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A study on the cytotoxicity of carbon-based materials

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ABSTRACT

With an aim to understand the origin and key contributing factors towards carbon-induced cytotoxicity, we have studied five different carbon samples with diverse surface area, pore width, shape and size, conductivity and surface functionality. All the carbon materials were characterized with surface area and pore size distribution, X-ray photoelectron spectroscopy (XPS) and electron microscopic imaging. We performed cytotoxicity study in Caco-2 cells by colorimetric assay, oxidative stress analysis by reactive oxygen species (ROS) detection, cellular metabolic activity measurement by adenosine triphosphate (ATP) depletion and visualization of cellular internalization by TEM imaging. The carbon materials demonstrated a varying degree of cytotoxicity in contact with Caco-2 cells. The lowest cell survival rate was observed for nanographene, which possessed the minimal size amongst all the carbon samples under this study. None of the carbons induced oxidative stress to the cells as indicated by the ROS generation results. Cellular metabolic activity study revealed that the carbon materials caused ATP depletion in cells and nanographene caused the highest depletion. Visual observation by TEM imaging indicated the cellular internalization of nanographene. This study confirmed that the size is the key cause of carbon-induced cytotoxicity and it is probably caused by the ATP depletion within the cell.

1. Introduction

Among carbon-based materials, sp^2 hybridized carbons are the most common forms and largest fraction of carbon materials fall into this category. In sp^2 carbons, the skeletons are formed by the alternate hexagonal single and double bonds between the carbons atoms and graphite is the ideal crystalline sp^2 carbon material. The key skeletal structure of graphite-like sp^2 materials remains the same, however, with more chemical and structural alternations of their morphology. The examples of such carbons include nano-carbons, like carbon nanotube or graphene, porous carbon, like activated microporous or mesoporous carbons or non-porous carbons, like carbon soot or carbon fiber [1,2]. In today's perspective, a very large number of sp^2 carbon-based materials are employed in biological and biomedical platforms, including drug delivery, artificial implants, imaging agents or poison arresters. Like any other foreign materials, biocompatibility of these materials always remains questionable and the past results obtained so far often contain conflicting or counter-intuitive results and explanations that prohibit any generalized conclusion.

Carbon materials have been shown to have toxic effects. The toxicity effects could possibly arise from the shape and size, surface functionality, hydrophilicity, porosity, surface conductivity or often the toxic guest species that are associated with these materials during processing. Carbon nanotubes (CNTs) are one of the early sp^2 carbon materials that were studied for possible toxicity and since then, the popular term 'nanotoxicity' has evolved. Albeit conflicting results that have been obtained on CNT based toxicity, it has almost been universally accepted that CNTs are toxic [1,3–7] and their toxicity is a strong function of degree of agglomeration, functionalization and metallic catalyst contents [8–10]. It was demonstrated that the sulfonic acid and meta-phthalic acid functionalized CNTs reveal much reduced cytotoxicity compared to non-functionalized CNTs and hydrophilicity was identified as the key factor [11]. Recently, Shi et al. [12] provided the visual evidence that the asymmetric shape of CNTs causes cell penetration. Graphene is an sp^2 -hybridized and 2D carbon nanomaterial that has already demonstrated its potential in biomedical platforms. The malicious effect of graphene has already been demonstrated from cellular to ecological studies. Researchers revealed that the sharp edge of graphene penetrates through the cellular membrane [13] and apparently the 'acicular' shape of the object causes cell penetration and possible toxicity. Riding et al. [14] demonstrated the mechanistic understanding of nanocarbon toxicity in biochemical level on both pro- and eukaryotic cells with the help of multi-beam synchrotron radiation-based Fourier-transform infrared imaging (SR-FTIRI) at diffraction-limited resolution. Similar to CNTs, functionalization of graphene with hydrophilic entities also proved to reduce its cytotoxic behavior [15]. Likely, hydrophilicity causes the nanomaterials to be well dispersed in water and statistically lowers the direct cell-material interactions, thus reducing deposition of the materials onto the cell

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membrane. Additionally, hydrophilic surfaces interact with proteins through water molecules, thereby minimizing protein degradation [16]. On the contrary, a hydrophobic surface directly interacts with cells, causing conformational changes and denaturation of protein, [16] thereby resulting in diverse cellular malfunctions [17].

Other than nanocarbons materials, bulk or monolithic carbons are also used in biomedical applications. Oral delivery of activated carbon suspensions has long been used as a poison arrester for accidental toxin ingestion or drug overdose [18,19]. Recently, there is a significant growth in the application of mesoporous carbons in drug delivery for a diverse array of drugs; both bulk [2,20–26] and nano-sized [27–29] mesoporous carbons have been employed for these purposes. In our previous work, we have demonstrated that mesoporous carbon material was non-toxic and its benign nature was confirmed through cell viability, protein adsorption and hemolysis studies [30]. Although cytotoxicity studies did not reveal significant toxicity to HeLa cells, higher surface area carbons were found to be slightly more toxic compared to lower surface area carbons. While employing porous carbons or even porous silica materials in biological platforms, different parameters also appear to play significant role in causing toxicity to cells, like surface area [31–33] surface functionality [33] (likely due to the surface hydrophilicity/hydrophobicity) or even the conductivity related to electron transfer mechanisms [33].

Considering all the apparent contributing factors towards toxicity and with greater implementation of carbon materials in biological and biomedical platforms, there is a need for a generalized toxicity study of different varieties of carbon materials with proper controls. Additionally, it has been demonstrated that two distinct phenomena cause cytotoxicity, oxidative stress of the cell causing reactive oxygen species (ROS) generation and reduction of cellular activity by adenosine triphosphate (ATP) depletion resulting in ultimate cell apoptosis or necrosis. For carbon-based materials, these two phenomena were not investigated in detail, and to the best of our knowledge, the exact root of cellular toxicity of carbons materials is not known. In this phase of work, we have selected five types of carbon materials with variation in size and shape, surface area, surface functionality and conductivity. The carbon materials that we selected for this study are non-porous carbon (C1), highly porous commercial carbon with very high surface area (C2), mesoporous carbon with low surface area and large pore width (C3), graphite powder with highest conductivity (C4) and nanographene with moderate surface area and high conductivity (C5) as a representative of nano-carbon. We did not include carbon nanotube (CNT) in our study as it was already studied several times in the past and several additional control parameters associated with CNTs would make the conclusion of study more complex. We employed Caco-2 (human colon carcinoma cell line) as a model cell to study the cytotoxicity as this type of cell line is regarded as a model cell for intestinal epithelia cells [34]. All the materials were characterized by porosity analysis, surface functionality and conductivity by X-ray photoelectron spectroscopy (XPS) and particle size and shape determination by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). We have examined the cytotoxicity study by a colorimetric assay, ATP depletion by a luminescent assay, reactive oxygen species (ROS) generation by a fluorescence assay, and visual inspection of cell internalization by light and electron microscopy (TEM).

2. Materials and methods

2.1. Carbon materials and characterization

Among the materials studied in this phase of work, non-porous carbon (C1) and mesoporous carbon (C3) were synthesized in the laboratory. The key principle of synthesis of C1 is very similar to that of reported in the literature [35] without the activation step. Typically, 10 mL of furfuryl alcohol is polymerized by adding 5 mL of 0.1 M *p*-toluenesulfonic acid dissolved in tetrahydrofuran as a catalyst. In order to avoid a vigorous reaction, the addition of the two reagents is controlled in a very slow fashion (~10 mL/h) and the reaction mixture is cooled in an ice bath. The reaction is continued for 48 h till it solidifies to a dark green, semi-solid mass. This mass is carbonization in a tube furnace in N_2 atmosphere up to 1000 °C and at a ramp rate of 10 °C/min and subsequent cooling in the same N_2 flow. Mesoporous carbon was also synthesized according to our previously published procedure [30]. Typically, 50 g resorcinol (carbon precursor) and 40 g F127 (template or structure dictating agent) were dissolved in a 20:1 volume ratio of water and ethanol. Resorcinol was cross-linked with 48 mL of formaldehyde along with 60 mL 6 M HCl as a catalyst. Upon separating the polymer layer from the solvent, it was carbonized under N_2 flow in a porcelain boat in the specified conditions of 0 °C to 400 °C at a rate of 1 °C/min and 400 °C to 1000 °C at a rate of 2 °C/min and subsequent cooling in the same N_2 flow. High surface area, commercially-available activated carbon (C2) and graphene (C5) were obtained from ACS Material and employed as received. Graphite powder was obtained from MTI Corporation and also employed as received.

The porosity, including BET surface area and pore size distribution of all the materials was analyzed by the standard N_2 adsorption-desorption at 77 K in Quantachrome's Autosorb iQ instrument. Scanning electron microscopic (SEM) images were obtained in an Evex Mini-SEM II HR-3000 model. The TEM images were obtained in a Carl Zeiss Libra 120 TEM operating at 120 kV. X-ray photoelectron spectroscopy (XPS) data was obtained in a Thermo-Fisher K-alpha instrument XPS system operating at monochromatic Al K-α as an X-ray anode. The X-ray energy employed was 1486.6 eV and resolution of 0.5 eV.

2.2. Toxicity studies

All toxicity studies were undertaken with Caco-2 cells (ATCC© HTB-37). Caco-2 is a human epithelial cell line. The cells were grown at 37 °C, 5% CO_2, and 100% humidity in DMEM (Life Technologies, Grand Island, NY) media that was supplemented with 10% fetal bovine serum (Atlantic Biologicals, Atlanta, GA) and 1% penicillin and streptomycin (Life Technologies, Grand Island, NY). Cell toxicity was measured with an MTT Assay, as described earlier [30], except experiments were conducted with Caco-2 cells and not HeLa cells.

ATP was measured with the CellTiter-Glo® Luminescent Cell Viability Assay from Promega (Madison, WI). Caco-2 cells were seeded at a density of 2×10^5 cells/mL with 100 μL per well in a white 96-well plate and allowed to grow for 24 h. Carbon materials were made in 100 and 300 μL/mL solutions in DMEM supplemented media and sonicated with a Heat Systems Ultrasonic XL sonicator (Plainville, NY) for 5 min. Each carbon was added to the wells in a volume of 25 μL. At different times, the cells were equilibrated to room temperature for 30 min [36,33], followed by addition of the CellTiter-Glo® reagents, per manufacturer instructions. The chemiluminescence was read after a 10 minute incubation of the reagents [36,33] on a Thermo Fluoroskan Ascent FL (Grand Island, NY). 9% Triton-X was used as a positive control and phosphate buffer saline (PBS) (Life Technologies, Grand Island, NY) was used as a negative control.

ROS was measured with CellRox® Green fluorogenic reagent (Thermo-Fisher, Grand Island, NY). Cells were treated the same as the ATP assay except the cells were seeded into a black 96-well plate. After equilibration to room temperature, the CellRox® reagents were added to the cells and carbon samples and incubated for 30 min at 37 °C. The media was removed and the cells were washed with PBS three times. The fluorescence (excitation: 485 nm/emission: 520 nm) was read on a Synergy Mx microplate reader (BioTek, Winooski, VT). Hydrogen peroxide at 0.25% and 0.5% was used as positive controls and PBS as a negative control.

2.3. Cell imaging

Light microscopy images were prepared as described earlier for the ATP assay except the cells were seeded into a clear 96-well plate and