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Study of the cytotoxicity of CCVD carbon nanotubes

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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 tests (neutral red assay for the cell viability and MTT assay—tetrazolinium salt—for the cell metabolic activity). No toxicity was found for any sample, although a slight dilution effect may exist for two of them.

In the heart of the dispute about the possible negative effects of nanotechnologies and the potential toxicity of nano-scale 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 possible applications of CNTs, it is striking to note the lack of data on their possible bio-toxicity. 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) [3–5]. 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 CNTs are forming smaller bundles, with a length generally ranging between a few tens to hundreds of micrometers. The diameter distribution is ranging between less than 1 nm to 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 $\mu\text{g}\cdot\text{m}^{-3}$. It is only very recently in 2004 that the last 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

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pulmonary toxicity of single-walled CNTs (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 granulomas. 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 granulomas 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.

We report here the study of the cytotoxicity of different samples of carbon nanotubes synthesised by CCVD. We took advantages of two existing standard tests, Neutral Red assay for the cell viability and MTT assay (tetrazolinium salt) for the cell metabolic activity. These tests 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 injected in the human body to be carried to targeted places by the blood.

The CNTs were prepared by Catalytic Chemical Vapour Deposition using bimetallic MgO-based catalysts [5, 12]. $Mg_{1-x}(Co_yM_{1-y})_xO$ catalysts were prepared by combustion synthesis [12]. 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 [13]. 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 $5^\circ C/min$, maximum temperature $1000^\circ C$, no dwell). This resulted in a mat of composite powder (which density de-

pended on the composition of the catalyst), which was then treated with a concentrated aqueous HCl solution (12M) to separate the CNTs by dissolving all the remaining oxide material, as well as unprotected metal particles [5, 12]. The suspensions were filtered and washed on cellulose nitrate membranes (Whatman, $0.45 \mu m$) with deionised water until neutrality. The samples were dried overnight in air in an oven at $80^\circ C$, yielding different amounts of CNTs according to the composition of the corresponding catalyst. The samples were characterised by electron microscopy (scanning and transmission); the specific surface area (noted S_e , $m^2 \cdot g^{-1}$) 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). The main characteristics of the different CNTs samples are detailed in Table I. Fig. 1 shows typical electron microscope images of the CNTs samples before the elimination of the oxide catalyst (which can be seen in SEM images: 1a, c, d). These images reveal a high density of CNTs in all three samples. Fig. 1b (TEM) shows that the filaments observed by SEM are in fact small-diameter bundles of CNTs. Careful analysis of high-resolution 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 more than 75% 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 4 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).

Primary cultures of human umbilical vein endothelial cells (HUVEC) were prepared and cultured as previously described [14]. HUVEC were submitted to the samples by the way of fragments of sterile (autoclaved: $121^\circ C - 20$ min) material. HUVEC were seeded at a density of 6,000 cells per cm^2 in 96-well microtiter plates (Nunc, Denmark) and the culture was maintained at $37^\circ C$ 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

TABLE I Characteristics of the CNTs samples. The catalyst composition is the elemental one, although these catalysts are not solid solutions [CC]

Reference	Catalyst composition	S_e ($m^2 \cdot g^{-1}$)	C_e (wt %)	Outer \varnothing (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

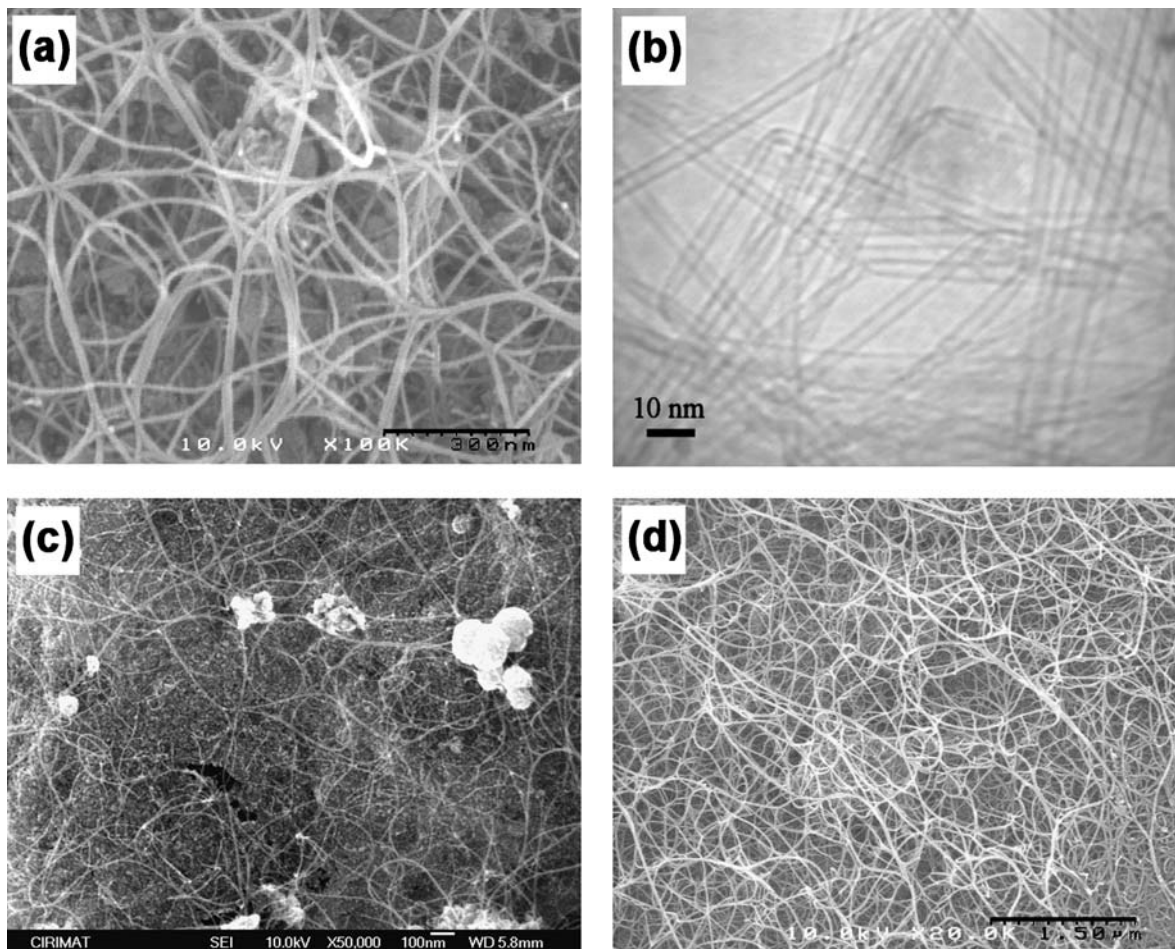


Figure 1 Typical images of the CNTs samples before the elimination of the oxide catalyst (which can be seen in the background). Sample A observed by SEM (a) and TEM (b); note the small diameter of the bundles of CNTs. Typical SEM images of samples B (c) and C (d).

5 cm² of the sample surface to 1 mL of IMDM (Iscove Modified Dulbecco's Medium) according to the standards of indirect cytotoxicity. Samples were treated in borosilicate glass tubes at 37°C for 120 h and 5% CO₂ 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. When HUVEC confluency was reached, the culture medium was removed and replaced by the samples at various dilutions [100% (v/v), 50% (v/v), 10% (v/v), 1% (v/v)] in the culture medium and supplemented with 10% FCS (Foetal Calf serum) for 24 h at 37°C and 5% CO₂. At the end of the incubation period, the supernatants (material or control samples) were discarded, and two different assays [15–18] 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.

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 test 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 below the dotted line at 75% of the response of the blank sample may be considered as cytotoxic. The results of the cells viability test (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 decreasing with the dilution, as confirmed by a significant statistical difference between diluted samples (measures indicated by a star in Fig. 3a) and the samples considered as “pure” (100%). The cell metabolic activity test (Fig. 3b) confirmed that none of the sample shown a cytotoxic effect but revealed no obvious dilution effect. 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

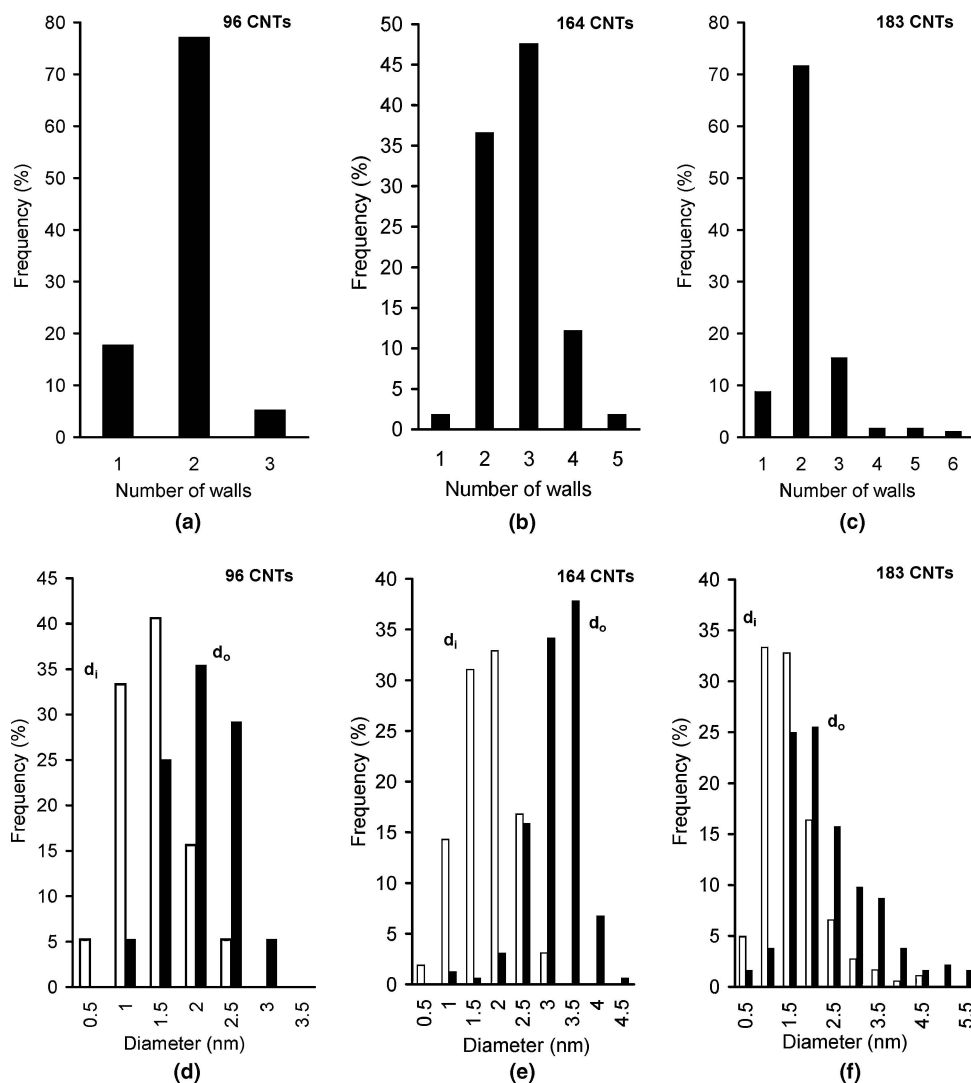


Figure 2 Distribution of the numbers of walls (a–c) and the inner (d_i) and outer (d_o) diameter (d–f) for each sample (whole population of CNTs). Sample A (a, d, respectively), sample B (b, e, respectively) and sample C (c, f, respectively).

to modifications of their environment. Indeed, they are known to respond quickly to various stimuli (biochemical, mechanical) and are thus good indicative cells [19] of a possible cytotoxicity effect.

The biocompatibility tests performed in this study required the use of very small concentrations of CNTs ($5 \text{ cm}^2 \cdot \text{mL}^{-1}$ for the most concentrated 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 $0.5 \mu\text{g} \cdot \text{mL}^{-1}$ for sample A, $0.64 \mu\text{g} \cdot \text{mL}^{-1}$ for sample B and $0.9 \mu\text{g} \cdot \text{mL}^{-1}$ 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 question of the validity of these widely used standard tests may be questioned in the case of 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 [20]; 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

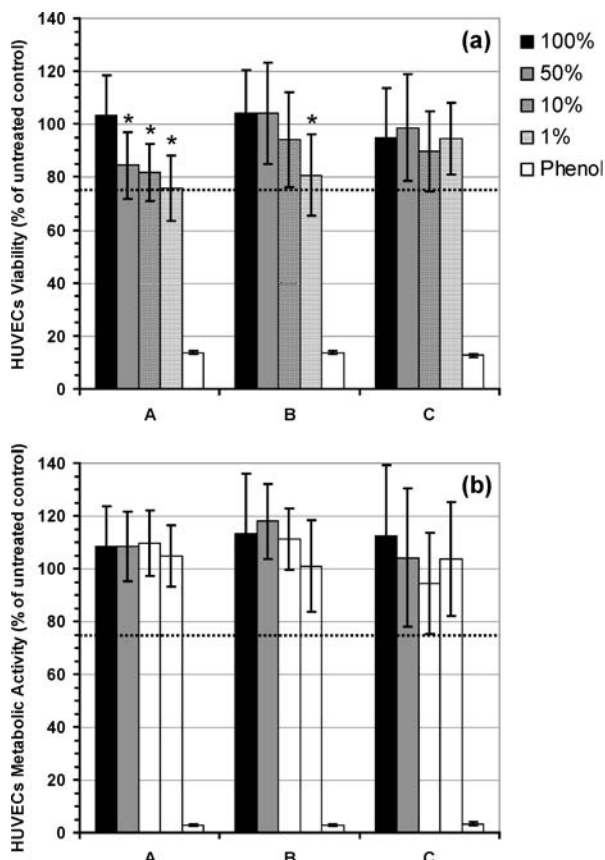


Figure 3 HUVEC Viability (a) and Metabolic activity (b) tests for samples A, B and C. Results expressed in percentage of the answer of the untreated control sample and compared to phenol (cytotoxic reference); A star (*) indicates that the value is statistically different from sample 100%.

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 possible 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 Pantarotto, the real CNTs concentration is unknown and in the case of Maynard, it is mainly the contamination with Fe which is responsible for the toxicity); it is possible (although statistically relevant for samples A and B) that in the present study the possible dilution effect observed 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 the cells has not been investigated in this study but would be important to know. 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 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 1 μm long), probably synthesised by the arc-discharge process (not indicated) 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 may make an important difference. A microscopy study of the HUVEC after the cytotoxicity test would give important information about the possible endocytosis of non-functionalised CNTs.

In summary, the cytocompatibility study of three CCVD-synthesised CNTs samples was investigated by two standard tests (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 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 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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