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Differential biocompatibility of carbon nanotubes and nanodiamonds

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Abstract

Carbon nanomaterials are being produced in increasingly larger quantities for many applications due to their novel characteristics such as enhanced thermal, electrical, mechanical, and biological properties. However, there is a lack of data on biological interactions to assess their biocompatibility before they will be accepted as non-toxic in industrial or biomedical arenas. In the present study, we examined both neuronal and lung cell lines for biocompatibility in aqueous suspensions of carbon nanomaterials, such as nanodiamonds (NDs), single- and multi-walled carbon nanotubes (SWNTs, MWNTs), and carbon black (CB), at concentrations ranging from 25–100 μg/ml for 24 h. Our results indicated that these carbon nanomaterials displayed differential biocompatibility in these two different cell lines. The greatest biocompatibility was found after incubation with NDs and both cell types followed the trend: ND>CB>MWNT>SWNT. Macrophages were found to be more sensitive to the nanomaterials with up to five times the generation of reactive oxygen species after incubation with MWNTs or SWNTs. However, there was a lack of ROS generation from either cell line incubated with ND-raw, as well as intact mitochondrial membranes, suggesting that NDs may be useful as a benchmark nanoparticle non-toxic control in replacement of CB, and should be further investigated for use in medical applications.

Keywords: Carbon nanomaterials; Cell culture; Viability; MTT; MMP; ROS

1. Introduction

Carbon nanomaterials are being produced in increasingly larger quantities for many applications due to their novel characteristics, such as enhanced thermal, electronic, mechanical, and biological properties [1]. In biological systems, they have been used as delivery vehicles [2,3], targeted cancer therapies, tissue scaffolds [4,5], biosensors, and more [6–11]. It is envisaged that nanodiamonds (ND) may be particularly well suited for biological applications that require optical transparency, chemical inertness, hardness, and high specific area [6,12]. Therefore, in view of their biological applications, it is necessary to understand the biocompatibility or toxicity of carbon nanomaterials in either cell-based systems or animal models.

Previous studies in our AFRL laboratory with *in vitro* cell culture models (macrophages, germ-line stem cells, liver cells, PC-12 cells) have shown that nanoparticles can induce size, composition, and concentration-dependent toxicity [13–18]. These same factors are likely to influence carbon nanomaterials' biocompatibility or toxicity. Recent studies suggest that the biocompatibility of carbon-based nanomaterials depends strongly on mass, purity, aspect ratio, and surface functional groups. Jia et al., found that low mass and pure fullerenes (C_{60}, >99.9% purity) were more biocompatible than higher mass and less pure multi-walled carbon nanotubes (MWNT, >95% purity) or single-walled carbon nanotubes (SWNT, >90% purity) in guinea pig alveolar macrophages [19]. Magrez et al., found that human lung tumor cell lines were more biocompatible with high aspect ratio MWNTs than compared to carbon nanofibers (CNF) or carbon black (CB) with lower aspect ratios, while acid functionalization increases the toxicity of both CNF and MWNT [20]. The changes in biocompatibility of these carbon nanomaterials, in relation to size or surface chemistry, can be explained by the high density of reactive bonds on carbon black and carbon

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nanofibers compared to MWNT [20]. One study using relatively large synthetic abrasive diamond powders (100 nm) (that were electron-beam irradiated and annealed for fluorescence, then incubated with kidney cells for 3 h at a concentration of 400 μg/ml) showed very low cytotoxicity for the diamond nanoparticles after they were internalized by the cells [7]. Our work with much smaller 2–10 nm acid or base-purified nanodiamonds at concentrations of up to 100 μg/ml for 24 h shows high biocompatibility in N2A cells [21]. Together these studies suggest that many factors contribute to the biocompatibility of carbon nanotubes while much less is known about the biocompatibility of NDs. Many studies have examined the biocompatibility of diamond surfaces [22,23], but simple extrapolation of surface biocompatibility data to diamond nanoparticles in solution has been shown to be impossible [9,24].

Other studies that have used *in vitro* cell culture models focused on lung or skin cells due to the risk of exposure in occupational or commercial settings [25–27]. However, it is unclear whether these nanomaterials can reach the nerves associated with these organs either through internalization through the skin and contact with olfactory nerves or translocation across the blood-brain barrier. In the present study, we examined both neuronal (neuroblastoma) and lung (alveolar macrophage) cell lines for biocompatibility in aqueous suspensions of carbon nanomaterials (*e.g.* ND, SWNT, MWNT, CB) at concentrations ranging from 25–100 μg/ml for 24 h. We further examined the morphological and subcellular effects of these nanomaterials on mitochondrial membrane permeability and reactive oxygen species (ROS) generation.

2. Materials and methods

2.1. Nanomaterials characterization

Multi-walled carbon nanotubes (MWNTs) were purchased from Tsinghua University, Beijing, China while single-walled nanotubes (SWNTs) were received from Rice University. Nano-sized carbon black was from Cabot (CB) and micron-sized cadmium oxide (CdO) was from the Fluka Chemical Company. Nanodiamonds (NDs) were generously supplied by NanoCarbon Research Institute Ltd. in Japan and were synthesized according to previously reported detonation techniques [28,29]. Nanomaterials were UV-sterilized, then diluted to stock concentrations of 1 mg/ml in deionized water. Characterization of the carbon nanomaterials size and morphology was performed with transmission electron microscopy (TEM) on a Hitachi H-7600 instrument. Purity was analyzed with inductively-coupled plasma-optical emission spectroscopy (ICP-OES) on a Thermo-Elemental IRIS Advantage ICP.

2.2. Cell culture protocols

Neuroblastoma cells, a neuronal phenotype, were generously provided by Dr. David Cool's laboratory at Wright State University (Dayton, Ohio) and rat alveolar macrophages (NR8383 CRL-2192) were purchased from ATCC (Manassas, VA). Cells were grown in an atmosphere of 5% CO_2 and 37 °C according to standard cell culture techniques [14]. Growth media for the neuroblastoma cells was DMEM/F12 supplemented with 10% normal fetal bovine serum (FBS) and growth media for the macrophages was Ham's Nutrient Mixture F-12K (Kaughn's Modification) media supplemented with 20% FBS. Both medias also contained 1% penicillin–streptomycin (ATCC). Other cell culture supplies included 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical Company, St. Louis, MO), 10x Phosphate buffered saline (pH 7.4) and 2.5% trypsin (Gibco Invitrogen™ Corporation, Carlsbad, CA) and rat tail collagen (type 1, UPSTATE, Waltham, MA).

Both neuroblastoma cells and macrophages were seeded in 24-well plates at a concentration of 250,000 cells/ml or approximately 130,000 cells/cm^2 while the plates for macrophages were first coated with type-1 rat tail collagen. After a desired growth period to approximately 80% confluence, cell cultures were dosed with freshly prepared nanoparticle working solutions at a concentration of 25–100 μg/ml in cell culture media without serum for neuroblastoma cells or media with 10% serum for macrophages to reduce proliferation. The 3-[4,5 -thylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay was conducted to assess cellular viability based on mitochondrial function [30]. After 30 min of incubation with MTT, a purple color developed within the cells, indicating the cleavage of the tetrazolium salt (MTT) by active mitochondria in live cells. The purple-colored product (formazan crystals) was extracted into solution with acidified isopropanol for homogeneous staining, and the absorbance was measured on a Spectromax 190 microplate reader from 570–630 nm after centrifugation to remove the nanoparticles. The percent reduction of MTT was compared to controls (cells not exposed to nanoparticles), which represented 100% MTT reduction. For fluorescent labeling of mitochondrial membrane permeability, cells were plated at 100,000 cells/ml into 2-chambered slides (Mīt-Σ-Ψ, Biomol). Fluorescence was visualized with TRITC and FITC filters on an Olympus IX71 epifluorescent microscope. Oxidative stress was measured in relation to the generation of reactive oxygen species (ROS). Prior to dosing cells with nanoparticles, the fluorescent probe 2′,7′-dichlorohydrofluorescein diacetate (DCHF-DA, Sigma) was applied under a light controlled environment as described by Wang and Joseph [31]. After nanoparticle treatment, the fluorescent intensity from each well was measured with a 485 nm excitation filter and a 530 nm emission filter on a SpectraMAX Gemini Plus microplate reader (Molecular Devices) equipped with SOFTmax Pro 3.1.2 software (Molecular Devices Corporation). The positive control, hydrogen peroxide (30% H_2O_2, Fisher Scientific), was used to assess the reactivity of the probe and showed a dose-dependent increase in ROS production.

3. Results and discussion

3.1. Nanomaterials characterization

The wide range of primary sizes and shapes of carbon nanomaterials were investigated with transmission electron microscopy (Fig. 1A–D). Individual cubic nanodiamonds (NDs) with sizes ranging from 2–10 nm (Fig. 1A) were smaller than more