REVIEW ARTICLE

Effect of particle agglomeration in nanotoxicology

Arie Bruinink · Jing Wang · Peter Wick

Abstract The emission of engineered nanoparticles (ENPs) into the environment in increasing quantity and variety raises a general concern regarding potential effects on human health. Compared with soluble substances, ENPs exhibit additional dimensions of complexity, that is, they exist not only in various sizes, shapes and chemical compositions but also in different degrees of agglomeration. The effect of the latter is the topic of this review in which we explore and discuss the role of agglomeration on toxicity, including the fate of nanomaterials after their release and the biological effects they may induce. In-depth investigations of the effect of ENP agglomeration on human health are still rare, but it may be stated that outside the body ENP agglomeration greatly reduces human exposure. After uptake, agglomeration of ENPs reduces translocation across primary barriers such as lungs, skin or the gastrointestinal tract, preventing exposure of "secondary" organs. In analogy, also cellular ENP uptake and intracellular distribution are affected by agglomeration. However, agglomeration may represent a risk factor if it occurs after translocation across the primary barriers, and ENPs are able to accumulate within the tissue and thus reduce clearance efficiency.

Keywords Agglomeration · Nanoparticles · Toxicity · Translocation · Uptake

A. Bruinink (✉)
Laboratory for Biointerfaces, Empa, Swiss Federal Laboratories for Materials Science and Technology, Lerchenfeldstrasse 5, 9014 St. Gallen, Switzerland
e-mail: arie.bruinink@empa.ch

J. Wang
Analytical Chemistry, Empa, Swiss Federal Laboratories for Materials Science and Technology, Überlandstrasse 129, 8600 Dübendorf, Switzerland

J. Wang
Institute of Environmental Engineering, ETH Zurich, Stefano-Franscini-Platz 3, 8093 Zurich, Switzerland

P. Wick
Laboratory for Particles - Biology Interactions, Empa, Swiss Federal Laboratories for Materials Science and Technology, Lerchenfeldstrasse 5, 9014 St. Gallen, Switzerland

Introduction

The production and widespread use of engineered nanoparticles (ENPs) in various fields of application will cause a concomitant increase in ENP emission and exposure. ENPs are intentionally produced and designed with very specific properties related to shape, size, surface properties and chemistry. Understanding their effects on environmental and human health is therefore of increasing interest and as a result, the number of publications dealing with possible ENP toxicity has been steadily growing in the past few years (Haynes 2010).

To a certain extent, organisms have found ways of handling the abundant, naturally occurring NP, but whether this is also the case for ENPs needs to be verified. There is evidence that besides dosage, the toxicity of ENPs is determined by their size, shape, surface and what is adsorbed on that surface (e.g., ions, biological components) (Fadeel and Garcia-Bennett 2010; Kendall et al. 2011; Kendall and Holgate 2012; Nel et al. 2009). Furthermore, it has become evident that the degree of ENP agglomeration not only has an effect on distribution in various environmental compartments (i.e., air, water, soil; Fig. 1) (Keller et al. 2010; Mädler and Friedlander 2007), and thus the route of uptake by humans (Asgharian and Price 2007; Geiser and Kreyling
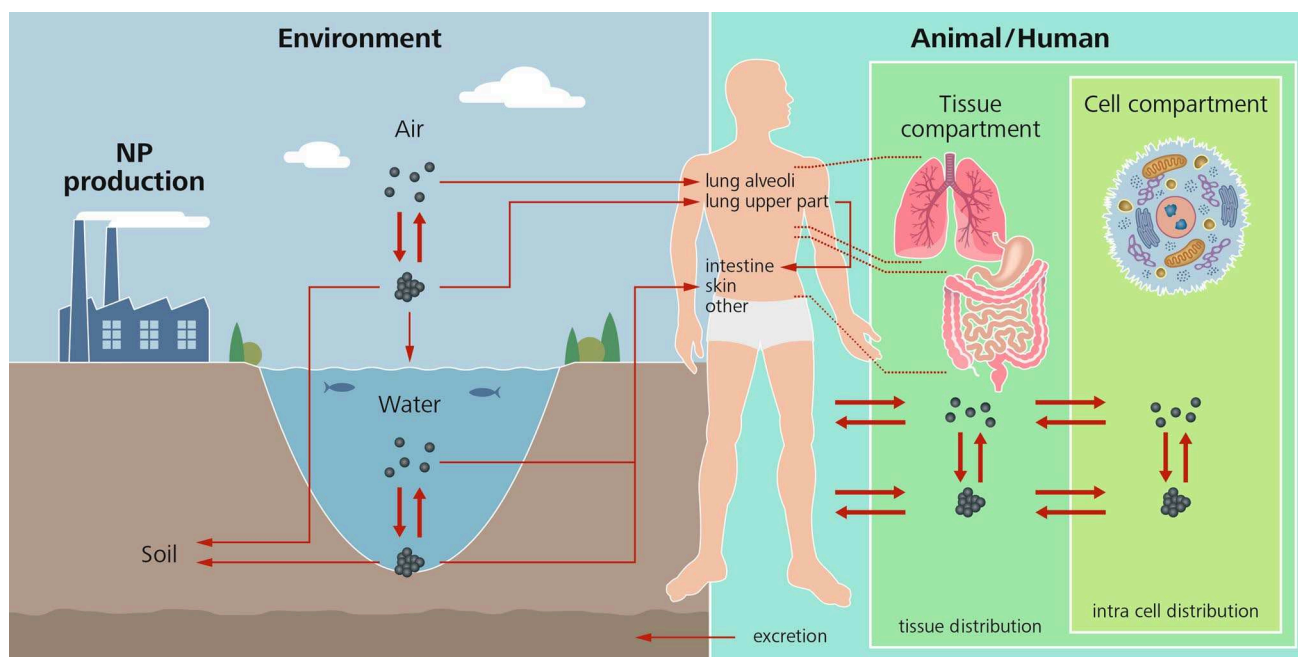
Fig. 1 Schematic and simplified pathway of the release, exposure and tissue distribution of ENPs to their final cellular site of action. *Thin arrows* material flow; *Thick arrows* reaction equilibrium and barrier transfer

2010; Kreyling et al. 2009), but also on the distribution and biological effects of ENPs within the whole body and individual cells (Andersson et al. 2011; Fraczek et al. 2008). Agglomeration may also lead to misleading results in cell culture studies evaluating ENP cytotoxicity (Wittmaack 2011a, b), because it may result in sedimentation of the ENPs onto the cells, drastically increasing their exposure to the ENPs and changing what the cells are exposed to (i.e., single particles versus various sized agglomerated ENPs).

In this review, we discuss the implications of ENP agglomeration on human exposure, transport within the body, accumulation and toxicity.

Definitions of nanoparticles, agglomeration, and aggregation

According to ASTM E2456-06, NP are defined as a "subclassification of ultrafine particle with lengths in two or three dimensions greater than 0.001 μm (1 nm) and smaller than about 0.1 μm (100 nm) and which may or may not exhibit a size-related intensive property" (ASTM E2456 2006). In practice, this definition cannot be used because ENPs are not produced in one defined size but around a certain size. Depending on the material and method of production, the size distribution is wide or narrow and may even be variable. To take this into account, a practical definition of NP was proposed by Kreyling et al. in (2010), based on the volume specific surface area, also known as the Brunauer–Emmett–Teller–specific surface area. In the

case of NP, this needs to be larger than 60 m^2/cm^3 (Kreyling et al. 2010). More recently, the EU commission recommended defining a nanomaterial as, "A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1–100 nm. In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50 % may be replaced by a threshold between 1 and 50 %" (Potocnik 2011). The same recommendation stated that "A material which, based on its number size distribution, is a nanomaterial should be considered as complying with this definition even if the material has a specific surface area lower than 60 m^2/cm^3."

ENPs are present individually or as part of larger entities composed of agglomerated or aggregated ENPs. The single ENP is termed as the primary entity and the agglomerated or aggregated ENP as secondary. In 2009, the European Food Safety Authority (Barlow et al. 2009) defined agglomerate and aggregate as follows: "An agglomerate is a group of NP (such as primary NPs) held together by weak forces, such as van der Waals forces or electrostatic forces. An aggregate is a group of NP (such as primary NPs) held together by strong forces, such as those associated with covalent or metallic bonds". Generally, in the case of an agglomerate, the external surface area is similar to the sum of the surface areas of the individual components, and in

the case of an aggregate, this area is significantly smaller. Consequently, it was recently suggested that "agglomerate" should be defined as: "a collection of weakly bound particles or aggregates where the resulting external surface area is similar to the sum of the surface areas of the individual components" and that "aggregate" is as "a particle comprising of strongly bound or fused particles" (ISO/TC229 2013). The degree of agglomeration is defined by $A = D/d$, in which A stands for the parameter of NP agglomeration, D for the agglomerate median diameter, and d for the median diameter of the NP (Shin and Lee 1997). In contrast to aggregates, secondary NPs as an agglomerate may preserve some of the properties of the primary NPs, such as high surface area and reactivity. Furthermore, because agglomerates are held together by weak forces, they may disintegrate in biological environments and thereby change their potential effect, in contrast to aggregates.

Effect of medium, particle mobility and sedimentation on agglomerates

After the release of ENPs into air or water, exertion of different forces determines their final distribution in the medium. On the one hand, gravitational forces induce ENPs to sediment, but on the other hand, the direction of the ENP movement can be either random Brownian particle diffusion or the same as that of the medium carrying the ENPs.

Sedimentation

When the specific gravity of the ENP constituents is larger than that of the surrounding medium, the terminal sedimentation velocity of a particle can be computed from the balance of gravity, buoyancy and drag forces. When ENPs form large agglomerates, the sedimentation velocity increases and sedimentation can be an important mechanism for ENP transport (Hinderliter et al. 2010; Teeguarden et al. 2007). However, agglomerates often have a porous structure that entraps the surrounding medium within, resulting in an effective density of the agglomerate that is significantly different from that of the raw material and producing a sedimentation velocity that is lower than that of compact spheres of the same size. Sedimentation is the key process by which ENPs are removed from the air and water compartments.

Brownian particle diffusion

The main transport mechanism of ENPs is Brownian particle diffusion. As this force is stronger for smaller particles, diffusion is a much more effective transport mechanism for ENPs than for micrometer-sized particles or agglomerates

(Mädler and Friedlander 2007). Hinderliter et al. (2010) showed that in motionless medium, small particles (much smaller than 10 nm), especially are transported by diffusion and large particles (much larger than 200 nm) by sedimentation. Particles between these size ranges are affected by both sedimentation and Brownian diffusion.

Motion of the carrier medium

If there exists relative motion between the medium and ENPs, ENPs experience a drag force which results in a retarding or opposing force to the relative velocity between the particle and medium. In the case of agglomerated ENPs, the drag force acting on the agglomerate is larger than that on a compact sphere of the same material volume (or mass, assuming the uniform material). This is because a compact sphere has minimal surface area for a given volume, thus having less interaction with molecules of the surrounding medium.

In summary, agglomeration increases the likelihood of ENPs being removed from the air and water compartments and as a result reduces the chance of them being taken up by biological systems. Motion of air or water significantly counteracts the sedimentation process.

Fate of ENPs in the environment

Effect of agglomeration on ENP distribution in air

Data summarized by Kuhlbush et al. (2011) show the reality of release and exposure of ENPs, and of unintentionally produced NPs, and their agglomerates in workplaces. For instance, measurements at workplaces manufacturing TiO_2 ENPs, depending on the phase of the reactor, showed a peak at approximately 50–100 nm and/or approximately 400–500 nm as revealed by analysis of the total particle number–size distribution. The larger sizes are thought to reflect ENP agglomeration (Lee et al. 2011). They made similar observations in silver ENP manufacturing workplaces.

Even during production, ENPs may form agglomerates and aggregates in the ambient atmosphere. Agglomeration of ENPs in air is greatly affected by electrostatic and van der Waals forces. The electrostatic repulsion/attraction forces are primarily dependent on the surface charge and the homogeneity of that charge. In air, strongly charged ENPs with the same charge will repel each other, whereas those of opposite charge will attract, promoting agglomeration. In the absence of a surface charge, van der Waals forces have a key role in promoting agglomeration when ENPs are in close contact, because of Brownian motion, shear forces, turbulence, etc. Van der Waals forces result

from the quantum mechanical movement of electrons giving rise to a small but important dipole in the particle, thereby inducing a dipole moment in the atoms of the adjacent particle (Nel et al. 2009).

In the case of vehicle emissions, the main factor determining the transport speed and direction of particles is the prevailing wind (Morawska et al. 2008). Charron and Harrison (2003) observed a decrease in approximately 10,000 normalized counts/cm^3 for particles in the range of 30–100 nm in vehicle emissions and a modal shift toward smaller values with increasing wind speed. NP-contaminated air will mix with uncontaminated air, resulting in particle dilution (Shi et al. 1999) and decreasing the opportunity for NPs to agglomerate. The chance of agglomeration occurring is maximal at locations with increased ENP concentrations (i.e., the site of production) (Stahlmecke et al. 2009). Agglomeration not only reduces the number of entities (individual particles, agglomerates) per cubic meter but also increases the sedimentation velocity, as mentioned earlier. Other possibilities of atmospheric ENP removal are rain (Garcia-Nieto et al. 1994) and adsorption to material surfaces or attachment to other particles (Schneider and Jensen 2009). Thus, dilution by mixing, sedimentation, wash out and adsorption result in reduced human exposure to ENPs.

Studies of the transport and transformation of ENPs in air after their release are still scarce. Initial steps to elucidate the various complex processes occurring in air have been made in laboratory-scale experiments (Kuhlbusch et al. 2011; Walser et al. 2012). Theoretical and numerical calculations may help improving prediction of the fate of particles in air and from that the potential size and type of exposure of biological systems.

Effect of ENP surface properties and the composition of water on agglomeration

ENPs entering the aquatic environment may remain as single, well-dispersed particles, reflecting their high colloidal stability, but often they tend to agglomerate in water. For instance, Keller et al. (2010) found that in water, TiO_2 and CeO_2 ENPs with primary particle sizes (PPS) of 27 nm size globules and of 8 × 67 nm-sized rods, respectively, directly agglomerate to sizes (secondary particle size; SPS) of approximately 200 nm. In a series using other ENPs, similar observations were made by Lin et al. (2010). If the agglomerates (and aggregates) are large enough, their mobility decreases and sedimentation is more likely to occur, resulting in ENP removal from the aquatic environment. Besides sedimentation, ENPs are eliminated from this compartment by attachment to an (macro-sized) immobile material, by dissolution processes, or by chemical reactions. Agglomeration/aggregation to larger entities may increase not only the likelihood of sedimentation but also

of being taken up by aquatic lower organisms, as will be discussed later.

Factors influencing agglomeration/aggregation in the aquatic environment are manifold (Chowdhury et al. 2012; Elimelech and Omelia 1990; Guzman et al. 2006; Karakoti et al. 2008; Keller et al. 2010; Lecoanet et al. 2004; Lin et al. 2010; Loosli and Stoll 2012; Nel et al. 2009; Shih et al. 2012; von der Kammer et al. 2010). The agglomeration process is primarily determined by the material's surface charge, but other parameters such concentration (Allouni et al. 2009), size (Bae et al. 2010), shape, including surface structure (Andersson et al. 2011) and chemical composition (Ahamed et al. 2008) are also important.

In water, besides the repulsive electrostatic forces, which act on ENPs in air, an additional effect may determine whether ENPs agglomerate or not (Fig. 2). Water will adhere to ENPs with a hydrophilic surface, forming a steric bumper layer that makes it difficult for ENPs to come into contact and thus inhibit agglomeration (hydrophilic repulsion) (Nel et al. 2009). The hydrodynamic diameter in a description of ENP size takes this bound water into account. By reducing the surface net charge (e.g., by changing the pH toward the isoelectric point), the electrostatic forces and hydrodynamic diameter will be reduced and concomitantly the repulsion. At a surface charge value [expressed as zeta-potential (ζ)] <25 mV, it is assumed that water is less bound to the particle surface and electrostatic repulsion is weak. In this situation, ENP agglomeration is favored (Guzman et al. 2006; Jiang et al. 2009). A more precise scale with regard to ζ and colloidal stability is given by Lin et al. (2010). In line with this (Andersson et al. 2011), evaluating different TiO_2 ENPs in phosphate-buffered saline solution seldom observed individual ENPs with a ζ between 20 and 24 mV. However, it should be noted that the correlation between low ζ magnitude (defined by electrophoretic mobility) and low dispersion stability does not always exist (von der Kammer et al. 2010).

Besides the aforementioned material characteristics, agglomeration is directly affected by environmental parameters, the most important of which are temperature (Karakoti et al. 2008), pH as discussed (Guzman et al. 2006; Limbach et al. 2008; von der Kammer et al. 2010) and water chemistry [ionic strength, presence of relevant monovalent and divalent ions, concentration of natural organic matter (NOM), etc.]. The effects of water chemistry will be discussed in more detail.

Ionic strength

Charged ions present in the aqueous medium (such as Na^+) are known to potentially influence the hydrodynamic diameter (Jiang et al. 2009) and thus hydrophilic repulsion. Ottofuelling et al. (2011) showed that in the presence of
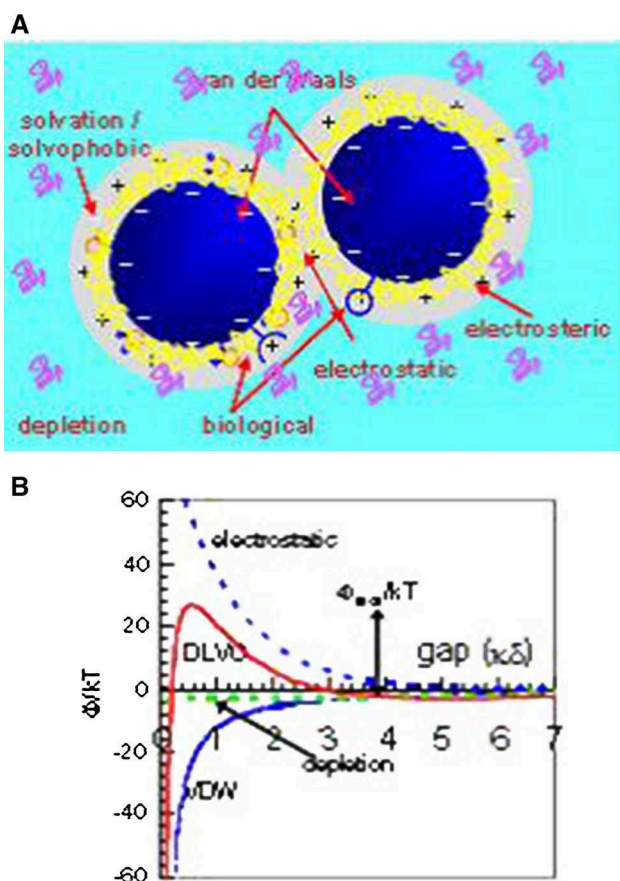
Fig. 2 ENPs in an organic material containing solution and the forces determining agglomeration considering van der Waals (vDW), electrostatic and depletion (as result of osmotic pressure) forces (Velegol 2007). Binding of organic material alters the surface charge and the layer of water molecules that are bound to the particle (**a**). Solvation/solvophobic forces determine the binding/repulsion of water molecules and by that the steric bumper layer diminishing or inhibiting agglomeration. **b** correlation between the size of the gap between particles and the height of the vDW, electrostatic and the force described by the Derjaguin–Landau–Verwey–Overbeek (DLVO) force theory obtained by a combination of the vDW and electrostatic forces. [Reprinted with permission of (Velegol 2007) ©SPIE 2007]

SO_4^{2-}, despite the revealed high negative ζ, the TiO_2 particles were agglomerated. The extent of the effect of ions on agglomeration/aggregation is dependent on their type and concentration (von der Kammer et al. 2010). Ions of the same type as the ENP (e.g., after dissolution of ENP material) will affect agglomeration. For instance, (Bae et al. 2010), using phosphate-buffered saline showed that silver ions contributed to the silver ENP agglomeration rate being maximal if the ionic ratio was >25 %.

Natural organic matter

NOM may influence the surface property and charge of ENPs and thus affect their agglomeration/deposition properties (Fig. 2). Depending on their own size and characteristics, these NOM may reduce (Baalousha 2009; Chen and Elimelech 2008; Keller et al. 2010) or induce (Quik et al. 2012) agglomeration.

Synergistic effects of multiple factors

By simultaneously evaluating the effect of two different factors on agglomeration behavior, von der Kammer et al. (2010) showed that interactions are complex even in simple defined systems. Because the composition of natural water varies (e.g., (Brunelli et al. 2013; Tsuda et al. 2010), predicting final agglomerate sizes and fate after the introduction of ENPs becomes rather challenging (Brunelli et al. 2013; Lin et al. 2010; von der Kammer et al. 2010). Therefore, ENP concentration and agglomerate size distribution have to be assessed case by case.

In summary, after introduction to the aquatic environment, ENPs and other NPs generally tend to agglomerate and thus to sediment. Dissolved components such as salts and organic matter may greatly affect the latter process.

Effect of uptake by and interaction with lower aquatic organisms on agglomeration

In the aquatic environment, ENPs and their agglomerates may interact with the aquatic fauna and as a result alter the degree of agglomeration. For instance, micrometer-sized ENP agglomerates may deagglomerate and disperse under the influence of bacteria. Horst et al. (2010) studied the effect of an environmental strain of the bacterium *Pseudomonas aeruginosa* on the SPS of agglomerated TiO_2 ENPs (PPS: 16 nm; initial SPS: 0.2–18 μm) and showed that in the presence of the bacteria, the frequencies of medium and large agglomerates (6–12 and 12–18 μm, respectively) were either sharply reduced or eliminated, respectively, whereas that of small agglomerates (0–6 μm) increased. This observed deagglomerating effect may be related to interaction with bacterial surfactants, as several strains of bacteria are known to produce molecules with surfactant properties (Bento et al. 2005).

The uptake of ENPs by aquatic animals is mainly restricted to adsorption, normal feeding and/or water filtration through the gills. For example, crustacean filter feeders have a high efficacy for accumulating relative low concentrations of entities in the size range of their common food (e.g., algae, bacteria) particularly around ≥0.5 μm (Gophen and Geller 1984). Therefore, these animals could potentially take up various types of agglomerated NP, including ENPs. For instance, agglomerated TiO_2 ENPs (PPS: 30 nm; SPS: 360 nm) can be taken up by the filter-feeding/grazing freshwater microcrustacean *Daphnia magna* and accumulate in the gut (Hartmann et al. 2012). The same species

have been reported to ingest and accumulate agglomerates of C60 at SPS >0.45 μm and of nanosized CNTs (Baun et al. 2008; Petersen et al. 2009). After exposing the filter-feeding organism crustacean *Thamnocephalus platyurus* to aqueous suspensions of the fullerenes C60 and C70, agglomerates with SPS of 5–10 μm were found in the gut, being an order of magnitude larger than the suspended fullerene agglomerates (SPS: 517 ± 21 and 656 ± 39 nm) (Patra et al. 2011). The excreted fullerene agglomerates were even larger (in the 10–70 μm range) and remained agglomerated even after 6 months if stored in pure water at room temperature. Similar observations were made by Roberts et al. (2007), who treated *Daphnia magna* with well-dispersed lipid-coated carbon nanotubes and found that the excreted nanotubes were no longer water soluble and agglomerated because of digestion of the lipid coating that had previously allowed the nanotubes to remain dispersed in suspension. Excretion of CNT is reported to be enhanced by subsequent feeding with algae (Petersen et al. 2009). These examples clearly show that not only the characteristics/composition of water affect the degree of ENP agglomeration but also the organisms that the aquatic environment hosts. Their importance has so far not been investigated.

Uptake by humans and toxicity of ENPs

Effect of particle/agglomerate size

ENPs that are released into the environment can be taken up by humans through two major routes: the respiratory tract, with an estimated surface of 1400 m^2, and the gastrointestinal tract, with an estimated surface of 200 m^2 in adult humans. As the third largest interface between the body and its environment, with an estimated surface of 1.9 m^2 in adult humans, skin is also exposed to NPs, sometimes willingly in large amount (e.g., sunscreen). However, so far there is not any evidence that a significant amount of NPs can enter the body through the skin, as long as they are not specially produced to overcome this barrier (Lademann et al. 2013).

Uptake by the respiratory tract

With every breath, aerosolized particles will be inhaled. Besides knowing what is inhaled, it is important to know where inhaled particles will be deposited. Based on the presence of ciliated lung epithelial cells, the respiratory tract can be divided into the upper, tracheobronchial system and the deeper, alveolar system without such cells. In the upper part, mucus and collected deposits are transported by the mucociliary "escalator" toward the pharynx and

swallowed (mucociliary clearance), but the alveoli are not covered by ciliated cells. As a result, deposited particles are removed very slowly and mainly by alveolar macrophages (Geiser and Kreyling 2010; Semmler et al. 2004).

Where particles and their agglomerates are deposited in the lung depends on their size (Fleming et al. 1996). Using eight human volunteers and 2.7-, 3.6- and 5.4-μm radioactive mannose particles, Glover et al. (2008) showed that the 5.4-μm particles were mostly deposited extrathoracically (e.g., in the nose: 70 %) and only 3.7 %, but 7.3 % of the 2.7-μm particles, reached the alveolar region. Particles under 10 nm in diameter are effectively removed from inhaled air in the tracheobronchial system, with little or no penetration into the pulmonary (alveolar) region (Asgharian and Price 2007). In the alveoli, it is predominantly particles in the range of 10–3,000 nm (maximum ≈ 20–40 nm) that are deposited (Creutzenberg 2012; Geiser and Kreyling 2010). Thus, the sizes of particles and their agglomerates that are predominantly present in ENP manufacturing workplaces (see section "Effect of uptake by and interaction with lower aquatic organisms on agglomeration") will maximally deposit in the region of the lung without mucociliary clearance.

After deposition in the alveolar region of the lung, particles and their agglomerates come into contact with pulmonary surfactants (Gasser et al. 2010). Besides nonprotein surfactant molecules (e.g., phosphatidylcholine), these comprise surfactant proteins A, B, C and D, with surfactant protein A being the most prevalent (Griese 1999; Ruge et al. 2011). The hydrophilic surfactant proteins A and D, especially contribute to pulmonary defence (Kishore et al. 2006). Comparing two magnetite-based sub-micrometer-sized particles, Ruge et al. (2011) found that, in vitro, surfactant protein D, especially adsorbs to hydrophobic particles with ζ of +3 mV (PPS: 150 nm) and surfactant protein A to hydrophilic ones with a ζ of +25 mV (PPS: 130 nm).

The binding of surfactant proteins may affect the fate of ENPs in two ways. In the first place, they may (similar to NOM in the aquatic environment) circumvent (further) ENP agglomeration or force agglomerates to deagglomerate (Maynard 2002). However, on the other hand, as reported for surfactant protein D (especially in the presence of 2 mM calcium), these surfactants may also promote agglomeration (Kendall et al. 2013). There is in vivo experimental evidence from Creutzenberg et al. (2012) of an increase in agglomerate size after instillation of rat lungs with TiO_2 ENPs (PPS and SPS: 180 nm), which suggests that in the lung the tendency to agglomerate predominates.

In the second place, the adsorbed components have an effect on the way that ENPs are eliminated from the pulmonary region. For instance, Ruge et al. (2011) showed that in the presence of surfactant proteins, magnetite-based sub-micrometer-sized particles are increasingly taken up by alveolar macrophages. This is supported by in vivo findings.

Kendall et al. (2013) showed that in surfactant D knockout mice, the uptake by alveolar macrophages of various types of polystyrene latex microspheres with sizes in the range of 80–500 nm and ζ ranging from -20 to -40 mV was reduced.

Phagocytosis by alveolar macrophages followed by their migration into the bronchial system is proposed as a key mechanisms of particle clearance from the alveolar region (Geiser and Kreyling 2010; Möller et al. 2008). By using iron microparticle inhalation studies, Lehnert and Morrow showed that after 24 h, more than 90 % of the lavaged particles were associated with alveolar macrophages (Lehnert and Morrow 1985). Similarly, in mice inhalation studies using gold ENPs (PPS: 21 nm; SPS in lavage: ≤ 100 nm and in macrophages >100 nm), after 24 h, approximately 83 % of the entities were associated with macrophages (Geiser et al. 2013). Furthermore, they found that approximately 2 % of the particles were located in epithelial cells, which are in agreement with the assumption that translocation of NPs into lung tissue is another, although minor, route of alveolar clearance (Geiser and Kreyling 2010). The translocation velocity toward the lung and other tissues is affected by material characteristics, including surface chemistry and particle size, with smaller particles being more rapidly translocated from the lung into various other tissues (Geiser and Kreyling 2010; Kreyling et al. 2009).

Uptake by the gastrointestinal tract

Initially, the food and fluid that are concomitantly consumed will affect the degree of ENP agglomeration (Peters et al. 2012). Furthermore, binding to indigestible food components diminishes the chance of ENPs being retained in the body. Throughout the gastrointestinal tract (stomach, small intestine and large intestine), ENPs and their agglomerates are subjected to different pH, varying from approximately 1.5 (stomach), 6 (duodenum) to approximately 7.4 (terminal ilium) (Fallingborg 1999). In the stomach, acid-sensitive ENPs will probably be eliminated by dissolution. ENPs with a critical isoelectrical point within the pH range of 1.5–7.4 will probably (further) agglomerate in those regions of the gastrointestinal tract with this pH if the ENP concentration is high enough locally or, more likely, through attachment to food components. Surfactant molecules (e.g., the typical lung surfactant proteins A, B, C and D), liver-produced bile and surfactant lipoprotein lamellar structures (the so-called surfactant-like particles) are released into the lumen of the gastrointestinal tract where they play among others an immunoregulatory role and digestion-promoting and/or uptake promoting roles regarding useful food components (Akella and Deshpand 2013; Mahmood et al. 2003; Rubio et al. 1995). As in the lung, these surfactants may also affect the degree of agglomeration, but so far it has not been reported how these surfactants depress or promote agglomeration. As mentioned in section "Effect of uptake by and interaction with lower aquatic organisms on agglomeration", the presence of bacteria in the small and large intestines may also decrease the degree of ENP agglomeration. Thus, various factors influence the degree to which ENPs agglomerate and hence their availability for uptake.

Animal studies (unfortunately only investigating the final stage regarding ENP uptake after ingestion) suggest that in the gastrointestinal tract, smaller entities are more likely to be taken up than larger ones. For instance, Hillyer and Albrecht (Hillyer and Albrecht 2001) evaluating the fate of colloidal gold ENPs (PPS: 4, 10, 28 and 58 nm) after oral administration (mice) for 7 days found that the smaller ENPs were more readily taken up. Jani et al. (1990) evaluated the extent of absorption by rats after daily gavage for 10 days of radiolabeled polystyrene particles (PPS: 50–3000 nm) and found that 34 % of the 50-nm particles and 26 % of the administered 100-nm particles were taken up, using the retained radioactivity in the body as an index. No evidence of a significant uptake of particles with PPS >300 nm was found. Schleh et al. (2012) also reported a size dependency for ENP uptake. They evaluated the gold content in the circulation 24 h after a single intraesophageal application (rats) of 1.4–200 nm (SO_3^-) functionalized negatively charged (-20 to -40 mV) gold ENPs. Of the 1.4-nm particles, 0.37 % but only 0.01 % of the 200 nm particles was found in the circulation (Schleh et al. 2012). It is interesting to note that by comparing (COO^-) and (NH_3^+) functionalized 2.8-nm gold ENPs, they found that almost 3-fold more of the negatively charged ENPs were taken up. To our knowledge, there are no reports so far on the effect of agglomeration on gastrointestinal ENP uptake, but it may be assumed that agglomerates behave like large particles, which may reduce or even prevent their uptake.

In summary, the uptake of particles and agglomerates through the lung is restricted to those reaching the alveoli, being predominantly in the nanometer-submicrometer range (maximum $\approx$ 20–40 nm). The possibility of being taken up in the alveoli, and also in the gastrointestinal tract, is reduced with increasing size, with (nearly) no particle/agglomerate uptake at sizes above 0.3 μm.

Effect of ENP agglomerate/particle size on tissue distribution

The effect of particle size and/or degree of agglomeration on tissue distribution has been the focus of several studies. Animals have been exposed by inhalation or lung instillation, by esophageal application or by injection (intravenous or intramuscular). Regarding lung exposure, for instance, Kreyling et al. (2009) could not find a difference in iridium retention in the various investigated tissues 24 h after inhalation

of iridium ENP agglomerates (PPS: 2–4 nm; SPS: 20 and 80 nm) by rats, taking the difference in ENP uptake by the lung into account. Schlech and et al. (2012) evaluated gold ENPs (PPS: 1.4, 5, 18, 80 and 200 nm) and showed that the 1.4- and 18-nm sizes, especially, remained in the carcass 24 h after esophageal application. Of the 1.4- and 5-nm ENP, approximately twice the amount of gold was retained in the kidney in comparison with spleen plus liver, and the small amount of the 80- and 200-nm ENPs taken up tended to accumulate predominantly in the liver plus spleen.

In contrast to exposure through the lung or intestines, injection of ENPs circumvents the barriers to enter the body. Intravenously injected ENPs are widely distributed through the body, and the extent of ENP tissue distribution is greatly modulated by particle and agglomerate size. By measuring total organ gold content, Keene et al. (2012) found that 24 h after intravenous injection of mice with gold ENPs (PPS: 5–8 nm) at different degrees of agglomeration/aggregation (SPS at moment of injection: 5–8, 30–200 or 500–2,000 nm) resulted in different tissue distributions. Injection of dispersed single ENPs resulted in accumulation primarily in the liver ($\approx$60 %) and spleen ($\approx$25 %), whereas after injection of small agglomerates, gold was found to be nearly equally distributed and nearly exclusively in the liver, spleen and lung. The application of large agglomerates gave rise to gold accumulation predominantly in the spleen ($\approx$65 %) and lung ($\approx$25 %). De Jong et al. (2008) injected rats intravenously with dispersed gold particles of 10, 50, 100 and 200 nm (PPS; with no quantitative information regarding SPS); of these, the 10 nm ENPs had the most widespread organ distribution (brain, thymus and testis). A similar result was found by Hirn et al. (2011), performing a comparable experiment. In order to determine the particle size limitation regarding ENP transfer into tissues, Yaehne et al. (2013) used chicken embryo chorioallantoic membrane as a blood vessel–tissue model. They took as the index for ENP transfer the degree of retention of carboxy-polysterene particles (PPS: 20, 50, 100 and 250 nm) by the blood vessel–tissue. In this model, an inverse correlation was found between particle size and uptake velocity, with 250-nm particles not being taken up. Uptake was nearly restricted to particles with a slightly negative (<-10 mV) or positive ζ. Furthermore, interestingly, a similar particle size threshold for crossing the placental barrier is reported (Wick et al. 2010).

As soon as particles are transferred into a tissue or if they are directly injected into it (e.g., intramuscularly), the extracellular matrix (ECM) may limit particle and agglomerate distribution by adsorption and filter phenomena (Goodman et al. 2008). The latter is, especially significant with increased particle and agglomerate sizes. For instance, in the case of single wall (SWCNT) and multiwall (MWCNT) carbonnanotubes, Fraczek et al. (2008) found that after

intramuscular injection, the larger MWCNT agglomerates (PPS: diameter 5–20 nm; length: 300–2,000 nm; SPS: 5–300 µm) remained at the site of injection, whereas the smaller SWCNT agglomerates (PPS: diameter 2–3 nm; length: 30–50 nm; SPS: 5–35 µm) were taken up by macrophages and transported to the lymph nodes. A study by Popović et al. (2010) investigating the translocation of fluorescent silica-based ENPs of different sizes (PPS: 12, 60, 125 nm) showed that after intravenous administration into tumor-bearing mice, only the 12-nm particles had migrated significantly from the blood vessel into the tumor. Thus, agglomerate size may play an important role in particle distribution within the tissue, but as far as we know there are no reports on the extent to which agglomerate size changes distribution within tissues.

In summary, not only ENP transfer into the body but also tissue distribution is greatly affected not only by surface chemistry but also particle and agglomerate size, with the smallest ones having the most widespread systemic distribution. There is evidence that particles of $\geq$250 nm have a very low to negligible likelihood of being transferred from the bloodstream into tissues. Increased particle and agglomerate size seems to greatly limit the dispersion within the tissue, but the extent to which agglomerate size changes distribution within the tissue is still unknown.

Effect of ENP agglomerate/particle size on transport into and distribution within the cell

The cell membrane is an important barrier between the extracellular and intracellular space. Rothen-Rutishauser et al. (2006) showed that erythrocytes were able to take up particles (non-agglomerated polystyrene and gold particles, TiO_2 agglomerates) $\leq$0.2 µm in size but not bigger ones. Because erythrocytes do not have phagocytic or endocytotic abilities, this finding strongly suggests that such uptake occurs through an energy independent, diffusion-like process. There is evidence that overall size of the particle affects cellular uptake, independent of whether this particle consists of a single unit or of a cluster of particles (aggregate/agglomerate) (Rothen-Rutishauser et al. 2006). In general, uptake processes (Fig. 3) are, however, energy dependent and may occur through different pathways (Fig. 3).

Using non-agglomerated latex particles in the size range of 50–1000 nm and non-phagocytic B16 cells, Rejman et al. (2004) showed that the 50–100 nm beads were taken up very rapidly in contrast to larger particles, possibly by a receptor-mediated process. In line with this, by comparing monodispersed negatively charged polystyrene particles (d-α-tocopheryl polyethylene glycol 1,000 succinate-coated and uncoated) in the size range of 25–500 nm, Kulkarni and Feng (2013) reported that uptake was maximal at approximately 100 nm (PPS), as measured using
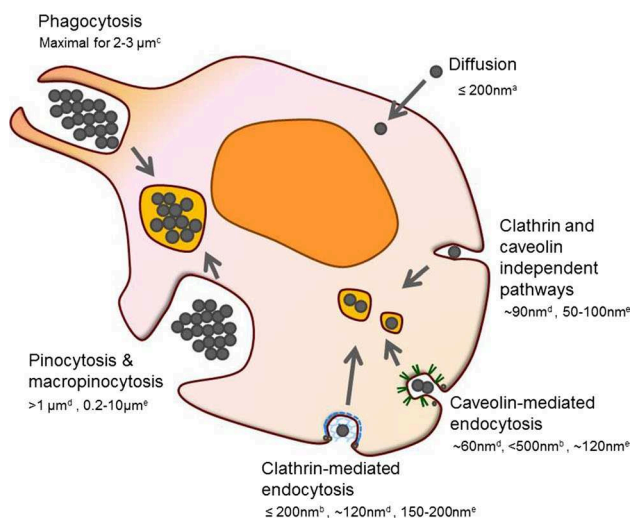
Fig. 3 Important pathways of cellular uptake of ENPs and their agglomerates. Depending on the uptake mechanism, ENPs are located in membrane-bound vesicles or are free 'floating' in the cell (Kettiger et al. 2013). [a](Rothen-Rutishauser et al. 2006); [b](Rejman et al. 2004); [c](Champion et al. 2008); [d–e]Size of the endocytic vesicle taken as index; [d](Conner and Schmid 2003), [e](Kumari and Mayor 2010)

Caco-2 and MDCK cells. Rejman et al. (2004) suggest that internalization of microspheres with a diameter <200 nm involves clathrin-coated pits. Orr et al. (2011) showed that only small SiO_2 ENP agglomerates (PPS: 100 nm; SPS: <200 nm) were taken up by clathrin-dependent endocytosis of macrophages. In the latter uptake, the macrophage scavenger receptor A seems to play an important role. With increasing size, a shift to a mechanism that relies on caveolae-mediated internalization became apparent (Rejman et al. 2004) and was the predominant pathway of entry for 500-nm particles. Particles as large as 500 nm were internalized by melanoma cells, with uptake largely blocked by endocytosis inhibitors, and additionally, no uptake was seen at 4 °C. Thus, it may be generally stated that there is a particle size dependency regarding the uptake mechanism (independent of whether it is composed of a single unit or many). However, it must be generally assumed that particles and their agglomerates are taken up by multiple mechanisms, with only some preferences for one or other pathway depending on particle (-agglomerate) surface and size characteristics (Bhattacharjee et al. 2012; Zhu et al. 2013). The cellular uptake of entities >1 μm is reported to be reduced. Andersson et al. (2011) showed that by treating A549 lung cells with anatase and rutile TiO_2 ENP agglomerates (PPS: 20–60 nm; SPS: in the sub- and micrometer range), the submicrometer-sized agglomerates tended to be more attached to cell surfaces than the micrometer-sized agglomerates. Mainly, small agglomerates <1 μm are found inside cells. Agglomeration (excepting except "soft"

agglomeration) to micrometer sizes hinders some particles from penetrating the outer cell membrane because of their intrinsic physical properties. Phagocytic cells, such as macrophages and intestinal epithelial cells, are reportedly able to take up larger entities (≤1.9 μm: (Yue et al. 2010); Caco-2 cells: ≤10 μm (Desai et al. 1997)).

Note that besides size, particle transfer from the extra- to the intracellular space is on the one hand dependent on the cell type and on the other hand dependent on the surface charge (Lynch et al. 2009). For instance, of 150-nm polymeric particles with a charge ranging from −40 to +35 mV, those with the highest charge, and, especially the positively charged ones, were found in macrophages after intravenous injection in mice (He et al. 2010).

Location of ENPs after cellular uptake

Although, as far as we know, only qualitative measurements have been reported, some tendencies regarding intracellular distribution can be described. Except for small particles, which may "diffuse" through the cell membrane, ENPs will be engulfed by the cell membrane at the moment they enter the intracellular compartment. This is supported by many reports showing ENPs and their agglomerates being (mainly) within vesicle-like structures (AshaRani et al. 2009; Drescher et al. 2011; Horie et al. 2010; Sadauskas et al. 2007). In addition, Brandenberger et al. (2010) reported that with prolonged exposure, ENPs were preferentially seen in larger sized vesicles such as lysosomes).

Within the cell, the location of ENPs is largely dependent on particle size. For instance, AshaRani et al. (2009) treated human glioblastoma U251 cells with well-dispersed silver ENPs (PPS: 6–22 nm) and reported that besides the presence of single and agglomerated ENPs in the cytoplasm, single ENPs were also present in organelles, such as mitochondria, and in the cell nucleus. Ahlinder et al. (2013) showed that after treating lung epithelium A549 cells with dispersed and agglomerated titanium or Goethite iron oxide ENPs, these entities were seen inside the cells (PPS TiO_2: 20–80 nm; PPS α-FeO(OH): 11 × 11 × 62 nm rods; agglomerated ENP SPS: size range 30 nm–3.2 μm). However, inside the nucleus (in some cells), only single ENP "dots" were observed. The same observation was made by Jugan et al. (2012), evaluating the effects of titanium ENPs (PPS: 12 nm) on A549 cells 4 h after treatment; no particles were seen in the nucleus in the case of larger ENPs (PPS: 21–142 nm). Shukla et al. (2011), evaluating the uptake and distribution of TiO_2 particles in a size range (single or agglomerate) between 70 and 330 nm in human epidermal cells, also observed some single, small particles (<100 nm) inside the nucleus. However, Kühnel et al. (2009), evaluating the uptake of slightly larger particles (i.e., non-agglomerated (PPS: 145 nm) and

agglomerated tungsten carbide-based particles (SPS: up to 400 nm) by rainbow trout gill cell line RTgillW1 cells, found that ENPs were localized within the cells but not in the nucleus. Analogously, Andersson et al. (2011) reported that after uptake by A549 epithelial cells, submicrometer TiO$_2$ ENP agglomerates (PSP: 20–80 nm; SPS 0.2–0.8 μm) were preferentially located in the cytoplasm near organelles such as mitochondria but not within them. Thus, it seems that there is a certain threshold for distribution inside organelles, including the nucleus, being probably in the range of 100 nm, which is near the aforementioned size threshold at which ENP may pass through the cell membrane in a diffusion-like way.

Clearance of ENPs from the body

An important aspect, especially in the case of chronic exposure, is the extent and efficiency of ENP removal from the body. Fabian et al. (2008) reported that 24 h after injecting rats intravenously with agglomerated TiO$_2$ ENPs (PPS: 20–30 nm; SPS: ≈1 μm), the highest levels were seen in all investigated organs. TiO$_2$ levels in the liver were maintained for at least 28 days, being the last day of measurement. In the spleen, lung and kidney the TiO$_2$ levels slightly decreased from days 1 to 14 and seemed unstable until day 28. This suggests that large ENP entities were only marginally cleared and trapped within these organs. In line with this, aspiration studies by Shvedova et al. (2014) showed that after treatment with carbon nanotubes (PPS: 65 nm × 1–3 μm), carbon fibers (PPS: 80–160 nm × 5–30 μm) or asbestos (PPS: 160–800 nm × 2–30 μm) by bolus dosing through pharyngeal aspiration and inhalation 5 h/day for 4 days, particles could still be visualized in the lung at 1-year post-exposure. By evaluating the fate of gold ENPs with different SPS (PPS: 1.4, 5, 18, 89 and 200 nm with agglomerates removed by filtration), Hirn et al. (2011) reported that 24 h after intravenous dosing of rats, the smallest sized ENPs (PPS: 1.4 nm, to lower degree 2.8 nm) were maximally excreted by the urinary and hepatobiliary systems, as concluded from concentrations found in the urine and small intestine. It is known that the upper threshold particle size for the kidney glomerular filter is approximately 6–8 nm (Longmire et al. 2008). So entities larger than this will not be renally excreted and are primary excreted through the hepatobiliary system (Longmire et al. 2008). As mentioned before, the liver is a key organ in which larger sized ENP entities accumulate. Intravenously injected ENPs are primarily taken up by Kupffer cells in the liver and by macrophages in other tissues. In the lung alveoli also, as mentioned earlier, macrophages are mainly responsible for clearance. The uptake by phagocytic cells is limited to particles <2 μm (Yue et al. 2010). However, non-degradable entities taken up by Kupffer cells are thought to be retained in the body (Longmire et al. 2008). Particles taken up by hepatocytes are potentially excreted (Longmire et al. 2008). The threshold particle size for excretion by the hepatobiliary system seems to be approximately 200 nm (Hirn et al. 2011). Thus, clearance of ENPs is largely defined by particle size and may be (nearly) absent for particles >200 nm.

Effect of ENP agglomeration on toxicity

As described in section "Location of ENPs after cellular uptake", after ENP exposure in vitro, small entities less than 100 nm in size can be found in organelles such as mitochondria and the nucleus, in addition to the cytoplasm, and thus potentially may directly affect energy production and the DNA, resulting in disturbed cell functionality/viability. Although in most cases, decreased cytotoxicity with increasing size of particles or agglomerates has been reported, several reports have contrary findings (Table 1). In the case that those few cells with ENPs in mitochondria and nucleus (Ahlinder et al. 2013; AshaRani et al. 2009; Shukla et al. 2011) are affected maximally, this small number of cells may be negligible in the context of functionality measurements of the whole cell culture. As a result, in toxicity evaluations using cell cultures, other factors may predominate and be responsible for the observed contradictory effects of ENP agglomeration. For instance, the mean ENP agglomerate size and its distribution may change or differ according to the culture medium, ENP concentration and incubation/exposure period (e.g., (Allouni et al. 2009; Murdock et al. 2008; Prasad et al. 2013). As a result, cells are exposed to different size distributions, with consequences for the experimental outcome. Furthermore, in the case of comparison of ENP samples with different mean agglomeration sizes but with size distributions that are (significantly) overlapping, no clear statements can be made regarding their effects. Only an in-depth appropriate characterization of the dispersion in cell culture will give meaningful results and enable correct statements regarding cytotoxicity (Warheit 2008). Besides this, for both outcomes (i.e., increase and decrease in toxicity because of agglomeration), the observed effects in cell cultures may be influenced by factors that are only indirectly related to ENP agglomeration. For instance, dissolution rates of dispersed and agglomerated ENPs differ, as reported for silver (Kittler et al. 2010) (Elzey and Grassian 2010). Furthermore, sedimentation of agglomerated ENPs may (time dependent) increase exposure and thus the apparent toxicity (Limbach et al. 2005; Obarzanek-Fojt et al. 2014; Teeguarden et al. 2007). In addition, ENPs and their agglomerates may interfere with assays assessing the functional state of the cells (Belyanskaya et al. 2007; Hirsch et al. 2011; Monteiro-Riviere et al. 2009).

Table 1 Examples of the possible effects of agglomeration on the toxicity of ENPs for mammalian cells in culture

Mate-rial	Size	Animal species and cell type	Treatment	Effects	References
SiO$_2$	No albumin: PPS 30 nm, PPS: 42, SPS: 281 nm and PPS: 64 nm, SPS: 137 nm. Rhodamine-labeled ENP: PPS: 34 nm, SPS: 153 nm With albumin: PPS = SPS: 42- and 64-nm rhodamine-labeled ENP: PPS = SPS: 34 nm	Human BEAS-2B lung bron-chial epithelial cells	50–200 µg/mL; 24 h	Agglomeration increases toxicity No albumin: increase IL-6 and IL-8, LDH release Maximal effects by rhodamine-labeled ENP With albumin reducing agglom-eration: negligible effects	(Gualtieri et al. 2012)
SiO$_2$	Hydrodynamic diameter: no albumin: PPS = SPS: 38 nm; with serum: PPS: 38 nm, SPS: ~5 µm	Mouse 3T3 fibroblast cells	5–100 µg/mL; 24 h	Agglomeration reduces toxicity (XTT-test)	(Drescher et al. 2011)
SiO$_2$	PPS: 30, 48, 118 and 535 nm. Monodis-persed	Mouse HEL-30 keratinocytes	10–200 µg/mL; 24 h	Expressed in µg/mL, larger parti-cles were less toxic (LDH and MTT tests)	(Yu et al. 2009)
Ana-tase/rutile TiO$_2$	PPS: 21 nm (range 15–60 nm): SPS: dispersion 1: ~110 and ~285 nm, dis-persion 2: ~765 nm	Human TK6 lymphoblasts; monkey kidney Cos-1 fibro-blasts; human embryonic epithelial cells	0.1–75 mg/cm^2 (corres-ponds to 0.6–360 mg/mL); 2, 24 and 48 h	Agglomeration increases toxicity Dispersion 2: DNA damage in all three cell lines Dispersion 1: no effect on geno-toxicity	(Magdolenova et al. 2012)
Anatase TiO$_2$	PPS: 7, 20, 200 nm, SPS: ranging from 75 to 180 nm	Human HaCaT keratinocyte cells; human A549 lung cells	30–100 µg/mL; 24 h	Increased particle/agglomer-ate sizes tend to lower ROS, caspase 3 but increased lipid peroxide levels	(Horie et al. 2010)
Anatase and rutile TiO$_2$	Anatase: PPS: 20 nm, SPS: 5.8 µm and PPS: 66 nm, SPS: 0.6 µm Rutile: PPS: 3 × 5 nm, SPS: 0.7 and 1.9 µm and PS: 6 × 80, SPS: 6.1 µm	Human A549 lung cells	5–200 µg/mL; 24 h	Rutile particles and 0.6-µm anatase agglomerates maximally increased IL-8. Only 0.6- but not 5.8-µm anatase and the rutile agglomerates induced lipid peroxidation	(Andersson et al. 2011)
Anatase and anatase–rutile TiO$_2$	Anatase: (a) PPS: 3.9 nm, SPS: 6.1 µm, (b) PPS: 6.7 nm, SPS: 6.5 µm, (c) PPS: 222 nm, SPS: 6.2 µm; anatase–rutile: (a) PPS: 22 nm, SPS: 12 µm, (b) PPS: 6.7 nm, SPS: 6.5 µm	Human Caco-2 colon cells	10–160 µg/cm^2 culture dish; 4 and 24 h	Anatase–rutile particles are most toxic (LDH release) Reduction of total cell activity directly correlated with particle SSA	(Gerloff et al. 2012)
Anatase TiO$_2$	PPS: 15–30 nm, SPS: mean 285 nm (up to 2 µm)	Human nasal epithelial cells	10–100 µg/mL; 24 h	No genotoxicity or cytotoxicity	(Hackenberg et al. 2010)
Anatase TiO$_2$	Anatase: PPS: 20 nm, SSA: 112–153 m^2/g; rutile: PPS: 3.2 nm, SSA: 112 m^2/g	Human A549 lung epithelial cells; human dermal fibro-blasts	3 µg -3 mg/mL; 1–48 h	Anatase was more cytotoxic	(Sayes et al. 2006)
Anatase–rutile TiO$_2$	PPS: 21 nm, SPS: 130–135 nm and 990–1240 nm	Human A549 epithelial cells; human HepG2 hepatic cells; human THP1 monocytes	10–100 µg/mL; 2–72 h	Marginal toxicity (MTT test, cell death) No SPS size effects on toxicity	(Lankoff et al. 2012)

Table 1 continued

Material	Size	Animal species and cell type	Treatment	Effects	References
SWCNT	Different suspensions with different degree of SWCNT dispersion	Human MSTO-211H mesothelioma cells	7.5–30 µg/mL; 72 h	Agglomeration increases toxicity (↓DNA quantity and cell activity)	(Wick et al. 2007)
Tungsten-carbide	PPS: 56 nm, with FCS: SPS: 145 nm Without FCS SPS: 400 nm	Rainbow trout RT-W1 gill cells	7–30 µg/mL; 3–72 h	With FCS (smaller agglomerates) ENP are more toxic (Alamar blue, CFDA-MA uptake/conversion)	(Kühnel et al. 2009)
Silver[a]	PPS 23 nm, SPS: 43, 190, 490, 110, 1,499 nm	Blood	14–110 µg/mL	Large agglomerates of silver nanoparticles cause less hemolytic toxicity than small agglomerates after 3 h (assuming still negligible degree of ENP dissolution)	(Zook et al. 2011)

PPS Primary particle sizes, *SPS* secondary particle size, *FCS* fetal calf serum, *SSA* specific surface area

[a] Only short-term effect of silver ENP was included in order to avoid interfering dissolution effect

Thus, the issue regarding the effects of agglomeration on cytotoxicity remains controversial. Improved cell culture models, improved characterization of (time dependent) ENP agglomeration, concentration at the cell membrane surface and knowledge regarding the limitations of the tests used may help to overcome the current impasse.

The relevance of in vitro studies in nanotoxicology to the in vivo situation is still disputable (Han et al. 2012). The aforementioned aspects may explain this but also effects may be evoked indirectly by changing cell–cell interactions (e.g., by changing the state of polarization of macrophages (Lucarelli et al. 2004). Macrophages are the cell predominantly in contact with ENPs and are known to affect the functionality of other cells (Holt et al. 2010; Tuan et al. 2008). Such effects might only be seen in vitro in more complex co-culture systems (see (Soma et al. 2000; Tao and Kobzik 2002)). Currently, much effort is being made to develop such systems (Alfaro-Moreno et al. 2008). Besides improving the prognostic value of in vitro tests, one way of solving this issue may be to evaluate the toxic effect of ENPs and their agglomerates directly in animal studies. However, the extent to which ENP agglomeration is able to modify the effects of ENPs on the health of mammals, including humans, has been scarcely investigated. The very few published reports all point in slightly different directions; for instance, agglomeration has no effects (Gosens et al. 2010) or the toxic effect is enhanced by agglomeration (Ispas et al. 2009) (Mutlu et al. 2010). The reason may be that animals are a "black box" without knowledge regarding the fate of ENP material after exposure. The agglomeration status may be altered at each step of the pathway from exposure, uptake, distribution in the body and interaction/uptake by cells. However, for a correct estimation of hazard, an estimate of particle concentration (and degree of agglomeration) at the site of action is crucially important (Fissan et al. 2013). One method of obtaining a more precise picture may be using simulation models, including assumptions on each of the values of transfer coefficients and particle association/agglomeration and dissociation constants, being connected to the arrows, indicated in Fig. 1. First steps in this direction have been made, for example, by correlating workplace release and lung uptake (Zhang et al. 2012), modelling particle deposition in relation to particle size (Hussain et al. 2011), modelling the distribution of particles in the extracellular matrix (Stylianopoulos et al. 2010) and modelling the cellular effects of drugs based on their translocation into the target cell compartment and activation/inactivation dependent concentration–effect relationships (Bruinink 2008). In these models, it should be taken into account that: (1) in vivo effects may only be seen in the long-term and after accumulation (Chen et al. 2011; Shvedova et al. 2014); and (2) the site of action in relation to the location of exposure may be an important

issue for the outcome because not only cellular uptake of the particles may give rise to adverse effects. For instance, Donaldson et al. (2010) suggest that fibers taken up by the lung may clog the pores of the pleural membrane as either single fibers or as agglomerates and cause adverse effects as seen after asbestos exposure. These effects may only be seen in vivo after lung exposure.

Thus, reports so far on the effects of agglomeration on toxicity remain contradictory. Improved cell culture models and computer models simulating the whole pathway between human exposure and effect induction may help to understand the key processes and to overcome the current situation.

Concluding remarks and recommendations

From the various reports (as discussed here), it may be stated that in general, animals, including humans, can deal with and are well protected against microparticles and micrometer-sized agglomerates. Uptake through the lung is limited to particles and agglomerates that are able to reach the alveolar region (being in the nanometer to submicrometer range). In the gastrointestinal tract, only particles and agglomerates less than 300 nm in size are able to enter the body. Within the body, particles and agglomerates again show a size limitation for transfer from the circulation into the tissue. This threshold seems to approximately 250 nm. In all cases, surface chemistry plays an important role in passage through barriers (e.g., limited transfer of positively charged particles through the intestine–body barrier; limited transfer of negatively charged particles through the blood vessel wall). After being transferred into tissue, increased size of ENP agglomerates not only strongly reduces translocation within the tissue but also diminishes uptake by cells. Large (>10 μm) microparticles will in general not be taken up. Furthermore, the chance of ENPs freely entering the cell and its organelles by energy-independent diffusion is strongly reduced by agglomeration because for this the threshold size seems to be approximately 100 nm.

When comparing the threshold particle size for tissue uptake and clearance, a very critical particle and agglomerate size become apparent: approximately 200–250 nm, which represents the upper threshold for being transferred into tissue and for being eliminated from the body. This means that ENPs entering tissue and thereafter forming agglomerates with diameters greater than this threshold size may accumulate. In the case of chronic ENP exposure, toxic threshold concentrations may in the long-term be reached locally, even if after a single exposure only a negligible amount is retained in the tissue, because of agglomeration. Unfortunately, current investigations on the effect of ENPs on health have only been of the effect of a single

exposure, although chronic exposure (with the change in accumulation) is the more relevant situation. So far, knowledge regarding agglomeration-based ENP accumulation and its relevance is still missing, so we strongly recommend including this aspect in future investigations.

Regarding toxicity, no conclusive statement regarding the effect of agglomeration can be made, either for in vitro or in vivo. The currently available in vitro literature is controversial, and literature regarding the effect of agglomeration in vivo is nearly nonexistent. To improve the validity of in vitro investigation-based statements, besides in-depth characterization of the ENPs used (including size distribution), narrowing the agglomerate size distribution and using the appropriate tests for evaluating cellular effects, it is recommended to make precise assumptions on the particle sizes and concentrations (including degradation products) that cells truly encounter during the treatment period. Finally, it is recommended to select and use in vitro systems that mimic more precisely the in vivo situation, thus enabling a more correct prognosis to be made. Regarding in vivo effects, a possible step in improving predictions of hazard is to use computer modelling to simulate the whole pathway from human exposure to exposure of single cells within the body, which should help identify critical knowledge gaps in making a comprehensive assessment of the effects of ENPs on human health.

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