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14. ABSTRACT
The exponential growth in the employment of nanomaterials (NMs) and the subsequent need to evaluate their safety have given rise to the field of nanotoxicology; which examines the cellular consequences following exposure to nano-sized particles. Initial nanotoxicological studies were limited by a lack of both available materials and accurate biodispersion characterization tools; however, the years that followed were marked by the development of enhanced capabilities on both the synthesis techniques and characterization technologies. In fact, these established NM-based tools and techniques are now standard practice for the evaluation of NM properties and their solution behavior. Paralleling advances in characterization, modifications of physical parameters, such as size, morphology, or coating, were able to individually influence the depth of NM-dependent physiological response studies. As such, a major research focus to date has been on the development of correlations between toxicological effects and specific particle original exposure and their biodynamic characteristics. While great strides have been made to advance the field, nanotoxicology is currently at a crossroads and faces a number of unique obstacles and technical limitations, such as the full descriptive requirements and standardization of dosimetry and its correlation to true exposures, rates of dissolution, surface modification dynamics for assuring to establish safety exposure limits. This review will discuss both the progress and future directions of nanotoxicology: highlighting key previous research successes and identifying challenges facing the field today.

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Contemporary Review

CONTEMPORARY REVIEW

At the Crossroads of Nanotoxicology *in vitro*: Past Achievements and Current Challenges

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ABSTRACT

The exponential growth in the employment of nanomaterials (NMs) has given rise to the field of nanotoxicology; which evaluates the safety of engineered NMs. Initial nanotoxicological studies were limited by a lack of both available materials and accurate biodispersion characterization tools. However, the years that followed were marked by the development of enhanced synthesis techniques and characterization technologies; which are now standard practice for nanotoxicological evaluation. Paralleling advances in characterization, significant progress was made in correlating specific physical parameters, such as size, morphology, or coating, to resultant physiological responses. Although great strides have been made to advance the field, nanotoxicology is currently at a crossroads and faces a number of obstacles and technical limitations not associated with traditional toxicology. Some of the most pressing and influential challenges include establishing full characterization requirements, standardization of dosimetry, evaluating kinetic rates of ionic dissolution, improving *in vitro* to *in vivo* predictive efficiencies, and establishing safety exposure limits. This Review will discuss both the progress and future directions of nanotoxicology: highlighting key previous research successes and exploring challenges plaguing the field today.

Key words: nanotoxicology; characterization; physicochemical parameters; safety regulation; Dosimetry; *in vitro* models

Recent advances in material science have resulted in the creation of particles in the nano-scale range. However, years of research in this field, coupled with technological advances, have brought about a transformation in nanomaterial (NM) synthesis capabilities (Guo and Wang, 2013). The unique physicochemical properties associated with engineered NMs, including primary size, core composition, morphology, porosity, surface chemistry, and reactivity, differentiate them from their bulk counterparts and make NMs attractive for use in commercial and scientific applications (Kessler, 2011; Salata, 2004). Currently over 1600 consumer products are on the market that incorporate NMs, with applications spanning energy, electronic, medical, commercial, industrial, and research sectors. However, one considerable drawback associated with NM-based applications is the unintentional, and sometimes detrimental, cellular consequences that can occur following NM exposure (Oberdörster *et al.*, 2005a). Due to this cytotoxic potential, the safety of NMs should be thoroughly assessed prior to their inclusion in nano-based consumer products and technologies.

The critical need to assess the safety of NMs and identify their cellular responses have given rise to the field of nanotoxicology, which has now been in existence for over a decade (Oberdörster *et al.*, 2005a). During this time, the field has grown tremendously, matured, and made substantial progress

in elucidating potential health hazards associated with NM exposure in a physiological environment (Love *et al.*, 2012; Nel *et al.*, 2006). The observed cellular reactions associated with NM exposure have identified a differential response that can be directly linked to their distinct physicochemical parameters (Podila and Brown, 2013; Sharifi *et al.*, 2012); demonstrating that minute changes in NM properties can alter subsequent behavior and cellular interactions. Although nanotoxicology has seen some revolutionary accomplishments, certain critical areas require attention and key challenges must be overcome to ensure continued advancement.

This milestone provides an excellent opportunity to look back at the last decade of nanotoxicology, celebrate the firm establishment of the field, evaluate the foremost research accomplishments, and diagnose current barriers. Major areas highlighted include the research initiative to link specific physicochemical properties to a resultant bioeffect, the development of enhanced NM characterization tools, and the generation of standardized protocols and reference materials. Moreover, this Review explores current challenges unique to nanotoxicology, such as developing accurate toxicological and characterization assessments, arriving at a consensus on the best dosimetry metric, improving *in vitro* models, and the lack of occupational exposure guidelines for NMs. Through addressing these obstacles, we believe, nanotoxicology will firmly cement its future research directions and ensure continued development.

THE RISE OF NANOTOXICOLOGY

In 2005, the International Life Sciences Institute Research Foundation/Risk Science Institute assembled a working group of experts with the goal of assessing the current state of NM toxicological knowledge and generating guidelines for the progression of this newfound field (Oberdörster *et al.*, 2005a). This working group established the original mission of screening for 'hazard identification' following NM exposure, and shaped the next few years of fundamental research which evaluated the toxic potential of simple nano-constructs, such as metal oxides and carbon structures (Braydich-Stolle *et al.*, 2005; Hussain *et al.*, 2005; Oberdörster *et al.*, 2005b). Another major result of the 2005 expert panel was that the primary routes of NM exposure were agreed upon and identified as oral, dermal, inhalation, and injection (Oberdörster *et al.*, 2005a), which drove the focus of *in vitro* and *in vivo* model selection to accommodate these areas of higher NM exposure.

Most importantly, this panel recognized that slight differences in physicochemical properties could elicit a unique bioresponse and led the call for NM characterization techniques and standard procedures. Initial challenges were faced in modifying standard characterization and toxicological procedures to account for the distinctive attributes associated with engineered NMs, such as their insoluble nature and agglomeration tendencies (Oberdörster *et al.*, 2005b; Hussain *et al.*, 2009). However, the initial hazard assessment, development of fundamental characterization tools, and recognition of appropriate cellular models established a solid framework for future innovation and the ability to accommodate more advanced engineered NMs.

ADVANCES IN CHARACTERIZATION TECHNIQUES

Due to the fact that NMs possess distinctive physicochemical properties in comparison to their bulk counterparts, developing advanced characterization procedures is necessary to accurately capture and quantitatively assess these parameters. To address that challenge, characterization equipment was developed and procedures optimized to monitor key NM properties, both as acquired stock materials and in biologically relevant environments. Nanotoxicology has helped drive this characterization revolution through the call for standardized procedures and in-depth assessment prior to inclusion in nano-based applications (Bouwmeester *et al.*, 2011; Warheit, 2008). However, even though characterization techniques have progressed greatly over the past decade, further improvements are necessary to accommodate newly developed and more advanced classes of NMs.

Initial NM Characterization

As no 2 batches of NMs contain identical physicochemical characteristics, extensive characterization has become a necessary practice following synthesis. Standard particle-based parameters evaluated during characterization include primary size, shape, surface charge, porosity, composition, and structure (Murdock *et al*, 2008; Richman and Hutchison, 2009; Sapsford *et al.*, 2011). From these material assessment values, other critical information can be ascertained including particle surface area, degree of agglomeration, and size distribution; all of which can influence cellular interactions. Several classes of equipment are critical in effectively characterizing NMs, including microscopy, spectroscopy, spectrometry, and light scattering devices. NM characterization techniques and measures are the focus of a number of review articles (Baer *et al.*, 2010; Lin *et al.*, 2013; Sayes and Warheit, 2009).

Evaluating the Nano-Cellular Interface

Full characterization goes beyond initial evaluation of specific NM physicochemical properties and includes assessing the degree and means of interaction with the surrounding cellular system; commonly referred to as the 'nano-cellular interface' (Nel *et al.*, 2009). Developing advanced and accurate ways to characterize the nano-cellular interface has posed a considerable challenge; however, advances have been made to identify intracellular fate and NM behavior *in vitro*. Experimental endpoints for interface evaluation include deposition efficiency, rates of NM internalization, final cellular location, degree of intracellular agglomeration, visualization of NM-cell binding patterns, and development of the protein corona (Table 1). These interactions have been strongly correlated to observed cytotoxicity, stress responses, and gene modulation (Wu *et al.*, 2013); making this interface a critical research focus. For example increased deposition efficiency directly correlates to the delivered NM dosage and resultant cytotoxicity (Cohen *et al.*, 2014). Similarly, studies have demonstrated that increased NM internalization leads to augmented cell stress and cell death (DeBrosse *et al.*, 2013; Dowding *et al.*, 2013). Moreover, it has recently emerged that the protein corona surrounding NMs is 'what the cell sees' and not only dictates the formation of the nano-cellular interface, but influences membrane interactions, deposition, mechanism of endocytosis, and stress reactions (Gunawan *et al.*, 2014; Monopoli *et al.*, 2012). Therefore, it is clear that characterizing the nano-cellular interactions is a critical step in the development of a fundamental understanding of nanotoxicology.

Current Characterization Challenges

To keep pace with the rapid development of synthesis and to expand understanding of NM behavior, new characterization

TABLE 1. Characterization Tools for the Nano-Cellular Interface

Behavior/Property	Tool	Reference
Deposition efficiency	ICP-MS	Mitrano *et al.* (2012)
Internalization	ICP-MS, TEM	Mitrano *et al.* (2012)
Cellular location	Microscopy, TEM	Braydich-Stolle *et al.* (2012)
Intracellular agglomeration	TEM, Hyperspectral Imaging	Stacy *et al.* (2013)
NM-binding Patterns	Microscopy	Darwiche *et al.* (2013)
Protein corona	MS, SDS-PAGE	Winzen *et al.* (2015)

tools and procedures need to be established. First, it would be advantageous to modify current standards, such as dynamic light scattering (DLS) and transmission electron microscopy (TEM), in order to perform with a higher sensitivity and in a more relevant, biological environment. Additionally, the presence of inter-particle interactions, which strongly influence NM behavior, are well documented and are known to be dependent on multiple factors, such as charge, surface moieties, core composition, particle concentration, and environmental factors (Min *et al.*, 2008; Nel *et al.*, 2009). Although complex thermodynamic models have been developed to predict these interactions as a function of inter-particle distance, no equipment exists to quantitatively and reliably assess these quantum forces.

One major limitation of assessments of the nano-bio interface is that they only record one moment in time. As this interface is a dynamic, ever-changing entity, these snapshots only provide a glimpse of how NMs interact within a cellular system and not a complete portrayal of their behavior (Alkilany *et al.*, 2013). Therefore, we believe, that to truly elucidate the multifaceted relationship between a physiological environment and NMs, enhanced, real time imaging techniques will need to be developed that can accommodate particles on the nano-scale. Expanding characterization mechanisms introduces the potential to generate new information that is currently impossible to ascertain with regards to both inter-particle and the nano-cellular interfaces.

UNIQUE CHALLENGES ASSOCIATED WITH THE NATURE OF NMs

As NMs possess distinctive physicochemical properties, establishing and validating metrics for toxicological analysis has encountered several technical challenges. Although traditional toxicology has well-established protocols in place (Derelanko and Auletta, 2014), the translation of these practices to nanotoxicology are typically less effective and yield greater experimental error, in part due to their insoluble nature which generates altered transport and toxicokinetic profiles. The following section of this Review highlights 2 of these current challenges: the need for standardizing analytical protocols and consideration for the sub-classification of carbon- and metal-based NMs.

Modification and Standardization of Toxicity Protocols

During evaluation, the use of standard toxicity assays for NMs may produce erroneous results due to interference arising from NM behavioral patterns including, an insoluble nature (Kumar *et al.*, 2012), the ability to agglomerate (Liu *et al.*, 2011), particle sedimentation (Cho *et al.*, 2011), unique forms of uptake and retention (Shete *et al.*, 2014), and the formation of a protein corona (Gunawan *et al.*, 2014). For example, NMs have been shown to interfere with the standard 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide viability assay, producing unreliable data unless additional controls are in place (Laurent *et al.*, 2012). The acknowledgement of this problem demonstrates the need to design, agree upon, and establish a standardized set of protocols specific for nanotoxicology, in order to increase the applicability and reliability of current and future efforts. The need for protocol reform has been acknowledged, with a number of international initiatives currently underway to research and optimize procedures for nanotoxicological evaluation (Love *et al.*, 2012; Oomen *et al.*, 2014).

Carbon Versus Metal NMs: Different Classes of Particles—Different Behavior

Further complicating the generation of standardized protocols is the fact that different classes of NMs, such as carbon-based versus metallic, behave dramatically different. Carbon nanotubes (CNTs) and metallic NMs are renowned for their intrinsic properties of high tensile/mechanical strength and exceptional thermal/electrical conductivity, respectively (Hopley *et al.*, 2014; Matejka and Tokarsky, 2014). The usage rate of CNTs is growing exponentially with applications including composites, paints, polymer films, and microelectronics (De Volder *et al.*, 2013). Nanotoxicological evaluation of CNTs is further hindered by dispersion and stability issues (Bouchard *et al.*, 2012). As shown in Figure 1, while CNTs and metallic NMs both possess dimensions on the nano-scale, they display dramatically different physical traits, which result in fundamental differences in NM behavior, the nano-cellular interface, and resultant bioeffects. For example, both metallic NMs and CNTs induce the production of reactive oxygen species (ROS); however, exposure to metal NMs leads to apoptosis while CNTs produce inflammation and fibrosis (Manke *et al.*, 2013). Increased apprehension over health concerns surrounds CNTs owing to increased biological persistence and non-degradability, similar to asbestos (Aschberger *et al.*, 2010). On the contrary, the primary concern associated with metal NMs is their rapid dissolution into ions, which disrupts cellular functions (Khan *et al.*, 2012; Maurer *et al.*, 2014). Currently, even more complex novel materials are being produced which have incorporate nanocarbon and nanometals, further complicating bio-exposure responses (Poulsen *et al.*, 2015; Zhang and Wang, 2007).

CORRELATING PHYSICOCHEMICAL PARAMETERS TO CYTOTOXICITY

From early nanotoxicological assessments, it became clear that cellular responses were strongly dependent on specific NM physicochemical properties (Podila and Brown, 2013; Sharifi *et al.*, 2012). Although the identification of this phenomenon was straightforward, the elucidation of these unique correlations has presented a major challenge. Advances in material science have allowed for the precise synthesis of NMs with tunable target parameters, including primary particle size,

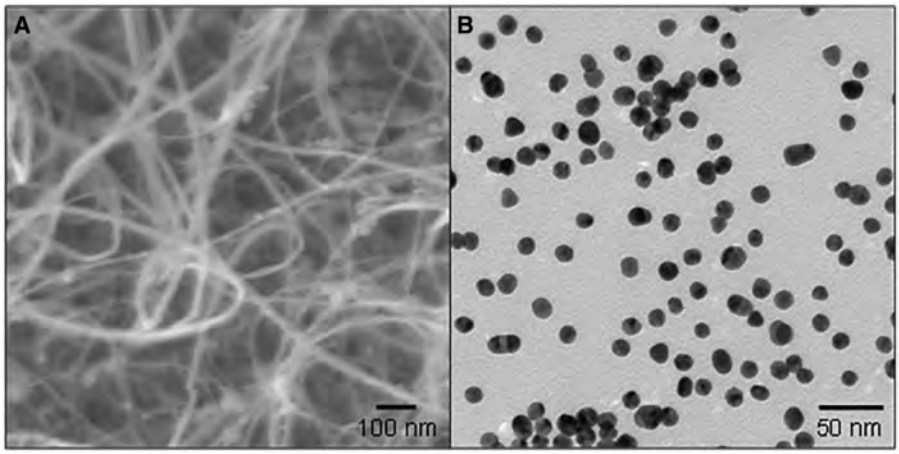

FIG. 1. Comparing the properties of CNTs and metallic NM. TEM images of (A) CNTs and (B) gold nanospheres, demonstrate the fundamental differences in the physical property of these classes of NMs. From these images the fundamental differences in structure are apparent, even though both are classified as NMs. These discrepancies are responsible for the differential responses observed following cellular exposure.

morphology, core material, and surface coatings, allowing for direct comparison. A succinct summary of what is currently known concerning target NM properties is presented in Figure 2. As the correlation of properties to toxicological outcomes is a critical area, there are a number of reviews focusing on this area (Aillon *et al.*, 2009; Fubini *et al.*, 2010; Rivera *et al.*, 2010).

Size

Primary particle size is arguably the most critical property pertaining to a nanotoxicological response, as it significantly influences both the mode and extent of cellular interaction. Previous studies have identified that the form of endocytosis, extent of NM internalization, and final intracellular fate are all dependent on particle diameter (Jiang *et al.*, 2008). Focusing specifically on cytotoxicity, the majority of studies have arrived at a similar conclusion: that smaller particles induce a higher degree of cell death (Haase *et al.*, 2011; Hsiao and Huang, 2011; Sohaebuddin *et al.*, 2010). Moreover, these results were mirrored by a size-dependent increase in ROS generation, loss of mitochondrial integrity, and increased secretion of pro-inflammatory cytokines. However, some exceptions have been noted, including zinc oxide, with no change in toxicity seen when NMs are compared with the micron sized counterpart (Warheit *et al.*, 2007).

Surface Chemistry

As a NM's surface coating is the driving force for both inter-particle interactions and formation of the nano-cellular interface, surface chemistry regulates the mechanism of cellular contact, degree of NM internalization, and resultant cytotoxicity (Chen *et al.*, 2011). As different surface moieties possess distinctive charges, numerous studies have examined the role of NM surface charge in cytotoxicity and stress responses. These studies have identified that positively charged NMs, independent of composition, are more efficiently internalized and generate an augmented toxic response over negatively and neutrally charged particles (Bhattacharjee *et al.*, 2010; Yu *et al.*, 2012; Zhu *et al.*, 2012). Efforts to elucidate a correlation between a specific surface chemistry and target bioeffects are currently underway, but the results are not as clear-cut as seen with charge. Currently, surface functionalization does not directly predict cytotoxicity, but many of the adverse NM implications can be mitigated or removed through coating processes. For example, surface chemistry influences the ionic dissolution rate (Bhattacharjee *et al.*, 2010), the kinetics of cellular internalization (Untener *et al.*, 2013), and observed particle stability, and behavior (Suntivich *et al.*, 2013). Due to these responses, surface modification is being explored as a means to elicit target bioeffects and ensure biocompatibility.

Composition

Although the addition of various surface coatings has been investigated to mask the influence of internal NM composition, the degree and mechanism of cytotoxicity can still be traced back to a NM's elemental core (Sohaebuddin *et al.*, 2010). Innate cytotoxic properties, the ability to disrupt chemical and biological molecule processes, and kinetic rates of ionic dissolution are all factors highly dependent on core material (Chang *et al.*, 2012; Hussain *et al.*, 2005). In general, these investigations identified certain elements, including Cu, Zn, Co, Ag, Ni, and Cd, as more cytotoxic than others, such as Au, Ti, and Fe. Recent reports have further explored the mechanism behind this composition dependent toxicity and identified a primary cause of death to be an augmentation of oxidative stress induced by the generation of ions from the NM surface (Xia *et al.*, 2008).

Morphology

Current nanotoxicology research efforts are focused on linking NM shape to a biological outcome. The knowledge gap pertaining to morphological dependence is because the ability to uniformly and consistently synthesize NMs of target shapes was only recently developed. Although a number of studies are beginning to emerge, there still exist a number of technical challenges and conflicting reports to consider before this effect can be fully clarified. It has been shown that spherical particles are internalized to a higher degree, in less time, and requiring less energy than rod or fiber shaped particles (Champion and Mitragotri, 2006). However, while nanorods may display reduced rates of cellular internalization, their larger contact area results in greater membrane and receptor interaction, which introduces a new set of unique cellular consequences to be explored (Lu *et al.*, 2010). For copper oxide NMs, the rate of ionic dissolution and degree of cytotoxicity varied between spherical, rod, and platelet morphologies (Misra *et al.*, 2014). As new NM morphologies are continually emerging, the dynamic, developing correlations between geometric shape and bioeffects

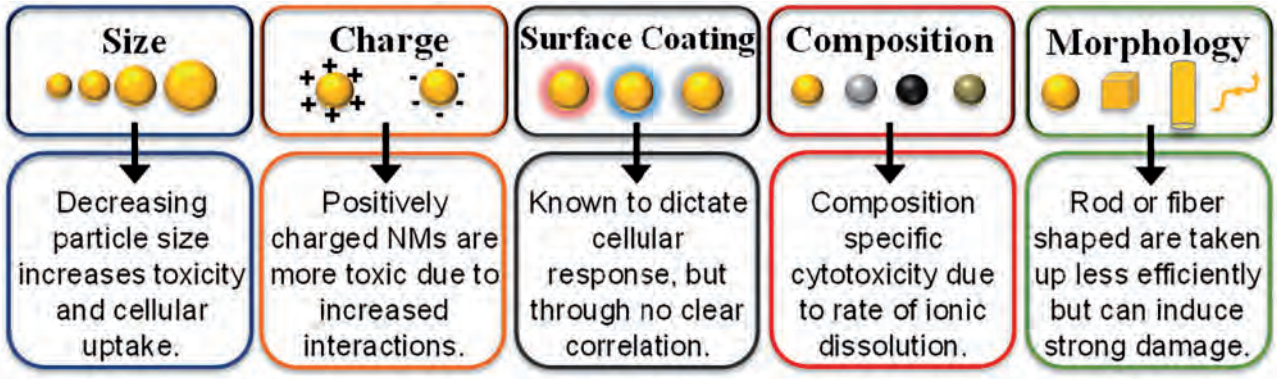

FIG. 2. Key physicochemical parameters of NMs and a summary of the current state of knowledge on how these properties influence nanotoxicity. Cellular responses are known to be dictated by specific NM properties. The most predominantly investigated include primary size, charge, surface chemistry, core composition, and morphology.

will be of tremendous importance for the design of nano-based applications.

MECHANISMS OF CYTOTOXICITY

In addition to identifying the NM properties that induce cytotoxicity, current investigations are underway to evaluate the mechanisms behind these responses. Results of these inquiries have found that excess production of stress response and modifications to protein and gene expression precedes cellular death (Carlson *et al.*, 2008; Nel *et al.*, 2006). Recent examinations have extended beyond stress and cytotoxicity to include the activation of immune and inflammatory reactions, modifications to gene regulation, alterations to protein expression and production rates, and alterations to signal transduction; all of which have been shown to be directly impacted by NM exposure (Braydich-Stolle *et al.*, 2010; Comfort *et al.*, 2011). For example, NM exposure resulted in altered cellular response to epidermal growth factor signal transduction, producing modified cellular behavior (Comfort *et al.*, 2011). In addition to activation of the stress response and production of stress-dependent proteins, NM exposure has resulted in modified gene regulation, in the areas of stress, toxicity, signal transduction, disease regulation (Comfort *et al.*, 2014a; Ng *et al.*, 2015; Sharifi *et al.*, 2013). The dramatic alteration to genetic and protein profiles have been shown to alter the phenotype and basal functionality of the cellular systems; demonstrating the far-reaching implications of non-toxic NM exposure. These pathways combine to generate a multi-layered cellular response that the scientific community is only beginning to piece together.

NM STANDARDS AND OCCUPATIONAL GUIDELINES

Generation of NM Standard Materials

The generation of NM reference standards was recently accomplished by the National Institute of Standards and Technology (NIST), with these standards serving as a benchmark for material comparison and method validation. Owing to the variability introduced by the physical and reactive properties of NMs, this was a tremendous achievement. In addition to undergoing a high degree of technical material scrutiny, these standards were specifically selected and designed to address current research needs, barriers to innovation, and utilization in nano-based applications. To date, the library of NIST nano-sized reference materials consists of polystyrene spheres of 60 and 100 nm, Au nanospheres of 10, 30, and 60 nm (Fig. 3), single wall CNTs, and 25 nm TiO_2, with Ag, CeO_2, and SiO_2 currently under evaluation.

Establishment of a Risk Assessment Framework

One utilization of standard materials is in the establishment of regulatory and occupational exposure limits (OELs), which are set in place by agencies such as OSHA and the National Institute for Occupational Safety and Health (NIOSH). Currently, limited occupational guidelines are established for NMs; however, due to the reactivity and energetic behavior of NMs their OELs will likely be orders of magnitude lower than their bulk counterparts. For example, nano-sized TiO_2 has a permissible exposure limits of 0.3 mg/m^3, whereas in bulk form that limit is 15 mg/m^3 (NIOSH, 2011). Several factors have contributed to this regulatory lag, including poor characterization, variable NM characteristics, irrelevant dosages and cell models, and conflicting reports on bio-responses. Taken together, these variations and limitations have made it unfeasible to set regulatory limits for NMs with any degree of confidence (van Broekhuizen *et al.*, 2012). However, in an effort to inform consumers, a Nanorisk framework was published in 2007 which described the development of a systematic process to assess environmental health and safety risks associated with exposures to products containing nanoscale materials (www.nanoriskframework.com).

Furthermore, a workshop was recently conducted to improve the development and evaluation of OELs for engineered NMs (Gordon *et al.*, 2014). Given the limited amount of available data in this area, one focus of this workshop was to identify current practices for establishing OELs from various stakeholders in an attempt to develop novel and alternative approaches. Based upon comparative results of intratracheal instillation studies with TiO_2 NMs, a bridging approach that incorporated subchronic and long-term studies was suggested as a means to estimate OELs (Warheit, 2009). Following the conclusion of this workshop, the major outcomes were made available to the scientific community, including a summary of the findings of bridging strategies for OEL development, and the identified future research areas (Warheit, 2013).

PRESENT CHALLENGES

Throughout this Review, we have touched upon several contemporary limitations facing the field of nanotoxicology. For

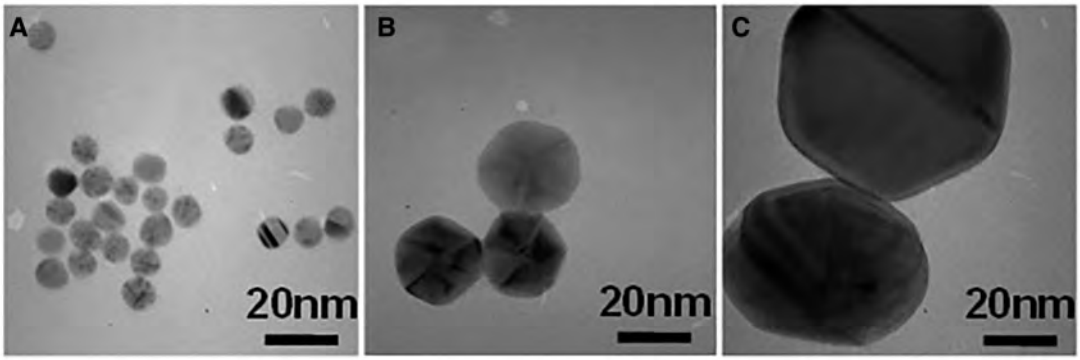

FIG. 3. Representative TEM images of NIST nanogold reference materials of primary particle size A, 10 nm; B, 30 nm; C, 60 nm. The development of standardized NMs is of great advantage to nanotoxicology, as it provides a benchmark material with well-documented physicochemical characterization and detailed biological responses following exposure.

example, we discussed the challenges with NM characterization and the need for advanced assessment tools which are accurate at lower concentrations and in a physiologically relevant fluid. Additionally, even though the development of toxicity protocols has greatly progressed, particle interference and unique challenges associated with NMs, remains a leading cause of erroneous results and conflicting conclusions. The limitations brought on through characterization and analysis challenges have severely restricted the ability to establish OELs for most NMs. These are all areas that we anticipate significant forward progress to be made in the near future.

Recently, the National Academy of Sciences Committee emphasized the critical need for development of reliable and validated screening tools to elucidate toxicity pathways of complex systems and address mechanistic issues (National Research Council, 2013). Accordingly, a current challenge is the design of simple, *in vitro* models that reliably predict *in vivo* effects following a NM challenge. In order to increase the relevance of *in vitro* studies, current study experimental design protocols require development of a new and expanded framework to include the following: (1) rigorous NM characterization and estimation of behavior throughout the life-cycle; (2) dose-response behavior at relevant human exposure levels, applied with appropriate dosemetrics; (3) selection of cell models that accurately reflect routes of NM exposure; (4) time course assessments that span from acute to chronic durations; and (5) utilization of proper benchmark controls to improve interpretation of study outcomes. Finally, to address this long-term goal, it will be critical to develop high throughput *in vitro* screening mechanisms that integrate relevant exposure routes, plausible NM dosages, and appropriate *in vivo* comparisons for validation. Therefore, the following sections will focus on the major challenges of implementing a NM dosimetry metric and improving *in vitro* to *in vivo* correlations.

The Question of NM Dosimetry

Even though numerous nanotoxicological studies have been published, development of well-characterized NM dosimetry remains a controversial topic. The fundamental rule in toxicology is that the magnitude of a biological response directly correlates with dose and time; meaning with high enough concentrations everything can be toxic (Johnston *et al.*, 2013). Therefore, understanding NM dosimetry is critical for properly interpreting results and developing NM risk assessments and OELs. Dosimetry parameters under consideration include target dose, exposure methodology, dose characterization, and dose metric. Proper terminology and consistency in experimental design is essential to avoid misconception and to generate reproducible results. For example, in *in vitro* studies, the term dose describes the quantity of NMs that reaches the biological target, whereas the expressions, treatment or exposure are more appropriate to describe the amount initially input into the system.

The selection and standardization of a dose metric has been widely debated; with possible dosing approaches including mass, surface area, and particle number. Currently, many studies employ dosimetry based on a mass per volume concentration, such as μg/ml, which introduced inconsistencies when results are compared across different cell culture conditions. However, many have presented data to argue that for NMs, the most appropriate metric for comparing biological effects is based on equivalent, exposed surface area (Duffin *et al.*, 2007; Donaldson *et al.*, 2008; Monteiller *et al.*, 2007; Waters *et al.*, 2009). Although there is no standard metric agreed upon by the community as a whole, it is safe to conclude that each metric must be characterized or calculated, so this information is available as the nanotoxicology field continues to develop. Moreover, it is also critical that NM exposure concentrations, regardless of dosimetry metric, be normalized by the total surface area of cells or tissue.

When designing a nanotoxicological study, the target dose and exposure range must be carefully considered and take into account both real world concentrations and available dosimetry models. The deposition efficiency, or the fraction of NMs expected to reach the target of interest is a critical variable for experimental consideration. NM and chemical dosimetry are fundamentally different due to the modified transport potential and gradients associated with the insoluble nature of NMs. This fact greatly increasing the complexity of predicting and estimating the delivered dose following a NM challenge. Traditional *in vitro* models require NMs to be dispersed in biological media which induces agglomeration and adsorption of biomolecules (Grabinski *et al.*, 2011; Lundqvist *et al.*, 2008; Mukhopadhyay *et al*, 2012); resulting in modification to the diffusive and sedimentation forces and overall transport of the NMs (Cho *et al.*, 2013; Teeguarden *et al.*, 2007). A recent study demonstrated more predictable dosimetry using and inverted exposure scenario, in which NMs were only deposited through diffusion, thus removing the variable of agglomerate density and sedimentation (Cho *et al.*, 2011). Although this approach

verified the presence of inconsistencies introduced by NM agglomerates, this set-up is not feasible for all cellular type evaluation. An alternate approach to reduce sedimentation is to introduce NMs to a cell culture under dynamic flow conditions, which results in diffusion-driven deposition of NMs (Toy *et al.*, 2011; Ucciferri *et al.*, 2014).

In vitro systems should also be optimized to represent a target exposure route, as this will affect dosimetry and resultant deposition metrics. For example, NMs exposed through an inhalation method occurs in the gas phase, therefore, an appropriate *in vitro* model to mimic inhalation should deliver NM aerosols to cells grown at an air-liquid interface. Due to the small size and low mass of nano-sized aerosols, designing exposure systems for dosing cells grown at the air-liquid interface has presented a unique challenge (Aufderheide, 2005). However, with the use of an external force, such as electric field or temperature gradient, air-liquid interface chambers which maintain optimal cell conditions have been successfully generated, thus allowing for an environment that more accurately mimics a physiological lung exposure scenario (Grigg *et al.*, 2009; Saffari *et al.*, 2012; Volckens *et al.*, 2009).

Great progress has been made in both understanding and predicting NM dosimetry through the theoretical *in vitro* Sedimentation and Diffusion Deposition (ISDD) model (Hinderliter *et al.*, 2010; Khanbeigi *et al.*, 2012). Assumptions in the ISDD mathematical model include a static upright system, spherical NM morphology, and uniform agglomerate size and density. Agglomerate density is approximated from an assumed fractal dimension, which is a dimensionless number describing the packing density of NMs. As limited techniques are currently available for measuring the fractal dimension of NM agglomerates in biological media, it is necessary to validate these theoretical estimations by measuring the NM dose. In addition to the success of the ISDD system, other models are being developed and implemented, such as the Multiple-Path Particle Dosimetry model, which specializes in approximating inhalation dosimetry and deposition (Cassee *et al.*, 2002).

Even beyond the challenges already presented, dosimetry is further complicated by additional situations uniquely associated with NMs. For example, soluble/semi-soluble NMs, such as copper and silver, readily oxidize and release ions over time introducing a second means of interaction with the cells (Comfort *et al.*, 2014b). Characterizing the rate of ion release and effective ion and NM exposure rates has becomes essential for nanotoxicological studies (Han *et al.*, 2012). Moreover, correlating *in vitro* to *in vivo* dose poses a considerable challenge as degree of agglomeration and behavior of NMs varies significantly between cell culture media and physiologically fluids (Maurer *et al.*, 2014). By altering the properties and behavior of NMs, these environmental-specific discrepancies will undoubtedly affect both NM transport rate to a target tissue or organ and received dose. Taking all these NM-dependent variables under consideration is not surprising that the establishment of dosimetry standards and exposure guidelines have presented major obstacles. Regardless of the many challenges inherently associated with NM dosimetry, a key takeaway message from this Review is that experimental designs must include detailed dosimetry and characterization aspects in order to support further progress towards goals in nanotoxicology and developing risk assessment frameworks.

Transitioning from Validated In Vivo *to* In Vitro *Studies*

When assessing the influence of a NM on a physiological system, the most accurate and predictive data will of course be obtained through *in vivo* analysis. However, several intrinsic drawbacks are associated with the utilization of *in vivo* models, including high cost, extended duration, ethical concerns, and an inability for high-throughput processing. Due to these constraints, as well as the sheer number of particles that require screening, *in vitro* assessment is currently the primary means of evaluating the safety and behavior of NMs (Arora *et al.*, 2012). However, to date the validity of *in vitro* analyses remains disputable due to a considerable gap between collected *in vitro* data and accurate *in vivo* predictions (Han *et al.*, 2012; Rushton *et al.*, 2010; Sayes *et al.*, 2007). Extensive work has been performed on the material side to improve this correlation through more efficient and reproducible synthesis procedures; however, a NM-based approach to this problem is contingent upon the yet unresolved question of dosimetry (Zhang *et al.*, 2012). *In vitro* models remain critical to the field of nanotoxicology, and are necessary to serve as a quick-screening procedure of shorter duration to assess immediate hazards associated with the continuous generation of new, innovative classes of NMs. Contrary to focusing on the material side of this challenge, we believe that significant progress on overcoming this correlation gap can be achieved through enhancement of the *in vitro* model design and exposure techniques.

Perhaps one of the most significant decisions for *in vitro* studies is the relevance of the cell model under investigation. Many NM studies have been performed on cell lines that are not representative of a primary NM route of exposure or even significant secondary targets such as liver of kidney models. Owing to the fact that tissues and organs are multicellular by nature, include immune system elements, have 3 spatial dimensions, and exist within a number of different and complex physiological fluids, perhaps the best way to bridge the *in vitro* to *in vivo* gap is to design cell models that are closer in nature to tissue/organ systems. System modifications can be introduced in order to achieve a more relevant *in vitro* model, including the development of co-culture models that incorporate proper immune cell lines (Braydich-Stolle *et al.*, 2010; Kasper *et al.*, 2011), design of 3D cellular systems (Hoelting *et al.*, 2013; Kim *et al.*, 2010), inclusion of physiological relevant fluids (Braydich-Stolle *et al.*, 2014; Comfort *et al.*, 2013), and extended durations to evaluate chronic exposure (Comfort *et al.*, 2014a). Not only does the introduction of these modifications bring an *in vitro* model closer to an *in vivo* system, but they allow for the evaluation of NM behavior and characterization as a function of time in a more realistic physiological environment. For example in a 3D culture, cellular exposure to NMs is predominantly dependent upon the transport capabilities of the particles into the cellular mass; similar to nano-sized objects traveling throughout a body (Hoelting *et al.*, 2013; Kim *et al.*, 2010). Moreover, when dispersed in biological fluids, as opposed to media, NMs were found to behave substantially different with modified agglomeration tendencies, rates of ionic dissolution, and cellular internalization (Braydich-Stolle *et al.*, 2014; Comfort *et al.*, 2013). Although each of these cell model modifications has been successfully utilized, the incorporation of these new variables each introduces unique challenges. Furthermore, there simply exist some *in vivo* scenarios that are impossible to perfectly replicate in a cellular model. When transitioning from primary cell types collected from animals to immortalized cells derived from cell lines, it requires considerations including the cell ratio in a co-culture and their biological functionality in simulating the relevant defense responses such as phagocytosis and inflammation. Therefore, the design and implementation of an enhanced cellular microenvironment that encompasses all

these elements, while possible, is a major undertaking and will require considerable time and resources to not only generate but optimize.

As previously discussed, designing an *in vitro* system to mimic exposure routes will improve both dosimetry and toxicity predictions. As inhalation is one of the primary routes of NM introduction, the development of an accurate exposure mechanism would provide better *in vitro* to *in vivo* correlations. Lung exposure is of great interest as previous studies have demonstrated a lack of clearance following inhalation, suggesting long term nanotoxicological implications may be a major concern (Moller *et al.*, 2008). During inhalation, particles remain dispersed in air, and arrive at the alveoli without encountering a fluid. Therefore, traditional 'submerged' cell culture and exposure systems would fail to accurately capture this exposure route and introduce extreme variability as NMs incur significant alterations to their characteristics and behavior in solution (Liu *et al.*, 2011; Murdock *et al.*, 2008). To overcome this challenge, scientists have designed aerosol chambers to expose cell cultures at the air-liquid interface which feeds cells from underneath of the supporting microporous membrane and allows deposition of airborne NMs in a conditioned environment, thus more accurately mimicking lung exposure scenarios. Multiple laboratories have built aerosol exposure chambers or modified commercially-available chambers based on needs, including particle type, size, dosage, gravimetric/electrostatic deposition capabilities, and toxicological endpoints (Huh *et al.*, 2010; Jeannet *et al.*, 2015; Xie *et al.*, 2012). However, as these exposure chambers can be highly specific in design and in operating procedures, reproducible, and quantifiable dose metrics are critical for particle deposition assessments and for comparisons of results. Furthermore, current NM literature-consists primarily of acute exposure studies with a limited set of endpoints. To truly elucidate NM behavior and resultant consequences in a biological system, these time points need to be carried out on a longer time scale. Once cell-based systems have been optimized to incorporate these fundamental parameters, a profound increase in the predictive capability of *in vitro* system should transpire.

Design of High-Throughput In Vitro Models for Nanotoxicological Evaluation

As previously discussed, new and novel classes of engineered NMs are being exponentially produced. Furthermore, to truly evaluate the safety of NMs, a large number of experimental analyses must be carried out, including cytotoxicity, stress activation, immune response, modulation of protein and gene production, and characterization of the nano-cellular interface. Examining these 2 statements together, it becomes apparent that to keep up with the pace of NM development and fully identify safety concerns, a rapid, high-throughput screening approach is needed (Macarron *et al.*, 2011). Due to the time constraints associated with *in vivo* experimentation, a high-throughput design for NM evaluation must utilize an *in vitro* model (Arora *et al.*, 2012). To ensure that a developed, high-throughput system can be accurately extrapolated to *in vivo* predictions, it should contain variables to improve basic cell models, as previously discussed in this Review. One emerging technology for rapid assessment of NMs is an engineered microfluidic chip with perfused chambers that incorporates cellular models (Valencia *et al.*, 2012; Watson *et al.*, 2014). Due to the ability to engineer key design aspects, such as the simulation of cell to cell interactions, the introduction of dynamic flow, the ability to apply cell patterning, the addition of porous substrates, and the option to incorporate 3D growth, microfluidic devices have been shown to simulate a more relevant physiological environment than standard *in vitro* cultures (Bhataia and Ingber, 2014); potentially offering a forward direction for the rapid safety evaluation of NMs.

CONCLUSIONS

Nanotoxicology has witnessed some truly revolutionary advances that resulted in a vast transformation of the field in recent years. Investigations now go well beyond the early question of 'Do NMs induce a cytotoxic response?' and focus on the mechanisms behind these cellular implications and modifications to the biological system in the absence of cellular death. New synthesis and characterization technique standards have made it possible to correlate specific physicochemical properties to observed responses, investigate the behavior and interactions of NMs in a cellular environment, and allow for the generation of reference materials. However, amidst this progress, major limitations are still plaguing the field today. Future efforts should focus on the continued improvement of characterization and synthesis to ensure the highest degree of uniformity within a NM set, the establishments of dosimetry guidelines and procedures, the improvement of cell-based models to improve behavior and evaluation, and the generation of risk assessment metrics for nano-size materials. With new innovative resources continually arising, we are confident that these challenges will not only be met with resolve, but overcome, bringing about continuing transformations and advancements in the coming decade of nanotoxicology research.

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