PERSPECTIVE

A Predictive Toxicological Paradigm for the Safety Assessment of Nanomaterials

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ABSTRACT The rate of expansion of nanomaterials calls for the consideration of appropriate toxicological paradigms in the safety assessment of nanomaterials. We advocate a predictive toxicological paradigm for the assessment of nanomaterial hazards. The predictive toxicological approach is defined as establishing and using mechanisms and pathways of injury at a cellular and molecular level to prioritize screening for adverse biological effects and health outcomes *in vivo*. Specifically as it relates to nanomaterials, a predictive approach has to consider the physicochemical properties of the material that leads to molecular or cellular injury and also has to be valid in terms of disease pathogenesis in whole organisms.

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The production of engineered nanomaterials represents a scientific breakthrough in material design and the development of new consumer products that are expected to impact almost every industrial and manufacturing sector, including medicine. It is anticipated that commercialization of nanomaterials and nanoenabled devices will grow into a $1 trillion industry by 2015.[1] While the successful implementation of this disruptive technology is important for commercial applications and the growth of the global economy, one also needs to consider the potential health and environmental impact of these materials.[1] One reason is that some of these materials will eventually come into contact with biological structures and processes that frequently occur at the nanometer scale or through the display of nanostructured biological surfaces. A second reason is that little is known about how the novel physicochemical properties that make engineered nanomaterials so attractive for use in consumer products may relate to the interactions that take place at the nano/bio interface.[2] Such interactions could be biologically inert, advantageous for improving biological function (*e.g.*, tissue engineering or sustaining phytoplankton growth), or could pose a biological hazard that culminates in human disease or toxicological impacts on the environment. Concern about nanomaterial safety is currently receiving much attention in the scientific, academic, industrial, and regulatory communities as well as in the debate on the impact of the implementation of nanotechnology on humans and the environment.[3−7]

In order to assess nanomaterial hazards, reliable and reproducible screening protocols are needed to test basic materials as well as consumer products made from them. Achieving this goal has proven to be quite challenging because of the large number of new nanomaterials that are produced continually, their host of novel physicochemical properties, and uncertainty in how those properties may relate to biological outcomes. There are a vast number of biophysicochemical interactions that could be generated when nanomaterials of different composition, size, surface area, shape, dispersibility, aggregation, crystallinity, surface functionalization, wettability, surface coating, and so on make contact with biological fluids, proteins, lipids, DNA, cell membranes, lysosomes, mitochondria, and nanometer-scale biological processes.[2] While we are fortunate that none of these biophysicochemical interactions have resulted in human disease or in ecological harm, there is experimental evidence that nanomaterials may pose some hazard that could be interpreted in terms of possible toxicological pathways or mechanisms of injury (Table 1).[8−11] Therefore, it is clear that a platform needs to be developed to deal with the vast number of biophysicochemical interactions that could take place at the nano/bio interface, and that one approach is to use the mechanisms of injury that are linked to disease pathogenesis or *in vivo* toxicological outcomes. Currently, there is considerable debate about how to proceed with nanomaterial toxicity testing, with the major discussion points centering around which toxicological end points to screen for, the comprehensiveness of the screening effort, the correct balance of *in vitro* (cellular and molecular) *versus in vivo* (animal or whole organism) testing, the cost of the effort, and who should be responsible for screening and safety assessment of nanomaterials.[2,4,12,13] Attempts to use traditional toxicological assays and models have resulted in conflicting and sometimes irre-

TABLE 1. Experimental Examples of Major Toxicological Pathways That Could Lead to Nanomaterial Toxicity

toxicological pathway	example nanomaterials	reference
membrane damage/leakage/thinning	cationic NPs	11
protein binding/unfolding responses/loss of function/fibrillation	metal oxide NPs, polystyrene, dentrimer, carbon nanomaterials	11, 31, 41
DNA cleavage/mutation	nano-Ag	42
mitochondrial damage: e^- transfer/ATP/PTP opening/apoptosis	UFP, cationic NPs	11, 16, 17
lysosomal damage: proton pump activity/lysis/frustrated phagocytosis	UFP, cationic NPs, CNTs	11, 16, 17, 28
inflammation: signaling cascades/cytokines/chemokines/adhesion	metal oxide NPs, CNTs	10, 28
fibrogenesis and tissue remodeling injury	CNTs	28, 39, 43
blood platelet, vascular endothelial and clotting abnormalities	SiO_2	44
oxidative stress injury, radical production, GSH depletion, lipid peroxidation, membrane oxidation, protein oxidation	UFP, CNTs, metal oxide NPs, cationic NPs	10, 11, 16, 17, 28

producible results such that is not yet a widely accepted screening platform.

In addition to the biophysicochemical complexity and lack of consensus about nanomaterial safety testing, it is important to consider that the scale at which this technology is growing could push this research area from ~800 nanomaterials currently known to numbers in the 10^4 range within a decade.[14] Thus, the rate of expansion must be considered when deciding what constitutes an appropriate toxicological paradigm so as to avoid the conundrum of the chemical industry, where among the >40 000 industrial chemicals, fewer than 1000 have undergone toxicity testing. One of the major factors contributing to this backlog is the high cost and length of time to complete even a single toxicological screen through animal testing. A recent report by the National Research Council of the National Academy of Sciences (NAS) set forth a vision of a required paradigm shift in the approach to toxicological testing in the 21st century, including the development of robust scientific platforms that can be used to screen a large number of toxicants simultaneously in order to prioritize the decisions that need to be made for *in vivo* testing.[15]

A platform needs to be developed to deal with the vast number of biophysicochemical interactions that could take place at the nano/bio interface.

A Predictive Approach for Oxidative Stress Screening. We advocate a predictive toxicological paradigm for the assessment of nanomaterial hazard (Figure 1).[3] We define a predictive toxicological approach as establishing and using mechanisms and pathways of injury at a cellular and molecular level to prioritize screening for adverse biological effects and health outcomes *in vivo*.[3] Specifically as it relates to nanomaterials, a predictive approach has to consider the physicochemical properties of the material that lead to molecular or cellular injury and also has to be valid in terms of disease pathogenesis in whole organisms. Evidence that such a mechanistic approach is possible comes from the study of the adverse health effects of ambient ultrafine particles (UFP).[9,16−20] The physicochemical properties of the ambient nanoparticles, including their small size and large surface area, allow for redox cycling of organic chemicals and bioavailability of transition metals and also play a role in their ability to generate reactive oxygen species (ROS) and to promote pro-inflammatory effects in cellular targets such as macrophages, epithelial cells, and dendritic cells, which participate in a number of disease pathogenesis in the lung and cardiovascular systems.[18,19,21] According to this oxidative stress paradigm, advanced levels of oxidant injury promote the development of disease by exerting pro-inflammatory effects in key target cells.[3,22] Accordingly, our studies have shown that there is a direct relationship between the UFP content of redox cycling organic chemical compounds (such as polycyclic aromatic hydrocarbons), the ability of the particles to generate ROS, promotion of cytokine and chemokine production at cellular level, and the ability of the particles to trigger allergic inflammation and growth of atherosclerotic plaques in animal models.[9,16,17,19,20,23] Excellent Pearson correlation coefficients have been established between the UFP capacity to generate ROS abiotically and ability to induce oxidative stress responses (*e.g.*, heme oxygenase 1 expression) in epithelial cells and macrophages.[19] Similar trends can now be shown to be developing in relating the abiotic and cellular oxidative stress effects to disease models such as allergen sensitization/allergic inflammation or atherosclerosis in animals.[18,20]

A recent paper questions our predictive paradigm by invoking different disease outcomes for particles that share a common *in vitro* mechanism.[12] The predictive paradigm does not necessarily state that the pro-oxidative effect of a particle such as UFP in and of itself can specify the exact disease outcome, which depends on a number of variables other than the injury mecha-

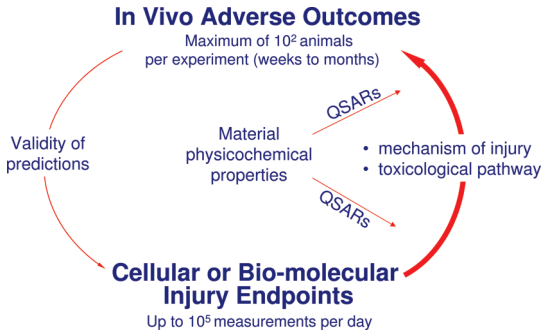

Figure 1. Elements of our proposed predictive toxicological paradigm for nanomaterial hazard testing. We define a predictive toxicological approach as establishing and using *in vitro* mechanisms and pathways of injury that are directly related to the physiochemical properties of nanomaterials as well as to disease mechanisms *in vivo*. The *in vivo* output is used to validate the *in vitro* screening method as being "predictive" and therefore valid for screening large batches of materials to obtain quantitative structure−activity relationships that can also be applied to *in vivo* observations. The high volume of screening activity *in vitro* therefore serves to prioritize the *in vivo* approach, where fewer measurements can be made. While the *in vitro* observations cannot stand alone, an established link to *in vivo* outcomes could speed up the *in vivo* testing procedure in spite of the logistical limitations.

nism. Instead, we propose that where a link has been established between a mechanistic pathway (such as oxidative stress) at the cellular level and an *in vivo* outcome (such as allergic airway inflammation or atherosclerosis), *in vitro* studies can help to predict the hazard potential of a series of ambient particles that differ in composition based on collection site and other factors. This is not to say that an engineered nanoparticle with pro-oxidant activity and capable of initiating pro-inflammatory cascades will necessarily lead to the same disease outcome because of the many other variables that apply. For example, if it is demonstrated that inhalation of the engineered nanoparticle can lead to airway inflammation, it would be straightforward to determine whether there is a link between the ability of the same particle to induce oxidative stress and inflammation in a target cell type. This target cell can then be used for a number of *in vitro* studies to accelerate the rate of information gathering about which physicochemical characteristics may be linked to the *in vivo* disease process and therefore reduce the number of animal studies to test that prediction.

Oxidative stress represents a dynamic equilibrium between antioxidant defense mechanisms that act to restore redox equilibrium and injurious cellular responses that can lead to toxicological outcomes. This concept is encapsulated in the hierarchical oxidative stress paradigm, which posits that ROS production leads to incremental cellular responses that can be classified as antioxidant defense, pro-inflammatory effects, and cytotoxicity (Figure 2A).[3,20,22] This model has enabled us to set up an integrated series of cellular screening assays that quantify (i) the induction of a homeostatic antioxidant defense pathway, which is mediated by transcriptional activation of the antioxidant response element in phase II enzyme promoters (Tier 1);[3,9,16,22] (ii) cytokine and chemokine expression through transcriptional activation of the gene promoters by redox-sensitive mitogen-activated protein (MAP) kinase and the transcription factor nuclear factor kappa-B (NF-κB) signaling cascades (Tier 2);[20,22] and (iii) activation of cytotoxic cell death (also known as toxic oxidative stress) through mitochondrial perturbation (Tier 3).[10,23] We have previously demonstrated that it is possible to compare the *in vitro* hazard potential of engineered nanomaterials with ambient UFP by conducting cellular assays that reflect each of the tiers of oxidative stress (Figure 2B).[9−11] Thus, the oxidative stress paradigm has proven useful in comparing the level of oxidant injury based on the ability of engineered nanomaterials that generate ROS spontaneously or in the context of a biological environment (Figure 2).[3] These pro-oxidative effects depend on nanomaterial properties such as an electronically active surface (*e.g.*, semiconductors, doped particles with an expanded band gap), the presence of transition metals or redox cycling organic chemical impurities (*e.g.*, carbon nanotubes), photoactivation leading to the generation of electron−hole pairs (*e.g.*, TiO_2), dissolution of the particle surface, and shedding of toxic metal ions (*e.g.*, ZnO and chalcogenides).[3,9−11] Thus, it is possible to envisage oxidative stress screening paradigms that compare metal, metal oxide, quantum dot, carbon nanotube, fullerene, and semiconductor nanomaterials for their potential to induce cellular injury through oxidative stress effects (Figure 2B). Proof-of-principle confirmation was recently provided by comparing three metal oxides (ZnO, CeO_2, and TiO_2), of which ZnO was clearly the most toxic in bronchial epithelial and macrophage cell lines based on particle dissolution and shedding of toxic Zn^{2+}.[10] Not only did ZnO nanoparticles generate H_2O_2 and superoxide radicals but they induced a Tier-2-like response that involved increased IL-8 and tumor necrosis factor-alpha (TNF-α) production by bronchial epithelial cells and macrophages, respectively.[10] These are the same cytokines that are found in the bronchoalveolar lavage fluid of welders exposed to incidental metal oxide particles (including ZnO) during welding.[1,24,25] This exposure could lead to an acute inflammatory response in the lung known as metal fume fever.[26] We propose that this represents a Tier-2-like effect *in vivo*, a notion that is further confirmed by genomics studies conducted on the blood cells of boilermakers before and after exposure to welding fumes.[27] The genetic footprint that emerged from these studies shows increased expression of oxidative stress genes, cytokine gene expression (including IL-8), and induction of programmed cell death.[10] Moreover, metal fume fever is characterized by a conditioning effect, such that prior exposure to a lesser amount of welding fumes can prevent an

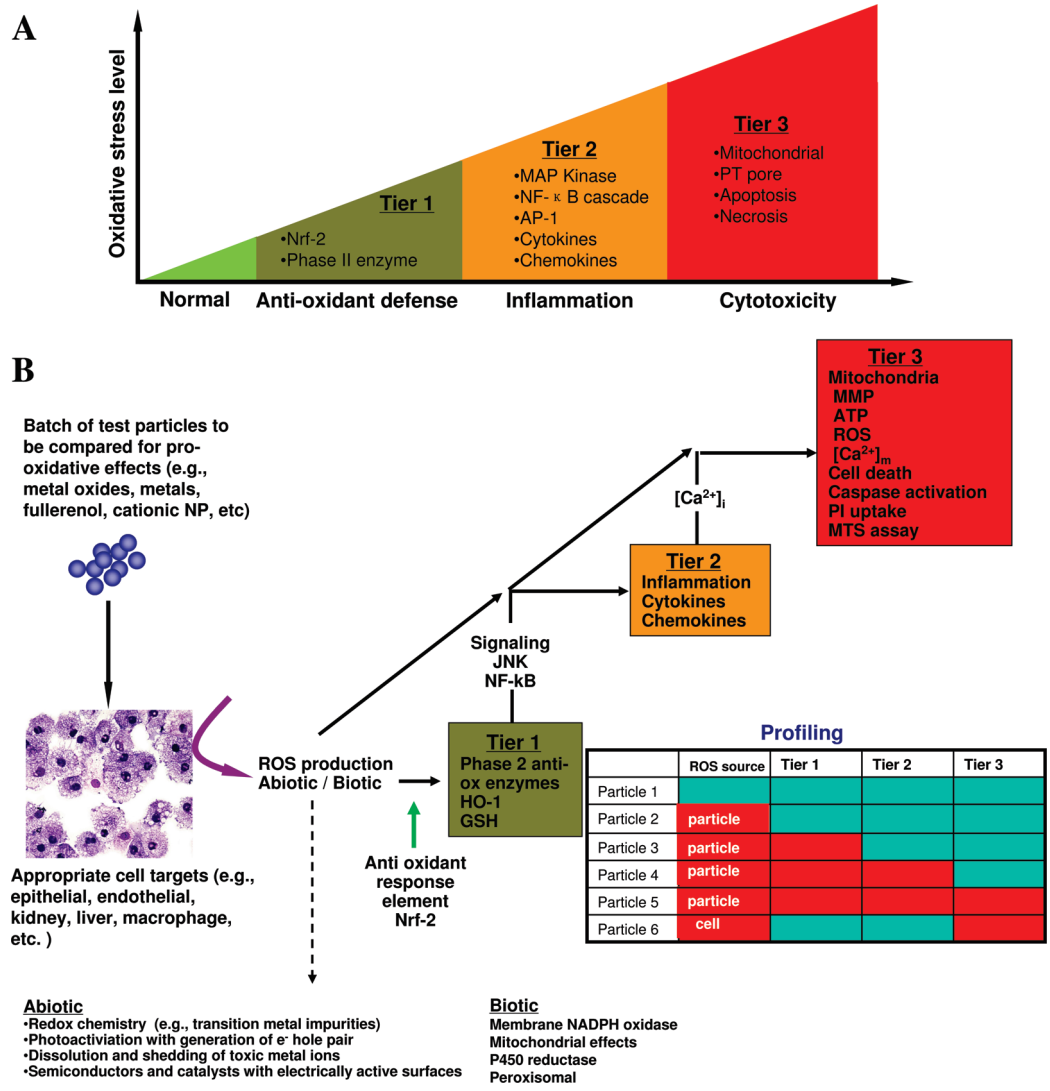

Figure 2. Use of hierarchical oxidative stress assessment to make predictions about nanomaterial hazard as an example of a predictive paradigm. (A) Nanomaterial-induced oxidative stress involves an incremental series of cellular responses that are encapsulated in the hierarchical oxidative stress paradigm. The different levels of cellular response can be classified as anti-oxidant defense, pro-inflammatory effects, and cytotoxicity. Each of these response tiers are initiated by specific biological sensors and activation mechanisms. In Tier 1, the transcription factor Nrf2 is activated to enhance the expression of phase II enzymes, which attempts to restore redox equilibrium. If the level of oxidant injury increases (Tier 2), cells express pro-inflammatory cytokines by activating signaling pathways such as the mitogen-activated protein kinase (MAPK) and nuclear factor-kappa B (NF-κB) cascades. At the highest level of oxidative stress (Tier 3), interference in mitochondrial inner membrane electron transfer or changing open/closed status of permeability transition pore could lead to effects on ATP synthesis and release of pro-apoptotic factors. (B) Implementation of the hierarchical oxidative stress paradigm to screen batches of engineered nanomaterials that are capable of reactive oxygen species production under experimental conditions. The ability to rank particles according to the oxidant potential could speed up the utility of the linked *in vivo* model to predict whether to test the most hazardous material first, what experimental dose to consider, what possible *in vivo* biological end points could be included, which nanomaterial properties are most likely to prove toxic *in vivo* (in the case of using combinatorial libraries), and so on. It is important to note that triggering of the different tiers of oxidative stress could have unique disease outcomes depending on the organ that is involved and the method of exposure. For instance, a Tier 2 response by inhaled ZnO nanoparticles could lead to rapid and transient induction of cytokines and chemokines, which, due to the dissolution of these materials, leads to an acute onset pulmonary inflammatory condition (metal fume fever) that rapidly dissipates. However, the induction of a Tier 2 response by ambient ultrafine particles that are retained in the lung and may also undergo systemic consultation could result in a different disease profile due to changing effects of the mucosal immune system or enhancing chronic vascular inflammation in atherosclerotic plaques.

acute secondary attack. This is reminiscent of a Tier 1 oxidative stress effect. We therefore propose that our oxidative stress screening assay is predictive of *in vivo* toxicological scenarios following the inhalation of engineered ZnO and ambient UFP nanoparticles. In addition to animal allergen sensitization/allergic inflammation and atherosclerosis models that reflect the induction of oxidative stress by UFP,[20] we are establishing additional disease models that may illustrate particle-induced oxidative stress, under the appropriate circumstances.

A General Approach for Predictive Toxicolgical Screening. Having demonstrated that a predictive toxicological approach for the assessment of nanomaterial hazards is possible, the key questions become (i) whether it is possible to develop additional predictive screening paradigms for nanomaterial toxicity, and (ii) whether it is possible to integrate the assays in Tiers 1−3 into a more rapid and high-throughput screening process to assess large batches of nanomaterials. With respect to (i), it is important to emphasize that we do not advocate that oxidative stress is the only predictive toxicological paradigm; several toxicological pathways or mechanisms may emerge over time.[3] These could include injury paradigms that are connected to frustrated phagocytosis (*e.g.*, in mesothelial surfaces),[28] changes in protein structure and function (*e.g.*, loss of enzymatic activity, protein unfolding response[29]), immune activation (*e.g.*, through exposure of cryptic epitopes or immunostimulatory effects[30]), fibrogenesis and tissue remodeling, blood clotting, vascular injury, neurotoxicity (*e.g.*, oxidative stress, protein fibrillation[31]), and so on (Table 1).[3] While only time will tell whether these injury mechanisms may evolve into clinical disease profiles, it is important to establish experimentally whether *in vivo* injury is possible in order to implement proper screening procedures that can prevent such a scenario. Although it is not possible at this stage to provide a detailed blueprint of how to develop a fully mature predictive system, we are beginning to appreciate the infrastructural needs to develop *in vitro* and *in vivo* quantitative structure−activity relationships (QSARs) to build such a model.

The first requirement for establishing a predictive toxicological paradigm is the need for rapid and high-volume *in vitro* data generation to assist decision-making for planning and carrying out the animal experiments (Figure 1).[15,32] The cost and the time required to perform these *in vivo* studies have proven to be one of the major stumbling blocks in the safety assessment of industrial chemicals, resulting in <2% having undergone toxicity testing. In our opinion, it is advantageous to establish *in vitro* QSARs and then to use the information on dose, kinetics, nanomaterial physicochemical properties, and quantifiable biological response outcome to plan and to execute *in vivo* experiments. This also has the advantage of being able to relate any adverse health effects or evidence of pathology to known mechanisms of injury.[15,32] This is not to say that primary *in vivo* screening procedures cannot or should not be performed, but where there is such a choice, it would be advantageous to build in a mechanism of injury or link to a toxicological pathway that can be exploited for establishing *in vivo* QSARs. The validity of this approach has been proven in the elucidation of chronic peritoneal inflammation by multiwall carbon nanotubes (MWCNTs).[28] In this case, the authors made use of the physicochemical properties of asbestos fibers to develop their hypothesis that long, stiff, and biopersistant MWCNTs may induce chronic mesothelioma inflammation *via* a mechanism of frustrated phagocytosis.[28]

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The second infrastructural requirement for a predictive paradigm is the development of rapid throughput or high-throughput screening (HTS) procedures to evaluate and to quantify the toxicological pathways shown in Table 1.[33−35] This requirement also relates to our earlier question of whether it is possible to combine individual assays in the hierarchical oxidative screening pathway to make an integrated screening procedure that facilitates HTS. In order to achieve this integration, it is important to consider combining cellular injury pathways that can be assessed by screening platforms such as epifluorescence microscopy, luminescence, UV/vis spectroscopy, fluorescence spectroscopy, fluorescence microscopy, multiplex immunoassay techniques, and real-time polymerase chain reaction (PCR). One approach would be to quantify cellular expression of a phase II enzyme (*e.g.*, NADPH reductase as a representative Tier 1 response) in parallel with cytokine/chemokine detection in the cellular supernatant (Tier 2) as well as a quantifiable mitochondrial response (Tier 3). It is possible to automate this screening by robotic handling of the sequential measurements. Another approach would be to find resulting common molecular or cellular responses that lead to cytotoxicity *via* injury pathways that involve oxidative stress, mitochondrial injury, membrane damage, stimulation of intracellular calcium flux, and so on (Figure 3).

The third infrastructural requirement is the acquisition, synthesis,

and characterization of standard reference nanomaterial libraries that can be used in the high-throughput system to elucidate the material properties that are most likely to lead to biological injury. One example is the construction of a standard reference nanomaterial library that incorporates the major classes of nanomaterials that are currently produced. It is important to link the library development to a nanomaterial classification that allows toxicological mechanisms to be interpreted in terms of intrinsic material properties, such as their classification as conductors, semiconductors, or insulators.[36] In addition to a reference library, it will be helpful to establish combinatorial nanomaterial libraries that exhibit systematic variation of material properties that are most frequently involved in injury, including size, shape, charge, crystallinity, metal doping, dissolution, porosity, surface area, wettability, and so forth. Examples of how this can be achieved have been provided recently and will require the cooperation of nanomaterial scientists who can contribute to library development.[37,38] Finally, it will be necessary to integrate all of the above data in a computerized expert system that utilizes machine learning, pattern recognition, cognitive neural networks, and fuzzy logic to generate QSARs that can be used for making predictions as well as clarifying where knowledge may be lacking.

Development of a Standard Reference for Nanomaterials. On the basis of our proposed predictive paradigm for nanomaterial hazard assessment, the NSF- and EPA-funded University of California Center for the Environmental Impact of Nanotechnology (CEIN) is developing a multidisciplinary and broad-based model of predictive toxicology premised on QSARs and nanomaterial injury mechanisms at the molecular, cellular, organismal, and ecosystem levels.[39] We are establishing a limited number of standard reference and combinatorial nanomaterial libraries for materials produced in the largest volumes and therefore more likely to come into contact with the environment. We consider the physicochemical properties of these materials that allow them to spread to the environment, become bioavailable through cellular/organismal uptake, and perform biocatalytic activities that could lead to toxicity in bacteria, yeasts, algae, protozoa, mammalian cells, and a series of trophic life forms that can be studied in terrestrial, fresh water, and marine mesocosms. We use mechanisms and biological pathways of injury that can be used to perform HTS with a view to facilitate *in vivo* toxicological procedures that are cost-effective and useful for rapid screening. All of the above nanomaterial physicochemical properties and biological and toxicological data are being fed into a computerized self-learning system to establish QSARs that help with risk predictions. This model building is carried out in collaboration with multiple partners nationally and internationally.

It is important to discuss the potential shortfalls of a predictive toxicological approach. Even if a link is established between *in vitro* and *in vivo* toxicological outcomes, the generation of disease in humans or ecotoxicological scenarios is dependent on real-life exposures at toxicologically relevant doses. Fate and transport as well as exposure assessment are key ingredients that are not covered in the predictive toxicological paradigm and are required for proper risk assessment. There are also toxicological scenarios that might unfold only in the course of long-term or chronic exposures and that depends on initiation and promoter events that cannot be simulated by a one-step toxicological mechanism. An example is the oncogenic events that are required to transform chronic granulomatous peritoneal inflammation in response to asbestos fibers (and possibly MWCNTs) into a mesothelioma.[28,40] While a screening assay for the generation of frustrated phagocytosis in response to long, biopersistant, and stiff asbestos fibers may be useful to predict chronic mesothelial inflammation, this response profiling is unlikely to cover mutagenic events that lead to malignancy. This may require another predictive model such as p53 gene knockout to elucidate that aspect.[40] While false-positive *in vitro* toxicological results (*e.g.*, glass fibers and kaolin) are sorted out by subsequent *in vivo* testing, there is

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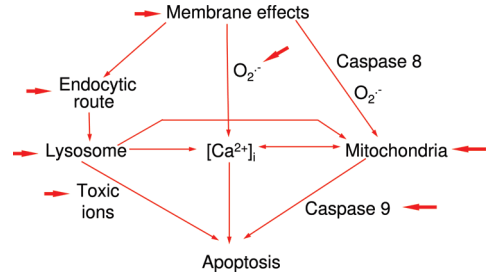

Figure 3. For *in vitro* high-throughput testing, using mechanisms of injury to formulate quantitative structure−activity relationships that incorporate hazardous nanomaterial properties, it is necessary to consider using cellular responses that are shared by a number of toxicological pathways. For instance, if the screening is developed to detect cytotoxicity, one might consider apoptosis as a final common pathway that integrates oxygen radical generation, $[Ca^{2+}]_i$ release, caspase activation, lysosomal damage, liberation of toxic metal ions, and mitochondrial perturbation. Each of these are quantifiable responses that are tied into currently known nanoparticle cytotoxic pathways. The arrows indicate some of the initiation points of cellular toxicity by nanoparticles.

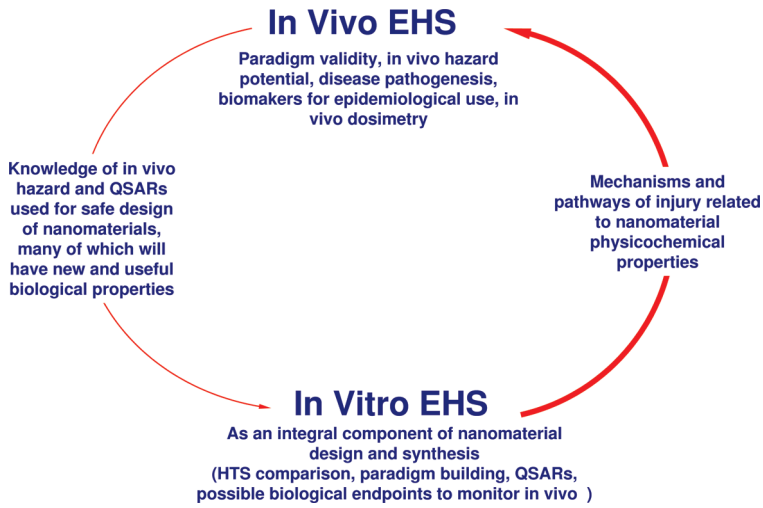

Figure 4. Ideal nano environmental health and safety paradigm will be a predictive safe paradigm where we will be able to use knowledge of the quantitative structure−activity relationships and potentially hazardous nanomaterial properties to perform safe design of nanomaterials during product research and development.

a concern about false-negative data generation *in vitro*. If a disease mechanism is involved that does not show up in the HTS, we could be faced with an unanticipated disease.[12] This is where computer-based learning, artificial intelligence, and establishment of knowledge domains could be helpful to predict additional information that is needed to study nanomaterial safety. It may also be possible to develop multistep predictive paradigms. In spite of the above pitfalls, we do not know of other substitutes that can currently generate the knowledge that will be required to grow on par with the expansion of the nanotechnology enterprise. In our opinion, the earlier we start gathering knowledge of the toxicology of nanomaterials, the more prepared we will be in dealing with the avalanche of new materials that are being developed. The ideal nano environmental health and safety (EHS) paradigm will be one in which our knowledge of potentially hazardous nanomaterial properties and QSARs will be developed to such an extent as to enable safe-design nanomaterials at the point of their conception (Figure 4). This will eliminate many of the concerns of the costs of nanomaterial hazard testing because the design of better materials will improve their market value.

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