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## A non-damaging purification method: decoupling the toxicity of multi-walled carbon nanotubes and their associated metal impurities

Christian Darne<sup>\*a</sup>, Alexandre Desforges<sup>b</sup>, Nawal Berrada<sup>b</sup>, Caroline Fontana<sup>a</sup>, Yves Guichard<sup>a</sup>, Laurent Gaté<sup>a</sup>, Dominique Bégin<sup>c</sup>, François Le Normand<sup>d</sup>, Fabrice Valsaque<sup>b</sup>, Jaafar Ghanbaja<sup>b</sup>, Jérôme Gleize<sup>e</sup>, Brigitte Vigolo<sup>\*b</sup>

Multi walled carbon nanotubes (MWCNTs) are purified by a gas-phase method allowing selective removal of the metallic impurities (Fe and Al catalyst residues) while the MWCNTs remain untouched. Contrary to works using standard purification methods, our approach allows to separately study toxicity due to metallic impurities and that of MWCNTs. Both raw and purified MWCNTs are thoroughly characterized by several techniques including transmission electron microscopy, Raman spectroscopy, thermogravimetric analysis, X-ray photoelectron spectroscopy and adsorption volumetry. The results show that the purification method is efficient and the MWCNT characteristics are well controlled. We study the oxidative stress cell response induced by the MWCNT sample after purification. Cytotoxicity and genotoxicity are investigated using *in vitro* micronucleus and comet assays. Our results show that the metallic impurities of the used MWCNTs are not involved in the toxic potential of these CNTs. Consequently, these used CVD-produced MWCNTs alone are responsible for the observed toxic response. This presented approach, applied to other source of CNTs, could be useful for determining the involvement of their metallic impurities in the observed toxic response.

### Introduction

Carbon nanotubes (CNTs) are promising materials for a variety of applications in nanotechnology, energy conversion, high performance composites and also in the biomedical field. CNTs have been advantageously used for drug delivery, cancer therapy, diagnostic tools, biosensors or tissue engineering<sup>1-3</sup>. Even if CNTs are incorporated into devices and materials for current practical uses, the question of their toxicity is still under debate. Researchers have shown that CNTs often provoke a toxic response such as pulmonary inflammation, oxidative stress, granuloma or fibrosis<sup>4-9</sup>. However, CNTs are a complex material and among the numerous potential parameters, those responsible for the observed toxicity are difficult to identify and the mechanisms involved are far from clear<sup>10-13</sup>. The conflicting results in the literature regarding toxicity induced by exposure to CNTs could be explained by the variety of CNTs that exists and their respective behavior, which is strongly dependent on the chemical treatments to which they are subjected. To reach a greater degree of certainty regarding the human health and environmental risks associated with CNTs, standards of well characterized CNTs for which the parameters can be separately modified have to be established<sup>14,15</sup>.

CNTs are produced using different methods leading to diverse structures, morphologies, sizes and variable contents of impurities including carbonaceous and metallic by-products<sup>16</sup>. Generally, post-synthesis chemical treatments are needed to overcome processing issues of as-produced CNTs. Surface

functionalization of CNTs is an important aspect for biomedical applications of CNTs. Grafting functional groups to the CNT surface is normally used to aid their dispersion or to obtain a specific functionality<sup>17,18</sup>. These chemical treatments, usually conducted using strong acids, are reported to dramatically modify the CNT toxic response. For instance, contamination through hydrochloric, nitric or sulfuric acid treatment (creation of carboxylated CNT fragments or halogenated contaminants) can induce toxic effects<sup>19,20</sup>. Elsewhere, CNT toxicity is often reported to be reduced after functionalization<sup>21-25</sup>. Apart from functionalization, purification is another chemical treatment commonly applied to the CNT samples. Standard purification methods as well involve strong acid treatments which are necessary to eliminate the metallic impurities<sup>26,27</sup>. In order to use purified CNTs for biomedical research, it is crucial that we study their toxic effects since the metal-based catalysts remaining in the CNT samples have been shown to induce their own effects<sup>28-30</sup>. Especially, transition metals are normally used for Chemical Vapor Deposition (CVD) processes which produce most available CNTs.<sup>31,32</sup> Multi-walled CNTs (MWCNTs) instead of single-walled (SWCNTs) or double-walled CNTs (DWCNTs) are widely chosen for applications since their cost production is lower. The choice of the studied MWCNT sample was dictated by its similarities with the MWCNT industrial samples which are used in commercial products. Indeed, the CNTs produced in large amount are synthesized by using iron as catalyst deposited on an alumina substrate. The particularity of MWCNT samples is that they contain no carbon impurities leading to 'non-protecting' metal-based particles contrary to SWCNTs and

DWCNTs which also often require higher amount of catalyst content to be synthesized. For CVD-produced MWCNTs, the amount of metal-based impurities is usually around 10 wt.%, as in the chosen MWCNT sample. Previous studies have shown that iron impurities and other kinds of nanoparticles contained in CNT samples can themselves lead to toxic effects<sup>31–33</sup>. While other studies claim that the metal-based impurities have no effect on toxicity<sup>34–36</sup>. A better understanding of human and environmental risk of CNTs requires to better control the factors that can be responsible for the toxic effects.

In this study, we were able to investigate separately the toxic effects of metal-based impurities (Fe and Al) independently from those of the used CVD-produced MWCNTs. Such a study was possible thanks to the in-house development of an original and highly selective purification method<sup>37</sup>. To the best of our knowledge, the proposed approach which has allowed to decouple toxicity of CNTs and that of their associated metallic impurities, has not been reported yet. Our chemical treatment, which is based on the use of a gas-phase halogen, efficiently eliminates metallic impurities from CNT samples while allowing both the CNT morphology and their structural quality to be preserved. The MWCNTs were purified by heating the powder in a chlorine atmosphere. The purified MWCNTs were then characterized using several techniques (transmission electron microscopy (TEM), Raman spectroscopy, thermogravimetric analysis (TGA), X-ray photoelectron spectroscopy (XPS) and adsorption volumetry) that showed that their characteristics were particularly well preserved. The cytotoxicity and genotoxicity of raw and purified MWCNTs were then studied using *in vitro* micronucleus and comet assays. We also studied the cellular oxidative stress after MWCNT treatment.

## Materials and methods

### Purification

For the CNT synthesis, alumina (particle size: 40–80 nm) was impregnated with the catalyst precursor (Fe(NO<sub>3</sub>)<sub>3</sub>·9H<sub>2</sub>O) with a loading rate of 20 wt.% in iron. After water evaporation, the solid material was calcined at 350 °C, and finally reduced in situ under hydrogen flow (100 mL min<sup>-1</sup>) at 400 °C for 1 h to produce the catalyst. Experimentally, a medium temperature (640 °C), a relatively low C<sub>2</sub>H<sub>6</sub>-to-H<sub>2</sub> molar ratio (60/40), a flow rate of 100 mL/min and a reaction time of 4 h were found to produce almost exclusively MWCNTs (>98%, determined by statistical image analysis) with a yield of 10 g per gram of catalyst. Hydrogen seems to play an important role in limiting the formation of amorphous carbon impurities along with carbon nanotubes<sup>38</sup>.

Potassium permanganate (KMnO<sub>4</sub>) was provided by VWR Chemicals and hydrochloric acid (HCl, concentration 37 %) and sulfuric acid (H<sub>2</sub>SO<sub>4</sub>, concentration 96 %), sodium chloride (NaCl) were purchased from Sigma Aldrich. The raw MWCNTs (r-MWCNT) were purified using a two-step method described in details elsewhere<sup>39</sup>. In brief, the MWCNT powder was first heated under chlorine (150 mL/min in N<sub>2</sub> as the carrier gas, total pressure 1atm) for 20 min at 500 °C, 600 °C, 650 °C, 700 °C, 800°C and 1100 °C. Chlorine was formed in situ by oxidation of

HCl with KMnO<sub>4</sub>. For environmental and safety reasons, once the treatment has ended, unreacted chlorine was neutralized in 3 successive vials containing a sodium hydroxide solution (17 wt.%) and decomposed in hypochlorite and chloride ions according to the following chemical reaction:



This phenomenon was confirmed by the presence of a yellow characteristic color in the 2 first vials; the last one being colorless. Added to this neutralization and to ensure that no chlorine was released to the atmosphere, a specific and sensitive test was carefully performed during each purification experiment. This test uses potassium iodide (KI) and it is based on the following oxidation-reduction reaction:  $2\text{KI} + \text{Cl}_2 \rightarrow 2\text{KCl} + \text{I}_2$ . Appearance of a brown spot due to rapid formation of iodine eventually revealed any trace of chlorine. After the chlorine-based treatment, a hydrochloric acid (4 N) washing step was performed at room temperature for 2 h. After washing (until the pH reached neutrality), filtration and drying, the purified samples were ready for analysis. The MWCNTs purified at 800°C and later used for toxicity analysis are referred to as p-MWCNT.

For the TEM and High Resolution TEM (HRTEM) analyses, a drop of the suspension of MWCNTs dispersed by sonication in ethanol was deposited on a holey carbon grid (200 mesh size) and then examined using a JEOL ARM 200F cold FEG apparatus (operating voltage of 80 kV). Typical images are extracted from a batch of at least 25 images taken at different locations on the TEM grid to guarantee a representative description of the MWCNT sample. Their sizes (diameter and length) were evaluated from a statistical analysis on many TEM images. TGA was carried out with a Setaram Setsys evolution 1750 Thermal Gravimetric Analyser. Temperature was increased from room temperature to 900°C at 5 °C/min under dry air (20 mL/min).

Raman spectroscopy characterization was performed using a LabRAM HR 800 micro-Raman spectrometer with an incident wavelength of 632.8 nm at a power of 0.25 mW/μm<sup>2</sup>. To guarantee reproducibility, at least three spectra were recorded on different areas for the same sample. For data analysis, for each sample, the I<sub>D</sub>/I<sub>G</sub> intensity ratio was calculated from the maximum height of the D and the G band after subtracting a baseline.

XPS measurements were recorded using a monochromatic AlK<sub>α</sub> source and hemispherical detector VSW (pressure 5.10<sup>-9</sup> mbar). For the experiments, the raw and the purified CNTs powder was mixed with KBr powder and then pressed. The Cl/C atomic ratio was determined from the Cl1s and Cl2p peaks as described in ref.<sup>39</sup>. The estimated precision of Cl/C is 0.1 % for the sample treated at 1100°C. For the sample treated at 800°C, detection of chlorine was difficult because it is close to the detection limit of the apparatus, the precision of the Cl/C ratio is probably higher in that case.

Adsorption isotherms at 77.3 K were determined using krypton on both raw and purified MWCNTs. Adsorption on the surface of the metallic particles can be neglected compared to that occurring at the surface of CNTs because their amount is relatively low (maximal value for the raw MWCNTs 14.6 wt. %) compared to that of CNTs and their density is from 2 (alumina) to 4 (iron) times greater than that of the MWCNTs. The

isotherms were normalized by the amount of carbon content (from TGA) in the samples.

### Toxicity experiments

The MWCNT concentrations are expressed in amount of MWCNTs per culture surface ( $\mu\text{g}$  of MWCNT/ $\text{cm}^2$ ); this unit offers several advantages when we work with insoluble solid materials like the MWCNTs. Indeed, these latter have high tendency to aggregate and sediment on cell and they are rarely homogeneously dispersed in the solution. This metering system allows to take into account only 2 criteria: the cell density (number of cells/unit of culture surface) and the concentration (quantity of material/unit of cell culture surface) regardless of the volume or the support used.

For the concentration of  $7.5 \mu\text{g}/\text{cm}^2$ , in ROS and cell viability experiments, to ensure that  $7.5 \mu\text{g}$  of MWCNTs are in contact with 5000 cells, we have used a solution at a concentration of  $12.75 \text{ mg}/\text{L}$  of MWCNTs. A solution of  $26.24 \text{ mg}/\text{L}$  of MWCNTs was used for the comet assay and a solution at  $16.5 \text{ mg}/\text{L}$  of MWCNTs for the micronucleus assay.

Due to their high aspect ratio (length to diameter), typical of fibers, the choice of which MWCNT concentrations to study was directed by those used for asbestos when investigating induction of genotoxic effects and cell transformation<sup>40</sup>.

**Cell culture.** V79 cells (lung fibroblast from Chinese hamster, ATCC, USA, reference CCL-93) were grown in Dulbecco's MEM (DMEM; Invitrogen, France), supplemented with 10 % fetal calf serum (Dutscher, France) and 0.5 % Penicillin/Streptomycin ( $5000 \text{ U}-5000 \mu\text{g}/\text{mL}$ , Invitrogen, France). The cell culture was carried out in a humidified chamber at  $37^\circ\text{C}$  with 10 %  $\text{CO}_2$ , as recommended by the distributor.

V79 cells were selected for this study as they are one of the cell models recommended in OECD (Organization of Economic Co-operation and Development) guideline n° 487 for use in the *in vitro* micronucleus assay.

**MWCNT dispersions.** In order to obtain a homogeneous suspension (estimated visually and from optical microscopy, see supporting information Figure S1), the samples were placed in water supplemented with 0.1 wt. % of bovine serum albumin (BSA) and sonicated, in the cup horn of a 400 W Branson Sonifier S-450D (Branson Ultrasonics Corp, Danbury, CT) containing ice-cold water, for 7 min at 25 % amplitude in a pulse mode (2s on/1s off). 1/50 of the MWCNT suspensions were then added to the cell culture medium (see below).

The V79 cells were treated with the prepared dispersions of the CNT samples with concentrations ranging from  $0.47$  to  $7.50 \mu\text{g}/\text{cm}^2$ . As the metal content of both the raw and the purified MWCNT samples was already known (by TGA), metal-free MWCNT concentrations can be calculated by subtracting the metal content. The concentration could be expressed by the total MWCNT sample amount or the metal-free MWCNT amount in  $\mu\text{g}$  per  $\text{cm}^2$  of cell culture surface.

**Free radical generation.** Intracellular localization of reactive oxygen species (ROS) was detected with the 5-(and-6)-chloromethyl-2'-7'-dichlorodihydrofluorescein diacetate fluorescence probe (CM-DCFDA, Molecular Probe, Invitrogen).

DCFDA derivatives detect a variety of ROS, including hydrogen peroxide, peroxy radicals and peroxyxynitrite anions<sup>41</sup>.

$1.5 \times 10^3$  V79 cells were treated with  $0.47$  to  $7.50 \mu\text{g}/\text{cm}^2$  of MWCNTs for 24 h in a 96-well plate (six replicate wells per condition). Following the protocol described by Guichard et al.<sup>42</sup>, a sample of nano-size anatase titanium dioxide ( $\text{TiO}_2$ ) was used as a positive control (see supporting information Figure S2). At the end of the treatment, the wells were washed with PBS and  $50 \mu\text{L}$  of CM-DCFDA was added to give a final concentration of  $5 \mu\text{M}$ . The plate was then incubated at  $37^\circ\text{C}$  for 30 min. Wells were then washed with PBS and the fluorescence was recorded using a microtiter plate reader (Synergy HT, BioTek) with excitation at 485 nm and emission detection at 530 nm. ROS induction was expressed by the fold change in the measured fluorescence intensity of treated cells relative to the control (cell culture without MWCNTs). Data were expressed as the average of the means  $\pm$  SEM (standard error). Experiments were repeated three times.

**Cell viability.** Cells ( $1.5 \times 10^3$  cells/well) were seeded in 48 wells of a 96-well plate for 24 h. The cell cultures were then treated for 24 h in the culture medium without (control) or with MWCNT suspensions at concentrations ranging from  $0.47$  to  $7.5 \mu\text{g}/\text{cm}^2$  of the cell culture surface (six replicate wells per condition). The remaining 48 wells received the same CNTs suspensions but were not seeded with cells in order to check for possible interactions between formazan reagent and CNTs, at it has been described<sup>40</sup>, even if this has not been demonstrated for the WST-1 (Water soluble Tetrazolium, Roche Diagnostics, France) reagent that we used. After treatment, 1/10 (v/v) WST-1 reagent (see also supporting information Figure S3) was added to each well for 3h. The plates were then centrifuged at 4500 rpm for 5 min to eliminate the majority of CNTs and thus avoid interference of the CNTs with the reading. The supernatant was transferred to new 96-well plates and optical density (OD) was recorded at 450 nm and 690 nm with a microtiter plate reader (Synergy HT, BioTek, France). The delta OD (OD<sub>450 nm</sub> - OD<sub>690 nm</sub>) was then calculated. At least three independent experiments were conducted for each condition. In our experiments, the optic density has not significantly decreased in the wells without cells.

**Genotoxicity.** Two types of assay were used to evaluate the genotoxicity of CNTs in V79 cells: the comet assay and the micronucleus assay.

**Comet assay.** The comet assay is a sensitive technique for the detection of DNA damage in eukaryotic cells to identify both single and double strand breaks in alkaline condition ( $\text{pH} > 13$ ). The formamidopyrimidine glycosylase (FPG)-modified comet assay was used to evaluate this oxidative DNA damage. The FPG enzyme, a glycosylase, identifies and specifically cuts modified bases such as 8-oxoguanine from DNA, producing apurinic sites that are converted into strand breaks by the associated AP-endonuclease activity. Therefore, DNA strand breaks detected by the FPG modified comet assay therefore provide a measure of oxidative DNA damage<sup>43</sup>. We followed the procedure from Collins et al.<sup>44</sup>, with minor modifications. In brief, two duplicate comet slides were made for each treatment. One slide was treated with FPG and the other one with the FPG buffer only. The two slides were subsequently treated in a same manner.

V79 cells ( $1 \times 10^5$  cells/well) cells were treated for 24 h with the MWCNT samples at concentrations ranging from 0.47 to 7.50  $\mu\text{g}/\text{cm}^2$ , or with methylmethane sulfonate (MMS, Sigma-Aldrich, France) at 0.25 mM as a positive control, or with the medium alone.

Approximately 20,000 cells were mixed in 600  $\mu\text{l}$  of 1 % low melting agarose (LMA, Sigma Aldrich, France) and the mixture was transferred onto a slide pre-coated with normal melting agarose (NMA 1 %, Sigma Aldrich, France). Slides were then immersed in lysis solution (NaCl 2.5 M,  $\text{Na}_2\text{EDTA}$  100 mM, Tris 10 mM with 1 % Triton X-100 and 10 % of fresh DMSO) and kept in the dark for 1 h at 4  $^\circ\text{C}$ . The slides were next drained and incubated in the dark for 30 min at 37  $^\circ\text{C}$ , in either an enzyme buffer alone or in an FPG (5 U/mL) in enzyme buffer (HEPES 40 mM, KCl 0.1 M,  $\text{Na}_2\text{EDTA}$  0.5 mM, pH 8). The slides were immersed in a cold alkaline solution (NaOH 300 mM,  $\text{Na}_2\text{EDTA}$  1 mM, pH 13) for 20 min. Electrophoresis was then performed in the same buffer at 0.7 V/cm for 40 min to allow the fragments of damaged DNA to migrate towards the anode. The slides were then washed with Tris-HCl 0.4 M for 15 min and stained with propidium iodide (2.5  $\mu\text{g}/\text{mL}$ ).

Slides were examined at 200X magnification under a fluorescence microscope. Images of 100 randomly selected comets were acquired and analysed for each sample (Comet assay IV, Perceptive Instruments, UK) in order to evaluate the % tail DNA used as a measure of DNA damage. The presence of MWCNTs did not interfere with the reading at the concentrations tested. Each experiment was repeated three times independently. In V79 cells, the mean values of the median  $\pm$  SEM obtained for the positive control (0.25 mM MMS) were  $7.4 \pm 1.1$  and  $69.2 \pm 2.9$ , respectively, after 24 h of treatment with or without FPG, respectively.

**Micronucleus test.** V79 cells ( $2.5 \times 10^4$ ) were seeded in Labtek® slides (Nunc A/S, Denmark). After 24 h, the cells were treated with either MWCNTs at concentrations ranging from 0.47 to 7.50  $\mu\text{g}/\text{cm}^2$  or with positive control (0.25 mM MMS), or with medium alone for 24 h (V79 cell doubling time: 14-18 hours). At the end of the treatment, cells were washed with PBS (phosphate buffer saline, Invitrogen, France) and fixed in methanol for 15 min. Slides were washed in PBS, drained and received one droplet of ProLong Gold antifade reagent® with DAPI (Molecular Probe, Invitrogen, France). About 2000 cells were analyzed for the presence of micronuclei (MN) at each concentration. The presence of CNTs did not interfere with the reading. Each assay was repeated three times. Cell proliferation/division was assessed by analysis of the mitotic index (% of mitotic cells, see supporting information Figure S4).

**Statistics.** Results are expressed as means  $\pm$  SEM (standard error of the mean) from three independent experiments (and six replicates in each). A logarithmic transformation was applied to the biological data. For each assay and for each CNT solution, independently, a mixed linear regression model was applied first to judge the concentration effect, followed by a post hoc test (with Bonferroni correction) to assess the difference between control and concentrations. A second mixed linear model was also applied to measure simultaneously the dose-effect response of both raw and purified CNTs. These two models included a random "experiment" effect to take into

account the variability between experiments. Statistical analyses were performed using Stata 14.0 software. The statistical significance threshold was set at 5 %.

## Results

### Purification of carbon nanotubes

The morphological characteristics of the raw MWCNTs (r-MWCNT) have been determined from TEM analysis. The used MWCNTs have no more than 10 walls and are quite flexible (Figure 2). Their diameter is between 10 and 80 nm and their length varies from several  $\mu\text{m}$  to several tens of  $\mu\text{m}$ . These MWCNT characteristics were not modified after the purification treatment. The used MWCNTs contain small amount of carbon impurities and in agreement with the synthesis method (see experimental section). From TGA, the metal-based impurity content of r-MWCNT was approximately 14.60 wt. %. These impurities come from the alumina support and iron. From the elemental analysis, the raw MWCNT sample contains 5.0 wt. % of Al and 2.6 wt. % of Fe in agreement with the metal oxide ( $\text{Fe}_2\text{O}_3$  and  $\text{Al}_2\text{O}_3$ ) content found by TGA.

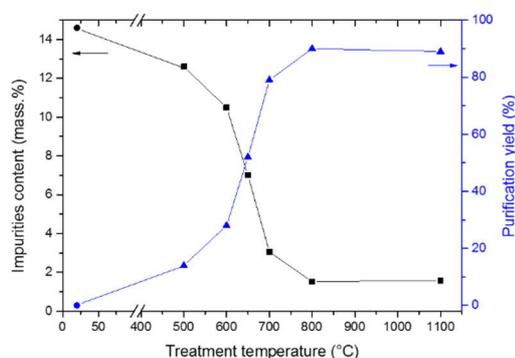
The raw MWCNTs (r-MWCNT) were treated with the home-developed chlorine-based purification method in order to remove the metallic impurities. Optimization of the purification was achieved by especially varying the temperature of the chlorine treatment. Good control of the impurity content in the CNT samples could be obtained as shown in Figure 1.

The purification yield  $Y$  is defined as:

$$Y = \frac{m_f - m_i}{m_i} \times 100$$

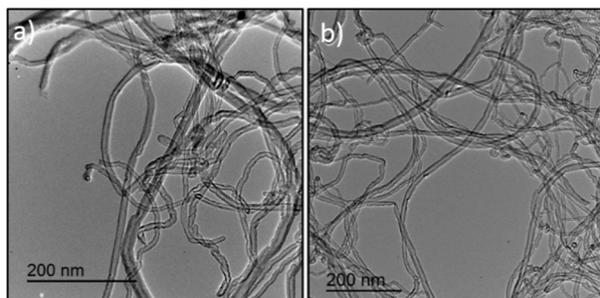
where  $m_f$  and  $m_i$  are the metallic content (wt.%) of the purified sample (p-MWCNT) and the raw sample (r-MWCNT) determined by TGA, respectively.

At 800 $^\circ\text{C}$  and above, a metallic impurity content as low as 1.52 wt. % was reached. The purification yield was then about 90 %. Since the MWCNT sample contained a negligible part of carbonaceous impurities (from TEM observations), the raw sample contained 85.40 wt.% of MWCNTs and the purified sample contained 98.48 wt.% of MWCNTs, the rest of the sample being the mineral impurities.



**Figure 1.** Impurity content measured by TGA in the samples before and after purification and the corresponding purification yield.

In the samples purified at temperatures of 800°C and higher, the remaining metallic impurities were certainly well-protected by a carbon covering (Figure 2a) and inaccessible to chlorine, which prevented their removal. Such impurities, barely observable by TEM, might be inaccessible and would therefore not react with the cells *in vitro*. In the purified samples (Figure 2b), the MWCNTs did not appear to have been damaged qualitatively; a careful TEM observation of their walls did not reveal any significant increase in the amount of defects.



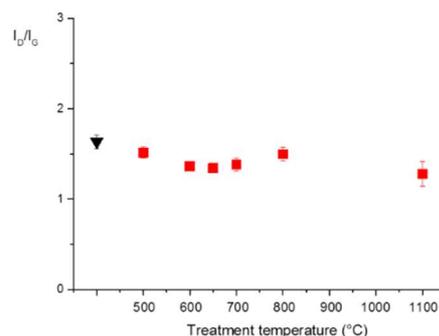
**Figure 2.** Typical TEM bright field micrographs of (a) the raw MWCNT sample and (b) the MWCNT sample purified at 800°C.

These observations are consistent with the results from Raman spectroscopy (Figure 3). No increase in  $I_D/I_G$  ratios was observed after purification over the whole temperature range investigated.

The atomic Cl/C ratio determined by XPS were below 0.1 and around 1.3 % respectively for the MWCNTs purified at 800°C and 1100°C. The chlorine content is therefore quite low for both samples.

Standard adsorption volumetry, carried out by using nitrogen as the adsorbent, was used to determine specific surface areas. The well-known BET (Brunauer–Emmett–Teller) method yielded values of 150-160 m<sup>2</sup>/g for both raw<sup>38</sup> and purified MWCNT samples. Further investigations, by using Kr as the adsorbent on p-MWCNT, revealed the presence of uniform surfaces meaning that, after purification, the MWCNTs had a good structural quality. The steps in the Kr isotherms (see supporting information, Figure S5) may be assigned to clean and regular surfaces as adsorption sites have similar adsorption energies for each adsorbed gas layer. When the shape of an isotherm shows one or more steps, the sample surface onto which adsorption occurs has uniform parts<sup>45</sup>. This result is in

agreement with the Raman spectroscopy demonstrating that no defects were introduced through application of the purification treatment.



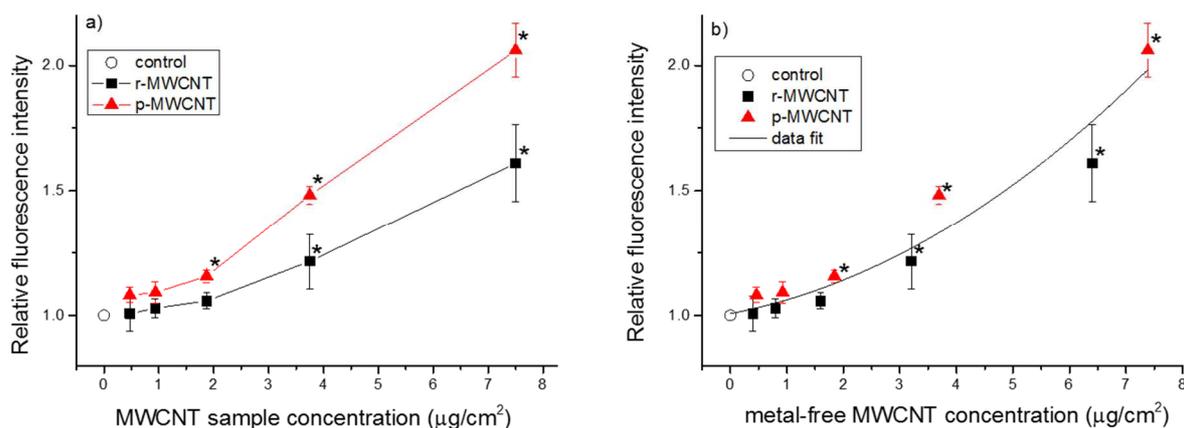
**Figure 3.**  $I_D/I_G$  ratios from Raman spectroscopy for the raw (black triangle) and purified MWCNT samples (red square).

For the following part of the work, we study the toxicity effects using the MWCNT sample purified at 800°C. This sample was selected as it did not present any morphological, chemical or structural modifications after purification.

### Toxicity

The concentrations used in the prepared dispersions are within the same range as those previously used in our laboratory for toxicity studies on asbestos fibres<sup>40</sup>. Here, we have investigated the toxic response of the raw and the purified MWNTs by considering the real carbon nanotubes content.

Intracellular ROS detection was performed after exposure to raw and purified MWCNTs. Figure 4 shows the recorded relative fluorescence intensity plotted as a function of the sample concentration (Fig. 4a). In Figure 4b, the MWCNT contents of raw and purified samples were recalculated by subtracting their respective metallic content (as determined by TGA). Both samples were able to induce a ROS production which, as expected, became more significant when sample concentration increased. Results from Figure 4a suggest that p-MWCNT was more reactive than r-MWCNT since the two curves differ significantly ( $p=0.045$ ). When the ROS production is plotted as a function of the concentration of only the MWCNTs, i.e. without any impurities (Fig. 4b), both MWCNT samples show a similar effect and the curves do not differ significantly. The fit in Figure 4b was derived from the data recorded for the two samples and the reliability factor of 0.86 is satisfactory.



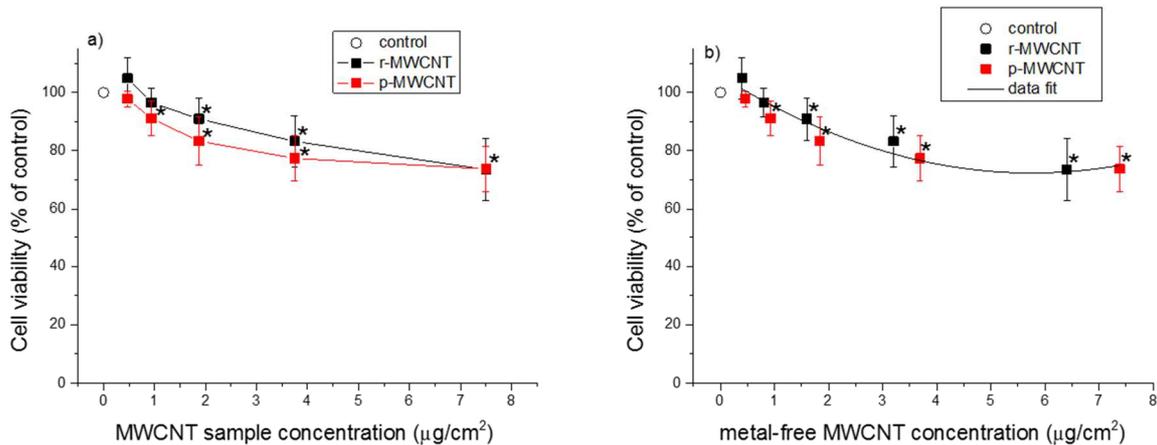
**Figure 4.** ROS production after 24 h of treatment of V79 cells with r-MWCNT (black square) and p-MWCNT (red triangle). a): ROS production as a function of the MWCNT sample concentration (including the metal-based impurities) and b): as a function of the MWCNT concentration alone (excluding metal-based impurities). \*statistically significant compared to control ( $p < 5\%$ ).

Cell viability as the percentage of the control value after 24 h of treatment with r-MWCNT and p-MWCNT is shown as a function of the CNT concentration in Figure 5. The observed decreases in cell viability are dose-dependent and the maximum decreases are 26.4 % and 26.2 % for r-MWCNT and p-MWCNT respectively at a CNT sample concentration of 7.5  $\mu\text{g}/\text{cm}^2$  (Figure 5a). As shown in Figure 5b, variation in cell viability as a function of the metal-free MWCNT concentration can be satisfactorily described using the same behaviour (data fit reliability 0.86).

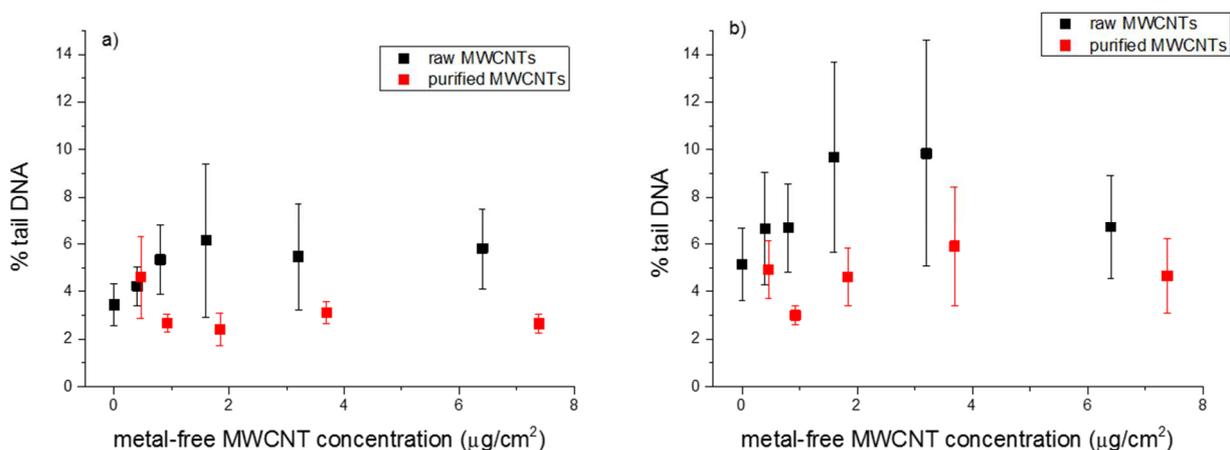
Genotoxicity is an important aspect of cellular toxicity since it can lead mutagenesis or apoptosis of the cells, potentially jeopardizing their expansion and resulting in cancer. In this work both comet and micronucleus (MN) assays, two reliable methods described in ref.<sup>45</sup> have been performed to study the genotoxicity effects of MWCNTs before and after purification. The results of the comet assay are expressed as the percentage of tail DNA for cells exposed to the two samples (r-MWCNT and p-MWCNT) with and without the addition of the enzyme (FPG)

(Figures 6a and 6b, respectively). Taking into account the measurement error, the comet assay yielded roughly the same results with both MWCNT samples. No significant level of DNA damage was induced by the (raw or purified) MWCNTs themselves with or without FPG. As expected, treatment with FPG led to an increase in the response but without any significant difference from the control, which also presented a higher tail DNA value. As FPG is a bacterial repair enzyme that recognizes and cuts certain modified bases, some base changes not induced by the test compound will also be detected and will generate nonspecific breaks that can be visualized by an increase of the level of breakage in the negative control.

Induction of MN in the V79 cells was assessed after 24 h of treatment with raw and purified MWCNTs (Figure 7). For both samples, a significant increase in the number of micronucleated cells is detected, meaning that the MWCNTs show similar genotoxic effects without any influence of the purification treatment.



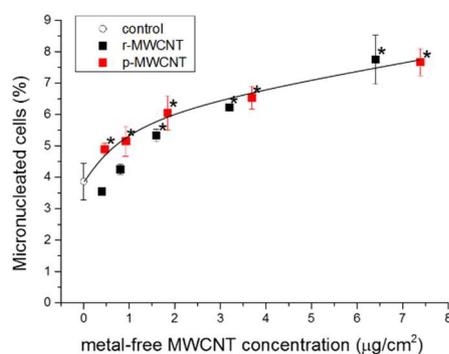
**Figure 5.** Percentage of V79 cells after 24 h of treatment with r-MWCNT and p-MWCNT. a) without and b) with normalization of the sample concentration to the MWCNTs. \*statistically significant decrease in cell number compared to the control ( $p < 5\%$ ).



**Figure 6.** Comet assay after 24 h of treatment in V79 cells. Percentage of DNA in tail as a function of metal-free r-MWCNT or p-MWCNT concentration without (a) and with FPG (b).

## Discussion

Contrary to other commonly studied particles (asbestos fibres and metallic oxide particles), the non-carbon impurities present in the CNT samples lead to biased results particularly for the comparison between non-purified and purified CNT samples toxicity. Especially, since some CNT samples can contain several tens of weight percent of non-carbon impurities, studying toxicity effects induced by CNTs implies to carefully separate as much as possible the CNT response from that of their impurities. Nevertheless, this aspect is seldom considered even if it directly impacts the dose-effect recorded and its comparison between CNT samples especially for purification investigations.



**Figure 7.** Micronucleated V79 cells after 24 h of treatment ( $\sim 1.7$  cell-division cycles for V79 cells) as a function of metal-free raw and purified MWCNT concentration. \*statistically significant decrease in cell number compared to control ( $p < 5\%$ ).

### Purification treatment: a gentle non-destructive method

Thanks to the purification method that we applied, metal-based impurity particles could be removed from the MWCNT sample while preserving the physical and chemical characteristics of the starting MWCNTs. Raman spectroscopy performed on the samples clearly showed that the D/G ratio did not increase after purification, which means that neither significant CNT damage nor functionalization has occurred by the chlorine-based treatment in agreement with HRTEM images (see supporting information Figure S6). However, as MWCNTs are not highly functionalized Raman spectroscopy might not be sensitive enough to detect any modification in the structure of these samples. Complementary techniques that are more sensitive to MWCNT surface modifications, such as XPS and adsorption volumetry (to date rarely investigated), are required to deeply investigate the state of the MWCNTs, especially after any chemical treatment. Results from XPS showed that p-MWCNT contains a very low Cl content (around 0.1 atomic %).

### Metal-based impurities

The raw MWCNT sample was easily purified at a temperature that is quite low compared to temperatures required for the same purification method for other CNT samples whose metal impurities are known to be quite well protected by carbonaceous shells. For arc discharge as-produced single walled carbon nanotubes (SWCNTs), chlorine-based purification conducted at 950°C for 2 h leads to a purification yield of metallic impurities of 65.2 %<sup>39</sup>. Under the same conditions, SWCNTs produced by the chemical vapor deposition (CVD) HiPco process, where the carbon layer covering the metallic particles is known to be thinner, the purification yield reached 87.5 %<sup>37</sup>. The purification yield for double walled carbon nanotubes (DWCNTs) whose impurities are embedded in large carbonaceous particles, was as low as 67 % under the same experimental conditions<sup>46</sup>. The accessibility of the metallic particles is the main limiting factor to remove them by any chemical treatment. In this work, according to the purification conducted on the MWCNTs, any metallic impurity particles that were not or were only poorly protected are probably removed from the sample. The negligible amounts of catalyst residues are probably highly protected by carbon layers, and, thus any metallic remaining particles would certainly not interact with cellular material.

### Toxic effects

As CNTs are particles with at least one dimension of less than 100 nm<sup>47</sup>, they are classified as High Aspect Ratio Nanomaterials (HARN). Since CNTs can be considered like fibers, their potential adverse effect is an important question which has drawn attention of researchers. Particle and fibre toxicity is often related to the capacity of these materials to induce oxidative stress in cells<sup>48</sup>. The presence of CNTs, near or inside cells, may lead to ROS production and to an overloading of the cell antioxidant defense system. This phenomenon can induce toxic effects such as alteration of DNA (genotoxic effects) which can ultimately lead to tumor development.

To take into account the question of the toxicity and carcinogenic potential of CNTs, we analyzed the ability of

MWCNTs to induce cell cytotoxicity, oxidative stress and genotoxic effects. Added to residual metal catalyst, the other reported parameters which can induce toxic effects are length, rigidity and surface functionalization. Moreover, the observed results also depend on the cell system used. As already discussed, the advantage of the used purification method is that it does not induce any morphological (length) and functionalization of the MWCNTs. Regarding rigidity, this parameter is rarely discussed for *in vitro* tests. In the works from Palomäki *et al.*<sup>49,50</sup> macrophages were treated with both types ("long rigid" and "long tangled") MWCNTs. Higher secretion of interleukin 1-family cytokines was observed for needle-like MWCNTs. Catalan *et al.* have shown that straight CNTs can be more toxic than flexible CNTs above 50 µg/cm<sup>2</sup>.<sup>51</sup> However, the toxicity induced by flexible CNTs including MWCNTs is as well non negligible. To give an overview of the reported *in vitro* CNT cytotoxicity, Table 1 gathers some examples of toxicity (cell viability) of many kinds of CNTs. From Table 1, CNT toxicity strongly depends on cellular system used *in vitro*; for a same CNT sample, *in vitro* toxicity can be different (see for example NM-403, refs.<sup>52,53</sup>). Among the studied MWCNT samples, our MWCNTs show a significant toxicity.

**Reactive oxygen species and cell viability.** It has been reported that both raw and purified CNTs are able to induce ROS production in V79 cells. The measured stress intensity has been shown to depend on the cell type, with raw MWCNTs inducing an ROS level in V79 cells<sup>54</sup> (also used in this study) similar to that observed here. In the present study, the CNT purification treatment had no effect on cell ROS production (Fig. 4b). This result could be an indication that neither the structure nor the surface of CNTs were modified by the purification method, a conclusion we also drew from the multi-technique characterization we performed. The value of the observed ROS level is difficult to compare to other works since results previously reported are mostly expressed in terms of the mass of the whole nanomaterial sample (i.e including both impurities and CNTs). However, Kagan *et al.* and Zhou *et al.* did not notice any difference between raw- and purified SWCNT whereas Pulskamp *et al.* observed ROS production for raw SWCNTs and the absence of ROS in purified SWCNTs<sup>32,55,56</sup>. These conflicting results that the method used for the CNT purification is determinant since it could in some cases induce CNT surface modifications such as surface functionalization which is known to reduce CNT reactivity<sup>57</sup>. We have shown that both raw and purified CNTs induce a decrease of cell viability with a similar trend and magnitude (Fig. 5). Our results clearly indicate that cytotoxicity from CNTs is not modified by the purification treatment, and in agreement with other works, that contaminants do not seem to be the main factor explaining CNT toxicity<sup>13,58,59</sup>. In a previous study, similar toxicity study was conducted on a MWCNT sample synthesized by the same method (by Dr. D. Bégin's group). The two MWCNT samples are flexible, they have similar length and diameter, the catalyst nature is also the same, only the catalyst respective content is slightly different. Fe content is of 2.6 and 4.2 wt.% and Al content is of 5 and 7.2 wt.% for the MWCNTs used in this work and the sample used in the work by Darne *et al.*<sup>54</sup> In that study, MWCNT sample was purified by a standard purification method consisting in a 2-step treatment<sup>60</sup>. The as-produced raw MWCNT powder was first treated with KOH (1 M) at 80°C in order to remove the alumina support. The resulting material

was washed several times with distilled water and then treated with a solution of HNO<sub>3</sub> (4 M) at 110°C under reflux during 2 h in order to remove the remaining iron particles. The cell viability response had revealed that these purified MWCNTs had a greater toxicity than the raw MWCNTs while in the present study, the response is roughly the same for CNTs before and after the applied purification treatment<sup>54</sup>. This result could be

related to the preservation of the CNT walls<sup>61</sup> through the applied chlorine-based purification method contrary to the standard ones which usually induce chemical and/or morphological modifications through the harsh conditions used for the purification treatment.

Table 1. Comparison of cell viability for different types of CNTs and for the MWCNT sample used in this work. The used cell system and the treatment conditions are also recalled.

Ref.	CNT type	Catalyst nature and content (% wt.)	Cell type	Viab./Cytotox. assay	Treat. time (h)	Concentration range	Concentration for which cell toxicity > 25 %
this work	r-MWCNT	5 Al, 2.6 Fe	V79	WST-1	24	0.47 – 7.5 µg/cm <sup>2</sup> (0.8 - 12.75 µg/mL)	7.5 µg/cm <sup>2</sup> (12.75 µg/mL)
	p-MWCNT	1.52 Fe			24		
62	MWCNT	Fe	Hek	Neutral Red	24	0.1 - 0.4 µg/mL	0.4 µg/mL
63	SWCNT	90 C	Alveolar macrophages (Guinea Pig)	MTT	6	1.41 – 28.25 µg/cm <sup>2</sup>	1.41 µg/cm <sup>2</sup>
64	MWCNT	0.6 Ni		Trypan blue exclusion	24	1.41 – 22.6 µg/cm <sup>2</sup> 40 and 400 µg/mL	none
56	SWCNT	2.8 Co, 4.2 Mo	NR8383	WST-1	24	3.1 – 62.5 µg/cm <sup>2</sup> (5 - 100 µg/mL)	62.5 µg/cm <sup>2</sup> (100 µg/mL)
	SWCNT	1.3 Co, 1.2 Ni					none
	MWCNT	99.8 C					none
	MWCNT	1.86 Fe, 0.55 Mo					none
65	SWCNT	HiPco, 10 Fe	A549	Alamar Blue MTT Commasie Blue	24 24 24	3.125 – 800 µg/mL	400 µg/mL 800 µg/mL 800 µg/mL
66	MWCNT	98 C	RLE Rat lung epith. cells	MTT	24	10 – 150 µg/mL	50 µg/mL
67	MWCNT	0.18 Fe	A549 BEAS-2B	WST-1	72	25-200 µg/mL	200 µg/mL 200 µg/mL
68	MWCNT	0.19 Fe, 0.14 S, 0.07 Co, 0.03 Al	RAW 264.7 A549	MTT	24	2.5 – 100 µg/mL	25 µg/mL 25 µg/mL
69	MWCNT-1 MWCNT-2	99 C 99 C	C6 rat glioma cells	MTT	24	25 – 400 µg/mL	400 µg/mL none
31	SWCNT SWCNT	1.1 Cr, 0.7 Fe, 0.3 Mo 9.3 Fe, 4.8 Co, 1.2 Mo	A549	Cell counting kit-8 (Formazan)	24	10 – 100 µg/mL	90 µg/mL 20 µg/mL
13	MWCNT	Narrow short (NS) 1.19 Ni, 0.85 Fe	THP-1	Cell titer assay (MTS, Formazan)	24	5 - 50 µg/mL (0.32 – 15.62 µg/cm <sup>2</sup> )	none
		NS purified (NSp)					none
		Large short (WS) 0.51 Fe, 0.47 Ni					50 µg/mL (15.62 µg/cm <sup>2</sup> )
		WS purified (WSp) 0.4 Ni					none
		Narrow long (NL) 0.82 Ni NL purified (NLp) 0.46 Ni					none
NS, NSp, WS, WSp, NL, NLp	Mice alveolar macrophages	none					
70	SWCNT	>60 C, 0.4 Co, 0.15 Mo	A549	N.A.	24	1 - 50 µg/mL	50 µg/mL

Table 1 (continued). Cell viability after treatment with CNTs of different types in comparison with this work. The used cell system and the treatment conditions are also recalled.

Ref.	CNT type	Catalyst nature and content (% wt.)	Cell type	Viab./Cytotox. assay	Treat. time (h)	Concentration range	Concentration for which cell toxicity > 25 %
71	MWCNT	purified	BEAS-2B	WST-1	24	24 µg/cm <sup>2</sup>	none
72	SWCNT	>90 C	V79	Cell titer assay (MTS, Formazan)	24	50 -200 µg/mL	none
	MWCNT-8	>95 C – D:8 nm					200 µg/mL
	MWCNT-20	>95 C – D:20 nm					none
	MWCNT-50	>95 C – D:50 nm					none
	SWCNT	>90 C					none
MWCNT-8	>95 C – D:8 nm	A549	none				

	MWCNT-20	>95 C – D:20 nm					none
	MWCNT-50	>95 C – D:50 nm					none
52	MWCNT	NRCWE-026 (14.9 Al) NM-401 NM-402 (2.4 Al <sub>2</sub> O <sub>3</sub> , 1.3 Fe <sub>2</sub> O <sub>3</sub> ) NM-403 NRCWE-006 NRCWE-040 (0.56 NiO) NRCWE-043 (1.2NiO) NRCWE-046	FE1 Muta mouse lung epithelial cell	Cell count	24	7.1 – 113.2 µg/cm <sup>2</sup> (12.5 – 200 µg/mL)	none 28.3 µg/cm <sup>2</sup> (25 µg/mL) 113.2 µg/cm <sup>2</sup> (200 µg/mL) 113.2 µg/cm <sup>2</sup> (200 µg/mL) 7.1 µg/cm <sup>2</sup> (12.5 µg/mL) 28.3 µg/cm <sup>2</sup> (50 µg/mL) none 113.2 µg/cm <sup>2</sup> (200 µg/mL)
51	MWCNT	Mitsui-7 (NRCWE-006)- straight NRCWE-007-tangled (0.5 Ni, 0.08 Fe)	BEAS-2B	Trypan blue	24	5 – 350 µg/cm <sup>2</sup>	10 µg/cm <sup>2</sup> (38 µg/mL) 10 µg/cm <sup>2</sup> (38 µg/mL)
73	MWCNT	99.8 C	RAW 264	Alamar blue	24	100 µg/mL	none
74	SWCNT	Raw (<30 Fe) Purified (<15 Fe) Highly purified (<2 Fe)	MeT-5A	Trypan blue	24	5 µg/mL	5 µg/mL none none
75	MWCNT	MWCNT-A + Pluronic (surfact.) MWCNT-B (Mitsui-7) + Pluronic (surfact.)	Undiff. HL-60 Diff. HL-60 Undiff. HL-60 Diff. HL-60	WST-1	24	25 – 100 µg/mL	none 100 µg/mL none 25 µg/mL
53	MWCNT	NM-403 NRCWE-055	NR8383	WST-1	24	2.5 – 80 cm <sup>2</sup> /cm <sup>2</sup> * 9 – 300 cm <sup>2</sup> /cm <sup>2</sup> *	2.5 cm <sup>2</sup> /cm <sup>2</sup> (1.85 µg/cm <sup>2</sup> **) 9 cm <sup>2</sup> /cm <sup>2</sup> (2.06 µg/cm <sup>2</sup> **)

\*cm<sup>2</sup> of NP specific surface/cm<sup>2</sup> of culture; \*\* converted concentration in µg/cm<sup>2</sup>

**Carbon nanotube genotoxicity.** Finally, we have evaluated the genotoxic effect by the CNTs through two complementary *in vitro* tests: the comet assay and the micronucleus assay. The comet assay has been validated as a sensitive method for quantifying DNA strand breaks and evaluating the genotoxic potential of xenobiotics<sup>76</sup>. The comet assay is a sensitive test for detecting ability of chemicals to generate single-strand and/or double-strand breaks of DNA<sup>77</sup>; here, it modified by using an enzymatic treatment in order to selectively detect DNA damaged by base modification<sup>78,79</sup>. The *in vitro* micronucleus assay assesses, in interphasic cells, appearance of micronuclei formed by chromosome breakage events (clastogenic effects) or chromosome losses (aneugenic effects) during nuclear division<sup>80</sup>. In agreement with a previous study<sup>54</sup>, for V79 cells, we found that neither raw nor purified MWCNTs induced significant DNA breakage or FPG-sensitive base modification in V79 cells in the comet assay we performed. After 24h of exposure, Cavallo et al. observed similar results in the A549 cells<sup>81</sup>. Moreover, in the micronucleus assay we performed, CNTs were able to induce some chromosomal disorders. No difference was observed between raw and purified material confirming that the toxicity and the genotoxicity of the CNTs are probably due to the CNT themselves. Sargent *et al.* studied and also reported this effect in their works and they claimed that “The similarity of carbon nanotubes to microtubules suggests a potential to interact with cellular biomolecules, such as the mitotic spindle”<sup>82</sup>. Such interactions can induce mitotic spindle modifications that lead to micronucleus formation as observed in our work.

## Conclusions

In this work, we were able to study the cytotoxic and genotoxic effects of a MWCNT sample by discriminating between the effects of metallic impurities and those of the MWCNTs. Our findings are important since this type of investigation is typically difficult to conduct as physical and chemical characteristics of CNTs are usually modified by the standard purification treatments. However, we have shown that when using our gentle gas phase purification method, the structure and morphology of MWCNTs are not modified. We have shown that Fe and/or Al do not play any role in the *in vitro* cytotoxicity or genotoxicity of the used MWCNT sample. This approach could be applied to other kinds of CNTs to investigate their own toxic response. However, this study shows that CNTs can induce significant toxic responses, which reinforces the precautionary principle in CNT handling.

## Conflicts of interest

There are no conflicts to declare.

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