Toxicology 297 (2012) 1–9

Contents lists available at SciVerse ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

Assessing the relevance of *in vitro* studies in nanotoxicology by examining correlations between *in vitro* and *in vivo* data

Xianglu Han[a,*], Nancy Corson[a], Pamela Wade-Mercer[a], Robert Gelein[a], Jingkun Jiang[c], Manoranjan Sahu[d], Pratim Biswas[d], Jacob N. Finkelstein[b,a], Alison Elder[a], Günter Oberdörster[a]

[a] Department of Environmental Medicine, University of Rochester, Rochester, NY, USA
[b] Departments of Pediatrics and Radiation Oncology, University of Rochester, Rochester, NY, USA
[c] State Key Joint Laboratory of Environment Simulation and Pollution Control, School of Environment, Tsinghua University, Beijing, China
[d] Department of Energy, Environmental and Chemical Engineering, Washington University in St. Louis, St. Louis, MO, USA

ARTICLE INFO

Article history:
Received 6 January 2012
Received in revised form 23 February 2012
Accepted 23 March 2012
Available online 2 April 2012

Keywords:
Nanoparticle
TiO_2
LDH
Protein carbonylation
Dose–response analysis
Sigmoidal
Steepest slope

ABSTRACT

There is an urgent need for *in vitro* screening assays to evaluate nanoparticle (NP) toxicity. However, the relevance of *in vitro* assays is still disputable. We administered doses of TiO_2 NPs of different sizes to alveolar epithelial cells *in vitro* and the same NPs by intratracheal instillation in rats *in vivo* to examine the correlation between *in vitro* and *in vivo* responses. The correlations were based on toxicity rankings of NPs after adopting NP surface area as dose metric, and response per unit surface area as response metric. Sizes of the anatase TiO_2 NPs ranged from 3 to 100 nm. A cell-free assay for measuring reactive oxygen species (ROS) was used, and lactate dehydrogenase (LDH) release, and protein oxidation induction were the *in vitro* cellular assays using a rat lung Type I epithelial cell line (R3/1) following 24 h incubation. The *in vivo* endpoint was number of PMNs in bronchoalveolar lavage fluid (BALF) after exposure of rats to the NPs *via* intratracheal instillation. Slope analyses of the dose response curves shows that the *in vivo* and *in vitro* responses were well correlated. We conclude that using the approach of steepest slope analysis offers a superior method to correlate *in vitro* with *in vivo* results of NP toxicity and for ranking their toxic potency.

© 2012 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

The exciting field of nanotechnology has seen a rapid expansion in recent years. However, there is a growing concern about potential risks posed by the technology due to exposure of humans and the environment (Stern and McNeil, 2008).

Current toxicological research on nanotechnology mainly focuses on the adverse effects of one of the building blocks of the technology – nanoparticles (NPs). There are several challenges that seem to be special to nanotoxicology when evaluating the adverse effects of NPs. One challenge is whether to use mass as a dose metric for assessing NP toxicity. Since the activity of NPs is determined to a large extent by NP surface properties, there has been efforts to determine if a dose metric based on surface area is preferable rather than one based on mass (Brown et al., 2001; Oberdörster et al., 2005a,b, 2007; Teeguarden et al., 2007; Monteiller et al., 2007; Wittmaack, 2007; Stoeger et al., 2007).

Another challenge is to validate *in vitro* assays that have been well established for assessing toxicity of chemicals. There is an urgent need for quick and reliable *in vitro* screening assays to replace or reduce the slow, costly and ethically controversial animal testing that might be required due to the rapid development and commercialization of nano-enabled products. However, some traditional *in vitro* assays have been found to generate misleading results because some of the nanomaterials could interfere with the assays (Wörle-Knirsch et al., 2006; Casey et al., 2007; Belyanskaya et al., 2007; Han et al., 2011). In addition, NPs, because of their large specific surface area, could adsorb essential nutrients in cell culture medium, making it difficult to interpret some cytotoxicity results (Guo et al., 2008).

When evaluating NP toxicity using *in vitro* assays, *in vivo* relevance is an essential criterion for accepting their utility. The *in vivo* relevance can be questioned because of the differences between *in vitro* and *in vivo* conditions. These differences warrant developing novel methods to define equivalent doses between *in vitro* and *in vivo* exposures in order to improve correlations between the two testing systems.

One difference is the high concentrations/dose used in most traditional *in vitro* studies. An extremely high dose rate (dose administered per unit of time) is another issue of *in vitro* studies

* Corresponding author at: Department of Environmental Medicine, University of Rochester, 575 Elmwood Ave., MRBx Building, Box 850, Rochester, NY 14642, USA. Tel.: +1 989 638 8168; fax: +1 989 638 9933.
E-mail address: XHan2100@gmail.com (X. Han).

0300-483X/$ – see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.tox.2012.03.006

because the full dose is delivered as a bolus in traditional *in vitro* assays. The dose rate in such studies is much higher than in *in vivo* inhalation studies in which animals are exposed to a low concentration of chemical or particle *via* inhalation for an extended period of time (hours, days, weeks, or longer). Another important difference is the wide use of dispersants *in vitro* but not necessarily *in vivo* (*e.g.*, inhalation of pristine NPs generated from dry powders) (Park et al., 2009). NPs dispersed in cell culture medium would adsorb some components in the medium while NPs inhaled into the lung would adsorb components of pulmonary surfactant. However, the use of high doses and high dose rates *in vitro* by itself does not invalidate *in vitro* assays. The *in vitro* data could still be useful as long as the data are confirmed to correlate well with *in vivo* results. In particular, *in vivo* dosing of the respiratory tract by intratracheal instillation or oro-pharyngeal aspiration are also bolus-type delivery methods and results should correlate better with *in vitro* dosing.

There have been some studies that addressed the issue of the *in vivo* relevance of some *in vitro* assays for evaluating the toxicity of NPs (Sayes et al., 2007) or ambient particulate matter (Seagrave et al., 2005). Both found poor correlations. However, these findings do not necessarily indicate intrinsic flaws of the *in vitro* assays for predicting *in vivo* toxicity. Instead, there can be multiple reasons for the differences between *in vitro* and *in vivo* results (Seagrave et al., 2005). Our group (Rushton et al., 2010) has proposed an alternative approach of slope analysis and found a good *in vitro*–*in vivo* correlation when applied to the data in the paper of Sayes et al. (2007).

The objective of this work is to test the hypothesis that results of *in vitro* assays correlate with acute *in vivo* effects when an appropriate response metric is used. Therefore, in this work, we continue to address the relevance of *in vitro* assays by comparing results of *in vitro* and *in vivo* studies for NPs of different sizes based on a slope analysis of dose–response curves. In addition to the graphical method used in our previous work to determine the maximum response per unit of the dose (steepest slope) (Rushton et al., 2010), we introduce a new method to derive the steepest slope – a mathematical method. We report in this work that using this approach showed a good correlation of acute toxicity between *in vitro* and *in vivo* results. Future work needs to also consider extension to long-term effects.

2. Methods

2.1. Materials

NPs used for the main study of this work includes different TiO_2 NPs (for correlation study), and for assay validation only a silver NP, and a copper NP. A series of anatase TiO_2 NPs of different sizes (3, 7, 10, 16, 30, 50, 53, and 104 nm) were used for the correlation study. These NPs were synthesized from titanium tetra-isopropoxide (TTIP, 97%, Aldrich) either in a diffusion or premixed flame aerosol reactor (Jiang et al., 2007). These two methods for preparing NPs are commonly used, although it was unknown if both methods could generate particles with different toxicological activities. Thus, NPs generated by both methods were considered as different NPs even if they had the same average size. The other NPs (P-25, TIO_2, Ag-40, and Cu-40) were only used for validating the protein carbonylation/oxidation assay (see Supplemental Information).

2.2. Physicochemical characterization of NPs

The hydrodynamic sizes of the anatase TiO_2 NPs in different media were measured using dynamic light scattering (DLS) technology (intensity distribution of suspended NPs) with a Malvern ZetaSizer (ZetaSizer Nano ZS). Number or volume distribution of the particles would generally incur larger errors because they are not direct measurements; instead, they are derived from the directly measured intensity distribution. The NPs were sonicated using a cuphorn sonicator for 15 s twice (Sonics VCX 750 with maximum output 750 W and a frequency of 20 kHz, Newtown, CT) at 29% maximum output. Suspension and cell culture media included saline (pH 5.9), phosphate buffered saline (PBS) with a pH of 7.4 (1.14 g of Na_2HPO_4, 0.72 g of NaH_2PO_4, and 8.18 g of NaCl in deionized water, pH adjusted to 7.4 using 10 N NaOH), RPMI 1640 cell culture medium with no phenol red (Invitrogen GIBCO® 11835), and RPMI medium containing 1% fetal bovine serum (FBS, Hyclone, SH30070.03). For each size of anatase TiO_2 NP, the same concentration was used (2 μg/ml). Measurements were repeated at least 6 times to ensure a stable reading; if not, measurements would be repeated further until there is no obvious time trend of the measured sizes as judged by examining the last 6 measurements. In addition, primary particle sizes and agglomeration state were also measured using transmission electron microscope. BET isotherms (Autosorb-1, Quantachrome) were used to determine the specific surface area of the NPs (Brunauer et al., 1938).

2.3. Rationale of choosing in vitro and in vivo endpoints

There is increasing evidence that the induction of oxidative stress through generation of reactive oxygen species (ROS) plays a critical role in the mechanism of toxicity induced by NPs (Brown et al., 2001; Xiao et al., 2003; Hussain et al., 2005; Nel et al., 2006; Long et al., 2006; Sayes et al., 2006; Federici et al., 2007; Limbach et al., 2007). Based on this, most of the endpoints we selected for the *in vitro* and *in vivo* analyses were related to oxidative stress or inflammation. The oxidative stress related endpoints in three testing systems were ROS generation in a cell-free system, protein carbonylation in cultured cells, and the number of polymorphonuclear neutrophils (PMNs) in lung lavage fluid. In addition, we also included measurement of released lactate dehydrogenase (LDH) from cultured cells. Though LDH release is caused by cellular membrane damage which may or may not be related to oxidative stress, the LDH assay is a very commonly used assay and warrants evaluation for rapid screening of NP toxicity. TiO_2 particles could interfere with the LDH assay by adsorbing LDH molecules (Han et al., 2011), however, there is only minor interference at low and intermediate concentration so the assay will still be useful as long as its *in vivo* relevance *can be shown*.

The endpoints chosen for this study are general and broad-based because the purpose is to choose relatively simple screening assays that are widely applicable so that the toxicity of a large number and variety of different NPs can be evaluated.

2.4. Cell-free ROS assay

The intrinsic capability of the NPs in generating reactive oxygen species (ROS) was evaluated using a cell-free ROS assay (Jiang et al., 2008). The assay was performed in the dark to avoid any interference from photocatalytic activities of the NPs. The dye 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (CalBiochem, 287810) is deacetylated and oxidized into a fluorescent product in the presence of NP-generated free radicals. The fluorescence was detected using a spectrofluorometer (absorbance/emission maxima, 485 nm/535 nm).

Three to six groups (including controls) were used for these NPs with concentration ranges from 0 to 100 μg/ml and a dose spacing factor of 2 or 3. When expressed as NP surface area, the highest concentration of 100 μg/ml is equivalent to 30 cm^2/ml for the largest TiO_2 NP and 426 cm^2/ml for the smallest NP. Since ROS level in the cell-free assay increased linearly with increasing NP concentration (see Section 3), the slope of this increase for each NP was obtained using linear regression. In addition to mass concentration (μg/ml), surface area concentration (cm^2/ml) was also used to express the slopes. After calculating the mass based slope for each NP, the surface area based slope was calculated by dividing the mass based slope by the specific surface area (SSA) of the NP.

2.5. Cell culture

A rat lung epithelial Type I like cell line, R3/1 (Koslowski et al., 2004), was used for the cellular *in vitro* studies. Cells were maintained in RPMI 1640 cell culture medium (without phenol red) containing 10% fetal bovine serum (FBS) and passaged every three or four days. For *in vitro* assays, cells with a density of 10^5 per ml were seeded in plates, grown to subconfluency (70–80% confluency), and exposed to NPs in RPMI 1640 with 1% FBS for different time durations. Though plates with wells of different sizes (6-, 12-, or 24-well plate) were used, when seeding cells into a plate, the same cell density was used and a constant depth of cell culture medium was maintained. NPs were probe sonicated in RPMI 1640 with 1% FBS on ice for 10 s twice.

2.6. LDH assay

The LDH assay was performed according to the Sigma LDH assay kit. The assay works by measuring the catalytic activity of LDH in the reaction: $\text{NADH} + \text{Pyruvate} \leftrightarrow \text{NAD}^+ + \text{Lactate}$, where NADH stands for reduced β-nicotinamide adenine dinucleotide. The decreasing rate of NADH – measured as decreasing absorbance at 340 nm over 2 min – is used as an indicator of LDH activity.

Eight groups (including controls) were used for this study with concentrations ranging from 0 to 200 μg/ml for larger particles (50 nm), 0–100 μg/ml for medium sized particles (10 nm and 30 nm), and 0–50 μg/ml for smaller particles (3 and 7 nm). The spacing factor was 2 or 2.5.

2.7. Protein carbonylation study

In this study, enzyme-linked immunosorbent assay (ELISA) was used for detecting protein carbonyls following their reaction with dinitrophenylhydrazine (DNPH) (Buss et al., 1997).

Download English Version:

https://daneshyari.com/en/article/2595919

Download Persian Version:

https://daneshyari.com/article/2595919

Daneshyari.com