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Electronic platform for real-time multi-parametric analysis of cellular behavior post-exposure to single-walled carbon nanotubes

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ABSTRACT

Single-walled carbon nanotubes (SWCNTs) implementation in a variety of biomedical applications from bioimaging, to controlled drug delivery and cellular-directed alignment for muscle myofiber fabrication, has raised awareness of their potential toxicity. Nanotubes structural aspects which resemble asbestos, as well as their ability to induce cyto and genotoxicity upon interaction with biological systems by generating reactive oxygen species or inducing membrane damage, just to name a few, have led to focused efforts aimed to assess associated risks prior their user implementation. In this study, we employed a non-invasive and real-time electric cell impedance sensing (ECIS) platform to monitor behavior of lung epithelial cells upon exposure to a library of SWCNTs with user-defined physico-chemical properties. Using the natural sensitivity of the cells, we evaluated SWCNT-induced cellular changes in relation to cell attachment, cell–cell interactions and cell viability respectively. Our methods have the potential to lead to the development of standardized assays for risk assessment of other nanomaterials as well as risk differentiation based on the nanomaterials surface chemistry, purity and agglomeration state.

1. Introduction

Nanomaterials implementation in a variety of fields from microelectronics (Ouyang et al., 2002), to photo-optics (Li and Zhang, 2009), aerospace (Baur and Silverman, 2007), energy (Frackowiak and Béguin, 2001), sensors (Merkoçi et al., 2005), bioimaging (Barone et al., 2005), and drug delivery (Bianco et al., 2005) has raised awareness of their occupational safety and health-posed issues (Barillet et al., 2010; Golin et al., 2013). Current available techniques to assess *in vitro* toxicity of nanomaterials such as silica (Clément et al., 2013), silver nanoparticles (Speranza et al., 2013), carbon- (Gui et al., 2011) or metal-oxide-based (Vittori Antisari et al., 2013) rely on the functionality, affinity and/or selectivity of a biological recognition elements (e.g., biosensor, antibodies, cellular membrane, organelles or DNA etc.) as well as the processing power and detection capabilities of micro and opto-electronics (Mulchandani and Bassi, 1995; Zhao et al., 2014). Such techniques record nanomaterial-induced changes to single or a population of cells (for instance generation of reactive oxygen species (ROS) following exposure to silver nanoparticles (Gliga et al., 2014) or changes in cellular viability and proliferation post-exposure to gold (Jain et al., 2014) or titanium dioxide (Jaeger et al., 2012) etc.) at discrete, user-controlled time points (e.g., 12, 24 or 48 h) and mainly through invasive, laborious and costly assays that require intensive and time-sensitive manipulation or handling of the samples (Kostarelos et al., 2007; Nowak et al., 2014).

Recently it was however found that some of these techniques are less applicable and reliable for assessing toxicity of carbon nanotubes (CNTs), fullerenes (C_{60}), carbon black (CB), or quantum dots (QD) (Dhawan and Sharma, 2010; Monteiro-Riviere et al., 2009). For instance, results showed that CNTs' high surface area, high adsorption capability, high catalytic activity and their characteristic optical properties could interfere with the reagents used for toxicity detection affecting their emission capability (Kroll et al., 2009; Monteiro-Riviere et al., 2009; Worle-Knirsch et al., 2006). Specifically, several studies showed that the suitability and accuracy of assays relying on catalytic and affinity biosensors such as tetrazolium salt and neutral red (Dhawan and Sharma, 2010) routinely used to evaluate cellular viability, become questionable due to the adsorption or binding affinity of the reagents onto the CNT surfaces (Kroll et al., 2009; Monteiro-Riviere et al., 2009; Worle-Knirsch et al., 2006). Such limitations in the current CNT-induced hazard assessments (Monteiro-Riviere et al., 2009) as well as the continuous development of different CNT forms and shapes with various functionalities and physico-chemical properties

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(Dong et al., 2013a; Marcolongo et al., 2007) do not allow for high-throughput and efficient toxicity assessment to be standardized and thus lead to minimum regulations of such nanomaterials exposure limits (Rogers-Nieman and Dinu, 2014). Specifically, according to Occupational Safety and Health Administration (OSHA), CNT exposures currently fall under the category of "particles not otherwise regulated" at a limit concentration of 5 mg/m^3 particles (Erdely et al., 2013; Lee et al., 2010). If CNTs are to reach their full potential for biotechnological applications (Bianco et al., 2005), new and scalable methods that allow for accurate cyto and genotoxicity evaluations need to be developed and implemented. Further, such methods should also allow for real-time assessment, minimum false positives, risk analysis of a variety of concentrations of nanomaterial being used for exposure, as well as risk correlations based on the nanomaterial length (Sato et al., 2005), diameter (Nagai et al., 2011), aggregation (Wick et al., 2007), impurities content (Aldieri et al., 2013), and/or surface chemistry (Saxena et al., 2007), just to name a few.

In this study, we implemented a rapid, non-invasive, high throughput, real-time continuous monitoring platform to detect CNT-induced changes in the behavior of confluent model human lung epithelial cells regularly used to investigate toxicity of nanomaterials of carbon (Gliga et al., 2014; Rogers-Nieman and Dinu, 2014; Siegrist et al., 2014). Our approach relied on an electric cell impedance sensing (ECIS) platform that used cells immobilized onto gold electrodes as a proxy to assess SWCNT-associated risk exposures as well as help perform risk analysis and risk differentiation based on the nanotubes' physico-chemical properties. By relying on the natural resistivity of the cells and the restrictions in the current pathways as imposed by the cell plasma membrane, comprehensive and multi-parametric analysis of the cellular behavior, cell attachment and cell–cell interactions were provided. ECIS platform was previously employed to monitor cellular changes upon exposure to digitoxin (a cardiac glycoside with anticancer potential; (Eldawud et al., 2014), cytochalasin D (a cytoskeletal inhibitor) (Opp et al., 2009) or sodium arsenate (a toxin responsible for cell retraction and changes in cytoskeleton) (Xiao et al., 2002a), all under user-controlled conditions. Our experimental procedure does not only capitalize on bioengineering means to provide parallel analysis of the cellular behavior upon exposure to a library of CNTs, all with high-sensitivity, in real-time and with minimum sample invasion, but further overcomes the limitations and concerns associated with the CNT interactions with the biological sensing elements. Further, our analysis could be extended to evaluate toxicity of other nanomaterials in a high-throughput and non-invasive fashion thus extending the ability for standardizing nanomaterial risk evaluation.

2. Materials and methods

2.1. Formation of the single-walled carbon nanotubes (SWCNTs) library

Acid treated single-walled carbon nanotubes (SWCNTs) were obtained by liquid phase oxidation of commercial (i.e., pristine) SWCNTs purchased from Unidym Inc. Specifically, the pristine SWCNTs were treated in a mixture of 3:1 (V/V) concentrated sulfuric (Fisher Scientific, 96.4%) and nitric (Fisher Scientific, 69.6%) acids for either 3 or 6 h to obtain SWCNTs with different degree of O-related functionalities and lengths (Campbell et al., 2013; Dong et al., 2013b). Upon time elapse, the SWCNTs/acids mixture was diluted in deionized water, filtered through a GTTP filter membrane (0.2 μm, Fisher Scientific) and washed extensively with deionized water to remove impurities or dissociated metal catalysts.

2.2. Characterization of the SWCNTs library

Raman spectroscopy was used to investigate the physical and chemical properties of the SWCNTs. For this, SWCNT powders (pristine or acid treated SWCNTs, both 3 and 6 h were assessed) were deposited onto clean glass slides and scanned using a Raman spectrometer (CL532-100, 100 mW, USA) and a 532 nm green laser with a spot size of <0.01 mm^2 directed though a 50X objective. Detailed scans were recorded in the 100 to 3200 cm^{-1} range; low energy laser (i.e., <0.5 mV) and short exposure time (i.e., 10 s) were maintained throughout data acquisition to prevent unexpected heating effects of the samples.

Energy dispersive X-ray spectroscopy (EDX) was employed for quantitative elemental analysis of the SWCNTs. Dry samples (pristine and acid treated SWCNTs) were mounted onto carbon tape and their elemental composition was evaluated using a Hitachi S-4700 Field Emission Scanning Electron Microscope with a S-4700 secondary detector and backscattered electron detector (in a single unit).

Atomic Force Microscopy (AFM) was used in air tapping mode to investigate the lengths of pristine and acid treated SWCNTs (Dong et al., 2013a; Marshall et al., 2006). Briefly, commercial Si tips (Asylum Research, AC240TS) were employed under their original manufacturing resonance frequency varying from 50 to 90 kHz. During the scanning process, the topography, phase and amplitude images of the SWCNT samples were collected simultaneously; a minimum of 3 scans were obtained for each SWCNTs sample being investigated and at least 30 individual SWCNTs were measured for an average of their length distribution.

Analysis of SWCNTs agglomeration state was performed using a dynamic light scattering device (DLS, Delsa Nano-Particle Analyzer, Beckman Coulter) that evaluated the dynamic fluctuations in the intensity of the scattered light as caused by the particles' Brownian motion (Cheng et al., 2011; Schwyzer et al., 2013, 2012). For this, suspensions of 50 μg/ml pristine and acid treated SWCNTs (both 3 and 6 h) were prepared in Dulbacco Minimum Essential Media (DMEM, Invitrogen) with 5% Fetal Bovine Serum (FBS, Invitrogen) and analyzed at 20 °C. For each sample, 150 measurements were recorded and the mean particle diameter was calculated using Stokes–Einstein relationship based on Photon Correlation Spectroscopy and it was corrected by analyzing the intensity, volume, and number distribution data collected for each samples using analytical software Delsa Nano-version 2.21/ 2.03.

2.3. Cell culture and cell exposure to the SWCNTs library

Immortalized human lung epithelial cells (BEAS-2B) purchased from American Type Culture Collection (ATCC) were cultured in DMEM medium supplemented with 5% FBS, 2 mM L-glutamine and 100 units/ml penicillin/streptomycin (Invitrogen). Cells were passaged regularly and kept in 5% CO_2 at 37 °C. To prepare the pristine and acid treated SWCNTs (both 3 and 6 h) for cellular exposures, the samples were first dispersed by sonication in deionized water to eliminate all large agglomerates (visual assessment). Subsequently, the samples were filtered using a 0.2 μm pore filter membrane, resuspended in DMEM media with 5% FBS, and again sonicated for 2 min to form stable suspensions.

2.4. Electric cell impedance sensing (ECIS)

Real-time analysis of cellular behavior post-exposure to SWCNTs (pristine and acid treated, both 3 and 6 h) was performed using an electric cell impedance sensing (ECIS, Applied Biophysics) platform. In one set of experiments, two 8W10E+ ECIS arrays with 40 gold electrodes each were simultaneously employed to provide concomitant measurements of 16 samples, all at multiple