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Nanomaterial Toxicity Testing in the 21st Century: Use of a Predictive Toxicological Approach and High-Throughput Screening

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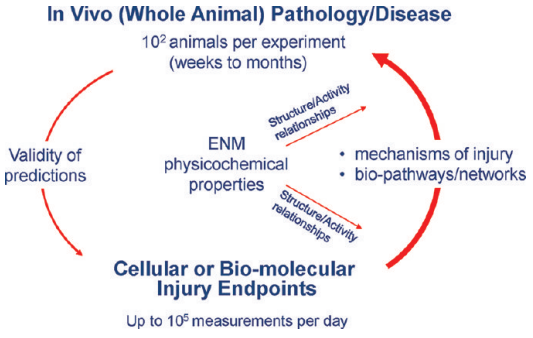
CONSPECTUS

The production of engineered nanomaterials (ENMs) is a scientific breakthrough in material design and the development of new consumer products. While the successful implementation of nanotechnology is important for the growth of the global economy, we also need to consider the possible environmental health and safety (EHS) impact as a result of the novel physicochemical properties that could generate hazardous biological outcomes. In order to assess ENM hazard, reliable and reproducible screening approaches are needed to test the basic materials as well as nanoenabled products. A platform is required to investigate the potentially endless number of biophysicochemical interactions at the nano/bio interface, in response to which we have developed a predictive toxicological approach. We define a predictive toxicological approach as the use of mechanisms-based high-throughput screening in vitro to make predictions about the physicochemical properties of ENMs that may lead to the generation of pathology or disease outcomes in vivo. The in vivo results are used to validate and improve the in vitro high-throughput screening (HTS) and to establish structure–activity relationships (SARs) that allow hazard ranking and modeling by an appropriate combination of in vitro and in vivo testing. This notion is in agreement with the landmark 2007 report from the US National Academy of Sciences, "Toxicity Testing in the 21st Century: A Vision and a Strategy" (http://www.nap.edu/catalog.php?record_id=11970), which advocates increased efficiency of toxicity testing by transitioning from qualitative, descriptive animal testing to quantitative, mechanistic, and pathway-based toxicity testing in human cells or cell lines using high-throughput approaches. Accordingly, we have implemented HTS approaches to screen compositional and combinatorial ENM libraries to develop hazard ranking and structure–activity relationships that can be used for predicting in vivo injury outcomes. This predictive approach allows the bulk of the screening analysis and high-volume data generation to be carried out in vitro, following which limited, but critical, validation studies are carried out in animals or whole organisms. Risk reduction in the exposed human or environmental populations can then focus on limiting or avoiding exposures that trigger these toxicological responses as well as implementing safer design of potentially hazardous ENMs. In this Account, we review the tools required for establishing predictive toxicology paradigms to assess inhalation and environmental toxicological scenarios through the use of compositional and combinatorial ENM libraries, mechanism-based HTS assays, hazard ranking, and development of nano-SARs. We will discuss the major injury paradigms that have emerged based on specific ENM properties, as well as describing the safer design of ZnO nanoparticles based on characterization of dissolution chemistry as a major predictor of toxicity.



Introduction

There is considerable debate about how to proceed with ENM toxicity testing, with the major discussion points centering around which toxicological endpoints to assess, the rigor of the screening effort, the correct balance of in vitro (cellular and molecular) versus in vivo (animal or whole

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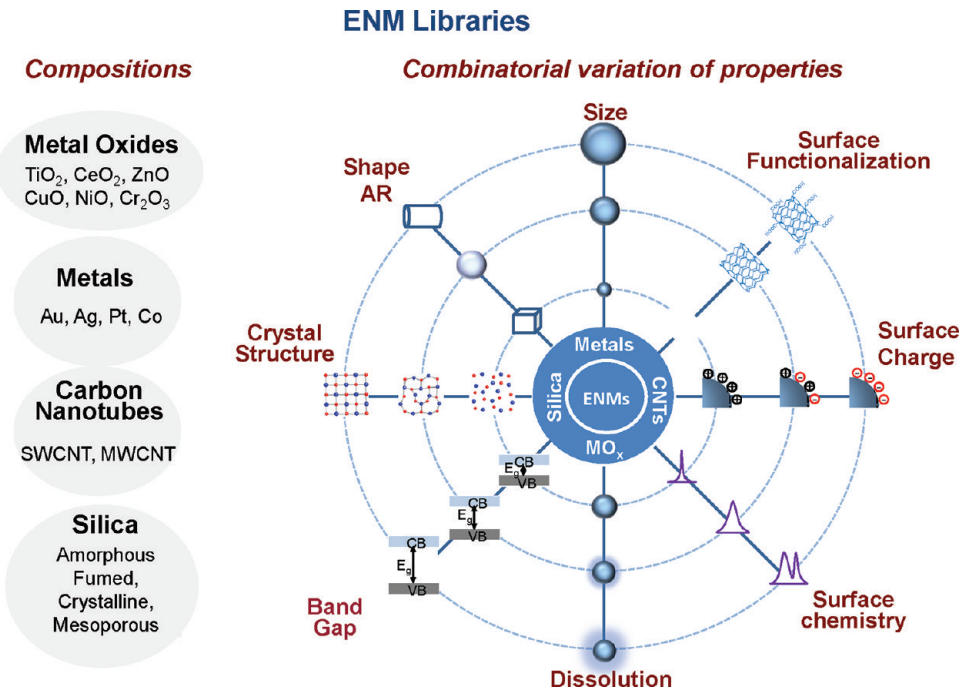

FIGURE 1. Use of compositional and combinatorial ENM libraries, including metals, metal oxides, carbon nanotubes, and silica-based nanomaterials, to perform mechanism-based toxicological screening that links material composition and systematic variation of specific properties to biological outcome.

organism) testing, the cost of the effort, and who should be responsible for overseeing nano EHS governance.[1−3] Therefore, a platform is required to investigate the potential vast number of biophysicochemical interactions at the nano/bio interface, and the approach implemented by the UC Center for the Environmental Implications of Nanotechnology (UC CEIN) as well as the UCLA Center for NanoBiology and Predictive Toxicology (UCLA CNPT) is to assess molecular and cellular injury mechanisms that can predict environmental and pulmonary hazard potential, respectively.

Key Ingredients for Developing Predictive Toxicology Through HTS Approaches

In the absence of standard reference materials for nano safety analysis, we implemented our own in-house synthesized and acquired compositional and combinatorial ENM libraries for high content data generation. Chemical composition is important because it determines the surface chemistry, surface energy, redox status, photoactivation potential, surface defects, charge, and surface groups that define "surface reactivity". Material chemistry and surface coatings also determine the wettability, suspension stability, solubility, shedding of toxic ions, and durability as key determinants of hazard generation. Although nano size and the accompanying increase in surface area contribute to surface reactivity, other physicochemical properties (e.g., dimension, shape, state of agglomeration, crystal structure, surface charge, and surface coating) are important modifiers of the effects of surface reactivity and composition. Thus, ENM toxicity can be attributed to relatively nonspecific biological responses to material size, shape, and biopersistance but could also result from specific biological interactions generated from a reactive surface, ligands, or release of toxic ions that impact specific toxicological pathways.[4] In light of this complexity, we have acquired well-characterized compositional libraries that include metals, metal oxides (MO_x), carbon nanotubes (CNTs), and different silicas to obtain mechanistic information that could be used for HTS, hazard ranking, and animal validation studies. Moreover, with the identification of potentially hazardous properties by different materials compositions, we have also introduced combinatorial ENM libraries in which specific properties are systematically changed to investigate quantitative SARs (Figure 1).

To fulfill the first key ingredient of having a well-characterized nanoparticle library, we initially synthesized a small MO_x library in which the physicochemical properties of TiO_2, CeO_2, and ZnO nanospheres were rigorously characterized before implementation of single-parameter toxicological screening looking at cytotoxicity, oxygen radical generation, or pro-inflammatory responses in cells.[5] Collectively, these assays demonstrated a high level of toxicity for

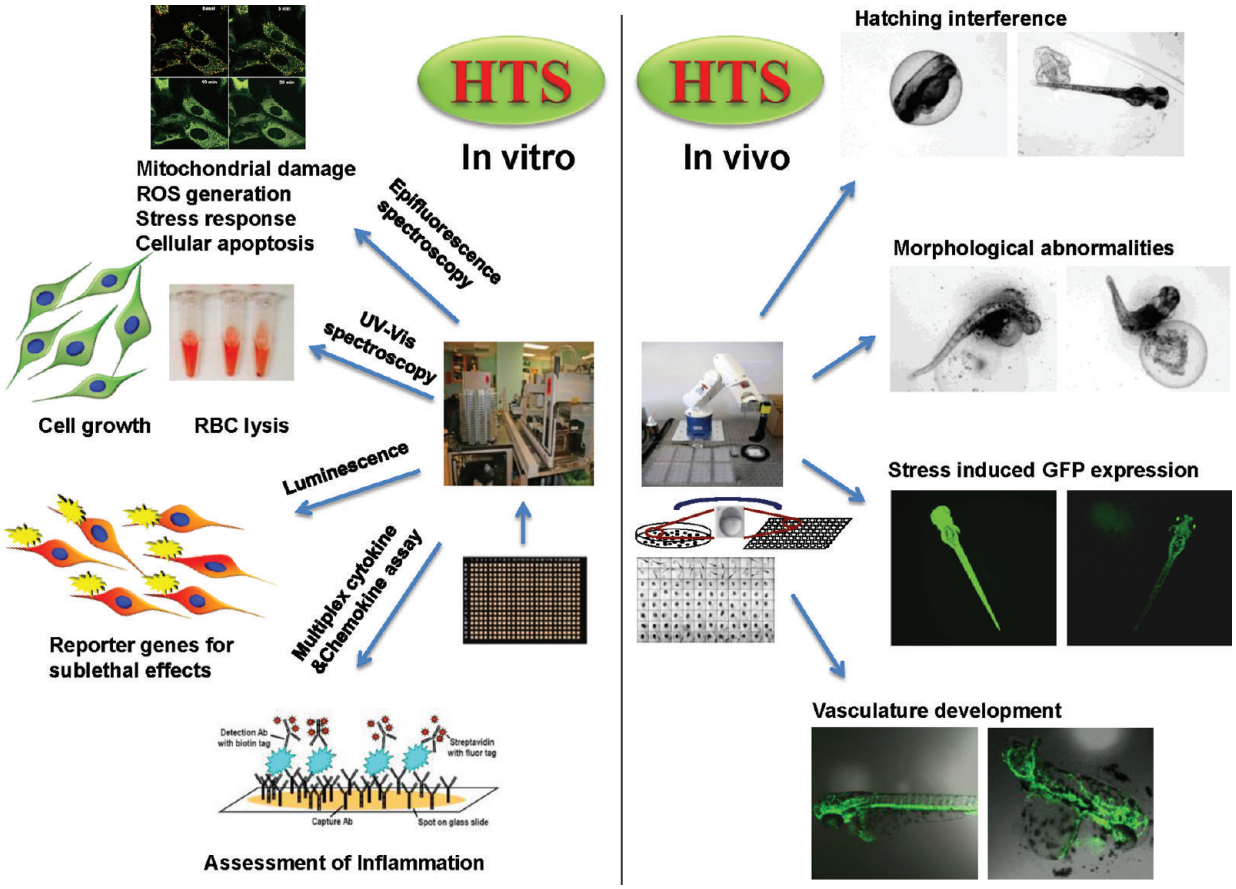

FIGURE 2. Examples of in vitro and in vivo HTS assays and response readouts in cells and zebrafish embryos.

ZnO but not TiO_2 and CeO_2 in mammalian cells.[5] This toxicity is the result of ZnO nanoparticle dissolution leading to the release of toxic Zn^{2+}, capable of generating reactive oxygen species (ROS), mitochondrial perturbation, IL-8, IL-6, and TNFα production, and induction of cytotoxicity. Many of these cellular injury responses are recapitulated by the acute pro-oxidative and pro-inflammatory effects of nano-ZnO in the rodent lung.[6] Moreover, the same injury pathways are triggered in humans during the development of metal fume fever, an occupational disease in welders. On the basis of the involvement of oxidative stress as a key mechanism of ENM toxicity, we developed a multiparametric HTS assay to track lethal and sublethal oxidative stress responses to MO_x.[7] This assay, which will be discussed in more detail later, allowed us to increase the number of materials that can be screened, including the performance of toxicological assessment of materials that generate ROS secondary to afferent injury mechanisms. This also allowed us to establish nano-SARs for metal and metal oxide nanoparticles, as well as the introduction of combinatorial libraries to assess the contribution of particle size, dissolution, shape, aspect ratio, and material bandgap (Figure 1). A similar approach was used to develop a predictive toxicological paradigm for the pro-fibrogenic effect multiwall CNTs (MWCNTs), starting with the raw (as-purchased) material and then introducing purified and carboxyl-functionalized tubes.[8,9] We also developed compositional and property-based libraries to study silica and nano-Ag toxicity. Silica ENM hazard is discussed in an independent contribution by Professors Brinker and Zink in this special edition.

The second infrastructure requirement for a predictive toxicological approach is the development of appropriate HTS approaches to quantitatively assess dose- and time-dependent cellular injury responses that are predictive of in vivo adverse outcomes (Figure 2). Biological, medical, pharmaceutical, and toxicological research has illustrated how a systems biology approach can be used for HTS. Although the current approach to the risk assessment of toxic substances (such as chemicals) is heavily reliant on apical adverse health effects in animal models, a description of adverse health outcomes does not provide a robust science-based platform for performance of predictive toxicological modeling. Although it is plausible that new screening paradigms could be discovered by proteomics or genomics-based

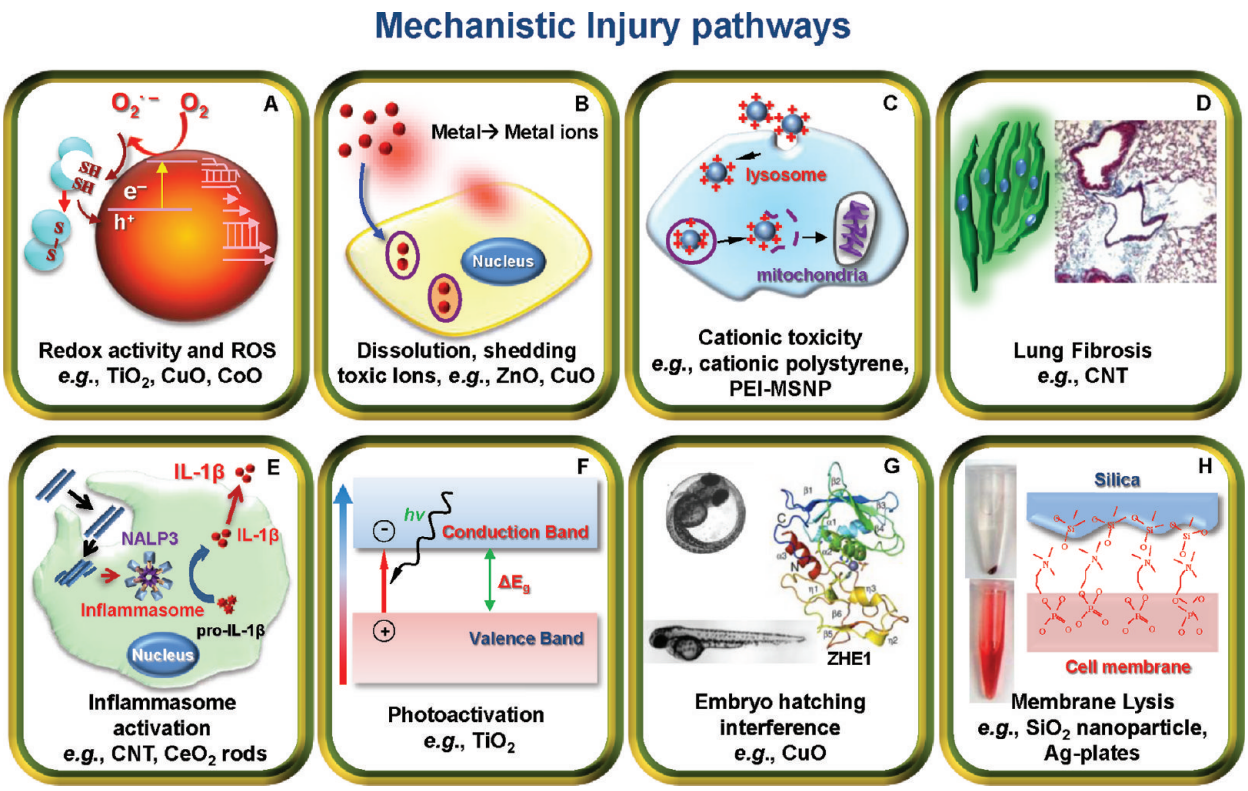

FIGURE 3. Examples of mechanistic injury responses for HTS. A, Oxidative stress; B, dissolution and release of toxic metal ions; C, cationic injury to surface membrane and organelles; D, pro-fibrogenic responses to CNT; E, inflammasome activation by long-aspect-ratio materials; F, photoactivation and influence of bandgap; G, Zebrafish embryo hatching interference; H, cell membrane lysis by surface reactivity.

approaches, the high data volume requires data-reduction and pathway-based approaches before the discovery can be used for HTS development. The most expeditious platform currently is the use of mechanistic cellular injury responses that can be linked to disease pathogenesis (Figure 3). Quantitative assessment of these toxicological pathways allows HTS to be used for in vivo prediction making, thereby limiting animal use as the primary test platform. Although the predictive potential of in vitro-to-in vivo comparisons is still in its infancy, a number of cellular injury-response mechanisms have emerged that can be quantitatively implemented to assess hazardous ENM properties (Figure 3). A few of these toxicological mechanisms have allowed us to establish our first generation of nano-SARs for toxicological modeling. Although to date most nano-SARs are premised on known toxicological pathways, it is likely that discovery platforms like proteomics and genomics will provide new screening paradigms in the future.

Considering be third key ingredient, namely, the use of mechanistic injury pathways, one of the best HTS examples that have emerged from our studies is screening for biological ROS production and oxidative stress in response to the bandgap of MO_x nanoparticles, electron/hole pair generation, surface defects, chemical dissolution, and release of toxic metal ions, etc. (Figure 4). We used a cocktail of compatible fluorescent dyes to develop an epifluorescence-based approach that screens for a functionally connected series of sublethal and lethal oxidant injury responses in cells (Figure 4 and 5).[7] This multiparameter HTS assay, which is performed by robotic handling of 384-well plates, contemporaneously assesses total cell number and nuclear size (using the DNA intercalating dye, Hoechst 33342), mitochondrial membrane potential (MMP, using 5,5′,6,6′-tetrachloro-1,1′,3,3′ tetraethylbenzimidazolylcarbocyanine iodide, aka JC-1), intracellular calcium flux (using Fluo-4), and increased membrane permeability in dying cells (using propidium iodide (PI).[7] An example of the triggering of multiparameter responses at different time points and over a wide range of ENM doses is illustrated in Figure 5. The functional link between these cellular responses is explained in Figure 4, which demonstrates that the assay is also capable of assessing ENMs that generate ROS secondary to surface membrane injury or organellar damage. Although similar hazard ranking could be obtained in single-parameter assays (e.g., MTS, LDH release, or cellular

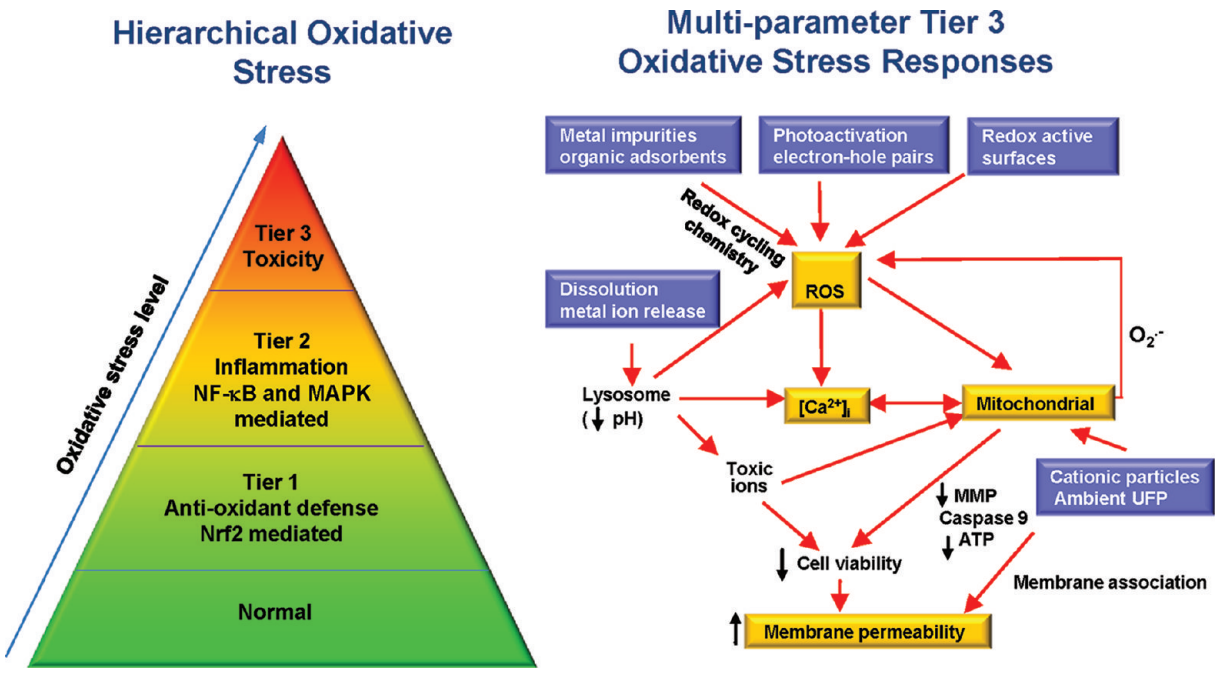

FIGURE 4. Cellular biology of oxidative stress and implementation of a multiparametric HTS assay premised on tier 3 oxidative stress parameters. The right-side panel provides examples of specific material properties that are capable of engaging a series of interrelated response parameters.

ATP content), the high-volume, time-related HTS data sets allowed us to develop machine-learning and hazard-ranking tools that are capable of establishing nano-SARs. In addition to the oxidative stress, other injury mechanisms have emerged for HTS, including inflammasome activation by long-aspect-ratio materials, cytokine and chemokine production, photoactivation, membrane injury (in response to cationic or reactive material surfaces), pro-fibrogenic responses, transcriptional activation of reporter genes by oxidative stress signaling pathways or DNA damage, hatching enzyme inhibition, and transgenic heat shock protein expression in zebrafish embryos (Figure 3).[6,8−12] All of the above responses can be developed into automated, high-throughput screening through readout procedure such as epifluorescence microscopy, luminescence-based reporter gene activity, membrane lysis assays, multiplex cytokine assays, brightfield microscopy, and fluorescence microscopy (Figure 2). In addition to mammalian cellular screens, we have also implemented HTS screening in a fish gill cell line, bacteria, yeasts, and whole organisms. The zebrafish screening platform will be discussed later.

The fourth requirement for a predictive paradigm is the development of a data analysis framework that includes in silico tools for data analysis, data transformation, machine learning, and modeling of HTS data (Figure 6). Because this area of expertise will be dealt with in an accompanying manuscript by Professors Cohen and Rallo in this edition, it is sufficient to mention that, because HTS data are highly dimensional, we use dimension-reduction (or feature-extraction) methods to project the data onto lower-dimensional feature spaces for visual interpretation. One visual approach is cluster analysis to extract similarity patterns and perform ENM grouping based on homologous biological responses or linkage to physicochemical properties.[13] Heat-map clustering plays an important role in HTS data analysis.[7,12] Heat maps provide ordered representations of data that facilitate the identification of similarity patterns of large data sets. A complementary approach is the use of topological preservation methods that are capable of maintaining, in the projected space, the distances measured in the original feature space. The self-organizing map (SOM) algorithm provides a valuable computational tool for data transformation.[14] Compared with heat maps, SOM projections facilitate the discovery of imbedded relationships between variables by providing topologically ordered representations of the input space. The clustering of the data can be visualized over the SOM grid by displaying the distances between cluster centers (distance matrices) or by projecting features onto the different component planes that allow for a more detailed evaluation of the parameters in these planes.[13,14] QSARs have been defined as statistical models that relate structural or property descriptors of a chemical to its biological activity. Relative to the chemical world, where SARs can be developed based on the availability of large

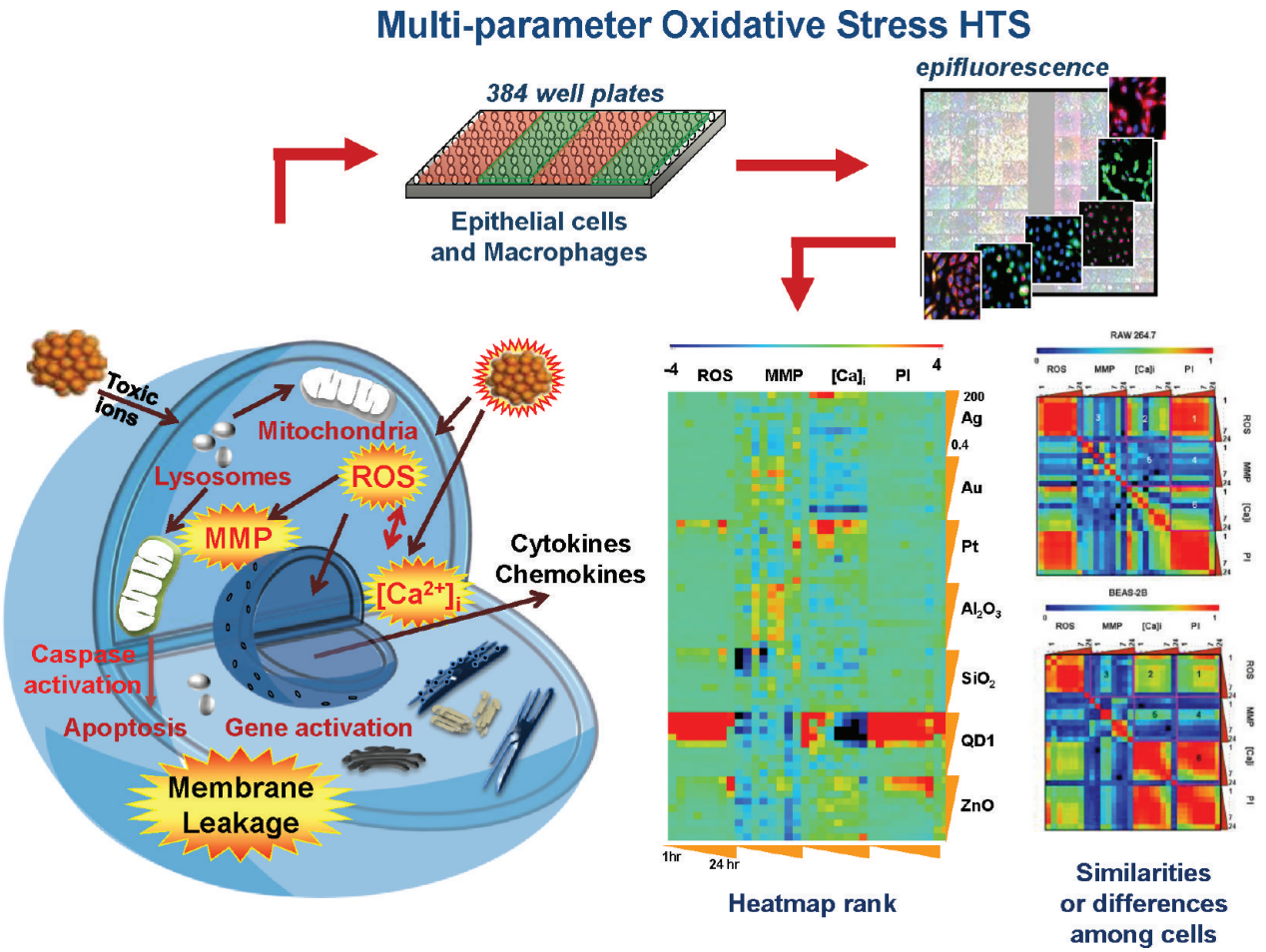

FIGURE 5. Multiparametric HTS analysis by automated epifluorescence microscopy, showing that screening at 10 different concentrations and at multiple time points (1−7 and 24 h) can be used for hazard ranking by heatmaps and similarity analysis.

databases for both physicochemical properties and toxicology, nano-SAR development entails a different approach that requires even larger data sets that need to consider the 3D composition of ENMs. Our HTS assays now provide data in a multivariate context (e.g., concentration, exposure times, and sublethal and lethal biological responses), allowing us to develop a first generation of nano-SARs that supplement those in the literature, most of which rely on the physicochemical properties of nanoparticles.

Use of Predictive Toxicology to Understand ENM Pulmonary Hazard

Inhalation toxicology is the study of pulmonary injury as a result of the inhalation of toxic fumes, gases, particles, and fibers. The lung is an important target organ in the occupational exposures that could follow the aerosolization of ENMs during their synthesis and handling. The study of ENM toxicity in the lung is particularly challenging from the perspective that it is difficult and expensive to simulate real-life inhalation exposures. Although there is evidence that incidental occupational exposures to ZnO and quartz particles can cause metal fume fever and pulmonary fibrosis in humans, respectively, we do not have a comprehensive understanding of how to perform a pulmonary hazard ranking of large series of ENMs. Moreover, the labor intensity and high cost of performing aerosolized inhalation for each new material is not logistically feasible. Thus, inhalation toxicology could benefit from a predictive toxicological approach, toward which we outline two scenarios.

The first example is the development of a predictive paradigm for MO_x nanoparticles, which are widely used in cosmetics, sunscreens, catalysts, textiles, and solar batteries. Many of these materials are produced as powders that can be inhaled and are known in their dissolved states to pose some hazard due to the redox activity or shedding of toxic metal ions. In the initial exploration of three MO_x nanoparticles, ZnO stood apart from TiO_2 and CeO_2 nanospheres for its ability to generate intense pulmonary inflammation as a

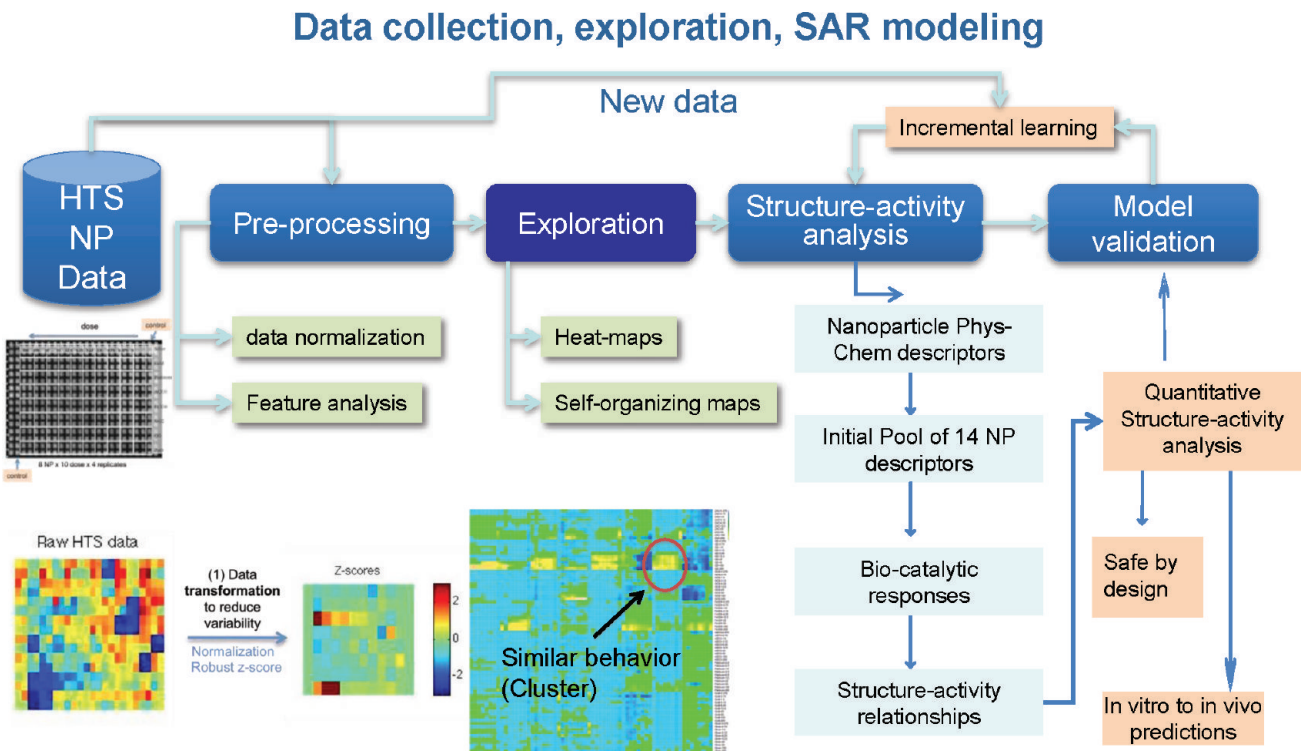

FIGURE 6. HTS data analysis framework. The data are preprocessed by normalization and feature analysis tools and then used to generate nano-QSARs according to property-based descriptors. The diagram shows how a metal and MO_x library was used with a pool of 14 nanoparticles descriptors that yielded four descriptors that could accurately classify materials as toxic and nontoxic (Figure 8). In vitro predictions are validated by inhalation toxicology studies in rodents (Figure 11) and can also be used for safer design (Figure 12).

result of dissolution and induction of a characteristic series of cytokines and chemokines that are also produced in bronchial epithelial cells and macrophages.[5] From a mechanistic perspective, intracellular release of Zn^{2+} induces oxidative stress, which is the basis for the multiparametric assay described above.[7] The induction of oxidative stress in the lung as well as systemically by dissolving ZnO particles can be imaged in live mice, using transgenic expression of the heme oxygenase 1 (HO-1) gene promoter linked to a luciferase reporter (Figure 7). With the exception of relatively few MO_x, the pulmonary hazard potential of most these materials has not been systematically explored. Following the introduction of the multiparametric HTS assay, it is now possible to develop SARs for hazard ranking in vivo and prediction making. As an example, we have used a logistic regression model to develop a nano-SAR capable of estimating the probability of nine MO_x nanoparticles being toxic or nontoxic based on multiparametric data.[15] Thus, the screening of Al_2O_3, SiO_2, Fe_3O_4, CeO_2, Co_3O_4, TiO_2, ZnO, CuO, and WO_3 nanoparticles in bronchial epithelial cells at concentrations of 0.375–200 μg/mL, hourly for 7 as well as 24 h, established a decision boundary according to which 4 of 14 material reactivity descriptors achieved a 100% classification accuracy.[13] According to this classification, the enthalpy to separate the MO_x atoms (atomization energy), the periodic table number of the metal, the nanoparticle volume (in solution), and the primary particle size were deemed accurate descriptors of the particles' ability to induce toxic oxidative stress (Figure 8). MO_x-induced oxidative stress predicts inflammogenic potential in the lung.

Recently, a conceptual framework was suggested in which the conduction (E_c) and valence band energies (E_v) of MO_x could be reactivity descriptors to develop a theoretical framework that explains the generation of oxidative stress when these particles come into contact with cells. One possibility is that the overlap of the E_c and E_v with biological redox potential of cells could constitute the basis for electron transfers that predicts the MO_x's oxidative stress potential. The cellular redox potential is under homeostatic regulation by a series of redox couples (Figure 8) that maintain the potential at −4.12 to −4.84 eV. Oxidizing or reducing substances could disturb this redox equilibrium by increasing ROS production and/or decreasing antioxidant levels. The immediate response is to trigger an Nrf2-mediated antioxidant defense at the lowest level (tier 1) of oxidative stress to restore the redox disequilibrium (Figure 4). HO-1 expression

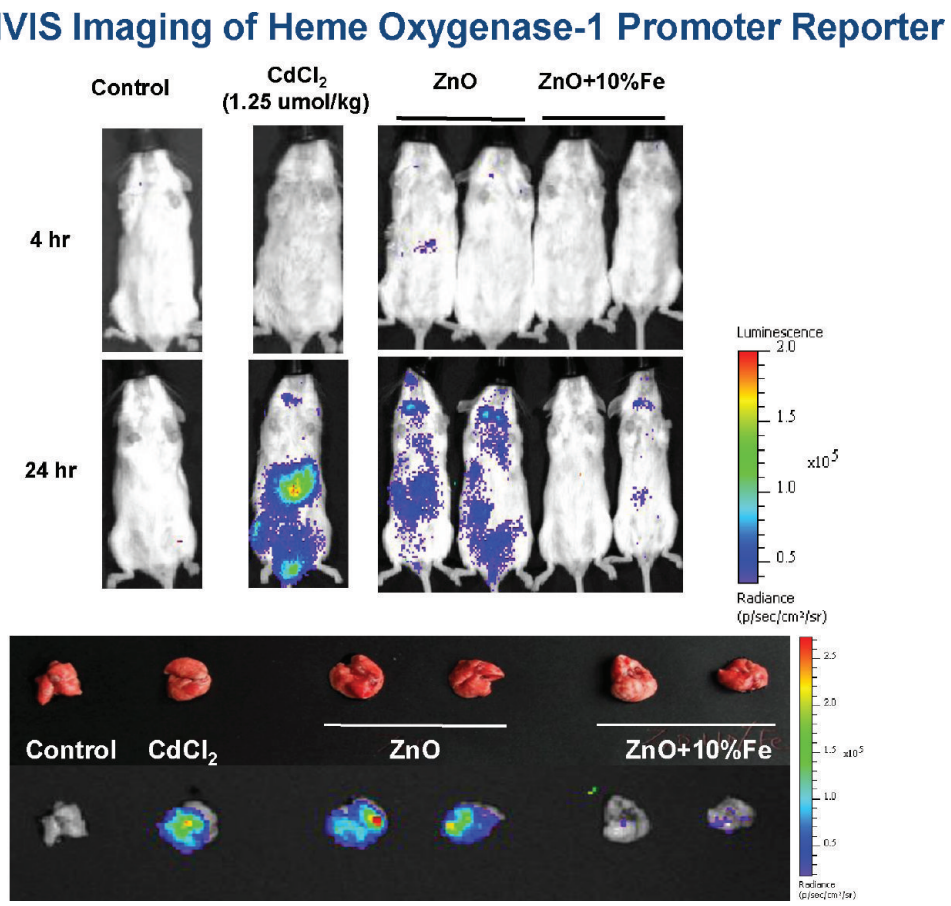

FIGURE 7. Live animal imaging to demonstrate ZnO-induced oxidative stress in the lung. Transgenic mice expressing the luciferase (luc) gene controlled by the heme oxygenase-1 (HO-1) promoter were used for live imaging of animals receiving oropharyngeal aspiration of undoped and Fe-doped ZnO as well as $CdCl_2$ (positive control). The IVIS image shows undoped ZnO-induced strong signals in the lung, liver, and intestines while Fe-doping leads to signal reduction. Subsequent sacrifice and ex vivo imaging of the lungs confirmed the in vivo findings.

is an example of a tier 1 response (Figure 7).[4] If this defense fails, the escalation of oxidative stress could shift the cellular response to activate pro-inflammatory signaling pathways (tier 2) or mitochondrial-mediated cytotoxicity (tier 3) (Figure 4). On the basis of our hypothesis that the relationship of bandgap energy to biological redox potential constitutes one of the parameters for predicting MO_x hazard potential, we developed a nano-SAR that projects the overlap of theoretical and experimentally determined E_c and E_v values with cellular redox potential for MO_x (Figure 8).[16] In an initial modeling exercise using 24 MO_x nanoparticles, we have been able to show that, for 5/7 materials showing bandgap overlap, there is an accurate prediction of their ability to generate toxic oxidative stress in the multiparameter assay (Figure 8). Moreover, our preliminary results indicate that the materials with bandgap overlap and ability to generate oxidative stress (e.g., Co_3O_4, Cr_2O_3, Mn_2O_3, and CoO) also induce high levels of acute pulmonary inflammation compared to materials that do not show bandgap overlap.[16] We expect that this will lead to nano-SARs that will allow us to classify MO_x nanoparticles into materials with low, medium, or high toxicological potential in the lung. However, we need to explain why TiO_2, which shows bandgap overlap but no HTS toxicity, and ZnO, which does not show bandgap overlap but does show toxicity, do not follow the bandgap hypothesis. It may be necessary to introduce solvation energy and photoactivation activity to develop a more complete picture.

The second predictive toxicological paradigm for pulmonary toxicology was developed for multiwall carbon nanotubes (MWCNTs). A number of tube characteristics determine the induction of chronic granulomatous inflammation, fibrosis, genotoxicity, cytotoxicity, and oxidative stress in the lung by single-wall and MWCNTs. This includes properties such as the length of the tubes or stacks, hydrophobicity, state of agglomeration, metal impurities, durability, and surface chemistry. Collectively, these properties are difficult to study in isolation when developing SARs.

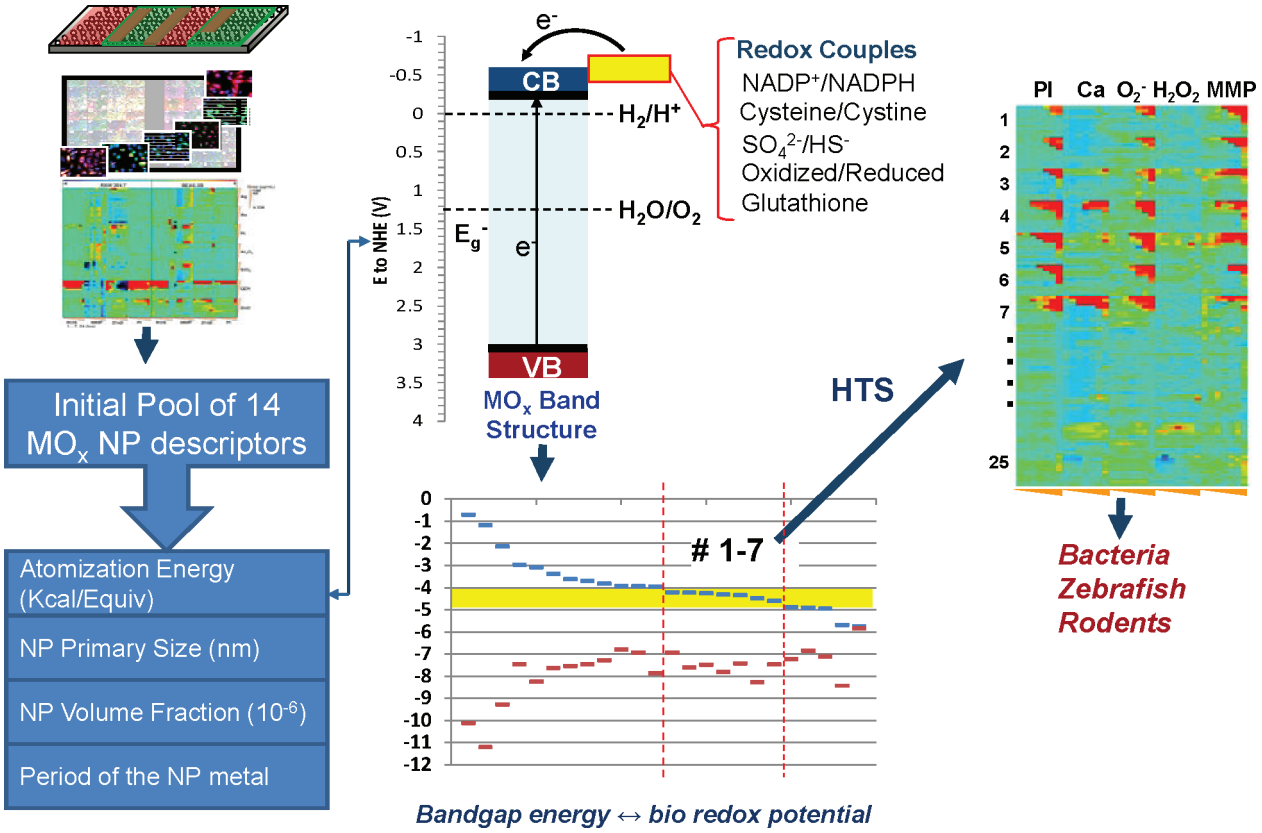

FIGURE 8. Correlation of MO_x toxicity to bandgap energy structure. The conduction band (CB, blue bar) and valence band (VB, red bar) energy of metal oxide nanoparticles are experimentally determined and compared to biological redox potential (zone highlighted in yellow). CB overlap with the biological redox potential could facilitate electron transfer to the redox couples that collectively determine the cellular redox potential. The disruption of the cellular redox equilibrium results in oxidative stress as defined by the multiparametric HTS assay.

One major advance was the development of a quantitative dispersal method for studying pro-inflammatory responses in tissue culture cells that are also relevant to fibrosis potential in the lung (Figure 9).[8,18] The dispersal was accomplished by using bovine serum albumin (BSA) and dipalmitoylphosphatidylcholine (DPPC) as surrogate lung lining fluid components capable of dispersing MWCNTs in a quantitative fashion.[8] Development of a quantitative dispersal index allowed us to perform a series of cellular assays that predict fibrogenic potential and pulmonary fibrosis induced by CNTs. We have recently demonstrated that the dispersal state of raw ("as-prepared" or AP), purified (PD), and carboxylated (COOH) MWCNTs play an important role in determining TGF-β1, PDGF-AA, and IL-1β production in vitro and in vivo. These biomarkers act synergistically in cell–cell communication and collagen deposition in the lung. Epithelial transition to a mesenchymal and pro-fibroblastic phenotype, in cooperation and under the influence of IL-1β production in macrophages, plays a role in collagen deposition in various disease states.[9] The effect of MWCNT dispersion in impacting these responses was most noticeable for AP- and PD-MWCNTs, which are more hydrophobic and unstable in aqueous buffers than the hydrophilic COOH-MWCNTs.[9] Well-dispersed AP- and PD-MWCNTs were readily taken up by epithelial cells and alveolar macrophages (AM) and induced more prominent TGF-β1 and IL-1β production in vitro, as well as TGF-β1, IL-1β, and PDGF-AA production in vivo, than nondispersed tubes. The excellent correlation between in vitro and in vivo biomarkers confirmed their utility for predicting the pulmonary fibrosis potential of MWCNTs. Because IL-1β production is mechanistically dependent on assembly of the NALP3 inflammasome to cleave pro-IL-1β, this presents a robust mechanistic paradigm for high-throughput discovery and development of nano-SARs that provide toxicological predictions based on the hydrophobicity, suspension stability, state of purity, and surface functionalization of CNTs. Our preliminary data indicate that tube dispersal by BSA plus DPPC enhances cellular uptake and lysosomal injury, leading to inflammasome activation.[18] Communication between the lysosome and inflammasome could constitute a final common pathway for predicting the pulmonary fibrosis potential of other

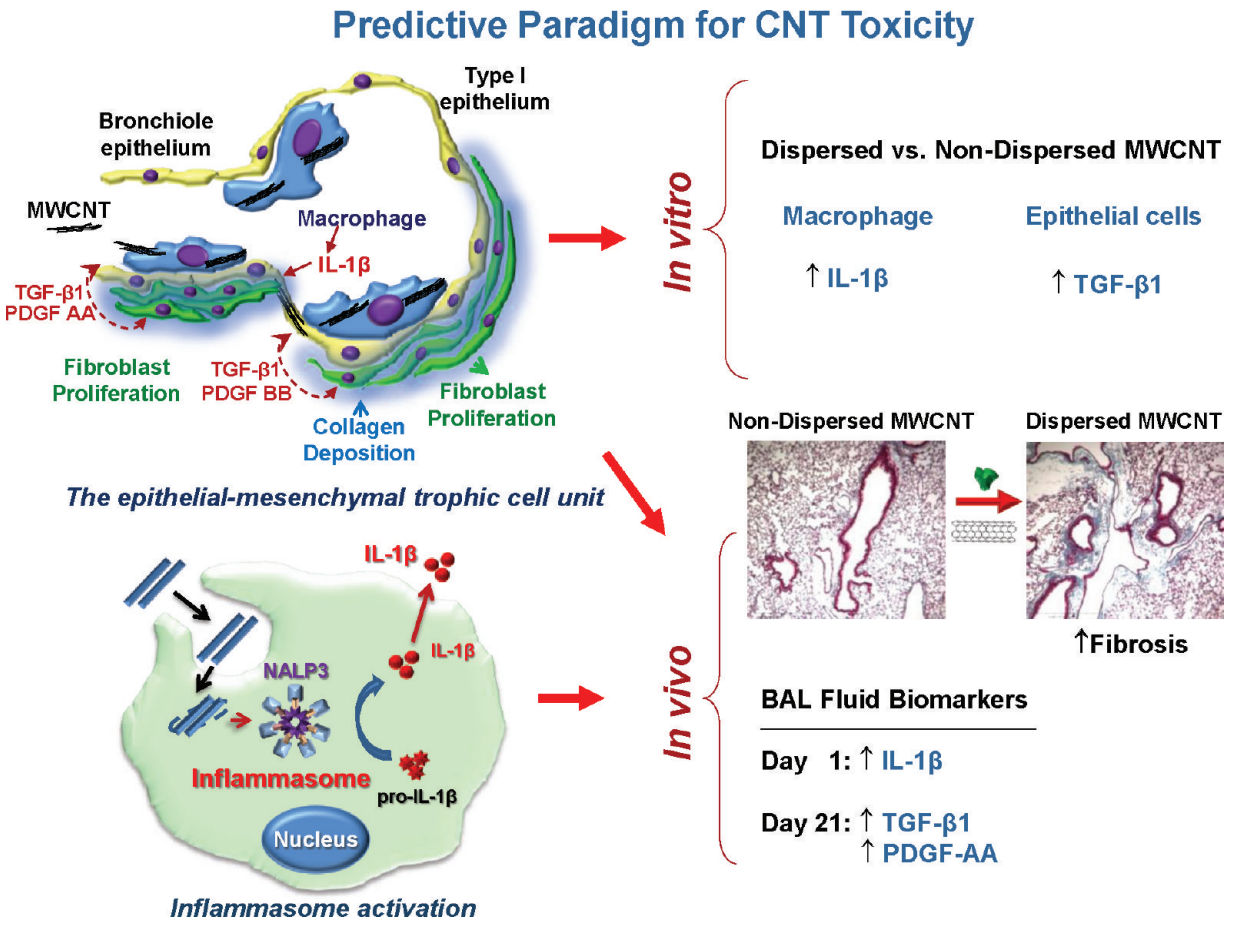

FIGURE 9. Predictive toxicological paradigm for fibrogenic effects of MWCNTs. Cellular assays were developed based on the synergistic cellular responses in the epithelial-mesenchymal trophic cell unit, which are predictive of the fibrogenic potential of MWCNTs in vivo. Well-dispersed MWCNTs induced more prominent pro-fibrogenic responses in vitro as well as in vivo than nondispersed tubes.

long-aspect-ratio materials, including MO_x. We are developing multiplex high-throughput assays for the cytokines and growth factors that predict the pulmonary hazard potential of CNTs, MO_x nanowires, and Ag nanowires (in progress).

Use of Predictive Toxicology to Understand Environmental Hazard Potential

Although nanotechnology is used for environmental cleanup, some ENMs could pose an environmental hazard potential. The mission of the UC CEIN is to develop a broad-based predictive scientific model to understand how the physicochemical properties of ENMs determine the materials' spread to the environment, bioaccumulation, trophic transfer, and catalysis of potentially hazardous outcomes at cellular, organism, and ecosystem levels (Figure 10).[17] In accordance with our predictive toxicological approach, this multidisciplinary Center makes use of well-characterized compositional and combinatorial ENM libraries to study these materials' potential to engage toxicological pathways in cells, bacteria, and environmental organisms based on quantifiable biophysicochemical interactions at the nanobio interface. Where possible, we use high-throughput approaches to establish SARs for prediction-making of primary ENMs' impact at different trophic levels in freshwater, seawater, and terrestrial ecosystems. In silico data transformation and decision-making tools are involved in data integration to provide hazard ranking, exposure modeling, risk profiling, and construction of quantitative nano-SARs.[17]

Toxicity testing in fish is recognized as a gold standard for ecotoxicology. Zebrafish, which has been used for modeling of biochemical and molecular disease mechanisms in humans, is a promising model for environmental risk assessment, including toxicological assessment of ENMs. Because of the embryo's transparency and well-characterized developmental stages, the zebrafish presents an excellent opportunity to develop high-content screening. Accordingly, we have developed an HTS approach to assess hatching interference by ZnO nanoparticles.[6] ZnO nanoparticle dissolution interferes with the hatching enzyme, ZHE1, which contains

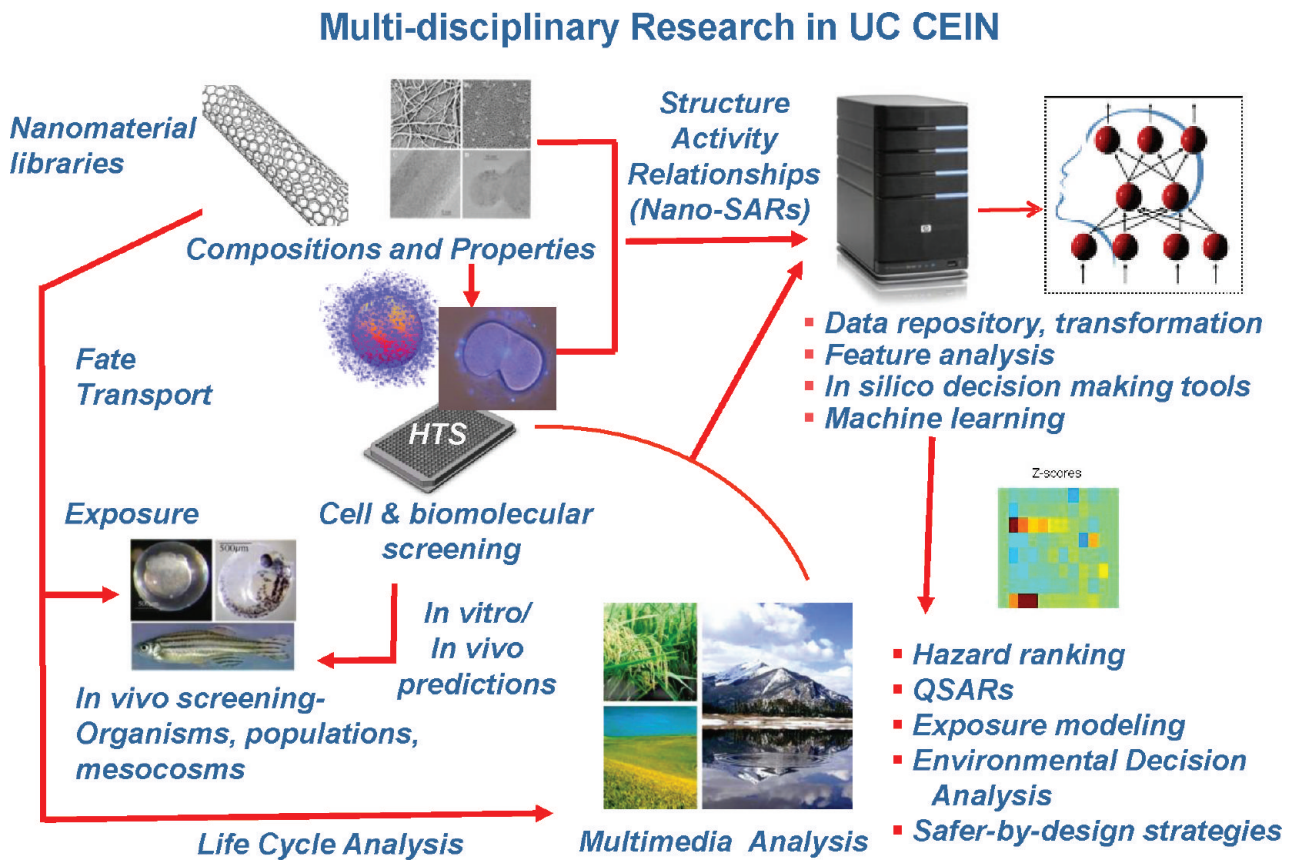

FIGURE 10. Multidisciplinary research utilizing a predictive toxicological approach, including ENM libraries, HTS, ecosystem toxicity, and computational predictions to facilitate the safe implementation of nanotechnology in the environment in UC CEIN.

three histidines in the active center that are ligated by Zn^{2+}. It has been proposed that for different metals this constitutes a hierarchical affinity binding series that controls metalloprotease activity in the following order: Mn(II) < Fe(II) < Co(II) < Ni(II) < Cu(II) > Zn(II).[10] Accordingly, we have used the zebrafish to develop a predictive toxicological paradigm that explains hatching interference by metal and MO_x nanoparticle libraries (Figure 2). A pilot study demonstrated that there is good correlation between the hazard ranking in our multiparameter HTS assay and the toxicological outcomes in zebrafish embryos.[15] One exception was nano-Ag, which was clearly more toxic in the embryos than to mammalian cells, suggesting species differences in the nano-Ag susceptibility. Moreover, while the hatching interference by MO_x nanoparticles (e.g., CuO, ZnO, and NiO) could be reversed by diethylene triamine pentaacetic acid (DTPA), this chelator did not reverse the nano-Ag effect.[10] Nano-Ag induces a high rate of morphological abnormalities (e.g., pericardial edema, bent spines, and tail malformations) that were not observed for the transition MO_x. These data suggest differences in the mechanism of action of Ag and the transition metals. Although we suspect that nano-Ag hatching interference is secondary to its impact on developmental pathways, the transition metals appear to target ZHE1 directly. We have produced recombinant ZHE1 enzyme to perform an abiotic assay with a cleavable substrate; we hope to use this to develop a SAR that can be used to predict MO_x toxicity in zebrafish embryos.

During the toxicity screening of the aforementioned materials, it was necessary to perform a visual examination of each embryo under a dissecting microscope. This approach is labor-intensive and limits the number of observations and statistical analyses that can be obtained. Thus, we developed a HTS approach that makes use of pick-and-plate robotics to speed up embryo placement in 96-well plates to increase the number of observations (Figure 2). In addition, we implemented two high-content platforms for image capture, allowing retroactive analysis and scoring of the data.[10] The first of these platforms utilizes bright-field image analysis, capable of capturing phenotypic and developmental abnormalities in embryos and larvae. The second, fluorescence-based platform, captures reporter gene expression in transgenic animals, including stress responses such as a heat shock protein (HSP) expression, using the *hsp70*

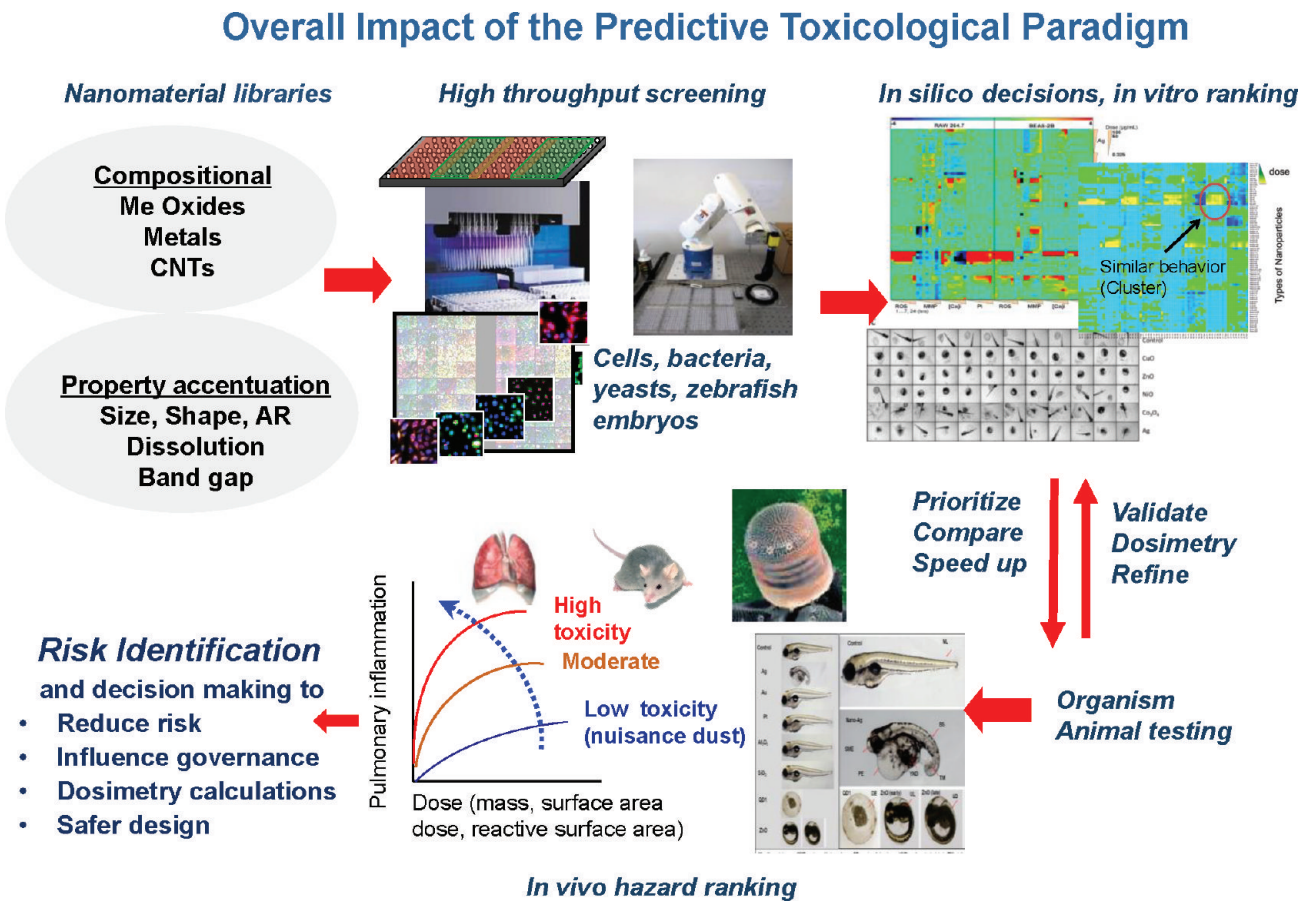

FIGURE 11. Wider implementations of predictive toxicological profiling through the use of ENM libraries, high-throughput hazard ranking, and SARs. The predictive approach assists the logistical planning and execution of costly animal studies that are often required for regulatory decision-making. Once a prediction is established, most analyses can be carried out in vitro.

promoter fused to green fluorescent protein (e.g., *hsp70*:eGFP) reporter.[10] Our collaborators in the UC CEIN and UCLA CNPT have also developed a phenotype-recognition system that allows rapid image scoring based on vectorial descriptors.[19] This technical innovation allows HTS of up to 24 MO_x in one experiment, and we are utilizing these data together with inductively coupled plasma mass spectrometry (ICP-MS) analysis and the recombinant ZHE1 assay to develop a nano-SAR for hatching interference based on metal periodicity and dissolution characteristics. This innovation will allow comprehensive dose–response analysis of materials with proven toxicological effects, with the ability to extrapolate the findings to environmental exposure levels. Interestingly, the induction of *hsp70*:eGFP expression in transgenic zebrafish larvae requires a log-fold higher concentration of nano-CuO and -ZnO than does that for hatching interference.[10]

To study the species differences for nano-Ag toxicity, we have introduced a rainbow trout gill epithelial cell line (RT-W1) to perform a comparative multiparametric HTS analysis of oxidative stress responses to nanosize Ag spheres, plates, and wires.[20] We also compared the results to toxicity outcomes in zebrafish embryos. Nano-Ag is clearly more toxic in RT-W1 than in mammalian epithelial cells. Interestingly, Ag nanoplates were considerably more toxic than spheres and wires when performing a multiparametric HTS assay in RT-W1. The same trend was observed in studying embryo lethality and hatching interference in zebrafish embryos. Although Ag ion shedding and bioavailability in cells and embryos could explain the effect of Ag spheres, the increased toxicity of the nanoplates is dependent on direct cellular and embryo contact. To explain the high surface reactivity of Ag nanoplates, high-resolution transmission electron microscopy (TEM) demonstrated the presence of stacking faults and point defects on the plates' surfaces, which were absent in the Ag spheres. Surface coating with cysteine could reduce the surface reactivity, with a reduction in cellular as well as embryo toxicity. This study demonstrates the importance of crystal defects in nanoparticle toxicity in addition to the established roles of Ag ion release in the toxicity of Ag nanospheres. The excellent correlation

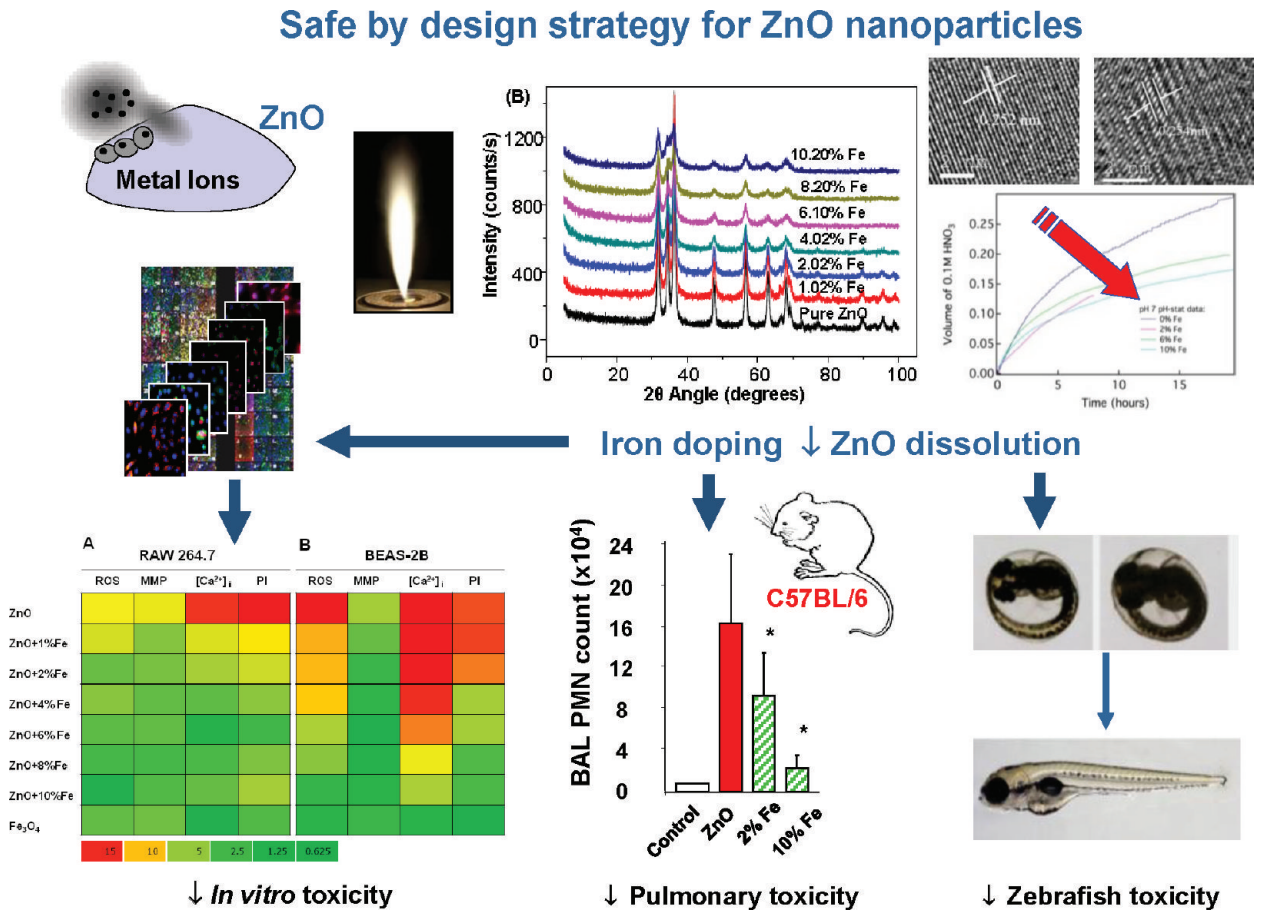

FIGURE 12. Use of Fe-doping as a safe-by-design strategy for ZnO nanoparticles. Dissolution and Zn^{2+} shedding is considered a major mechanism of ZnO toxicity. Iron-doped ZnO nanoparticles were synthesized and shown to exhibit the lower rate of dissolution in biological and environmental media. Cellular HTS and in vivo studies in zebrafish embryos and the rodent lung confirmed a reduction in toxicity by Fe-doping.

between the in vitro and in vivo toxicological assessment illustrates the utility of a fish cell line together with zebrafish embryos for developing a predictive environmental paradigm.[20]

Wider Implementations of a Predictive Toxicological Approach for ENMs

HTS and a predictive toxicological approach can speed up hazard ranking and nano EHS decision-making to be commensurate with the number of new materials that are being introduced (Figure 11). HTS and in vitro hazard ranking also assist in the logistical planning and execution of more costly animal experiments. This could impact regulatory decision-making, which is often premised on animal data as the primary data source. HTS of compositional and combinatorial ENM libraries have yielded the first generation of SAR modeling tools to assist environmental decision-making and predicting in vivo outcome (Figure 11).

We have demonstrated an approach for the safer design of ZnO nanoparticles by hypothesizing that a slower dissolution rate will decrease this material's toxicity (Figure 12). Since mixed zinc–iron oxides are more resistant to proton-assisted dissolution than pure zinc oxides, we investigated the possibility of using iron doping to reduce dissolution. Using flame-spray pyrolysis, Prof. Lutz Mädler (Germany) generated a library of ZnO nanoparticles with an increasing atomic percentage of iron uniformly distributed throughout the crystal matrix.[6,7] As postulated, crystal field splitting by the 3d orbital of the substituted Fe^{2+} enhanced the aqueous stability and reduced the dissolution of the doped particles in biological and environmental media.[7] When assessed in the multiparametric HTS assay, iron-doped ZnO showed a significant reduction in cytotoxicity with an increasing atomic % of iron. When tested in the zebrafish platform, doped particles had a decreased effect on hatching interference and the generation of morphological abnormalities. The importance of ZnO dissolution in generating acute pulmonary inflammation in rodents was also confirmed by a significant reduction in acute pro-inflammatory and pro-oxidative effects of iron-doped ZnO in the lungs of rodents.[6]

Challenges and Outlook

Even if a link is established between in vitro and in vivo toxicological parameters, adverse outcomes in humans and the environment are dependent on real-life exposures at toxicologically relevant doses. Fate and transport as well as exposure assessment are key ingredients not incorporated in the predictive toxicological paradigm. There are also toxicological scenarios under chronic exposure conditions that could involve multiple steps that are not properly simulated in a single-step toxicological exposure. One example is the oncogenesis against a background of chronic granulomatous inflammation, e.g., the development of mesothelioma following asbestos exposure. While an in vitro screening assay for cytokines may be useful for predicting pulmonary inflammation in response to CNTs,[9] this response profiling does not address mutagenesis. Even though false-positive in vitro toxicological results (e.g., glass fibers and kaolin) can be sorted out by in vivo testing, there is a concern about false-negative in vitro outcomes. It is also possible that we could miss disease outcomes that are not captured by the in vitro injury events selected for HTS. This will require continuous improvement and introduction of proteomic and genomics discovery platforms to widen the scope and build a systems biology approach. It is also possible that machine learning will help to identify systems biology approaches to improve nano safety screening. In spite of the above pitfalls, we do not know of any substitutes currently that can replace the high-throughput predictive approach for ENM safety evaluation in the 21st century.

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FOOTNOTES

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REFERENCES

1 Nel, A.; Xia, T.; Madler, L.; Li, N. Toxic potential of materials at the nanolevel. *Science* **2006**, *311*, 622–627.

2 Nel, A. E.; Madler, L.; Velegol, D.; Xia, T.; Hoek, E. M.; Somasundaran, P.; Klaessig, F.; Castranova, V.; Thompson, M. Understanding biophysicochemical interactions at the nano-bio interface. *Nat. Mater.* **2009**, *8*, 543–557.

3 Xia, T.; Li, N.; Nel, A. E. Potential health impact of nanoparticles. *Annu. Rev. Public Health* **2009**, *30*, 137–150.

4 Meng, H.; Xia, T.; George, S.; Nel, A. E. A predictive toxicological paradigm for the safety assessment of nanomaterials. *ACS Nano* **2009**, *3*, 1620–1627.

5 Xia, T.; Kovochich, M.; Liong, M.; Madler, L.; Gilbert, B.; Shi, H.; Yeh, J. I.; Zink, J. I.; Nel, A. E. Comparison of the mechanism of toxicity of zinc oxide and cerium oxide nanoparticles based on dissolution and oxidative stress properties. *ACS Nano* **2008**, *2*, 2121–2134.

6 Xia, T.; Zhao, Y.; Sager, T.; George, S.; Pokhrel, S.; Li, N.; Schoenfeld, D.; Meng, H.; Lin, S.; Wang, X.; Wang, M.; Ji, Z.; Zink, J. I.; Madler, L.; Castranova, V.; Lin, S.; Nel, A. E. Decreased dissolution of ZnO by iron doping yields nanoparticles with reduced toxicity in the rodent lung and zebrafish embryos. *ACS Nano* **2011**, *5*, 1223–1235.

7 George, S.; Pokhrel, S.; Xia, T.; Gilbert, B.; Ji, Z.; Schowalter, M.; Rosenauer, A.; Damoiseaux, R.; Bradley, K. A.; Madler, L.; Nel, A. E. Use of a rapid cytotoxicity screening approach to engineer a safer zinc oxide nanoparticle through iron doping. *ACS Nano* **2010**, *4*, 15–29.

8 Wang, X.; Xia, T.; Addo Ntim, S.; Ji, Z.; Lin, S.; Meng, H.; Chung, C. H.; George, S.; Zhang, H.; Wang, M.; Li, N.; Yang, Y.; Castranova, V.; Mitra, S.; Bonner, J. C.; Nel, A. E. Dispersal state of multiwalled carbon nanotubes elicits profibrogenic cellular responses that correlate with fibrogenesis biomarkers and fibrosis in the murine lung. *ACS Nano* **2011**, *5*, 9772–9787.

9 Wang, X.; Xia, T.; Ntim, S. A.; Ji, Z.; George, S.; Meng, H.; Zhang, H.; Castranova, V.; Mitra, S.; Nel, A. E. Quantitative techniques for assessing and controlling the dispersion and biological effects of multiwalled carbon nanotubes in mammalian tissue culture cells. *ACS Nano* **2010**, *4*, 7241–7252.

10 Lin, S.; Zhao, Y.; Xia, T.; Meng, H.; Ji, Z.; Liu, R.; George, S.; Xiong, S.; Wang, X.; Zhang, H.; Pokhrel, S.; Madler, L.; Damoiseaux, R.; Lin, S.; Nel, A. E. High content screening in zebrafish speeds up hazard ranking of transition metal oxide nanoparticles. *ACS Nano* **2011**, *5*, 7284–7295.

11 Xia, T.; Kovochich, M.; Liong, M.; Zink, J. I.; Nel, A. E. Cationic polystyrene nanosphere toxicity depends on cell-specific endocytic and mitochondrial injury pathways. *ACS Nano* **2008**, *2*, 85–96.

12 Zhang, H.; Xia, T.; Meng, H.; Xue, M.; George, S.; Ji, Z.; Wang, X.; Liu, R.; Wang, M.; France, B.; Rallo, R.; Damoiseaux, R.; Cohen, Y.; Bradley, K. A.; Zink, J. I.; Nel, A. E. Differential expression of syndecan-1 mediates cationic nanoparticle toxicity in undifferentiated versus differentiated normal human bronchial epithelial cells. *ACS Nano* **2011**, *5*, 2756–2769.

13 Liu, R.; Rallo, R.; George, S.; Ji, Z.; Nair, S.; Nel, A. E.; Cohen, Y. Classification NanoSAR development for cytotoxicity of metal oxide nanoparticles. *Small* **2011**, *7*, 1118–1126.

14 Rallo, R.; France, B.; Liu, R.; Nair, S.; George, S.; Damoiseaux, R.; Giralt, F.; Nel, A.; Bradley, K.; Cohen, Y. Self-Organizing Map Analysis of Toxicity-Related Cell Signaling Pathways for Metal and Metal Oxide Nanoparticles. *Environ. Sci. Technol.* **2011**, *45*, 1695–1702.

15 George, S.; Xia, T.; Rallo, R.; Zhao, Y.; Ji, Z.; Lin, S.; Wang, X.; Zhang, H.; France, B.; Schoenfeld, D.; Damoiseaux, R.; Liu, R.; Lin, S.; Bradley, K. A.; Cohen, Y.; Nel, A. E. Use of a high-throughput screening approach coupled with in vivo zebrafish embryo screening to develop hazard ranking for engineered nanomaterials. *ACS Nano* **2011**, *5*, 1805–1817.

16 Zhang, H.; Ji, Z.; Xia, T.; Meng, H.; Low-Kam, C.; Liu, R.; Pokhrel, S.; Lin, S.; Wang, X.; Liao, Y.; Wang, M.; Li, L.; Rallo, R.; Damoiseaux, R.; Telesca, D.; Madler, L.; Cohen, Y.; Zink, J. I.; Nel, A. E. Use of Metal Oxide Nanoparticle Band Gap to Develop a Predictive Paradigm for Acute Pulmonary Inflammation based on Oxidative Stress. *ACS Nano* **2012**, *6*, 4349–4368.

17 Godwin, H. A.; Chopra, K.; Bradley, K. A.; Cohen, Y.; Harthorn, B. H.; Hoek, E. M.; Holden, P.; Keller, A. A.; Lenihan, H. S.; Nisbet, R. M.; Nel, A. E. The University of California Center for the Environmental Implications of Nanotechnology. *Environ. Sci. Technol.* **2009**, *43*, 6453–6457.

18 Wang, X.; Xia, T.; Duch, M. C.; Ji, Z.; Zang, H.; Li, R.; Sun, B.; Lin, S.; Meng, H.; Liao, Y. P.; Wang, M.; Song, T. B.; Yang, Y.; Hersam, M. C.; Nel, A. E. Pluronic F108 Coating Decreases the Lung Fibrosis Potential of Multiwall Carbon Nanotubes by Reducing Lysosomal Injury. *Nano Lett.* **2012**, DOI: 10.1021/nl300895y.

19 Liu, R.; Lin, S.; Rallo, R.; Zhao, Y.; Damoiseaux, R.; Xia, T.; Lin, S.; Nel, A.; Cohen, Y. Automated Phenotype Recognition for Zebrafish Embryo Based in Vivo High Throughput Toxicity Screening of Engineered Nano-Materials. *PloS One* **2012**, *7*, e35014.

20 George, S.; Li, S.; Ji, Z.; Thomas, C. R.; Li, L.; Mecklenburg, M.; Meng, H.; Wang, X.; Zhang, H.; Xia, T.; Hohman, J. N.; Lin, S.; Zink, J. I.; Weiss, P. S.; Nel, A. E. Surface Defects On Plate-Shaped Silver Nanoparticles Contribute To Its Hazard Potential In A Fish Gill Cell Line And Zebrafish Embryos. *ACS Nano* **2012**, *6*, 3745–3759.