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Non-specific interaction of carbon nanotubes with the resazurin assay reagent: Impact on *in vitro* assessment of nanoparticle cytotoxicity

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ABSTRACT

In vitro cytotoxicity assays are essential tools in the screening of engineered nanomaterials (NM) for cellular toxicity. The resazurin live cell assay is widely used because it is non-destructive and is well suited for high-throughput platforms. However, NMs, in particular carbon nanotubes (CNT) can interfere in assays through quenching of transmitted light or fluorescence. We show that using the resazurin assay with time-point reading of clarified supernatants resolves this problem. Human lung epithelial (A549) and murine macrophage (J774A.1) cell lines were exposed to NMs in 96-well plates in 200 μL of media/well. After 24 h incubation, 100 μL of supernatant was removed, replaced with resazurin reagent in culture media and aliquots at 10 min and 120 min were transferred to black-wall 96-well plates. The plates were quick-spun to sediment the residual CNTs and fluorescence was top-read (λ_{Ex} = 540 nm, λ_{Em} = 600 nm). The procedure was validated for CNTs as well as silica nanoparticles (SiNP). There was no indication of reduction of resazurin by the CNTs. Stability of resorufin, the fluorescent product of the resazurin reduction was then assessed. We found that polar CNTs could decrease the fluorescence signal for resorufin, possibly through oxidation to resazurin or hyper-reduction to hydroxyresorufin. This effect can be easily quantified for elimination of the bias. We recommend that careful consideration must be given to fluorimetric/colorimetric *in vitro* toxicological assessments of optically/chemically active NMs in order to relieve any potential artifacts due to the NMs themselves.

1. Introduction

Clearer understanding of the toxicological behavior of nanomaterials (NM) is emerging with an increasing number of studies utilizing *in vitro* methodologies for toxicological assessments (Rodriguez-Yanez et al., 2012; Yang and Liu, 2012). Many of the assays utilize colorimetric and fluorimetric detection methods. One such assay, the resazurin assay is utilized to measure cell viability, based on the reduction of blue, non-fluorescent resazurin to pink, fluorescent resorufin by metabolically active cells (O'Brien et al., 2000). The cellular reduction of resazurin occurs by metabolic enzymes located in the mitochondria, cytosol and the microsomal fractions (de Fries and Mitsuhashi, 1995; Gonzalez and Tarloff, 2001). The decrease in the magnitude of resazurin reduction below control levels indicates cytotoxicity (loss of cell viability). The test is simple, rapid, versatile, cost-effective and shows a high degree of correlation with cytotoxicity assessed by other methods, such as MTS (Riss and Moravec, 2004).

Some drawbacks of conducting resazurin-based cytotoxicity assessments have been previously identified (De Jong and Woodlief, 1977; Goegan et al., 1995; O'Brien et al., 2000). First, resazurin can be reduced by antioxidant components of cell culture media such as ascorbic acid, cysteine or dithiothreitol, giving rise to higher background levels (De Jong and Woodlief, 1977). The apparent rate of reduction of resazurin is also sensitive to the presence of protein in the cell culture medium (Goegan et al., 1995). Moreover, an extensive hyper-reduction of resorufin (pink) by

Abbreviations: AB, Alamar Blue; CNT, carbon nanotubes; CTB, CellTiter-Blue; DLS, dynamic light scattering; DMEM, Dulbecco's modified eagle medium; FBS, fetal bovine serum; FTIR, Fourier transform infrared spectroscopy; FU, fluorescence units; ICP-AES, inductively coupled plasma atomic emission spectroscopy; NM, nanomaterials; S_{BET}, Brunauer–Emmett–Teller surface area; SiNP, silica nanoparticles; TEM, transmission electron microscopy.

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metabolically active cells to a final non-fluorescent product hydroresorufin (colorless) has also been observed, with a potential for an underestimation of cell activity (O'Brien et al., 2000).

As recently documented, numerous assays are susceptible to interference from test compounds, including particulates, such as NMs. Chemical interactions of NMs, such as single-wall carbon nanotubes (CNT), carbon black or carbon nanohorns with reporters in test assays or their inherent optical properties can interfere with the analytical methods which utilize absorbance, fluorescence and luminescence techniques (Casey et al., 2007; Doak et al., 2009; Geraci and Castranova, 2010; Isobe et al., 2006; Kroll et al., 2009, 2012; Monteiro-Riviere et al., 2009; Ong et al., 2014; Oostingh et al., 2011; Worle-Knirsch et al., 2006). For example, single-wall CNTs chemically interact with 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide (MTT), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), resazurin (Alamar Blue; AB/CellTiter-Blue; CTB), Neutral Red, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-1) and Coomassie blue, leading to unreliable results (Casey et al., 2007; Isobe et al., 2006; Monteiro-Riviere et al., 2009; Worle-Knirsch et al., 2006).

Within the standard framework it is vital that each assay measurement is free of artifacts due to the presence of the NMs. In this communication we specifically focus on the effects of CNTs on fluorescence and identify a simple approach to relieve the confounding effects of CNTs in the resazurin-based assay, including physical quench and chemical interference, so that reliable and consistent assessment of CNT toxicity can be achieved.

2. Methods

2.1. Test materials

Single-wall CNTs, CNT-1 and CNT-2 were obtained from the laboratory of Dr. Benoit Simard (NRC, Ottawa, ON, Canada). Multi-wall CNTs, CNT-3 and CNT-4 were obtained from Sun Nanotech (Beijing, China). Single-wall CNTs were synthesized by a pulsed laser-oven method using cobalt and nickel as catalysts (Kingston et al., 2004). Multi-wall CNTs were produced by chemical vapor deposition using iron as catalyst. Multi-wall CNTs had a diameter of 10–30 nm and >80% purity.

All of the CNTs were previously characterized for specific surface area and pore volume (S_{BET}; Table 1S), size (TEM; Fig. 1S), metal content (ICP-AES; Table 2S), surface functionalities (FTIR; Fig. 2S) and the hydrodynamic diameter in liquid media (DLS; Table 3S) and published elsewhere (Kumarathasan et al., 2012, 2014). Single-wall CNTs and multi-wall CNTs were surface-modified by an oxidation process following our previously reported procedure (Kumarathasan et al., 2012). In brief, the oxidized materials had 30–80% lower content of metal species (e.g. Ni, Fe, Co, Mo), contained polar surface –COOH groups, had shortened length and a decreased specific surface area (Kumarathasan et al., 2012), as well as showed lower tendency to flocculate and had smaller hydrodynamic diameter, than their pristine CNT counterparts (Kumarathasan et al., 2014). From here on, pristine single-wall CNTs, oxidized single-wall CNTs, pristine multi-wall CNTs and oxidized multi-wall CNTs will be referred to as CNT-1, CNT-2, CNT-3 and CNT-4, respectively.

Amorphous silica nanoparticles; SiNP-1 (10–20 nm, cat # 637238) and SiNP-2 (12 nm, cat # 718483) were obtained from Sigma–Aldrich Canada Co. (Oakville, ON, Canada). Briefly, from the Sigma–Aldrich product specification sheets and certificates of analysis, SiNP-1 was determined to be an amorphous nanopowder, with 20 nm average size particles (SAXS) and 30 ppm trace metals content (ICP), while SiNP-2 was determined to be an amorphous nanopowder, with 12 nm primary particle size (TEM), 210 m^2/g surface area (S_{BET}) and 30.7 ppm trace metals content (ICP). Standard Reference Materials (SRMs); SiO_2 (respirable cristobalite, SRM-1879a), TiO_2 (titanium dioxide, SRM-154b) were from the National Institute of Standards and Technology (Gaithersburg, MD, USA).

CTB (resazurin) reagent was purchased from Promega (Fitchburg, WI, USA). Cell culture media and supplements were obtained from Hyclone (Logan, UT, USA). All other reagents were purchased from Thermo-Fisher (Nepean, ON, Canada).

2.2. Preparation of particles for testing

To prepare stocks, CNTs and all additional particles were weighed and re-suspended in sterile particle preparation buffer (Tween-80, 25 μg/mL; NaCl, 0.19% w/v) to final concentration of 3 mg/mL and 10 mg/mL, as required, using a Dounce glass homogenizer (Nadeau et al., 1996). The particle suspensions were sonicated in ice-cold water for 20 min using a Branson 1510 water bath sonicator (Branson, Shelton, CT, USA) and homogenized with 25 strokes of the homogenizer piston. The particle suspensions were aliquoted into sterile centrifuge tubes with O-ring seals and sterilized in an Isotemp water bath (Thermo-Fisher) at 56 °C for 30 min (Vincent et al., 1997). For experiments, particle suspensions were diluted with the appropriate serum-free culture medium and particle preparation buffer to make final particle concentrations to be applied to the cell cultures, which were sonicated for additional 10 min prior to dosing. Note, that TiO_2 particles were washed three times with methanol and three times with phosphate buffered saline prior to the preparation of the stocks (Vincent et al., 1997).

2.3. Cell culture

A549, human alveolar type II epithelial cells and J774A.1, BALB/c murine macrophages (ATCC, Manassas, VA, USA) were maintained in DMEM/high glucose culture medium containing phenol red and 2 mM L-glutamine (Hyclone, SH30022.01). Ten (10) % fetal bovine serum (FBS) (Hyclone, SH3039603) and 50 μg/mL of gentamicin (Sigma, G1397) were added into T-75 culture flasks and cells were kept in a humidified incubator at 37 °C, 5% CO_2, 100% humidity until confluence. For passaging, A549 cells were routinely detached using 0.25% trypsin with 0.2 g/L ethylenediaminetetraacetate (EDTA) (HyClone, SH30042.01) while the J774A.1 cells were detached using a cell scraper.

2.4. Cell exposure to particles

For experiments, A549 and J774A.1 cells were seeded in black-wall 96-well plates at cell densities of 2×10^4 and 4×10^4 cells/well, respectively and cultured for 24 h in phenol red-free DMEM/high glucose media (HyClone, SH30284.01) supplemented with 10% FBS and 50 μg/mL of gentamicin prior to exposure to particle suspensions. Following incubation, cells were exposed to 0, 10, 30 and 100 $\mu g/cm^2$ doses of CNTs (equivalent to 0.0165, 0.05, 0.165 mg/mL) using the Liquidator 96 instrument (Mettler-Toledo, Columbus, OH, USA) in serum-free media. The final content of each well was 200 μL, with 5% FBS. The cells were then incubated for additional 24 h.

2.5. Conventional resazurin assay

The conventional CTB assay was conducted using Zephyr liquid handling instrument (Calyper, Hopkinton, MA, USA). Briefly, 100 μL of supernatant was discarded from each well and replaced with 50 μL of phenol red-free, serum-free cell culture media containing CTB (resazurin) reagent (40%) for a final ratio of (1:7.5