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A comparison of three dispersion media on the physicochemical and toxicological behavior of TiO_2 and NiO nanoparticles

Erika R. Gutierrez [a], Richard M. Kamens [a], Michael Tolocka [b], Kenneth Sexton [a], Ilona Jaspers [c,*]

[a] Department of Environmental Sciences and Engineering, Gillings School of Global Public Health, Chapel Hill, NC, USA
[b] Department of Chemistry and Biochemistry, Rowan University, Chapel Hill, NC, USA
[c] Center for Environmental Medicine, Asthma, and Lung Biology, School of Medicine, University of North Carolina-Chapel Hill, Chapel Hill, NC, USA

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ABSTRACT

Nanomaterials represent a burgeoning field of technological innovation. With the onset of environmental release and commercial product exposure associated with nanomaterial manufacture and proliferation, the concomitant effects on human health remain unknown and demand further investigation. Agglomeration of nanomaterials in biologically relevant media used in *in vitro* methods further complicates dosing in toxicological study. *Objective:* to compare the effects of *in vitro* dispersion techniques on the physicochemical and toxicological dosimetry of TiO_2 (<50 nm) and NiO (<20 nm) nanoparticles and some resulting toxicological endpoints to test for potential effects. *Methods:* three media were prepared for A549 and 16hbe14o cells with varying concentrations of TiO_2 and NiO nanoparticles. Physicochemical effects were analyzed with dynamic light scattering, ICP-MS, SEM, and TEM. Toxicological effects were determined after stimulation of cells with nanoparticles for 4 and 24 h followed by analysis of inflammatory and oxidative stress markers with ELISA and RT-PCR. Our data show that dispersion media differentially affect physicochemical properties and toxicological endpoints. Therefore, we conclude that *in vitro* nanotoxicology models that use re-suspension methods of exposure yield inconsistent and misleading biological results due to physicochemical variation of particle characteristics and transport processes.

1. Introduction

Nanomaterials represent a growing field of technological innovation. With the onset and continuing increase of environmental release associated with nanomaterial manufacture and proliferation, the concomitant effects on human health need to be determined [8,27,25]. Of particularly expedient concern is the effect on respiratory health, since inhalation presents an important route of exposure. The occupational health effects encumbered by workers during nanoparticle manufacture and remediation remain unquantified, as are the risks to consumers of nanomaterial-based products [24,19].

Currently, the most common and cost-effective method to study the respiratory health effects of nanoparticles involves the use of *in vitro* toxicological models combined with a resuspension technique [1]. Nanoparticle resuspension techniques involve the direct addition of nanoparticles to cell culture medium. However, fluctuations between physicochemical characteristics in the colloidal state lead to the agglomeration of nanoparticles in solution, which consequently presents inherent difficulties to the exposure and accurate evaluation of toxicity [12]. These dynamic interactions of, and between particle size, chemical composition, surface modifications, and particle number that lead to agglomeration will elicit an irregular and non-uniform deposition pattern [23,6,12,16]. In addition, processes of particle deposition onto the cell monolayer change with the size and status of the agglomerated particles [17].

At large particle sizes or agglomerates (d_p [particle diameter], or d_{ag} [agglomerate diameter] > 100 nm), sedimentation processes dominate. In the nanomaterial range (10–100 nm), a combination of both sedimentation and diffusion processes occur. At particle sizes d_p < 10 nm, the process of deposition onto the cell monolayer involves properties related to the mobility of the particle in solution through Brownian motion and diffusion [6,3]. To date, the majority of *in vitro* resuspension studies have used doses that suggest agglomeration rates and physical processes that involve sedimentation onto the cell monolayer, assume uniform deposition, and/or ignore the physical process leading to nanoparticle uptake into the cell monolayer. Some degree of agglomeration takes place

* Corresponding author at: Center for Environmental Medicine, Asthma, and Lung Biology, CB#7310, 104 Mason Farm Road, Chapel Hill, NC 27599, USA. Tel.: +1 919 966 8657.
E-mail address: ilona_jaspers@med.unc.edu (I. Jaspers).

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during manufacture and in the air in exposure conditions, which have been characterized [5]. The goal is to use methods, which achieve and control representative degrees of agglomeration and resulting size and to maintain these conditions during toxicity exposure tests. Therefore, issues of quality control arise when the dose is extrapolated to lower, more environmentally and biologically relevant levels, chronic and sub-chronic exposure levels, and the primary and/or agglomerated particle size is $\leqslant$100 nm [17,11]. Thus, the consistency of the exposure is at stake due to the mixture of physical processes occurring in solution: diffusion, Brownian motion, and sedimentation, which control the behavior, their natural agglomeration state, and exposure of the nanoparticles to the cell monolayer [24]. These complications present obstacles for risk assessment and exposure regulation since the exposure may no longer be representative of particles in the nano-scale range, but the sub-micron to micro-range, as well as vary between exposure in different dispersants, cell culture media, and media depth used for the exposure [24,17,20].

Numerous methods and techniques have been developed to resolve the issue of agglomeration. Surfactants are a common additive to control the degree of agglomeration and micelle formation, since there are many biological components of the cell culture medium system that can play a dual role as a de-agglomerating agent and as a cellular nutrient [18,15]. On the other hand, supposedly passive, non-toxic xenobiotic surfactants are used to de-agglomerate nanoparticles in cell culture medium. There are three types of surfactant-based methods employed in common use to control agglomeration: serum or serum proteins, biologically based pulmonary surfactants such as DPPC and Survanta [13,21], and non-biologically based surfactants [14]. The efficacy of these surfactants has not been inter-compared nor has their subsequent effect on biological endpoints been assessed.

Choosing a particular dispersion method may implicate downstream effects on both the exposure science and the toxicological endpoints. In this study, three different de-agglomerating agents have been chosen for comparison due to their widespread use in research labs and usage in nanotoxicology publications. The following surfactants were used for comparison: (1) fetal bovine serum (FBS), to reflect the traditional cell culture medium surfactants, (2) a synthetic surfactant and non-ionic block copolymer, F68, and (3) a semi-synthetic, plant-derived, but naturally occurring human lung surfactant 1,2-dipalmitoyl-sn-glycero-3-phospho choline (DPPC) in bovine serum albumin (BSA). Nickel oxide (NiO) and titanium dioxide (TiO_2) metal oxide nanoparticles were chosen due to their unknown and great potential for human health effects, increased environmental and airborne proliferation, occupational safety hazards, and role in atmospheric chemistry [8–10,22]. Therefore, our research objective is to compare the effects of dispersion and exposure techniques on the physicochemical and toxicological dosimetry of TiO_2 (<50 nm) and NiO (<20 nm) nanoparticles and resulting toxicological endpoints. The selected toxicological endpoints are meant to serve as an example and proof of principle of the importance of exposure method choices, and not to be suggestive of an all-inclusive set of the many possible toxicological effects.

2. Materials and methods

2.1. Reagents and supplies

Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Norcross, GA). F-12K 1× (Ham's F-12K Nutrient Mixture, Kaighn's Modification with L-glutamine) was obtained from Mediatech, Inc. (Manassas, VA). Minimal Essential Medium (MEM) was bought from Gibco (Invitrogen, Carlsbad, CA). Bovine serum albumin and penicillin/streptomycin solution were obtained from Invitrogen (Carlsbad, CA). DPPC and 10% Pluronic F-68 Solution were obtained from Sigma Aldrich (St. Louis, MO). MEM was bought from Invitrogen (Carlsbad, CA). Titanium (IV) dioxide (TiO_2) and nickel (II) oxide (NiO), were obtained from Sigma–Aldrich (St. Louis, MO). The reported mean diameters were TiO_2, <50 nm and surface area 35–65 m^2/g, and NiO, <20 nm.

2.2. Cell culture and dispersion media composition

A549 cells were cultured in F-12K supplemented with 10% FBS and 1% penicillin/streptomycin. 16hbe14o cells were cultured in MEM supplemented with 10% FBS and 1% penicillin/streptomycin. All cells were grown to confluency in their respective and traditional medium in 24-well plates. At confluency, cells were treated with the respective dispersion media and nanoparticles described hereafter.

Three media were prepared for A549 cells: "F12K + FBS" is composed of F-12K cell culture medium/10% FBS/1% penicillin/streptomycin, "F12K + F68 Media" is F-12K cell culture medium/0.1% Pluronic F68 nonionic surfactant/1% penicillin/streptomycin, and "BSSM" is F-12K cell culture medium/0.01% 1,2-dipalmitoyl-sn-glycero-3-phosphocholine, semisynthetic (DPPC)/0.6 mg/mL BSA/1% penicillin/streptomycin. BSSM is modeled after the biocompatible medium developed by [13]. The BSSM in this study uses the same surfactant composition as Porter et al. but is adapted to use the appropriate A549 cell culture nutrient mixture.

Two media were prepared for 16hbe14o cells: "MEM + FBS" is MEM cell culture medium/10% FBS/1% L-glutamine/1% penicillin/streptomycin and "MEM + F68" is MEM cell culture medium/0.1% F68 nonionic surfactant/1% L-glutamine/1% penicillin/streptomycin.

Each dispersant contained varying concentrations (0, 0.01, 0.1, 0.5, 10, 100 μg/mL) of –TiO_2 or NiO nanoparticles.

2.3. Preparation of dispersion media with nanoparticles

1-mg of nanoparticles were weighed and transferred into 100-mL of dispersion medium. The resulting solution was sonicated in a 125-watt Branson Ultrasonic water-bath sonicator for 30 min. The solution was immediately diluted to the desired concentration; if serial dilutions were made, vortexing for 1 min on high occurred between each dilution. All dispersion medium containing nanoparticles were stored in the dark and experimentations were initiated within 30 min of sonication. All solutions were re-vortexed if more than 20 min elapsed since the initial sonication and/or vortexing. After 24 h, solutions were discarded.

2.4. Physicochemical analysis

2.4.1. Dynamic light scattering (DLS) and ζ-potential

Measurement of particle size with DLS and solution stability via ζ-potential was determined with a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). The size distributions were multimodal for concentrations of 1 and 10 μg/mL and unimodal for 0.01, 0.1, and 0.5 μg/mL. The period of time between sonication and exposure was the same for particle sizing and for exposure to the cells. The gap between sonication and DLS measurement as well as cell exposure was kept to a minimum (<30 min) and derived from the fact that the nanoparticle sonication and cell culture equipment were located in different laboratories.

2.4.2. Inductively coupled plasma mass spectrometry (ICP-MS)

Quantitative measurement of nanoparticle concentration and metal contamination was determined with a Varian 820-MS ICP-MS (Agilent Technologies, Santa Clara, CA). Nanoparticle