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Nanosafety: a Perspective on Nano-Bio Interactions

Bengt Fadeel and Arturo A. Keller*

Engineered nanomaterials offer numerous benefits to society ranging from environmental remediation to biomedical applications such as drug or vaccine delivery as well as clean and cost-effective energy production and storage, and the promise of a more sustainable way of life. However, as nanomaterials of increasing sophistication enter the market, close attention to potential adverse effects on human health and the environment is needed. Here a critical perspective on nanotoxicological research is provided; the authors argue that it is time to leverage the knowledge regarding the biological interactions of nanomaterials to achieve a more comprehensive understanding of the human health and environmental impacts of these materials. Moreover, it is posited that nanomaterials behave like biological entities and that they should be regulated as such.

1. Nanosafety: Slow Train Coming?

Nanotechnology permeates every aspect of modern life, and more nanomaterial-enabled products reach the market every year.[1] It is thus important to ensure that the promise of nanotechnology does not come at a cost to human health or the environment. Considerable efforts have been invested over the past 15 years to address the human health and environmental impacts of engineered nanomaterials, yet some experts have painted a somber picture, concluding that, "despite much research, mechanistic understanding remains limited."[2] However, recent studies have provided important insights with regard to the interactions of engineered nanomaterials with biological systems including so-called coronation (the formation of a layer of biomolecules on nanomaterial surfaces) and transformation (dissolution or degradation of nanomaterials in the environment or in the human body). In the present perspective, we will discuss some of the key lessons learned in nanosafety research in the past 15 years. However, the sheer number of publications during this period[3,4] prevents an exhaustive account of nano(eco)toxicology, and we will therefore highlight selected examples. Some authors have suggested that nanotoxicology "has emerged to address the dark shadows of nanomedicine."[5] On the contrary, careful toxicological evaluation of novel nanomedicines (including vaccines) serves to promote the safe use of this class of compounds. Biomedical applications of nanomaterials are not addressed here, but toxicological studies aimed at understanding and minimizing adverse effects of nanomaterials in the occupational and/or consumer setting are also relevant for (future) biomedical applications as they may teach us valuable lessons about nano-bio interactions. Overall, we suggest that the purpose of nanotoxicology is to draw nanomaterials out of the "shadows," while ensuring that hazardous materials are weeded out. The topic has been reviewed by several other experts,[6–9] and the question has been raised as to whether nanosafety research is on the right track.[3] We believe so, but there are issues that need to be dealt with in a forthright manner such as the "asbestos analogy." There is also the perennial question of "novelty" which may have done the field of nanosafety research a disservice as it seems to distract from the fact that nanoparticles are not necessarily novel for humans or for the environment.[10]

2. Revisiting the Asbestos Analogy

The asbestos-like pathogenicity of multi-walled carbon nanotubes (MWCNTs) was suggested in a pilot study 15 years ago.[11] In the latter study, MWCNTs (50 μg) were injected directly into the abdominal cavity of mice leading to inflammation (neutrophil exudation) and the formation of lesions called granulomas; this was seen for samples containing long fibers but was less pronounced for short MWCNTs. Hence, in this sense, the so-called fiber pathogenicity paradigm appeared valid also for carbon nanotubes. Indeed, subsequent studies confirmed that not only the length of the fibers but also their rigidity correlated with the "inflammogenic" potential of MWCNTs.[12] However, while asbestos fibers are notoriously biopersistent, carbon nanotubes are susceptible, under certain conditions, to enzymatic biodegradation.[13,14] Thus, while some MWCNTs have been classified as possibly carcinogenic to humans, the lack of evidence (animal data) precludes generalization across all types of carbon nanotubes.[15] Despite this fact, the asbestos analogy seems to have taken on a life of its own.[16] The problem with this analogy is that it overshadows real progress in nanosafety research.

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Hence, it is true that long and rigid MWCNTs may replicate asbestos-induced mesothelioma following their instillation into the pleural cavity of mice.[17] However, we risk throwing out the baby with the bathwater if we lump all carbon nanotubes into one category.[18,19] We also run the risk of overlooking important research showing, for instance, that surface modification (functionalization) can alleviate the pathogenicity of MWCNTs.[20] Moreover, functionalization of MWCNTs serves to enhance renal clearance in mice.[21] Thus, even though MWCNTs are less susceptible to biodegradation when compared to SWCNTs, these materials may nevertheless be excreted from the body (under certain conditions). It is noted that early work on PEGylated SWCNTs showed no toxicity; however, "nude" (immunodeficient) mice were utilized, which precludes any general conclusions.[22] Several lessons can thus be gleaned from the past 15 years of investigations: material characterization is of utmost importance, the choice of in vivo model influences the outcome, careful studies of nanomaterials may unearth new mechanisms in biology, and, finally, establishing "ground truth" even for a single category of nanomaterials is a demanding task.

The human body constantly interfaces with the external environment, and biological barriers exist to protect us and preserve homeostasis. The skin is perhaps the most obvious and most visible of these barriers, but the gastrointestinal epithelium and pulmonary epithelium are equally important barriers between us and the outside world. Moreover, internal barriers exist that protect vulnerable organs: the blood-brain barrier (BBB) protects the brain, and the placenta protects the unborn child.[23] Needless to say, the instillation of particles directly into the abdominal or pleural cavity (see above) circumvents key barriers. Nevertheless, evidence for the translocation of nanoparticles across the air-blood barrier has also been documented, although the fraction of translocated particles is small. Hence, in a recent study involving fourteen volunteers, translocation of inhaled gold nanoparticles was observed as early as 15 min after exposure in some subjects and was present in most of the subjects at 24 h.[24] The authors estimated that less than 0.5% of the gold nanoparticles were translocated into the circulation, which is largely in agreement with previous studies in rats.[25] Unsurprisingly, size is an important determinant of particle translocation to the blood, but the fate of inhaled nanoparticles is also influenced by the adsorption of biomolecules, as shown in a study by Choi et al. in which nanoparticles were instilled into the lungs of mice.[26] More recent studies have shown that nanoparticle cycling between different cell types in the lungs and interactions between particles and biomolecules (lipids) originating from the cells may influence toxicological outcomes.[27] In summary, it is evident that "nanotoxicology" requires a deep understanding not only of the tested materials and their transformations but also of the biological systems. It is also important to address exposure: most toxicological studies are "acute" studies in which cells or tissues are subjected to a single acute dose, while "chronic" or repeated exposure studies are also warranted in order to understand the potential impact on human health and the environment. Early work revealed that chronic exposure of the human (non-malignant) BEAS-2B bronchial epithelial cell line to SWCNTs caused malignant transformation in vitro and tumorigenicity in vivo upon injection of transformed cells into immunodeficient mice.[28] Other investigators have demonstrated that low-dose chronic exposure of the immortalized human HaCaT keratinocyte cell line to Ag nanoparticles elicited a sustained cellular stress response in the absence of toxicity.[29]

3. Nanomaterials as Biological Entities

The immune system is designed to protect us from harm. To this end, immune cells express receptors with which to detect and respond to pathogens.[30] T cells and B cells of the adaptive immune system express receptors with exquisite specificity for certain motifs, while cells belonging to the innate immune system (our first line of defense against foreign intrusion) express so-called pattern recognition receptors which are more promiscuous. Indeed, recent studies have shown that some receptors also "sense" engineered nanomaterials. For instance, T cell immunoglobulin mucin 4 (Tim4) has been identified as a receptor for MWCNTs.[31] Tim4 was thus found to play a role in the recognition of MWCNTs by murine peritoneal macrophages and was shown to promote granuloma development in mice following the intraperitoneal injection of MWCNTs. In a recent protein structure-based in silico screen, the immune receptor sialic acid immunoglobulin-like binding lectin-14 (Siglec-14) was identified as a human MWCNT-recognizing receptor provoking inflammation.[32] The chirality of nanoparticles can also influence receptor binding. Xu et al. reported that left- and right-handed gold nanoparticles differed in terms of their ability to provoke immune responses.[33] Both particles bound to specific adhesion receptors on immune cells, but the left-handed enantiomer displayed a higher affinity.

Furthermore, recent work revealed that chirality-dependent protein coronas (see below) correlated with tissue accumulation and clearance rates in vivo, and evidence was provided for a chirality-dependent functionality of lipoproteins including ApoE and ApoA1, acting either as opsonins or dysopsonins upon adsorption to nanoparticles.[34]

The inflammasomes are key sentinels of the innate immune system that respond to endogenous or exogenous "danger" signals.[35] Inflammasome assembly in the cytosol of innate immune cells such as macrophages leads to the activation of a proteolytic enzyme called caspase-1 with processing of its substrates pro-IL-1β and pro-IL-18 and subsequent secretion of IL-1β and IL-18. Fifteen years ago, several groups reported that asbestos fibers, crystalline silica (quartz), and aluminum salts trigger the NLRP3 inflammasome.[36–38] Further studies revealed that SiO_2 and TiO_2 nanoparticles also elicit NLRP3 inflammasome activation,[39] although it is noted that very high doses (200 μg mL^{-1}) were applied in the latter study. Subsequent work showed that MWCNTs trigger the NLRP3 inflammasome,[40] and a very recent study revealed that low-dose exposure to SiO_2 nanoparticles triggers the NLRP3 inflammasome in the absence of priming with microbial agents.[41] Thus, NLRP3 emerges as a key cytosolic "sensor" of nanomaterials. Recent work has shown that non-canonical inflammasome activation involving caspase-11 (not caspase-1) mediates the adjuvanticity (i.e., the ability to augment immune responses) of polymeric nanoparticles.[42] The authors were able to define a specific size (50 nm) as being optimal for inducing immune effects. Thus, receptors on the cell surface or in the cytosol are also capable of responding to synthetic nanomaterials.

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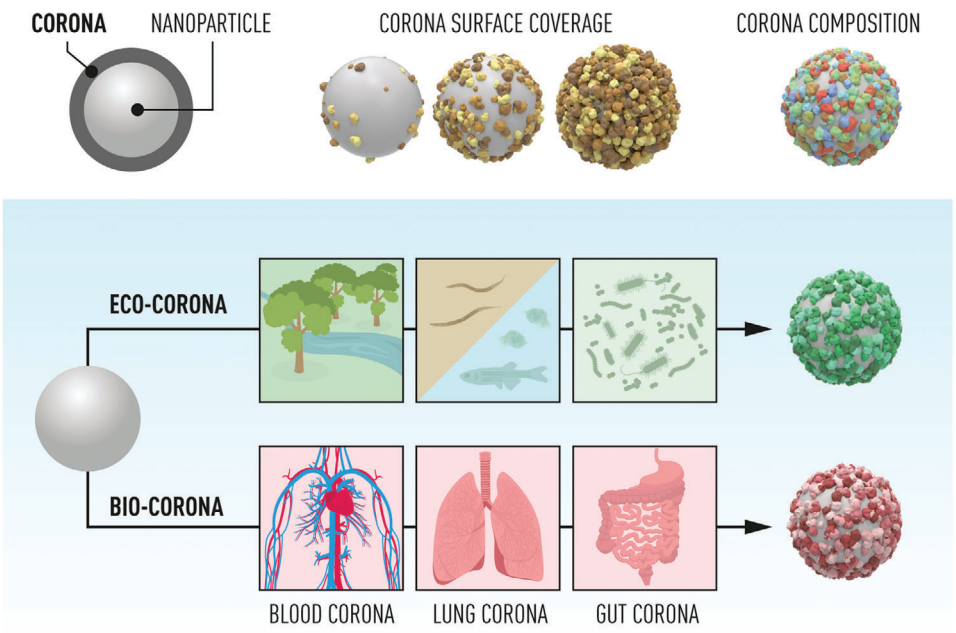

Figure 1. Nanoparticle "coronation." The cartoon shows the surface-adsorbed layer of biomolecules (aka corona) which may vary with respect to surface coverage and in terms of the types of biomolecules (i.e., proteins, lipids, and others). Nanoparticle eco-coronas, consisting of natural organic matter, as well as extracellular polymeric substances, are formed both in terrestrial and aquatic ecosystems. Nanoparticle bio-coronas of varying composition are also formed in the human body following exposure through ingestion or inhalation, or upon injection of the particles into the bloodstream.[51]

These studies suggest that engineered nanomaterials display certain commonalities with biological entities (such as receptor-mediated recognition and cellular uptake), clearly showing that nanomaterials are not small molecules (chemicals), and yet nanomaterials are currently regulated as chemicals. However, even though every nanomaterial has a well-defined chemical composition, nanomaterials are more than chemicals, as they are defined by a host of other physicochemical properties such as size, shape, etc. Furthermore, nanomaterials are endowed with a biological "identity" that evolves in the body or in the environment; this surface-adsorbed layer of proteins, lipids, and other biomolecules is commonly referred to as the nanomaterial "corona".[43]

4. Transformation of Nanomaterials

The "corona" (**Figure 1**) has been studied in considerable detail in recent years, and the composition of the protein corona, in particular, has been cataloged by several groups.[44,45] However, the emphasis has been on the corona that is formed extracellularly (or in the natural environment) while less is known about the fate of the corona in cells. Recent work has shown that polystyrene (PS) nanoparticles and the associated corona are separated following cellular uptake such that the PS nanoparticles are found in recycling endosomes whereas the protein corona is found in multivesicular bodies.[46] This shows that macrophages can decode the synthetic and biological "identities" of nanoparticles. Nanomaterials introduced into the environment are invariably coated with a heterogeneous mixture of environmental components including humic substances which are formed through the decomposition and transformation of plant and microbial residues.[47] Indeed, as noted by the latter authors, "proteins are not always the most abundant constituents in the eco-corona, especially if formed outside an organism." Interestingly, a recent study focusing on the trophic transfer of gold nanoparticles in an aquatic food chain consisting of microalgae, daphnids, and zebrafish demonstrated that the eco-corona, formed mostly from fish plasma, affects the dissolution of the particles, which, in turn, may affect particle distribution and transfer.[48] Whether the eco-corona that is formed in the environment (**Figure 1**) could have implications for human health is not well understood, though one may speculate whether the eco-corona could enter the food chain.[49] Does the composition of the corona tell us anything about the cellular uptake and biodistribution of nanoparticles? Several studies have tackled this question, in most cases by addressing one ligand and one receptor at a time. However, in a recent landmark study, an unbiased approach was taken in which pooled genome-wide knockout screens were applied to identify the receptor(s) involved in the cellular uptake of serum-coated gold nanoparticles.[50] The authors found that the low-density lipoprotein (LDL) receptor was responsible for cellular uptake in vitro while nanoparticle accumulation in vivo correlated with LDL receptor expression in different organs. Conceptually, it may be instructive to consider nanoparticles cloaked in biomolecules as mimicking LDL (natural, cholesterol-transporting nanoparticles). Hence, recent studies have revealed that engineered nanomaterials can be recognized both as native structures via pattern recognition receptors and by virtue of the surface-adsorbed "corona" of biomolecules.

The so-called Trojan horse-type entry/dissolution mechanism is well known for oxides of metals such as Zn, Cu, Co, and Mn.[52] Hence, these nanoparticles are internalized as particles, but once they are trafficked to lysosomes they dissolve, leading to the

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release of large amounts of metal ions. This means that both particles and soluble ions must be factored in when addressing the toxicity of such materials. Further evidence of the dissolution of nanoparticles was provided in a recent comprehensive study in which molybdenum (Mo)-based nanoparticles were found to undergo transformation in the liver following intravenous injection in mice.[53] The authors demonstrated that this led to the incorporation of Mo into Mo-dependent enzymes, thus increasing the specific activities of those enzymes in the liver. Combining three powerful analytical techniques, single-particle inductively coupled mass spectrometry, single-cell mass cytometry (also known as CyToF), and synchrotron X-ray absorption spectrometry, other investigators explored the fate of Ag nanoparticles in a human leukemic T cell line (used as a model of T lymphocytes).[54] The authors found that the transformation of Ag nanoparticles was dominated by sulfidation, which can be viewed as a detoxification pathway. Thus, the transformation of nanoparticles is not always detrimental. However, while sulfidation can serve as a detoxification process for Ag nanoparticles,[55] sulfidation may not be sufficient to fully detoxify CuO nanoparticles.[56]

Humans are colonized by microorganisms that dwell in and on the body. To paraphrase the singer-songwriter Madonna, "we are living in a microbial world." The gut microbiome, in particular, has been found to play a crucial role in maintaining host health by contributing to various physiological processes, such as digestion, metabolism and immune function, whereas disruption to the gut microbiome (dysbiosis) is thought to be linked to a variety of human diseases.[57] Therefore, exposure to nanomaterials could lead to adverse effects on human health via the gut microbiome. Recent work has shown, using zebrafish as a model system, that oral exposure to graphene oxide (GO) displaying a "corona" of microbial metabolites triggered a type 2 immune response, which was found to occur through the activation of the aryl hydrocarbon receptor (AhR), an important sensor of environmental cues.[58] Thus, GO can modulate the crosstalk between the microbiome and the immune system via the AhR. This means that the microbiome should be factored in when addressing the impact of nanomaterials on human health. Furthermore, rare earth oxides, exemplified by La_2O_3, were found to elicit an imbalance of the microbiome in the lungs of mice.[59] Another recent study demonstrated that inhalation co-exposure to carbon black and ozone prompted distinct changes in the lung and gut microbiomes in mice.[60] The plant microbiome is believed to be a key factor in determining plant health. In a seminal study, gold nanoparticles which are widely believed to be stable in the environment were found to undergo dissolution in a wetland mesocosm (i.e., a simulated ecosystem). Specifically, aquatic plants (macrophytes) and their associated microbiomes were shown to serve as a major sink for nanoparticle accumulation and transformation.[61] Taken together, nanomaterial transformations (coronation, dissolution) may occur in the environment as well as in the human body, which may greatly impact the subsequent biological responses to these nanomaterials.

5. Nanosafety: Towards Safe-by-Design

In this perspective, we briefly highlighted recent discoveries in the nanosafety field with emphasis on nano-bio interactions (i.e., interactions of nanomaterials with biological systems). However, there are remaining challenges with respect to the environmental and human health impacts of nanomaterials. Hence, while there has been significant progress in assessing the likely exposure of ecological receptors to the most widely employed nanomaterials, and while their effects on aquatic ecosystems have been assessed, albeit with considerable uncertainty,[62] unexpected risks are likely to come from novel, multi-component nanomaterials, engineered to make them more mobile and more bioavailable. Even though inadvertent (occupational) exposure, as well as intentional (consumer and medical) exposure to nanomaterials, is on the rise, the types of nanomaterials that are currently released to the environment are rather limited.[1] However, the so-called agri-tech revolution, driven in part by nanotechnology,[63] means that a significant increase of novel nanomaterials in agriculture seems likely, not only for pest and disease control, but also for the delivery of nutrients and other active ingredients. Trophic transfer from food crops to humans and other ecological receptors has not been studied systematically and warrants more attention. Another area of concern is the use of novel nanocomposites for water treatment.[64] While these nanomaterials promise significant benefits, there is a concern that a fraction may enter the water distribution system, in which case this may pose unknown risks. Additional safeguards need to be put in place to ensure the safe development of water treatment using nanomaterials. More futuristic scenarios include the use of nanomaterials in ocean fertilization or other forms of geoengineering for climate remediation.[65] Here, the potential long-term impact on ecological systems needs to be carefully considered.

The importance of validating in vitro (cell culture) models and verifying the findings obtained in such models using in vivo (animal) models is well understood, but differences between different animal models are often overlooked. However, as with the variability in disease susceptibility in humans, various strains of mice exhibit differences as a function of their genetic background. Jones et al. examined the clearance of nanoparticles from the blood in Th1-biased mouse strains (such as C57BL/6) versus Th2-biased mouse strains (such as BALB/c) following i.v. injection and found significant differences in nanoparticle clearance.[66] Pulmonary inflammation in response to CdSe/ZnS quantum dots and MWCNTs has also been shown to be mouse strain dependent.[67,68] Moreover, Scoville et al. provided compelling evidence, using 25 different inbred mouse strains, that genetic background contributes to variations in the inflammatory response to Ag nanoparticles.[69] The authors also performed genome-wide association mapping to identify potential candidate susceptibility genes. To sum up, the choice of animal models is important (even though animal models may not always be the right choice). The results of these studies suggest that individual variability needs to be considered in the risk assessment of nanomaterials. The microbiomes in the gut, in the lungs, and on the skin should also be factored in if we are to understand the impact of nanomaterials on human health.[70]

The importance of nanomaterial characterization cannot be overstated, and this applies to ecotoxicology as well as human toxicology investigations. The material characterization must be sufficiently detailed, particularly as certain material properties that may be predictive of toxicity, such as band gap energy, are not included in the routine workflow, as previously pointed out by others.[2] Furthermore, transformations in the environment

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and in other biological systems influence the biological impact of nanomaterials, and analytical methods to monitor these transformations are needed.[71] We suggest that safe-by-design approaches should be tailored to address distinct transformation behaviors of nanomaterials in the environment and in the human body.

Finally, in silico (modeling) approaches including the computational deconvolution of transcriptomics, proteomics, and metabolomics data, have been incorporated into the toxicological toolbox in recent years.[72] In particular, toxicogenomics approaches have increasingly been applied to understand the biological effects of nanomaterials but also to enable the grouping of nanomaterials on the basis of their mechanism-of-action (as deduced from gene expression profiles). In a recent study, transcriptomic responses ("signatures") to lithium cobalt oxide across taxonomic groups were studied, revealing commonalities and differences.[73] In other recent work, evidence was presented for a common and conserved gene expression pattern across biological models for a range of nanomaterials.[74] This is good news, as these findings suggest that the biological responses to novel materials are not necessarily unprecedented or "novel". Instead, host responses to pathogens and nanoparticles may well be conserved which means that it should be possible to predict the outcomes of nanomaterial exposure. Moreover, if responses to nanomaterials can be predicted, they can also be prevented, either through targeted interventions or through the purposeful (re)design of the materials.[75]

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

bio-corona, eco-corona, nanomaterials, nanosafety, safe-by-design

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