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The impact of nanomaterial characteristics on inhalation toxicity

Frank S. Bierkandt, Lars Leibrock, Sandra Wagener, Peter Laux and Andreas Luch *

During the last few decades, nanotechnology has evolved into a success story, apparent from a steadily increasing number of scientific publications as well as a large number of applications based on engineered nanomaterials (ENMs). Its widespread uses suggest a high relevance for consumers, workers and the environment, hence justifying intensive investigations into ENM-related adverse effects as a prerequisite for nano-specific regulations. In particular, the inhalation of airborne ENMs, being assumed to represent the most hazardous type of human exposure to these kinds of particles, needs to be scrutinized. Due to an increased awareness of possible health effects, which have already been seen in the case of ultrafine particles (UFPs), research and regulatory measures have set in to identify and address toxic implications following their almost ubiquitous occurrence. Although ENM properties differ from those of the respective bulk materials, the available assessment protocols are often designed for the latter. Despite the large benefit ensuing from the application of nanotechnology, many issues related to ENM behavior and adverse effects are not fully understood or should be examined anew. The traditional hypothesis that ENMs exhibit different or additional hazards due to their "nano" size has been challenged in recent years and ENM categorization according to their properties and toxicity mechanisms has been proposed instead. This review summarizes the toxicological effects of inhaled ENMs identified to date, elucidating the modes of action which provoke different mechanisms in the respiratory tract and their resulting effects. By linking particular mechanisms and adverse effects to ENM properties, grouping of ENMs based on toxicity-related properties is supposed to facilitate toxicological risk assessment. As intensive studies are still required to identify these "ENM classes", the need for alternatives to animal studies is evident and advances in cell-based test systems for pulmonary research are presented here. We hope to encourage the ongoing discussion about ENM risks and to advocate the further development and practice of suitable testing and grouping methods.

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1. Introduction

The use of engineered nanomaterials (ENMs) is increasing continuously and represents an indispensable technology in the current times. This so-called "new" material is applied in different sectors like the automotive industry, consumer goods or medical applications. At the time of writing this article, more than 1600 "nano-containing" consumer products were registered in the Nanotechnology Consumer Products Inventory (CPI), an inventory providing the currently best available overview of nano-enabled consumer products introduced to the global market.[1] Due to their ubiquitous presence, human exposure to ENMs cannot be fully prevented. Exposure may occur *via* ingestion of food,[2] direct dermal contact[3] while using tools and consumer products,[4,5] and inhalation of airborne particles.[6,7] Out of these exposure routes, inhalation is assumed to entail the most harmful potential. From the 15th century onwards,[8] several adverse health effects have been associated with exposure to airborne materials such as coal,[9] quartz,[10] diesel particles,[11] asbestos fibers[12] or ultrafine particles (UFP) in general.[13] The potential health risk of inhaled particulate matter is underscored by the example of ambient air pollution, which is thought to have accounted for about three million deaths in 2012 according to a WHO estimation.[14,15] Although these statistics also comprise the adverse effects of other air pollutants, a carcinogenic effect was attributed to nano-sized carbon-core particles in the exemplary case of diesel engine emissions.[16] With the ongoing elucidation of the toxicological mechanisms of particulate matter, some nano-specific regulations have already been implemented, *e.g.*, for cosmetics,[17] food

German Federal Institute of Risk Assessment (BfR), Department of Chemical and Product Safety, Max-Dohrn-Strasse 8-10, 10589 Berlin, Germany.
E-mail: Andreas.Luch@bfr.bund.de; Tel: (+49) 30 18412-4538

contact materials,[18] and biocides.[19,20] Nevertheless, the relevance of the "nano" dimension for adverse health effects remains questionable.[21] The current knowledge indicates that for granular biodurable particles without known specific toxicity (GBPs), such as carbon black nanoparticles (CBNPs)[22] or titanium dioxide (TiO_2),[23] dust overloading of the lungs represents the main mechanism for carcinogenicity. However, the clearance of GBP materials from the lungs may differ between individual substances as recent results with barium sulfate ($BaSO_4$) demonstrate.[24] A toxicological risk assessment requires not only the consideration of particle size but also careful attention to other NM characteristics which may impact the dissolution in the lysosomes of pulmonary macrophages, and thereby the retention in the lungs upon long-term inhalation. Although some *in vivo* studies indicate that ENMs are able to cross biological barriers, such as the pulmonary air–blood barrier, more efficiently than the corresponding bulk materials,[7,25] other data dissent such a difference.[26] The fact that most materials with a primary particle size in the nanometer range are inhaled as micrometer-sized agglomerates, formed due to van der Waals forces,[27–33] further adds to the observation that NMs generally behave like fine dusts, *i.e.*, particulate matter 2.5 (PM2.5). So far, it is still subject to debate whether deagglomeration takes place under physiological conditions. Another uncertainty is represented by a potential *in vivo* dissolution of NMs. While, for instance, cerium oxide (CeO_2) exhibits a low solubility in physiological media *in vitro*,[34] dissolution *in vivo* was shown to be significantly higher.[26,35,36] Additionally, differences in the physiological properties of different cells (*e.g.*, acidity) may also have an effect on NM behavior. Up to now, no standard protocol is available for the general solubility testing of NMs. The translocation of NMs to secondary target organs such as the liver, heart, spleen, or kidney has been reported to occur subsequently to pulmonary uptake.[37,38] However, it remains uncertain whether particles are transferred as such or in dissolved form with a subsequent *de novo* particle formation upon arrival at the final destination.[39–41] The toxicokinetics of the inhaled NMs, including total uptake, biodistribution and the dose received per organ, is therefore of paramount importance for a reliable risk assessment and has been addressed in several recent publications.[42,43] This review is focused on the factors determining the toxicokinetic behavior of airborne ENMs. The procedures for a thorough characterization of ENM properties and exposure scenarios, which are an indispensable prerequisite for the preparation and evaluation of toxicokinetic studies, are presented as well. Furthermore, recently developed *in vitro* systems for biokinetic studies, aimed to investigate toxicological parameters under physiological conditions, are described. The elucidation of toxicokinetic mechanisms shall help to understand why certain ENM properties exert toxicological effects. Besides the identification of potential hazards, this information may contribute to group ENMs in order to achieve a more comprehensive risk assessment.

2. Nanomaterial characteristics and exposure

2.1. Characteristics

Historically and based on their different origins, nano-sized materials are often discriminated into intentionally produced ENMs and UFPs, the latter of which are incidentally generated.[44] For a thorough exposure and toxicity assessment of ENMs, the discrimination between both is required. The understanding of the complex features of ENMs, evolving with their increasing applications and different surface coatings ranging from inorganic/organic layers over polymers to specific biological molecules, is of utmost importance to comprehend their life cycle and fate as well as to assess specific responses observed in toxicological investigations.[45] In the future, attention regarding a potential hazard should also be focused on fibres[46] since there are hints on the potential carcinogenic effects of some of these materials.[47] Fibers can be rigid, rod-like or more flexible bundle-like structures and are usually described by their aspect ratio. Multi-walled or single-walled carbon nanotubes (MWCNT/SWCNT) or asbestos are examples of this morphology.[48] Similarly, nanoplatelets exhibit a significantly increased aspect ratio.[49,50] Besides their shape, ENMs exhibit further physico-chemical properties which need to be characterized as they are thought to determine the material-specific hazard. The relevant physical properties include size, specific surface area, agglomeration or aggregation, surface morphology, crystal structure and solubility in different media. The chemical properties on the other hand comprise impurity profile, reactivity, surface charge, zeta potential and particle stability.[51,52] An overview of ENMs and their properties with regard to risk assessment is provided by Stefaniak *et al.*[52]

2.2. Routes of exposure

Considering the different routes of ENM exposure—dermal contact, ingestion and inhalation—inhalation is regarded as the main route of concern.[53,54] In the following, we describe three main areas as potential sources for inhalation exposure of humans:

Workplace: Exposure to airborne ENMs is of most relevance in the field of occupational health. The possible events include production, handling and transport of ENMs, during the development of nano-enabled products and also during work processes with ENM-containing products. The examples for activities with possible particle release are any handling of powders, embedding materials into matrices, vacuum cleaning, wet milling, filtration, weighing of materials, *etc.*[55] Knowing the process leading to ENM exposure is of high relevance as it may drastically influence the exposure characteristics. Kuhlbusch *et al.* give an overview about ENMs relevant for work-related activities, which include, besides others, carbon black (CB), CNTs, fullerenes, MWCNTs, silver (Ag), silicon dioxide (SiO_2) and metal oxides like TiO_2, CeO_2 or aluminum oxide (Al_2O_3).[55]

Consumer products: A variety of consumer products are also likely to produce respirable airborne ENMs in proximity to the user. For exposure assessment, it is of high relevance whether the ENMs are released as part of the intended use. Exposure to airborne ENMs may occur through the use of sprays and powders, mostly applied in cosmetics, cleaning or care products.[56–60] Nevertheless, different authors showed that "nano"-labeled spray products do not necessarily contain ENMs,[56,57] while a release of nano-sized airborne particles during application has been shown for others.[56,61] The spraying process itself was identified as the main factor determining the particle size. Propellant sprays produce significantly smaller particles in comparison with pump sprays. This indicates, on the one hand, that consumer products may produce nano-sized airborne particles even though the liquid formulation does not contain any ENMs. On the other hand, it becomes evident that the specific application procedure of each product determines the exposure scenario. Although more studies on aerosol formation by consumer products are currently being conducted,[62] the market introduction of new products and still inconclusive data call for further research.

Environment: The sources of ENMs in the environment are ubiquitous and remain difficult to identify or even to quantify. As recently reviewed by John *et al.*, ENMs may be released into the ambient air by similar processes as in occupational or daily life scenarios.[63] The release of ENMs may occur primarily at phases downstream to the production, such as particle recovery, spray drying or milling. Furthermore, leakage during the production may introduce particles into the environment.[63] ENMs may further be accidentally released into ambient air during the handling and transport of particles. Furthermore, emissions may occur during the application of products containing or releasing ENMs, either by the intended use itself or through abrasion by material degradation under various ambient influences.[64–66] TiO_2, for example, may be released from façade coatings, or during waste treatment such as recycling, landfill disposal, or incineration.[67]

Further sources of airborne ENMs in the environment are exhaust gas catalysts which are often produced with nano-sized metal oxides such as CeO_2.[68,69] Keller *et al.* estimated that the release in ambient air represents less than 1.5% of the total global ENM production, the smallest share of ambient emissions compared to disposal in landfills or into water.[70] Nevertheless, due to the particular risk of adverse effects due to particle inhalation, the significance of this process should not be underestimated.[63] In ambient air, however, UFPs arising from traffic and industrial emissions, domestic heating, but also from the formation of precursor gases from volcanic activities or vegetation still represent a considerable amount of nano-sized particles.[71–73] The overlapping size range of ENMs and UFPs therefore complicates their characterization. ENM exposure is the least researched route with respect to exposure measurements and assessment and still poses a challenge due to its comparably low concentrations and limited analytical methods. Thus, the discrimination between ENMs and background particles which has to be ensured for ENM exposure and toxicity testing still represents an outstanding issue.[55]

2.3. Metrics for exposure assessment

A valid assessment of ENM toxicity requires an exposure assessment and, in preparation thereof, the definition of the most relevant exposure metrics to measure. As reviewed by Tsai *et al.*, particle mass was originally used for exposure assessment in working places, and is currently primarily considered in the context of statutory regulations.[74] The benefit of this property is on the one hand its stability during the life cycle, from release to uptake. On the other hand, all other regulations on chemicals are also based on mass concentration. Due to the ratio of size to mass, however, it is subject to debate to what extent bulk mass concentration is applicable for ENMs.[54,75–77] Particle number and surface area were suggested as more suitable indicators. These metrics, in contrast, may change during the life cycle, making the linkage from release to exposure more difficult. For occupational exposure assessments, advanced measurement strategies have been established during the last 10 years, as proposed, *e.g.*, by the National Institute for Occupational Safety and Health (NIOSH),[78] the Organization for Economic Co-Operation and Development (OECD),[79] the International Organization for Standardization (ISO)[80] or Brouwer *et al.*[81] The tiered approaches suggest the gathering of information about a specific ENM use or an exclusion of ENM release as the first step. If ENM release is anticipated, an increased concentration of the nano-sized material has to be ascertained and differentiated from any background dust originating from the work space or ambient environmental sources (step 2). The strategies for a detailed background characterization have been summarized by Kuhlbusch *et al.*[55] and include the measurement of time series (temporal approach), discrimination by comparing a place representative for background concentration with an ENM exposed area (spatial approach), comparative studies with and without ENMs, and a size resolved chemical and/or morphological analysis.[78,82–86] Once a significant release has been recognized, the monitoring of further metrics like particle number, particle size distribution, or surface area concentration is proposed (step 3).[79,87] Aerosol sampling for chemical, morphological and gravimetric analysis complements the online obtained instrumental data. To date, no clear evidence for a step change in hazardous properties relating to the nano dimension of a particle has been revealed.[88–90] Therefore, the discussion about the relevance of other physicochemical parameters such as shape, surface reactivity, and solubility as described above is still ongoing.

After identifying the valuable metrics for exposure assessment, it is important to apply suitable measurement techniques in order to gain the required information. As each analytical technique has its limitations, different measuring principles should be applied—if possible in combination—to gain comprehensive insight into the characteristics of an aerosol.[55,91] For a thorough particle characterization, the measurement of particle sizes, mass size distribution, number

concentration and surface area concentration is required. In order to retain particles for further characterization, a sample of the aerosol should be retained. A listing of suitable instruments for aerosol characterization is given by Maynard *et al.*[91] and Plitzko *et al.*[92]

As the concentration and nature of aerosols can alter over time and space,[55,56,93] the ENM characteristics in terms of size, shape, composition or solubility may differ. Therefore, the characterization of the aerosol fate is an essential prerequisite for exposure and toxicological assessment.[94] The site of ENM exposure remains a critical issue. The latest developments have therefore moved towards the application of personal monitors to monitor and assess the aerosol a worker is actually being exposed to.[91,95,96]

Since workers are more likely to be exposed to ENMs compared to consumers, more effort has been directed to the exposure assessment in occupational environments. Nevertheless, there are several studies that have determined the exposure to ENMs from consumer products such as sprays.[57–59,61,97,98] An approach suitable to evaluate toxicity has to consider specific release scenarios and the life cycle of ENMs for a realistic exposure assessment. For a further investigation of ENM exposure in daily life scenarios, it might be reasonable to adopt testing approaches that are already established for workplace monitoring.[97]

2.4. Exposure conditions for toxicity testing

For the evaluation of ENM toxicity, the application of a realistic dose is crucial.[21] The results on the adverse effects of ENMs were obtained in a number of studies at unrealistic high doses.[77,99–102] Furthermore, toxicological studies on ENMs should be performed with nano-sized particles in order to investigate a potential size-related effect. In most situations, ENMs will rapidly form aggregates and agglomerates.[94,103] Thus, depending on the type of exposure scenario, *e.g.*, local or temporal variation, exposure to larger agglomerates, as, for example, achieved by dry particle dispersion, might provide more realistic results.[104] Additionally, the agglomeration behavior is strongly influenced by the environment in which a particle is released.[105] As a consequence thereof, the toxicological properties of a material might be influenced by the respective medium, *e.g.*, workplace or wet room air. The aggregates and agglomerates of ENMs are not necessarily stable in case a change of the external environment occurs. Such a change may trigger disaggregation or de-agglomeration of particles, leading to an altered material deposition in the respiratory tract. Therefore, the specific environmental conditions have to be considered for exposure and toxicity assessments. Generally, the employed aerosolization techniques, such as brush dust feeding, spark discharge, or nebulization systems, produce aerosols exhibiting different characteristics, such as agglomeration state or liquid constituents. In addition, the kind of aerosol generation impacts on particle size and characteristics as discussed elsewhere.[106] A large number of studies exist describing the use of aerosol generators fitting for the respective study design which depends on the desired ENM size, their state of mono-/polydispersity or degree of humidity.[107–109] When generators are not able to produce the desired aerosol characteristics, combined methods have to be used, *e.g.*, the addition of a differential mobility analyzer to obtain a monodispersed aerosol.[109] The generating mechanisms typically used are nebulization, atomization, electrospraying, and brush dust feeding.[59,107–112] Aerosol generation from nanofibers and nanotubes may require particular consideration. More detailed information on fiber generation has been reported by Oberdörster *et al.*[111] and Polk *et al.*[112]

For the investigation of ENM fate, the influence of other substances on material alteration needs to be considered. This aspect is necessary for assessing their potential effects as human exposure to ENMs hardly occurs without alteration by any chemical substance and might lead to significantly changed characteristics. Substances causing ENM modification may originate either from the products they are used in, *e.g.*, as sprays, or from reactions in the atmosphere. The evaporation of the droplets with suspended ENMs during the use of "nano" sprays, for example, is often a rapid process depending on the droplet composition. It has been suggested that evaporation can lead to agglomeration of the ENMs within the droplet, forming larger particles with a different physical and physiological behavior.[59] This may also cause other constituents to dry on the particles' surface. This carrier mechanism, also called "Trojan Horse" mechanism,[113,114] may carry substances *via* ENMs into compartments that are not reached in the absence of these materials. It is therefore important to understand the interplay of particulates and dissolved substances in droplets of liquid aerosol clouds in order to assess potential risks to humans.

It can be summarized that the characteristics and exposure conditions for ENMs have a major influence on their toxicological behavior. Therefore, a thorough characterization of the exposure atmosphere is required to allow conclusions on possible toxicological effects.

3. NM grouping

As the use of NM grows and the variety of their modifications increases even more, complete testing of each and every individual NM regarding possible hazards would be an enormous and almost unending endeavor. For chemicals, in order to facilitate and economize risk assessment – in addition to reducing unnecessary testing – the grouping of substances is already a well-established procedure.[115,116] Due to their more complex characteristics, such grouping is still under discussion for NMs. For them – in contrast to chemicals – more diverse compositions occur, exhibiting various size distributions, shapes, agglomeration/aggregation states or surface modifications. In addition, these characteristics can change over time while a NM ages or is exposed to different environments which may, for example, influence the ENM surface. To nevertheless apply a classification system to NMs, several concepts have been suggested. These are, for example, based on

the NM's morphology (*e.g.*, size and shape), its chemical composition (*e.g.*, metal and metal oxides), its biokinetics and persistency (*e.g.*, solubility, uptake, and fate), its modes of action, *i.e.*, the observable, secondary effects (*e.g.*, frustrated phagocytosis) or its underlying mechanism (*e.g.*, toxic release and Trojan Horse effect), or its source-to-adverse-outcome-pathway (SAOP).[117–119] So far no decisive strategy has been established as no NM characteristic has been identified as the sole cause for the observed toxicological effects. Instead, these approaches overlap reflecting the connection of the physico-chemical properties or the toxicological effects, as the NM solubility and persistence, for instance, are inevitably related to their chemical composition but also to any surface modification. This interdependency adds to the complexity of identifying and categorizing the NM characteristics which are already impeded by the NM diversity and their aging processes. Therefore, grouping strategies using tiered approaches and the separation of the NM characteristics into intrinsic, system-dependent, and composition-related properties are suggested.[120,121] The assessment and mapping of NMs according to these factors in combination with computational analysis, *i.e.*, principal component analysis (PCA), may eventually be able to help select categories and sort the NMs.[122]

Looking at the toxic effect of a NM, generally four groups of underlying mechanisms can be seen which are also reflected in the aforementioned grouping approach criteria and caused by (a) the contact with the NM and are determined by its physical properties (*e.g.*, shape, volume and surface), (b) the dissolution behavior, *i.e.*, the release of toxic chemicals of the NM, (c) the carrier mechanism, *i.e.*, the transportation of toxic substances adsorbed on the NM's surface into otherwise inaccessible regions and (d) the biological/toxic effect of the NM itself or its coating. Additionally, NMs can exhibit several of these effects in combination. In order to develop risk assessment that is capable of handling the immense amount of NMs – including their various modifications – the link between mode of action, underlying mechanism, and physico-chemical characteristics has to be elucidated. As inhalation is a likely exposure route in the day-to-day routine, focus should be put on airborne NM and especially ENM utilized in consumer products, *e.g.*, in sprays or powders. To limit the scope of this work, we therefore mainly focus on airborne/inhaled ENMs and provide an overview of ENM characteristics and the related toxicity.

4. Influence of particle characteristics on toxicity and toxicokinetics of inhaled nanomaterial

The elucidation of particle biokinetics following inhalation is pivotal in order to understand NP toxicity.[123] For airborne ENMs and particulate matter in general the human respiratory system with its large surface area and relatively thin air–blood barrier represents a potent entry into the body and secondary organs *via* the blood circulation. As daily exposure to airborne particles has occurred since the early days of human evolution, protection and clearance mechanisms against such substances have evolved in the different parts of the respiratory system.[124] Depending on the ENM characteristics these defenses are more or less effective and the deposition, uptake and fate of the inhaled material can be influenced.

4.1. Influence of size and shape on ENM deposition and clearance

Besides, of course, the anatomic structure of the specific respiratory system, the aerodynamic properties, *e.g.*, mass, aerodynamic diameter, and shape, are the main factors determining the mechanisms for the deposition of inhaled NMs (see Fig. 1).[125–127] The elucidation of these mechanisms and the related NM characteristics is therefore essential to understand and possibly forecast the deposition of NMs and derive potential risks.

The influence of these physical properties as primary characteristics is evident by following the human respiratory pathway from the nasopharynx *via* the tracheobronchial regions into the deeper lungs. First of all, material with main aerodynamic diameters between 5 and 10 μm is deposited *via* the mechanism of impaction in the nasopharyngeal region, comprising the nose, mouth, pharynx, larynx, and olfactory bulb.[128–132] Impaction, which to a lesser extent also occurs in the subsequent regions, affects these NMs as they are incapable of following the airstream within the airway – due to their inertia – and collide with the walls.[129,133] ENMs that are able to pass the nasopharynx traverse the tracheobronchial region with its increasing number of branches and finally reach into the bronchioles. Here, primarily ENMs with aerodynamic diameters between 1 to 5 μm are deposited by sedimentation processes which generally affect particles in more

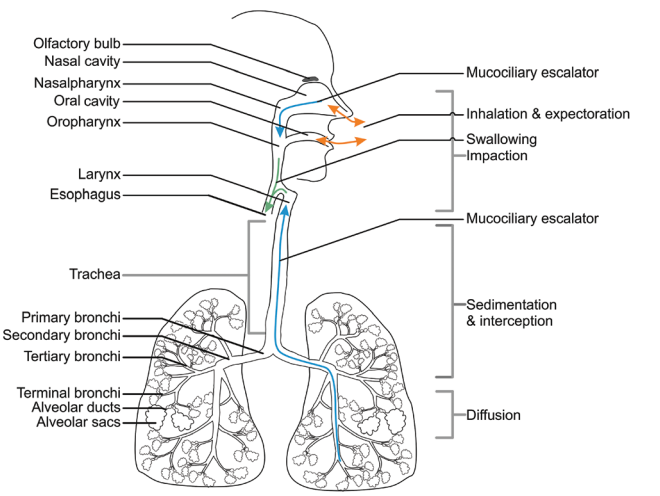

Fig. 1 Schematic view of the human respiratory system and depiction of the mechanism involved in the inhalation, deposition and clearance of airborne ENMs.

horizontal, narrower segments with lower airflow velocities by gravitational pull.[129–131,134] Smaller ENMs will be carried further and ENMs with diameters between 10 and 20 nm will eventually be deposited in the alveoli *via* a diffusion mechanism caused by minimal gas flow, Brownian motion, and collisions with air molecules. Considering non-spherical ENMs, the deposition also depends on their shape. ENMs with at least one elongated dimension, so-called high aspect ratio nanoparticles (HARNs), will – depending on the airway – collide with the airway walls in an interception mechanism despite being theoretically small enough to remain airborne. In conclusion, their physical properties, especially aerodynamic diameter and aspect ratio, influence the ENMs' ability to penetrate further into the respiratory system and thereby render them possible to deploy any toxic potential there. The place of deposition and therefore their physical characteristics can also influence any subsequent ENM clearance. This includes nose-blowing or – if the ENMs were deposited further in the respiratory system – transport towards the nasopharynx and subsequent swallowing or expectorating. This transport from trachea, bronchi or bronchioles – the mucociliary escalator – is based on the respiratory epithelium and its lining fluid produced by its goblet cells.[135] The deposited material is adsorbed and transported by the beating of its ciliated cells to the pharynx. The speed of the transport varies depending on the physiological conditions and activity, and is fastest in the upper respiratory system. Depending on the place and deposited material, the clearance from the nasopharynx alone, for instance, can result in clearance half-times from tens of minutes to several hours.[132,136]

4.2. Influence on macrophagic phagocytosis and overload

As no ciliated cells – and therefore no mucociliary escalator – are present in the alveolar region, the deposited ENMs may only be removed by solubilization or macrophagic phagocytosis.[137,138] In the latter case the macrophages may in turn be mobilized and subsequently cleared which again depends on the ENM properties. The macrophagic phagocytosis which comprises the stages of particle recognition, attachment, and phagocytic uptake[139] was found to be influenced by the ENM size, their morphology, their dimensions and their agglomeration state.[45,140] The observed size dependency was connected to the first stage, the particle recognition stage. Particle size and agglomeration state dependency was observed in *in vitro* as well as *in vivo* studies but no definite conclusion could be drawn yet. While Stearns *et al.* found a time-dependent internalization of ultrafine, almost exclusively agglomerated TiO_2 NPs (50 nm) into A549 cells,[141] Geiser *et al.* reported only low levels of phagocytosed, mostly non-agglomerated TiO_2 NPs (20 nm) after inhalation by rats.[142] The reasons for this may be the differences in the models studied, *i.e.*, *in vivo vs. in vitro*. Besides size and agglomeration, the ENM surface is also a factor to influence the steps of phagocytosis. Therefore, changes, such as any coating or any corona surrounding the ENM, impact any subsequent cellular reaction. The formation of a protein corona, for instance, represents an important post-manufacturing change to ENMs which is related to the inhalative process and can increase macrophagic phagocytosis.[143] Further investigations of the protein corona formation during inhalation showed that although a large total quantity of proteins is bound, most of them can only be found in low numbers.[144] This can lead to an enrichment of low abundant proteins and may equalize the influence of other ENM features on their cellular uptake. This is of special importance regarding the applicability of intratracheal instillation tests.[145]

Certain conditions, occurring above a certain particle load, can reduce mobility and macrophage-related clearance as was first reported by Morrow in 1988.[146] Under these conditions, an impairment of mucociliary clearance causes particle accumulation in the lungs linked to elevated particle distribution to lung-associated lymph nodes. Several studies with regard to clearance under these so-called overload conditions in humans were conducted and resulted in retention half-times between around 100 and over 700 days.[147–149] In animal experiments, retention half-times up to 240 days after long-term exposure under overload conditions were reported for rats.[150] In addition to impaired clearance, secondary effects such as inflammation, fibrosis and cancer may be induced.[17] Initially, volume- or mass-/concentration-based approaches including various thresholds were suggested and were, for instance, based on the hypothesis that macrophage mobility is largely impaired above 6% and completely stopped at more than 60% of the volumetric particle load for the rat lung.[146] In opposition to these, the total particle surface area was suggested as an appropriate metric for the measurement of overload conditions by others.[151,152] In this approach, a threshold of 0.02–0.03 m^2 nanoparticle surface area per gram lung is assumed based on the relation between polymorphonuclear cells (PMNs) and lung burden.[152] In addition to particle volume or surface area, a small particle diameter seems to facilitate lung overload. The retention half-life of 500 days for 20 nm TiO_2 NM in the lungs was found to be significantly higher compared to that of 250 nm TiO_2 NM, having a retention half-time of 170 days.[153] Furthermore, there was stronger inflammation observed with the small particles in contrast to the larger ones. Studies on other nano-GBPs such as carbon black, TiO_2, polystyrenes, cobalt (Co), and nickel (Ni) are in support of this.[154] Overload conditions may also influence the clearance of other contaminants like microorganisms and different studies indicate that in situations in which the alveolar macrophages are already loaded with UFPs or ambient particles, the clearance and killing of microorganisms, *e.g.*, *Streptococcus pneumoniae*, is impaired.[155–157] A reduced internalization probably caused by increased oxidative stress is assumed to be the reason. The NM shape was also observed to strongly impact the phagocytic clearance. When a particle cannot be successfully engulfed by macrophages, for example, HARNs, any further clearance step is hindered. Furthermore, this inability to encapsulate these particles can lead to an increased toxicity *via* frustrated phagocytosis as explained later.

4.3. Translocation from the pulmonary region into secondary organs and subsequent effects

If not being cleared, ENMs can cross the pulmonary barrier, reach the blood stream and therefore be translocated into secondary organs.[7,158] This again can depend on their particle size, surface area, or surface charge, as was recently, for instance, shown for gold (Au) ENM.[159] Furthermore, the retention in secondary organs was also found to change and different results depending on the method of ENM administration, *e.g.*, intratracheal instillation or intravenous injection, indicate an effect of differences in protein corona formation. Secondary organs such as the liver, kidney, brain, or cardiovascular system may subsequently suffer over time. Adverse effects as a result of such NP translocation and respective retention have been associated with, for instance, several neurodegenerative pathologies like Alzheimer's and Parkinson's disease, or simply with physiological aging.[160] On the other hand, ENM clearance *via* the mucociliary escalator as well as ingestion by grooming in animal testing leads to material translocation to the nasopharyngeal region and enabled subsequent swallowing.[161]

4.4. Differences in nanomaterial solubility and impact on their fate and toxicity

As mentioned before, nano-sized materials can generally be divided into soluble and poorly soluble. And although solubility as a property of the inhaled, deposited ENMs was not discussed so far, it has to be taken into account when their fate and toxicity are assessed.

In the case of airborne ENMs, any dissolution after inhalation and deposition in the respiratory tract naturally affects the ENM persistence and subsequent fate in the organ and entire organism.[162] Besides the aforementioned mucociliary clearance and following excretion, the dissolution of the deposited ENMs therefore contributes to clearance from the respiratory tract. Dissolved material can access the blood stream and eventually the body.[163] Depending on the solubility, the International Commission on Radiological Protection (ICRP) proposed three different classes to categorize particulate matter which differ by their pulmonary clearance in humans: soluble material exhibits a retention half-time of less than 10 days, partly soluble matter has a retention half-time between 10 to 100 days and poorly soluble material has retention times over 100 days.[164] The European Center for Ecotoxicology and Toxicology of Chemicals (ECETOC) proposed a dissolution half-time in artificial lung fluids slower than any macrophage-mediated clearance as the definition for poorly soluble particles (PSPs) in the respiratory tract.[162,165–169]

Besides clearance and fate, the dissolution also impacts the toxicity and several elements used in ENMs are known to be toxic in ionic form, *e.g.*, Ag, zinc (Zn), or copper (Cu). Dissolution can therefore also induce toxicity based on the release of the ionic, toxic constituents as shown for various ENMs. The effects of indium (In) containing ENMs, for instance, which are commonly used in microelectronics and are associated with increased In body fluid levels as well as the development of interstitial lung diseases in workers,[170] were elucidated by Gwinn *et al.*[171] The underlying mechanism based on phagocytosis and subsequent dissolution in the macrophage media was identified by inhibition of either the internalization or the phagolysosomal acidification. Reduced toxicity in both cases could be related to the blocking of phagocytosis in the former experiment, while ENM uptake still took place in the latter and decreases in toxicity could thus be attributed to an inhibited dissolution. For zinc oxide (ZnO) nanowires, a macrophage-mediated dissolution after phagocytosis was also found and resulted in toxicity similar to that of ionic zinc.[172] The cause of the ZnO nanowire toxicity was therefore concluded to be related to this pH-dependent dissolution rather than their high aspect ratio. Such intracellular ion release after ENM internalization and induction of cytotoxicity and oxidative stress was also proved for normally low-soluble Co-containing particles in BEAS-2B cells.[173] It was demonstrated that a much higher total cobalt amount was found after treatment with particular Co_3O_4 than with soluble $CoCl_2$, the toxicity indicating a more rapid internalization for the ENM. Additionally, based on similar quarter maximal inhibitory concentrations (IC_{25}) observed in both cases, the toxic effects are suggested to be dominantly related to intracellular dissolution. Despite being insoluble in the physiological pH environment, these nano-sized materials can therefore, after being dissolved in macrophages, still induce toxicity or reach the blood stream. These observations, *i.e.*, a potential stronger endocytic ENM uptake in combination with toxic effects after intracellular solubilization, also underline again that the nano-sized character may affect the toxicity without directly causing it.

Dissolution as well as its speed are generally influenced by a variety of factors which are related either to the ENM characteristics (*e.g.*, size, chemical composition, and surface chemistry) or – as seen in the aforementioned examples – to the properties of the respective location (*e.g.*, solvent and pH).[174] While a theoretical connection between ENM size and dissolution generally applies, the process of dissolution is complex.

Regarding the impact of ENM properties, the aforementioned size-dependent dissolution was, for instance, investigated for Ag ENM for epithelial and macrophage cells, and increased dissolution as well as subsequent toxicity were reported for smaller ENMs.[175] Additionally, the surface coating was also scrutinized and a reduced dissolution for polyvinylpyrrolidone (PVP)-coated NMs was seen compared to citrate-coated Ag ENMs. Further ENM-related aspects like morphology and curvature, agglomeration state or surface modification by adsorbed species or ENM coatings can affect the ENM simultaneously and have opposing effects. Due to all influencing factors, predictions are hard to make but the following trends can generally be seen as long as only one aspect is examined at a time.[176] Dissolution of smaller ENMs is faster. Positively curved (convex) structures exhibit a higher solubility than

negatively curved (concave) ones, which is connected to the ENM morphology and their surface porosity. A higher degree of aggregation results in lower solubility. These observations are based on aspects like diffusion layer and surface area as well as thermodynamic properties. Furthermore, adsorbed molecules on the surface can function as a hindrance or promoter of dissolution. This last feature illustrates that EMN characteristics and the properties of the site in question are highly connected, as the adsorbed species depend on the environment and the pathway the ENM traversed. The formation of a protein corona is a good example as it – by itself – depends on the ENM characteristics, especially several surface-related properties, *e.g.*, surface charge, curvature or chemical surface modification, but is also influenced by the proteins present at the site of exposure.[177] Then again, it influences the subsequent ENM behavior and can, for instance, change the following cellular uptake, dissolution and eventual toxicity. This linkage and its complex impact on any resulting toxicity for inhaled ENMs were recently again demonstrated for ZnO nanowires. Their interaction with human epithelial cells has been investigated with regard to an adsorbed, natural pulmonary surfactant.[178,179] Although a higher ENM internalization into the cells was observed for nanowires that had adsorbed the surfactant, a reduced dissolution speed was found. Although an initial toxic response was therefore decreased due to this reduction, the elevated uptake eventually led to an overall increased toxicity.

As mentioned before, the site of dissolution and the related environment the ENM passed before are reflected in the parameters that also affect the ENM dissolution, and any subsequent fate as well as toxicity. These parameters, *e.g.*, solvent, pH, possible adsorptions on the surface, and already existing constituent concentration, vary between species, organs and cells.[163,180,181] For instance, varying macrophage-mediated dissolution rates for different species were found in a comparison between humans and canines for cobalt oxide (Co_3O_4) particles,[182] but similar capacities were found in humans and rabbits with regard to manganese oxide (Mn_2O_3).[183] For In-containing ENMs, a difference between cell types could also be observed as only macrophages, but not epithelial cells, exhibited more efficient dissolution and strong toxicity after ENM internalization.[171] For Ag ENMs, the analysis even revealed different dissolution rates and toxicities between cell lines of the same cell type, *e.g.*, epithelial cells.[184] In the case of the aforementioned corona formation, a relation to the exposure route is evident. Any already dissolved ENM material, on the other hand, is connected to the cell's history, Similarly, other intracellular conditions, such as pH, are related to its current status.

The complexity of the dissolution behavior which depends on a variety of ENM-related characteristics and conditions based on the environment and dissolution site is schematically shown in Fig. 2. The fact that some ENM characteristics can have an impact on the clearance and fate, while in turn being influenced by the ambient cell, underscores the need for detailed investigation.

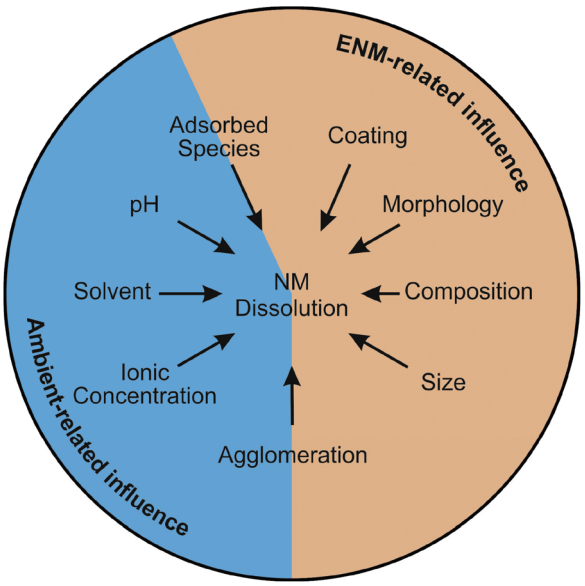

Fig. 2 Parameters influencing NM dissolution, which are related to the ENM characteristics (right side) or related to the conditions of the system.

4.5. Species extrapolation

Besides being impacted by the NM characteristics, deposition as well as any following clearance are also influenced by the respective organism. This is evident in their unlike anatomy, which results in differences between animals, *e.g.*, rats and mice, and humans, for instance, regarding the airflows in the nasopharyngeal region or in the respiratory system. For this reason, findings such as the translocation into the olfactory bulb reported for ZnO-NPs in rats,[185] or brain damage observed in canines due to the translocation of UFPs *via* the olfactory bulb[186,187] need to be cautiously considered with respect to any human toxicological risk assessment. Furthermore, in a comparative inhalation study between rats, mice, and hamsters by Bermudez *et al.*, species-specific distinctions were also found regarding the clearance under overload conditions.[188] They observed a more effective clearance for hamsters and a more severe toxicological impact on rats. Sayers *et al.* also recently investigated the different depositions of micro- and nano-sized C_{60} particles after 13 weeks of inhalation by rats and mice with identical concentrations.[189] While similar retention half-times were observed for both C_{60} particle sizes in mice, in rats the nanomaterial resulted in slower clearance kinetics compared to the micro-sized material. Generally, these differences between species regarding anatomy, clearance, and any resulting response and toxicity, reported elsewhere,[190–194] have to be considered for toxicological studies. This underscores the importance of the appropriate tests and questions if, for instance, overload conditions – observed for rodents – are equally applicable to humans.[165,195] In particular, rats seem to be more susceptible to NP exposure in terms of inflammatory responses and tumor formation.[196] This discussion of whether or not interspecies comparison is

applicable for inhalation toxicity is ongoing[197] and bioassays as well as mathematical models try to overcome the difficulties to allow interspecies extrapolation.[198,199] Therefore, the application of the appropriate test system is pivotal to correlate the data with human exposure and risk assessment, and recent advances in the development of such systems will be presented later on.

4.6. Toxicity/modes of action

As indicated by the highlighted linkages between ENM characteristics and their behavior, the toxicity of ENMs is not only connected to their nano-sized property but instead affected by a variety of characteristics. These can, as was stated before, be categorized into intrinsic, system-dependent and composition-related properties. Related to their route of exposure, dissolution, clearance, and possible translocation, nano-sized materials can cause local as well as systemic toxicity, and epidemiological studies have, for instance, linked environmental UFPs to increased mortality.[200,201] As mentioned before, dosimetric considerations of NMs changed from the classical view of bulk mass or concentration to, for instance, surface area as the metric. Due to the observation that the mass dose is only a surrogate for the part of a particle dose that actually induces toxicity, the term biologically effective dose (BED) was adopted for nanotoxicology.[162] The term was originally established in radiobiology[202] and in nanotoxicology refers to that toxicologically active fraction of the retained dose that really induces an adverse effect. This shift puts the focus on the respective individual NM characteristics as the cause for the observed toxicological effects, *e.g.*, the long, biopersistent fibers within the overall inhaled asbestos. The understanding of the underlying mechanisms enables a better risk assessment and can allow the development of optimized, less toxic ENMs, *i.e.*, safer by design. The addition of polystyrene coatings to CNTs, for instance, was shown to be able to reduce their toxicity while keeping the structure intact.[203] But the elucidation of the coatings and their diverse effects is still ongoing as was observed for the coating of CBNPs with benzo[*a*]pyrene, 9-nitroanthracene, or other polycyclic aromatic hydrocarbons (PAHs).[204] While the first two lowered the ROS release in various cell lines, likely due to the reduced surface area, the last one led to an increased toxicity *via* apoptosis. Together, this indicates that the biological effect of these coated CBNPs is connected to the interplay of the surface area reduction and the toxicity induced by the coating itself. This complexity of the ENM properties and their relation to a toxic response illustrates that appropriate test systems are required which do not only address the "nano"-size characteristics.

Therefore, the different modes of action and mechanisms for NM toxicology have to be analyzed and assessed. The complexity of this task is further increased when looking at oxidative stress, for example. Its formation in response to ENM treatment has been observed for a variety of nano-sized materials[205,206] and is regarded as the imbalance between the incidence of the so-called reactive oxidative species (ROS) and their depletion by antioxidant processes and reagents. ROS are always present under physiological conditions and normally well regulated in cells. While different reactions are responsible for causing such imbalances, their mechanisms can be categorized into (I) an increased formation of ROS directly due to the NM introduced into the cell, (II) a more indirect way by affecting the mitochondria which are involved in cellular respiration, or (III) quenching of cellular antioxidants.[207] This abnormal increase of ROS after NM internalization is considered to be the primary nanotoxicological process leading to increased oxidative stress and responsible for various secondary pathophysiological effects.[208] The importance of oxidative stress also becomes evident in the possible generation of DNA damage accompanying an inflammatory reaction triggered by inhaled ENM.[209,210] One example is the aforementioned, so-called frustrated phagocytosis, *i.e.*, the impaired clearance of HARNs from the alveolar region by macrophagic phagocytosis. As these elongated NMs cannot be completely internalized and processed by the macrophages and their phagolysosomal agents, an increased production and release of toxic agents into the environment ensues. This release from affected macrophages includes ROS which in turn can induce an elevated inflammatory reaction in the affected area. This effect is not only governed by the length of the NM and the respective organism's capacity but also influenced by their specific morphological properties. For instance, while MWCNTs generally can also induce frustrated phagocytosis,[48] curled MWCNTs were shown to be rather cleared instead.[211] On the other hand, NM-mediated toxicological effects are often associated with genotoxicity, either directly or indirectly. Direct genotoxicity may occur when ENMs penetrate into the cell nucleus and directly interact with DNA or cellular proteins that are involved in chromosome segregation. And while, for instance, some TiO_2 NMs are able to induce genotoxicity, inconsistencies between various studies of aquatic organisms illustrate the complexity of the effect and the assessment itself.[212] Indirect genotoxicity, on the other hand, may occur when the ENM in question causes imbalances of the cell's oxidative state, for instance, by depletion of cellular antioxidants,[213,214] which again can cause DNA damage.[215]

5. Test systems for nanomaterial inhalation

As stated before, numerous ENM and ENM modifications need to be assessed and—even considering the aforementioned grouping approaches—the required amount of tests necessitates the use of new and alternative test systems. Besides the use of test animals for the investigation of toxicological effects and elucidation of the respective ENM characteristics, the development of novel *in vitro* models has therefore become an important endeavor in toxicology research in recent years.

Besides the choice of test animal, it is – as discussed before – crucial to develop an *in vitro* system which is able to mimic the complex *in vivo* situation as closely as possible by consider-

ing the different mechanisms affecting nanotoxicity. This challenge becomes very difficult as even cells from the same origin can respond differently.[216–218] This is also known for coculture systems[219] as well as for 3D cell models.[218] The next part of this review summarizes the different cellular systems available and their application in the toxicological assessment of ENMs.

Although *in vitro* and *in silico* tests are nowadays commonly used for toxicity testing, animal experiments are still the mainstay for studying chemicals as well as inhalable ENMs.[220] A variety of technical guidelines (TGs) for such studies have been developed by the OECD, for instance, TG 412,[221] TG 413,[222] or TG 453.[223] While TG 412 addresses subacute inhalation toxicity, TG 413 gives recommendations about chronic and long-term toxicity testing. TG 453 combines chronic studies with carcinogenicity studies. All TGs recommend rats as the test animal of choice with an exposure time of 6 hours per day at 5 days per week. This is suggested for three or more different doses and one negative control group. The exposure duration amounts to 28 days and 90 days as recommended in TG 412 and TG 413, respectively, and 12–24 months in TG 453, depending on whether the chronic design is applied solely or in combination with the carcinogenicity study design. The assessment of particle translocation, particle biokinetics and particle organ burden based on TG 412 was already conducted in many projects addressing regulatory concerns.[26,224–226] TG 413 and TG 453 have so far been applied to the long-term toxicity assessment of TiO_2.[227]

Besides the inhalation studies following these OECD TGs, *in vivo* studies have also been conducted by intratracheal instillation.[220,226] Compared to inhalation, intratracheal instillation is cheaper and easier to use and allows for a more controlled application. In contrast to inhalation, where the test substance is administered as an aerosol or vapor *via* the nose, mouth, or nose and mouth, for intratracheal instillation, a bolus injection is given directly into the lungs.[225] Since the local dose, administration and possible system-dependent alterations resemble actual life situations more accurately, inhalation is the preferred exposure route for the toxicity testing of respirable substances. To address animal welfare and high costs of animal experiments, the quest for novel *in vitro* methods emerged. These shall be based on the 3R principles from Russell and Burch which stressed the replacement, reduction and refinement of animal testing.[228,229]

5.1. Exposure methods

Besides the right choice of the respective cell model, depending on the question to be answered, the type of ENM application has been the subject of discussion during the last few years. Standard *in vitro* toxicity studies were carried out under submerse culture conditions where the particles were dispersed and subsequently apically applied on the cells.[219] As these conditions do not reflect the real inhalation process where particles are airborne and not dispersed in a medium, different particle exposure methods were designed. Hence, studies now often use ALI systems[230–232] which allow the cells' exposure to ENM-containing aerosols.[233] In addition, to enable the assessment of the effects of airborne ENMs, ALI systems offer other advantages, like more realistic inhalation conditions or a reduced change of particle characteristics which lead to an exposure system closely mimicking the *in vivo* situation.[233] Thus, different ALI systems have been introduced in the last few years, the Vitrocell and the Cultex system being the primarily applied ones.[232,234–236] Because particle deposition in both systems is based on sedimentation and gravitation,[237] the deposited particle mass is relatively low.[218] To overcome this, Lenz *et al.* developed the so-called ALI cell exposure (ALICE) system which allows particle deposition rates greater than 50%.[237] In addition, exposure systems using electrostatic particle deposition[238,239] or thermophoretic forces[240] were designed to increase the deposition rate or to improve particle deposition on cells, respectively.

5.2. Single cell line *in vitro* models

As nano-sized materials are deposited in the bronchial and primarily the alveolar regions of the lungs,[131] research and development into novel *in vitro* models focused on cell lines from different origins, all of them exhibiting advantages and disadvantages (see Table 1). One of the most commonly used cell lines for toxicological investigations of inhalable ENMs is the A549 cell line which was derived from a human lung adenocarcinoma.[230,233,241–243] A549 cells were used by Demokritou *et al.* to study, for example, the toxicological effects of CeO_2 ENMs on cell lines and on animals and it was found that CeO_2 does not cause cytotoxicity *in vitro*, but *in vivo* cytotoxicity was clearly observed.[244] Other researchers, such as Mülhopt *et al.*, used this cell line and found a particle concentration-dependent lactate dehydrogenase (LDH) release after wood stove exhaust exposure which indicates a particle-related cytotoxicity on A549 cells.[233] Furthermore, Oeder *et al.* conducted a metabolomics and transcriptomics study where increased oxidative stress and proinflammatory signaling in A549 cells were observed after exposure to heavy fuel oil, while adverse effects in protein biosynthesis, cellular stress, cell adhesion, and cell junction were observed with diesel fuel.[245] Although A549 cells represent a human alveolar model, justifying the widespread use, they have the disadvantages of being of carcinogenic character and not forming tight junctions,[243,246] which sets them apart from true-to-life situations.

To overcome this drawback, the later developed BEAS-2B cell line was derived from a non-tumorigenic, immortalized human bronchial epithelia cell line by Reddel *et al.* in 1988, using an adenovirus 12-SV40 hybrid virus.[247] The functionality of its tight junctions was then investigated by Noah *et al.*[248] As they closely resemble an actual human lung, BEAS-2B cells were used in many studies to examine the cellular effects of different particles at the air–liquid interface (ALI) as well as under submerse exposure conditions.[233,249–251] Recent application fields for BEAS-2B cells cover investigations regarding the toxicity of e-cigarettes and environmental particulate matter. Oeder *et al.* showed with their metabolomics and transcriptomics study that diesel fuel and heavy fuel oil can change many cellular processes like protein biosynthesis,

Table 1 Studies regarding the development and application of different cell lines (top), of cocultures (center) and of different 3D models (bottom)[a]

Cell lines/ coculture/3D model	Origin	Characteristics	Nano-sized material	Exposure	Tested method/end points	Ref.
A549	Human lung adenocarcinoma	No tight junctions,[219] carcinogenic, model for type II[243]	CeO_2; SiO_2-coated CeO_2	Submerse	Cytotoxicity	244
			CeO_2	Submerse	TEER, cytotoxicity, inflammation, genotoxicity, oxidative stress	249
			CeO_2	ALI	Cytotoxicity, inflammation, genotoxicity, oxidative stress, gene expression	218
			TiO_2, CeO_2	Submerse ALI	Cytotoxicity, inflammation, genotoxicity, oxidative stress	219
			Fluorescein sodium	ALI	Cytotoxicity	233
			Heavy fuel oil and diesel fuel exhaust	ALI	Proteomics, metabolomics	255
			CuO	ALI	Cytotoxicity, inflammation	216
			CeO_2	ALI	TEER, cytotoxicity, genotoxicity	256
BEAS-2B	Human bronchial epithelium	Tight junctions, non-carcinogenic	CeO_2	ALI	Cytotoxicity, inflammation, genotoxicity, oxidative stress, gene expression	218
			CeO_2	Submerse	TEER, cytotoxicity inflammation, genotoxicity, oxidative stress	249
			Heavy fuel oil and diesel fuel exhaust	ALI	Transcriptomics	255
			CeO_2	Submerse	Cytotoxicity, inflammation, gene expression	257
			ZnO-NMs	Submerse	Cytotoxicity	251
			Cigarette and e-cigarette smoke	ALI	Genotoxicity	232
16HBE14o-	Human bronchial epithelium	Cilia,[258] tight junctions[259]	TiO_2-NMs	Submerse	Inflammation, oxidative	241
			TiO_2, Ag, SiO_2	Submerse	Cytotoxicity, inflammation, TEER	254
Calu-3	Human bronchial adenocarcinoma	Tight junctions, microvilli, carcinogenic	(SiO_2-coated) CeO_2, (SiO_2-coated) ZnO	Submerse	Cytotoxicity, TEER, immunofluorescence staining	253
			SWCNT, MWCNT	Submerse	Cytotoxicity, TEER	252
			clarithromycin microparticles	ALI	Cytotoxicity, TEER	260
hAELVi	Human alveolar epithelium	Tight junctions, non-carcinogenic	n.d.	n.d.	n.d.	246
THP-1 & A549	Human monocytic Human alveolar epithelium		CeO_2, TiO_2	Submerse, ALI	Cytotoxicity, inflammation, oxidative stress	219
THP-1 & 16HBE14o- & HLMVEC	Human monocytic Human bronchial epithelium Human lung microvascular endothelial cells		TiO_2, Ag, SiO_2	Submerse	Cytotoxicity, inflammation, oxidative stress, TEER	254
THP-1 & A549 & Ea.hy926 & HMC-1	Human monocytic Human alveolar epithelium Human endothelia cells Human mast cells		SiO_2 rhodamine-labeled, DEPM	ALI	Cytotoxicity, inflammation, oxidative stress TEER, fluorescence measuring of translocation	231 and 261
MucilAir™	Human lung bronchial epithelium		CeO_2	ALI	Cytotoxicity, inflammation, oxidative stress, genotoxicity, gene expression	218
			CeO_2	Submerse	Cytotoxicity, inflammation, oxidative stress, genotoxicity	249
			MWCNTs	ALI	Cytotoxicity, inflammation, cilia beating frequency, mucociliary clearance, gene expression	262
EpiAlveolar	Human lung alveolar epithelium		n.d.	n.d.	n.d.	263
PCLS	Rat		PVP-coated Ag, ZnO, quartz microparticles	Submerse	Cytotoxicity, inflammation, multiphoton microscopy, cell proliferation	264
			CeO_2	ALI	Cytotoxicity, inflammation, oxidative stress	265
			CeO_2	ALI	Cytotoxicity, inflammation, oxidative stress	266
	Mice		SiO_2	Submerse	Inflammation	267
	Human		CeO_2, SiO_2, ZnO, Ag, MWCNTs	Submerse	Cytotoxicity, inflammation, oxidative stress	268

Table 1 *(Contd.)*

Cell lines/ coculture/3D model	Origin Characteristics	Nano-sized material	Exposure	Tested method/end points	Ref.
Organ-on-a-chip	GIT/Caco-2, HT29-MTX; liver/HepG2/C3A	Carboxylated polystyrene	Submerse	Dextran translocation, immunofluorescence staining	269
	Lung/primary human airway epithelial cell, goblet cells, ciliated beating cells	n.d.	n.d.	Inflammation, gene expression, cilia beating frequency	270
	Lung/primary human airway epithelial cells; GIT/Caco2	n.d.	n.d.	TEER	271

[a] ALI – air–liquid interface; Caco2 – human colon adenocarcinoma cell line; CuO – copper oxide; DEPM – diesel exhaust particulate matter; GIT – gastrointestinal tract; HepG2/C3A – human hepatoma cell line; HT29-MTX – human colon adenocarcinoma cell line; LDH – lactate dehydrogenase; n.d. – no data; PCLS – precision cut lung slices; PVP – polyvinylpyrrolidone; TEER – transepithelial electrical resistance. For cocultures, the combined cell lines are indicated by &.

metabolic pathways, and cellular connections and can cause oxidative stress as well as inflammation signaling.[245] Thorne *et al.* reported a study comparing the genotoxicity of cigarette and e-cigarette smoke. They were able to show that cigarettes cause clear genotoxicity by inducing DNA double strand breaks whereas e-cigarette smoke does not show any genotoxicity.[232] Kuper *et al.* compared the toxicity of CeO_2 ENMs between different *in vitro* models showing that the exposure of A549 and BEAS-2B to CeO_2 ENMs resulted in a slight increase in genotoxicity for the BEAS-2B cells whereas MucilAir as a 3D cell model was not affected.[249] In addition to the above-mentioned cell lines, other bronchial cell lines such as Calu-3[252,253] and 16HBE14o- are also applied to study the interactions of lung cells with nanoparticles.[241,254] Since the deposition of nano-sized airborne material primarily occurs in the alveolar region and subsequent retention or translocation is one of the key factors for toxicological risks of ENMs, alveolar epithelia cell models may represent a suitable model for the toxicity testing of inhalable ENMs. The recently developed cell line hAELVi combines the advantages of a non-tumorigenic human alveolar epithelia cell model with a functional intact barrier, and this cell line should therefore be considered for future studies of ENM inhalation toxicity.[246]

5.3. Coculture models

To better understand the toxicology and translocation mechanisms of ENMs on the one hand and better mimic the complex human respiratory system on the other, the interaction of different pulmonary cells has to be considered. The development of coculture and 3D systems has therefore been a focal point of interest and endeavors during the last few years. While the cell lines used for coculture models are grown as 2D monolayers separated by a membrane, the different cells in 3D systems are combined to generate histological structures that resemble the *in vivo* situation more closely. For instance, Loret *et al.* investigated the toxic and inflammatory potential of airborne TiO_2 and CeO_2 particles using a coculture system containing A549 and THP-1 cells.[219] Under ALI as well as submerse conditions, these ENMs can induce cytotoxicity, inflammation and oxidative stress. Furthermore, the authors could show that the coculture system is more sensitive regarding an inflammatory response than the A549 monoculture. Hoet *et al.* on the other hand generated a tricultured model comprising 16HBE14o-, THP-1 and HLMVEC cells and found that immune cells have a strong influence on the integrity of the intracellular barrier.[272] Furthermore, the system has been proved to be a suitable tool to determine cytotoxicity, oxidative stress and the inflammation potential of TiO_2, SiO_2 and Ag nanoparticles.[254] In addition, a coculture system consisting of the four cell types A549, THP-1, Ea.hy926 and HMC-1 was developed by Klein *et al.* and was shown to closely mimic the alveolar barrier found *in vivo*.[231] Although submerged testing usually led to overestimated toxic effects, ALI conditions indicated it to be an appropriate physiological model to examine the translocation of SiO_2 nanoparticles.[254] Besides SiO_2 ENMs, only diesel exhaust particulate matter was investigated using this system but – likely due to low level exposure – no significant changes in viability or inflammatory response were observed.[261]

5.4. Three-dimensional cell models and organ-like systems

In addition to cocultures, new 3D cell models have been explored as more complex and advanced models. These models are designed to emulate the *in vivo* structure. MucilAir™ presents a 3D bronchial model system that includes ciliated beating cells as well as mucus producing goblet cells.[273] This particular model allows for the testing of toxicity and the ability to cross the biological barriers of many chemicals as demonstrated by Reus *et al.* and Sauer *et al.*[228,274] Using the MucilAir™ system, the particulate effects of CeO_2 on cytotoxicity and genotoxicity as well as inflammation could also be observed by Kooter *et al.* and Kuper *et al.*[218,249] Another promising model was developed by Walles *et al.* using a vascularized scaffold derived from a porcine jejunal segment called BioVaSc which was subsequently incubated with fibroblasts and human primary bronchial cells. Model characterization revealed a 3D system mimicking the human airway which is composed of airway epithelia cells, basal cells, goblet cells, and ciliated cells. Because of these high similarities, an evaluation of whether these cells represent a more appropriate system for pulmonary toxicity studies should be conducted.[275] As inhalable nano-sized materials primarily deposit in the bronchial and alveolar regions of the lungs, alveolar cell

models are needed as well to address the toxicological risk of respirable NMs. To study NM toxicity on 3D alveolar cells, the so-called EpiAlveolar model was developed and depicts a system composed of macrophages and epithelia cells located at the apical side, and endothelia cells at the basal side. The system is therefore likely to mimic the *in vivo* alveolar region and may in the future be used to evaluate questions regarding particle retention, clearance and toxicity.[263]

The cultivation of whole organs has been a complex endeavor for more than 60 years.[276] But only in the 1990s, when Siminski *et al.* developed a method to cut whole organs after agarose embedding, precision cut lung slices (PCLS) have been established.[277] Nowadays, PCLS can be generated from rats,[264] mice,[267] and humans.[278] Sauer *et al.* investigated 16 ENMs on rat PCLS including CeO_2, SiO_2, ZnO, Ag and MWCNTs and found a cytotoxic and inflammatory potential of CeO_2, MWCNTs and TiO_2.[268] In addition, upon ion release, Ag and ZnO ENMs caused tissue destruction, an observation which was absent in the case of more insoluble ENMs like TiO_2, CeO_2, SiO_2, and MWCNTs. The reduction of cell viabilities in PCLS was also reported for Ag and ZnO ENMs by Hirn *et al.*, whereas micro-sized quartz particles showed no toxicity.[264] Moreover, PCLS were used to assess the toxic potential of a CeO_2-based catalyst but only small effects were seen for oxidative stress pathways and tumor necrosis factor alpha (TNF-α) and adenosine triphosphate (ATP) metabolism.[69,266] Taking also the microfluid model systems of the organ of interest into account, more advanced concepts to emulate the *in vivo* situation were developed.[279] These so-called organs-on-a-chip were, for example, used to analyze the translocation of 50 nm polystyrene particles across a gastrointestinal tract model. The lowered occurrence of effects on subsequent liver cells revealed that the gastrointestinal tract represents a strong barrier for most of these particles.[269] Only a small fraction that crossed the barrier resulted in an aspartate aminotransferase release which indicated liver cell damage. Another organ-on-a-chip model which imitates the human lung was developed by Benam *et al.*[270] Due to the inclusion of mucus producing goblet cells, ciliated beating cells, and functionally intact tight junctions, this lung model resembles the *in vivo* situation very well but studies are actually still outstanding. In addition, Henry *et al.*[271] as well as Jain *et al.*[280] recently developed new organ-on-a-chip models with features required for mimicking pulmonary structures. But the impact of these organ-on-a-chip models on nanotoxicology testing also remains to be evaluated.

In summary it can be stated that cell lines provide a standardized, relatively easy-to-use and well-established utility to study the toxicity of respirable ENMs. Due to their limited ability to resemble *in vivo* 3D structures and to mimic intercellular communication, the usability as models for ENM inhalation remains questionable. Coculture and 3D models which feature such multi-cell type buildups and interactions may resemble the *in vivo* situation much better. Furthermore, such systems can emulate the *in vivo* spatial structure and organization, which is necessary for any organ function. Nevertheless, even these newly developed models do not enable a complete artificial reproduction of the highly complex lung structure. PCLS, on the other hand, may provide an opportunity to overcome this issue since they are directly derived from the lungs and thus contain all different kinds of lung cells within the respective 3D structure. However, as the generation of PCLS is clearly more complicated and more expensive than standard cell culturing, it remains to be clarified if they will be broadly established in the field of nanotoxicology. For that reason, the organ-on-a-chip method seems to be a more practical tool whose microfluidic variants even enable the imitation of the blood flow. The organ-on-a-chip therefore has the potential to introduce an applicable and feasible *in vitro* platform to study the pulmonary toxicology of nano-sized materials. However, further development and evaluation are needed and may include the combination with PCLS.

6. Summary and outlook

These newly established test systems or the ones still in development will be needed to assist in the enormous task to investigate the ENM characteristics and assess their toxicokinetics. The decreased size of ENMs in comparison with their bulk materials was initially considered to be a decisive factor for their divergent biokinetic behavior and assumed to provoke additional toxic effects. As summarized here, mechanisms relevant for particle toxicity like deposition, clearance, or translocation were found to significantly influence particle toxicology as well.[89,281] Then again, these biokinetic properties can be attributed to further more detailed particle characteristics such as shape, surface area, reactivity, or solubility (see Fig. 3). These characteristics are often interrelated with one another while some are affected by the ambient conditions at the site of exposure. These findings certainly call for the re-evaluation of exposure limits. So, in the case of nano-sized GBPs, for example, a 4-fold higher inflammatory potency has been assumed for microscaled material when the parameter "mass" is used instead of "surface area" as the dose metric.[16] And while size is one of these characteristics influencing processes such as deposition or dissolution, there is as yet no convincing evidence for the presence of a nano-specific toxicity following inhalation, and no step change is found when going from micro- to nanoparticles.[89,282] Biological responses and hazards that were reported for ENMs, for example, oxidative stress, inflammation, or proliferation, have also been found for non-nano-sized materials.[283] These observations clearly argue against the differentiation of "perilous" nano-sized and "uncritical" micro-sized material which is often taken as the basis for regulations and recommendations so far. With regard to carcinogenicity, the phenomenon of lung overload is assumed as the main mechanism for both nano- and micro-sized GBPs, at least based on the available animal data.[146,284] However, other observations, such as the *in vivo* dissolution of inhaled, low-soluble ENMs, *e.g.*, a rapid lung clearance of $BaSO_4$, still show the need for further research.[285] As dis-

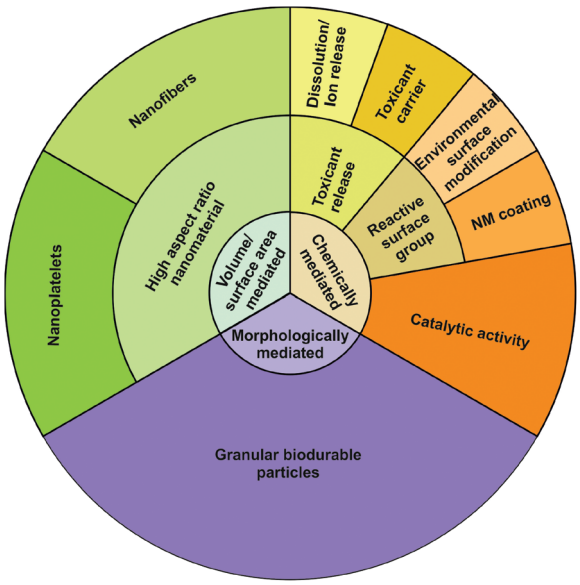

Fig. 3 ENMs can be grouped according to toxicity-related characteristics into: (1) particles that express a *chemically mediated toxicity* which can be categorized further into (a) materials releasing toxic compounds, either by dissolution of the respective NMs or *via* toxicants carried by NMs, (b) particles carrying a reactive group on their surface, either originating from their coating or aging, or surface adsorbed compounds, and (c) particles that possess a high catalytic activity themselves; (2) non-chemically toxic GBP materials whose mode of action is often connected to an induced overload (*morphologically mediated toxicity*); and (3) high aspect ratio nanomaterials (HARNs) that induce frustrated phagocytosis (*volume/surface area mediated toxicity*). Besides fibrous materials of a certain size and rigidity, platelets of a certain size can also be assigned to this category.

solution can lead to an entirely different distribution pattern in extrapulmonary organs in comparison with the translocation of insoluble particles, a closer look at these processes under physiological conditions is required. With regard to particle shape, the discovery that the carcinogenic effects observed in asbestos and connected to its high aspect ratio and rigidity[12] can also be seen in other fibrous materials is alarming.[161,286] Due to their often small aerodynamic diameter, these objects are able to penetrate deep into the respiratory system.[287–289] As mentioned before, the potential carcinogenic properties of such materials are understood to be related to their high aspect ratio and rigidness.[290] Most importantly, numerous fibrous materials such as MWCNTs,[291] silicate nanotubes (SiO_2-NTs),[292] glass fibers,[293] or silicon carbide whiskers (SCWs)[294] were shown to possess carcinogenic potency. However, other non-fibrous HARN materials like graphene platelets were also shown to exhibit such effects[295] and have been reported to induce frustrated phagocytosis and inflammatory responses as well.[296,297] In consideration of these findings summarized above, a grouping of nanomaterials[117] according to toxicity-related characteristics seems a suitable way to facilitate toxicological risk assessment.[90,119,298,299] To address the changes that occur during the ENM uptake *in vivo*, such as agglomeration and protein corona formation, a more pathway-related approach may be preferred to group nano-sized materials according to their complete life cycle ranging from formation *via* uptake until possible modification in the different target tissues of an organism.[119,298] Clearly, further research will be necessary to understand all of these modes of action, their mechanisms and the individual linkages between the specific ENM characteristics. As this paper illustrates, the topic at hand is extensive, and complex, and thus calls for a clearer proactive classification of new ENMs.

Conflicts of interest

There are no conflicts of interest to declare.

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