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Investigation of the cytotoxicity of CCVD carbon nanotubes towards human umbilical vein endothelial cells

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Abstract

The cytotoxicity of different samples of carbon nanotubes synthesised by catalytic chemical vapour deposition was investigated towards human umbilical vein endothelial cells, using two cytotoxicity standard assays (neutral red assay for the cell viability and MTT assay—tetrazolinium salt—for the cell metabolic activity). No cytotoxicity was found for any sample.

Keywords: Carbon nanotubes; Chemical vapor deposition; Biocompatibility

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1. Introduction

In the heart of the dispute about the possible negative effects of nanotechnologies and the potential toxicity of nanoscale objects, the question of the cytotoxicity of carbon nanotubes (CNTs) is very relevant. Before CNTs can be used in medical applications such as drug-delivery agents for example, their innocuity towards the human body has to be assessed. Compared to the abundant literature dealing with the synthesis, characterisation and potential applications of CNTs, it is striking to note the lack of data on their possible bio-toxicity. CNTs are unique tubular 1D-objetcs which can be described as a rolled hexagonal carbon network, ended by half-fullerene caps. They can be single-walled (SWNTs) or multi-walled (MWNTs) in the case of concentric tubes arranged in a Russian dolls-like structure. It is important to keep in mind that CNTs can be produced by different methods, the main three ones being the electric arc-discharge [1], Laser ablation [2] and catalytic chemical vapour deposition (CCVD) [3], [4] and [5]. Each method gives CNTs of different kind and all require the use of metals as catalysts. As a general trend, arc-produced CNTs tend to be about 1 μm long and to gather into large bundles up to more than 100 CNTs. These CNTs are generally very straight and, said to be free of structural defects. They are produced together with carbon soot containing amongst others fullerenes and graphitic particles. Laser ablation produces purer samples (containing fewer by-products) and the CNTs form very long and large bundles, up to hundreds of μm long. CCVD can be used to produce a wide variety of different CNTs ranging from SWNTs to MWNTs (possibly containing structural defects). When the number of walls is low (less than 5), CCVD CNTs are generally forming thin bundles, with a length generally ranging between a few micrometers to hundreds of micrometers. The diameter distribution is ranging between less than 1 nm and 4–5 nm, as opposed to the arc-discharge and laser ablation methods where the diameter distribution is narrower and mainly centred around 1.3 nm. Each synthesis method produces a different quality of CNTs, the term of quality involving the chemical purity (residual catalyst—mainly transition metals—and presence or not of other forms of carbon species such as amorphous carbon and graphitic-like particles) and the structural quality of the CNTs. The control of the number of walls of the CNTs and the diameter distribution can also be seen as a sort of purity (in terms of selectivity).

The first study (published in 2001) by Huczko et al. [6] was focusing on the comparison between arc-discharge produced CNTs and asbestos fibres. CNTs were introduced in guinea pigs by intratracheal instillation (suspension in sterile saline media with addition of surfactant). The results of the bronchoalveolar lavage examinations 4 weeks after the instillation revealed no change in the pulmonary function and did not induce any measurable inflammation in bronchoalveolar space, concluding to the innocuity of the tested samples.

Shvedova et al. [7] have investigated the assessment of raw-CNTs cytotoxicity using human keratinocyte cells, showing that the dermal exposure to raw CNTs may lead to dermal toxicity due to accelerated oxidative stress in the skin, mainly because of important amounts of iron in the studied samples (HiPCO CNTs, CCVD process). Another study by the same group [8] has compared the aerosols formed during the handling of different CNTs samples (HiPCO, laser ablation) and has shown that the airborne concentrations were generally lower than 53 $\mu g\ m^{-3}$. It is only recently in 2004 that two reports on the toxicological effects of CNTs have been issued, both based on a comparative toxicological approach and intratracheal instillation route of exposure to examine the pulmonary toxicity of SWNTs. Lam et al. [9] focused on the histopathological alterations in mice 7 and 90 days after the exposure to CNTs, produced by two

different CCVD methods and containing various amounts of residual catalytic metals (Fe, Co, Ni, Mo, etc.). They reported that CNTs could be more dangerous than quartz particles and that all the CNTs tested induced lung lesions characterised by the presence of granulomae. They also suggested that the metal contaminants could not be the only cause. Warheit et al. [10] studied the ability of CNTs to induce pulmonary inflammation as well as to alter lung cellular proliferation. They confirmed the formation of granulomae after instillation of SWNTs (laser-ablation produced soot containing about 60% SWNTs) and revealed that the unique mechanical and chemical properties of CNTs make them more persistent in biological systems. Pantarotto et al. [11] reported the translocation of water soluble SWNT derivatives across cell membranes and have shown that cell death can be induced by functionalised CNTs (bioactive peptides), depending on their concentration in the media. Very recent results of in vitro tests performed on human embryo kidney cells [12] suggest that SWNTs can induce cell apoptosis but also that these cells are able to isolate CNTs-attached cells from the unaffected ones by secretion of "isolation" proteins. it has also been shown that CNTs can generate an inflammatory response of the human immune system, both via the classical and alternative pathways [13].

We report here the study of the cytotoxicity of different samples of carbon nanotubes synthesised by CCVD. We took advantages of two existing standard assays, Neutral Red assay for the cell viability and MTT assay (tetrazolinium salt) for the cell metabolic activity. These assays are optimized for indirect cytotoxicity and we adapted them to test the effect of the CNTs on cells. Human umbilical vein endothelial cells (HUVEC) are especially good candidates for these tests, because CNTs are often thought to be used for drug-delivery [14] and would be injected in the human body to be carried to targeted places by the blood.

2. Experimental

2.1. Synthesis of CNTs

The CNTs were prepared by catalytic chemical vapour deposition using bimetallic MgO-based catalysts [5] and [15]. $Mg_{1-x}(Co_yM_{1-y})_xO$ catalysts were prepared by combustion synthesis [15]. The different compositions were as follows: $x = 0.01$ with $y = 0.75$ and M = Mo (sample A) or W (sample B); $x = 0.05$ with $y = 0.9$ and M = Mo (sample C). Magnesium and cobalt nitrates were dissolved in deionised water and the required amount of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ or $Na_2WO_4 \cdot 2H_2O$ was added to the solution. Citric acid was used as the fuel, using the stoichiometric proportion [16]. For each experiment, 1.5 g of the starting oxide was reduced in a H_2–CH_4 mixture (18 mol.% CH_4, heating and cooling rates 5 °C/min, maximum temperature 1000 °C, no dwell). This resulted in a mat of composite powder (which density depended on the composition of the catalyst), which was then treated with a concentrated aqueous HCl solution (12 M) to separate the CNTs by dissolving all the remaining oxide material, as well as unprotected metal particles [5] and [15]. The suspensions were filtered and washed on cellulose nitrate membranes (Whatman, 0.45 µm) with deionised water until neutrality. The samples were dried overnight in air in an oven at 80 °C, yielding different amounts of CNTs according to the composition of the corresponding catalyst. The samples were characterised by electron microscopy (scanning electron microscopy and transmission electron microscopy using a JEOL

2010 operated at 120 kV); the specific surface area (noted S_e, $m^2 g^{-1}$) was measured by the BET method using N_2 adsorption at liquid N_2 temperature (Micromeritics FlowSorb II 2300) and the carbon content (noted C_e, wt. %) was obtained by elemental analysis (flash combustion).

2.2. Evaluation of the CNTs' cytotoxicity

Primary cultures of human umbilical vein endothelial cells (HUVEC) were prepared and cultured as previously described [17]. HUVEC were submitted to the samples by the way of fragments of sterile (autoclaved: 121 °C–20 min.) material. HUVEC were seeded at a density of 6000 cells per cm^2 in 96-well microtiter plates (Nunc, Denmark) and the culture was maintained at 37 °C after cell plating until confluency was reached. At confluency the medium was replaced by the material samples. To obtain such samples, fragments of sterile material were immersed in culture medium (IMDM) in order to obtain 5 cm^2 of the sample surface (estimated from the measure of the specific surface area of each CNTs sample) to 1 mL of IMDM (Iscove Modified Dulbecco's Medium) according to the standards of indirect cytotoxicity [18]. Samples were treated in borosilicate glass tubes at 37 °C for 120 h and 5% CO_2 without stirring according to the standard procedures. Borosilicate tubes containing IMDM with either no material or a solution of phenol at a concentration of 64 g L^{-1} (known to be cytotoxic) were processed under the same conditions to provide negative and positive controls, respectively. After this step, all samples were centrifuged at 1200 rpm (180*g*) and the supernatant was recovered, according to the standard procedures. In the case of CNTs, centrifugation at such a low acceleration for very dilute suspensions is not able to withdraw the CNTs from the suspensions. Suspensions were supplemented to 10% FCS (Fetal Calf serum) and final dilutions were achieved using IMDM containing 10% FCS to obtain the 50% (v/v), 10% (v/v) and 1% (v/v) samples. When HUVEC confluency was reached, the culture medium was removed and replaced by the samples (100µL) at various dilutions [100% (v/v), 50% (v/v), 10% (v/v), 1% (v/v)] in the culture medium (0.33 cm^2 wells containing approximately 30,000 endothelial cells at the time of the assay) and supplemented with 10% FCS for 24 h at 37 °C and 5% CO_2. At the end of the incubation period, the supernatants (material or control samples) were discarded, and two different assays [19], [20], [21] and [22] were performed: cell viability (Neutral Red assay) and cell metabolic activity (MTT assay). The intensity of the colours obtained (red and blue respectively) is directly proportional to the viability and metabolic activity of cell populations and inversely proportional to the toxicity of the material. For the statistical analyses, values are expressed as mean ± SD. The statistical significance was tested by an unpaired Student t test and $p < 0.05$ was considered significant.

3. Results and discussion

Using the same CCVD conditions, three different kinds of CNTs were prepared from three different catalysts because we wanted to investigate both the possible influence of the nature of the catalytic metal (Co combined to either Mo (samples A and C) or W (sample B)) and of the amount of catalytic metal (catalyst used to synthesize sample C contains a higher content of catalytic metal than catalyst used to prepare sample A). Fig. 1 shows typical electron microscope images (SEM, TEM) of the CNTs samples. SEM images were obtained on the raw CCVD samples, i.e. before the elimination of the oxide catalyst which can be seen in the background of SEM images: 1(a, c, d). These images reveal a high density of CNTs in all three samples. TEM images (Fig. 1b and inset of Fig. 1d) show that the filaments observed by SEM are in fact

individual or small-diameter bundles of CNTs. Careful analysis of high-magnification TEM images allowed the measure of the number of walls and diameter of the CNTs (Fig. 2). Sample A (Fig. 2a, 96 individual CNTs imaged) contains CNTs having between 1 and 3 walls, with ca. 80% double-walled CNTs (DWNTs) and outer diameters ranging from 1.1 to 3.2 nm (Fig. 2d). Sample B (Fig. 2b, 164 individual CNTs imaged) contains CNTs having between 1 and 4 walls, with 37% DWNTs, 48% triple-walled CNTs (TWNTs) and 12% CNTs with four walls. The outer diameters are ranging from 1.1 to 4.3 nm (Fig. 2e). Sample C (Fig. 2c, 183 individual CNTs imaged) contains CNTs having between 1 and 6 walls, with 9% single-walled CNTs (SWNTs), 72% DWNTs and 15% TWNTs. The outer diameters are ranging from 0.7 to 6.3 nm (Fig. 2f).

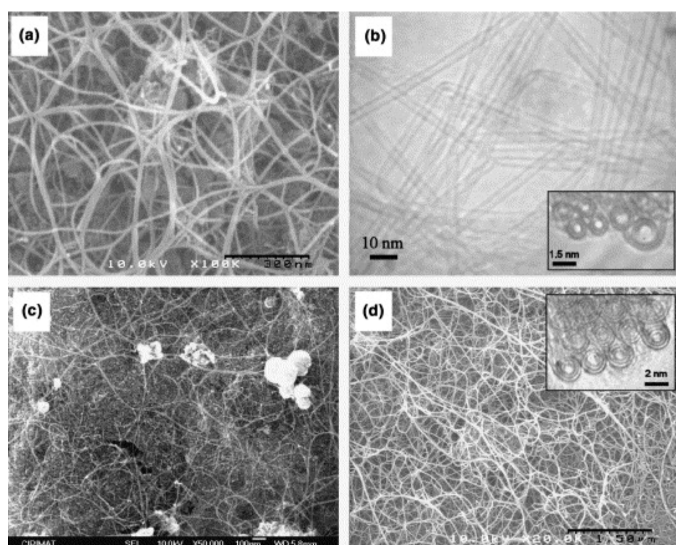

Fig. 1. Typical images of the CNTs samples. SEM images were obtained on the raw CCVD samples, before the elimination of the oxide catalyst (which can be seen in the background). Sample A observed by SEM (a) and TEM (b); note the small diameter of the bundles of CNTs. Typical SEM images of samples B (c) and C (d—the inset is a TEM image showing a cross-section of some of the few triple-walled CNTs found in this sample).

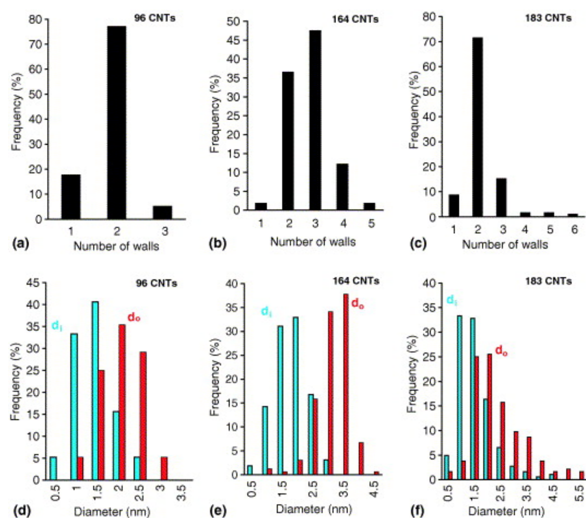

Fig. 2. Distribution of the numbers of walls (a–c) and the inner (d_i) and outer (d_o) diameter (d–f) for each sample (whole population of CNTs). Sample A (a) and (d), respectively, sample B (b) and (e) respectively and sample C (c) and (f), respectively.

The main characteristics of the CNTs samples are summarized in Table 1: they have a specific surface area ranging from 550 $m^2 g^{-1}$ (sample C) to more than 1100 $m^2 g^{-1}$ (sample A). The carbon content is higher than 90 wt.% (corresponding to more than 97.8 at.%) for samples A and B and close to 80 wt.% (equivalent to more than 95 at.%) for sample C. The diameter distribution widens from sample A (1.2–3.2 nm) to sample C (0.7–6.3 nm).

Table 1.
Characteristics of the CNTs samples

Reference	**Catalyst composition (nm)**	S_e **($m^2 g^{-1}$)**	C_e **(wt.%)**	**Outer ∅(nm)**	
				Range	**Mean value**
A	$Mg_{0.99}(Co_{0.75}Mo_{0.25})_{0.01}O$	1110	92.8	1.2 – 3.2	2.01
B	$Mg_{0.99}(Co_{0.75}W_{0.25})_{0.01}O$	790	90.4	1.0 – 4.3	3.11
C	$Mg_{0.95}(Co_{0.90}Mo_{0.10})_{0.01}O$	550	80.2	0.7 – 6.3	2.37

The catalyst composition is the elemental one, although these catalysts are not solid solutions [15].

Data obtained from the biocompatibility studies following incubation of cells with CNTs suspensions were expressed for Neutral Red and MTT assays as a percentage of the values obtained from cells incubated with negative control extracts. Phenol (64 g L^{-1}) was used as a toxic control. Fig. 3 compares the results (given in percent of the response to the assay compared to an untreated control blank sample) of the cell viability (Neutral Red assay, Fig. 3a) and cell metabolic activity (MTT assay, Fig. 3b). Any response lower than the dotted line at 75% of the response of the blank sample may be considered as cytotoxic. The results of the cells viability assay (Fig. 3a) indicated that none of the samples was found to be cytotoxic, although for samples A and B the error bars sometimes extend below the cytotoxicity threshold (75%). There may be a slight dilution effect for samples A and B, with the HUVEC viability slowly decreasing with the dilution, as confirmed by a significant statistical difference between diluted samples (results marked with a star in Fig. 3a) and the maximum concentration (100%). The cell metabolic activity assay (Fig. 3b) confirmed that none of the sample showed a cytotoxic effect but revealed no obvious dilution effect in this case. HUVEC were chosen in this study because of their possible future contact with CNTs when intravenous route is envisaged and because such cells are very sensitive to modifications of their environment. Indeed, they are known to respond quickly to various stimuli (biochemical, mechanical) and are thus good indicative cells [23] of a possible cytotoxic effect.

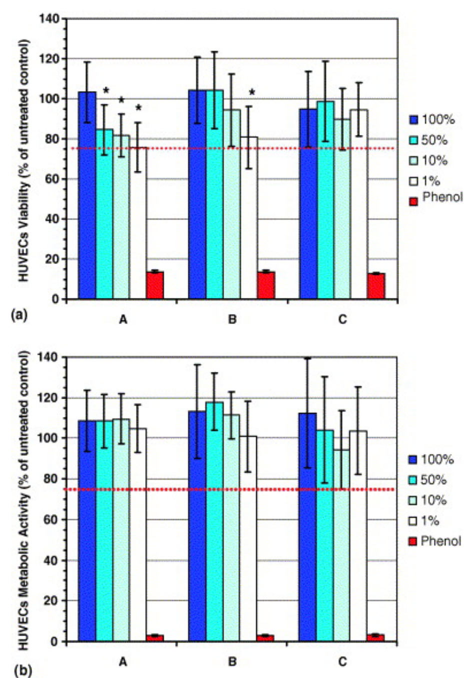

Fig. 3. HUVEC viability (a) and metabolic activity (b) assays for samples A, B and C. Results expressed in percentage of the answer of the untreated control sample and compared to phenol (cytotoxic control reference); a star (*) indicates that the value is statistically different from sample 100%.

The biocompatibility tests performed in this study required the use of very small amounts of CNTs (5 cm^2 of CNTs per mL for the non-dilute suspension). Because the specific surface area of each CNTs sample was different (and very high), the concentration in weight per volume was different for each of them. The concentrations for the more concentrated suspensions were thus close to 0.5 $\mu g\ mL^{-1}$ for sample A, 0.64 $\mu g\ mL^{-1}$ for sample B and 0.9 $\mu g\ mL^{-1}$ for sample C. Several successive dilutions were required to reach such low concentrations and systematic errors thus accumulate. Although the samples were prepared very carefully, the validity of these widely used standard tests may be questioned in the case of very high specific surface area nanomaterials such as CNTs.
The question of the chemical purity of the samples has already been raised: is the toxicity coming from the CNTs themselves or from residual catalysts still present in the purified samples? Lam et al. [9] suggested that the metal contaminants could not be the only cause of the toxicity, in agreement with the results of this study because the only sample showing no cytotoxicity at any concentration is the one which contains the more residual metal nanoparticles. These metal nanoparticles are in fact encapsulated by graphene-like layers and thus efficiently protected against their environment [24]; the metals should thus never be in contact with the biological material. If the toxicity does not come from carbon-encapsulated residual catalyst, it may rather come from the structural characteristics of the CNTs, such as their gathering into bundles of different diameter and their outer diameter distribution. In terms of bundling, one can imagine that the lower the concentration of the dispersion, the higher the dispersion of the CNTs and thus the smaller the diameter of the bundles. A lower concentration would favour a higher dispersion of the CNTs which may improve their interactions with the cells and thus possibly lead to some toxicity. The question of the outer diameter distribution of the CNTs is important as well. The response of the three samples to the tests does not show any clear correlation with the mean diameter of the CNTs populations. In fact, this is probably because the diameter of the CNTs has no relationship with the size of the HUVEC, which are adherent cells of cobblestone morphology in such culture conditions, with a mean size usually ranging from 30 to 50 µm on the culture support. Such small variations of the CNTs outer diameter distribution should thus have no consequence. About the effect of concentration, Pantarotto et al. [11] and Maynard et al. [8] also worked with different concentrations of CNTs and both claimed a lower toxicity when the concentration was decreased (in the case of Maynard, it is mainly the contamination with Fe which is responsible for the toxicity); it is also possible (although statistically relevant for samples A and B) that in the present study the potential dilution effect could be only fortuitous and may fall within the experimental error. The results of the MTT assay would agree with this second explanation. Experiments to confirm this observation are currently in progress.
The inflammatory response of these cells has not been investigated in this study but would be important to know. However, results obtained on sample A indicate that the DWNTs can activate the human complement via both the classical and alternative pathways. The C_{1q} protein, which is involved in the classical pathway, adsorbs readily onto CNTs [13]. We do not have any data about the possible endocytosis of CNTs by the cells but Pantarotto et al. [11] have reported that functionalised CNTs are able to cross the cell membrane and to accumulate in the cytoplasm or even reach the nucleus without being toxic for the cell at low enough concentration. The exact concentration (in weight per volume) of the CNTs suspensions was not given but it was reported that the toxicity appeared with increasing the concentration, which is different from the results presented here. However, there are two main differences between these studies because (i) Pantarotto et al. [11] used very short CNTs (0.3 to 1µm long), and (ii) these CNTs were functionalised by bioactive peptides. The length of the CNTs used in this work is more likely to

be ranging between 5 μm and up to 100 μm, which makes an important difference. A microscopy study of the HUVEC after the cytotoxicity test would give important information about the possible endocytosis of these non-functionalised CNTs.

4. Conclusion

In summary, the cytocompatibility study of three CCVD-synthesised CNTs samples was investigated by two standard assays (Neutral Red and MTT assays). It revealed no cytotoxic effect according to the standards of the method. However, we have observed that for the CNTs having the highest specific surface area, a cytotoxicity effect (decrease of HUVEC viability) seems to appear slowly with increasing the dilution of the suspension. This dilution effect may be related to the amount of CNTs present in the different suspensions (the higher the specific surface area, the lower the amount of CNTs) and/or to bundling effects. Although these results do not indicate any cytotoxicity towards HUVEC, and because each CNTs sample is different (presence of residual metals or additional carbon contamination such as graphitic-like particles and/or amorphous carbon deposits), the handling of CNTs should always be done in safe conditions by wearing gloves and appropriate respiratory protection. The results of the study of the cytotoxicity of a given sample could probably not be extrapolated without risk to another sample prepared using a different technique or even from a different catalyst for the same synthesis process. Potential physical damages to the cells and the likely chemical stability of CNTs in the human body incite to carefulness but the results of this study however suggest that the vectorisation of substances by CNTs via injection in the blood circulation seems conceivable.

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