Opinion

Challenges in assessing nanomaterial toxicology: a personal perspective

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Nanotechnology exploits the fact that nanoparticles exhibit unique physicochemical properties, which are distinct from fine-sized particles of the same composition. It follows that nanoparticles may also express distinct bioactivity and unique interactions with biological systems. Therefore, it is essential to assess the potential health risks of exposure to nanoparticles to allow development and implementation of prevention measures. Risk assessment requires data concerning hazard and exposure. Several challenges face the field of nanotoxicology in obtaining the necessary data for assessment of the bioactivity of nanoparticles. They include: (1) the vast number of nanoparticle types to be evaluated, (2) the need to use nanoparticle doses and structure sizes in cellular and animal test systems which are relevant to anticipated workplace exposures, and (3) artifactual *in vitro* results due to absorption of nutrients or assay indicator compounds from the culture media. This 'opinion' reviews the progress made in the field of nanotoxicology in recent years to overcome these challenges. © 2010 John Wiley & Sons, Inc. *WIREs Nanomed Nanobiotechnol*

Nanotechnology is the manipulation of matter on a near-atomic scale to produce new structures, materials, and devices. Nanoparticles are structures having one dimension less than 100 nm. Nanoparticles exhibit unique physicochemical properties, which can differ dramatically from fine particles of the same composition. These unique properties can be exploited in a wide number of novel applications and products. Such applications include cosmetics, sunscreens, antimicrobial products, paintings, coating, electronics, sensors, structural materials, sporting goods, energy storage devices, conductive fabric, bone grafting, medical imaging, and targeted drug delivery. The number of nanoparticles being developed for incorporation into nanomaterials for a wide variety of commercial products is growing rapidly.[1] Indeed, nanotechnology is expected to grow into a trillion dollar industry employing millions of workers worldwide within the next decade.[2] Due to the small size and low density of nanoparticles, aerosolization is likely during energetic processes. Such aerosolization has been documented during vortexing,[3] weighing,[4] or sonication[4] of carbon nanotubes (CNT). Therefore, worker exposure is anticipated during production, use, and disposal of nanoparticles.[5] Since nanoparticles exhibit unique physicochemical properties and can be aerosolized. It is essential to conduct hazard and exposure assessment to support risk assessment and allow the development of recommendations for exposure limits and the implementation of prevention strategies.

A major challenge in hazard assessment in nanotechnology is the large and rapidly growing number of possible nanoparticles to be tested for biological activity. For CNT, there are four synthesis processes. Raw CNT then can be purified by heat or acid treatment to remove catalytic metals. CNT can vary in width, being single-walled, double-walled, or multi-walled, as well as in length. Lastly, CNT can be functionalized with a variety of chemical groups. Thus, for CNT alone, there can be hundreds of different test materials with different physicochemical properties, possibly exhibiting substantially different

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bioactivity. Because it is not feasible to conduct hazard assessment for every possible nanoparticle, there is the need to develop a matrix of relationships between specific physicochemical properties and resultant bioactivity. Such a matrix would allow prediction of possible health effects in the absence of complete toxicity testing. A matrix of properties versus bioactivity could be applied to a control banding analysis. Control banding is a qualitative strategy to design a program to control exposures when no relevant Occupational Exposure Limits are available. The basis for a control banding approach is the ability to evaluate a particular industrial process and group potential chemical exposures according to similarities in physical and chemical characteristics of the material involved; the handling and processing tasks in which the chemical is used; and the tasks within the process where exposures might occur. Gathering and analyzing these basic factors would lead to decisions on the types of control strategies that would be appropriate for each of the potential exposures. There are various control banding models being practiced today, all of which have the basic elements of employing good occupational hygiene practices; using engineering controls, which may be supplemented by personal protective equipment; containing the chemical process; and seeking the advice of an occupational health and safety specialist.[6] The concept of control banding as an approach for the risk management of engineered nanoparticles was proposed by Schulte et al.[7] A preliminary and useful step in the control banding process involves placing the candidate nanomaterial, in this case the CNT, into a relative hazard category. This step is often referred to as hazard banding. The hazard category, or banding approach, is needed because of the lack of complete toxicological data on CNT. The appropriate hazard band for a CNT could be determined by using information available from analogous materials. For example, a comparison of the pulmonary fibrotic response of an equal mass exposure to single-walled carbon nanotubes (SWCNT) and quartz has been reported in a mouse.[8] Results indicate that SWCNT cause interstitial fibrosis more rapidly and at a lower mass dose than the known fibrogenic quartz particles. In addition, SWCNT have the potential to be aerosolized as respirable particles. Therefore, SWCNT would be placed in a high hazard category and appropriate controls would be prudent. Such comparative toxicology data would not be available for all nanoparticles. Therefore, having a matrix of properties correlated to biological activity, as proposed above, would add great value to the process. Once a material is placed in a hazard band, the control banding model would next factor in process parameters, such as the physical form of the material, the quantity and frequency of the handling of the material, the physical energy of any processing steps, and an assessment of the potential severity of exposure. At the conclusion of this overall hazard assessment, a target control level, or band, is selected. Controls capable of maintaining exposures within the specified band are then selected and implemented, with periodic verification that the controls are operating properly. To develop this property-response matrix, biological scientists and toxicologists must form collaborations with material scientists, who would synthesize sets of nanoparticles with well-defined physicochemical properties for bioactivity testing. Examples of a testing set would be nanoparticles having the following characteristics:

1. Same composition and size but different shape
2. Same composition, size, and shape, but different crystallinity
3. Same size and shape, but different chemistry
4. Same composition, size, and shape, but different surface functionalization
5. Same composition, size, and shape, but with or without catalytic metals removed.

In this manner, one could develop a mechanistic understanding of what nanoparticle properties affect bioactivity. Therefore, it is critically important to fully characterize the physicochemical properties of the nanoparticle being evaluated for biological activity. The importance of particle characterization to meaningful toxicological evaluation is demonstrated by the following three examples. First, Porter et al.[9] reported that aspiration of multi-walled carbon nanotubes (MWCNT) resulted in transient pulmonary inflammation with persistent fibrosis. In contrast, Mitchell et al.[10] reported no pulmonary response following inhalation of MWCNT. The test material used in the Porter et al. study was well characterized as MWCNT, exhibiting a multi-lamellar structure under high resolution transmission electron microscopy (TEM).[9] In contrast, the material used in the Mitchell et al. study was from a commercial supplier and has been criticized as being 'stacked carbon cups' rather than MWCNT, explaining the different biological response.[11] Second, Porter et al.[12] and Hamilton et al.[13] have demonstrated the importance of nanoparticle shape to bioactivity, reporting that TiO_2 nanowires are more bioactive in both cellular and animal models than nanospheres of the same composition and diameter. Third, the importance of

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contamiating catalytic metals in the ability of SWCNT to generate reactive oxygen species and cause oxygen stress and cytotoxicity has been demonstrated in macrophages and keratinocytes *in vitro*.[14,15]

Classically, hazard assessment uses dose-response data from chronic animal inhalation exposure studies where the dose rate of deposited particles is relatively low. Considering time and cost factors, pulmonary exposure to particles is often by pharyngeal aspiration or intratracheal instillation of a single bolus of particles, i.e., a high dose rate. A critical question is the relevance of pulmonary response to a bolus dose of particles versus inhalation of particles over an extended time period. To resolve this question, information concerning deposition, fate, and clearance of nanoparticles after a bolus or inhaled exposure is critical. Information concerning the rate of translocation of nanoparticles from the lung to systemic organs is also vital. Although data are not extensive, information exists that biological responses to a bolus dose of nanoparticles may reflect responses following short-term inhalation at the same total lung burden. For example, pulmonary responses, transient inflammation, and rapid onset but persistent fibrosis following a 4-day inhalation of SWCNT are qualitatively similar to those reported after pharyngeal aspiration of SWCNT in a mouse model.[8,16] Indeed, responses to inhalation of a well-dispersed aerosol of SWCNT were fourfold greater than aspiration of an equal mass of poorly dispersed SWCNT. However, aspiration of a well-dispersed suspension of SWCNT gave quantitatively similar results as inhalation of the same lung burden of SWCNT.[16,17] Quantitative similar transient lung inflammation and injury were also reported for aspiration versus short-term inhalation of MWCNT in a mouse model.[9,18] Lastly, the degree of systemic microvascular dysfunction resulting from an equal lung burden of either intratracheally instilled or inhaled fine TiO_2 has been reported to be identical.[19,20]

As shown in Figure 1, pulmonary exposure to fine TiO_2 as a bolus instilled dose of 100 μg or a lung burden of 90 μg inhaled over 8 h caused nearly identical inhibition of the ability of systemic arterioles to respond normally to dilator infusion 24 h post-exposure. These results support the usefulness of bolus exposures to evaluate the relative ranking of biological reactivity of nanoparticles. Such initial potency evaluations would then be used to determine which nanoparticles should be tested more completely in a 90-day inhalation study.

Even if bolus *in vivo* exposure proves to be a reasonable alternative to chronic inhalation studies, such experiments remain costly and labor intensive.

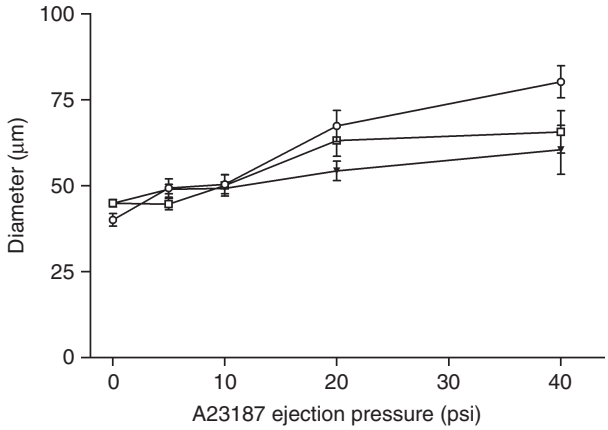

FIGURE 1 | Inhalation and IT instillation exposure to fine TiO_2 similarly impair systemic endothelium-dependent arteriolar dilation 24 h after exposure to a similar degree. Dilation in response to arteriolar infusion of the calcium ionophore, A23187, was monitored by intravital microscopy of an exteriorized vascular bed from an anestitized rat with nerves and vessels intact. For inhalation exposure, the dose metric is the measured particle mass (μg) deposited in the lungs. For instillation exposure, the dose metric is the measured particle mass placed in suspension and then instilled. ○ = Control, □ = 100 μg instillation, ▼ = 90 μg inhalation.

Therefore, there is a need to develop high throughput, low cost, *in vitro* assays, which are predictive of *in vivo* response. Oxidative stress is a widely held paradigm for initiation and progression of disease.[21] A recent study has compared the ability of *in vitro* assays for oxidant generation to predict *in vivo* inflammatory responses of the lung after exposure to a set of metal nanoparticles.[22] For eight distinct nanometals, acellular generation of reactive species correlated with *in vivo* inflammatory potency with an R^2 of 0.74–0.79, using an oxidant sensitive dye or electron spin resonance spectroscopy, respectively. However, cell-mediated reactive species production in response to this nanoparticle set showed an even stronger prediction of *in vivo* inflammatory potency ($R^2 = 0.95$). Although *in vitro* reactive species generation was very predictive of *in vivo* bioactivity for nanometals, such predictivity did not hold for SWCNT. Kagan et al.[14] demonstrated that removal of catalytic metals from SWCNT greatly reduced oxidant generation *in vitro*. Furthermore, purified SWCNT (metals removed by acid treatment) failed to stimulate inflammatory cytokine production by macrophages in culture.[8] Although purified SWCNT were negative using *in vitro* assays of oxidant stress, they were potent in causing rapid and persistent pulmonary fibrosis.[8,16,17] Although *in vitro* oxidant production failed to predict the *in vivo* bioactivity of SWCNT, *in vitro* assays for fibroblast proliferation and collagen production following *in vitro* exposure to SWCNT

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appear to predict the pulmonary fibrotic activity of SWCNT seen in mouse models.[23] Therefore, knowledge of the mechanisms by which a given nanoparticle induces a biological response is essential for the development of predictive *in vitro* assays.

A second major challenge to nanotoxicology is the use of exposure doses and particles sizes in bioassay systems which reflect airborne exposure levels and structure sizes in workplaces producing and using nanoparticles. Collecting workplace exposure data specific to an engineered nanoparticle of interest is complex and challenging. A principal challenge facing the industrial hygienist is the access to instruments and analytical methods sensitive enough to detect, and specific enough to measure the nanoparticle. This challenge is compounded by the need to provide data specific to the nanoparticle in the presence of other airborne particles that may have similar elemental composition or possibly of similar particle size. Initial investigations focused on a particle size-based approach, i.e., sampling and measuring particles in the nanometer size range. Currently, a variety of commercially available air sampling instruments exist that can characterize airborne particulate aerosol using a number of metrics, including mass, number concentration, size distribution, number concentration by size, and surface area. Because of the need for diverse data, exposure studies use a combination of pump and filter-type air sampling along with an instrumental approach that uses combinations of direct-reading instruments that provide information on particle number, size distribution, and surface area. Since none of the instruments are yet small enough to be worn by a worker, collection of personal breathing zone samples is not possible. Therefore, area sampling is conducted. Figures 2 and 3 give examples of the size and complexity of some of the instruments used to collect a broad range of data on nanoparticles. Regardless of the metric and method selected for exposure monitoring, it is critical that measurements be taken before production or processing of a nanomaterial to obtain background nanoparticle exposure data. Measurements made during production and processing can then be evaluated to determine if there has been an increase in particle number concentrations in relation to background measurements, and whether that change represents worker exposure to the nanoparticle. Current state-of-the-art characterization of workplace exposure to nanoparticles involves a multi-faceted approach incorporating many of the sampling techniques mentioned above. National Institute for Occupation Safety and Health (NIOSH) has developed the Nanoparticle Emission Assessment Technique to qualitatively determine the release of engineered nanoparticles in the workplace. This approach is helpful for the initial evaluation of workplaces where engineered nanoparticles are manufactured or used. If nanoparticle release is

FIGURE 2 | Example of field application of instruments needed for real-time measurement of number, mass, size distribution, and surface area.

FIGURE 3 | Examples of different sampling instruments used to measure occupational exposure to nanoparticles including the determination of real-time particle number concentrations and size-fractionated mass concentrations.

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found, then a more comprehensive and quantitative approach may be adopted.[24] The first step to characterizing workplace exposures would involve identifying the source of nanoparticle emissions. A condensation particle counter used in parallel with an optical particle counter/size analyzer (OPC) provides acceptable capability for this purpose. It is critical to determine ambient or background particle counts before measuring particle counts during the manufacturing, processing, or handling of engineered nanoparticles. However, investigators need to be aware that background nanoscale particle counts can vary both spatially and temporally depending on the unique conditions of the workplace. If nanoparticles are detected in a process area at elevated concentrations relative to background particle number concentrations, then a pair of filter-based area air samples should be collected for particle analysis via TEM and for determining mass concentration. TEM can provide an estimate of the particle size distribution and, if equipped with an energy dispersive X-ray analyzer, a determination of elemental composition can be made to identify the nanoparticle. This final analysis is more definitive in identifying and characterizing the actual nanoparticle being evaluated and provides a more useful description of actual exposure in the workplace.

A technical problem when one conducts cell culture exposure to nanoparticles or pulmonary exposure of animal models by pharyngeal aspiration or intratracheal instillation is that nanoparticles agglomerate into micrometer-size particles when suspended in biological media, such as phosphate-buffered saline. This agglomeration is an issue because evidence indicates that the structure size of nanoparticles delivered to the *in vitro* test system or lung is critical to the biological response. For example, Wang et al.[23] reported that well-dispersed SWCNT caused proliferation of lung fibroblasts in culture, whereas poorly dispersed SWCNT exhibited no effect. Intratracheal instillation of rats to a well-dispersed suspension of nano-carbon black proved eightfold more inflammatory than an equal mass of poorly dispersed carbon black nanoparticles.[25] Similarly, Mercer et al.[17] found that well-dispersed SWCNT were fourfold more potent than poorly dispersed SWCNT in causing alveolar interstitial fibrosis. The ability to distinguish the pulmonary response of nanoparticles versus fine particles of the same composition depends on the use of well-dispersed nanoparticles. For example, Warheit et al.[26] reported that pulmonary inflammation in response to fine or nanosize TiO_2 was similar. In this study, structure sizes of test suspensions of fine and nano-TiO_2 were both $\approx$2 μm as determined by dynamic light scattering. In contrast, Sager et al.[27] used well-dispersed suspensions of TiO_2 and found nanoparticles to be 40-fold more inflammatory than an equal mass of fine particles of TiO_2. They also reported that well-dispersed nano-TiO_2 moved more rapidly into the alveolar interstitium than fine TiO_2. The importance of nanoparticle dispersion in the evaluation of nanoparticle fate after pulmonary exposure was also supported in a study of MWCNT exposure. Well-dispersed MWCNT were shown to move across alveolar epithelial cells into the interstitial space, lymphatics, subpleural tissues, and intrapleural space.[9] In contrast, poorly dispersed MWCNT (3-μm structure agglomerates) failed to move across alveolar epithelial cells.[28]

Because the degree of nanoparticle dispersion affects bioactivity, development of effective dispersion media is a high priority goal in nanotoxicology research. Criteria for an acceptable dispersion medium are as follows:

1. Ability to minimize agglomeration of nanoparticles in suspension
2. Biological compatibility
3. Failure to mask surface reactivity of the particle.

Organic solvents effectively meet the first criteria. However, they are often toxic to cells and lung tissue, so are of no use in hazard assessment studies. Albumin (5–10%) has been used to disperse nanoparticles for *in vitro* studies.[29,30] However, albumin may alter the surface properties of nanoparticles, such as SWCNT. Indeed, SWCNT suspended in albumin (5–10%) were actively phagocytosed by macrophages,[29,30] yet no phagocytosis by macrophages was noted in the absence of albumin.[8] Furthermore, the exposure of mice by aspiration of SWCNT dispersed without albumin resulted in the low uptake of SWCNT by alveolar macrophages *in vivo*.[17] Evidence indicates that albumin avidly adsorbs onto the surface of SWCNT, thus making these nanoparticles recognizable by scavenger receptors.[30] Therefore, dispersion appears to change cell–nanoparticle interactions. One could argue that upon inhalation nanoparticles would deposit on the alveolar surface and interact with alveolar lining fluid rather than 10% serum albumin. Using this logic, Sager et al.[31] used diluted alveolar lining fluid obtained by bronchoalveolar lavage as a dispersion medium. They reported that this was highly effective in dispersing nano-carbon black and nano-TiO_2 particles. Porter et al.[32] measured the albumin (0.06%)

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and disaturated phosphatidyl choline (0.01 mg/mL) levels in this diluted alveolar lining fluid to develop an artificial alveolar lining fluid. They found this to be an excellent dispersion medium for MWCNT and nano-carbon black or TiO_2. Pulmonary exposure to actual or artificial diluted alveolar lining fluid did not cause inflammation or lung injury and was thus biocompatible.[31,32] In addition, suspension of fine quartz in diluted alveolar lining fluid did not alter the pulmonary responses to quartz exposure (inflammation, cytotoxicity, and air/blood barrier damage), indicating that the concentrations of albumin and disaturated phosphatidyl choline in the dispersion medium were too low to mask the reactive quartz surface.[31,32] Therefore, artificial diluted alveolar lining fluid appears to be an excellent dispersion medium for pulmonary nanotoxicology studies.

It is essential that lung burdens of nanoparticles used in animal models be relevant to lung burdens anticipated in nanotechnology workers. Thus far, only a few studies of workplace exposure to nanoparticles are available in the literature. Analysis of a laboratory producing MWCNT found peak airborne total dust levels of 400 $\mu g/m^3$ associated with weighing and blending processes.[33] Another study reported total airborne particulate levels of CNT plants to be as high as 320 $\mu g/m^3$ during oven opening and catalyst preparation.[34] Significant aerosolization was also reported during CNT spraying, preparation, and sonication procedures.[34] High airborne levels (710–6700 $\mu g/m^3$) have been recorded during nanometal reactor clean out operations.[35] In addition, high airborne levels of inhalable dust containing carbon were measured during wet saw cutting of carbon nanofibers composites.[36] From the measurements of airborne nanoparticle concentration in workplace air, one can estimate the lung burden of exposed workers as follows:

Deposited dose = (airborne concentration) × (minute ventilation) × (exposure time) × (deposition fraction).

Minute ventilation in an adult male at rest is 7500 mL/min. However, minute ventilation can increase to 20,000 mL/min during light work.[37] Deposition fraction, i.e., the fraction of inhaled particles which deposit in the lung, varies with the aerodynamic diameter of inhaled structures, i.e., agglomerate size, as described in humans by the Task Group on Lung Dynamics.[38] For an average worker, exposure duration could be calculated as (8 h/day) × (5 days/week) × (48 weeks/year) = 1920 h/year. To relate anticipated work lung burden to experimental lung burdens in animal models, it is useful to normalize lung burden for alveolar epithelial surface area. Values for alveolar epithelial surface area in humans and common animal model species were reported by Stone et al.[39] and are given in Table 1. It is also important that structure sizes of nanoparticles in cell culture or animal exposures approach those reported in workplace air. To date, only a few studies have provided such data. Airborne structure size of ≈400 nm was reported during the cutting of carbon nanofiber composites.[36] Han et al.[33] provided an electron micrograph showing the distribution of airborne MWCNT structures found in a production laboratory. Relatively dispersed structures, similar to those reported in the workplace,[33] have been achieved in an animal inhalation exposure study with MWCNT as shown in Figure 4, where count medium aerodynamic diameter is ≈410 nm.[40] Similar

TABLE 1 | Alveolar Epithelial Surface Area

Species	Cell Surface Area (m^2/lung)
Human	102
Rat	0.4
Mouse	0.05

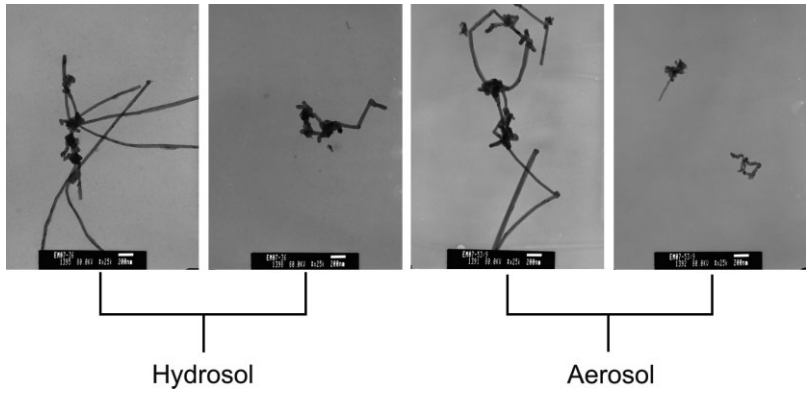

FIGURE 4 | Structure sizes of MWCNT. Left panels are electron micrographs of MWCNT suspended in diluted artificial alveolar lining fluid and used to expose mice by pharyngeal aspiration.[9] Right panels are electron micrographs generated from a dry MWCNT sample for inhalation exposure of mice.[18,40] Note the similarity of well-dispersed structures. Scale bars = 200 nm.

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MWCNT structures were obtained by Porter et al.[9] who used a diluted artificial alveolar lining fluid to suspend MWCNT for aspiration into mouse lungs (Figure 4). These results indicate that with care one can expose cells or animal models to structure sizes of nanoparticles representative of those found in workplace air.[18,40]

Nanoparticles exhibit a high surface area per mass. Therefore, nanoparticles can rapidly bind proteins present in suspension media forming a protein corona around the nanoparticle.[41] This affinity for nanoparticles to adsorb chemicals could represent problems for *in vitro* investigation of the cytotoxicity of nanoparticles. For example, SWCNT and MWCNT have been reported to be cytotoxicity to macrophages and lung epithelial cells.[42,43] In contrast, other *in vitro* studies have reported low cytotoxicity for purified CNT where catalytic metals have been removed.[8,14,15,29,44] These conflicting results were due to an artifact in the studies reporting high cytotoxicity, i.e., CNT bound to the dye used to assay for cell death.[45,46] Likewise, CNT have been reported to depress cell proliferation *in vitro*.[47,48] However, induction of fibroblast proliferation has been reported using low concentrations of SWCNT in culture.[23] It was found that at higher doses CNT adsorbed essential nutrients, such as folic acid, from the culture media giving artifactual cell death.[47,48] Such adsorption of nutrients and assay reagents can be avoided by using relatively low concentrations (μg/mL) of nanoparticles for *in vitro* assays. Our laboratory recommends using *in vitro* doses (μg/surface area of cultured cells) representative of *in vivo* lung burdens (μg/alveolar epithelial cell surface area).

In summary, at first the challenges in nanotoxicology appear daunting. They include the sheer number of nanoparticle types to be evaluated for potential adverse bioactivity, the need to use doses and structure sizes in cellular and animal test systems which mimic workplace aerosols found in nanotechnology facilities, and possible interference of nanoparticles with assay systems due to absorbance of nutrients and assay indicator compounds from the culture media. However, material scientists are collaborating increasingly with toxicologists to provide well-characterized set of nanoparticles for biological testing. With increasing understanding of relationships between physicochemical properties and bioactivity, a matrix can be constructed to assist in predicting the relative bioactivity of a nanoparticle. Then, control banding principles can be applied to develop prevention strategies for the safe commercial use of not yet tested nanoparticles. Furthermore, increased mechanistic understanding of biological effects of exposure to nanoparticles will assist in the development of predictive, high throughput, low cost, *in vitro* assays for nanoparticle screening. Development of protocols for workplace assessment of aerosolized nanoparticles is progressing. This would allow scientists to conduct *in vitro* and *in vivo* toxicology studies using nanoparticle concentrations and structure sizes relevant to workplace exposures. With well-conducted hazard and exposure assessment studies, risk assessment will be possible. Since control technology (HEPA filters, local exhaust ventilation, and respirators) appears effective for nanoparticles,[33–35,49] prevention strategies can be implemented to allow the safe development and growth of the nanotechnology industry.

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