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Interactions of Engineered Nanomaterials in Physiological Media and Implications for *In Vitro* Dosimetry

Joel Cohen, Glen DeLoid, Georgios Pyrgiotakis, and Philip Demokritou*

Center for Nanotechnology and Nanotoxicology, Department of Environmental Health, Harvard School of Public Health, Boston, MA

Abstract

In vitro toxicity assays are efficient and inexpensive tools for screening the increasing number of engineered nanomaterials (ENMs) entering the consumer market. However, the data produced by *in vitro* studies often vary substantially among different studies and from *in-vivo* data. In part these discrepancies may be attributable to lack of standardization in dispersion protocols and inadequate characterization of particle-media interactions which may affect the particle kinetics and the dose delivered to cells. In this study, a novel approach for preparation of monodisperse, stabilized liquid suspensions was presented and coupled with a numerical model which estimates delivered dose values. Empirically derived material- and media-specific functions were derived for each media- ENM system that can be used to convert administered to delivered doses. The interactions of ENMs with a variety of physiologic media were investigated and the importance of this approach was demonstrated by *in vitro* cytotoxicity assays using THP-1 macrophages.

Keywords

nanotoxicology; liquid suspension; DLS; dosimetry

1 INTRODUCTION

The physicochemical properties of engineered nanomaterials (ENMs) (mobility, quantum size effects, surface area, surface energy) differ substantially from those of the corresponding bulk materials sharing the same chemical composition and atomic structure. These properties endow ENMs with exceptional conductivity, reactivity, and optical sensitivity, and hence superior functionality in a variety of consumer products, including sporting goods, tires, catalysts, microelectronics, cleaners, paints, cosmetics and pharmaceuticals. These same properties also result in interactions with biological systems that vary vastly from those of their micron-sized counterparts, which can make them unsafe for humans and for the environment (Nel et al., 2006).

*corresponding author: Philip Demokritou, Phone #: (617) 432-7990, pdemokri@hsph.harvard.edu.

Declarations of Interest

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The most common exposure pathways for these ENMs include ingestion (pharmaceutical products, food), dermal contact (occupational exposure, cosmetics), injection (nanomedicines and drug delivery mechanisms), and inhalation (occupational and consumer exposure). These varied exposure pathways each present unique challenges to biological systems, and may result in diverse toxicological outcomes. For example, recent studies suggest that inhaled nanoparticles have the potential to pass from the lungs into the bloodstream and enter extrapulmonary organs, leading to increased cardiovascular morbidity and mortality (Choi et al., 2010, Brain, 2009, Mills et al., 2009). Although preliminary evidence demonstrates the potential for ENMs to cause adverse biological effects, the underlying toxicity mechanisms are not currently well understood (Oberdorster, 2007).

A number of studies have endeavored to develop efficient and inexpensive screening tools to correlate mechanisms of biological activity and toxicity with ENM characteristics such as size, shape, and surface area (Rallo et al., 2011, Shaw et al., 2008, Krewski et al., 2010). Due to the high cost and time required for *in vivo* toxicity studies, most of these efforts have focused on *in vitro* methods (Lai, 2011, Balbus et al., 2007). High-throughput *in vitro* toxicity assays have recently been employed to assess multiple toxicity endpoints, in multiple cell lines, of libraries of ENMs over a range of exposure times and concentrations (George, 2011).

In addition to the refinement and standardization of *in vitro* and *in vivo* methods, several aspects of the delivery of ENMs in liquid suspension to cultured cells, typical of *in vitro* toxicity studies, require further analysis. First, commercial ENM nanopowders are limited in diversity of physicochemical and morphological properties (usually to a few sizes for a given composition) making systematic, parametric studies of the relationships between ENM properties (size, surface, composition, shape, charge, etc.) and biological outcomes impossible.

Second, ENMs suspended in culture media may flocculate, agglomerate or dissolve, and interact with serum components (Fadeel, 2010, Jones and Grainger, 2009, Verma and Stellacci, 2010), which can alter their biological properties. More importantly, administered doses may differ substantially from the doses actually delivered to cells. Furthermore, comparison of *in vitro* doses to those administered by inhalation is difficult, which can result in large differences in effective dose between *in vitro* and *in vivo* studies. These limitations may explain some of the disparities reported in the literature between *in vivo* and *in vitro* ENM studies (Fadeel, 2010, Fischer and Chan, 2007).

Typical comparisons of biological response to ENM exposure employ administered dose metrics based on the ENM properties as measured in the dry powder form (e.g., mass or surface area per volume), without taking into account particle-particle and particle to physiologic fluid interactions in the suspension liquid suspension (Oberdorster et al., 2005, Jiang, 2008, Rushton et al., 2010, Wittmaack, 2007, Oberdorster et al., 1994). These interactions depend largely upon the dispersion protocol; the particle characteristics, including primary particle size and shape, chemical composition and surface chemistry (Ji et al., 2010, Jiang, 2009, Murdock et al., 2008, Zook et al., 2010); and the liquid media properties such as ionic strength, specific conductance, pH, and protein content ((Lee et al.,

2011, Bihari et al., 2008, Elzey, 2009, Murdock et al., 2008, Zook et al., 2010, Wiogo et al., 2011, Laxen, 1977). ENM interactions, in turn, lead to agglomeration in liquid media, which alters the total number of free particles in suspension and the total surface area available for interaction with cells *in vitro*, as well as the mass transport of ENMs (i.e. sedimentation and diffusion coefficients) which directly impacts delivery of particles to cells (Teeguarden et al., 2007). The importance of such particle dynamics in an *in vitro* system is demonstrated by the observation that rapidly settling particles elicit cytokine secretion within minutes of application, whereas slow or non-settling particles may take several hours to elicit a similar response (Teeguarden et al., 2007).

Finally, the methods used to disperse nanoparticles in culture media for *in vitro* studies, which can substantially affect their physical and chemical properties – and hence their biological activities, differ widely between laboratories (Roco, 2010). Clearly a harmonized (standardized and shared) protocol for nanoparticle dispersion is required if the efforts of the many laboratories performing these studies are to produce data that is congruous and cumulative.

In this study, the particle transformations and kinetics of a panel of industrially-relevant ENMs currently under investigation by the Organization for Economic Cooperation and Development (OECD) (OECD, 2010), and their implications on *in vitro* dosimetry were investigated. These ENMs were dispersed using a standardized sonication protocol in a variety of typical cell culture media formulations. Empirical functions for converting administered dose to dose delivered to adherent cells in a 96-well microplate format were derived for a variety of commonly used metal oxide ENMs dispersed in physiological fluids including cell culture media with or without either fetal bovine serum or serum albumin. The proposed standardized dispersion protocol and empirical dose metric functions reported herein may be useful for *in vitro* nanotoxicity studies, enabling consistent, reproducible preparations of stabilized, monodisperse ENM suspensions and reliable estimates of the particle dose delivered to cells as a function of time. The utility of this approach on *in vitro* nanotoxicity studies was demonstrated using adherent PMA-matured THP-1 macrophage cells and a standard fluorescence microscopy-based live/dead cytotoxicity assay.

2 EXPERIMENTAL SECTION

2.1 Nanomaterials and characterization of ENM powders

ENMs investigated in this study are listed in Table 1. ENMs included both commercially available and in-house generated ENMs of varied composition, surface chemistry, and primary particle size. In-house particles were generated using the Harvard Versatile Engineered Nanomaterial Generation System (VENGES), as previously described (Demokritou et al., 2010, Sotiriou et al., 2011)

Specific surface area, *SSA*, defined as the area per mass (m^2/g), was determined by the nitrogen adsorption/Brunauer-Emmett-Teller (BET) method (Micrometrics Tristar Norcross, GA, USA). The equivalent primary particle diameter, d_{BET} , was calculated, assuming spherical particles, as:

$$d_{BET} = \frac{6}{SSA \times \rho_p} \quad (1)$$

where ρ_p is the particle density, which was obtained for each particle from the densities of component materials, at 20°C, reported in the CRC handbook of Chemistry and Physics (CRC, 2010).

Particle diameter was also determined by X-ray diffraction and reported as d_{XRD} (nm). Particle morphology and size were further characterized by scanning and transmission electron microscopy (SEM/TEM).

2.2 ENM dispersal and characterization in liquid suspensions

ENM dispersion was performed using a protocol based on those established by OECD and NIST (OECD, 2010, Taurozzi, 2010), including calibration of sonication equipment to ensure accurate application and reporting of delivered sonication energy (DSE) in J/ml (Taurozzi et al., 2010). This protocol included identification and subsequent utilization of the material-specific critical sonication energy required to achieve monodisperse solutions at the lowest agglomeration state, as measured by dynamic light scattering (DLS). Probe sonication was performed in deionized water (DI H₂O) to minimize reactive oxygen species generation via sonolysis, to minimize ionic strength and specific conductance, and hence particle interactions, during sonication, and to avoid denaturation of proteins in the final cell delivery media. Stock solutions were then diluted in various physiological fluids prior to characterization for various particle and media parameters, or application to cells for toxicological evaluation. The dispersal protocol is illustrated schematically in Figure 1, and is described in detail in the following sections:

Sonication and Critical DSE determination—The critical DSE (DSE_{cr}) of an ENM is defined as the delivered sonication energy (in J/ml) required to achieve a monodisperse solution at the lowest particle agglomeration state in deionized water. The DSE_{cr} was determined for each ENM as follows. ENMs were dispersed at 1 mg/ml in 10 ml of solute in 50 ml, 24 mm diameter conical polyethylene tubes, by sonication with a Branson Sonifier S-450A (Branson Ultrasonics, Danbury, CT) fitted with a flat tip 1/8 inch probe (maximum power output of 400 W at 60 Hz, continuous mode, output level 1). The probe tip was immersed to a depth of 5 cm in the sample suspension, and the sample tube was bathed in ice during sonication (except during calibration) to minimize heating of the particles. The system was calibrated by the calorimetric calibration method described by Taurozzi (Taurozzi et al., 2010), whereby the power delivered to the sample was determined to be 3.14 W. The delivered sonication energy (DSE) was determined from the calibrated delivered power over a range of sonication times. Dispersions were analyzed for hydrodynamic diameter (d_H), polydispersity index (PdI), zeta potential (ζ), and specific conductance (σ) by dynamic light scattering (DLS) using a Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK). Plots of hydrodynamic diameter as a function of DSE exhibiting asymptotic de-agglomeration trends were derived for each ENM and used to determine the material specific DSE_{cr} , corresponding to the lowest agglomeration state (minimum d_H).

Preparation of ENM dispersions in physiological media—For each ENM, a stock solution of 1mg/ml was prepared by sonicating the ENM powder in DI H₂O as described above for a time corresponding to the material-specific DSE_{cr} . The stock solution was then diluted in either RPMI alone, RPMI + 10% fetal bovine serum (FBS), or RPMI + 1% bovine serum albumin (BSA) to the final concentration (0, 6.25, 12.5, 25, 50, or 100µg/ml), and vortexed for 30 seconds. ENM dispersions in media were analyzed by DLS as described above. pH was measured using a VWR symPHony pH meter (VWR International, Radnor, PA, USA).

2.3 Dose calculations

All dose calculations were based on a media volume, V , of 0.1 ml, and mass concentration, γ , of 50 µg/ml.

Administered dose calculation—Administered mass dose, M_A (µg), was calculated as:

$$M_A = \gamma_A \times V \quad (1)$$

where γ_A is the mass concentration (µg/ml), and V is the volume of media (ml).

Administered particle number dose, N_A (#), was calculated from M_A , hydrodynamic radius, r_H (nm, determined by DLS for ENMs in suspension), and material density, ρ (g/cm³, assuming spherical, non porous agglomerates with densities equal to those of the corresponding original particles) as:

$$N_A = \frac{M_A}{\left(\frac{4}{3}\pi r_H^3\right) \times \rho} \quad (2)$$

Administered surface area dose, SA_A (cm²), was calculated as:

$$SA_A = \left(4\pi r_H^2\right) \times N_A \quad (3)$$

Delivered dose (D_D) calculation—Delivered doses in terms of mass, M_D , number of particles, N_D , and surface area, SA_D , were calculated from administered doses as:

$$M_D(t) = f_D(t) \times M_A, \quad (4)$$

$$N_D(t) = f_D(t) \times N_A, \quad (5)$$

and

$$SA_D(t) = f_D(t) \times SA_A, \quad (6)$$

where $f_D(t)$ is defined as the fraction of administered particles deposited at time t , onto adherent cells at the bottom of a cell culture well.

Calculation of deposited fractions $f_D(t)$ —The *in vitro* sedimentation, diffusion and dosimetry (ISDD) model proposed by Hinderliter et. al. (Hinderliter et al., 2010) was used to calculate numerically, for each ENM-media combination, the fraction of administered particles that would be deposited onto cells as a function of time $f_D(t)$. The particle hydrodynamic diameter, d_H , as measured by DLS and listed in Table 2 was used as input to the model. Additionally, the following parameters were used as input to the ISDD numerical model: Media column height, 3.3 mm; temperature, 310K; media density, 1.00 g/ml; viscosity, 0.00069 Pa s for RPMI and 0.00074 Pa s for RPMI/10%FBS and RPMI/1%BSA (Hinderliter et al., 2010); administered (initial suspension) particle concentration, 50 $\mu\text{g/ml}$; particle density, as listed in Table 1.

For each ENM-media combination, the model-derived $f_D(t)$ was fit to a Gompertz sigmoidal equation as follows:

$$f_D(t) = 1 - e^{-\alpha t}, \quad (7)$$

where t is time (h), and α is a particle- and media-specific deposition fraction constant (h^{-1}). The deposition fraction constant α (h^{-1}) was calculated for each ENM-media combination.

Solving Equation 7 for the time t at which the fraction $f_D(t)$ of administered particles is delivered yields:

$$t = -\ln(1 - f_D(t)) / \alpha, \quad (8)$$

Equation 8 was used to calculate the time required for delivery of 99% of the administered dose, t_{99} , for each ENM-media combination using the specific deposition function constants, α , and an $f_D(t)$ value of 0.99.

2.4 In vitro toxicity assessment

RPMI, calcein AM, ethidium homodimer-1, Hoechst-33342, and HCS CellMask Blue were obtained from Life Technologies (Carlsbad, CA). Penicillin/Streptomycin, HEPES and BSA were obtained from Sigma Aldrich (St. Louis, MO), and FBS and heat-inactivated FBS were obtained from Atlanta Biologicals (Atlanta, GA). All incubations were performed at 37°C/5% CO_2 unless otherwise specified. Assays were repeated three times for each ENM.

THP-1 monocytes were cultured in RPMI supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, and 10 mM HEPES. THP-1 cells were matured according to the method described by Daigneault et. al. (Daigneault et al., 2010) as follows: Cells were resuspended at a $3.2 \times 10^5/\text{ml}$ in RPMI/10%FBS containing 200 nM PMA, dispensed into 96-well black-walled imaging plates (BD, Franklin Lakes, NJ) at $8.0 \times 10^5/\text{cells/well}$ in culture media with 200 nM PMA, incubated for 3 days, washed with PBS, and incubated for an additional 5 days in RPMI/10% FBS (without PMA).

PMA-matured THP-1 macrophages were incubated for either 4 or 24 hours with 100 $\mu\text{l/well}$ of ENM dispersions at varied concentrations between 0.0 and 50.0 $\mu\text{g/ml}$ in either RPMI

alone, RPMI + 1%BSA or RPMI+10%FBS. Following ENM exposure cells were washed with HBSS, incubated for 30 min. with 1.0 μ M CalceinAM, 2.0 μ M ethidium homodimer-1, 2.0 μ g/ml Hoechst 33342, and 2.5 μ g/ml HCS CellMask Blue in HBSS, and finally covered with PBS.

Confocal fluorescence microscopy was performed using a BD Pathway 855 High-Content Bioimager with a 20X NA075 objective (Olympus), with laser auto-focus, 2 $\times$ 2 binning and flat field correction. Green channel images (calceinAM) were acquired using 470/40 band-pass (BP) excitation, 515 long-pass (LP) dichroic and 515 LP emission filters, red channel images (ethidium homodimer-1) using 560/20 BP excitation, 595 LP dichroic and 645 LP emission filters, and blue channel images (Hoechst/CellMask Blue) using 350/20 BP excitation, 400 LP dichroic, and 435 LP emission filters. Three non-overlapping 20X image fields were acquired for each well to produce a total of six sets of images (3 fields $\times$ duplicate wells) per condition.

Image analysis and quantification was performed using custom software developed in MATLAB[®] (The Mathworks, Natick, MA). Individual cells were identified in Hoechst/CellMask Blue images (blue channel) by watershed segmentation as previously described (DeLoid et al., 2009, Sulahian et al., 2008). Viable cells were identified based on green (calceinAM) and red (ethidium homodimer-1) image channel pixel values corresponding to each set of cell pixels. A threshold for calceinAM images, below which cell pixels were categorized as calcein negative, was selected as the minimum pixel grayscale value at which all brightly staining cells were positive. The threshold for ethidium homodimer-1, above which cell pixels were categorized as ethidium-homodimer-1 positive, was selected as the maximum grayscale value at which only pixels within brightly stained nuclei were positive. Threshold values were maintained throughout analysis of all experiments with all ENMs. Cells in which more than 50% of pixels were calceinAM positive, and in which less than 5% of pixels were ethidium homodimer-1 positive, were scored by the software as viable. All other cells were scored as non-viable. The total number of viable and non-viable cells for each treatment/media combination was obtained by summing viable and non viable cell counts for the three fields in each of the duplicate wells.

3 RESULTS

3.1 Characterization of raw ENM powders

The panel of ENMs investigated in this study consisted of industry-relevant metal oxides of varied primary particle sizes, shapes and surface chemistries. ENM properties in powder form are summarized in Table 1 and include SSA , d_{BET} and d_{XRD} . In addition, as shown in the representative TEM/SEM images in Figure S-1, these ENMs appeared to form chain-like aggregates in nanopowder form.

3.2 Characterization of ENM liquid suspensions – Dispersion Protocol

Critical DSE—For each ENM, the DLS determined hydrodynamic diameter (d_H) was plotted as a function of the DSE. For all ENMs, d_H decreased with increasing DSE toward a horizontal asymptote, representing a minimal agglomeration state. From each curve, an ENM-specific DSE_{cr} was estimated by determining the DSE value at which the dispersed

ENMs were within 10% of their observed minimum d_H value. (theoretical d_H at $DSE = +\infty$). An example of the aforementioned asymptotic curve and its estimated DSE_{cr} for SiO_2 (EVONIK, $d_{\text{BET}} = 55$ nm) is shown in Figure 2. Table 2 summarizes the estimated DSE_{cr} for all investigated ENMs. DSE_{cr} values varied from 161 to 242 J/ml, and required between 8.5 and 12 minutes with the Branson 450 analog sonicator at the settings described in methods (data not shown).

In order to validate the reproducibility of our calorimetric calibration and estimation of DSE_{cr} , calibration, sonication and DLS analysis were performed with a second sonicator (Vibra-Cell VCX 130, Sonics and Materials, Inc. Newtown, CT). The Vibra-Cell model yielded agglomeration sizes and DSE_{cr} values (data not shown) for various ENMs consistent with those obtained using the Branson 450 sonicator (data not shown), verifying that the proposed calorimetric approach for the precise estimation of DSE, enables reproducible results with different sonicators (Taurozzi et al., 2010).

Dispersion stability over time—The size distribution (by DLS) of dispersions created at and below DSE_{cr} , of a representative ENM dispersion (EVONIK SiO_2 , $d_{\text{BET}} = 55$ nm) over a 24-hour period are shown in Figure 3. Dispersion at the DSE_{cr} (161 J/ml, Figure 3A) produced a monodisperse suspension of small agglomerates. In contrast, sonication below the DSE_{cr} (9 J/ml, Figure 3B) produced polydisperse suspensions of significantly larger agglomerates. In addition to achieving minimal agglomeration, sonication at the DSE_{cr} also produced dispersions with stable size distributions, whereas the size distribution of dispersions generated below DSE_{cr} changed substantially over time (Figure 3A and 3B). Similar results were obtained for all other ENMs investigated in this study (data not shown).

Agglomeration in physiological fluids—Measured properties of ENM dispersions (hydrodynamic diameter (d_H), polydispersity index (PdI), zeta potential (ζ), and specific conductance (σ), in DI H_2O and the three media formulations studied are summarized in Table 3. In general, d_H , PdI , and ζ were both material- and media-specific. The pattern of agglomerate sizes in the various dispersion media differed qualitatively between ENMs with negative ζ values and those with positive ζ values in DI H_2O . ENMs with negative ζ values in DI H_2O (SiO_2 and Ag/SiO_2 ENMs) formed much larger agglomerates in media containing 10% FBS, than in either RPMI alone, RPMI with 1% BSA or DI H_2O . In contrast, materials with positive ζ values in DI H_2O (Fe_2O_3 , CeO_2 , TiO_2) formed markedly larger agglomerates (up to micron scale) in RPMI alone, intermediate-sized agglomerates in RPMI containing either 1% BSA or 10% FBS, and smaller agglomerates in DI H_2O .

3.3 Dosimetry analysis and empirical deposition fractions, $f_D(t)$

Administered dose—Total administered mass, M_A , surface area, SA_A , and particle number, N_A , doses for each ENM-media system are summarized in Table 4. Note that since N_A is inversely proportional to r_H^3 (Equation 2), larger agglomerates in the dispersions result in much smaller administered numbers of particles. Furthermore, since SA_A is proportional to $r_H^2 \times N_A$ (Equation 3), or $r_H^{2/3}$, larger agglomerates in the dispersion are associated with smaller administered surface area values, for a given administered mass dose. For example, the approximately 3.5 fold larger agglomerate size, as indicated by the DLS-determined r_H ,

for the RPMI/10%FBS media, compared with that observed in RPMI alone, for VENGES 10Ag/SiO₂ ($d_{BET}=5.3\text{nm}$), is accompanied by a roughly 20 fold lower N_A ($4.05\text{E}+07$ vs. $8.08\text{E}+08$), and a 2.7 fold lower SA_A (0.231 cm^2 vs. 0.629 cm^2).

Delivered dose- empirical deposition fraction equations—The particle- and media-specific deposition fraction constant, $\alpha(\text{h}^{-1})$, and the time required to deliver 99% of the administered dose to cells, $t_{99}(\text{h})$, for each ENM-media system, are summarized in Table 5 and Figure 4. In addition, representative deposition fraction curves for 10Ag/SiO₂ ($d_{BET}=5.3\text{nm}$), SiO₂ ($d_{BET}=11.9\text{nm}$), TiO₂ ($d_{BET}=25\text{nm}$), and CeO₂ ($d_{BET}=30\text{nm}$) in each media formulation are presented in Figure 5. Notably, both the deposition fraction curve shape, deposition fraction constant α , and t_{99} , were greatly influenced by material density. Materials of relatively high density, such as TiO₂ ($\rho=4.23\text{g/cm}^3$), and CeO₂ ($\rho=7.65\text{g/cm}^3$) had relatively steep deposition fraction curves and corresponding large α values, as well as relatively short t_{99} times, compared to materials of lower density such as SiO₂ ($\rho=2.2\text{g/cm}^3$) (Table 5, Figure 4, Figure 5).

It is also clear that deposition kinetics are greatly dependent on dispersion media and the associated material agglomeration states. For example, the delivery of 10Ag/SiO₂ to cells in media containing FBS, in which particles exist in relatively large agglomerates (larger d_H), occurs at a much faster rate (large α and short t_{99}), than in RPMI alone or in RPMI with 1% BSA (Table 5, Figure 4, Figure 5).

3.4 In vitro cytotoxicity of ENM suspensions

In order to assess the roles of ENM property transformations and deposition kinetics in ENM-cell interactions and biological effects, *in vitro* cytotoxicity in adherent PMA-matured THP-1 macrophages was evaluated over a range of administered and delivered doses for ENMs in each of the three media formulations, at 4 and 24 hours. Cell viability following exposure to $50\text{ }\mu\text{g/ml}$ 10Ag/SiO₂ ($d_{BET}=5.3\text{nm}$) in each media type, for 4 and 24 hours, is depicted in Figure 6. Cell viability is also presented alongside corresponding administered and delivered dose metric values for each exposure time in Table 6. Toxicity data were collected and analyzed for all other ENM-media combinations, various doses and exposure times and will be presented by the authors in a forthcoming manuscript.

Although 1-way ANOVA revealed statistically significant differences between percent viability of cells treated with 10Ag/SiO₂ ($d_{BET}=5.3\text{nm}$) in the three different media formulations at four hours (overall $p < 0.05$), neither Tukey's nor Bonferroni's multiple comparison tests identified significant differences in cytotoxicity within any pair of dispersal medias. Nevertheless, particles dispersed in RPMI/10%FBS appeared to be more toxic than those dispersed in either RPMI alone, or RPMI/1% BSA. For an identical administered mass dose ($M_A = 5.0\text{ }\mu\text{g}$), the administered surface and number doses were substantially lower in RPMI/10% FBS ($SA_A = 0.231\text{ cm}^2$, $N_A = 4.05\text{E}+07$) than in either RPMI alone ($SA_A = 0.629\text{ cm}^2$, $N_A = 8.08\text{E}+08$) or in RPMI/1% BSA ($SA_A = 0.815\text{ cm}^2$, $N_A = 1.76\text{E}+09$). In contrast, however, the delivered surface dose at 4 hours was greater for particles in RPMI/10%FBS ($SA_D = .205\text{ cm}^2$) than for cells in either RPMI alone ($SA_D = .087\text{ cm}^2$) or in RPMI/1% BSA ($SA_D = .140\text{ cm}^2$), and delivered mass dose was markedly greater in

RPMI/10% FBS ($M_D = 4.440$ g) than in RPMI alone ($M_D = 0.691$ g) or with 1% BSA ($M_D = 0.859$ g), consistent with the higher cytotoxicity observed with particles dispersed in RPMI/10%FBS. No significant differences were detected by 1-way ANOVA, between viability of cells treated for 24 hours in the different media formulations, although a similar trend was observed, consistent with the same relative differences in administered and delivered surface and mass doses.

4 Discussion

Recent research has demonstrated that ENM polydispersity and agglomeration stability and polydispersity can have profound impacts on ENM toxicity *in vitro* (Liu et al., 2009, Tedja, 2011). Harmonized methods for *in vitro* nanotoxicity assessments must therefore include ENM dispersion preparation and characterization protocols that ensure fairly monodispersed and stabilized ENM suspensions suitable for *in vitro* cellular studies. The sonication protocol proposed here includes the important step of identifying the material specific DSE_{cr} , which defines the sonication energy necessary to establish a monodisperse suspension with minimal agglomeration (Fig. 2) that maintains its size distribution over time (Fig. 3). Both the monodispersity and the stability of ENM dispersions over time influence the particle mobility and delivery of particles to cells. Therefore, it is of paramount importance to ensure that the dispersion protocol delivers sonication energy at or above the DSE_{cr} in order to achieve monodisperse ENM suspensions with particle size distributions that remain stable for the duration our toxicological evaluation.

This study shed new light on particle interactions in various commonly used dispersion media for a panel of industry relevant nanosized metal oxide particles. Consistent with the previous reports in the literature, particle-particle interactions were influenced by primary particle properties (primary particle size, surface chemistry), as well as media characteristics (specific conductance, protein content) (Ji et al., 2010, Jiang, 2009, Murdock et al., 2008, Bihari et al., 2008, Zook et al., 2010). For example, a sonication energy (DSE_{cr}) of 161 J/ml was required to achieve a monodisperse solution ($PdI = 0.188$) of minimal agglomeration size ($d_H = 123.8$ nm) for VENGES SiO_2 ($d_{BET} = 11.9$ nm) in DI H_2O , in which a zeta potential of -22.1 mV was observed. Hydrodynamic diameter and zeta potential remained relatively stable when diluted in to RPMI alone ($d_H = 114.5$ nm, $\zeta = -25.4$ mV). However, when dispersed in either RPMI/10%FBS or RPMI/1%BSA, zeta potential was decreased by approximately 50% ($\zeta = -12.3$ mV in RPMI/10%FBS, $\zeta = -14.6$ mV in RPMI/1%BSA), consistent with the formation of protein coronae surrounding particles and adding bulk to agglomerates, as described by Lundqvist et al (Lundqvist et al., 2008). Increased bulk and decreased electrostatic repulsion between particles, due to the presence of proteins on the agglomerate surface, may explain the observed increase in agglomeration size in these media ($d_H = 123.8$ nm in DI H_2O , $d_H = 203.97$ nm in RPMI/10%FBS, $d_H = 143.8$ nm in RPMI/1%BSA). In contrast VENGES Fe_2O_3 ($d_{BET} = 4.8$ nm) required a higher DSE_{cr} of 242 J/ml in DI H_2O , a media of low specific conductance ($\sigma = 0.0148$ mS/cm), to effectively break down agglomerates and achieve a monodisperse suspension. When diluted in RPMI alone, a media of high specific conductance ($\sigma = 11.0$ mS/cm), the zeta potential decreased from 27.8 mV to -16.7 mV. This dramatic change in electrostatic charge may explain the striking increase in agglomeration size observed in RPMI ($d_H = 4333$ nm). According to the

extended DLVO theory (Derjaguin, Landa, Verwey, and Overbeek), the stability of a colloidal suspension depends upon the net balance of all forces acting on the suspended particles: electrostatic, van der Waals, steric, electrosteric, depletion, solvation etc. In suspension, electrostatic repulsion is a major force preventing particle aggregation via the formation of the Electrical Double Layer (Derjaguin, 1941). In media of high specific conductance, the electrical double layer surrounding the particles is compressed, resulting in a decreased zeta potential. This decrease in repulsive electrostatic forces allows van der Waals attractions to dominate and ENM agglomeration to occur. In RPMI/10%FBS and RPMI/1%BSA, the formation of protein coronas around agglomerates and the resulting steric hindrance between particles may explain the relatively smaller agglomerate sizes (225.5nm in RPMI/10%FBS, 937.4nm in RPMI/1%BSA). A similar pattern of particle transformation was observed for CeO₂ and TiO₂ ENMs, consistent with previously reported transformations of these ENMs in various media (Ji et al., 2010, Sehgal et al., 2005).

Appropriate characterization of ENM suspensions are required for accurate determination of administered doses. Most often, primary particle diameter (d_{BET}) is used for estimating administered dose. However, this approach does not account for the influence of particle interactions and agglomeration in physiological media (Rushton et al., 2010, Wittmaack, 2007). The variable particle agglomeration sizes and resulting administered doses for ENMs in different media formulations presented in Table 4 demonstrate the importance of agglomeration state in calculating dose. From these data it is clear that the media- specific agglomeration state of ENMs in suspension influences the administered number and surface doses (N_A and SA_A), with implications for the subsequent fate and transport of the ENMs, their delivery to cells, and ultimately their toxicity. Differences between traditionally reported administered doses, based on the d_{BET} , and those based on the media-specific r_H values may explain some discrepancies in the literature. Agglomeration and partico-kinetics can also influence particle mobility and the subsequent dose delivered to cells. For each ENM in our panel, the material- and media- specific deposition fraction constant α , was highly variable and dependent upon particle characteristics such as density and agglomeration state (Table 5, Figure 5). Our *in vitro* cytotoxicity data for VENGES 10Ag/SiO₂ (d_{BET} =5.3nm) underscored the importance of utilizing these types of media-particle characterization-based dose metrics when evaluating cytotoxicity. Significant decreases in cell viability were observed following 24 hours of exposure, compared with only 4 hours for all three ENM-media systems (Figure 6, Table 6). Comparisons of various administered and delivered doses demonstrated that particle interactions in liquid media and the subsequent deposition kinetics significantly influence the timing and extent of cytotoxic events observed *in vitro*. Taken together, our data suggests that delivered doses estimated from properly characterized ENM dispersions may be more physiologically relevant than traditionally reported primary particle size-based administered doses. This physiological relevance, combined with conceptual simplicity of the particle-media specific deposition constant, α , and the ease with which it can be determined, make this approach an attractive option for use in the design of *in vitro* cellular assays.

In future studies we plan to extend our investigation to deposition fraction constants and delivered dose metrics for additional industrially relevant ENMs. Additional studies are also

necessary to confirm the delivery doses estimated here, for which only indirect supportive evidence has been presented to date. It will also be necessary to investigate the kinetics and mechanisms involved in the internalization of particles by cells, including actively phagocytic cells such as macrophages (of which many varieties of primary and cell line macrophages should be examined), as well as other cell types whose interactions with particles may differ substantially from those with macrophages and from each other.

5 Conclusions

Our results demonstrate that particle kinetics and property transformations in physiological fluids have important implications for *in vitro* dosimetry, and should be central to the design and interpretation of cellular toxicological studies of ENMs. Harmonization of ENM dispersion protocols should include proper reporting of dispersion protocols and delivered sonication energy, which should be sufficient to establish stable monodisperse suspensions. The importance of considering the particle dose actually delivered to cells is also demonstrated, and the fact that this is not typically done may explain some of the discrepancies in the nanotoxicology literature. Furthermore, it is critical that *in vitro* toxicity protocols employ relevant exposure times derived from robustly characterized ENM- and media-specific particle-kinetics. The importance of particle-kinetics in various physiological media for a panel of industrially relevant nano-sized metal oxides was demonstrated. A simple exponential deposition function was proposed (Equation 8), and a particle- and media-specific constant, α , was obtained for each ENM-media system. This simple empirical function can be used to estimate delivered particle doses at specified times. In future studies, it will also be necessary to investigate the kinetics of particle internalization by cells, and to link this to particle and media specific parameters. Evaluation of various administered, delivered, and internalized dose metrics will help to illuminate the mechanisms by which ENM induce cellular toxicity, and improve the consistency, relevance and validity of *in vitro* toxicity evaluations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Characterization and dispersion protocol.

Figure 2. Hydrodynamic diameter as a function of DSE for EVONIK SiO₂ ($d_{\text{BET}}=55\text{nm}$). Critical DSE: energy required for minimal agglomeration.

Figure 3. Particle size distribution for dispersions of SiO₂ ($d_{\text{BET}}=55\text{nm}$) in RPMI at various time points and DSE levels; A. sonication at the critical DSE (161 J/ml), B. sonication below critical DSE (9 J/ml).

Figure 4.
Time to deliver 99% of administered dose to cells (t_{99}) for each ENM-media combination.

Figure 5.

Fraction of administered dose deposited, f_D , as a function of t (h) for various ENMs in the three media formulations; A. VENGENS 10Ag/SiO₂ ($d_{\text{BET}}=5.3\text{nm}$); B. VENGENS SiO₂ ($d_{\text{BET}}=11.9\text{nm}$); C. EVONIK TiO₂ ($d_{\text{BET}}=25\text{nm}$); D. VENGENS CeO₂ ($d_{\text{BET}}=30\text{nm}$).

Figure 6. Cytotoxicity of VENGES 10Ag/SiO₂ (d_{BET}=5.3nm) at 4 and 24 h in the three media formulations at a mass concentration of 50 µg/ml.

Figure 7.

Comparison of delivered mass (μg) and surface area (cm^2) doses for various ENM dispersed in RPMI at a mass concentration of $50\mu\text{g/ml}$ following 24 hours of exposure.

Table 1

ENM powder properties. SSA: (specific surface area) by nitrogen adsorption/Brunauer-Emmett-Teller (BET) method. d_{BET} : particle diameter determined from SSA and particle density, ρ , as described in methods.

d_{XRD} : particle diameter by X-ray diffraction.

Material	SSA (m ² /g)	d_{BET} (nm)	d_{XRD} (nm)	ρ (g/cm ³)
VENGES SiO ₂	231	11.8	NA	2.2
VENGES 10Ag/SiO ₂ *	200	9.9	6 (75 wt%) ** 27(25 wt%)	3.029
VENGES 10Ag/SiO ₂ *	375	5.3	6.9	3.029
VENGES 2Ag/SiO ₂ *	350	5.7	NA	2.365
VENGES 25Ag/SiO ₂ *	290	6.8	8.1	4.272
VENGES CeO ₂	152	13	9	7.65
VENGES CeO ₂	66	30	20	7.65
VENGES Fe ₂ O ₃	239	4.8	2(95 wt%) ** 30(5 wt%)	5.242
EVONIK SiO ₂	200	14	NA	2.2
EVONIK SiO ₂	50	55	NA	2.2
EVONIK TiO ₂	50	21	33	4.23

Note:

* denotes composite material XAg/SiO₂, where X is equal to the mass percentage of Ag deposited onto SiO₂ particles.

** denotes crystal size measured by x-ray diffraction is bimodal

Table 2

ENM Critical DSE

Material	DSE _{cr} (J/ml)
VENGES SiO ₂ d _{BET} =11.9nm	161
VENGES 10Ag/SiO ₂ d _{BET} =9.9nm	242
VENGES 10Ag/SiO ₂ d _{BET} =5.3nm	242
VENGES 2Ag/SiO ₂ d _{BET} =5.7nm	242
VENGES 25Ag/SiO ₂ d _{BET} =6.8nm	242
VENGES CeO ₂ d _{BET} =13nm	242
VENGES CeO ₂ d _{BET} =30nm	242
VENGES Fe ₂ O ₃ d _{BET} =4.8nm	242
EVONIK SiO ₂ d _{BET} =14nm	161
EVONIK SiO ₂ d _{BET} =55nm	161
EVONIK TiO ₂ d _{BET} =25nm	161

Table 3

Properties of ENM dispersions. d_H : hydrodynamic diameter, Pdl: polydispersity index, ζ : zeta potential, σ : specific conductance

Material	Media	d_H (nm)	Pdl	ζ (mV)	σ (mS/cm)	pH
VENGES SiO ₂ d_{BET} =11.9nm	DI H ₂ O	124 ± 7.19	0.188 ± 0.013	-22.1 ± 1.04	0.0102 ± 0	6.05 ± 0.46
	RPMI	115 ± 2.40	0.273 ± 0.025	-25.4 ± 2.00	10.4 ± 0.29	7.77 ± 0.12
	RPMI/10%FBS	204 ± 13.5	0.542 ± 0.004	-12.3 ± 0.066	12.1 ± 0.033	8.05 ± 0.13
	RPMI/1%BSA	144 ± 5.59	0.653 ± 0.032	-14.6 ± 0.20	11.2 ± 0.088	7.85 ± 0.082
VENGES 10Ag/SiO ₂ d_{BET} =9.9nm	DI H ₂ O	198 ± 9.08	0.256 ± 0.004	-33.4 ± 6.64	0.0097 ± 0.002	7.35 ± 0.13
	RPMI	158 ± 5.29	0.365 ± 0.017	-27.7 ± 0.75	11.3 ± 0.066	8.43 ± 0.15
	RPMI/10%FBS	220 ± 29.3	0.453 ± 0.084	-11.5 ± 0.17	11.3 ± 0.033	8.46 ± 0.22
	RPMI/1%BSA	155 ± 21.4	0.656 ± 0.18	-13.8 ± 1.60	9.66 ± 0.64	8.21 ± 0.19
VENGES 10Ag/SiO ₂ d_{BET} =5.3nm	DI H ₂ O	133 ± 2.33	0.353 ± 0.029	-24.5 ± 0.72	0.0359 ± 0	8.55 ± 0.21
	RPMI	157 ± 18.1	0.287 ± 0.025	-21.7 ± 0.67	10.9 ± 0.15	8.28 ± 0.073
	RPMI/10%FBS	427 ± 73.3	0.594 ± 0.102	-9.8 ± 0.20	10.8 ± .13	8.33 ± 0.10
	RPMI/1%BSA	121 ± 56.5	0.548 ± 0.061	-13.3 ± 0.84	12.8 ± 0.28	8.26 ± 0.12
VENGES 2Ag/SiO ₂ d_{BET} =5.7nm	DI H ₂ O	137 ± 0.76	0.31 ± 0.0011	-25.2 ± 0.55	0.0151 ± 0	7.9 ± 0.084
	RPMI	301 ± 17.6	0.392 ± 0.036	-24.6 ± 0.24	11 ± 0.10	8.43 ± 0.10
	RPMI/10%FBS	516 ± 11.3	0.511 ± 0.055	-10.7 ± 0.058	10.4 ± 0.22	8.42 ± 0.091
	RPMI/1%BSA	149 ± 11.3	0.509 ± 0.087	-13.4 ± 0.44	12.7 ± 0.15	8.34 ± 0.082
VENGES 25Ag/SiO ₂ d_{BET} =6.8nm	DI H ₂ O	122 ± 1.14	0.396 ± 0.005	-24.8 ± 1.43	0.0487 ± 0.004	9.13 ± 0.11
	RPMI	198 ± 9.22	0.4 ± 0.011	-26.8 ± 0.87	12.9 ± 0.33	8.29 ± 0.12
	RPMI/10%FBS	310 ± 13.9	0.488 ± 0.040	-14.5 ± 4.62	9.41 ± 0.26	8.03 ± 0.079
	RPMI/1%BSA	156 ± 17.2	0.449 ± 0.064	-15.5 ± 0.033	11.5 ± 0.066	8.19 ± 0.10
VENGES CeO ₂ d_{BET} =13nm	DI H ₂ O	136 ± 0.404	0.362 ± 0.005	35.5 ± 1.00	0.0123 ± 0	5.56 ± 0.089
	RPMI	1678 ± 260	0.475 ± 0.053	-10.1 ± 0.239	11.1 ± 0.12	7.92 ± 0.087
	RPMI/10%FBS	235 ± 5.92	0.253 ± 0.010	-14.3 ± 0.433	10.4 ± 0.057	8.16 ± 0.098
	RPMI/1%BSA	369 ± 6.46	0.338 ± 0.0138	-15.4 ± 0.583	11.4 ± 0.133	7.95 ± 0.079
VENGES CeO ₂ d_{BET} =30nm	DI H ₂ O	127 ± 16.4	0.353 ± 0.110	34.5 ± 3.15	0.022 ± 0	7.4 ± 0.04
	RPMI	1376 ± 371	0.149 ± 0.130	-9.77 ± 0.497	11 ± 0.152	7.95 ± 0.069

Material	Media	d_H (nm)	PdI	ζ (mV)	σ (mS/cm)	pH
	RPMI/10%FBS	227 $\pm$ 3.25	0.243 $\pm$ 0.0132	-12.0 $\pm$ 0.329	11.9 $\pm$ 0.0883	8.19 $\pm$ 0.073
	RPMI/1%BSA	552 $\pm$ 37.3	0.406 $\pm$ 0.120	-14.7 $\pm$ 0.408	10.7 $\pm$ 0.490	7.94 $\pm$ 0.065
VENGES Fe ₂ O ₃ d_{BET} =4.8nm	DI H ₂ O	151 $\pm$ 9.14	0.449 $\pm$ 0.016	27.8 $\pm$ 2.42	0.0148 $\pm$ 0.002	7.03 $\pm$ 0.088
	RPMI	4333 $\pm$ 400	0.357 $\pm$ 0.020	-16.7 $\pm$ 0.23	11 $\pm$ 0.12	8.45 $\pm$ 0.094
	RPMI/10%FBS	226 $\pm$ 4.20	0.213 $\pm$ 0.014	-13.4 $\pm$ 0.60	11.4 $\pm$ 0.033	8.46 $\pm$ 0.11
	RPMI/1%BSA	937 $\pm$ 104	0.612 $\pm$ 0.069	-15.6 $\pm$ 0.93	11.4 $\pm$ 0.32	7.74 $\pm$ 0.086
EVONIK SiO ₂ d_{BET} =14nm	DI H ₂ O	166 $\pm$ 4.27	0.195 $\pm$ 0.040	-36.3 $\pm$ 2.79	0.0239 $\pm$ 0	7.23 $\pm$ 0.079
	RPMI	147 $\pm$ 0.78	0.122 $\pm$ 0.008	-32.2 $\pm$ 0.0019	11.8 $\pm$ 0.29	8.43 $\pm$ 0.098
	RPMI/10%FBS	227 $\pm$ 2.50	0.302 $\pm$ 0.002	-12.2 $\pm$ 0.25	11.3 $\pm$ 0.17	8.43 $\pm$ 0.081
	RPMI/1%BSA	210 $\pm$ 0.90	0.738 $\pm$ 0.038	-15.6 $\pm$ 0.49	11 $\pm$ 0.18	8.19 $\pm$ 0.11
EVONIK SiO ₂ d_{BET} =55nm	DI H ₂ O	262 $\pm$ 2.42	0.16 $\pm$ 0.0029	-38.6 $\pm$ 0.19	0.00867 $\pm$ 0	7.18 $\pm$ 0.075
	RPMI	244 $\pm$ 1.83	0.165 $\pm$ 0.008	-36.5 $\pm$ 1.77	11.1 $\pm$ 0.26	8.38 $\pm$ 0.077
	RPMI/10%FBS	277 $\pm$ 32.7	0.474 $\pm$ 0.021	-12.7 $\pm$ 0.066	11.8 $\pm$ 0.12	8.36 $\pm$ 0.080
	RPMI/1%BSA	290 $\pm$ 11.0	0.475 $\pm$ 0.046	-16.6 $\pm$ 0.57	12.3 $\pm$ 0.15	8.14 $\pm$ 0.074
EVONIK TiO ₂ d_{BET} =25nm	DI H ₂ O	241 $\pm$ 6.37	0.209 $\pm$ 0.011	25.8 $\pm$ 3.82	0.0368 $\pm$ 0.005	7.28 $\pm$ 0.19
	RPMI	2867 $\pm$ 74.8	0.194 $\pm$ 0.043	-11.4 $\pm$ 0.351	12.5 $\pm$ 0.18	8.47 $\pm$ 0.13
	RPMI/10%FBS	471 $\pm$ 0.97	0.193 $\pm$ 0.028	-10.9 $\pm$ 0.55	10.5 $\pm$ 0.42	8.45 $\pm$ 0.11
	RPMI/1%BSA	408 $\pm$ 14.4	0.196 $\pm$ 0.022	-9.75 $\pm$ 3.30	4.56 $\pm$ 3.27	8.33 $\pm$ 0.099

Administered doses. Total surface area (SA_A), and particle number (N_A) were calculated using Equations 1–3 and the estimated agglomerate density (ρ_a), for a volume, V , of 0.1 ml, a mass concentration, γ_A , of 50 $\mu\text{g}/\text{ml}$, and total mass of 5 μg , with particle radius, r , set to the particle agglomerate hydrodynamic radius ($r_H = d_H/2$) obtained from DLS for each of the three media formulations.

Table 4

Material	Media	ρ_a (g/cm^3)	r_H (nm)	SA_A (cm^2)	N_A
VENGES SiO_2 $d_{\text{BET}}=11.9\text{nm}$	RPMI	1.07	57.3	2.45	5.95E+09
	RPMI/10%FBS	1.03	103	1.42	1.09E+09
	RPMI/1%BSA	1.05	71.9	1.98	3.05E+09
VENGES 10Ag/ SiO_2 $d_{\text{BET}}=9.9\text{nm}$	RPMI	1.29	78.8	1.47	1.89E+09
	RPMI/10%FBS	1.23	110	1.10	7.25E+08
	RPMI/1%BSA	1.30	77.4	1.50	1.99E+09
VENGES 10Ag/ SiO_2 $d_{\text{BET}}=5.3\text{nm}$	RPMI	1.19	78.7	1.60	2.06E+09
	RPMI/10%FBS	1.09	214	0.642	1.12E+08
	RPMI/1%BSA	1.23	60.8	2.01	4.34E+09
VENGES 2Ag/ SiO_2 $d_{\text{BET}}=5.7\text{nm}$	RPMI	1.08	150	0.920	3.24E+08
	RPMI/10%FBS	1.06	258	0.549	6.55E+07
	RPMI/1%BSA	1.14	74.7	1.76	2.52E+09
VENGES 25Ag/ SiO_2 $d_{\text{BET}}=6.8\text{nm}$	RPMI	1.31	99.0	1.16	9.41E+08
	RPMI/10%FBS	1.23	105	1.16	8.39E+08
	RPMI/1%BSA	1.37	77.9	1.41	1.85E+09
VENGES CeO_2 $d_{\text{BET}}=13\text{nm}$	RPMI	1.22	839	0.146	1.66E+06
	RPMI/10%FBS	1.88	117	0.680	3.94E+08
	RPMI/1%BSA	1.64	185	0.496	1.16E+08
VENGES CeO_2 $d_{\text{BET}}=30\text{nm}$	RPMI	1.46	688	0.150	2.52E+06
	RPMI/10%FBS	2.61	114	0.953	5.89E+08
	RPMI/1%BSA	1.87	276	0.291	3.04E+07
VENGES Fe_2O_3 $d_{\text{BET}}=4.8\text{nm}$	RPMI	1.04	2167	0.0668	1.13E+05
	RPMI/10%FBS	1.29	113	1.03	6.48E+08
	RPMI/1%BSA	1.11	469	0.289	1.05E+07

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Material	Media	ρ_a (g/cm ³)	r_H (nm)	SA_A (cm ²)	N_A
EVONIK SiO ₂ d _{BET} =14nm	RPMI	1.37	73.3	1.49	2.22E+09
	RPMI/10%FBS	1.30	114	1.02	6.27E+08
	RPMI/1%BSA	1.31	105	1.09	7.92E+08
EVONIK SiO ₂ d _{BET} =55nm	RPMI	1.42	122	0.864	4.62E+08
	RPMI/10%FBS	1.39	139	0.780	3.23E+08
	RPMI/1%BSA	1.38	145	0.752	2.84E+08
EVONIK TiO ₂ d _{BET} =25nm	RPMI	1.10	1434	0.0949	3.67E+05
	RPMI/10%FBS	1.37	236	0.466	6.68E+07
	RPMI/1%BSA	1.40	204	0.523	9.99E+07

Table 5

Deposition fraction constant, α (h^{-1}), and time for delivery of 50% of dose, t_{50} (h), and 99% of dose, t_{99} (h), for each ENM-media system.

Material	Media	d_H (nm)	α (h^{-1})	t_{50} (h)	t_{99} (h)
VENGES SiO ₂ (d_{BET} =11.9nm)	RPMI	115 $\pm$ 2.40	0.0136	51.1	343
	RPMI/10%FBS	204 $\pm$ 13.5	0.00945	73.3	492
	RPMI/1%BSA	144 $\pm$ 5.59	0.0115	60.5	406
VENGES 10Ag/SiO ₂ (d_{BET} =9.9nm)	RPMI	158 $\pm$ 5.29	0.0162	42.8	287
	RPMI/10%FBS	220 $\pm$ 29.3	0.00984	70.4	473
	RPMI/1%BSA	155 $\pm$ 21.4	0.0120	57.9	388
VENGES 10Ag/SiO ₂ (d_{BET} =5.3nm)	RPMI	157 $\pm$ 18.1	0.0113	61.4	412
	RPMI/10%FBS	427 $\pm$ 73.3	0.00612	113	759
	RPMI/1%BSA	122 $\pm$ 56.5	0.0125	55.2	370
VENGES 2Ag/SiO ₂ (d_{BET} =5.7nm)	RPMI	301 $\pm$ 17.6	0.00923	75.1	504
	RPMI/10%FBS	516 $\pm$ 11.3	0.00624	111	745
	RPMI/1%BSA	149 $\pm$ 11.3	0.0117	59.1	397
VENGES 25Ag/SiO ₂ (d_{BET} =6.8nm)	RPMI	198 $\pm$ 9.22	0.0131	53.0	356
	RPMI/10%FBS	310 $\pm$ 13.9	0.00918	75.5	506
	RPMI/1%BSA	156 $\pm$ 17.2	0.0111	62.3	418
VENGES CeO ₂ (d_{BET} =13nm)	RPMI	1678 $\pm$ 260	0.0284	24.4	164
	RPMI/10%FBS	235 $\pm$ 5.92	0.0177	39.3	263
	RPMI/1%BSA	369 $\pm$ 6.46	0.0162	42.8	287
VENGES CeO ₂ (d_{BET} =30nm)	RPMI	1376 $\pm$ 371	0.0916	7.57	50.8
	RPMI/10%FBS	227 $\pm$ 3.25	0.0289	24.0	161
	RPMI/1%BSA	552 $\pm$ 37.3	0.0407	17.0	114
VENGES Fe ₂ O ₃ (d_{BET} =4.8nm)	RPMI	4333 $\pm$ 400	0.00669	104	695
	RPMI/10%FBS	226 $\pm$ 4.20	0.00933	72.3	498
	RPMI/1%BSA	937 $\pm$ 104	0.00810	85.5	574
EVONIK SiO ₂ (d_{BET} =14nm)	RPMI	147 $\pm$ 0.78	0.0119	58.2	390
	RPMI/10%FBS	227 $\pm$ 2.50	0.00755	91.7	615

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Material	Media	d_H (nm)	α (h ⁻¹)	t_{50} (h)	t_{99} (h)
EVONIK SiO ₂ (d_{BET} =55nm)	RPMI/1%BSA	210 ± 0.90	0.00588	118	791
	RPMI	244 ± 1.83	0.0207	33.5	225
	RPMI/10%FBS	277 ± 32.7	0.00784	88.4	593
	RPMI/1%BSA	290 ± 11.0	0.00758	91.5	614
EVONIK TiO ₂ (d_{BET} =21nm)	RPMI	2867 ± 74.8	0.0308	22.5	151
	RPMI/10%FBS	471 ± 0.97	0.0151	45.8	307
	RPMI/1%BSA	408 ± 14.4	0.0156	44.5	298

Summary of administered doses, delivered doses and cytotoxicity for 10Ag/SiO₂ (d_{BET}=5.3nm) dispersed in each media formulation (0.1 ml at 50 µg/ml, administered mass dose, M_A, = 5.0 µg).

Table 6

Media	S _A (cm ²)	N _A	4 Hours				24 Hours			
			M _D (µg)	S _{A_D} (cm ²)	N _D	Cell Viability (%)	M _D (µg)	S _{A_D} (cm ²)	N _D	Cell Viability (%)
RPMI	1.60	2.06E+09	0.221	0.0707	9.09+07	74.1	1.19	0.380	4.89E+08	6.49
RPMI/10%FBS	0.642	1.12E+08	0.121	0.0154	2.71E+06	70.5	0.683	0.0878	1.53E+07	16.6
RPMI/1%BSA	2.01	4.34E+09	0.245	0.0986	2.12E+08	94.4	1.30	0.523	1.13E+09	26.0