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Subject: *Environmental, Health, and Safety Research Needs for Engineered Nanoscale Materials* by the Nanotechnology Environmental and Health Implications (NEHI) Working Group of the National Science, Engineering, and Technology Subcommittee (NSET) on Behalf of the National Nanotechnology Initiative (NNI)

The Working Group on Nanotechnology Environmental and Health Implications (NEHI) has begun to address critical aspects of nanomaterials safety for humans, animals, and the environment. NEHI recently published a descriptive outline of research needs and objectives for determining necessary steps to insure environmental health and safety (EH&S) aspects of engineered nanomaterials.

According to the National Nanotechnology Initiative (NNI) and NEHI, nanomaterials are defined as *those materials purposefully manufactured with dimensions between 1 and 100 nanometers and that exhibit unique properties determined by their size*. Nanomaterials offer the promise of efficient interactions and potent chemical reactions. However, research demonstrates that nanochemicals often have an exponentially greater propensity for toxicity and proclivity to harm human cells and the environment than their bulk chemical counterparts do. Physicochemical properties of nano-sized chemicals differ from their micro-sized counterparts of the same composition. These differences are accounted for by the increased surface area of the nanochemicals with respect to traditional chemicals and the concomitant increase in the number of particles that humans may be exposed to per unit of mass. Because scientists cannot limit the potential of nanomaterials to helpful or even intended interactions, nanomaterials often cause toxic effects to human cells.

NEHI has created three principles to guide nanomaterials EH&S research. We appreciate the opportunity to address important aspects of each of these newly described principles.

1. Prioritize Research Based on the Value of Information: This principle is of utmost importance. In scientific research, value can be measured by way of the greater good and societal impact of said research. The value of scientific research in the biomedical field can be analyzed in terms of tax-payer/investor input and the contribution of this research towards disease prevention and treatment. The FDA's own figures illustrate a lack of acceptable value whereby 92% of all drugs that pass preclinical testing (most of which is animal experimentation) fail in human trials. Outdated methods of animal experimentation impede the progress of this field. Millions of dollars and nearly a decade of research are invested in each potential drug. Because animals do not exhibit diseases most often found in humans, artificial means of approximating the appearance of symptoms (with differing physiological causes) are carried out. Pharmaceuticals able to alleviate these artificial symptoms are moved on to the next stage of development. The logic of this methodology is clearly flawed.

Human-relevant *in vitro* and *in silico* methods do not share the pitfalls of animal experimentation and are allow scientists the ability to collect repeatable and relevant data. Cell-based, non-animal assays are capable of detecting toxic effects of traditionally sized chemicals and pharmaceuticals as well as nanomaterials. In several reports comparing the effects of nanosized chemicals to those of traditional microsized chemicals, animal experiments were not able to show critical toxicological differences, while cell-based assays clearly showed differences in toxic effects [1, 2]. Distress pathways are triggered when toxic chemicals are introduced into cell-based experiments whether the chemicals are nanosized or microsized; therefore, cellular response can be measured using truly comparable parameters.

In terms of "value of information", there is simply no comparison between data collected from human cell-based experiments and the data collected by outdated animal experiments. It is for this reason that PETA is optimistic that NEHI will agree that its principles of value-based research would be best actuated using human-relevant non-animal assays.

2. Leverage international and private sector research efforts: There is much to be done during this critical time period as regulatory agencies work to define, understand, categorize, and standardize production methods so that the risks of nanomaterials can be studied effectively. To this end, it is of increasing importance that data collection and reporting be performed using standardized procedures from the outset. Consistent use of recognized vocabulary should also be used so that research redundancy is avoided.

Because the abilities of research labs to conduct experiments have far surpassed the agility of regulatory agencies to regulate, define, and standardize acceptable methods of nanoparticle production, we are presented with a regulatory quagmire which must be dealt with in a conservative manner.

International harmonization should begin with the NEHI of the NNI advocating for a structured, weight of evidence based approach to nanomaterial toxicity testing. This tiered strategy should be published by the U.S. government and be rooted in rigorous *in*

vitro and *in silico* science. Available assays measuring known pathways indicative of stress and cytotoxicity in human cells should be the basis for this guidance. Nanomaterials destined for consumer products should pass tests such as those described above prior to further product development.

3. Use of adaptive management for nanomaterials EHS research: Because of the rapid advancement in the field of nanotechnology and the development of novel nanomaterials and assays, regulatory requirements should be capable of adapting rapidly to this field. Adaptive management will allow the field of nanotechnology to be the first field in recent history to avoid becoming entrenched in outdated test methods. PETA applauds this principle and hopes that its practical implementation steers regulatory testing further and further away from animal experimentation.

Metrology for Nanomaterials – Achieving a Standardized, Tiered Approach to Measurement:

Because nanomaterial production is not yet standardized, resulting nanomaterials are non-uniform and are riddled with various heavy metal contaminants. Use of appropriate, available metrological devices as well as continued advances in device development are needed so that purity, size, shape, distribution, structure, and surface area assessment is determined using high-resolution instruments. In order to reduce batch-to-batch variation in nanomaterials produced within the same manufacturing facility, as well as variability found in the same nanochemical made by different manufacturers, the use of high throughput methods of synthesis and analysis are recommended. In this way, consistent composition and quality of product would be greatly improved.

Standardized methods that allow manufacturers and researchers to know the exact composition and level of purity of the nanomaterial they are studying are paramount to attaining useful toxicity data. The best methods available to measure and test the purity of nanomaterials include scanning electron microscopy (SEM) with energy dispersive X-ray (EDX) analysis that measures nanomaterial size and detects dispersion, non-carbon-based contamination, and geometry of the nanomaterial. Transmission electron microscopy (TEM) answers questions pertaining to particle/bundle size, morphology and purity of the nanomaterial's surface while thermogravimetric analysis (TGA) analyzes a wide range of parameters, including compositional analysis, decomposition temperature, rate of decomposition – a quantitative measure of mass change associated with transition and thermal degradation and nanomaterial oxidation. Raman spectroscopy is able to identify carbon spectral features while x-ray photoelectron spectroscopy (XPS) can be used to determine surface elemental group composition and provides chemical state information for the first several layers of the sample surface.

Instruments such as scanning mobility particle sizers (SMPS) that can measure the size of particles between 3 and 1000 nanometers (nm) and scanning electron microscopes (SEM) that utilize an electron dispersive spectrometer (EDS) make nanoparticles countable and their chemical compositions discernable. A recently announced advance in nanoparticle detection for workplace safety comes from Dekati Ltd. with the advent of the Electrical

Dekati Industrial Hygiene Particle Sensor (EDiPS) (Finnish Presidency Conference – Safety for Success). This sensor is portable and offers real-time nanoparticle measurements to insure workplace safety. Dekati Ltd. expects the EDiPS to be available commercially by the end of this year.

Experiments aimed at elucidating the toxicity of these compounds using non-standardized methods result in uninterpretable toxicity data. These problems are recognized by researchers and regulatory agencies and have proven insurmountable by means of traditional low-throughput, expensive, and irreproducible animal-based toxicology methods.

Nanomaterials and Human Health:

NEHI reports that the National Toxicology Program (NTP) plans long-term rodent studies with the faulty notion that they will identify nanomaterials that are toxic to humans. The NTP is responsible for reams of uninterpretable rodent cancer assay data that cannot be extrapolated to humans [3, 4]. In fact, the rodent cancer data cannot even be extrapolated from male to female rodents or from mice to rats [5].

In addition, rodent studies failed to identify truly toxic substances such as cigarette smoke, asbestos, and benzene, resulting in unprotected human exposure [3]. These failures in animal experimentation continue to accrue costs associated with these toxic exposures. Now, an approach similar to this failed effort to detect carcinogens is planned for the field of nanotechnology.

For reasons outlined above, old-fashioned, irrelevant testing will fail even more miserably for nanomaterials than it has for traditional chemicals. Studies have been published showing that *in vivo* experiments are unable to differentiate the toxic potentials of traditional versus nano-sized chemicals. For example, *in vitro* assays have detected cytotoxic responses in human cells for nano-sized titanium dioxide, while showing that micro-sized titanium dioxide did not have the same toxic propensity [6-8].

Relevant, Repeatable, and Rigorous Nanoparticle Testing Methods:

Several recent peer-reviewed publications have focused on a set of predictive measures of cytotoxicity. Nanotoxicologists from several research groups stress the importance of assays that detect the generation of reactive oxygen species (ROS), measure the increase in cell's: oxidative stress, mitochondrial perturbation, inflammation response pathways, protein denaturation and degradation, and DNA damage [[1, 2, 9].

Andre Nel, PhD, is a well-respected nanomaterials expert and wrote an often-cited nanotoxicity review [2]. In his 2006 review entitled, *Toxic Potential of Materials at the Nanolevel,* Nel summarizes the most important aspects of toxicity testing by explaining that generation of ROS is among the most predictive of tests that can be done. In addition, Nel specifies that **the ultimate goal of the predictive approach to toxicity**

testing "would be to develop a series of toxicity assays that can limit the demand for *in vivo* studies, both from a cost perspective as well as an animal use perspective[2]." This notable scientist seems to recognize that animal experimentation has severe limitations and is problematic in this modern era.

Studies from Dr. Vicki Colvin's lab, utilize a series of *in vitro* human cell culture assays predictive of cellular responses to toxic chemicals. In a study entitled "*Nano-C_{60} cytotoxicity is due to lipid peroxidation,"* experiments were performed to assess cytotoxicity/cell viability, lactate dehydrogenate release, mitochondrial activity, DNA content, plasma membrane permeability, lipid peroxidation, glutathione production, and the ability to prevent oxidative damage by the addition of L-ascorbic acid. By changing the number of hydroxyl groups on the fullerene surface resulted in a reduction of toxicity by several orders of magnitude. These experiments show that fullerene toxicity can be rigorously tested by means of cost-effective, predictive, and relevant *in vitro* assays. In addition, potential toxicity of the fullerenes was lowered significantly by using these *in vitro* assays to target chemical aspects of the nanomaterials that contribute to toxicity. The author states that, **"*in vitro* testing provides a cost-effective means for such studies, and as this report illustrates, cell culture experiments are well suited for developing mechanistic models to inform material development."** In addition, the author explains that this study seeks **"to set a standard for future efforts to characterize the environmental and health impacts of other classes of engineered nanoparticles [10]."** The above studies clearly show that the most efficient (and humane) means of toxicity testing lie in modern, high-throughput *in vitro* assays.

Many groups have used human cell culture in concert with microarray experiments and cytotoxicity analyses, which allow detection of early signs of cellular toxicity. Known stress responses can be measured before and after exposure to nanomaterials thereby giving scientists clear indications of cellular responses.

The HμREL is a microchip device that allows the scientist to test a compound within a matrix of different cell types, linked via microfluidic channels. The HμREL can answer questions regarding how nanomaterials interact with and are metabolized by human tissues. Details of this work can be read in depth in Sin *et al*. 2004, entitled *The Design and Fabrication of Three-Chamber Microscale Cell Analog Devices with Integrated Dissolved Oxygen Sensors*. Systems such as these will allow scientists to test whether a nanomaterial is effectively targeted to a particular organ or cell, and whether it has detrimental effects on organs such as the kidney, liver, or heart. Using this novel technology will save not only human and animal lives, but also time, money, and resources [11].

Reliance upon unvalidated, unethical, and unpredictive animal experiments has stunted scientific capability and resulted in millions of wasted animal lives and reams of irrelevant data. Applying these antiquated methods to the burgeoning field of nanotechnology will result in data that are even less correlative because animal experiments have a demonstrated inability to differentiate between bulk and nano forms of chemicals in contrast to cost effective, non-animal test methods.

The Effective Approach to Nanomaterials Safety Testing:

We advocate a conservative approach for nanomaterials testing to ensure the safety of nanomaterials. A conservative approach is the only tenable solution for introducing nanomaterials onto the market. The aforementioned set of modern, *in vitro* testing methods should serve as the basis of a rigorous, reliable, and human-relevant system for nanomaterials testing.

Sincerely,

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