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Colony Forming Efficiency and microscopy analysis of multi-wall carbon nanotubes cell interaction

Jessica Ponti*, Renato Colognato, Hubert Rauscher, Sabrina Gioria, Francesca Broggi, Fabio Franchini, Cesar Pascual, Guido Giudetti, François Rossi

European Commission, Joint Research Centre, Institute for Health and Consumer Protection, Nanobiosciences Unit, Via E. Fermi 2749, 21027 Ispra, VA, Italy

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ABSTRACT

In this work, we present a complete physicochemical characterization of multi-wall carbon nanotubes (mwCNTs) in order to assess their potential toxicological effects in *in vitro* cell models using Colony Forming Efficiency (CFE) assay. We verified that Dimethyl Sulfoxide (DMSO) was a more suitable solvent to disperse mwCNTs compared to culture medium guaranteeing reproducibility in the preparation of testing dilutions. The CFE assay was carried out on five mammalian cell lines representing the potentially exposed and/or target organs for nanomaterials (lung, liver, kidney, intestine, skin), as well as on mouse fibroblasts cell line, which usually is considered a sensitive model to verify *in vitro* cytotoxicity of test compounds. A statistically significant toxic effect was found only in human alveolar basal epithelial cells and immortalized mouse fibroblasts, for which the interaction between mwCNTs and cells was additionally studied by Atomic Force and Scanning Electron Microscopy. In this study, we considered and suggested the CFE assay as a promising test for screening studies of cytotoxicity. In addition, combining *in vitro* tests with physicochemical analysis, this work underlines basic points to be considered when research on nanomaterials has to be carried out, to set up, in our opinion, well-defined and suitable experimental planning and procedures.

1. Introduction

Carbon-based nanomaterials (CBNs) are now among the most promising nanomaterials. Due to their extraordinary electronic and mechanical properties and their different forms such fullerenes, carbon nanoparticles, nanofibers and single-, double-, and multi-wall carbon nanotubes (mwCNTs) they are considered attractive for a large variety of industrial applications (Rivas et al., 2007). In particular, those ones that employ CBNs for DNA, proteins technologies and drug delivery for biomedical uses, as well as the production of innovative carbon-based intelligent textiles have attracted much attention (Wong Shi Kam et al., 2005; http://www.inteltex.eu; Rivas et al., 2007). Some of these materials are already under massive industrial production: for carbon nanotubes alone, novel production methods were introduced in the last two years considerably improving their synthesis and yield. Despite this, the information concerning potential hazards related to CBNs exposure remains sparse, incomplete and still under debate (Oberdörster, 2004; Lam et al., 2004; Ding et al., 2005; Oberdörster et al., 2005).

Many reviews report a relationship between exposure to CBNs and health consequences and come to the conclusion that, based on present knowledge, exposure to CBNs could place exposed workers to a possible risk resulting in cytotoxic response that goes from alterations in cell viability to inflammation and DNA damage (Donaldson et al., 2006; Rotoli et al., 2008; Chou et al., 2008; Genaidy et al., 2009; Rotoli et al., 2009). These effects are also supported by evidence of direct interaction of CNTs with cells (Monteiro-Riviere et al., 2005; Porter et al., 2007; Fenoglio et al., 2008; Muller et al., 2008).

Nevertheless, a limiting factor in the evaluation of CBNs *in vitro* toxicity responses is the selection of a suitable and reliable *in vitro* assay. In fact, most of the available biochemical assays show that CBNs can interact with commonly used assay dyes (e.g. MTT, XTT, Alamar Blue), thus producing low reproducibility or unreliable results (Wörle-Knirsch et al., 2006; Casey et al., 2007; Monteiro-Riviere et al., 2009). Published literature on CNTs toxicity often reveals unclear and/or contradictory results together with a generally high level of discrepancies. This may be attributed to several confounding factors, such as the use of not sufficiently characterized materials, and/or non-standardized protocols for *in vitro* treatments and methods for assessing toxicity endpoints. Furthermore, the potentially misleading information on the toxic effect of CNTs is increased by the fact that different units, for assessing

* Corresponding author at: Joint Research Centre, IHCP, NBS Unit, TP203, via E. Fermi 2749, 21027 Ispra, VA, Italy. Tel.: +39 0332785793; fax: +39 0332785787.
E-mail address: jessica.ponti@jrc.ec.europa.eu (J. Ponti).

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concentrations, are used in different works, thus making published data poorly comparable in terms of dose-effect relationships (Oberdörster et al., 2005; Monteiro-Riviere et al., 2009).

In this work, we present a study assessing the cytotoxicity of mwCNTs, following their physicochemical characterization in solution, in five mammalian cell lines representing the main organs potentially exposed to nanomaterials. A mouse fibroblasts cell line was also used. Combining *in vitro* tests with physicochemical characterization, this work underlines basic points to be considered when a study of the toxicity of nanomaterials has to be carried out and hence allows setting up defined and suitable experimental procedures for such studies (Oberdörster et al., 2005).

Cellular interaction of mwCNTs was studied in more detail in human alveolar basal epithelial cells and in immortalized mouse fibroblasts, the only two cell lines in which a statistical significant toxic effect induced by mwCNTs was observed. The *in vitro* test used in this work was the Colony Forming Efficiency (CFE), a clonogenic assay that measures the ability of a single cell to form a colony. This test has already been used to assess cytoxicity of cobalt, gold nanoparticles and single or mwCNTs in different *in vitro* systems. The CFE was previously optimized using immortalized mouse fibroblasts lung cells, rat brain endothelial cells, human cervix carcinoma cells, human hepatocytes and dog kidney cells (Ceriotti et al., 2007; Herzog et al., 2007; Ponti et al., 2009a,b; Gellein et al., 2009). At the moment the CFE assay is considered a promising test to study toxicity of CNTs and nanomaterials in general, as it makes use of no cellular dyes, that have been demonstrated to be a possible reason for invalid results due to their biochemical interaction with the nanomaterials tested (Casey et al., 2007; Monteiro-Riviere et al., 2009). In addition, the CFE is usually more sensitive than the conventional biochemical methods probably because this test does not measure a specific biological effect, but rather cell death in general (Ponti et al., 2006; Herzog et al., 2007).

2. Materials and methods

2.1. mwCNTs sample preparation and characterization

mwCNTs (Nanocyl® - 7000, Nanocyl S.A., Belgium) were produced via catalytic carbon vapor deposition process. A 10% (w/v) of metal oxide impurities was declared by the manufacturer after determination by thermogravimetric analysis (TGA) and confirmed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Perkin-Elmer SCIEX, Ontario, Canada) in our laboratory. Morphological analysis by Transmission Electron Microscopy (TEM) showed an average diameter of 10 nm and length in the range of 0.1–10 μm. The specific surface area, analyzed by gas adsorption technique according to Brunauer, Emmett and Teller (BET) was 250–300 m^2/g.

The first objective and challenge in this work was to select the most appropriate dispersant to have reproducible mwCNTs dispersions using a non-toxic solvent, at least at the testing concentration used, for our cell models. To this purpose, two stock suspensions of mwCNTs (10 mg/mL) were prepared: one in dimethyl sulfoxide (DMSO, Sigma Aldrich, Italy) and the other one in complete culture medium used for the dog kidney cell lines (MDCK) (Dulbecco's Modified Eagle Medium (DMEM) high glucose added with 10% (v/v) Fetal Bovine Serum (FBS) Australian Origin, 4 mM L-glutamine and 1% (v/v) pen/strep (Invitrogen, Italy). Since in this work we used six cell culture media similar in their chemical composition, the MDCK cell culture medium was selected as a representative of all six media to study the physicochemical properties of mwCNTs dispersions.

The two suspensions were characterized for dispersion, pipetting reproducibility and time of sedimentation by visible and near infra red light absorption (VIS-NIR) with a Thermo Nicolet Vision 300 spectrometer (Attal et al., 2006). mwCNTs bind by an interaction energy of ca. 500 eV/μm of tube–tube contact (Girifalco et al., 2000). Since the mwCNTs in the dispersions have a strong tendency to bundling and entanglement, the reproducible preparation was therefore an essential task. Stock suspensions in culture medium and in DMSO were diluted in the corresponding two solvents at concentrations of 0.125, 0.250, 0.500, 0.750, 1 and 2 mg/mL and analyzed at wavelengths in the range between 600 and 1000 nm. After selection of DMSO as the most suitable solvent, mwCNTs dispersibility, reproducibility of pipetting and sedimentation in complete culture medium were analyzed at dilutions of 1, 10 and 100 μg/mL and 10 and 100 μg/mL, respectively. The wavelength region from 600 to 1000 nm was used to characterize the reproducibility of pipetting and the absorption value at 650 nm was used to analyze mwCNTs sedimentation for times up to 180 s. The DMSO concentration in each solution tested with cells (1, 10 and 100 μg/mL) was kept at 1% (v/v), corresponding to the maximum non-toxic concentration for cells selected for this work. Furthermore, the dispersions of CNTs were screened for lipopolisaccharide (LPS) content by the Limulus Amebocyte Lysate (LAL) test. The analysis was performed on different dilutions (1, 10 and 100 μg/mL) starting from the 10 mg/mL stock dispersion of mwCNTs. The results did not indicate any LPS content (data not reported).

2.2. Cell culture conditions

Human keratinocyte cells (HaCaT) were originally supplied by the German Cancer Research Center (Germany), human alveolar basal epithelial cells (A549), human intestinal epithelial barrier cells (Caco-2), dog kidney cells (MDCK) and hepatocellular carcinoma cells (HepG2) were originally purchased from the American Type Culture Collection (ATCC). Balb/3T3 mouse fibroblasts stemming from the clone A31-1-1 were purchased from Hatano Research Institute (Japan).

Experimental cultures were prepared from deep-frozen stock vials and always maintained in a sub-confluent state. They were maintained in complete culture medium. HaCaT and HepG2 were cultured in DMEM high glucose (Invitrogen, Italy) added with 10% (v/v) Fetal Clone II serum (Hyclone, Celbio, Italy) and 1% (v/v) pen/strep (Invitrogen, Italy). MDCK were cultured in DMEM high glucose (Invitrogen, Italy) added with 10% (v/v) Fetal Bovine Serum (FBS) Australian Origin and 4 mM L-glutamine (Invitrogen Italy) and 1% (v/v) pen/strep (Invitrogen, Italy). Caco-2 were cultured in the same medium of MDCK but added with 10% (v/v) FBS North America origin. A549 were cultured in Ham F-12 added with 10% (v/v) Fetal Calf serum (Invitrogen Italy), 1% (v/v) pen/strep and 0.5% HEPES (Invitrogen, Italy). Balb/3T3 were cultured in Minimum Essential Medium (MEM) low glucose added with 10% (v/v) Fetal Bovine Serum (FBS) and 0.6% (v/v) pen/strep (Invitrogen, Italy). All the cell cultures were maintained in standard cell culture conditions (37 °C, 5% CO_2 and 95% humidity, HERAEUS incubator, Germany).

2.3. Colony Forming Efficiency assay

Cell viability, after exposure to mwCNTs, was studied by Colony Forming Efficiency assay. Cells were seeded at a density of 200 cells/dish (Balb/3T3, HaCaT, Caco-2, HepG2 and MDCK) and of 300 cells/dish (A549) in 3 mL complete culture medium (60 × 15 mm Petri dish, 19.3 cm^2 bottom surface area, Corning, Costar, Italy) at least in three replicates for each treatment. After 24 h, mwCNTs suspensions were directly added to the cell culture to obtain the appropriate final concentration of 1, 10 and 100 μg/mL, corresponding to 1.5×10^{-3}, 1.5×10^{-2} and 1.5×10^{-1} $\mu g/mm^2$ in the Petri dish used for the CFE experiments, respectively. After 72 h of exposure, the medium was changed with complete fresh culture medium that was renewed twice/week. After 7 days, cells were fixed for 20 min with 3.7% (v/v) formaldehyde solution (Sigma, Milan, Italy) in Phosphate Buffer Solution (PBS) (1×, GIBCO, Italy). Dishes were stained for 30 min with 4% (v/v) Giemsa solution (GS-500, Sigma, Italy) in ultrapure water. Colonies were manually scored under a stereomicroscope. Each experiment included a negative control (untreated cells in culture medium), a positive control (cells exposed to sodium chromate 1000 μM, CAS N. 10034-82-9 Sigma, Italy) and a DMSO solvent control (cells exposed to the same solvent contained in the stock mwCNTs suspension).

The results were normalised to the solvent control (DMSO 1%, v/v) and expressed as CFE(%) ((average of treatment colonies/average of solvent control colonies) × 100). The corresponding Standard Error Mean was calculated for at least 3 independent experiments and at least 3 replicates for each experimental point (SEM = (SD/√number of replicates)).

2.4. Statistical analysis

Raw data were normalised to the corresponding solvent control for each independent experiment. After normalisation, the statistically significant differences for CFE values versus DMSO 1% (v/v) were calculated by the one-way ANOVA analysis (GraphPadPrism4 statistical software, GraphPad Inc., CA, USA) that was also used to compare the experimental points of the independent experiments and to assess the statistical significant difference among the results obtained by the six used cell lines.

2.5. Microscopy analysis of mwCNTs and their interaction with cells

We investigated the morphology of mwCNTs in powder or dispersed in DMSO in the stock suspension as well as mwCNTs interacting with cells, using a Scanning Electron Microscope (SEM, FEI-Nova 600I Nanolab) and an Atomic Force Microscope (AFM, Agilent Technologies model 5500, USA).

SEM images were made using low acceleration voltages (10 KeV) and signals were revealed by both external and internal electron detectors.

AFM imaging using MagneticAC (MAC) type V cantilevers with a spring force constant of 0.4 N/m (Agilent, USA) was performed in MAC mode, a proprietary semi-contact scanning mode from Agilent Technologies. The cantilever, coated with magnetic material, oscillates under control of a magnetic field resulting in better, low-noise imaging compared to standard Alternating Contact Atomic Force Microscope (ACAFM) modes.

mwCNTs powder or a 10 μL drop of mwCNTs stock suspension in DMSO (10 mg/mL) were directly deposited on silicon or glass substrates for SEM and AFM