Evaluation of potential engineered nanomaterials impacts on human health: from risk for workers to impact on consumers

CHAPTER

Evaluation of potential engineered nanomaterials impacts on human health: from risk for workers to impact on consumers

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HIGHLIGHTS

- Although the occupational setting is the most likely situation in which low-dose, chronic exposure to ENM, mostly through inhalation, is expected, no occupational ENM-related disease has been reported yet, reliable exposure biomarkers have yet to be identified, and robust methodologies to define exposure itself have yet to be implemented.
- Safety-by-design approaches are considered important tools for risk mitigation and prevention for workers, and, potentially, also for consumers exposed to ENM.
- Consumers are being increasingly exposed to ENM, especially through ingestion, due to the presence of ENM in food, or cutaneous exposure, given the presence of ENM in several cosmetics.
- Due to the increasing variety of ENM produced and put on the market, categorization based on adverse outcome pathway approaches seems a promising strategy for a biologically relevant grouping.
- ENM high adsorption capability, a typical "nano" property, leads to the formation on the ENM surface of a biocorona, including proteins and other molecules present in the biological fluid in which the ENM is dispersed. The formation of the biocorona is a complex and dynamic process. The interaction of the ENM with cells and tissues, and hence its potential health effects, is strongly dependent on its biocorona that confers a new, evolving biological identity to the nanomaterial.

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KEY POINTS

1. No ENM-specific toxic effect on humans has been demonstrated yet.
2. Nanoscale particles are common in workplaces, and not necessarily associated with ENM production.
3. In workplaces exposure to ENM mainly occurs through inhalation, thus rendering airways and the lungs important, yet not exclusive, target organs.
4. Robust strategies to estimate exposure specifically related to ENM are yet to be identified thus justifying protective measures based on precautionary approaches.
5. SbD strategies are in high position in the hierarchy of risk mitigation measures. These are based on methods to minimize ENM-related occupational hazards working on the early steps of the production process through modifications of the design, production, exploitation, storage, and disposal.
6. Consumers are exposed to ENM mainly through ingestion or cutaneous application of widely marketed products, such as food additives and cosmetics, several of which contain nanosized components.
7. Although minor, a sizable fraction of ENM present in food additives is absorbed and distributed to other body compartments.
8. Conversely, no evidence of ENM absorption by intact skin has been obtained so far.
9. When interacting with body tissues, ENM adsorb bioactive molecules present in the biological fluids. As a result, the biological activities of both the ENM and the adsorbed molecule may change, with the complex acquiring a novel, tissue-dependent biological identity.
10. At the light of the ever increasing number and variety of ENM produced and marketed, preventive assessment of potential hazards requires grouping and categorization approached, which should be based on both ENM structural features and expected biological identity.
11. Research activity should pursue the AOP strategy, thus favoring a preventive assessment of ENM-related hazard.

10.1 INTRODUCTION—WHAT DOES THIS CONTRIBUTION DEAL WITH?

In this chapter we make an introductory appraisal of potential engineered nanomaterial (ENM) impact on human health. We speak of "potential ENM impacts on human health" because no specific toxic effect of ENM on humans has been demonstrated yet, in contrast with other unintentionally generated ultrafine particles. Moreover, as concluded by Krug (2014), "many good studies demonstrate, through careful analysis of the dose–response relationship, that we are operating in a safe area, since neither the effects shown nor the predicted environmental concen-

trations lead one to expect any impact on human health or the environment." To date, the situation does not seem substantially changed (Warheit, 2018).

Experimental studies on the effects of ENM on animals or in vitro models are being published at the impressing rate of more than 2000 per year, and an attempt to perform a critical review would be unreasonable. Moreover, the findings of these studies, especially those published some years ago, are often highly questionable, since many contributions do not take into account the "fundamental rules as are applied to toxicology" (Krug, 2014) and actually "contribute to the Babylonian plethora of low-value results that exists today" (Krug, 2014). However, those studies have fed the perception of an increased risk potential in the public opinion. At the same time, we have excluded from our contribution any discussion about the intrinsic limitations of in vitro studies and how these limitations can be overcome by the most advanced and sometimes sophisticated developments in the models adopted, such as 3D cultures, cocultures of different cell lines or primary cells, organoids, or organ-on-a-chip microdevices.

Our contribution will focus, instead, on two limited, yet critical, aspects of the rapidly developing field of nanotoxicology. We try to answer to the following questions. First, is there any documented risk for workers who produce, modify, handle, or in any sense exploit ENM at their workplace? Second, does the exposure to ENM in our everyday life of consumers have any potential consequence? Moreover, three subjects of great current interest, such as safety-by-design (SbD) strategies, adverse outcome (AO) pathway (AOP) approaches, and the relationship between synthetic and biological identities of ENM, will be also considered.

Emphasis is given to ENM widely present in industry or on the market, such as multiwalled carbon nanotubes (MWCNT) or TiO_2 and SiO_2 nanoparticles (NPs). Instead, intentional exposure to nanobiomedical devices, which are increasingly exploited or proposed in a variety of diagnostic and therapeutic applications, will not be considered.

10.2 ARE ENGINEERED NANOMATERIAL WORKERS AT RISK?

The occupational setting represents perhaps the most likely situation in which chronic exposure to a specific ENM may occur. This exposure usually occurs through the respiratory system where, due to their size, ENM can reach the alveoli (Borm et al., 2006). In animal models the local effects of ENM exposure are usually inflammation (Chou et al., 2008), intuitively related to the production of oxidant compounds, directly or indirectly dependent on the interaction between ENM and the tissue (Xia et al., 2006). On a chronic time scale, fibrosis commonly ensues (Sanchez et al., 2009).

In particular fiber-like materials, such as carbon nanotubes, behave like asbestos fibers in animals, and the mechanisms involved are actively investigated (Wang et al., 2017). Even though asbestos reminds of carcinogenic risks, available literature

on fibrous ENM as possible carcinogens is not conclusive. In its 2017 monograph on carcinogenicity of some ENM and fibers (IARC, 2017), the IARC concluded that no relevant data on human carcinogenicity of MWCNT were available to the ad hoc constituted working group. However, several studies documented oncogenic activity of MWCNT in experimental animals. Intrascrotal injection of nanotubes caused peritoneal mesothelioma, the typical asbestos-related cancer, in male rats (Sakamoto et al., 2009). These results were consistent with a previous contribution, reporting mesothelioma development in male $p53^{+/-}$ mice intraperitoneally injected with MWCNT (Takagi et al., 2008) and were successively confirmed in either male or female rats exposed through a single intraperitoneal injection of two distinct, although similar, MWCNT preparations (Nagai et al., 2011). A possible limit present in these studies consists in the route of administration adopted, since exposure to MWCNT is expected to occur through the airways. However, inhaled MWCNT had a promoting effect on 3-methylcholanthrene-initiated bronchioloalveolar adenoma and lung adenocarcinoma in male mice (Sargent et al., 2014). On the contrary, negative results were reported for subcutaneous administration of MWCNT in male mice (Takanashi et al., 2012). Analysis of mechanistic data available in literature led the IARC Working Group to conclude for a moderate mechanistic evidence for mesothelioma-related end-points for MWCNT and to a weak evidence for single-walled carbon nanotubes (SWCNT), due to the lack of data. The IARC Working Group underlined that "the mechanistic events relevant to genotoxicity, lung inflammation, and fibrosis as well as translocation to the pleura, are liable to occur in humans exposed to CNT by inhalation." It should be stressed that most of the positive studies exploited a specific MWCNT preparation (MWCNT-7 from Mitsui Ltd) and that IARC pointed to the existence of significant gaps in the comprehension of the mechanisms underlying carcinogenicity due to CNT heterogeneity and limited number of long-term studies. Thus while concluding that there is inadequate evidence in humans for the carcinogenicity of carbon nanotubes, IARC considered sufficient the available evidence to define that particular MWCNT preparation (MWCNT-7) carcinogenic in experimental animals, while evidence was considered inadequate for MWCNT other than MWCNT-7 (and closely similar preparations). The more limited availability of studies led IARC to define inadequate the evidence for the carcinogenicity of SWCNT. These elements led to the definition of MWCNT-7 as "possibly carcinogenic to humans (Group 2B)," according to the IARC grouping of carcinogens, while other MWCNT and SWCNT have been defined as not classifiable as to their carcinogenicity to humans (Group 3).

Another ENM produced in thousands of tons per year in several manufacturing processes is represented by NP of TiO_2. In a 2010 IARC monography TiO_2 is also categorized as a Group 2B carcinogen for humans (IARC, 2010); however, the monography concern both bulk and nanosized TiO_2. Moreover, none of the studies concerning human carcinogenicity considered the possible impact of particle size. IARC concluded that these studies (three epidemiological cohort studies and a single population-based case–control study) do not suggest an association between

occupational exposure and risk for cancer (either in the lung or in other sites). In contrast, evidence for carcinogenicity in animals is stronger, although somewhat inconsistent. Indeed, while two inhalation studies in rats and one in mice were negative, two other studies reported an increased incidence of tumors in rats exposed to nanosized TiO_2. Conversely, oral, subcutaneous, and intraperitoneal exposures were all negative. On the basis of these data, IARC concluded that "there is inadequate evidence in humans for the carcinogenicity of titanium dioxide" but that "there is sufficient evidence in experimental animals for the carcinogenicity of titanium dioxide" and that "titanium dioxide is possibly carcinogenic to humans (Group 2B)." Again, these conclusions are not specifically applied to nanosized or bulk TiO_2.

It should be recalled that possible consequences of ENM inhalation may not be limited to local lung effects. Indeed, the large alveolar surface constitutes a possible way of ENM absorption and translocation to distant organs. Although this mechanism is quantitatively poorly efficient (Kreyling et al., 2017a), it has been documented in experimental models and may be of great pathophysiological significance. Moreover, local inflammation may lead to the release of inflammatory mediators, in particular cytokines, in the blood. Recent evidence indicates that this indirect mechanism may be more important than direct effects of the ENM translocated from the lungs to the rest of the body (Ganguly et al., 2017).

The relevance of the respiratory route explains why the presence of ENM in aerosols or in dust derived from any phase of the productive process is considered an important parameter (Savolainen and Pietroiusti, 2017; Kuhlbusch et al., 2011). Experimental evidence has indicated that in a real-life scenario nanomaterials often agglomerate or adsorb to background particles, suggesting that they interact with the respiratory system under these forms (Brouwer, 2010; Ding et al., 2017). This peculiarity should be adequately considered when experimental conditions for in vitro testing of biological effects are planned. On the contrary, a common tendency in the field is to disperse the ENM as much as possible (ideally obtaining monodispersed suspensions) using natural or synthetic dispersing agents, which, on the other hand, may also have unwanted effects. On the other hand, it is possible that the surfactant produced by alveolar cells acts as a natural dispersant, thus increasing the fraction of monodispersed ENM. To complicate further the situation, the biological effects of monodispersed or agglomerated ENM may be different, and ENM agglomerates may be endowed with peculiar toxic effects not detected with the monodispersed counterparts (Rotoli et al., 2015).

In the workplace environment ENM may be present under different forms, monodispersed or included in homo- or hetero-agglomerates. This fact, along with the high possibility of confounding effects from the NPs present in the environment independently from the productive process, renders exposure assessment a very challenging issue. Thus robust exposure limits based on experimental evidence have not yet been established, and the recommended preventive approaches are still based on precautionary principles. The approaches proposed include modifying production processes, enforcing administrative means, and adopting personal pro-

tective equipment [see Savolainen and Pietroiusti, (2017) for an extensive discussion]. In particular in the more general context of SbD strategies, modifications of the production process should be conceived and implemented, on the basis of ENM physicochemical properties, to lower the risk acting on either the exposure potential or the ENM-associated hazards.

However, occupational exposure limits (OELs) have been proposed for some ENM (TiO_2 NPs, carbon nanotubes, and nanofibers, silver NPs, cellulose nanocrystals) (Schulte et al., 2018). Given the hundreds of different ENM that are being produced and marketed, substance-by-substance efforts to establish specific OEL are not feasible, and the OELs proposed should be considered as prototypes for risk assessment in the perspective of grouping and categorization of ENM (Schulte et al., 2018).

Lack of clear cut adverse effects of ENM upon occupational respiratory exposure has not prevented the search for exposure biomarkers. The first wave of studies, reviewed by Liou et al. (2015), did not offer consistent results. More recently, in a series of contributions (Pelclova et al., 2016a,b,c, 2017), Pelclova et al. have demonstrated that inflammatory markers, markers of DNA and protein oxidative damage, and lipid oxidative markers are all increased in the exhaled breath condensate of workers exposed to NP of TiO_2. The authors report that the "median particle number concentration in the production line ranged from 1.98×10^4 to 2.32×10^4 particles/cm^3 with approximately 80% of the particles <100 nm in diameter," thus suggesting a role for nanosized titanium dioxide, and a mass concentration between 0.40 and 0.65 mg/m^3, well below the proposed OELs (NIOSH, 2011; Morimoto et al., 2010). Most recently, Zhao et al. have investigated cardiopulmonary parameters among workers who were exposed to NP of TiO_2 to identify the related biomarkers. The total mass concentration of particles was 3.17 mg/m^3, 39% of which were NPs. Several markers of lung damage (SP-D and reduced pulmonary function), cardiovascular disease (VCAM-1, ICAM-1, LDL, and TC), oxidative stress (SOD and MDA), and inflammation (IL-8, IL-6, IL-1β, TNF-α, and IL-10) were found associated with occupational exposure, but only the surfactant protein SP-D showed a time (dose)–response pattern in the exposed workers (Zhao et al., 2018).

Exposure routes other than respiratory, such as the gastrointestinal system and the skin, are less important for workers than for final consumers (see below, impact of ENM on consumers). However, if ENM do not penetrate in the airway wall and are not persistent in the lung tissue, they are trapped in mucus, moved up to the pharynx by ciliated cells, and eliminated through swallowing, thus reaching the gastrointestinal system (Kreyling et al., 2013). Although cutaneous exposure to manufactured ENMs in the workplace has been tentatively quantified (Van Duuren-Stuurman et al., 2010), effective dermal penetration is, at best, uncertain (see later, impact of ENM on consumers). On the other hand, secondary exposures may also follow skin exposure, through unwanted airway contamination.

10.3 SAFETY BY DESIGN

The strategy of SbD {prevention through design (PtD) in the United States [National Institute for Occupational Safety and Health (NIOSH)]} is based on methods to minimize occupational hazards working on the early steps of the production process. In practice hazards should be anticipated and eliminated, or at least reduced, through modifications of the design, production, exploitation, storage, and disposal.

SbD strategy was not conceived specifically for ENM, but, since several years, the NIOSH has devoted much attention to SbD/PtD strategies for ENM workers. NIOSH launched a specific governmental supported initiative (NIOSH) and, more recently, has released a publication on "General Safe Practices for Working with Engineered Nanomaterials in Research Laboratories" (NIOSH, 2012), where Sbd/PtD strategies are highlighted. When applied to ENM, as for other materials, SbD approaches must take into account the whole life cycle and can target either exposure or hazard reduction (Geraci et al., 2015), thus minimizing risks to workers throughout the process (Schulte et al., 2008). In the hierarchy of controlling workplace hazards through (1) eliminating, substituting, or modifying the nanomaterials; (2) engineering the process to minimize or eliminate exposure to the nanomaterials; (3) implementing administrative controls that limit the quantity or duration of exposure to the nanomaterials; (4) providing for use of personal protective equipment (NIOSH, 2012), SbD strategies are thus in a higher position than the use of individual protection devices.

Several projects funded by the European Commission deal with the strategy of SbD. For instance, summing up the conclusions of the NanoImpactNet project, Hunt et al. (2013) state "Regulatory bodies should encourage an industrial and innovation approach by which midlife and end-of-life information must be fed into start-of-life (design) information, closing the life cycle of NMs."

For their successful exploitation, SbD strategies require an in-depth study of the structural determinants that underlie hazard or facilitate exposure. Indeed, several early attempts to apply a SbD strategy relied on the "fiber paradigm," the attribution to the fiber-like shape of an important portion of the hazard associated with the exposure to high-aspect ratio NPs (HARN), such as MWCNT (Donaldson et al., 2011; Czarny et al., 2014; Tsuruoka et al., 2015). These approaches may be applied to other HARNs (Allegri et al., 2016a). Mitigation of MWCNT-associated hazards has been also pursued through functionalization (Hussain et al., 2016; Fanizza et al., 2015; Deligianni, 2014; Chatterjee et al., 2014), which can modify several properties of the material, such as the tendency to agglomerate or protein adsorption (Allegri et al., 2016b). Examples of other potential applications of SbD strategies to ENM have been proposed for silica-based ENM (Lehman et al., 2016) and NPs of CeO (Davidson et al., 2016), while a critical appraisal of the state of the art has recently been provided by Hjorth et al. (2017).

10.4 IMPACT OF ENGINEERED NANOMATERIAL ON CONSUMERS

The gastrointestinal system and the skin represent the most important routes for non-professional ENM exposure (Pietroiusti, 2012). As far as the oral route is concerned, a percentage of nanosized particles are present in common food additives, such as TiO_2 (E171) and amorphous silica (E551). Less frequently, gastrointestinal system can also be exposed to silver (used as colorant, E174) and zinc oxide NP. Another way to introduce ENM through the oral route is due to food contamination by nanomaterials shed from packaging. In many types of modern biopolymer-based packaging materials fillers with at least one nanoscale dimension are present to improve the mechanical and barrier properties of the material, thus leading to the formation of nanocomposites (Ghanbarzadeh et al., 2015). Some NPs used as fillers for packaging materials, such as silver, TiO_2, or ZnO NP, have also antimicrobial properties (Ghanbarzadeh et al., 2015). Lastly, as recalled earlier, inhaled ENM, cleared from the airway through the mucociliar mechanism, is swallowed and, hence, reaches the gastrointestinal system.

Exposure estimates have been performed for the most widely used foodborne ENM and have yielded values of 0.036 and 1.8 mg/kg of body weight for a heavy-consumer's daily intake of, respectively, TiO_2 (Lomer et al., 2000) and SiO_2 NP (Dekkers et al., 2011). How much of the ENM intake is absorbed? In a recent study, Kreyling et al. radiolabeled TiO_2 NP and followed their fate after a single ingestion in rats. More than 99% of the ingested amount was rapidly eliminated in feces. However, 0.6% of the administered dose passed the gastrointestinal-barrier and about 0.05% was still detected after 7 days, with NP identified and quantified in several organs, such as the skeleton, uterus, spleen, brain, kidneys, lungs, and the liver (Kreyling et al., 2017b). These data should be considered at the light of Ti levels measured postmortem in human liver and spleen, which indicate that TiO_2 particles, including a significant fraction of NP, are present in human liver and spleen. Authors conclude that "The levels are below the doses regarded as safe in animals, but half are above the dose that is deemed safe for liver damage in humans when taking into account several commonly applied uncertainty factors" and that "health risks due to oral exposure to TiO_2 cannot be excluded" (Heringa et al., 2018).

Direct investigations on possible adverse effects of the foodborne ENM are still in limited number. Moreover, many of these studies have been performed with laboratory-grade materials, consisting of pure ENM preparations, rather than with food-grade materials, corresponding to those effectively used as food additives in real life, which contain a variable nanosized fraction. This discrepancy can be conspicuous. Thus at least for TiO_2 NP, it has been proposed that gastrointestinal effects should be studied with materials endowed with characteristics resembling those of food-grade TiO_2: (1) crystalline-phase: anatase, (2) isoelectric point: very

close to 4.1, (3) fraction of NPs comprised between 15% and 45%, and (4) a low specific surface area (around 10 m^2/g) (Dudefoi et al., 2017).

In vitro experiments on cells of gastrointestinal origin have indicated that while the acute toxicity of silica and TiO_2 NP is generally low, zinc oxide NP exhibits a moderate toxicity, likely attributable to the dissolution of NP in simulated gastrointestinal fluids (McCracken et al., 2013; Setyawati et al., 2015), and other ENM, such as silver NP, has a significant acute toxicity (Schneider et al., 2017). These effects are usually attributed to the availability of a significant amount of metal ions leading to ROS production and oxidative stress (Setyawati et al., 2015). Also animal studies indicate a low acute toxicity of TiO_2 NP (Jovanovic et al., 2016), with very high values of NOAEL [no observed adverse effect level (Warheit et al., 2015)]. However, for longer exposures, significant biological effects on intestinal cell function are observed. For instance, microvilli loss, impaired barrier function, and decreased nutrient transport are observed after "chronic" (5 days) but not acute (4 hours) exposure to TiO_2 NP of a Caco-2/HT29-MTX coculture model (Guo et al., 2017). This model is particularly interesting since the coculture is covered by a mucus layer, thus mimicking in this regard healthy intestinal mucosa. In the same model impaired glucose transport caused by exposure to TiO_2 NP has been recently described (Richter et al., 2018). In vivo experiments indicate that chronic oral exposure to TiO_2 NP produces also extraintestinal changes, such as hyperglycemia (Hu et al., 2016) and cardiac dysfunction (Hong et al., 2016), along with increased levels of Ti in several organs (Hu et al., 2016).

Besides the expected local effects, such as oxidative stress and inflammation, experimental evidence, reviewed by Bergin and Witzmann (2013), Pietroiusti et al. (2016), and Mercier-Bonin et al. (2018), suggests that ENM may exert important effects through their interaction with gut microbiota (the community of organisms living within the gastrointestinal tract). Since the concept of microbiota is by no way limited to the gastrointestinal system, although most available information concerns this district, it is possible that ENM interactions with the microbiota of other districts may also have biological relevance. An interesting example of interaction among intestinal epithelial cells, ENM, and microbiota has been recently offered by Richter et al. (2018). Glucose uptake was measured in the Caco-2/HT29-MTX coculture model cited above in the presence of TiO_2 NP, using control monolayers and monolayers cultured with the commensal *Lactobacillus rhamnosus* GG (*L. rhamnosus*). Reduction in glucose transport was observed along with microvilli damage in the absence but not in the presence of the beneficial bacteria.

Gastrointestinal tract is bathed by a variety of diverse secretions, from the mouth to the colon-rectum. The characteristics of these fluids, which are highly divergent, can affect the physicochemical features of ingested ENM, such as surface chemistry, dissolution (in case of metal or metal oxide NPs, such as Ag, CuO, or ZnO), agglomeration and, hence, the interaction of the material with the mucosa surface and its absorption (Jo et al., 2016).

Even more importantly, gastrointestinal fluids may affect the biological outcome of exposure to ENM in an additional way. Indeed, when suspended in bio-

logical fluids, which are complex solutions of low- and high-molecular weight compounds, ENM, given their high adsorbing capability due to their high surface/volume ratio, adsorb proteins, and other components. Thus the surface of the ENM, which actually interacts with cells and extracellular structures, is not the bare surface of the NP but, rather, a complex array of biological molecules derived from the biological fluid in which the NPs, or their agglomerates, are suspended. The characteristics and dynamics of this corona of molecules have been mainly investigated in reconstituted systems in which the ENM interacts with one or more proteins, hence the denomination of "protein corona." But, especially in the complex environment of the gastrointestinal tract, which is also rich of lipids and detergent molecules, it is highly likely that the ENM corona has a more complex, likely tract-specific, composition. Actually, one of the first studies on oral exposure to ENM documented the adsorption of bile salts to the nanomaterial and attributed to this interaction some biological effects (McCracken et al., 2013). The interaction between ENM and the body surface (in this case, the gastrointestinal mucosa and the mucus layer that cover wide portions of the mucosal surface) is profoundly affected by the composition of the corona. Conversely, the interaction with the mucus layer markedly limits the contact of the material with mucosal cells, its absorption, and systemic delivery (Mercier-Bonin et al., 2018). Additional complexity is given by the possibility that ingested ENM also interact with food matrices (Go et al., 2017).

In conclusion, for biological studies, the structural identity of the ENM (i.e., the structural determinants that influence its biological effects and should be thoroughly characterized to identify proper structure–activity relationships) should be considered together with ENM biological identity, that is the surface characteristics resulting from the interaction of the NP with the components of the biological fluid in which they are dispersed (see Section 10.6).

As far as the skin is concerned, many cosmetic products contain nanostructured components. In particular, NPs of TiO_2, SiO_2, and ZnO are present in many sunscreens. SiO_2 NP can be also present in toothpastes, antiwrinkling products or polishing creams, due to their high absorbing properties. Evidence for skin penetration of the most common ENM has been thus far negative (Krug, 2014). For example, experiments performed on animal models indicated that, as anticipated for occupational exposures, TiO_2 NPs contained in topical products are not absorbed by intact skin (Sadrieh et al., 2010). However, no systematic investigations have been performed on the possibility that ENM, contained in cosmetics, may reach basal epidermis cells, or even distribute to other organs through dermal vessels, upon exposure of damaged skin. This issue is not trivial, since, in populations characterized by high risk for skin cancer, sunscreens are used everyday, sometimes over large areas of body surface.

10.5 STRUCTURAL IDENTITY VERSUS BIOLOGICAL IDENTITY(-IES): THE ROLE OF BIOCORONA

Upon ENM dispersion in biological fluids, a layer of adsorbed molecules, present in the fluid, covers the NP surface. This dynamic layer is composed by a stable portion, called the "hard" corona, which directly interacts with ENM surface, and a more superficial portion, called "soft" corona. Protein corona is a highly dynamic structure and undergoes changes depending on the prolongation of the incubation or on other environmental conditions present during the interaction (Tenzer et al., 2013; Vilanova et al., 2016; Feiner-Gracia et al., 2017; Weiss et al., 2018). Given the high adsorption capability of ENM, the composition of this corona may be very complex. This has been demonstrated also for ENM widely present in productive processes and on the market. For instance, 115 and 48 proteins were identified through liquid chromatography-tandem mass spectrometry in the corona of, respectively, negatively charged 20 and 100 nm SiO_2 or TiO_2 NP dispersed in rat plasma (Shim et al., 2014). Protein variety was lowered when adsorption was investigated with arginine-coated SiO_2 NP (Shim et al., 2014). Improved analytical methods have led to the discovery of a larger variety of corona proteins. When dispersed in respiratory tract lining fluid, ENMs [specifically SiO_2 and poly(vinyl) acetate NPs] adsorb hundreds of different proteins [429 vs 698 proteins identified (Kumar et al., 2016)]. In this complex structures interactions occur not only between proteins and the ENM, but also among proteins themselves, giving rise to a "corona interactome" (Pisani et al., 2017a).

As expected, corona formation is strongly influenced by structural ENM properties, such size, shape, porosity and surface charge, composition, topography, or reactive groups (Sakulkhu et al., 2015; Tenzer et al., 2011; Ma et al., 2014; Paula et al., 2014; Clemments et al., 2015; Di Cristo et al., 2016), as well as by physicochemical characteristics of the dispersion fluid, such as pH (Titma, 2018).

The interaction between ENM and biomolecules, present in biological fluids, has complex, still incompletely characterized, effects on the biological reactivity of the NP. First, biocorona adsorption changes the surface properties of ENM and, hence, surface-dependent biological effects. For example, TiO_2 NPs, which are usually considered a low-toxicity ENM, catalyze photogenerated radical production and, hence, are endowed with phototoxicity. When the NPs are suspended in a protein-rich fluid, these effects are lowered in proportion of the protein-coated NP surface and, hence, of the protein concentration of the fluid (Garvas et al., 2015). However, the ability of TiO_2 NPs to produce oxidative damage is not limited to phototoxicity. Indeed, exposure to this ENM leads to oxidation of cell membrane lipids, an effect also mitigated by the protein corona (Runa et al., 2017). While ENM surface chemistry obviously affects protein corona formation and composition, different protein coronae may, conversely, affect other physicochemical

properties of the ENM, such as agglomeration tendency and, hence, toxicity (Mortensen et al., 2013; Allegri et al., 2016b).

Moreover, the protein corona may directly determine the interaction between ENM and cells or tissues. For instance, the presence of a protein corona markedly modifies ENM uptake by cells (or penetration in tissues) (Lesniak et al., 2012; Caracciolo et al., 2015; Shahabi et al., 2015; Aoyama et al., 2016; Binnemars-Postma et al., 2016; Mirshafiee et al., 2016; Saikia et al., 2016; Tavano et al., 2018) and toxicity in vitro (Panas et al., 2013; Docter et al., 2014; Fedeli et al., 2014; Liu et al. 2015; Orlando et al., 2017) or in vivo (Yoshida et al., 2015; Saikia et al., 2016). Different protein coronae explain why the biological effects of a given ENM change depending on the serum (human vs bovine) used for the dispersion of the material (Izak-Nau et al., 2013) or, simply, for cell culture (Pisani et al., 2017b). If the adsorbed protein has a specific, biologically relevant, role in that cell/tissue system, the interaction may also confer novel biological activities to the ENM. For instance, fibrinogen, a major component of plasma proteins, significantly enhances cytotoxicity and proinflammatory activities of SiO_2, carbon soot, and TiO_2 NP on murine alveolar macrophages in a dose-dependent manner (Marucco et al., 2016).

The interaction with ENM may also cause conformational alterations and/or oxidative damage (Jayaram et al., 2017) of adsorbed proteins of both hard and soft coronae (Wang et al., 2011). For example, adsorption to TiO_2 or SiO_2 NP causes characteristic, pH-dependent distortions of adsorbed bovine serum albumin (Ranjan et al., 2016; Givens et al., 2017). Indeed, at pH 2 but not at pH 4, the protein is completely unfolded when adsorbed on TiO_2 NP and markedly stretched when adsorbed to SiO_2 NP. Interestingly, structural anomalies or altered conformation of adsorbed proteins elicit specific cell responses (Jayaram et al., 2017; Borgognoni et al., 2015). If the protein is an enzyme, modification of its conformation may cause inhibition, stimulation, or more complex changes in its activity, depending on the adsorbing ENM (Deng et al., 2014). Furthermore, enzyme activation by ENM may have relevant pathophysiological consequences, even in the absence of cells. For example, very low concentrations of TiO_2 NP (50 ng/mL) trigger the blood contact system through FXII adsorption and activation, leading to the stimulation of kinin, complement, and coagulation cascades (Ekstrand-Hammarstrom et al., 2015). Interestingly, among plasma proteins, SiO_2 NPs exhibit a peculiar adsorption capability toward components of complement and coagulation pathways, together with lipoproteins (Tenzer et al., 2011). Proteins involved in innate immunity present in other biological fluids also exhibit a peculiar tendency to be adsorbed to ENM (Kumar et al., 2016).

Lastly, ENM corona is composed not only by proteins, but also by other components of the biological fluid in which they are dispersed. Indeed, in bronchoalveolar fluid or gastrointestinal secretion, lipids (Whitwell et al., 2016) or other organic molecules may constitute a significant portion of the surface layer. For this reason, the term biocorona seems more appropriate than protein corona. An important bioactive molecule which may enter ENM biocorona is bacterial

lipopolysaccharide (LPS), a common environmental contaminant and a powerful macrophage activator. Adsorption of LPS to various ENM, such as SiO_2 (Di Cristo et al., 2016), TiO_2 (Bianchi et al., 2015), Ag (Galbiati et al., 2018), and Au (Li et al., 2017) NP, powerfully enhances their proinflammatory effects, at least in vitro. What is more important, also the activities of the adsorbed molecule are quantitatively and qualitatively changed (Bianchi et al., 2015, 2017; Li et al., 2017), thus adding an additional layer of complexity to the mechanisms underlying ENM effects in biological systems.

Overall, even for widely used and deeply characterized ENM, the formation of biocorona provides the nanomaterial with a novel biological identity (Monopoli et al., 2012), directly responsible for ENM biological activities, the formation of which is influenced by ENM physicochemical features. This fact may have profound repercussions in toxicological studies, either in vitro or in vivo (Vranic et al., 2017; Monopoli et al., 2011; Tenzer et al., 2013; Wohlleben et al., 2016). More importantly, the composition of the biological fluids is obviously modified in several conditions of pathophysiological relevance. Therefore the biological identity of a given ENM and, hence, its effects on cells and tissues are expected to vary accordingly. These multiple identities greatly expand the complexity of a preventive estimation of possible health effects of a single ENM.

For example, after having characterized the interaction between polystyrene and TiO_2 NPs with human bronchoalveolar fluid, Whitwell et al. suggest that ENM may interact, together with surfactant-associated proteins (SP-A, -B, and -D), also with lipids and pulmonary surfactant, implying potential health effects for "people with chronic airway diseases such as asthma and chronic obstructive pulmonary disease (COPD), or those who have increased susceptibility toward other respiratory diseases" (Whitwell et al., 2016). Thus a more thorough investigation of ENM biological identities is needed to identify possible markers of enhanced susceptibility to adverse effects. Interestingly, a different composition of protein corona of SiO_2 NP dispersed in respiratory tract lining fluids derived from asthmatic or control subjects has been recently demonstrated (Kumar et al., 2017).

Finally, it should be remembered that ENM interaction with proteins or other organic molecules is by no means limited to extracellular organic fluids. For instance, silica NPs bind also intracellular proteins, in particular those with large unstructured regions, such as RNA-binding proteins and translation initiation factors (Klein et al., 2016; Vitali et al., 2018). Interestingly, ENM adsorption of intracellular proteins has been recently exploited for biotechnological applications (Fogli et al., 2017).

10.6 THE ADVERSE OUTCOME PATHWAY APPROACH

An AOP is a conceptual construction of a sequential chain of events, causally related, experimentally validated, and affecting increasingly complex levels of bio-

logical organization, which results in an AO. The AOP concept has been applied to AOs, considered relevant for risk assessment, which affect either health or environment (OECD, 2017). Between the molecular initiating event (MIE) and the AO, the pathway is described by several key events (KEs), linked by KE relationships (OECD, 2017).

Besides its importance in defining a frame to describe mechanistic relationship to explain toxic effects at organism or population levels, AOPs provide an approach to use relatively simple biological models, or even in silico modeling, to study KE linked to outcomes relevant for risk assessment. Originally developed for ecotoxicology (Ankley et al., 2010), AOPs are increasingly exploited in chemical toxicology (OECD, 2018). In particular, in 2012 OECD started a program on the development of AOPs, defined them as "the central element of a toxicological knowledge framework being built to support chemical risk assessment based on mechanistic reasoning" (OECD).

The AOP approach has been proposed as a powerful tool for linking predictive toxicology to ENM risk assessment (Schulte et al., 2018; Lai et al., 2018; Mirshafiee et al., 2017). Indeed, as underlined recently by Vinken (2018), besides helping in the identification of data gaps or the logical organization of available experimental data, AOPs may also favor categorization and grouping of toxicants, linking shared KEs and MIEs to common physicochemical features. This obviously requires an in-depth characterization of the ENM as an integral component of the AOP formulation. AOP properties are particularly important for ENM, since the fast introduction into the productive cycle and, subsequently, on the market of new nanomaterials makes practically unfeasible a one-by-one preventive toxicological analysis.

However, a correct adoption of AOP approach implies the satisfaction of several requirements. The most important is that MIE identification must be based on the assumption that sufficient experimental evidence exists to support the AOP and to document the ENM role. Therefore the definition of a robust AOP requires a series of experimental studies aimed at defining not only the MIE but also the KEs.

For this reason, examples of fully defined AOP involving ENM are still lacking, and none of the AOPs (endorsed, under review, or under progress) enlisted in OECD AOP knowledge base (https://aopkb.oecd.org/) involve specifically ENMs. Examples of proposed ENM-specific AOP concern model organisms such as *Danio rerio* [inhibition of egg hatching by CuO NPs (Muller et al., 2015)] and *Caenorhabditis elegans* (with effects at whole-genome level of MWCNT, combining system biology with possible identification of AOPs).

As far as possible impact on human pathology is concerned, the development of an ENM-related AOP seems more advanced for carbonaceous nanomaterials and, in particular, for carbon nanotubes. Wang et al. (2015) explored the possibility that a common AOP leads to fibrosis starting from respiratory exposure to different carbonaceous ENMs (three types of SWCNT, graphene, and two types of graphene oxide). They first performed in vitro studies on the production of inflammatory (IL-1β) and fibrogenic (TGF-β) cytokines by macrophages and airway epithelial

cells, and then correlated the results with data from experiments on animal models, where, besides cytokine production, the development of lung fibrosis was assessed (Wang et al., 2015). Authors concluded that the dispersal state and surface reactivity of ENM were important determinants of the profibrogenic AOP. The correlation of changes at gene expression level in vivo (proposed as KE) with apical endpoints (fibrosis and septal thickness) has indicated that the doses of MWCNT responsible for the KE are comparable to those validated for the final outcomes by the NIOSH (Labib et al., 2016). The AOP leading to inflammation and fibrosis upon exposure to MWCNT has been further dissected in mice, demonstrating that Stat-6 activation, rather than IL-1β production, is correlated with fibrotic changes (Nikota et al., 2017). Finally, in experimental animals the exposure to MWCNT leads to the development of mesothelioma [a well-documented effect, at least for fiber-shaped MWCNT (Suzui et al., 2016; Rittinghausen et al., 2014; Takagi et al., 2012; Sakamoto et al., 2009; Takagi et al., 2008; Nagai et al., 2011)]. However, the pathway involved is not completely defined (Kuempel et al., 2017) since the implied KE is not only those associated with inflammation and fibrosis, although, undoubtedly, these mechanisms are very important in the carcinogenic effects of MWCNT (Poland et al., 2008). Recent evidence suggests that another KE, consistent with the immunopathologic findings detected in mesothelioma patients, is the capability of MWCNT to induce a local immunosuppressive state, increasing the accumulation of monocytic myeloid-derived suppressor cells and thus contrasting the T-cell–dependent immune surveillance on tumor cells (Huaux et al., 2016).

An additional, important advantage of AOP approach is that, as an example cited earlier (Eom et al., 2015) demonstrates, it would easily include data from system biology techniques, which are increasingly exploited in nanotoxicological studies (Costa and Fadeel, 2016).

10.7 CONCLUSIONS

The knowledge-based body of data generated by systems toxicology (ST) and AOP analysis approaches will certainly improve the safety assessment of ENM, but the complex and multifaceted nature of events occurring at the nanobiointerfaces (at the cell, tissue, organ, and system level) also implies that the full replacement of in vivo assessment is not yet possible (Bergamaschi et al., 2015). To serve Risk Analysis, the data generated by ST should be validated in real exposure scenarios where more complex and unpredictable interactions can occur. Epidemiological data from human populations specifically exposed to ENM are currently very limited for many reasons (Guseva Canu et al., 2018). Epidemiological research and interventional studies, which are a necessary prerequisite for health programs and prevention, should lead to identification, selection, and validation of candidate biomarkers for generalized exposure and health effects surveillance (Bergamaschi et al., 2015). In such studies biomarkers could help to circumvent the issues of the

heterogeneity of ENM, making difficult to identify and recruit enough workers with the same exposure pattern, considering that exposures to different NPs may lead to the same pathway for disease, or share common mechanisms (Schulte and Hauser, 2012; Bergamaschi et al., 2017).

In conclusion, while we share the opinion that health decisions on a variety of nanomaterial types still await better scientific bases (Warheit, 2018), we wish to stress that, until now, diseases directly linked to ENM exposure "belong to the realm of possible risk (i.e. cannot be excluded, but are unlikely)" (Pietroiusti, 2012). However, it should be also stressed that our knowledge of the consequences of ENM interactions with specific bioactive molecules present in biological fluids or with the microbial populations resident in our body compartments (first of all in the gut) is still very incomplete. The elucidation of these interactions will likely represent the most important objectives of future research on the possible health impact of ENM.

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FURTHER READING

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