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REVIEW

Particokinetics *In Vitro*: Dosimetry Considerations for *In Vitro* Nanoparticle Toxicity Assessments

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The rapid growth in the use of *in vitro* methods for nanoparticle toxicity assessment has proceeded with limited consideration of the unique kinetics of these materials in solution. Particles in general and nanoparticles specifically, diffuse, settle, and agglomerate in cell culture media as a function of systemic and particle properties: media density and viscosity and particle size, shape, charge and density, for example. Cellular dose then is also a function of these factors as they determine the rate of transport of nanoparticles to cells in culture. Here we develop and apply the principles of dosimetry *in vitro* and outline an approach for simulation of nanoparticle particokinetics in cell culture systems. We illustrate that where equal mass concentrations (μg/ml) imply equal doses for dissimilar materials, the corresponding particle number or surface area concentration doses differ by orders of magnitude. More importantly, when rates of diffusional and gravitational particle delivery are accounted for, trends and magnitude of the cellular dose as a function of particle size and density differ significantly from those implied by "concentration" doses. For example, 15-nm silver nanoparticles appear ~4000 times more potent than micron-sized cadmium oxide particles on a cm^2/ml media basis, but are only ~50 times more potent when differences in delivery to adherent cells are considered. We conclude that simple surrogates of dose can cause significant misinterpretation of response and uptake data for nanoparticles *in vitro*. Incorporating particokinetics and principles of dosimetry would significantly improve the basis for nanoparticle toxicity assessment, increasing the predictive power and scalability of such assays.

Key Words: **nanomaterial; kinetics; dosimetry; *in vitro*; risk assessment; settling; agglomeration; diffusion.**

INTRODUCTION

Developing testing strategies that can meet the burgeoning demand to characterize the hazard potential of the considerable number of nanomaterials that have been or will be produced is one of the most significant challenges faced by the regulatory, research, and producer communities. *In vitro* studies, which have become an essential component of risk assessment–directed research paradigms for chemicals, pharmaceuticals, consumer products, and fine and ultrafine particulates, are an essential element of all tiered approaches for toxicity assessment of nanomaterials that have been proposed (Holsapple *et al.*, 2005; Nel *et al.*, 2006; Oberdorster *et al.*, 2005). Their inclusion reflects their evolution from systems principally for evaluating mechanism of toxicity to high-throughput systems for rapid and cost-effective screening of hazards posed by new products and environmental chemicals (Bakand *et al.*, 2005; Eisenbrand *et al.*, 2002).

Despite the considerable attention *in vitro* systems have received and their growing application to nanomaterial toxicity assessment (Braydich-Stolle *et al.*, 2005; Hussain *et al.*, 2005), little attention has been devoted to a critical examination of their suitability, particularly when it comes to particle solution dynamics and dosimetry. In contrast to soluble chemicals, particles can settle, diffuse, and aggregate differentially according to their size, density, and surface physicochemistry. These processes are expected to significantly affect the cellular dose. The definition of dose for nanoparticles in an *in vitro* system is therefore more dynamic, more complicated, and less comparable across particle types, than it is for soluble chemicals. Thus, there is a need to develop a more complete understanding of these processes, how particle and media characteristics affect them, and their potential impact on cellular dose *in vitro* before dose-response assessment for nanomaterials can be conducted adequately.

The purpose of this manuscript is to improve the basis for *in vitro* assessment of nanoparticle toxicity by advancing the understanding particle solution dynamics in cell culture media as they relate to dosimetry and dose-response assessment. We develop these ideas by introducing the concept of cellular dose *in vitro* as an important dose metric and by integrating aspects of material science, solution physics, and kinetics to present the factors and processes affecting the cellular dose for particles. The impact of improved *in vitro* dosimetry is illustrated in several examples including reinterpretation of published dose-response

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data. We conclude with a review of the experimental methods for measuring the dose *in vitro* and an outline for a complementary computational approach to *in vitro* nanomaterial dosimetry.

CHEMICAL DOSIMETRY: BASIC CONCEPTS

A fundamental principle of pharmacology discovered through efforts to develop a standard scale of chemical activity that transcends biological systems is that response is proportional to the concentration of the affecter molecule at the site of action (Hardman and Limbird, 2001). The dose-response paradigm for the field of toxicology is similarly predicated on this principle. The use of target tissue dose, rather than less specific measures of dose such as exposure or administered dose, has been shown to improve correlations between dose and response for drugs, chemicals, and inhaled gases and particles (Brown *et al.*, 2005; Schroeter *et al.*, 2006; Treinen-Moslen, 2001; Witschi and Last, 2001). Thus, there is high confidence in the use of target tissue dose for *in vivo* dose-response assessment, and it has become the gold standard for dose-response assessment in pharmaceutical safety assessment and chemical risk assessment (NRC, 1994).

Target tissue dosimetry is also an important but largely ignored aspect of the *in vitro* dose-response paradigm for industrial chemicals and pharmaceuticals. The target tissue/site in *in vitro* cell culture systems is the cell, or cellular targets such as receptors (Teeguarden and Barton, 2004), rather than a tissue remote from the site of administration. The prevailing assumption for *in vitro* systems is that the nominal media concentration of a test material is proportional to the cellular dose and is therefore a good measure of dose at the target site. This assumption is reasonably accurate for soluble chemicals when saturable processes such as metabolism and active transport do not influence cellular concentrations. Dose-response differences between two test chemicals can therefore be attributed to factors other than target tissue/site dose such as metabolism, partitioning, potency, efficacy, or the characteristics of its binding to a receptor. This paradigm has been widely and successfully used to assess the relative potency of drug candidates and the relative toxicity of industrial and environmental chemicals (Allen *et al.*, 2005; Bakand *et al.*, 2005; Eisenbrand *et al.*, 2002; Padron *et al.*, 2000). It has also been used to assess the relative potency of particulate matter, which is the subject of this analysis (Bakand *et al.*, 2005).

A NEW PARADIGM FOR PARTICLE DOSIMETRY *IN VITRO*

The factors and processes controlling cellular dose for particles, including nanoparticles, are different than for chemicals. Particles are affected by their solution dynamics: They settle, diffuse, agglomerate, and change surface charge/chemistry over time in solution, changing the nature of the particles and their transport to cells. These processes are affected by the properties of the particles themselves (e.g., size, density, and surface chemistry) as well as the solution (viscosity, density, presence of proteins, etc.). The definition of dose for nanoparticles in an *in vitro* system is thus more dynamic, more complicated, and less comparable across particle types, than it is for chemicals. For instance, particles of different size and density settle at different rates and these differences equate to differences in transport to adherent cells in culture. The extent of these differences and their impact on the toxicity assessment of particles in general, and submicron particles specifically, is not widely appreciated. Nonetheless, it is clear that a new paradigm for particle and nanomaterial dosimetry *in vitro*, one that accounts for these differences and focuses on the cellular dose rather than exposure, is needed if *in vitro* assays are to play an important role in hazard assessment for nanomaterials. Developing this approach will require a more systematic assessment of the differences between chemicals and particles in solution as they relate to differences in approaches for dosimetry *in vitro*. The remainder of this Forum Article is devoted to developing the necessary principles, illustrating their application and demonstrating their importance to developing a deeper quantitative understanding of the cellular-level response to nanoparticles.

PRINCIPLES OF DOSIMETRY FOR NANOMATERIALS

Multidimensional Aspects of In Vitro *Nanomaterial Dosimetry*

Chemical dosimetry *in vitro* is a problem of two dimensions: amount and time (duration and timing). Standard *in vitro* dose metrics such as concentration and area under the curve (AUC), reflect this dimensionality. Chemicals in solution have physicochemical properties (lipophilicity, solubility, etc.) that are typically well known and unchanged in solution. Chemicals do not have macroscale physical characteristics (e.g., shape and surface chemistry) that affect delivery, as nanoparticles do; thus, chemical concentration and the duration of exposure provides sufficient information on dose to compare response across a wide variety of chemicals and experimental conditions. In contrast, dosimetry for nanoparticles *in vitro* is a problem of multiple dimensions, including not only amount and time but also particle characterization: physical characteristics (e.g., size, shape, and agglomeration state), core particle, and surface chemistry. Of all these aspects, the physical and chemical characteristics have received the most attention (Oberdorster *et al.*, 2005). This initial emphasis was appropriate because few conclusions regarding the comparative toxicity of nanoparticles can be made without a clear understanding of the physical and physicochemical characteristics of the material. Oberdorster recently assessed the related issues of material characterization and dosimetry *in vitro* (Oberdorster *et al.*, 2005), presenting a characterization/dosimetry prioritization scheme focusing on

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characterization of the most important material properties. While physicochemical characterization is critically important, it should not be equated to dosimetry.

The other aspects of dosimetry *in vitro*, time and amount, have not been the subject of as thorough an analysis. That the time and amount dimensions are especially important for particles is easily demonstrated. Larger, denser particles are delivered to cells *in vitro* more rapidly and more completely over shorter durations than smaller, less dense particles. These differences in transport would be expected to impact the magnitude and timing of cellular responses (Fig. 1). Thus, there is a need to extend the paradigm for *in vitro* particle and nanomaterial dosimetry to these other elements and develop appropriate dose metrics for nanomaterials that are consistent with their unique characteristics and behaviors.

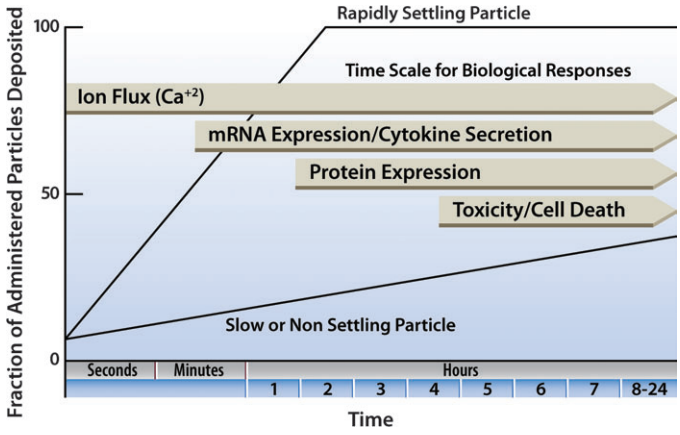

FIG. 1. Illustration of the influence of particle transport rate on cellular dose *in vitro* as a function of time overlaid with the time scale for common measures of biological response. Both the rate and extent of transport of nanoparticles vary with settling and diffusion rate and can differ significantly for test materials within the time frame of typical *in vitro* experiments. For instance, monodisperse 100-nm gold nanoparticles will settle completely in less than 1.5 h from media 1–2 mm deep.

Defining Dose for Nanoparticles In Vitro

Dose for nanoparticles *in vitro* can be defined at various levels of specificity with regard to the site of action and mode of action, reflecting administered dose at the most nonspecific level, apparent exposure at a more specific level, or cellular dose at the most specific (Fig. 2). Nominal media mass, surface area, or number concentrations are nonspecific metrics of dose (Fig. 2), better referred to as exposure. These are the principal metrics currently in use or proposed for use in conducting *in vitro* dose-response assessments (Braydich-Stolle *et al.*, 2005; Fubini *et al.*, 2004; Gurr *et al.*, 2005; Hussain *et al.*, 2005; Oberdorster *et al.*, 2005; Sayes *et al.*, 2006; Stringer *et al.*, 1996). These dose/exposure metrics are used chiefly because they are straightforward to calculate from material characteristics and experimental conditions and are easy to transform using the following relationships:

$$\text{Surface area concentration} = \frac{\text{mass concentration}}{\text{particle density}} \cdot \frac{6}{d} = \#\,\text{concentration} \cdot \pi d^2, \quad (1)$$

$$\#\,\text{Concentration} = \frac{\text{mass concentration}}{\text{particle density}} \cdot \frac{6}{\pi d^3} = \frac{\text{Surface area concentration}}{\pi d^2}, \quad (2)$$

where particles are assumed to be spherical, or can be represented as spheres, and d is the particle diameter in cm, surface area concentration is in cm^2/ml media, mass concentration is in g/ml media, # indicates particle number, and particle density is in g/cm^3.

In general, these metrics (surrogates) of dose are expected to poorly reflect the cellular dose. Cells respond to materials they come in contact with (delivered dose) or subsequently internalize, not to materials that remain suspended in the media over the course of an experiment. For example, membrane receptors on macrophages and epithelial cells commonly used *in vitro* mediate particle uptake and cellular response, such as the release of inflammatory markers (Becker *et al.*, 2002; Chen *et al.*, 1997; Iyer *et al.*, 1996). The macrophage membrane toll-like and scavenger receptors are two such receptor systems shown to mediate the inflammatory response to particulate matter (Inoue *et al.*, 2006; Obot *et al.*, 2002). Mass, surface area, or number of particles *delivered* to the sites of these receptors on the cell surface, or the corresponding AUC, better reflect dose at this site of action than nominal media concentration. The delivered dose also has the advantage of being directly scaleable and comparable to metrics of dose commonly used for particulates *in vivo*; for example, the delivered dose per surface area of cells in culture can be compared with the dose delivered/surface area of respiratory tract tissues. Intracellular sites of action such as the endosome, lysosome, or phagolysozome are best represented by more specific metrics of dose such as internalized mass, surface area, or number of particles or their amounts in specific intracellular compartments (Fig. 2). These dose metrics have the advantage of accounting for size or other particle-dependent differences in cellular uptake and can also be tailored to a mode of action, though in practice, they are difficult to measure.

Particokinetics In Vitro*: Processes Affecting Cellular Dose*

The processes, media properties, and characteristics of particles that impact transport of nanoparticles to cells *in vitro* can be organized broadly in three groups: those that affect diffusion, those that affect gravitational settling, and those that affect agglomeration. Table 1 summarizes these factors and contrasts their relevance for materials in the nano and supra-nano domains. Collectively, diffusion, gravitational settling,

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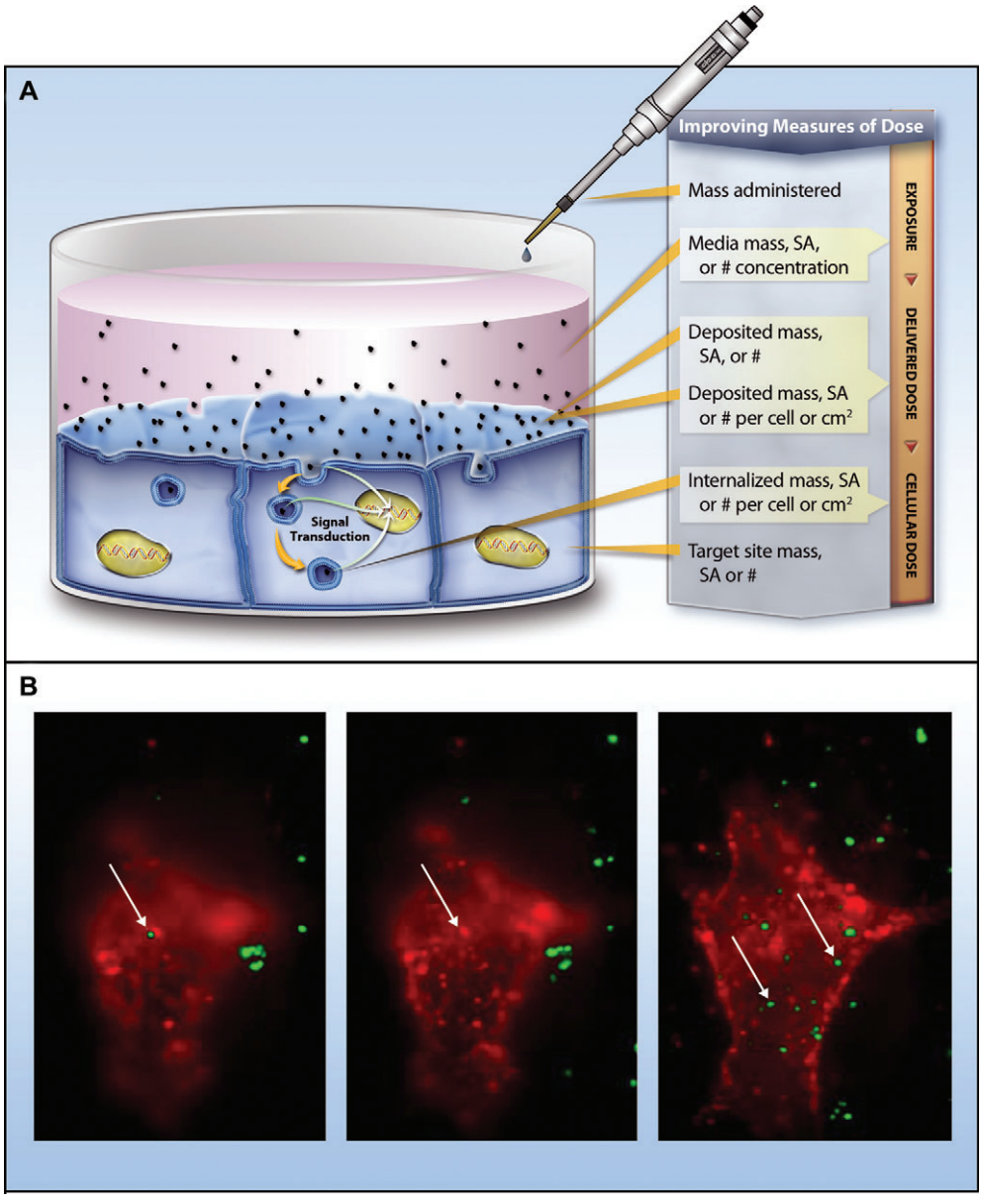

FIG. 2. Dose for nanoparticles *in vitro* increases in specificity and relevancy as dose measures move from administered amount to amount delivered to cells or internalized by cells (Panel A; SA, surface area; "#", particle number). Images (Panel B) of an alveolar epithelial cell (C10), grown in culture and exposed to fluorescence-tagged 500-nm amorphous silica particles, demonstrate the principles shown in A. The cell membrane is marked (red) by a membrane-specific fluorescent marker (wheat germ agglutinin), which was also internalized over time as an integral part of endocytotic vesicles. Panel B1 illustrates delivered dose: a silica particle (green) on the apical surface of the cell. The particle is no longer visible as the focal plane moves into the interior of the cell (Panel B2). Silica particles taken up into the cell are observed as the focal plane moves farther into the interior of the cell (Panel B3).

and agglomeration represent the solution dynamics of particles, which we call particokinetics here to distinguish our focus on dosimetry and kinetics of transport from the fluid dynamic and physical chemistry focus of the field of colloidal particle solution dynamics. These processes and the factors that affect processes are described below. Cellular-level processes that affect uptake or response are not considered here, but have been the subject of other reports (Chithrani *et al.*, 2006; Limbach *et al.*, 2005; Moss and Wong, 2006).

Diffusion is the spontaneous, passive movement of particles from areas of high chemical potential to low chemical potential. There is no net transport by diffusion in systems at equilibrium. Particles in cell culture systems are not necessarily in equilibrium. Particles adhere to cells or are taken up by cells, creating a concentration gradient in the unstirred layer immediately above the cells. This gradient drives diffusional transport of particles. Rates of diffusional transport are a function of particle size and the viscosity of the media; smaller particles diffuse

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TABLE 1
Particle and Media Characteristics Affecting Delivered Dose *In Vitro*

	Size (nm)		Affect on Nanoparticle Transport	
	< 1000	> 1000	Diffusion	Gravitational settling
Material property				
Size	±	+	↓ With ↑ diameter	↑ With square of diameter
Shape	±	+	Uncertain	Spheres most efficient
Density	±	+	—	↑ With density
Surface chemistry	+	+	Agglomeration[a]	Agglomeration
Zeta potential[b]	+	+	Agglomeration	Agglomeration
Concentration	+	+	Agglomeration	Agglomeration
Media property				
Density	±	+	—	↓ With ↑ media density
Viscosity	±	+	↓ With ↑ viscosity	↓ With ↑ media viscosity

[a]Agglomeration refers to affects on diffusion and gravitational settling that are secondary to changes in size and shape due to agglomeration.
[b]A measure of particle charge.

more rapidly than large particles. Most other media and particle characteristics (charge and surface chemistry) do not affect diffusional transport directly. Diffusional transport can be estimated from the diffusion coefficient (D, cm^2/sec):

$$D = \frac{RT}{N6\pi\mu d}, \tag{3}$$

where R is the gas constant (8.314 J/K/mol), T is temperature (K), N is Avogadro's number, μ is the solution viscosity (kg/m/sec), and d is the particle diameter (m). Calculated diffusion coefficients for a range of particle sizes are shown in Table S1 (Supplemental Data). Note that D is an inverse function of particle size and is not a function of particle density. The time required to diffuse a given distance in one dimension can be calculated from:

$$t = \frac{\langle \overline{r^2} \rangle}{2D}, \tag{4}$$

where $\langle \overline{r^2} \rangle$ is defined as the root mean squared distance or the distance that the average particle will travel (Bergqvist *et al.*, 1987; Einstein, 1905).

From Equation 4, it is apparent that defining a "diffusion velocity" is not straightforward. The time to diffuse a specified distance was found by Einstein to be inversely related to the square of the distance. For this reason, we present mean diffusion times for particles to diffuse a specific distance (i.e., 1 mm, ~1/2 the media depth of typical cell culture experiments) (Fig. 3), while remembering that this is only a mean time and some particles will take markedly less or more time to reach the cells. Since the diffusion coefficient is inversely related to particle size, delivery by diffusional transport is less important for larger particles (above ~100 nm); the time for a 1000-nm particle to diffuse 1 cm is ~3 years compared with only 1 day for 1-nm particles. Larger particles are delivered principally by gravitational settling.

Gravitational settling in solution is the product of opposing forces: gravity(↓), drag(↑), and buoyancy(↑). Buoyancy is the force exerted by the fluid that has been displaced, while drag is due to the viscous forces of the fluid on the moving particle. The gravitational force on a particle is a function of mass and therefore of particle size and density, while buoyancy is a function of particle volume (size) and the density of the liquid media (Bird *et al.*, 1960). Drag is a function of particle

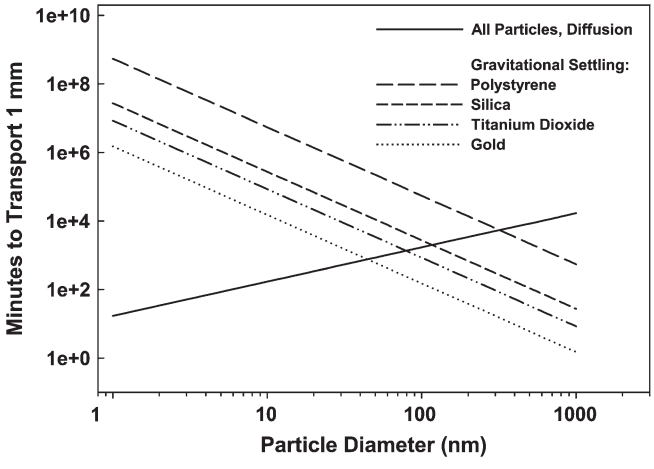

FIG. 3. The theoretical relationship between particle diameter and the time to diffuse (dotted line) or settle (solid lines) 1 mm in fluid with the characteristics of water. Transport times are presented for spherical, non-interacting materials spanning a 10-fold range in densities and were calculated as per Equations 3–5 using the following values: gas constant, 8.314 J/K/mol; viscosity, 0.00089 kg/m/sec; fluid density, 1.0 g/ml; acceleration due to gravity, 9.8 m/sec; temperature, 298 K.

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size, fluid viscosity, and particle velocity. The net effect is a particle size–, shape–, and density–dependent rate of delivery of particles from suspension in the media to cells. Terminal settling velocity can be calculated from Stoke's Law as:

$$v_{sed} = \frac{2g(\rho_p - \rho_m)d^2}{9\mu}, \quad (5)$$

where g is the acceleration due to gravity, ρ_p and ρ_m are the density of the particle and media, respectively, d is the particle diameter, and μ is the media viscosity (Crank, 1975).

Gravitational settling increases with particle density and the square of particle diameter. In contrast to diffusion, gravitational settling is the dominant form of particle delivery for large, dense particles. The rate of gravitational settling for spherical particles of selected sizes and densities in aqueous medium are presented in Table S2 (Supplemental Data). The rate of gravitational settling is significantly different across particle sizes and densities, although the settling velocities of 100-nm particles and smaller particles can be quite small. Protein binding in serum-containing media can also alter the hydrodynamic diameter of nanoparticles, with corresponding changes in particle density/buoyancy (Tirado-Miranda *et al.*, 2003) and therefore settling rates. Shape influences gravitational settling through effects on drag and buoyancy. Though nanoparticles have many shapes, fractal agglomerates, cubes, and rods, for instance, many can be adequately represented as spheres for the purpose of calculating gravitational settling rates. As a very general rule, particles with aspect ratios greater than 2, carbon nanotubes, for example, cannot be represented as spheres for settling calculations (Swaminatan *et al.*, 2006), and alternative approaches should be used (Herzhaft and Guazzelli, 1999).

Agglomeration is the adherence of single or groups of particles into larger masses due to attractive forces or chemical or mechanical binding (Maeakin, 1988). Irreversible agglomerates of primary particles are called hard aggregates. Examples of this type of agglomeration can be seen in electron micrographs of a number of metal oxides (Limbach *et al.*, 2005), C_{60} fullerenes (Fortner *et al.*, 2005), and carbon nanotubes (Lisunova *et al.*, 2006). Particles can also be manufactured as single particles; examples are amorphous silica and polystyrene beads. Both of these particles can interact with each other to form soft (reversible) agglomerates if there is a net attractive pair potential. Many nanoparticles are in some degree of agglomeration whether they are dry or in solution.

Agglomeration shifts the size class distribution of particles from its initial state to one with a larger mean and in some cases, greater dispersity. Agglomerates have a higher mass and volume than the individual particles they are composed of and have correspondingly higher gravitational and buoyant forces acting on them. Drag is also increased due to the higher volume and nonspherical shape. Rates of diffusional transport are lower for agglomerates. Agglomerates are not solid particles due to spaces between individual packed particles (Sterling *et al.*, 2005) and therefore have a lower density and surface area:mass ratio than the primary particles. The net effect is settling rates for agglomerates that are generally higher than the smaller primary particles, but may be higher or lower than a comparably sized single particle depending on the agglomerate shape and packing density (Johnson *et al.*, 1996).

Several factors influence the rate and extent of agglomeration. Particle concentration affects the rate and degree of agglomeration by influencing the rate of direct particle-to-particle interactions. Properties of nanoparticles such as zeta potential, shape, and hydrophobicity/hydrophilicity can also impact rates of agglomeration by influencing repulsive or attractive (adhesive) properties. Fluid characteristics (van Oss *et al.*, 1978) and the extent and method of mixing or sonication are characteristics of the experimental system that affect agglomeration. The presence of proteins on the particles can create a steric repulsive force and reduce the net attractive interactions between particles, altering the agglomeration state (Limbach *et al.*, 2005).

The net impact of these processes, which are usually ignored, is to complicate the relationship between simple measurable metrics of apparent exposure such as nominal media concentration and more relevant measures of delivered dose, for example, deposited particle surface area. Reliance upon these dose metrics will in some cases, as shown below, lead to incorrect conclusions regarding the comparative toxicity of the particles or the dynamics of cellular response.

THE IMPACT OF IMPROVED *IN VITRO* DOSIMETRY FOR NANOPARTICLES

Improving the basis for comparative dose-response analysis *in vitro* for nanoparticles through adequate consideration of particokinetics and application of the principle of target tissue dose (cellular dose *in vitro*) will have an impact in several major research areas: discovery of fundamental particle characteristics (e.g., size and physicochemistry) influencing toxicity and uptake, comparative nanoparticle toxicity, and the dynamics of cellular response to nanoparticles.

To illustrate the impact of dosimetry on *in vitro* toxicity assessment and to generalize the findings across particle size, we present a comparison of nominal media concentration dose on a mass or surface area basis for particles with densities equivalent to TiO_2 and Au. These dose metrics are also compared with nominal media concentrations adjusted to reflect relative rates of gravitational settling or total delivery (diffusional transport and gravitational settling) (Fig. 4). The adjusted doses are an approximation of the dose delivered to cells *in vitro*. Conceptually, if it takes particle A twice as long as the 1000-nm reference particle to travel by gravitational settling and/or diffusion 1 mm, the relative fraction of the nominal media concentration of particle A delivered is assumed to be 0.5. This fraction is the adjustment factor. The path length, 1 mm, is ~1/2 the media depth typically used in cell culture experiments,

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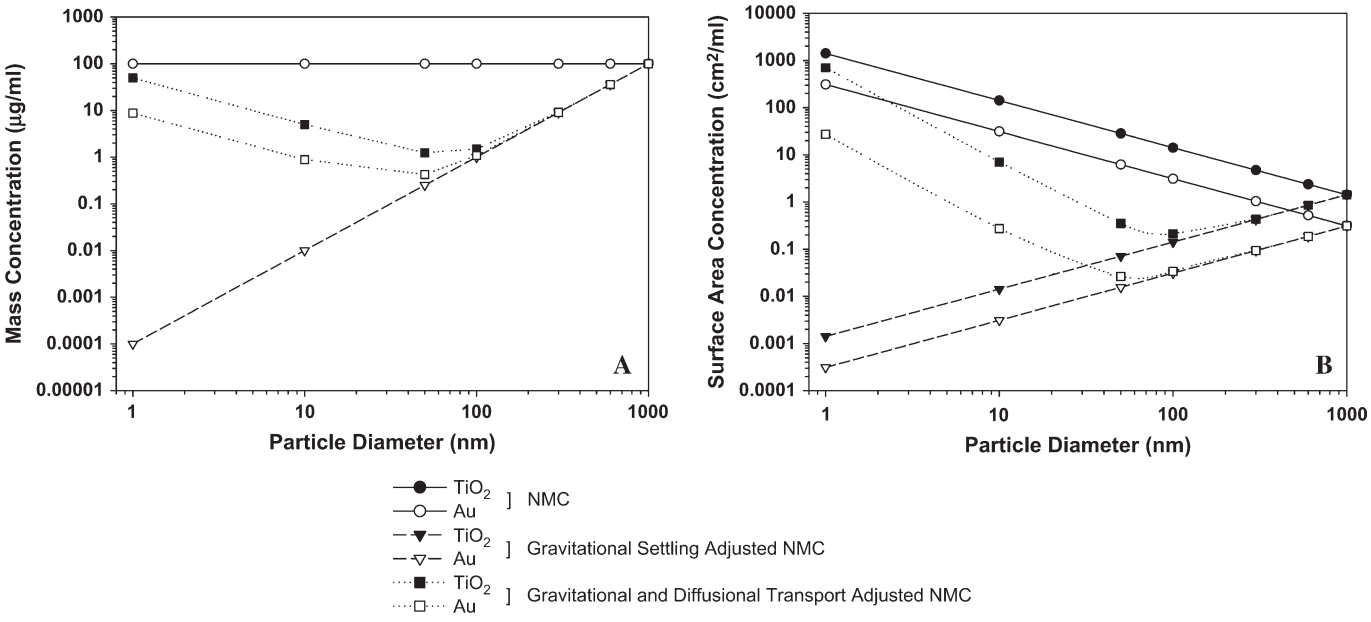

FIG. 4. The relationship between particle diameter and nominal (solid lines), gravitational settling (dashed lines), or gravitational settling and diffusional transport (dotted lines) rate–adjusted mass (A) or surface area concentration (B) dose for equivalent nominal media concentrations of 100 µg/ml TiO_2 (closed symbols) and Au (open symbols) nanoparticles. The TiO_2 line in Panel A is behind the Au line. Delivery rate–adjusted doses show both different trends and orders of magnitude differences when compared with nominal media concentrations. NMC, nominal media concentration.

where materials are applied a premixed particle suspension. The adjustment factor is independent of the length of the experiment. Including experimental duration and particle depletion would improve the dose adjustment, but is a more computationally intensive approach. A more detailed description of the dose adjustment is found in the ''Appendix'' section.

Equivalent nominal media mass concentrations imply equal doses for all particle sizes (Fig. 4A). Nominal media particle surface area concentrations decrease as a linear function of particle diameter (per Equation 1), differing nearly 3 orders of magnitude across particle size in the nanometer range for a given mass concentration (Fig. 4B). Cellular dose (estimated as described above) shows important differences when compared with nominal media concentrations (Figs. 4A and B, adjusted dose metrics). Adjusting only for differences in gravitational settling rates (as per Equation 6), cellular dose on a mass or surface area basis increases as a function of the square of particle diameter (Figs. 4A and B). These approximated cellular doses differ from those presumed from nominal media concentrations by up to 6 orders of magnitude and most importantly have distinctly different, and in the case of surface area, opposite, trends; nominal surface area concentration decreases, but delivery-adjusted surface area cellular dose increases as a function of particle diameter. Adjusting instead for particle size differences in gravitational and diffusional delivery (Equation 7), similar trends are observed for particles greater than ~50 nm, below which diffusional delivery is estimated to contribute more significantly than gravitational settling, and cellular dose increases as particle diameter decreases (Figs. 4A and B). The difference between nominal media concentrations and delivery-adjusted nominal media concentrations is as great as two orders of magnitude for these selected materials. Trends for particle number concentrations are similar (data not shown), but the differences are larger since nominal particle number concentration is inversely related to the cube of the particle diameter (per Equation 2).

In order to demonstrate the impact of using nominal media mass or surface area concentrations for dose-response analysis, an idealized dose-response data set was created. The data set reflects the leading hypothesis that cellular response is proportional to particle surface area (Oberdorster *et al.*, 2005). Response data were generated by multiplying the estimated delivered surface area (approximated as per Fig. 4, and Equation 7) of gold nanoparticles 1, 10, 100, or 1000 nm in diameter by an arbitrary response factor (3300 response units/cm^2 surface area). By design, the particles appear equally potent on a delivered particle surface area basis (Fig. 5, Panel A). Plotted against nominal media mass concentration, the 1-nm particles appear 100 times more potent than the 10- and 1000-nm particles, but 1000 times more potent than the 100-nm particles (Fig. 5, Panel B). The 10- and 1000-nm particles appear equally potent because the estimated transport rates are similar though one is diffusion driven (10 nm) and one is driven by gravitational settling (1000 nm) (also see Fig. 3). The estimated delivery rate of the 100-nm particle is lower because it is too large for effective diffusional transport and too small for effective gravitational settling. When plotted against nominal media surface area concentration, the 1-nm particles appear

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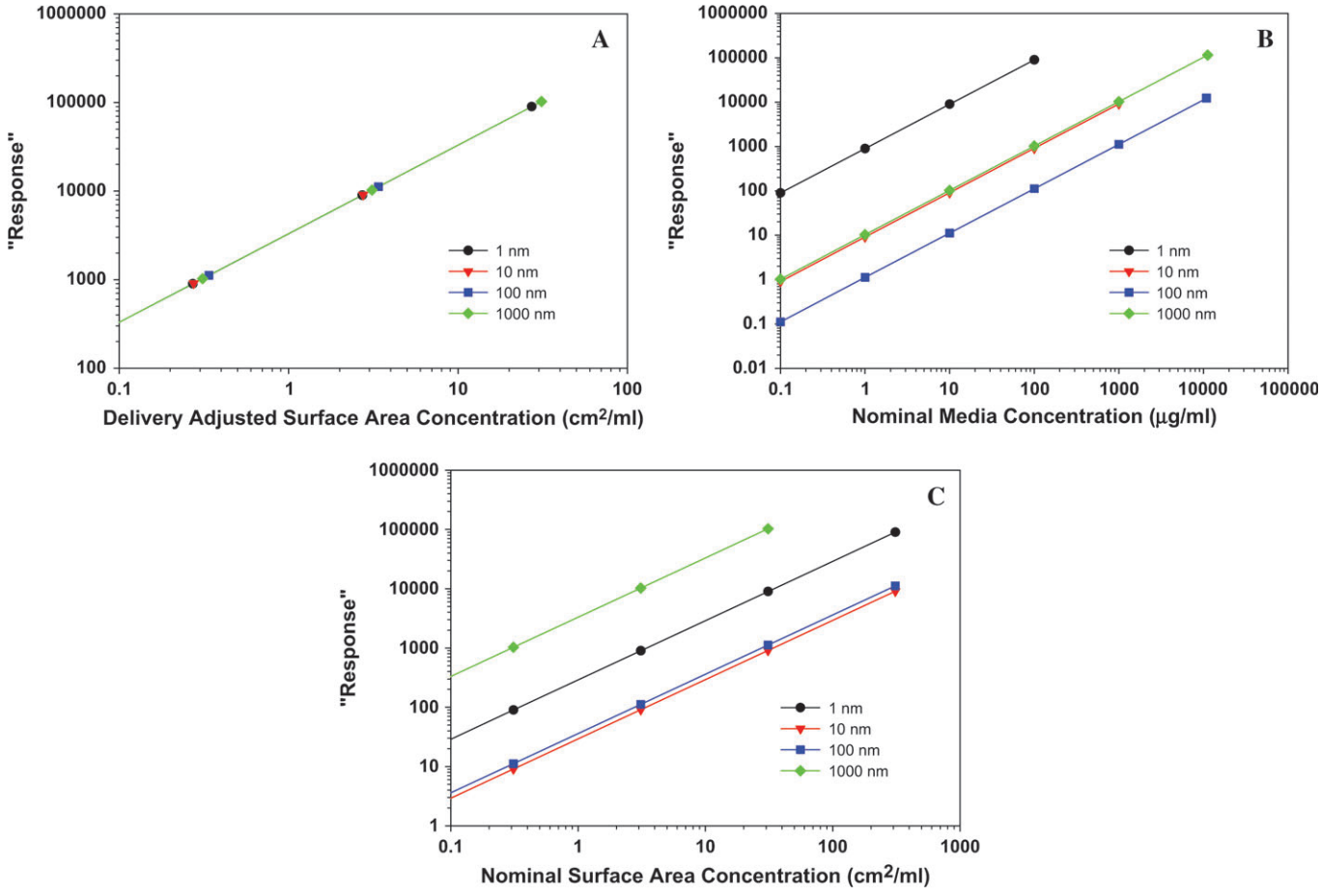

FIG. 5. Comparison of idealized dose-response data demonstrating that using nominal media mass or surface area concentrations obscures underlying dose-response patterns and the nanoparticle properties (here surface area) driving response. Response was assumed to be proportional to delivered surface area dose approximated by adjusting nominal media concentrations for estimated differences in diffusional and gravitational delivery rates (see "Appendix" section).

10 times more potent than the 10- or 100-nm particles (Fig. 5, Panel C), but 10 times less potent than the 1000-nm particles, the net result of differences in transport rates (see Fig. 3) and significantly smaller surface area concentrations for the larger particles.

Reevaluation of published nanoparticle dose-response data through application of the principle of cellular dose *in vitro* demonstrates the same effect. Hussain *et al.* (2005) reported cytotoxicity EC50's for 1000-nm CdO, 15- and 100-nm Ag nanoparticles, and 30- and 150-nm MoO_3 nanoparticles on a nominal mass media concentration basis in BRL 3 rat liver cells. We have extended their analysis by comparing the EC50's on a nominal media particle surface area/milliliter basis as well as adjusting the EC50's for approximated delivery rates as in Figure 3 (Table 2). When the nominal media surface area concentration of particles in the media is used as the dose metric, the 1000-nm CdO particles appear 2.5 to 4 orders of magnitude more potent than the Ag and MoO_3 nanoparticles, greater differences in potency than nominal mass media concentrations imply (Table 2). Hussain observed settling of particles in solution, pointing out that the dose may be higher for particles that settle (Hussain *et al.*, 2005) and that differences in settling may influence their apparent toxicities. When differences in particle delivery are estimated and accounted for, the EC50's for these particles are all within 2 orders of magnitude, (0.005–0.66 cm^2/ml). The order of potencies depends on the choice of dose metric. As measures of dose improve in their relationship to target-site dose, the difference in potency between these particles appear less.

These analyses illustrate that metrics of exposure such as nominal media mass or surface area concentrations are expected to obscure underlying dose-response patterns such as a dependency on surface area, and consequently our ability to discover the fundamental properties of nanomaterials driving response.

DOSIMETRY METHODS

Wider application of dosimetry in *in vitro* systems and realization of its attendant benefits to nanomaterial safety assessment will only be possible if technologies and methods are readily available to make the necessary measurements

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TABLE 2
The Impact of Selected Metrics of *In Vitro* Dose on Nanoparticle Toxicity Assessment

		EC50's			
				Delivery-adjusted surface area concentration (cm^2/ml)[a]	
Material	Particle diameter (nm)	Nominal media mass concentration (μg/ml)	Surface area concentration (cm^2/ml)	Gravity	Diffusion and gravity
CdO	1000	0.75	0.005	0.005	0.005
Ag	100	24	1.37	0.017	0.020
Ag	15	50	19.0	0.005	0.272
MoO_3	150	250	21.3	0.232	0.262
MoO_3	30	210	89.4	0.039	0.663

[a]Delivery adjustment according to Equation 6 (gravity) or 7 (diffusion and gravity), assuming a path length of 1 mm and normalized to the 1000-nm CdO particle, as described in the ''Appendix'' section. EC50's reported by Hussain *et al.* (2005) were determined in the rat liver cell line BRL 3A as the concentration producing a 50% increase in media lactate dehydrogenase.

directly or to estimate them computationally. The most obvious and available techniques are analytical in nature. They involve the use of instruments for either directly observing nanomaterials (transmission electron microscopy [TEM], scanning electron microscopy [SEM], Light Scattering, and epifluorescence) or measurements of mass (inductively coupled plasma–mass spectrometry [ICP-MS] and liquid chromatography MS/MS [LC/MS]), all of which can be transformed through simple calculations into commonly used metrics of dose. Computational methods for calculating cellular dose have yet to be developed, but the necessary mathematical basis for constructing such models exists. Ideally, experimentalists would have both methods available to them for experimental design and interpretation.

Experimental Dosimetry

Nanoscale materials present special problems to the analytical measurement of materials in or on cultured cells. Microscopy methods (TEM, SEM, confocal, etc.) have the advantage of providing direct measures, with limitations, of particle size, number, agglomeration state, and localization, on or within cells. For example, confocal microscopy was recently used to determine the rate and extent of macrophage uptake of 26-nm fluorescent microspheres (Moss and Wong, 2006). High-resolution fluorescence microscopy can detect particles (fluorophores) smaller than the diffraction limit, but may not provide sufficient resolution to distinguish small agglomerates from the individual particles. Unfortunately, conventional visible light microscopy is generally inadequate to resolve nanoscale particulates or agglomerates less than 200 nm. This limit is the result of diffracted light interfering with the resolution of objects. Nevertheless, the utility of optical microscopy to characterize the subcellular localization of particles greater than ~200 nm or of agglomerates should not be overlooked. The attachment of fluorophores to the surface of nanoparticles significantly increases the functional resolution as the diffraction limit does not apply when the emission of single fluorophores are imaged, although diffraction may occur with high concentrations of the fluorophore. However, it must be recognized that the fluorophore is a modification of the particulate surface that may alter the native kinetic and biological properties of the particle.

New advances in optical microscopy that break the diffraction limit and provide imaging resolution of ~20 nm have been applied to localization of proteins in live cells (Betzig *et al.*, 2006; Rust *et al.*, 2006). These and related approaches that utilize novel combinations of illumination, detection, and computation should be useful for characterizing the fate and disposition of fluorescent nanoparticles within living cells. Alternative methods involve use of multiphoton imaging, such as coherent anti-Stokes Raman spectroscopy, to provide three-dimensional images of nonfluorescent nanoparticulates (e.g., metal oxides) in cells or even tissue sections (Holtom *et al.*, 2001; Zheng *et al.*, 2004). These multimodal methods are attractive for their ability to provide high contrast without modification of the nanoparticulate surface.

Numerous analytical methods such as MS, ICP-MS and LCMS have high sensitivity for the inorganic or organic constituents of nanoparticulates. These approaches are generally destructive and thus provide little direct information on the cellular localization, primary particle size, or agglomeration state. The number of primary particles present in the sample can be estimated from the total analyte mass, the nominal particle size, and the number of atoms per volume particulate (Chithrani *et al.*, 2006) and transformed into the selected dose metric by simple calculation. An important limitation of these analytical approaches is potential contamination of the sample by elements not associated with the nanomaterial, that is, iron-containing proteins or zinc from the plastic culture vessels or media, which would complicate the measurement of iron or zinc oxide nanomaterials in cells (or tissues).

Radioactive isotopes can be used to overcome this limitation for organic and inorganic materials, but the use of stable isotopes incorporated into the nanomaterials at synthesis as recently proposed by Gulson and Wong (Gulson and Wong, 2006) is perhaps the most promising labeling strategy. The native properties of the nanoparticulate are maintained while increasing sensitivity and selectivity for the exogenous materials. Although expensive to produce, stable isotopes do not require special handling and disposal. The use of stable isotopes is not limited to metal-containing nanoparticulates as accelerator mass spectrometry has exquisite sensitivity for carbon-13 or carbon-14 and could be used to trace fullerenes

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labeled with these isotopes. Analytical methods applicable to aspects of nanoparticle characterization of nanoparticles other than dose can be found elsewhere (Oberdorster *et al.*, 2005).

Computational Dosimetry

To date, there has been little consideration given to computational methods for estimating cellular dose *in vitro* for particles of any size. This omission is somewhat surprising given the wide use of *in vitro* systems and the large investments made in developing computational methods for estimating target tissue dose *in vivo* for particulates and ultrafine particulates (Asgharian and Anjilvel, 1998; Morrow, PE 1966; Stober *et al.*, 1989; Tran *et al.*, 1999; USEPA, 2005). The multipath particle deposition (MPPD, http://www.ciit.org) and International Commission on Radiological Protection (ICRP) models are two publicly available models of particulate dosimetry in rodents (MPPD) and humans (MPPD and ICRP). The MPPD model successfully applies computational descriptions of diffusion and gravitational settling–dependent deposition of particles to predict dose to selected regions of the respiratory tract. The kinetics of particles in media is a similar and perhaps simpler problem; the medium, in this case a liquid rather than air, is at rest rather than flowing and there is little impact of the container. The solution dynamics of particles in liquids is well studied, and mathematical approaches for both diffusion and gravitational settling have been developed (Mason and Weaver, 1924). These approaches have yet to be formed into an approach for describing the particokinetics—the combined influence of diffusion and gravitational settling on particle delivery to cells *in vitro*. To address this need and provide researchers with a dosimetry tool to use prospectively in research design and retrospectively for data interpretation, we have undertaken the development computational particokinetics and dosimetry model for particles including nanomaterials in solution. We believe that the resulting computational model, and perhaps more importantly what is learned during the experimental and computational work supporting its development, will promote a more fundamental understanding of the processes that affect cellular dose and nanoparticle toxicity in the fashion that physiologically based pharmacokinetic (PBPK) modeling brought a deeper understanding of kinetics and mechanisms of toxicity to chemical risk assessment. Computational methods will eventually offer a good adjunct to, or, where they can be validated, substitute for analytical or observational methods for calculating nanoparticle dose metrics *in vitro*.

SUMMARY AND CONCLUSIONS

One grand challenge in nanotoxicology is to satisfy the urgent need for rapid hazard assessment of emerging nanoparticles through development of suitable high-throughput *in vitro* assays (Holsapple *et al.*, 2005; Nel *et al.*, 2006). There are several important attributes *in vitro* test systems should have. They should represent cell types targeted by nanoparticles through common routes of exposure (e.g., lung or skin epithelia, macrophages, or other elements of the reticuloendothelial system) and measure common modes of action such as oxidative stress and inflammation (Nel *et al.*, 2006). Cellular dose should be measurable or calculable. Test systems having these attributes will be valuable research tools for investigating the operative biology of nanoparticle toxicity, discovering the material properties that drive response as well as characterizing the comparative toxicity of particles for use in hazard screening. In all cases, the investigative and predictive power of *in vitro* assay systems will be restricted without adequate experimental and computational methods for nanoparticle dosimetry *in vitro*. Nanotoxicology needs a consistent, accurate, and biologically motivated approach for making comparisons of response using *in vitro* systems across nanoparticle size and type.

The concepts presented in this manuscript apply to particles in general, not only to particles in the nano domain. Because particle dosimetry for toxicological assessment appears to be most important at the boundary between the nano and supranano size domains, some discussion of the definition of ''nanomaterial'' or ''nanoparticle'' is valuable. By conventional definition, nanomaterials have at least one dimension of 100 nanometers or less. The definition originates in the material science and physics fields, where the unique properties—conductivity, chemical reactivity, physical chemistry, and catalytic properties—of these small structures were first recognized. The definition is appropriate, albeit too narrow. The rapid development and commercialization of new nanomaterial products including catalysts, cosmetics, drug delivery systems, tools for microbiology and medicine, semiconductors, and coloring agents include not just materials in the < 100-nm range, but those in the submicron range and those that form agglomerates larger than 100 nm. Their biological ''uniqueness'' is of course not likely to be constrained to sizes fitting the conventional definition of nanomaterials (< 100 nm), a fact implicit in the definition of nanomaterial, 1–1000 nm, used by the pharmaceutical industry (Sun *et al.*, 2004). Thus, for the purposes of dosimetry and toxicological assessment, these new materials should be more broadly viewed by the biological community as a largely unstudied new class of *submicron particulate products*, and consideration should be given to expanding the conventional material science definition of nanomaterials to include those in the range of 1–1000 nm.

Our analyses demonstrate that studies relying on nominal media concentrations involve large, unaccounted for differences in both the extent and rate of transport of surface area, particle number, and mass. These differences confound particle comparisons and likely obscure the underlying relationships between response and particle characteristics. Ideally, to test particle size as a determinant of response, the experimental design would deliver particles of different sizes to cells at the

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same rate of transport of surface area in one group and particle number in another throughout the duration of the study or equivalent amounts at the end of the study. Such a design allows examination of particle size as a determinant of response when the other principal variables (particle number and delivery rate) are controlled. Concentrations and media depths resulting in these experimental conditions could be determined analytically by considering differences in material delivery to cells. These principles apply equally to studies focused on specific measures of response such as cytokine release or those whose goal is a broad evaluation of biological response or mechanisms of toxicity through proteomic and transcriptomic analyses.

Nanoparticle cellular uptake studies would also benefit from careful consideration of nanoparticle delivery rates. Under conditions where uptake by cells is rapid relative to transport rates, the rate of delivery becomes the rate-limiting factor for particle uptake (Limbach *et al.*, 2005). Particle size– and concentration-dependent differences in macrophage uptake of nanoparticles have been shown (Chithrani *et al.*, 2006; Desai *et al.*, 1996; Moss and Wong, 2006). However, it is not clear if the results reflect a fundamental difference in rates of particle uptake or differences in particle number concentration in the test solutions. Nanoparticle uptake studies would benefit from equalizing particle number concentration or the transport rate of particles across the study, eliminating particle number/transport as a confounder.

The analyses presented here were conducted under several simplifying assumptions, the most important of which are that particles are noninteracting and monodisperse rather than agglomerates. The particle size and concentration dependence of agglomeration was not considered and would change the relationships presented here between particle size, concentration, and cellular dose for agglomerating nanoparticles. Other important particle characteristics such as zeta potential and mean hydrodynamic diameter and processes such as particle dissolution protein binding and mixing/sonication of suspensions, all of which can directly or indirectly affect particle delivery to cells, were also not considered and should be the subject of a more complete analysis. Thus, the differences in cellular dose across particle size should not be used directly or assumed to apply to suspensions of particles with significant agglomeration, potential for dissolution, or protein binding; they are used to illustrate principles and demonstrate the importance of these principles to dosimetry. The general conclusions of this work, however, and the principles the analyses demonstrate are valid for both agglomerates and monodisperse nanoparticle solutions.

Experimental measurement of cellular dose *in vitro* is often difficult or costly and as such arguably contrary to an inherent strength of *in vitro* studies. There cannot be an expectation that cellular dose will be measured directly in most *in vitro* studies. The capability is also not likely to be available to all research teams nor will it be available to private and government risk assessors who will have the need to interpret published studies that report particle characteristics but not measures of cellular dose. Development and deployment of a validated computational model for *in vitro* dosimetry would provide a tool for calculating cellular dose for *in vitro* studies to be used prospectively in research planning or retrospectively for interpretation of studies. Such a model would allow researchers to estimate more relevant measures of cellular dose from primary particle characteristics (particle size, density, concentration, and if available, surface charge), revealing more accurate dose-response relationships, improving the basis for comparative toxicity assessment of nanoparticles. Ultimately, it should be understood that discovering the fundamental relationships between the properties of nanomaterials and toxicological response will require the separation of the complex kinetics of nanoparticle delivery *in vitro* from the dynamics of response, which is made possible by integrated computational and experimental dose-response analysis. Because computational dosimetry will be possible only if the supporting data are available, experimentalists should consider the value of providing careful descriptions of their test system including media constituents, media depth and volume, dimensions of the wells, as well as a rigorous characterization of the test material and its state in solution (degree of aggregation, for instance). Computational dosimetry, cross study comparison, and interpretation of results will not be possible without high standards for reporting of material and test system characteristics.

SUPPLEMENTAL DATA

Tables S1 and S2 present the diffusion constants and gravitational settling rates calculated for a range of particle densities and diameters in an aqueous media with the properties of water and are available online at http://toxsci.oxfordjournals.org/.

APPENDIX

The adjustment factor is the ratio of approximated delivery rates, normalized to a 1000-nm referent particle:

$$\text{Adjustment factor}_{\text{gravity}} = \frac{\text{SR}_P}{\text{SR}_{\text{PN}}}, \tag{6}$$

$$\text{Adjustment factor}_{\text{gravity+diffusion}} = \frac{\text{DR}_P + \text{SR}_P}{\text{DR}_{\text{PN}} + \text{SR}_{\text{PN}}}, \tag{7}$$

where DR_P and SR_P are times to diffuse and settle 1 mm, respectively, for a particle size *P*, and DR_{PN} and SR_{PN} are the corresponding times to diffuse or settle 1 mm for the particle size that the doses are normalized to (in this manuscript, always a 1000-nm particle). The time to settle 1 mm was calculated from the gravitational settling rate that was calculated using Equation 5. Time to diffuse 1 mm was calculated from the diffusion coefficient (Equation 3), and the selected diffusion distance using Equation 4.

The basic concept is that if it takes particle A twice as long as the 1000-nm referent particle to travel by gravitation settling and/or diffusion 1 mm, the relative fraction of the nominal media concentration of particle A delivered is 0.5. One millimeter is ~1/2 the media height for culture conditions in our

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laboratory. The adjustment factor is applied by multiplying nominal media mass or surface area concentrations by the adjustment factor to arrive at the delivery- or transport-adjusted dose.

The adjustment is based on the absolute times to settle or diffuse 1 mm because comparable rates for diffusion and gravitational settling can only be calculated when the distance is stated; the rate of diffusion cannot be generalized in one dimension. The diffusion rate for a given distance is calculated as 1/time to diffuse that distance. We chose to adjust doses based on the time to settle or diffuse 1 mm rather than the corresponding rate (mm/time) to avoid confusions that might arise from reporting ''diffusion rates.'' The results are the same whether the normalization is based on imputed rates or time to settle or diffuse. Nonetheless, the ratio of delivery times is an approximation; the calculated diffusion rate assumes no interaction with other particles and is not concentration dependent. Direct measurement or estimation using computational model of settling and diffusion through cell culture media would improve the accuracy of the adjustment.

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