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In vitro toxicity of carbon nanotubes, nano-graphite and carbon black, similar impacts of acid functionalization

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ABSTRACT

Carbon nanotubes (CNT) and nano-graphite (NG) are graphene-based nanomaterials which share exceptional physicochemical properties, but whose health impacts are unfortunately still not well understood. On the other hand, carbon black (CB) is a conventional and widely studied material. The comparison of these three carbon-based nanomaterials is thus of great interest to improve our understanding of their toxicity. An acid functionalization was carried out on CNT, NG and CB so that, after a thorough characterization, their impacts on RAW 264.7 macrophages could be compared for a similar surface chemistry (15 to 120 $\mu g \cdot mL^{-1}$ nanomaterials, 90-min to 24-h contact). Functionalized nanomaterials triggered a weak cytotoxicity similar to the pristine nanomaterials. Acid functionalization increased the pro-inflammatory response except for CB which did not trigger any TNF-α production before or after functionalization, and seemed to strongly decrease the oxidative stress. The toxicological impact of acid functionalization appeared thus to follow a similar trend whatever the carbon-based nanomaterial. At equivalent dose expressed in surface and equivalent surface chemistry, the toxicological responses from murine macrophages to NG were higher than for CNT and CB. It seemed to correspond to the hypothesis of a platelet and fiber paradigm.

1. Introduction

Carbon-based nanomaterials share a unique place in nanotechnologies due to their exceptional electrical, thermal, chemical and mechanical properties. In the last decades, new allotropic forms of carbon were discovered and completed this group. In the present study, a focus will be made on carbon-based nanomaterials with three nanometric dimensions: carbon black (CB), two nanometric dimensions: carbon nanotubes (CNTs), and one nanometric dimension graphene or nano-graphite (NG).

CB is a traditional carbon material widely used as a pigment or reinforcing phase in tires. It has been considered as nanomaterial only since a few years. CB typically falls within the ISO definition of a nanomaterial (ISO/TS 80004-2:2015) with primary particle diameters between 10 and 100 nm. The health effects of CB have been extensively studied. The International Agency for Research on Cancer (IARC) has classified CB as possible carcinogenic to humans (Group 2B) (IARC, 2010). The IARC reviewed notably an in vivo study showing that lung cancer in rats caused by an exposure to CB started after signs of inflammation, cell injury and oxidative stress with the production of reactive oxygen species (ROS).

CNT and graphene are two carbon-based nanomaterials that have demonstrated exciting physicochemical properties since their late discovery, promising thus numerous industrial applications. Multi-walled and single-walled carbon nanotubes (MWCNT and SWCNT respectively) have been studied since the 1990s (Iijima, 1991; Iijima and Ichihashi, 1993). Graphene was first not thought to be a stable material until Novoselov et al. in 2004 managed to prepare and study a single graphene layer (Novoselov et al., 2004). MWCNT and SWCNT are now produced at an industrial scale, while it is still uneasy to produce graphene at large scale. Most industrials sell nano-graphite (NG), also called graphite nanoplatelet, which is composed of a few layers of graphene and has close physicochemical properties. Their mechanical resistance, electrical conductance and thermal stability are, however, decreasing with the number of graphene layers, i.e., the thickness of the NG.

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Compared with CNT or NG, CB is either used in toxicity studies as a negative control (Bottini et al., 2006; Lam et al., 2004; Schinwald et al., 2011) or a positive control (Di Giorgio et al., 2011; Pulskamp et al., 2007). Due to the late discovery of CNT and NG and the lack of comparable studies, the toxicity data are still incomplete for these nanomaterials. It has been shown that CNT and NG have the potential to trigger inflammation, cytotoxicity and oxidative stress (Chen et al., 2011; Schinwald et al., 2011; Zhang et al., 2012). However, there is no unique toxicity for one type of nanomaterial. There are multiple toxicity profiles depending on the nanomaterial physicochemical properties. It is therefore crucial to fully characterize the nanomaterials before any toxicity study.

Few studies have yet compared the biological effects of CNT and NG. Zhang et al. (2010) studied the in vitro impacts of NG and SWCNT. The cytotoxicity and oxidative stress in PC12 cell line (derived from a neuroendocrine tumor) were found globally equivalent. However, the cytotoxicity results were dependent of the test used: MTT or LDH. Using a MTT test with a different cell line (human hepatoma HepG2 cells) Yuan et al. (2011) observed a higher cytotoxicity for SWCNT than for NG. Nevertheless, MTT is controversial because of biases induced by the CNT (Belyanskaya et al., 2007; Wörle-Knirsch et al., 2006). In their in vivo study, Ma-Hock et al. (2013) assessed the biological impacts on Wistar rats of a head-nose inhalation of graphene, NG, MWCNT, and CB. Only local inflammation with no further toxicity was detected, with this order of gravity: MWCNT > graphene > NG > CB with CB not inducing any inflammation. Overall, the results remained inconsistent between the toxicological studies. One explanation for these inconsistencies could be the difference in surface chemistry. Indeed, NG and CNT were produced by different methods along the studies and exhibited different levels of metallic impurities and oxygen-containing groups.

One way to equalize the surface chemistry of carbon-based nanomaterials is to functionalize their surface by an acid treatment (Figarol et al., 2014). Acid functionalization of CNT is relatively easy to carry out and is of great interest for industrials. The CNT hydrophobicity is indeed decreased, so they become more dispersible in aqueous solvents. Moreover, this process purifies the CNT from their metallic impurities, residues of the catalysts used for their production and entrapped into the CNT structure that are dissolved after a strong acid treatment. NGs are often produced by the Hummers modified method (Hummers and Offeman, 1958) using strong acids to exfoliate graphite, resulting in thin flakes of oxidized NG. The biological impact of the oxygen-containing groups on CNT or NG is still not fully understood. In a previous study (Figarol et al., 2014), we demonstrated that surface acid groups increased the pro-inflammatory response and, to a lesser extent, the cytotoxicity of murine macrophages (RAW 264.7) exposed to functionalized CNT. This trend seemed to be dependent of a threshold in surface acid groups, related to the physicochemical properties of pristine CNT. Other studies showed contradictory results but did not isolate the effects of the CNT purification. In this way, even when selecting the same cell line (i.e., murine macrophages RAW 264.7), the results can be inconsistent. Dong et al. (2012) and Wang et al. (2012) found similarly that CNT acid functionalization enhanced the cytotoxicity and the pro-inflammatory response, while Zhang et al. (2012) observed a decreased cytotoxicity and only a slight increase in the pro-inflammatory response. On the contrary, Fraczek-Szczypta et al. (2012) detected no change in cytotoxicity but a decrease in cell proliferation due to the CNT acid functionalization. For NG or graphene, the results are more consistent. Oxidized graphene from Hummers method and acid functionalized graphene seemed to decrease the cytotoxicity and oxidative stress compared with exfoliated or pristine NG (Duch et al., 2011; Sasidharan et al., 2012, Sasidharan et al., 2011). The pro-inflammatory response was on the other hand either found to be increased by acid functionalization (Duch et al., 2011) or decreased (Sasidharan et al., 2012). Overall, the understanding of the biological impact of an acid functionalization is still incomplete. To our knowledge, only Zhang et al. (2012) compared the in vitro response of CNT and NG with surface acid groups. Differences in cellular uptake were observed between functionalized MWCNT, oxidized NG and nano-diamonds. However, the cytotoxicity of acid functionalized MWCNT, and oxidized NG were not significantly different. The pro-inflammatory response and oxidative stress were unfortunately not assessed.

In the present study, a first objective was to determine if acid functionalization impacts the in vitro cellular response of murine macrophages with a similar pattern for different shapes of carbon-based nanomaterials (i.e., nanotubes, nanoplatelets or nanospheres). Secondly, the biological impacts of acid functionalized MWCNT, CB, and NG showing a similar surface chemistry were compared.

2. Material and methods

2.1. Powders

The multi-walled carbon nanotubes (NC7000™, Nanocyl, called CNT) were synthetized by CVD (chemical vapor deposition) and have a diameter of 9.5 nm and a length of 1.5 μm according to the manufacturers. The nano-graphite (NG) was purchased from Graphene Supermarket. The flakes have a 12 nm average thickness and a 4.5 μm average particle size according to the manufacturers. Carbon black (CB) was purchased from Evonik Degussa (Printex® 85).

2.2. Acid functionalization

The acid treatment consisted in an oxidation by refluxing the carbon nanopowders in a solution of nitric and sulfuric acids (3:1 v/v). Functionalized nanomaterials were filtered (0.025 μm MF-Millipore Membrane) and rinsed until the pH reached 5. They were dried in an oven at 100 °C for 18 h. Concentrations in nanopowders, acid solutions and duration of the oxidation were optimized for each nanomaterial to obtain a comparable level of surface acid groups (see Supplementary material Table A1). Functionalized carbon-based nanomaterials were called CNTf, CBf and NGf in contrast to non-functionalized nanomaterials i.e., pristine nanomaterials (CNT CB and NG). The term pristine will be used even after the dispersion of nanomaterials into the biological medium even though their secondary properties are affected.

2.3. Physicochemical characterization

Morphologies of the carbon-based nanomaterials were observed using field-emission scanning electron microscopy (FEG-SEM, JEOL JSM 6500F, Akishima, Tokyo, Japan) at a 2 kV. A few milligrams of nanopowder were put on a carbon-coated holey film. Samples were coated with a 3 nm gold layer before FEG-SEM observations. CNT, CNTf, CB and CBf average diameters were measured using FEG-SEM images. ImageJ software was used to measure 100 diameters per picture (repeated three times). Average diameters were expressed as the mean of 300 measurements. Atomic force microscopy (AFM, JPK Nanowizard®) was used to confirm the dimensions of the NG and NGf. Samples were prepared by the sonication of a 10 $mg \cdot mL^{-1}$ NG or NGf suspension in distilled water (5 min, 30%, 3 mm probe, Branson Sonifier). One drop was deposited on a 1 cm^2 silicon wafer, spread by centrifugation (226 *g*, 2 min, Megafuge 16R, Thermo Scientific), and dried 10 min at 100 °C. Specific surface areas (SSA, $m^2 \cdot g^{-1}$) were determined by the Brunauer–Emmet–Teller (BET) method, using N_2 adsorption at 77 K after out-gassing at 110 °C (Micromeritics ASAP 2000).

After acid functionalization, increases in structural defects were analyzed by Raman spectroscopy (XploRA, Horiba Scientific) with a laser at 532 nm, a x50 objective, a 2400T network, 20 acquisitions of 20 s giving a spectra between 1000 and 2000 cm^{-1}. Around 1340 cm^{-1}, the D-band (D for disorder) is linked to the sp^3 hybridized carbon. Its intensity increases with ill-organized graphite structure (Belin and Epron, 2005). Around 1570 cm^{-1}, the G-band (G for