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Toxicity determinants of multi-walled carbon nanotubes: The relationship between functionalization and agglomeration

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ABSTRACT

The elucidation of toxicity determinants of multi-walled carbon nanotubes (MWCNT) is still incomplete. Functionalization with carboxyl groups is, however, commonly used to mitigate MWCNT toxicity, although the rationale for the mitigating effect has not been fully clarified yet. In this work, two optimized chemical vapor deposition methods were employed to obtain MWCNT of comparable length but different diameter, which were subsequently functionalized. For MWCNT of diameter larger than 40 nm, no detrimental effects on cell viability of macrophages were observed, while mild cytotoxicity was recorded for diameters between 15 and 40 nm, with a mitigating effect of functionalization. To investigate the factors responsible for the mitigation, we used the thinnest MWCNT preparation on different cell models, evaluating several endpoints, such as viability, production of nitric oxide (NO), expression of pro-inflammatory markers, the Trans-Epithelial Electrical Resistance (TEER), and clonogenic activity. Substantial mitigation of the changes caused by pristine MWCNT was observed not only with carboxyl- but also with amino-functionalized MWCNT, suggesting that negative or positive surface charge was not the main factor responsible for the effect. Instead, either functionalized preparation exhibited a stronger tendency to agglomerate that was strictly dependent on the presence of proteins. Moreover, we found that either carboxyl- or amino-functionalized MWCNT adsorbed a larger amount of serum proteins than pristine counterparts, with a distinctive pattern for each type of MWCNT. We propose, therefore, that the formation of larger agglomerates, dependent upon different protein coronae, contributes to mitigate the biological effects of functionalized MWCNT in protein-rich biological media.

1. Introduction

After their discovery by Iijima [29], carbon nanotubes (CNT) have been increasingly used in advanced industrial applications. Indeed, due to their excellent physico-chemical, electrical and mechanical properties, they are applied in numerous technological fields such as polymer composites, microelectronics, energy storage and sensors [18]. CNTs are graphitic hollow filaments of variable lengths, up to several hundred micrometers, depending on the production method. Single-walled carbon nanotubes (SWCNT) are composed of a single cylindrical sheet of graphene, while multi-walled carbon nanotubes (MWCNT) consist of several concentric, coaxial, rolled up graphene sheets [44]. The CNT diameter typically ranges from 0.4 to 3 nm for SWCNT and from 1.4 to 100 nm for MWCNT [62]. Chemical vapor deposition (CVD) is the most frequently used method for CNT synthesis and is the prevalent

Abbreviations: BET, Brunauer, Emmett and Teller; BSA, Bovine Serum Albumin; CFE, colony forming efficiency; CNT, carbon nanotubes; CVD, carbon vapor deposition; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EDS, energy dispersive X-ray spectrometry; FBS, Fetal Bovine Serum; FT-IR, Fourier transform infrared spectroscopy; MWCNT, multi-walled carbon nanotubes; NO, nitric oxide; SDS, sodium dodecyl sulphate; SDS-PAGE, SDS polyacrylamide gel electrophoresis; SWCNT, single-walled carbon nanotubes; SSA, specific surface area; TEER, Trans-Epithelial Electrical Resistance; TGA, thermogravimetric analysis; XRD, X-ray diffraction.

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technique for mass production of CNT due to its easy scaling-up [9].

Different preparations of MWCNT yield contrasting results about their bio-safety, as some preparations appear to be highly hazardous, while others seem harmless [13,24,47,51]. Thus, toxicity evaluation of these materials has to be taken on a case-by-case study, because CNT cannot be regarded as a simple chemical substance. Therefore, the investigation of MWCNT toxicity has to be designed according to their specific features and cannot adopt the same strategy of the conventional toxicology studies applied for general chemical compounds [42].

The conventional view point is that SWCNT exhibit significant cytotoxicity to human and animal cells through various mechanisms [12,33,40], whereas MWCNT are considered less active [32]. However, increasing evidences suggest that MWCNT can be inflammogenic and fibrogenic in rodents [11,20]. Moreover, a specific MWCNT preparation (MWCNT-7) has been recently classified as possibly carcinogenic to humans (Group 2B) by the International Agency for Research on Cancer, although other preparations of MWCNT were not classifiable respect to their carcinogenicity to humans [26]. These contrasting results can be attributed to the use of materials with different degree of purity and structural features [68], as well as to the conditions adopted for the *in vitro* studies [72] and the cell types tested for the assays [35]. Thus, despite several excellent reviews on the advancement in knowledge about CNT toxicity [2,16,19,36,37,46,63], the current understanding of the physico-chemical determinants of MWCNT toxicity is still incompletely settled [30].

In general, the toxicity of CNTs is attributed to their physico-chemical characteristics, such as length, diameter, shape, purity, surface area and surface chemistry [37,38], which, in turn, are remarkably influenced by the synthetic route. CNT contamination by catalyst residues is unavoidable during their production, so that the impact of residual catalyst metals may be also important [38]. Among the various determinants known to influence the biological activity of CNT, also functionalization with carboxylic groups has been considered and investigated. However, contrasting results have been reported in this case too. Functionalization enhances toxicity in airway epithelial BEAS-2B cells [10,65], while on the contrary, it has been demonstrated to suppress bioactivity in other epithelial models [39] and in macrophages [28]. Also *in vivo* models have yielded diverging data, with mitigating [31,58] or enhancing [64] effects reported for different endpoints.

When suspended in biological media, MWCNT adsorb macromolecules that form a corona and modify the properties of the material [41]. Protein adsorption is heavily influenced by surface curvature and surface area. Surface curvature is directly related to the outer diameter [27,52], while surface area is influenced by outer diameter, pore volume and surface chemistry. In particular, surface chemistry influences the adsorption of macromolecules to CNT depending on: (1) the available specific surface area (functionalized CNT may present higher or lower surface area values according to the functional groups which are attached on their sidewalls); (2) the solution pH, in relation to CNT pKa value (at pH below pK_{α} adsorption is increased); (3) the ionic strength in the solution (ionic strength increases adsorption) [14]. However, the relationship between protein corona, functionalization and MWCNT biological effects is still under investigation.

In this work, we tested the hypothesis that functionalization with carboxyl or amino groups affects the formation of protein corona and, hence, the biological effects of MWCNT. To this purpose, we preliminary examined four MWCNT preparations, three synthesized in our laboratory *via* CVD method and one commercial, of comparable length but different diameter and surface chemistry, and compared their effects on cell viability with those exhibited by a benchmark MWCNT preparation obtained from the Joint Research Centre (JRC) repository (Ispra, Varese, Italy). One of the preparations exhibiting changes in biological activity attributable to functionalization was then further investigated, studying both protein corona and several toxicological endpoints.

2. Experimental procedures

2.1. Supply and synthesis of carbon nanotubes

A thermal chemical vapor deposition (CVD) reactor was used to synthesize MWCNTs. The reactor consists of a horizontal quartz tube with 3.4 cm inner diameter and 100 cm length housed in a three-zone 80 cm long cylindrical furnace. Synthesis of CNT was performed by two approaches. In the first case, camphor and ferrocene were used as carbon source and catalyst, respectively, while acetylene as carbon source and iron particles supported on Al_2O_3 substrate as catalyst were used in the latter.

More specifically, for the preparation of the CNT1 sample (see Table 1), a Pyrex flask containing the reagent mixture, which consisted of camphor (96% purity in weight, Sigma–Aldrich, Athens, Greece) as carbon precursor and ferrocene (98% purity in weight, Sigma–Aldrich, Athens, Greece) as catalyst, in a 20/1 mass ratio, was connected to the tube nearby the nitrogen inlet. Nitrogen gas flow was used to carry the gas mixture of precursors toward the center of the furnace, where pyrolysis took place at 850 °C, and CNT were grown. For the preparation of CNT2 and CNT3 samples, the catalyst particles were placed on a ceramic boat inside the quartz tube and in the middle of the isothermal zone of the reactor. Firstly, a constant nitrogen flow rate was passed through the quartz tube to remove the air from the system, and, then, the reactor was heated at 700 °C under nitrogen flow. Subsequently, nitrogen was replaced by a mixture of acetylene/nitrogen.

Commercial MWCNT were obtained from Nanothinx S.A. (Patra, Greece). In particular, two types of commercial nanotubes were used in this study: NTX1 (pure MWCNT, REF) and NTX5 (MWCNT functionalized with –COOH groups, O-REF). The physical characteristics of these nanomaterials (according to the technical datasheet) are presented in Table 1. Additionally, a TEM image of the NTX1 nanomaterial is presented in Fig. 1e.

The NM-401 MWCNT preparation was obtained from the JRC Nanomaterials Repository hosting representative industrial nanomaterials (Ispra, Varese, Italy). This nanomaterial is classified as a representative test material (RTM) and includes a (random) sample from one industrial production batch sub-sampled into vials under reproducible (GLP) conditions, with the stability of the subsamples monitored. A detailed physico-chemical characterization of this material is provided in the specific JRC Report [54].

2.2. Carbon nanotubes purification and functionalization process

After the synthesis, the raw products were milled and exposed at atmospheric air flow at 400 °C for 1 h, aiming at the removal of amorphous carbon. Afterwards, they were purified with constant boiling of 5 M HCl in a Soxhlet extractor in order to remove the remaining metal particles. Finally, the purified CNTs were washed with distilled water and dried in oven. To activate the CNT surface with –COOH groups (O-CNT1, O-CNT2 and O-CNT3), an acid solution mixture of 6 M HNO_3:H_2SO_4 in a ratio of 1:3 was used. Then, the CNT/acid mixture (0.15 g CNTs/10 mL acid solution) was stirred for 48 h at 80 °C. The suspension was filtered, and the black powder was washed with ethanol, acetone and distilled water and, eventually, dried in oven.

For the preparation of $-CONHCH_2CH_2NH_2$ functionalized (amino) MWCNT (A-REF), MWCNT-COOH (O-REF) were stirred in a 20:1 mixture of thionyl chloride ($SOCl_2$) and dimethylformamide