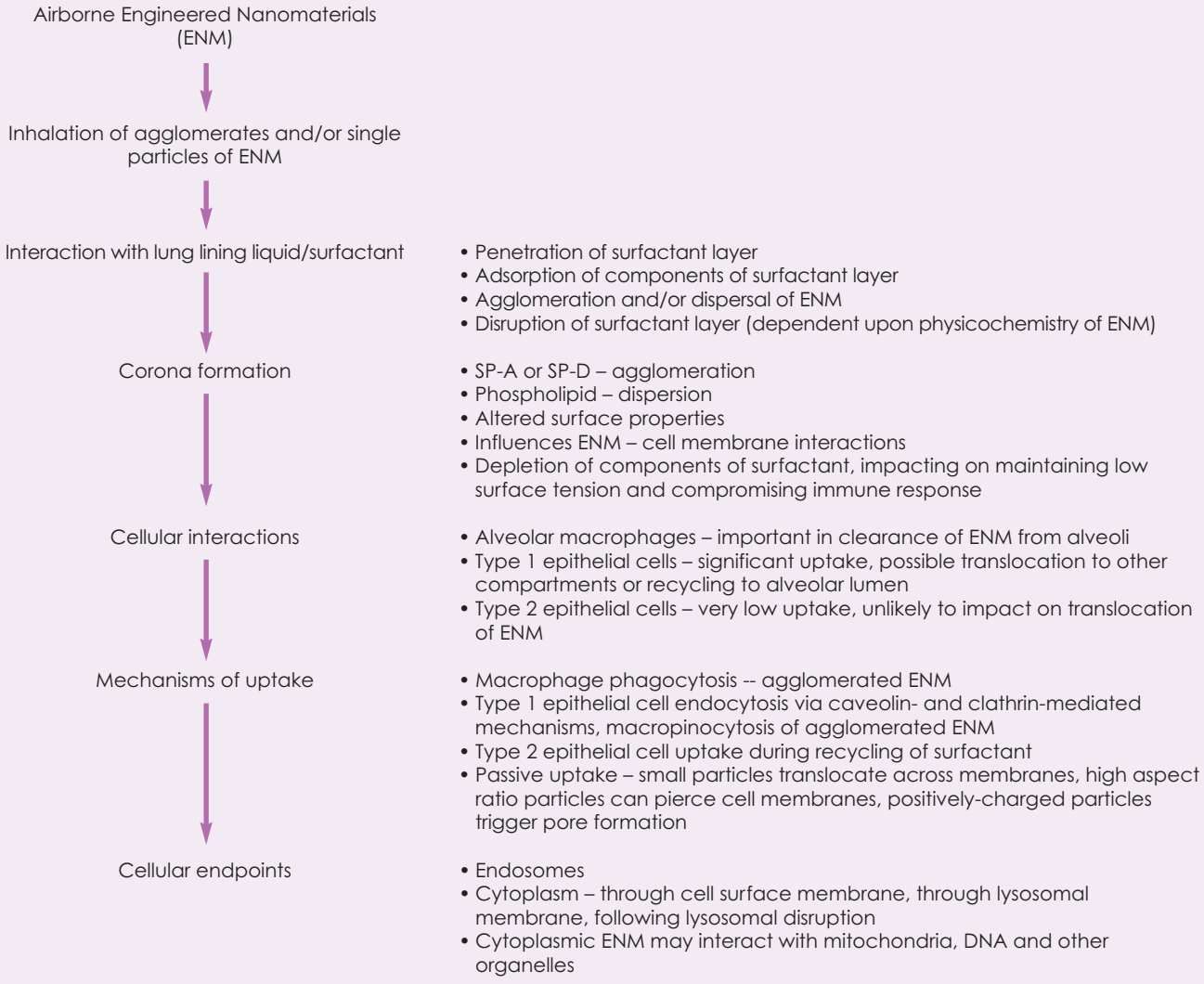
nanotubes could induce pulmonary responses similar to those observed with amphibole asbestos, which contains fibers that are long (> 20 μm) and durable.[32] Thus, such materials would not be very useful in drug delivery by inhalation. On the other hand, evidence has suggested that nanosized material is not as readily phagocytozed by macrophages,[5] possibly due to the mode of macrophage recognition of particulate material and also the inability for sufficient very small particles to cross the cell membrane passively. Therefore, nanoparticle agglomeration/aggregation at the lung liquid interface could promote normal phagocytic activity, as proposed above, particularly following opsonization of inhaled material by surfactant proteins to enhance macrophage phagocytosis, as shown *in vitro*.[28] The exact mechanisms are unclear. A series of studies *in vitro* and *in vivo* indicate that binding of the collagenous region of the surfactant protein to macrophage cell surface receptor CD91, an endosomal receptor molecule, can induce macrophage phagocytosis and pro-inflammatory mediator release, while binding of the carbohydrate recognition domain to the signal-regulatory protein alpha (SIRP-alpha) (CD172) receptor inhibits anti-inflammatory mediator production.[12]

The number of macrophages in a normal healthy lung is relatively few compared to alveolar epithelial cell numbers and induction of an inflammatory response to increase phagocytic cell numbers would be important if the normal macrophage and mucociliary clearance mechanisms were to take place. It has been shown in rats *in vivo* that inhaled nanoparticles (e.g., spark-generated 25 nm iridium, ^{192}Ir, concentration of 2.5 μg/cm^3 air, corresponding to a particle number concentration of 5×10^9 cm^3)[33] that are not internalized by macrophages can reach the pulmonary interstitium, suggesting that they may be internalized by, or pass between, epithelial cells and can translocate across the alveolar epithelium.[5,33,34]

Alveolar epithelial type 1 cells—the major target of inhaled nanoparticles

Inhaled nanoparticles that escape macrophage clearance and penetrate the lung lining liquid layer will reach the apical epithelial cell surface, where a number of processes can occur. The most significant target cell is the alveolar epithelial type 1 cell. These cells cover 95% of the epithelial surface area of the alveolus, with a large diameter (reaching 80 μm), but with a depth of less than 200 nm[35] and are situated

Transformation and fate of inhaled nanoparticles within the alveolus



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in close proximity to the microvascular endothelium. The alveolar epithelial-microvascular endothelial wall of the alveolus is less than 0.5 μm in depth, mainly to facilitate effective gas exchange and ion and fluid transport between the gas-blood compartments.[36,37] Type 1 cells are long-lived cells derived from their progenitor alveolar epithelial type 2 cells. They are metabolically active, producing mediators of inflammation and exhibiting endocytosis.[38, 39] Although there is relatively little *in vitro* work with nanoparticles using alveolar type 1 cells, the derivation of TT1 cells,[39] a unique, immortal, human alveolar epithelial type 1 cell line, has enabled a number of studies of nanomaterial interactions.

A high percentage (~80%) of TT1 cells *in vitro* internalize a range of nanomaterials, including polystyrene, silver and carbon nanotubes.[39-42] These nanomaterials are relatively non-toxic at low concentrations, although their shape, size and surface functionalization impart differential uptake. This involves both passive (directly through the cell membrane);[41-43] and active mechanisms (e.g., endocytosis, macropinocytosis). [39, 42, 43] The nanomaterials can be found both inside lysosomal structures as well as in the cytoplasm. The processes involved depend on the material under study. Silver nanowires (currently under development for optoelectronics) are endocytozed by type 1 cells[40] and can reach the cytoplasm. Interestingly, although dissolution to silver ions might be thought to cause toxicity, intracellular sulphidation of silver limits its dissolution to silver ions, possibly preventing overt toxicity.[30] Cationic, anionic or non-ionic grafted species of differentially functionalized multiwalled carbon nanotubes (MWNTs) rendered water soluble for medical applications, were not cytotoxic to TT1 cells. They were internalized by both endocytosis (including macropinocytosis of agglomerated MWNTs)[41] and direct penetration of the cell surface following exposure to 5 μg/ml. This is a relatively low dose for toxicity studies but would represent a "hot spot" exposure, possibly of occupational relevance in humans. It is important to appreciate that very little is known about human exposure to carbon nanotubes. These studies aim to better understand and anticipate their bioreactivity. Following endocytosis, MWNTs also penetrated lysosomal membranes to reach the cytoplasm, indicating important mechanisms of entry and translocation.[41] Although not quantified, it appeared that the cationic MWNTs showed a greater cellular interaction and cellular penetration than anionic MWNTs, likely reflecting attraction between the positively-charged MWNTs and negatively-charged cell membrane. However, studies with 50 nm polystyrene nanospheres showed that amine modification to render the particles cationic produced a very cytotoxic nanoparticle that penetrated the cell membrane by cell membrane pore formation.[43] Necrotic and apoptotic cell death occurred as indicated by increased cellular caspase 3/7 and 9 levels and LDH release. Neutral and carboxylated polystyrene of identical size were relatively non-cytotoxic, although all three polystyrenes were bioreactive, inducing pro-inflammatory mediator release into the tissue culture medium, determined by standard, enzyme-linked immunoassays. Both neutral and negatively-charged (carboxylated) 50 nm polystyrene nanoparticles were readily internalized by TT1 cells, mostly by passive mechanisms,[39] but also active mechanisms (involving clathrin-coated pits).[32] In contrast, when TT1 cell uptake of 100 nm neutral, positive and negative polystyrene nanoparticles was similarly investigated, fewer particles were internalized, and the mechanisms were mostly active endosomal pathways, not passive, indicating the significance of nanosize in particle uptake mechanisms.[39] Others have shown that the flux of amidine-modified polystyrene (positively-charged) nanoparticles across rat primary alveolar epithelial cells was nanosize dependent and 20-40 times that of similar sized, negatively-charged, carboxylated particles.[44] It was suggested that the mechanism involved was translocation via passive mechanisms through the plasma membrane, since ethylene glycol tetraacetic acid (EGTA) and other endosomal inhibitors did not affect nanoparticle flux across the epithelial barrier *in vitro*.[44] Interestingly, unlike amine modification,[33] amidine modification did not induce cell death,[44] possibly due to different charge density. Importantly, the same group noted profound differences in polystyrene nanoparticle uptake between alveolar epithelial cells isolated from rats and mice. In the mouse cell model, there was active uptake involving clathrin-and dynamin-dependent endocytosis, but in the equivalent rat model, uptake did not involve endosomal pathways. These findings indicate the importance of using human cells for such studies.[45] In combination, these studies highlight the vital role that alveolar type 1 cells are likely to play in the translocation of nanosized objects across the respiratory epithelium, as well as the possibility of modulating particle clearance from the lung.[46]

Alveolar epithelial type 2 cells—not important in nanoparticle translocation?

Alveolar epithelial type 2 cells are the secretory cells in the alveolus that produce lung surfactant, as well as other important molecules, including mediators of inflammation, antiproteases, lysozyme and antioxidants. Although alveolar epithelial type 2 cells are present in greater numbers than alveolar epithelial type 1 cells, they are smaller (~10 μm diameter). Just a small apical area contributes to the alveolar epithelial surface so that they form only ~5% of the total alveolar surface area.[35] Nevertheless, as these secretory cells are so important in maintaining alveolar homeostasis, and possibly might form a drug target,

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it is necessary to understand how these cells might respond to inhaled nanoparticles.

Primary human alveolar epithelial type 2 cells rarely internalize particulate material *in vitro*, including polystyrene nanoparticles[39]or other nanoparticulate material. This differs from the studies of primary rodent cells, described above.[44, 45] The reason for this is unknown, but may involve "protection" due to apical release of surfactant which binds nanomaterials. In addition, type 2 cells are not enriched with endocytic vesicles or lysosomes, although they do recycle surfactant. Uptake and internalization of used surfactant by type 2 cells is facilitated by SP-A, as illustrated using primary rodent type 2 cells *in vitro*, and in experimental animal (rodent) models *in vivo.*[12] Therefore, it is possible that nanoparticles might also be internalized during uptake of used surfactant by type 2 cells. Interestingly, considering the high percentage of 50 nm polystyrene nanoparticles that were passively taken up in the study of the human type 1 cell model described above, it is difficult to understand why significant numbers of type 2 cells do not internalize the same particles in a passive manner, particularly when using an identical protocol.

Other studies of the bioreactivity of polyethylene glycol (PEG)-coated gold (14 nm and 100 nm) with primary rat type 2 cells indicate there is very little particle uptake, which may also reflect surface functionalization, whereas rat macrophages were observed to internalize the gold particles.[47] Although type 2 cells do not avidly internalize particles, recently significant bioreactivity was observed following exposure to MWNTs, where short tubes (1.0 μm) were most bioreactive with alveolar type 2 cell epithelium, inducing high levels of IL-6 and IL-8 secretion. Therefore, even at a low concentration, 1 μg/ml of short MWNTs caused a 5-fold increase in IL-8 release, whereas long MWNTs had no effect. In contrast, MWNTs 20 μm in length induced the most striking responses in primary human alveolar macrophages, including marked cytotoxicity, approximately 50%, and pro-inflammatory mediator release, inducing 2-fold and 7-fold increases in IL-8 release at 1.0 and 10 μg/ml exposure.[48] There are numerous studies showing significant uptake of a wide range of nanoparticles by the human A549 adenocarcinoma cell which is used as a human type 2 cell surrogate. However, A549 cells are essentially cancer cells, which do not necessarily express surfactant and have different immune responses compared to primary human type 2 cells.[49, 50]

Summary

The interaction of nanoparticles at the air-liquid interface of the lung is complex. *In vitro* studies illustrate the significance of differential interactions by nanomaterials with components of lung lining liquid and highlight the impact of adsorption of lipids and proteins on particle penetration and subsequent cellular processing. Although it is generally believed that macrophages play an important role in particle clearance from the lung, they will not necessarily be able to provide an impenetrable barrier to nanomaterials that deposit at the gas-liquid barrier of the lung, even if sufficiently agglomerated following interaction with lung secretions. This reflects numerous factors, including the physicochemistry of the nanoparticles themselves, as well as the wide dispersion and penetration of particles to the epithelial surface, avoiding contact with macrophages. Alveolar type 1 cells avidly internalize nanoparticles, particularly by passive processes but also via active endocytosis, involving clathrin-mediated mechanisms and macropinocytosis (especially for high aspect ratio objects and agglomerates). While these mechanisms function mainly with smaller particles, depending on the particle shape and surface chemistry, direct piercing of the membrane can also occur. Alveolar type 2 cells do not avidly internalize nanoparticles but their bioreactivity may trigger inflammation and increased macrophage recruitment, which might in turn impact particle clearance. At present, it is not fully possible to predict how specific nanoparticle physicochemistry will impact interactions at the air-liquid interface. Therefore, it is vital to establish these mechanisms during development and use of novel products to avoid adverse health effects and optimize efficacy of therapeutic products.

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