CHAPTER

41

Engineered Nanomaterials: Biomarkers of Exposure and Effect

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INTRODUCTION AND BACKGROUND

The use of engineered nanomaterials (ENM) and their applications has grown dramatically since the turn of the 21st century because of multiple technological benefits of the use of material at nanoscale. More than 1600 "nanoenabled" products in commerce, all required workers for that to happen (http://www.nanotechproject.org/cpi/), and, according to recent estimates, 6 million workers will be potentially exposed to ENM in 2020 (Roco, 2011).

Nanotechnologies is in fact an umbrella name for a great number of technologies that utilize material at nanoscale for different purposes. For these reasons, nanotechnologies have been recognized as highly cross-cutting technologies, whose products, based on the use of nanomaterials, utilize physical and chemical properties of ENM, different from their chemically identical bulk counterparts (Savolainen et al., 2010).

However, during the production and use of ENM, there is the chance of exposure of workers, consumers, and the environment (Savolainen et al., 2010; Kuhlbusch et al., 2011; Valsami-Jones and Lynch, 2015). The effects of such exposure cannot be predicted based on our current understanding of chemicals, given the fact that material at nanoscale has both a particulate identity and a molecular identity, which are responsible for the potential biological effects. A recent review has summarized work conducted in relation to exposure to ENMs in the workplace and processes that may lead to such exposure (Kuhlbusch et al., 2011). For example, it could be shown that exposure to TiO_2 nanomaterials may arise during cleaning and maintenance operations as well as in the case of a failure of normal operation (Plitzko, 2009), to TiO_2 and silver during the reactor and vacuum pump operations (Lee et al., 2011), to carbon nanotubes (CNTs) during spraying, preparation, ultrasonic dispersion, wafer heating, and opening the water bath cover (Lee et al., 2010), or to precious metal nanoparticles (NPs) in a furnace room and in an electro-refining area (Miller et al., 2010).

Although it has been argued that size as such does not cause harmful effects (European Commission, 2011), a number of studies have convincingly shown that ENMs cause toxic effects not induced by chemically identical but larger particles (Rossi et al., 2010; Palomäki et al., 2011; Catalán et al., 2012). These effects are likely due to exposure to the unique features of ENM, either to their intrinsic properties or to their small entity that allows them to reach targets not reachable by their larger, chemically identical counterparts (Kreyling et al., 2009). An important issue in this context may also be the biocorona formed to surround NPs, and larger particles, once they reach biological environments (Monopoli et al., 2012).

Another major challenge in the assessment of hazards of ENM to experimental animals, humans, and environmental species is, that being in a particulate form, the behavior of ENM differ dramatically from that of traditional soluble chemicals having an impact, not only on the kinetics of ENM in biological environments but also on their potentially harmful effects (Pietroiusti et al., 2018).

The properties of nanomaterials cause marked challenges to the assessment of hazards of ENM via the lungs, but also via other exposure routes. However, in the lungs, when the NPs most readily reach the body, they become

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covered by biomolecules (Monopoli et al., 2012) rendering their kinetic behavior and effects more difficult to assess. The special features of the airways add to the complexity to the hazard assessment of ENM via the inhalational route. In general terms, assessing effects of ENM is demanding because the associations of harmful effects of ENM features (physicochemical and biological) are not well understood.

To this end, novel approaches for the prediction of material at nanoscale need to be developed (Kinaret et al., 2017a,b). There are currently a number of ongoing attempts to develop such hazard prediction tools and frameworks [www.nanosolutionsfp7.com/; www.guidenano.eu/; www.nanomile:eu-vri.eu/; www.sun-fp7.eu/]. Some of the known effects of ENM include those of titanium dioxide (National Institute of Occupational Safety and Health—NIOSH, 2011) and of metal oxides and metals (Saber et al., 2013), and carbon-containing materials induced pulmonary inflammation (Ryman-Rasmussen et al., 2009a; Palomäki et al., 2011; Mercer et al., 2013; Kinaret et al., 2017b). These effects include, among others, inflammation, granuloma formation, and fibrosis of the lungs (Rossi et al., 2010; Ryman-Rasmussen et al., 2009b; Saber et al., 2013). It has also been shown that both tangled and rigid rod-like CNTs (Mitsui-7) can reach the lung and subsequently the subpleural space and cause collagen deposition. Subpleural space is also the site of pulmonary mesothelioma initiation (Ryman-Rasmussen et al., 2009b; Mercer et al., 2013). However, only rigid, rod-like CNTs have been shown to induce mesothelioma in rodents (Takagi et al., 2012; Sargent et al., 2014). In addition, several fibrous and crystalline ENM have been shown to induce genotoxic effects in vivo and in vitro (Catalán et al., 2012; Kinaret et al., 2017a).

In the light of reports on the production of pleural effusion and pulmonary fibrosis following exposure to nanomaterials (Ryman-Rasmussen et al., 2009a,b), and also the carcinogenicity of CNTs (Chernova et al., 2017), search for biomarkers as tools to protect workers has been emphasized (Schulte and Hauser, 2012; Bergamaschi, 2012; Iavicoli et al., 2014; Bergamaschi et al., 2017). Being able to develop biomarkers of exposure to ENM would greatly increase the certainty of assessment of potential risks of exposure to ENM. In fact, it is quite obvious that the (ENM) biomarkers will become available when detailed molecular mechanisms of ENM-induced diseases can be better characterized. Until then, bridging data between in vitro and in vivo reactions are required. The understanding of possible diseases of particles is currently based on biokinetics (absorption, distribution, metabolism, excretion: ADME) viewed, with regard to the various ENM considering both their physicochemical characteristics and their interactions with biomolecules, and even considering particulate matter (PM) toxicity, such as asbestos. At the current state, these two areas are serving as starting points for knowledge and understanding within the new fields of molecular biology. Several topics have to be considered in the development of biomarkers of exposure for ENM, including foreign body recognition systems and/or immune systems, especially of innate nature. These studies are applicable to acute and chronic in vivo responses including virtually permanent deposition of PMs to the reticuloendothelial system (RES). In any case, for sound growth of the nanomaterial industry and protection of workers and users, promotion of new studies and usage of available data in a reasonable balance is practical and essential.

The development of novel biomarkers for ENM is also hampered by the lack of a systematic database on ENM toxicity, even though there is a plethora of detailed information on specific toxicity of several ENM. This renders ENM risk and safety assessment a challenge, especially because information on exposure to ENM in occupational setting or other environments is lacking for most of the materials (Kuhlbusch et al., 2011; Valsami-Jones and Lynch, 2015; Pietroiusti et al., 2018). This situation is reflected by the fact that there are no occupational exposure limits implemented for any of the ENM anywhere (Van Broekhuizen and Reijnders, 2011, Van Broekhuizen et al., 2012). It is not surprising that there are concerns regarding the safety of ENM in work places, consumer products, and the environment.

Epidemiological studies were crucial in identifying a correlation between exposure to different sizes of particles and fibers in causing a disease (Donaldson and Seaton, 2012). The search for biomarkers of exposure to, and effects of particles could only become possible once the elucidation of the molecular mechanisms involved in particle-induced diseases, including fibrosis, lung cancer, and mesothelioma (Mossmann, 2000; Grosse et al., 2014) had been established. Such correlations were identified later between ambient PM_{10} and ultrafine particles (UFP) and cardiovascular diseases (Dockery et al., 1993) or pulmonary function (Pope and Kanner, 1993). The relevance of oxidative and inflammatory markers due to exposure to particles and fibers and especially nanomaterials has been recently reviewed by Bergamaschi et al. (2017). In addition, in some cases, ions released from the NPs such as silver, gold, and iron can be measured in urine and in blood (Iavicoli et al., 2014).

From the outset, attempts have been directed to develop biomarkers of exposure to nanomaterials based on their ability to induce oxidative stress and inflammation (Gulumian et al., 2006; Johnston et al., 2013; Manke et al., 2013). Biomarkers of oxidative stress and inflammation have been shown to have an association with the biopersistence of particles and fibers (Searl et al., 1999) resulting in frustrated phagocytosis and oxidative cellular stress, especially in the lungs. The challenge

that has remained in the development of biomarkers of exposure to nanomaterials and other particulates has been the lack of specificity toward ENM. Hence, so far these biomarkers seem to work at a group level in a given epidemiological study, but they seem not to be suitable for the assessment of exposure of a given worker to ENM because so many exposures to different kind of PM, including fungi, bacteria, wood dust, and man-made mineral fibers among others induce a similar response (e.g., Savolainen et al., 2010; Bergamaschi et al., 2017).

The search for appropriate biomarkers for exposure to NPs will be of great relevance to the need for such exposures to be detected early enough in the process of toxicity and also pathogenicity. Successful identification of such biomarkers will prevent the recurrence of the experience with larger particles where exposures continued unabated for very long periods until pathological changes were observed, producing diseases such as fibrosis and cancer. In addition, earlier lessons of the benefits of biomarkers for oxidative stress and inflammation might help also in the search for biomarkers for ENM. It is, though, of importance to remember the lack of specificity of such biomarkers, and for that reason also search for biomarkers with a better specificity would be of value (Palomäki et al., 2011; Kinaret et al., 2017a,b).

CLASSIFICATION AND CHARACTERISTICS OF NANOMATERIALS

According to the definition given in the technical report of the International Standards Organization (ISO) (ISO/TR 14786, 2014), nanomaterials may include nanosized objects with one or more external dimension in the nanoscale. Nanomaterials may therefore be distinguished by their shape as either NPs (all three dimensions in the nanoscale), nanofibers (two dimensions in the nanoscale, including nanowires, nanotubes, and nanorods), or nanoplates (one dimension in the nanoscale).

Because of the multiplicity in chemical composition, shape, and size of NPs, as well as multiplicity in the effects they produce in in vitro cell cultures and in vivo—in pulmonary, cardiac, reproductive, renal, and cutaneous systems (Kumar et al., 2012)—it will therefore be useful to classify NPs into general groups: carbon-based, inorganic metal, or metal oxides as well as organic NPs, with the hope that biomarkers that might be specific to a certain group but not to other groups of NPs may emerge .

The classification of nanomaterials may be made as carbon-based NPs that include fullerenes, carbon nanofibers, graphene, and carbon black (CB); inorganic metal-based NPs that include gold and silver NPs; and metal oxide NPs that include among others titanium dioxide, zinc oxide, and cerium oxide; quantum dots (QDs); and finally organic NPs that include organic polymers.

Carbon-Based Nanoparticles: Fullerenes

Fullerenes are carbon-based allotropes in a hexagonal network of carbon atoms, which may be in the form of a hollow sphere, ellipsoid, tube, or plane. When in spherical cages, they may contain between 28 to more than 100 carbon atoms, the most widely studied of which are those containing 60 carbon atoms (C60), first synthesized by Kroto et al. (1985). When in layers, they are called graphenes, and when in hollow cylinders they are called CNTs, which may be single layer (SWCNT), double layer (DWCNT), or multiple layers (MWCNT). Finally, CB, composed of partially amorphous graphitic material, is mostly spherical in shape (Aitken et al., 2004).

Inorganic Nanoparticles

Inorganic NPs are particles in nanometric dimensions composed of pure metals or metal oxide, or are of metallic composition. The most common examples include gold, silver, aluminum, titanium, silica, tungsten, manganese, copper, cerium, iron, molybdenum, and palladium NPs. They are also synthesized in various geometries, examples being spherical, nanoshells, nanorods, tripods, tetrapods, nanocages, and star-shaped nanorice-shaped gold NPs (Chen et al., 2003; Chen et al., 2005; Nehl et al., 2006; Wang et al., 2006; Murphy et al., 2008).

Generally, QDs are fabricated from groups II–VI or groups III–V elements of the periodic table (Aitken et al., 2004). Examples include indium phosphate (InP), indium arsenate (InAs), gallium arsenate (GaAs), gallium nitride (GaN), zinc sulfide (ZnS), zinc–selenium (ZnSe), cadmium–selenium (CdSe), and cadmium–tellurium (CdTe) metalloid cores (Hines and Guyot-Sionnest, 1996; Dabbousi et al., 1997). Newer, heavier structures (e.g., CdTe/CdSe, CdSe/ZnTe) and hybrids composed of lead–selenium (PbSe) have also been synthesized (Kim et al., 2003).

Organic Nanoparticles

The highly branched and symmetrical molecules known as dendrimers are the most recently recognized members of the polymer family. Their unique branched topologies give dendrimers properties that differ substantially from those of linear polymers. Dendrimers were first synthesized by Vögtle in 1978 (Buhleier et al., 1978). They are three-dimensional globular, monodisperse, highly branched polymers prepared in a series of repetitive reactions from simple branched monomer units emitted from a central core with an exterior corrugated surfacing whose size and shape can be precisely

controlled. Dendrimers are fabricated from monomers using either convergent or divergent step-growth polymerization. Dendrimers' unique architecture enhances their ability to exhibit high functional structures (Zeng and Zimmerman, 1997; Pricl et al., 2003; Lee et al., 2005; Namazi and Adeli, 2005). Subsequently, a wide range of dendrimers of different structural classes are synthesized using divergent (built from the central core to the periphery) or convergent (built from the periphery toward the central core) strategies—using repeat units ranging from pure hydrocarbons to peptides, or coordination compounds.

Attempts to classify nanomaterials in their risk assessment for regulatory purposes have included those that are based on a set of performance metrics that measure both the toxicity and physicochemical characteristics of the original materials, as well as the expected environmental impacts through the product life cycle (Tervonen et al., 2008) or more recently based on features that control nanomaterial biological interactions (Castagnola et al., 2017) or on their structure–activity relationship (Gajewicz et al., 2018).

Toxicity Testing of Nanomaterials

The introduction of a radical overhaul for testing synthetic chemicals in animal models for their adverse effects on humans and the environment has recently been advocated. As such, design of integrated testing strategies has been proposed, using modern methods including cell culture techniques with the implementation of a combination of biochemical knowledge of cellular pathways with genomics, proteomics, and metabonomics (Hartung, 2009).

Although these in vitro techniques may be useful in the initial identification of the toxicity of NPs and in the elucidation of the mechanisms involved in their toxicity, long-term animal studies comparing the toxicity and carcinogenicity of certain types of NPs have been strongly advocated (Shi et al., 2013). The necessity for conducting such long-term studies has emanated from the fact that mineral particles and fibers in general and certain NPs in particular are shown to be biopersistent (Oberdörster, 2010).

In addition, these short-term biological tests are shown to produce false positives in the case of non-biopersistent fibers because although they may have effects in vitro, they do not persist long enough in the lungs for a sufficient dose to build up and produce effects in vivo (Donaldson and Tran, 2004). This possibility was confirmed with biopersistent and non-biopersistent nanofibers, where it was found that the biopersistence was influenced by both fiber dimensions and solubility (Searl et al., 1999). Moreover, concerns were raised as to the ability of short-term in vitro assays to accurately predict the in vivo effects of the functionalized products of inhaled CNTs (Zhang et al., 2013). However, once the functionalized side chains are removed and the biopersistent core structure is presented naked to the organism, both ex-A and ex-N NPs become identical (Sayes et al., 2007; Warheit et al., 2009).

DEFINITION AND MEANING OF BIOLOGICAL MONITORING AND ITS APPLICATION TO ENGINEERED NANOMATERIALS

Biological monitoring (BM) deals with the systematic or repetitive measurement of chemical or biochemical markers in fluids, tissues, or other accessible matrices from people exposed to or with past exposure to xenobiotics. BM can be used with the purpose of identifying potential hazards of new and emerging chemicals—potentially including ENMs—thus identifying groups at higher risk of health outcomes (Schulte and Hauser, 2012). The main objectives of such periodical measurements are (1) the assessment of individual or group exposure; (2) the identification of early, specific, nonadverse biological effect parameters that are indicative, if compared with adequate reference values, of an actual or potential condition leading to health damage; and, ultimately (3) the assessment of health risk to exposed subjects (Manno et al., 2010). Biomarkers are recommended for use in assessing the effects or exposure to harmful substances, specifically where there are low or intermittent levels of exposure, mixtures of toxicants that may act synergistically, or exposure resulting in disease with long latency period. Biomarkers are increasingly used as surrogate indicators of designated events in a biological system due to the inaccessibility of target organs; in spite of this limitation, it is thought that biomarkers are more directly related to the adverse effects that one attempts to prevent than any ambient measurement, and this supports the use of BM in risk assessment (Smolders et al., 2010). On the other hand, BM is more complex in terms of standardization and interpretative efforts as compared to ambient monitoring; the use of biomarkers requires a toxicological knowledge for their interpretation and ethical issues should be addressed as generally required in human studies.

Assessing even subtle health effects resulting from exposure to ENM is challenging for several reasons, including the heterogeneity of nano-objects in real life, and the lack of available tests with a known sensitivity and specificity to detect physiological and biological modifications clearly related to particle exposure.

Hazard studies have identified the most significant biological responses and target organ/systems affected by different ENM (Pappi et al., 2008; Aschberger et al.,

2010; Savolainen et al., 2010). Unfortunately, research over the past few years has not been conducted with the aim of identifying threshold exposures. The effects of ENM on human health are—to a large extent—unknown, and currently there is no report of any definitive human disease that is caused or worsened by ENM exposure. The current body of information on the human health effects potentially related to ENM comes from the studies on incidental NPs, air pollution epidemiology, and studies on occupational exposures with similarities to NPs such as welding fumes, ultrafine CB, or diesel exhausts (Madl and Pinkerton, 2009). Epidemiological studies have found hazardous respiratory effects from occupational exposure to some industrial processes involving generation of significant amounts of UFP, such as CB (Wellmann et al., 2006), fumed silica (Merget et al., 2002), metal oxides (Antonini, 2003; Luo et al., 2009), and fibers of concern.

The main effects attributed to ENM are (1) lung inflammation and fibrosis; (2) genotoxicity and DNA oxidative changes; (3) carcinogenic or procarcinogenic effects; and (4) vascular impairment resulting from endothelial activation, prothrombotic effects, and accelerated atherosclerosis. Although the respiratory and cardiovascular systems represent the main or the most studied targets of ENM, recent researches suggest that other organs can be indirectly affected by ENM exposure. Lung effects largely depend on the physicochemical characteristics and surface properties of instilled/inhaled particles. While the surface properties of insoluble particles are the main determinants of their interaction with biological systems, those ENM that rapidly dissolve into toxic ions lead readily to inflammation (Donaldson et al., 2013). Some ENM, such as titanium dioxide (TiO_2), copper oxide (CuO), ZnO and iron oxide NPs, cationic polystyrene, and C60 fullerene, have demonstrated prooxidative and proinflammatory properties both in vitro and in vivo, mainly related to their surface reactivity and chemical composition (Madl and Pinkerton, 2009), although not all NPs cause inflammation via a mechanism involving oxidative stress. The tissue response to CNTs following instillation or inhalation is characterized by transient inflammatory changes, oxidative stress, fibrosis (Shvedova et al., 2008), and cancer (Chernova et al., 2017).

Through a process of translocation across biological barriers, NPs can reach and deposit in secondary target organs where they may induce adverse biological reactions. Therefore, a correct assessment of NP-induced adverse effects should take into account the different aspects of toxicokinetics and tissues that may be targeted by NPs.

Cardiovascular effects have been assessed in workers handling TiO_2 nanomaterials, thus suggesting that exposure to particles with a diameter <300 nm might affect parasympathetic function leading to higher heart rate variability in workers (Ichihara et al., 2016). Acute exposure to TiO_2 NPs acutely alters cardiac excitability and increases the likelihood of arrhythmic events (Savi et al., 2014) and also induces myocarditis in mice (Hong et al., 2015).

Recent experimental findings, considering the role of new players in gut physiology (e.g., the microbiota), shed light on several outcomes of the interaction between ENM and gastrointestinal tract, fostering for long-term unpredictable consequences (Pietroiusti et al., 2017). Ruiz et al. (2017) have demonstrated that TiO_2 NPs exacerbate experimentally induced colitis and that TiO_2 in blood is significantly higher in human beings suffering from inflammatory bowel diseases during the acute phase of the disease. Few in vivo studies demonstrated that NPs may affect kidney function, leading to both tubular and glomerular changes (Iavicoli et al., 2016). The issue of potential effects of manufactured NPs on brain functions, given that NP's potential to induce oxidative stress, inflammation, cell death by apoptosis, or changes in the level of expression of certain neurotransmitters, has been recently reviewed (Bencsik et al., 2018).

Studies on the relationship between particulate pollutants (including elemental carbon) and health effects have generated a panel of circulating biomarkers reflecting inflammation end points, endothelial activation, platelet activation, oxidative damage to DNA and lipids, and antioxidant capacity (Loft et al., 2008; Møller and Loft, 2010).

Challenges of the Development of Biomarkers of Exposure to Engineered Nanomaterials Due to Their Biokinetics

Considering the behavior of ENM in a biological system, i.e., in a cell or an organ, as shown in Fig. 41.1, there are three issues of special interest: primary NPs, secondary aggregated NPs, and NPs firmly attached to the cellular matrix.

The first stage occurs before the exposure, the second at the site of absorption, and the third at the level of distribution, metabolism, and excretion and deposition at the potential final destiny of the particle, if any. At the third stage, the active dispersion mechanisms and attachment of the NP through bonding by the host with other insoluble foreign particles determine the final fate of the ENM. For the functionalized NPs, initial toxicity may depend largely on the functionalized side chain moieties that interact first with the organism's biomolecules. However, as shown in Fig. 41.2, metabolic activity of the host organism may eventually succeed in removing the side chains, leaving the biopersistent core structure, such as C_{60} for fullerene-based functionalized products.

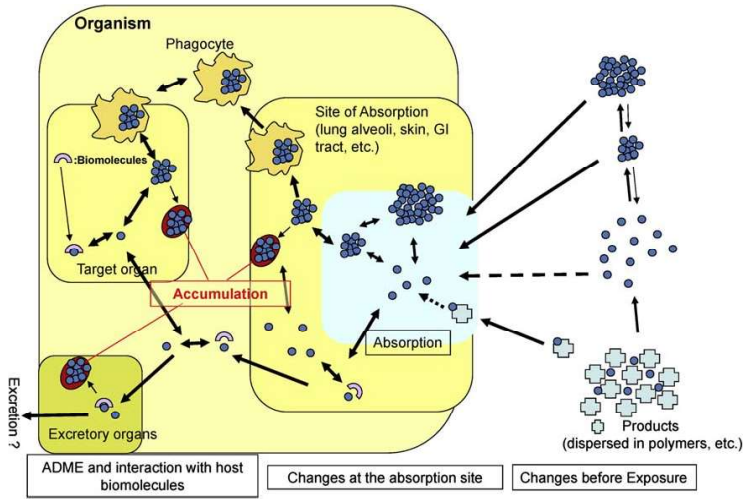

FIGURE 41.1 A pictorial representation of absorption, distribution, metabolism, excretion, and deposition of engineered nanomaterials in cells and tissues.

The diversity and complexity of an ever-increasing number of NM with varying physicochemical properties, and the complex and changeable nature of nanobiointeractions, make the biological behavior of NM not predictable on the basis of their inherent properties. Recent findings indicate that the physicochemical properties and the integrity of NPs can change dramatically following internalization by cells in vitro and in vivo, even for NPs with high colloidal stability such as polymer-coated gold NPs (Kreyling et al., 2015).

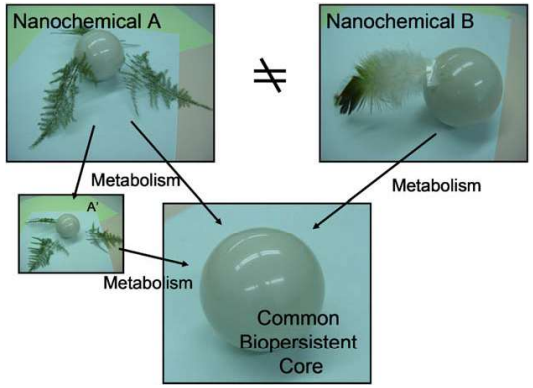

FIGURE 41.2 Symbolic scheme of metabolism of functionalized nanoparticles. When the side chain is different, then the molecules A and B are chemically and toxicologically different. However, once the functionalized side chains are removed and the biopersistent core structure is presented naked to the organism, both ex-A and ex-B nanoparticles become identical.

While the material-intrinsic properties determine the "synthetic identity," the context-dependent properties of the nanomaterial influence its "biological identity"—which is shaped, in part, by the adsorption of biomolecules that form a "corona" on the surface of NPs; the composition of this "bio-corona" depends on the particular biofluid and may exhibit dynamic changes as the NP crosses from one biological compartment to another (Monopoli et al., 2012; Fadeel et al., 2013). Moreover, particles of similar size and composition can raise qualitatively different effects in relation to their methods of synthesis, as for amorphous silica NPs (DiCristo et al., 2015).

Besides intrinsic characteristics of particles, the presence of contaminants can cause significant changes in their chemical identity, leading to increase in biological activity. This issue is clearly described by synergistic effect of NP and endotoxins on cytokine production and immune cells activation (Bianchi et al., 2015; Li and Boraschi, 2016). Such changes in surface characteristics and chemical identity have important implications for biomarker research because experimental setting may not be truly representative of particle–cell interactions in a real-life scenario. As a result, our current testing strategy is actualized by using simplified models, different dosing regimens, and exposure routes and so represents a compromise to understand what could happen in humans, which requires more caution in the interpretation of findings with consideration of the weight of evidence (Bergamaschi et al., 2015).

BIOLOGICAL INTERACTIONS RELEVANT TO BIOMARKERS OF EXPOSURE TO ENGINEERED NANOMATERIALS AT MOLECULAR, CELLULAR, AND ORGAN LEVEL

Biological interfaces of ENM are numerous and complex, existing both on the cell surface and inside the cell. When compared between primary (nonaggregated) and secondary (aggregated) NPs of the same origin, the target biomolecules may be different. In particular, the host defense mechanisms against the insoluble, i.e., biopersistent, NPs may be similar to those used by the cells against bacterial and viral infections. The most important differences between normal chemical toxicity and ENM toxicity are the activation of the innate immune system against foreign bodies, the subsequent influence on the acquired immune system, and the so-called indirect oxidative stress responses, which eventually lead to cell death (apoptosis and necrosis), cell proliferation and tissue modification (e.g., fibrosis), and indirect genotoxicity and carcinogenesis.

Studies on asbestos-induced noncancerous and cancerous diseases have revealed several key events at the molecular level in the cells. As for the morphological events, fiber carcinogenesis has mesothelial cells and phagocytic cells of the immune system as targets. The former target is subject to direct damage by the exposure, resulting in clastogenicity and indirect attack by phagocytes nearby. The indirect danger signal from the phagocytes protects damaged mesothelial cells from apoptosis, thus increasing the chances of the attacked cells to proceed to neoplastic forms. (Nagai and Toyokuni, 2010). The importance of the length and the aspect ratio (length/thickness) of the fiber in the frustrated phagocytosis has been thoroughly reported (Stanton et al., 1981; Pott et al., 1994: Roller et al., 1997). It is likely that the chronic active inflammatory lesions are more important than fibrous scar formation (Poland et al., 2008; Donaldson et al., 2010; Takagi et al., 2012). Condensation of secondary elements to the fibers, such as iron in the body for the Fenton reaction, is postulated as a direct mechanism or as an augmenting factor of fiber mesotheliomagenesis (Nagai et al., 2011).

Another classical example of particle-induced carcinogenesis is thorotrast (Mori et al., 1983). The size of thorium dioxide primary particles in emulsion, used as an X-ray contrast medium, is approximately 10 nm in diameter (Riedel et al., 1983). Primary particles are concentrated initially in the first line of macrophages to form larger clusters and when these macrophages die off by either alpha ray effect or by foreign body-based oxidative stress, the cluster is released to the intercellular space, and phagocytosed again by the second line of macrophage, resulting in the formation of larger clusters (Nishizawa et al., 1987). As a whole, the biological half-life time has been reported as over 400 years in some reports (Janower et al., 1968). It has been proposed that the carcinogenic effect is linearly proportional to radioactivity of the emulsion, indicating that the permanent retention of the particle in the RES is not responsible for the effect (Wesch et al., 1983). However, this event clearly shows that biopersistent ENM administered into the bloodstream or into a place where the particle eventually enters the bloodstream will be gradually concentrated into larger clusters and permanently trapped by the RES.

The recognition of foreign NM has been studied at the molecular level. The mechanisms of foreign body recognition share the same signal pathways and reactions to the recognition of bacteria and viruses, i.e., the innate immune system. NALP3/NLRP3 inflammasome was reported as a mediator of the sensing mechanism of foreign ENM via lysosomal damage and endogenous "danger" signals (Dostert et al., 2008; Palomäki et al., 2011). It seems important to identify both the direct oxidative stress by immediate responses to the ENM

and the indirect IFN signaling-mediated oxidative stress for the understanding of ENM generated cell injury and cell growth that may eventually lead to tissue/organ damage and carcinogenesis (Mullan et al., 2005; Chakrabarti et al., 2011).

The precipitating types of adjuvant, such as aluminum salts, are reported to induce cell migration to the site of coadministered antigen and induce cell killing so that the mesh of DNA from the dead cell activates the immune activity via distinct signaling pathways (Marichal et al., 2011). Diesel exhaust and pollen allergy enhancement might be considered as a similar phenomenon to the ENM attribution to the immune system—more toward adverse immune reactions, such as allergy, persistent immune activation triggering autoimmune diseases, and immune suppression (anergy) (Siegel et al., 2004; Marichal et al., 2011). The finding that the double-stranded DNA from the host tissue may take part in enhancing immunization of an externally applied antigen may add some consideration to the lifelong deposition of biopersistent ENM and the induction of production of biomolecules that could serve as bioindicators of exposure to ENM.

Protein scaffolding and nanomaterials is another aspect of biological interaction. An example is the acceleration of bone-implant connection as a result of accelerated bone formation by CNTs used at the interface of the implant and grafted tissue (Saito et al., 2008; Usui et al., 2008). The authors claim that there is minimal inflammatory reaction at the interface. These findings address the issue of whether primary particles deposited in the mesenchymal connective tissue would stimulate noninflammatory reaction, such as direct stimulation of fibroblast or mesenchymal stem cells to produce progressive pathology such as fibrosis, angiogenesis, noninflammatory granulation tissue formation, or bone formation. The systemic distribution of single fibers we found after MWCNT intraperitoneal injection poses an unanswered question whether tissue reaction would eventually take place in those deposition sites.

Examples of Given Biomarkers

Mesothelin is reported to increase in the serum of human patients with mesothelioma (Imashimizu et al., 2011) and in a rat study where multiwall CNTs were given via intraperitoneal exposure (Sakamoto et al., 2010). MIP1alpha is also reported to be secreted by macrophages treated with titanium dioxide (Xu et al., 2010). There are many other putative markers reported in relation to toxicity of NPs, such as CD146 and IMP3 (insulin-like growth factor 2 mRNA-binding protein 3) (Okazaki et al., 2013). Many of them are waiting for further validation.

Histological biomarkers for MWCNT of asbestos size against mesotheliomagenesis include our finding that the chronic inflammatory lesion consisting of MWCNT-laden activated macrophages without acute inflammatory cell infiltration, granuloma formation, and fibrotic scar formation (Fig. 41.3), considered as a phenotype of frustrated phagocytosis, is important for mesotheliomagenesis (Takagi et al., 2012). This phenotype is considered to be supported by a study on SWCNT (Mangum et al., 2006). This finding may be in good correlation with the acute phase biomarkers posted by several research studies (Poland et al., 2008; Murray et al., 2012). For a morphological picture demonstrating the impact of MWCNT on the mesothelial lining in the peritoneal cavity in sensitive mice strain refer Fig. 41.4 (Takagi et al., 2012).

Biomarkers of Exposure

Once inhaled, NPs can deposit in lung cells including alveolar macrophages and translocate through or between epithelial and endothelial cells into the blood and lymph circulation, potentially reaching sensitive target sites including bone marrow, lymph nodes, spleen, heart, and central nervous system. Translocation to many organs by ENM has been demonstrated and quantified (Geiser and Kreyling, 2010; Holgate, 2010), but its clinical relevance is not known. The likelihood of being taken up can vary dramatically among exposure conditions and by the behavior of particle aerosols in which nano-objects are commonly present and taken up as agglomerates and/or aggregates of various aerodynamic diameter. The internal dose of common chemicals is usually assessed by measuring both the amount of the substance and/or its metabolites or as a product of interaction with biomolecules. An ideal biomarker of exposure should be specific for the exposure of interest and also detectable in small quantities, measurable by noninvasive techniques, and capable of providing a positive predictive value to a specific health status (Mutti, 2001). Toxicokinetic and toxicodynamic data, which are available for a few ENM (e.g., metal NPs, fullerenes, and single-walled CNT) suggest that translocation rates from the portal-of-entry to secondary organs are very low (Kreyling et al., 2002; Choi et al., 2010; Geiser and Kreyling, 2010). So far, few studies have provided quantitative demonstration of ENM translocation from the lung. Recent research undertook biokinetic analysis in rats, using near-infrared imaging, to follow the fate of intratracheally instilled NPs of various size, surface modification, and core composition. Choi et al. (2010) found that noncationic NPs smaller than 34 nm in diameter, which did not bind serum proteins, reached the regional lymph nodes quickly. However, larger NPs are consistently retained in the lungs. When the ENM

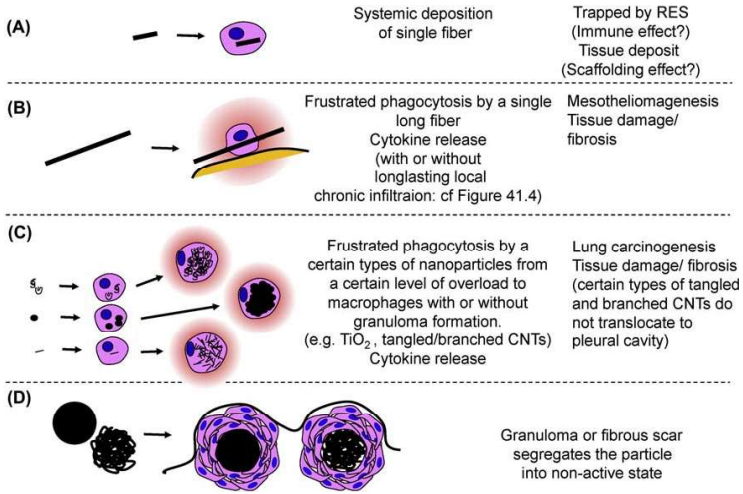

FIGURE 41.3 (A) A nanoparticle short or small enough to be phagocytosed by a single macrophage is easily removed from the initial exposure site, and either ends up trapped in the reticuloendothelial system or distributed systemically via bloodstream to renal glomeruli, choroid plexus, and other organs. (B) A relatively long fiber (cf. Stanton et al., 1981) such as asbestos, multiwall carbon nanotubes, and some single wall nanotubes are phagocytosed by more than one macrophage, resulting in the release of various cytokines and other inflammatory mediators without the formation of granulomas or scars. This state is reported to last for a considerably long period, resulting in mesotheliomagenesis in cases of the peritoneal/pleural cavity, and local fibrosis in pulmonary alveoli. (C) Certain types of nanomaterials, either primary or secondary particles, that are small enough to be phagocytized by a macrophage can induce frustrated phagocytosis from a certain level of overload to the macrophage. It is not well known what kind of condition leads to subsequent formation of foreign body granuloma. Lack of formation of such granuloma can be seen for a long period in some condition. In case of MWCNT, it is experienced that the fibers recovered from the pleural cavity are almost always the straight ones without branching. (D) Large aggregate and agglomerate are entrapped by a group of macrophages and result in formation of foreign body granuloma and eventually fibrous scarring. This reaction effectively segregates the foreign body from the inflammatory system and thus does not seem to contribute to mesotheliomagenesis and further expansion of diffuse fibrosis. These different types of engineered nanomaterials have a different potential for penetrating barriers and hence reaching different organs. They also have different ability to induce the synthesis of biomolecules that could serve as a biomarker of exposure to ENM.

diameter falls below 6 nm, and the particles are zwitterionic, about half of the NPs rapidly enter the bloodstream from alveolar airspace and are mostly cleared from the body by means of renal filtration. Thus, while the size seems of minor importance for the toxicity, it dramatically affects particle translocation and biokinetics.

Although some ENM have been demonstrated to easily cross biological barriers, the number of ENM entering the systemic circulation could be too low to lead to any significant internal dose, and hence not be detected in peripheral tissues in humans. There is a general consensus on the likelihood that particles whose aerodynamic diameter is between 0.1 and 1.0 μm reach the lower respiratory tract, but their deposition is low compared with particles with lower and higher aerodynamic diameter because of symmetric human lung morphology (Broday and Agnon, 2007). The daily dose of inhaled NP in humans is limited to a maximal NP aerosol concentration of $10^{12}/m^3$ (beyond that, coagulation rapidly occurs under ambient conditions), and the daily volume of air inhaled by an adult is $15\,m^3$. Assuming a particle deposition of 0.3, the daily deposition amounts to about 5×10^{12} NPs (Oberdörster, 2010). As a result, it may take years to reach an appreciable internal dose, whereas the biologically effective dose (i.e., the entity within any dose of particles in tissue that drives a critical pathophysiologically relevant form of toxicity, e.g., oxidative stress, inflammation, genotoxicity, or proliferation) may be achieved independently from the mass of inhaled particles (Donaldson et al., 2010).

Low-soluble ENM (e.g., CNT or TiO_2)—even though they can partially break down in contact with

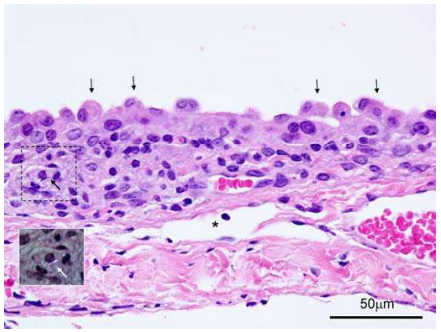

FIGURE 41.4 Atypical mesothelial hyperplasia of the tendinous portion of diaphragm of a mouse with 3 μg of MWCNT injected intraperitoneally (sampled at terminal sacrifice, i.e., 365 days after i.p. inoculation of the MWCNT (Takagi et al., 2012). (*Black arrows*: hobnail appearance of the atypically hyperplastic mesothelial cells. *Asterisk*: lymphatic drainage of the peritoneal cavity.) The polarized image is of the *dotted area*). (*White arrow*: an MWCNT fiber in a macrophage-like cell [birefringent]). *Cancer Science. Reproduced with permission.*

phagocytic cells—show a slow clearance leading to accumulation over time with exposure. For many particles, size and agglomeration state are influential in dictating toxicity; thus, particle dissolution and the release of ions—which would be expected to be greater for smaller particles—may affect organ distribution. Cho et al. (2013) studied the absorption, distribution, and excretion patterns of TiO_2 and ZnO NPs following oral administration. Zinc concentrations in blood, organs, and urine were higher than concentrations of titanium when NPs were administered orally for 13 weeks. The urine concentration of Ti in the TiO_2-treatment groups showed no significant differences compared with the control group; in contrast, the concentration of Zn in the urine of ZnO-treatment groups was significantly increased in the middle- and high-dose groups and showed positive trend dose-responses. In vivo, NPs are mostly retained in the liver, and fragments of the organic shell are excreted through the kidneys, probably due to proteolytic digestion (Kreyling et al., 2015).

Following liver accumulation, metal NPs show a protracted elimination and slow release of particles from the target organ into systemic circulation (Johnston et al., 2010), giving rise to detectable trace amounts in body fluids. Demonstration of translocation of ENM from lung into systemic circulation is theoretically possible for metallic NPs, which release metal ions or dissolve in biological media. Similar to metal species in welding fumes, metallic elements are measurable in blood and urine with appropriate analytical methods, giving an estimate of current or past exposure. For instance, Lee et al. (2012) measured silver concentration in blood and urine in Korean workers exposed to silver NPs, whereas in the study of Lee et al. (2015) molybdenum blood concentration was measured as a candidate biomarker for MWCNT exposure.

To estimate the dose retained at the portal of entry, it can be informative to assess the tissue dose reaching the target organ, e.g., the lung. Sampling of exhaled breath condensate (EBC) provides a matrix for the simultaneous monitoring of exposure and effects on target organ. As EBC mainly consists of water that is practically free of potentially interfering solutes, it is an ideal biological fluid for elemental determinations based on electrothermal atomic absorption spectroscopy or inductively coupled plasma-mass spectrometry. This novel approach has represented a significant advancement over the analysis of alternative media (blood, serum, urine, hair), which are not as reliable (owing to interfering substances in the complex matrix) and reflect systemic rather than lung (target tissue) levels, e.g., of pneumotoxic metallic elements (Goldoni et al., 2004). Particles of rutile and/or anatase were found in the EBC of exposed workers in 70% of the postshift samples, the mean concentration of titanium in production workers being 24.1 ± 1.8 μg/L, whereas in the research workers the values were below the limit of quantitation, e.g., 4.0 ± 0.2 μg/L. Thus, the concentration of titanium originating from TiO_2 in EBC might serve as a direct exposure marker in workers producing TiO_2 pigment; however, the stability of its concentrations in EBC not being influenced by current exposure support the use as a biomarkers of deposited dose following long-term exposure (Pelclova et al., 2015a).

Biomarkers of Effect

A biomarker of effect is defined as any measurable biochemical, physiological, or other alteration within an organism that, depending on magnitude, can be recognized as an established or potential health impairment or disease (Henderson et al., 1989). Biomarkers should not be considered as diagnostic tests, but rather as indicators reflecting early modifications preceding progressive structural or functional damage at the molecular, cellular, and tissue level, i.e., changes possibly leading to adverse effects but completely reversible on the removal from the exposure of concern. Biomarkers of effect can be used to evaluate whether a well-characterized exposure is associated with a shift in the distribution of relevant biochemical or functional end points, indicative of early changes in the target or critical organs/tissue. To assess such early events associated with exposure to ENM, the choice of potential

biomarkers can give insights on local and systemic oxidative stress, systemic inflammation, and inflammatory response in target organs, as in respiratory and cardiovascular systems.

Biomarkers of Lung Inflammation and Systemic Effects

Because airway inflammation is the main outcome investigated following NM exposure, inflammatory biomarkers deserve greater importance for biomonitoring purposes. Breath analysis has been proposed as a noninvasive approach that allows the identification of the inflammatory and oxidative stress biomarkers involved in the pathogenesis of various clinical conditions (Montuschi, 2007) and for investigating occupational lung diseases (Corradi et al., 2010), exposure to welding fumes (Gube et al., 2010) or particles from aircraft engines (Desvergne et al., 2016). Besides macromolecules (e.g., DNA or RNA), an increasing panel of biomarkers reflecting oxidative stress and inflammatory pathways can be determined in EBC. Thiobarbituric acid reactive substances, such as MDA—a product of membrane lipoperoxidation—and 8-isoprostane—a peroxidation product of prostaglandin metabolism—can be quantified; proinflammatory cytokines, such as leukotrienes B4 (LTB4), can be determined as biomarkers of inflammation. Inflammatory response in the airways is characterized by an influx of neutrophils, whose activation is associated with a respiratory burst resulting in overproduction of hydrogen peroxide (H_2O_2), changes in pH, and depletion of the glutathione (GSH) pool.

Various classes of proteins and volatile compounds can be measured in EBC, including the saturated hydrocarbons and oxygen-containing substances formed during the fatty acid lipid peroxidation of cell membranes. A wide variety of carbonyl compounds are generated as secondary oxidation products during respiratory burst. In particular, saturated aldehydes, such as 4-hydroxy-trans-nonenal (HNE) and 4-hydroxy-trans-hexenal (HHE), are formed by the peroxidation of omega-3 and -6 fatty acids, the basic components of cell membrane phospholipids. EBC concentrations of MDA, HNE, and HHE were significantly higher in workers exposed to TiO_2 aerosols as compared with unexposed people (Pelclova et al., 2015b). Oxidative stress biomarkers (MDA, HNE, HHE, C6—C10, 8-isoprostane, 8-OHdG, 8-OHG, 5-OHMeU, 3-ClTyr, 3-NOTyr, o-Tyr, and C11) were elevated in the EBC of workers exposed to NPs during iron oxide pigment production compared with control subjects (Pelclova et al., 2016).

Recently, Lacombe et al. (2018) performed an in-depth proteomics characterization of EBC; a total of 229 unique proteins were identified in EBC among which 153 proteins were detected in both EBC pooled samples. A detailed bioinformatics analysis of these 153 proteins showed that most of the proteins identified corresponded to proteins secreted in the respiratory tract (lung, bronchi).

Breath can be analyzed also in the gaseous phase (as exhaled breath). For instance, nitric oxide (NO) is produced by all cellular components of pulmonary inflammation (macrophages, epithelial cells, mast cells, lymphocytes, and granulocytes). Studying NO exhaled from the lower airways offers a unique possibility to study features of pulmonary NO metabolism noninvasively in all states characterized by lung inflammation. In a follow-up study, Wu et al. (2014) found an increase in fractional exhaled nitric oxide (FENO) among workers handling nano-TiO_2 powders, but not in all of the NM exposed categories investigated; the values recorded were over the threshold of 35 ppb, suggesting a chronic airways inflammation. Differences in FENO between workers of an MWCNT facility and nonexposed, with no difference in lung function or the pneumoproteins have been found by Vlaanderen et al. (2017).

Pneumoproteins, such as Clara cell protein (CC16) and surfactant-associated protein B (SP-B) in the serum, have been validated as markers of alveolo—capillary barrier integrity/permeability in human studies on gaseous/particulate pollutants (Broekaert et al., 2000; Gulumian et al., 2006). There is evidence that acute exposures to certain pulmonary irritants can cause a transient increase in serum CC16 levels, and limited evidence also suggests that a transient increase in serum CC16 levels can be caused by a localized pulmonary inflammation without impairment of pulmonary function. The biological interpretation of chronic changes in serum CC16 is less clear, owing to chronobiological variability. In workers in indium tin oxide production plants, significant positive relationships were found between S-In and surfactant protein A (SP-A), and surfactant protein D (SP-D) levels, sensitive markers of interstitial lung disease. SP-A and SP-D levels were elevated significantly in the workers with moderately high indium exposure (Liu et al., 2012).

Early events at vascular level can be assessed by a panel of circulating biomarkers reflecting inflammation end points, platelet activation, and antioxidant capacity (as assessed by the activity of Cu/Zn-superoxide dismutase, and glutathione peroxidase-1). Inactivation of antioxidant enzymes within erythrocytes, plasma interleukin-6 (IL-6), and soluble tumor necrosis factor-receptor II (sTNF-RII), investigated during a longitudinal study, showed a positive association with vehicle emissions tracers (Delfino et al., 2010). High-sensitivity C-reactive protein in plasma, plasma fibrinogen, and IL-6 could represent good candidates for BM of ENM.

Acute phase proteins, such as C-reactive protein (CRP), are commonly used as biomarkers of (inflammatory) disease, and associations have also been shown between inflammatory biomarkers including CRP and exposure to PM air pollution. 70-nm silica nanoparticles (nSP70) induced a higher level of acute phase proteins such as haptoglobin, CRP, and serum amyloid A (SAA) than larger silica particles (diameter >100 nm) (Higashisaka et al., 2011). In addition, the level of these acute phase proteins was elevated in the plasma of mice after intranasal treatment with nSP30. The same authors identified hemopexin (another acute phase protein) as a potential biomarker for predicting the effects of silica NPs (Higashisaka et al., 2012).

Following intratracheal instillation of titanium dioxide (TiO_2), CB, diesel exhaust particles and CNTs, tissue mRNA expression of acute phase protein, and plasma levels of Serum Amyloid 3 were increased. Interestingly, these inflammatory biomarkers significantly correlated with the magnitude of neutrophilic influx in bronchoalveolar lavage fluid, thus suggesting that lung inflammation is associated with the expression of biomarkers predictive of cardiovascular outcomes (Saber et al., 2013).

Exposure to NPs could enhance the adhesion of endothelial cells and modify the membrane structure of vascular endothelium, which plays an important role in the regulation of fibrinolysis. Radomski et al. (2005) showed that both urban dusts and engineered carbon particles, such as CNT and CB—except C60CS—stimulated platelet aggregation and accelerated the rate of vascular thrombosis in rat carotid arteries with a similar rank order of efficacy. All particles resulted in upregulation of GPIIb/IIIa in platelets. In contrast, particles differentially affected the release of platelet granules, as well as the activity of thromboxane-, ADP-, matrix metalloproteinase-, and protein kinase C-dependent pathways of aggregation. Exposure to nano Ag (0.05—0.1 mg/kg i.v. or 5—10 mg/kg i.t. instillation) enhanced platelet aggregation and promoted venous thrombus formation in rats (Jun et al., 2011). Therefore, assessment of platelet aggregation and expression of GPIIb/IIIa in platelets may be useful to evaluate possible prothrombotic effects induced by NM. Early systemic prothrombotic effects induced by fine particle (<2.5 μm) exposure were detected by the quantification of levels of the plasminogen activator inhibitor-1 (PAI-1) (Kilinc et al., 2011). Elevated PAI-1 is closely associated with enhanced thrombosis by impairing fibrinolysis; metal NPs have proven to increase PAI-1 expression in endothelial cells in vitro (Yu et al., 2010).

Vesterdal et al. (2010) showed that exposure of young and aged apolipoprotein E knockout mice (apoE−/−) to CB (Printex 90, 14 nm average size) by intratracheal instillation, resulted in modest vasomotor impairment, with a lack of association with nitrosative stress (3-nitrotyrosine), and without increases in the expression of vascular adhesion molecule and intercellular adhesion molecule (ICAM-1) on endothelial cells or in plaque progression.

Interestingly, workers exposed to a mixture of NM showed statistically significant changes across exposure risk classes for high sensitive C reactive protein levels and inflammatory cell activation (increased ICAM-1 in macrophages), IL-6, and fibrinogen. Moreover, the depression of antioxidant enzymes, namely SOD and GPx, were associated with NM handling (Liou et al., 2012).

Erdely et al. (2011) demonstrated that the pulmonary exposure to CNT triggered the induction of primary cytokines such as IL-6 and IL-1β, which regulate multiple pathways of the inflammatory cascade as well as secondary inflammatory mediators, chemokines (CCL2, 4, 19, 22; CXCL1, 2), which directly regulate leukocyte recruitment to the inflammatory site. Iavicoli et al. (2018) investigated the adverse effects induced by subchronic intravenous administration of palladium NPs (PdNPs) on the immune system of female Wistar rats by evaluating cytokines (e.g., IL-1α, IL-2, IL-4, IL-6, IL-10, IL-12), the granulocyte—macrophage colony-stimulating factor , the INF-γ, and TNF-α serum levels at different dose levels (0, 0.012, 0.12, 1.2, and 12 μg PdNPs/kg b.w. till 60 days). Subchronic exposure to PdNPs induced a decreasing trend in serum levels in most of the cytokines investigated, with the highest concentration (12 μg/kg) determining significant inhibitory effects.

In a small cohort of workers occupationally exposed to MWCNTs, Shvedova et al. (2016) found significant changes in the ncRNA and mRNA expression profiles between exposed (inhalable concentration 14.42 ± 3.8 $\mu g/m^3$; respirable concentration 2.83 ± 0.6 $\mu g/m^3$ as elemental carbon concentrations in breathing zone samples from workers) and nonexposed workers. A dysregulation of profile of genes involved in cell cycle regulation/progression/control, apoptosis, cell proliferation, and carcinogenetic pathways was characterized in eight workers having direct contact with MWCNT-containing aerosols in the previous 6 months (Shvedova et al., 2016). In a concomitant study on the same group (Fatkhutdinova et al., 2016), it was found that exposure to MWCNTs in the order of 3 times above the recommended exposure level proposed by the NIOSH (1 $\mu g/m^3$) caused significant increase in IL-1β, IL-4, IL-5, IL6, TNF-α, inflammatory cytokines, and KL-6 in sputum samples. Moreover, the level of TGF-β1 was increased in serum obtained from young (<30 years old) exposed workers. In the serum samples, levels of IL-1β, IL-4, IL-10, and TNF-α were significantly elevated in the MWCNT exposed group.

Vlaanderen et al. (2017) assessed 51 immune markers and three pneumoproteins in serum among 22 workers of an MWCNT producing facility and 39 age- and

gender-matched, unexposed controls, considering potentially confounding parameters (age, body mass index, smoking, and sex). These authors found significant upward trends for immune markers C–C motif ligand 20, basic fibroblast growth factor, and soluble IL-1 receptor II with increasing exposure to MWCNT.

Biomarkers of Oxidative DNA Damage and RNA Methylation

Panel studies and cross-sectional investigations on health effects of PM exposure have found consistent associations between exposure to combustion-derived particles and products of oxidative damage to DNA and lipids (Loft et al., 2008; Møller and Loft, 2010). Among the DNA oxidation products, 8-hydroxy-2-deoxyguanosine (8-OHdG) and 8-hydroxyguanosine (8-OHG), measured in DNA of peripheral blood cells and urine, have been the most studied. Base excision repair products of oxidative damage to DNA in urine seem to originate mostly from the oxidation of the deoxynucleotide pool and do not represent solely repairing/excretion of the oxidized-DNA guanine, but biomarkers of effective dose.

Urinary 8-OH-dG concentration has been investigated as a potential biomarker of oxidative stress in response to exposure to incidental NPs emitted from photocopiers, and it was found significantly increased as compared with background levels (Khatri et al., 2013). In EBC of workers occupationally exposed to TiO_2 aerosols, Pelclova et al. (2015b) found an increase in markers of oxidation of nucleic acids (including 8-hydroxy-2-deoxyguanosine [8-OHdG], 8-hydroxyguanosine [8-OHG], 5-hydroxymethyl uracil [5-OHMeU]). Conversely, in workers handling nanomaterials in 14 plants in Taiwan, 8-OH-dG urinary and plasma levels did not show significant differences compared with controls (Liou et al., 2012).

The DNA-damaging potential of many NPs of different composition (metals, metal oxides, silica, QDs, fullerenes, nanofibers, SW-, and MWCNT) has been demonstrated in vitro (Gonzalez et al., 2008; Singh et al., 2009; Magdolenova et al., 2013). NM can affect DNA also by direct mechanisms, including a mechanical interference with cellular and nuclear components, such as microtubules of the mitotic spindle (Gonzalez et al., 2008). Although interactions between the particles and the assay cannot be totally excluded, the use of Comet assay in human biomonitoring studies could provide valuable information for hazard identification of NM (Karlsson, 2010; Karlsson et al., 2015). Modified Single Cell Gel Electophoresis (SCGE) provides parallel information on oxidative DNA damage caused by NM, such as CNT, detecting oxidized/damaged pyrimidines and purines (Muller et al., 2008; Migliore at al., 2010), and revealing dose–effect and dose–response relationships between the mass concentration of NM and the frequency of micronucleated lymphocytes. Both tests have been applied in a cohort of nanomaterial workers in Taiwan, but there were no significant differences in changes between the exposed and control workers between baseline and the 6-month follow-up (Liao et al., 2014).

DNA methylation, a major genomic mechanism of gene expression control, can be affected by ROS, which are considered as one of the main cellular stressors generated by PM exposure as well as by some metals. Stoccoro et al. (2013) highlighted the ability of certain NPs to induce an impaired expression of genes involved in DNA methylation reactions leading to global DNA methylation changes, as well as changes of gene-specific methylation of tumor suppressor genes, inflammatory genes, and DNA repair genes, all potentially involved in cancer development. Moreover, some nanosized compounds are able to induce changes in the acetylation and methylation of histone tails, as well as microRNA (miRNAs) deregulated expression.

Brown et al. (2016) assessed the promoter methylation of inflammatory genes (IFN-γ and TNF-α) after MWCNT exposure and found a correlation between these changes and initial cytokine production. In addition, methylation of a gene involved in tissue fibrosis (Thy-1) was also altered in a way that matched collagen deposition. These authors also found that MWCNT exposure lead to DNA hypomethylation in the lung and blood, which coincided with disease development (i.e., fibrosis).

MiRNAs are noncoding small RNAs that regulate the expression of broad gene networks at the posttranscriptional level, interacting with several mRNA targets, and their use as possible biomarkers of the effects of acute and chronic environmental exposure has been suggested (Vrijens et al., 2015). Some nanosized compounds are able to induce selected miRNAs deregulated expression, and this may help in finding specific fingerprints. Ng et al. (2011) demonstrated that gold nanoparticles (AuNPs) altered the expression of 19 genes in human fetal lung fibroblasts, upregulating the miRNA-155 (miR-155) and downregulating the *PROS1* gene—a gene encoding a vitamin K-dependent plasma protein that functions as a cofactor for the anticoagulant protease, activated protein C to inhibit blood coagulation. Silencing of miR-155 established *PROS1* as its possible target gene. DNA methylation profiling analysis of the *PROS1* gene revealed no changes in the methylation status of this gene in AuNP-treated fibroblasts, whereas chromatin condensation and reorganization was observed in the nucleus of fibroblasts exposed to AuNPs.

Nagano et al. (2013) compared the effectiveness of serum levels of liver-specific or -enriched miRNAs (miR-122, miR-192, and miR-194) with that of conventional hepatic biomarkers (alanine aminotransferase and aspartate aminotransferase) as biomarkers for nSP70 induced liver damage in mice.

Toward Specific Biomarkers for Engineered Nanomaterials Exposure

Research of biomarkers reflecting exposure to certain particles and fibers of concern have already generated a large amount of data supporting the validity of intermediate end points to assess changes before clinically apparent disease occurs (Gulumian et al., 2006) and several biomarkers can reliably help in assessing exposure and effects of NM (Iavicoli et al., 2014; Bergamaschi et al., 2017). The challenge that remains for the use of biomarkers of exposure for NM is the lack of specificity of all the biomarkers developed so far that could hamper the applicability of biomonitoring to nano-object.

It should be recognized that the existence of nanospecific (i.e., size-dependent) effects is an arbitrary assumption because the threshold of 100 nm does not infer, per se, new properties, whereas a gradual change in surface reactivity could actually modulate biological interactions independently from size. Properties other than size, such as shape, surface area, surface charge, and reactivity, can more effectively describe the dose leading to biological effects, as in the classical particle and fiber toxicology science (Auffan et al., 2009; Fubini et al., 2010; Donaldson and Poland, 2013). This does not mean that there are no changes specifically induced by some particles at certain size thresholds, but these changes should be interpreted as mode of actions instead as "new" toxicological properties (Donaldson and Poland, 2013; Lynch et al., 2014). For instance, the more toxicity and DNA damaging potential of nanosized than microsized CuO particles in A549 cells has been attributed to the ability to deliver Cu^{2+} inside cells ("*Trojan horse*" effect). In contrast, the micrometer particles of TiO_2 caused more DNA damage compared to the NPs, which is likely explained by the crystal structures.

New achievements in biomarker discovery came from the study of the intercellular communication pathways (Valadi et al., 2007; Raposo and Stoorvogel, 2013; Lin et al., 2015). In vitro studies have revealed that cellular uptake of NPs provoked micro vesicular endosome (MVe) formation intracellularly; however, few studies have studied the biological consequences once the exosomes have been secreted extracellularly in vivo. On i.t. instillation (4 μg or 20 μg) with 43-nm diameter magnetic iron oxide nanoparticles (MIONs), Zhu et al. (2012) observed a dose-dependent generation of exosomes in the alveolar region of BALB/c mice. These exosomes were quickly eliminated from alveoli into systemic circulation and largely transferred their signals to the immune system, with activation of splenic T cells. Interestingly, the maximum dose used in this study is equal to a half-workday deposition mass in human lung tissue at the permissible exposure limit of iron oxide fume (at a concentration of 10 mg/m^3) suggested by the Occupational Safety and Health Standards (OSHA, USA). These findings suggest that respiratory exposure to MIONs can activate systemic T cells in susceptible individuals, through exosome-mediated signaling pathways.

Systems Toxicology, the integration of classical toxicology with quantitative analysis of large networks of molecular and functional changes, is also expected to provide information on the dynamic interaction between molecular components of biological systems and about the possible mechanisms, by assessing whether specific biological pathways are activated/perturbed by specific NM, thus identifying fingerprints and nano-specific end points useful for hazard identification and, ultimately, for risk assessment (RA) (Costa and Fadeel, 2016). Comparative proteomic studies have shown strong similarities in the pulmonary response to different ENM with known hazardous particles and fibers. One repeated aspiration study (4 μg per mouse, twice a week, for 3 weeks) allowed the identification of a pattern of 109 proteins representing cellular processes affected by both SWCNT and crocidolite asbestos; S100a9, a high-sensitivity marker of inflammation, can be proposed as a biomarker of human response to SWCNT exposure (Teeguarden et al., 2011). Mice exposed by pharyngeal aspiration to 40 μg CNT showed increased inflammatory blood gene expression and serum cytokines followed by an acute phase response (e.g., CRP, SAA-1, SAP). At 28 days, serum acute-phase proteins with immune function including complement C3, apolipoproteins A-I and A-II, and 1-macroglobulin were increased (Erdely et al., 2011). CNT exposure resulted in measurable systemic markers but lacked specificity to distinguish from other pulmonary exposures.

A toxicogenomic approach has been used in assessing specific mechanisms at the molecular level, identifying patterns of cellular perturbations in specific pathways, through identification and quantification of global shifts in gene expression in cell models challenged with ENM. Pulmonary exposure to CNT resulted in an elevated series of measurable potential biomarkers in blood, including genes expressed in the circulating blood cells and/or soluble proteins not unique to the type of particles (Erdely et al., 2009). In particular, MWCNT-induced gene upregulation of more than half of the tested genes in the lung related to inflammation, oxidative stress,

coagulation, and tissue remodeling and to a significant increase in the circulating blood gene expression of several biomarkers of neutrophil response. Interestingly, several genes were activated in the circulating blood cells but not in the lung, at least at 4 h after exposure to MWCNT, e.g., osteopontin (a marker of early mesothelioma), colony stimulating factor-1 (CSF-1), and insulin growth factor receptor 1. Exposure to CNT also triggered the induction of primary cytokines such as IL-6 and IL-1b, which regulate multiple pathways of the inflammatory cascade as well as secondary inflammatory mediators, and chemokines, which directly regulate leukocyte recruitment to the inflammation site.

Guo et al. (2012) analyzed mRNA expression profiles in lungs of mice exposed to 0–80 μg of MWCNT by pharyngeal aspiration until 56 days postexposure and identified sets of genes associated with human lung cancer risk and progression with significant odds ratios. C57BL/6 mice exposed to 18, 54, and 162 μg Printex 90 carbon black nanoparticles (CBNP) showed perturbation of pathways, networks, and transcription factors of predicted phenotypes (e.g., pulmonary inflammation and genotoxicity) that correlated with dose and time. Comparison to inflammatory lung disease models (i.e., allergic airway inflammation, bacterial infection, and tissue injury and fibrosis) and human disease profiles revealed that induced gene expression changes in Printex 90 exposed mice were similar to those typical for pulmonary injury and fibrosis. Very similar fibrotic pathways were perturbed in CBNP-exposed mice and human fibrosis disease models, thus supporting the use of toxicogenomic profiles in human health risk assessment of NPs (Bourdon et al., 2013).

Palomäki et al. (2015) performed proteomics analyses of human macrophages exposed to tangled or rigid, long MWCNTs, or crocidolite asbestos, using hyphenated techniques and concluded that not all types of CNTs are as hazardous as asbestos fibers.

Kinaret et al. (2017b) have systematically investigated transcriptomic responses of the THP-1 macrophage cell line and lung tissues of mice, specifically induced by several carbon nanomaterials (CNMs). They observed only a minute overlap between the sets of intrinsic property-correlated genes at different exposure scenarios, suggesting specific transcriptional programs working in different exposure scenarios. However, when the effects of the CNM were investigated at the level of significantly altered molecular functions, a broader picture of substantial commonality emerged. As a result, in vitro exposures can efficiently recapitulate the complex molecular functions altered in vivo.

High-throughput (omics) methods, if applied in a rigorous manner, hold great promise for the development of (novel) biomarkers and biomarker signatures. In addition, achievements in this field may make more consistent the regulatory approach to hazard assessment based on dynamic adverse outcome pathway (AOP) models. (Vietti et al., 2016; Labib et al., 2016). With the aim to evaluate the application of global gene expression data in deriving pathway-based points of departure for MWCNT-induced lung fibrosis, as a noncancer end point of regulatory importance, Labib et al. (2016) showed an early perturbation of similar biological pathways regardless from MWCNT types across the doses and postexposure time points studied. The authors also showed that transcriptional benchmark dose (BMD) values for pathways associated with fibrosis (4.0–30.4 μg/mouse) were comparable to the BMDs derived by NIOSH for MWCNT-induced lung fibrotic lesions (namely, 21.0–27.1 μg/mouse), thus suggesting that transcriptomic data can be used to derive acceptable levels of exposure to NM in product development (Labib et al., 2016).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

BM is an important component of the occupational and environmental health surveillance, especially when occupational and/or environmental exposure monitoring data are unavailable or difficult to obtain. The literature on short-term effects of air pollutants and the available literature on NM, suggest identifying multiple biomarkers—a biomarker profile—to assess both effects at the "portal of entry" (e.g., inflammatory changes, short-term respiratory changes, respiratory, eye or skin irritation) and systemic effects (e.g., heart-rate variability, platelet aggregation and prothrombotic changes, acute phase proteins) in susceptible subgroups of the general population exposed to incidental NPs or to a mixture of pollutants.

Table 41.1 summarizes a panel of biomarkers of exposure and effect potentially available for human biomonitoring studies aimed at assessing early effects and health outcomes.

In spite of the lack of validation of "nanospecific" biomarkers, it is proposed that at this stage that the sensitivity rather than the specificity of biomarkers should be privileged to identify potentially predictive biomarkers suggestive of the biological pathway and mechanistic changes underlying the causality of exposure conditions and association with hazards (e.g., at workplace) and also to identify predictive biomarkers (Bergamaschi et al., 2015). For the moment, all studies carried out so far (see the review by Liou et al., 2015) have used a cross-sectional design and consequently do not allow confirming the observed effects and understanding the dynamics of their occurrence and

TABLE 41.1 Appraisal of Biomarkers of Exposure or Effects Relating to Ultrafine Particle or Engineered Nanomaterial

Quality of Biomarker	
Biomarkers of exposure	• Exhaled particles and/or elements in EBC (estimate of the "deposited dose" or "target tissue dose") • Elements analysis in biological fluids (excretion, body burden) • Protein modification ("corona")
Biomarkers of effective dose/early effect	• Lipid peroxidation products in EBC or blood (MDA, TBARS, conjugated dienes, LTB4, F2- and 8-isoprostane) • DNA excision base products (8-OHdG, 8-oxo-Gua, 8-OHG) • Exhaled NO (FeNO) and nitrosative stress products (3-nitrotyrosine) • Carbonyl compounds (4-HNE, 4-HHE) in EBC • Serum pneumoproteins (CC16) • Platelet activation/aggregation and prothrombotic changes • Acute phase proteins: hsCRP, SAA, Haptoglobin, Hemopexin • IL-6 and sTNF-RII • Coagulation factors (fibrinogen, plasminogen activator inhibitor-1[PAI-1]) • Vascular adhesion molecules (VCAM-1) and intercellular adhesion molecule (ICAM-1)
Biomarkers reflecting alterations in cell structure/function	• Fibrogenic markers (KL-6 glycoprotein; MMP-1, MMP-7, MMP- 9) • Osteopontin (Early mesothelioma development) • Micronucleus • DNA strand breaks (Comet assay + FPG-ENDO III) • Epigenetic markers: DNA (hypo)methylation; MicroRNAs (miRNAs) • Extracellular micro- and nanovescicles, exosomes (EMVs' cargo characterization)

The table includes biomarkers validated in human studies on people exposed to different ultrafine or fine particulates or known fractions, and biomarkers specifically investigated in relation to ENM.
Note: *4-HNE*, 4-hydroxy-2-nonenal; *8-isoprostane*, 8-isoprostaglandin F2α; *8-OHdG*, 8-hydroxy-2′-deoxyguanosine; *8-OHG*, 8-hydroxyguanosine; *8-oxo-Gua*, 8-oxo-7,8-dihydroguanine; *CC16*, Clara cell protein; *EBC*, exhaled breath condensate; *FPG-ENDOIII*, lesions detected as sites in DNA sensitive to formamidopyrimidine DNA glycosylase and endonuclease III; *hsCRP*, high sensitivity C-reactive protein; *IL-6*, plasma Interleukin 6; *LTB4*, Leucotriene-B4; *MDA*, malondialdehyde; *NO*, nitric oxide; *SAA*, serum amyloid A; *sTNF-RII*, soluble tumor necrosis factor-receptor II; *TBARS*, thiobarbituric acid reactive substances.

duration. Consequently, this kind of study could only help to identify some "intermediate" biological changes of effects. Only the study of Lee et al. (2015) showed a reduction, though not clinically significant, in lung function parameters in a cohort of workers occupationally exposed to nanoscale CB. Longitudinal panel studies with repeated exposure and effect biomarker measurement are necessary to investigate whether this unspecific "intermediate" biological changes could be indicative or predictive of clinical effects and apply them in health surveillance programs. To provide a coherent approach and make future epidemiological research a reality, a well-defined framework is needed for the careful choice of materials, exposure characterization, identification of study populations, definition of health end points, and evaluation of the appropriateness of study designs, data collection and analysis, and interpretation of the results (Riediker et al., 2012). Moreover, the scientific, methodological, political, and regulatory issues that make epidemiological research in nanotechnology-exposed communities particularly complex. Standardization of data collection and harmonization of research protocols are needed to eliminate misclassification of exposures and health effects. Forming ENM worker cohorts from a combination of smaller cohorts and overcoming selection bias are also challenges (Guseva Canu et al., 2018).

In conclusion, BM of exposure and effect should represent a valuable component of an integrated strategy and a proactive approach to risk assessment and management. Hence, it is an opportunity for companies committed to the responsible development of nanotechnology and also an ethical obligation toward all populations of workers that everything is done to assure a safe working environment.

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