An integrated methodology across the dispersion preparation-characterization-in vitro dosimetry continuum for engineered nanomaterials

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ABSTRACT

Evidence continues to grow of the importance of in vitro and in vivo dosimetry in the hazard assessment and ranking of engineered nanomaterials (ENMs). Accurate dose metrics are particularly important for in vitro cellular screening to assess the potential health risks or bioactivity of ENMs. In order to ensure meaningful and reproducible quantification of in vitro dose, with consistent measurement and reporting between laboratories, it is necessary to adopt standardized and integrated methodologies for 1) generation of stable and minimally polydisperse ENM suspensions in cell culture media, 2) colloidal characterization of suspended ENMs, particularly properties that determine particle kinetics in an in vitro system (size distribution and formed agglomerate effective density), and 3) robust numerical fate and transport modeling for accurate determination of ENM dose delivered to cells over the course of the in vitro exposure. Here we present such an integrated comprehensive methodology for in vitro dosimetry, including detailed standardized procedures for each of these three critical steps..

Keywords: nanotoxicology; dosimetry; in vitro; dispersion

1 INTRODUCTION

The unique physicochemical properties of ENMs are being exploited for use in a growing variety of commercial nano-enabled products (NEPs), including electronics, cosmetics, and structural materials, as well as a wide variety of products for antimicrobial, agricultural, medical, therapeutic, and diagnostic applications. The rapid proliferation and commercialization of these ENMs and associated NEPs poses a potential risk of both occupational and consumer exposures to materials for which toxicological data are extremely limited. Moreover, the high degree of variability in physicochemical properties such as composition, size, morphology, surface topology, chemistry and modifications, crystallinity, and impurity content among these ENMs presents a substantial challenge to the nanotoxicology field. In addition, human exposure is not limited to pristine ENMs but also includes a wide variety of particles released from NEPs across their life cycle, including at the consumer use and disposal stages. Indeed, the potential for exposure from such life cycle particulate matter (LCPM) may exceed that of pristine ENMs. To complicate the matter further, the physi-cochemical properties and toxicological profiles of LCPM may differ greatly from those of the corresponding pristine ENMs. To address the vast and growing number and variety of ENMs entering the market, there is great need for fast, inexpensive, and *in vivo*-validated high-throughput screening strategies based on *in vitro* cellular assays. However, *in vitro* testing results from different labs often contradict one another, and even greater disparity is observed between *in vitro* and *in vivo* results. Much of this *in vitro* vs. *in vivo* disparity can be explained by the failure of simple cellular systems (which often use immortal, i.e., highly abnormal, cell lines) to adequately recapitulate the complex milieu of a mammalian organism. However, it is likely that mismatch of *in vitro* and *in vivo* doses, due to inadequate ENM characterization—and particularly failure to adequately account for fate and transport of ENMs *in vitro*—is a major contributor to these disparities[1-3].

In a typical *in vitro* cellular assay, ENMs are dispersed in cell culture media, and the resulting suspension is dispensed onto adherent cells in multiwell cell culture plates. Cellular responses are measured following incubation (typically 24 h) over a range of doses to establish a dose–response relationship. The doses most often used to define these relationships are measured as total administered mass, surface area or number of particles, or the per volume concentration of mass, surface area, or particles in the initial administered suspension. This *in vitro* hazard and bioactivity assessment approach has been widely and successfully used for industrial and environmental chemicals, as well as for drug candidates[4,5]. However, because ENMs in suspension are subject to ENM- and media-specific physicochemical transformations that affect their fate and transport, and thus the dose delivered to cells is a function of exposure time, it is not suitable for assessment of colloidal suspensions of ENMs.

When ENMs are suspended in cell culture media or physiological fluids, they may form agglomerates consisting of multiple protein-coated primary particles and trapped intra-agglomerate fluid. The extent and kinetics of

protein corona and agglomerate formation, and the size distribution and stability of agglomerates, are largely determined by the intrinsic properties of the primary ENM particles (i.e., composition, size, shape, surface chemistry) and the extrinsic properties of the fluid (i.e., ionic strength, pH, protein and other biomolecular types, and content)[6,7]. The degree of agglomeration and the stability of the resulting size distributions are also highly dependent on the methods used to disperse the ENMs in the liquid[1]. These transformations have critically important effects on nano-biointeractions between suspended ENMs and cells. Agglomeration reduces the total number of particles, as well as the total surface area of the suspended ENM available for interaction with cells. Indeed, cellular toxicity studies have demonstrated that agglomerated nanoparticles exert substantially different biological effects as compared with those exerted by well-dispersed nanoparticles of the same material[8,9].

Agglomeration also determines the key properties that determine the fate and transport of particles in suspension, namely size and effective density[2,10]. The agglomeration state of suspended ENMs can range from no agglomeration at all, with suspended forms having diameters on the same order as the primary particles, to formation of large micron-sized agglomerates containing hundreds or thousands of primary particles. Because the sedimentation rate of a particle in suspension is proportional to the square of its diameter, a tenfold difference in size results in a 100-fold difference in sedimentation rate, and roughly the same fold difference in delivered *in vitro* dose[10]. The other key determinant of transport is effective density. Because agglomerates can contain relatively large amounts of medium, the effective density of suspended ENMs can be much less than the density of the primary particle material, and it is often closer to the density of the medium[2]. Because the sedimentation rate is directly proportional to the difference between the medium and the agglomerate effective density, this can also have a large effect on the dose delivered to cells over time. It was recently demonstrated that buoyant ENMs present a special case and challenge for characterization and dosimetric analysis[11]. If the raw material density is lower than the medium density, as in the case of some conjugated polymers, nanobubbles and liposomes used in nanomedicine and food applications, the agglomerates do not settle, but instead rise or float away from cells over time, making the dose–response relationship impossible to determine. We have recently demonstrated that, in a standard cell culture system, buoyant polypropylene ENMs have no effect on cells, whereas in an inverted cell culture system, in which cells are oriented above the ENM suspension, the same ENMs produced dose-dependent increases in cytotoxicity and reactive oxygen species generation[11].

Dosimetry can affect interpretation of *in vitro* hazard ranking results. *In vitro* nanotoxicology is comparative in nature, often relying on previously studied nanomaterials as controls to provide hazard rankings among large panels of ENMs. However, studies often fail to sufficiently characterize colloidal properties of suspended materials or to account for effects of these properties on fate and transport and the exposure dose delivered to cells. The critical role of dosimetry in interpretation of *in vitro* nanotoxicology studies was highlighted in the seminal work of Wittmaack, who reported the formation and rapid settling and accumulation of micron-sized agglomerates of suspended nanostructured powders[12]. Importantly, Wittmaack pointed out that the resulting exceedingly high exposure doses could cause physical overload effects from overlying or internalized ENMs that could be misinterpreted as toxicity. Additional studies by Wittmaack reported that *in vitro* toxicity of SiO_2 nanoparticles was dependent upon the areal density of nanoparticle mass delivered to cells over the exposure duration, further emphasizing the importance of particle kinetics in interpretation of biological responses[13]. Thus, an ENM that forms large and dense—and thus rapidly sedimenting—agglomerates that quickly concentrate around cells may be reported as more toxic than one that forms smaller and less dense—and thus slower-settling—agglomerates, even though the latter material might well be more toxic than the former at equivalent delivered doses[3]. Recent studies report the impact of agglomerate properties on the time required for ENM delivery to cells *in vitro*[2,14], and demonstrate the subsequent impacts of dosimetry on hazard ranking of large panels of low-aspect-ratio ENMs[3].

We present here a multistep *in vitro* dosimetric protocol that enables nanotoxicologists to quantify delivered dose metrics as a function of time[14]. This protocol consists of three interconnected parts: (i) ENM dispersion preparation; (ii) ENM dispersion characterization; and (iii) numerical fate and transport modeling to derive delivered dose metrics (**Fig. 1**). We explain these in more detail below.

2 ENM DISPERSION PREPARATION

A reproducible dispersion preparation includes two key components: (i) calorimetric calibration of the sonication equipment and reporting of the delivered sonication energy (DSE) and duration in units of J/ml, and (ii) determination of the material-specific critical delivered sonication energy (DSEcr) required to achieve a suspension with the smallest possible agglomerates that are minimally polydisperse and maximally stable over time. Importantly, sonication must be performed in water rather than in culture media in order to avoid generation of reactive oxygen species by sonolysis and denaturing of proteins. To generate dispersions in media for use in experiments, the material is first sonicated in deionized water with a delivered sonication energy (J/ml) equal to DSEcr, and subsequently diluted in cell culture media to the desired initial concentration for application to cells (**Fig. 1**).

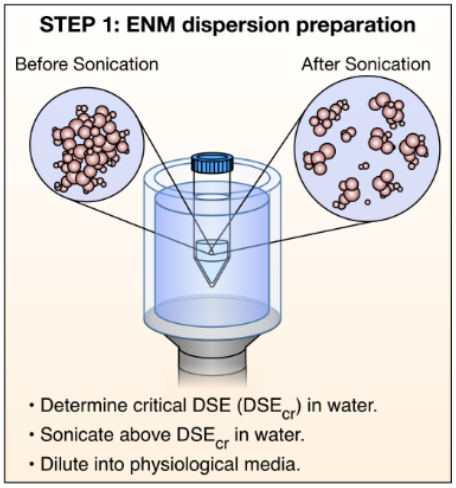

Figure 1: Step 1 of the *in vitro* dosimetric protocol: ENM Dispersion Preparation

3 ENM DISPERSION CHARACTERIZATION

Characterization has become an increasing concern in ENM toxicological testing. Robust characterization, including physical, chemical, and morphological properties of ENM powders, as well as colloidal properties of suspended ENMs, is essential for understanding biointeractions with cells. Lack of suitable ENM characterization, either of the pristine nanomaterials or of the dispersions, can call into question the validity and interpretability of *in vitro* toxicity data. Our protocol focuses on the colloidal properties of ENM suspensions that drive particle transport in liquid suspension, and thus determine the dose delivered to cells *in vitro*, namely the size (diameter) and effective density of the formed agglomerates. Although analytical ultracentrifugation (AUC) is the gold standard for measurement of ENM effective density, it requires relatively expensive equipment not available in many labs and is limited in terms of throughput. The VCM was developed by the authors to allow high-throughput measurement of effective density using a standard bench-top centrifuge and relatively inexpensive packed cell volume (PCV) tubes[2]. In brief, a known volume of suspension of a known ENM concentration is loaded into a PCV tube and centrifuged to collect the agglomerates in the capillary section of the tube. From the measured volume of the pellet and known volume of ENM, the effective density can be calculated as a weighted average of media and ENM.

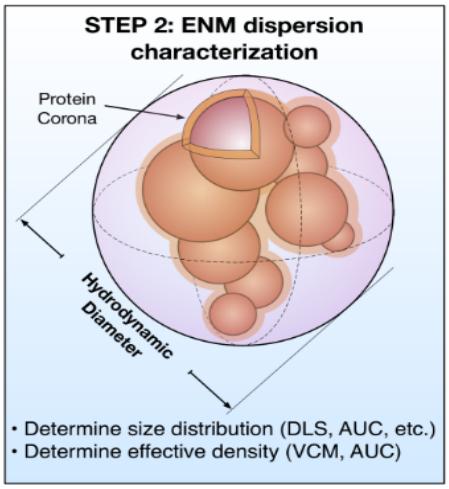

Figure 2: Step 2 of the *in vitro* dosimetric protocol: ENM Dispersion Characterization.

4 FATE AND TRANSPORT MODELING

With the stable suspension created and characterized, numerical modeling can then be used to compute the delivered dose metrics as a function of exposure time. The 1D model presented here, referred to as the DG fate and transport model, provides both deposition and concentration metrics, and concentration profiles of ENMs across the well as a function of time[10]. DG model also allows modeling of variable binding kinetics at the bottom of the well, accommodates simultaneous simulation of polydisperse suspensions, and allows modeling of dissolution for soluble or partially soluble ENMs. The program is implemented in MATLAB, and it typically runs in a few minutes before exporting dose metrics to an Excel file. The entire integrated methodology, as well as its core components, has previously been experimentally validated, as described in below in detail, for a variety of metal and metal oxide ENMs[10,14], and is suited to most low-aspect-ratio nanomaterials.

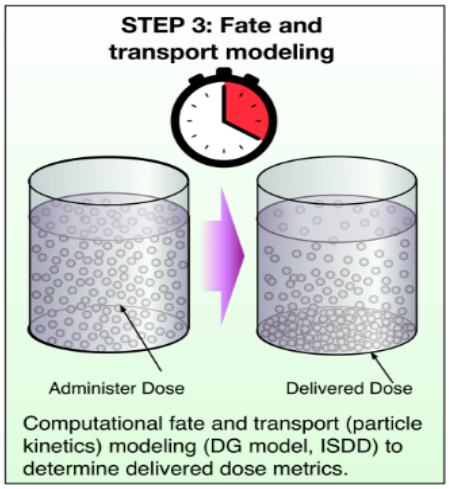

Figure 3: Step 3 of the *in vitro* dosimetric protocol: Fate and Transport Modeling

5 CONCLUSIONS

Several groups have recently proposed standardized dispersion protocols that result in reproducible and stable nanoparticle dispersions in media relevant for *in vitro* toxicity studies[15]. Review of these proposed standardized protocols highlights the key elements necessary for achieving and characterizing reproducible, stable, and relatively monodisperse suspensions for *in vitro* toxicity testing. Our protocol draws on best practices identified from those protocols, and it achieves similar results in terms of agglomerate size, size distribution, and agglomerate stability over time. As described in detail previously, the integrated methodology presented here, including ENM dispersion preparation, characterization, and dosimetry using the DG model, was validated by comparing predicted concentration profiles along the vertical axis for suspensions of metal oxide ENMs with empirical measurements of ENM concentration in thin cryosections from flash-frozen cylinders of ENM suspensions[10]. In addition, gold-standard 3D computational fluid dynamics (CFD) models were developed and used to validate the DG model and the integrated methodology presented here. Very close agreement was observed between empirical cryosection measurements and both the DG and CFD model predictions. The methodology had been further validated by our lab in terms of predicting biological outcome in an *in vitro* study of the hazard ranking of a large panel of low-aspect-ratio ENMs[3]. Details on the validation of this integrated *in vitro* dosimetry methodology can be found in papers previously published by the authors[1-3,10].

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