Cytotoxicity of Carbon Nanotubes on J774Macrophages Is a Purification-Dependent Effect

Silvia Lorena Montes-Fonseca, Erasmo Orrantia-Borunda, Alberto Duarte-Moller, Antonia Luna-Velasco, Manuel Román-Aguirre, Carmen González Horta, and Blanca Sánchez-Ramírez

Abstract

The cytotoxicity of the carbon nanotubes (CNTs) is an important factor for the manufacture of nanovaccines. The aim of this work was to evaluate the relationship of the purification method of CNTs in cellular toxicity using macrophages (MOs) from the J774 cell line. Viability test was performed with MTT assays at 24 h of exposure at concentrations of 0.06, 0.6, and 6 mg/L of unpurified (UP-CNTs) or purified (P-CNTs) CNTs by two different methods: (1) reflux with 3M HNO_3 and (2) sonication in H_2SO_4/HNO_3. Characterization and COOH content of CNTs was performed using scanning electron microscopy, raman spectroscopy, and titration with $NaHCO_3$. P-CNTs1 had lengths >100 μm and 2.76% COOH content, while P-$CNTs_2$ had lengths >1 μm and 7% COOH content. This last particle showed a lower toxic effect. The results suggest that the lenght and COOH content are important factors in the toxicity of the CNTs.

Introduction

Carbon nanotubes (CNTs) are cylindrical nanoparticles, which have unique structure and fascinating physical and chemical properties allowing them to be used in biotechnological applications [1, 2]. Due to their ability to penetrate plasmatic membrane [3], CNTs may be used as particulate substances carriers in biological systems [4, 5], as

well as in biosensors to detect cellular tumors [6], and for nanovaccine production [7, 8]. Nevertheless, these applications are limited by the high insolubility of CNTs, which conduct to generate CNTs deposition in cells, organs, and tissues, causing toxic effects [9]. Several studies have demonstrated that CNTs provoke cellular apoptosis and a decrease in viability in lung tumor cells [10], human fibroblasts [11], human T lymphocytes [12], and umbilical vein endothelial cells [13]. It has also been observed that CNTs induce the production of reactive oxygen species in human epidermal keratinocytes (HEKs) [14] and macrophages cells [15, 16]. Some authors attribute these effects to the hydrophobic nature of the CNTs, fabrication residues, and high surface area and size [17]. According to this last point, many authors converge with the notion that the length of CNTs is an additional factor for cellular toxicity. Poland et al. [18] found that intraperitoneal exposure ofmice tomultiwall CNTs with length of 20μm or longer resulted in asbestosis-like pathology.

Some authors recommend to purify the CNTs previous to test in a biological system, in order to eliminate the fabrication residues. Saito et al. [19] performed a purification technique using a mixture of concentrated H_2SO_4/HNO_3 3 : 1 v/v and sonication in a water bath for 22 h. They obtained shortened CNTs with length <1 μm and found that these can be easily dispersed in polar solvents such as ethanol, dimethyl sulfoxide, and dimethyl formamide. In addition, acid-treated CNTs are excellent candidates for functionalization with amino compounds [19] to be used in biomedical applications. However, studies about toxicity of these particles in biological systems have not been done. Therefore, the aim of this work was to evaluate the relationship of

the purification method of CNTs in cellular toxicity using macrophages (MOs) from the J774 cell line.

Methods

Synthesis and Purification of CNTs. CNTs were synthesized by spray pyrolysis, using toluene and ferrocene as the carbon source and the catalyst, respectively [20]. For the first group of CNTs (CNTs1) the synthesis time was 20 min and the purification was carried out with 0.1 g of unpurified CNTs (UP-CNTs1) and 150mL of HNO_3 3M. The mixture was dispersed by sonication for 90 min and refluxed for 24 h. Purified CNTs of the first group (P-$CNTs_1$) were filtered and washed with distilled water in an oven at 90◦C for 8h. For the second group of CNTs ($CNTs_2$) the synthesis time was 2 min and the purification method was as follows: 0.2 g of UP-$CNTs_2$ was suspended in 400mL of a mixture of concentrated H_2SO_4 (90%)/HNO_3 (70%) 3 : 1 v/v and sonicated in a water bath for 48 h. The resultant P-$CNTs_2$ were collected with a polytetrafluoroenthylene filter with 450nm pore size and washed four times with water and methanol, respectively. Finally, the P-$CNTs_2$ were dried at room temperature [19].

Characterization of CNTs. The CNTs were characterized by scanning electronic microscopy (SEM) in a JEOL SEM, model JSM-5800 LV equipped with an energy dispersive X-ray analyzer (for elemental analysis). The quality of the structure of CNTs was measured by Raman spectroscopy using a micro-Raman LabRAM HR, Horiba Jobn Yvon, coupled to Olympus BX-4 microscope. The laser line used to excite the sample was 632.8 nm and all measurements were performed at room temperature. The carboxyl groups (COOH) in the P-CNTs were measured by titration with $NaHCO_3$ base on a method established by Hu et al. [21], modified as follows: 0.1 g of P-NTC_1

and P-NTC_2 was stirred in 50mL of 0.05N $NaHCO_3$ aqueous solution. The mixture was then filtered through amembrane (pore size of 0.45 μm). The P-NTCs collected on the membrane were washed with deionized water to remove the $NaHCO_3$ residues. The combined filtrate and washings were added to 50mL of 0.05N aqueous HCl solution and boiled for 20 min to degas the CO_2 of the solution. After cooling to room temperature, the excess HCl in the solution was titrated with 0.05N aqueous NaOH solution to reach a neutral pH 7.00 [21].

Viability Tests in J774 MOs Cell Line. Cell viability was determined using 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays (Sigma, St. Louis MI, USA) in 96-well plates, as described previously [22]. Briefly, 10^5 cells were cultivated in DMEM-HG supplemented with 10% heat-inactivated bovine fetal serum, 100 IU/mL of penicillin, 100 μm/mL of streptomycin, and 2mM L-glutamine; MOs were interacted with UP-CNTs1, P-$CNTs_1$, UP-$CNTs_2$, and P-$CNTs_2$ in supplemented DMEM-HG at concentrations of 0.06, 0.6, and 6 mg/L and sonicated for 30min previous to cell interaction. Cultures were incubated for 24 h at 37◦C in humid atmosphere at 5% CO_2. MOs without stimulus were used as control. At 20 h of cultivation time, 0.1mg ofMTT dissolved in sterile phosphate-buffered saline was added to each well and incubated for 4 hmore. Cells were lyzed with acidified isopropanol and absorbances at 590nm were quantified using a BioRad ELISA microreader.

Statistical Analysis. Statistical analysis was carried out through the Minitab software and using a one-way ANOVA in order to determine the difference between the MOs interactions with different CNTs at all the concentrations used.

Results and discussion

Characterization of CNTs. The different groups of CNTs were characterized by SEM (Figure 1). The dimensions obtained for UP-$CNTs_1$ averaged 20 to 100nm in diameter and 120 μm in length (Figures 1(a) and 1(c)). The P-$CNTs_1$ obtained by reflux with HNO_3 3M showed better particle dispersion than UP-$CNTs_1$. Also open-end formations were favored in P-$CNTs_1$, but length was similar than for UP$CNTs_1$. Semiquantitative analysis of elemental composition (Table 1) showed a 2.77% decrease in Fe content between unpurified and purified samples, as well a slight increase in O content from 1.26 to 4.26%. On other hand, the dimensions for UP-$CNTs_2$ averaged 20–40nm in diameter and 30 μm in length (Figures 1(b) and 1(d)). P-CNTs2 obtained by sonication with H_2SO_4/HNO_3 3 : 1 v/v showed a considerable decrease of their length from 30 μm to <1 μm (Figure 1(f)). Likewise, the semiquantitative analysis of elemental composition showed a 1.42% decrease in Fe content and an increase in O content from 1.22 to 22.21%, in P-$CNTs_2$ (Table 1).

These results indicates that both purification techniques encourage to an effective removal of Fe from the surface of the UP-CNTs. However, the length and the O content are quite different between P-$CNTs_1$ and P-$CNTs_2$. Although P$CNTs_1$ showed an increase in O content, P-$CNTs_2$ had an increase of fivefold, indicating a greater addition of oxidized groups (COOH, OH, CO) on the surface of the CNTs [19, 23].

In order to confirm that the O content on the surface of the P-CNTs was related with the addition of COOH groups, these last were measured by tritration with $NaHCO_3$ as was indicated in the methodology section. The tritration results clearly showed that P-$CNTs_2$ had a higher percentage of COOHgroups (7%) than in P-$CNTs_1$ (2.76%). This indicates that the acid purification process combined with sonication (used for P-$CNTs_2$)

increased the formation of acid groups on the surface of P-CNTs due to the strong interaction of CNTs allowing open-end formations and promoting the oxidation of exposed carbon atoms [24].

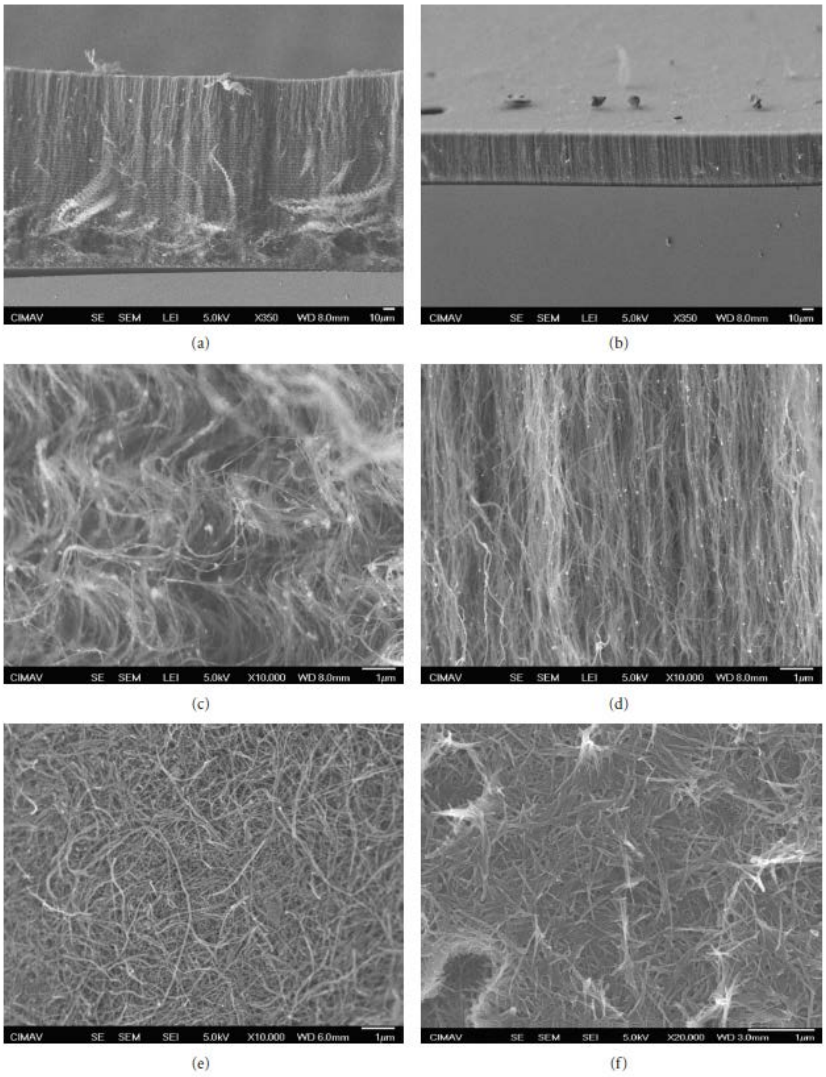

FIGURE 1: Photomicrophotographs of UP-CNTs and P-CNTs obtained by SEM. Microphotographs show length for UP-CNTs$_1$ and UP-CNTs$_2$ ((a), (b) resp.) and diameter for UP-CNTs$_1$ and UP-CNTs$_2$ ((c), (d) resp.). (e) and (f) are shown characteristics of P-CNTs$_1$ and P-CNTs$_2$ respectively.

On the other hand, the quality of the CNTs structures was determined by Raman spectroscopy. Figure 2 shows the raman spectra for UP-CNTs$_1$, UP-CNTs$_2$, P-CNTs$_1$, and PCNTs$_2$. Each of them consists of two characteristic bands, namely, D-band at 1338 cm^{-1} and G-band at 1600 cm^{-1}. The G-band is a characteristic feature of the

graphitic layers and corresponds to the tangential vibration of the carbon atoms, while the D-band is a typical sign for defective graphitic structures and is usually attributed to the presence of amorphous or disordered carbon in the CNTs samples. The comparison of the ratios of these two peaks intensities (I_G/I_D) gives a measure of the quality of the samples. If bands have similar intensity this indicates a high quantity of structural defects [25, 26].

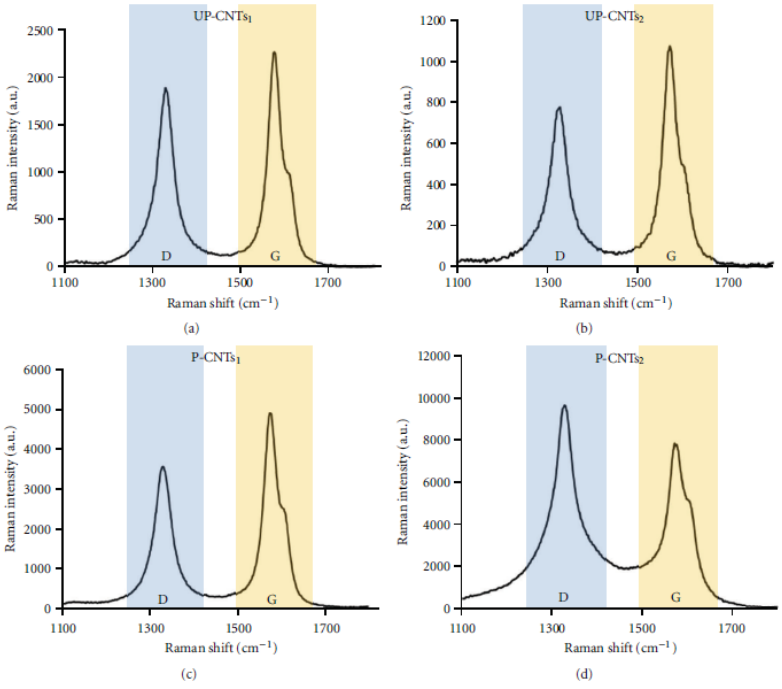

FIGURE 2: Raman spectra of CNTs samples. Different Raman spectra obtained from UP-CNTs$_1$ (a), UP-CNTs$_2$ (b), P-CNTs$_1$, (c) and P-CNTs (d). In each figure are shown the D-band at 1338 cm^{-1} and the G-band at 1600 cm^{-1} (laser excitation 632.8 nm).

Based on the Raman spectra obtained for CNTs, it was observed that the UP-CNTs$_1$ had a D-band intensity higher than that obtained for UP-CNTs$_2$, and I_G/I_D ratio obtained for UP-CNTs$_1$ (1.19) was lower than ratio observed for UPCNTs$_2$ (1.38). These data indicate that UP-CNTs$_2$ had better structural quality than UP-CNTs$_1$. In the case of the P-CNTs, a lower D-band intensity was obtained for P-CNTs$_1$; however I_G/I_D ratio (1.37) was higher compared with ratio obtained for P-CNTs$_2$ (I_G/I_D ratio =0.81). These results suggest that reflux purification process increases the structure quality of CNTs.

These results agree with the observation that P-$CNTs_2$ had more open-end formations than P-$CNTs_1$.

Cell Viability of J774 MQ Cell Line. Viability results of MOs that interacted with UP-CNTs and P-CNTs at concentration of 0.06, 0.6 and 6mg/L during 24 h are shown in Figures 3 and 4.MOs that interacted with UP-$CNTs_1$ showed a significant decrease to 40% of cell viability independent of the dose of UP-$CNTs_1$ tested, as reported recently by our investigation group [27]. In MOs that interacted with UP-$CNTs_2$ a dose-dependent toxic effect was observed, no significant cytotoxic effect was observed at 0.06 and 0.6 mg/L concentration, whereas cells that interacted with 6 mg/L had a significant decrease in cellular viability, when compared to control (Figure 3). These results indicate that length and quality structure of the CNTs contributes to cellular toxicity. The toxic effect was greater with$UP\text{-}CNT_1$ which were longer and more defectuous than UP-CNT_2. These results agree with Yamashita et al. [28], who demonstrated that long and thick CNTs cause DNA damage and severe inflammatory effects. Similarly Sato et al. [29] observed that CNTs of 825nm of length were more toxic than shorter CNTs. On the other hand, MOs that interacted with both groups of P-CNTs had a dose-dependent toxic effect. However, cell viability had a significant decrease in MOs that interacted with P-$CNTs_1$ at all concentration tested compared with control cultures (Figure 4). In the case of MOs that interacted with P-$CNTs_2$, a significant decrease in cellular viability was detected only with 6mg/L, and lower concentrations had no significant effect (Figure 4).

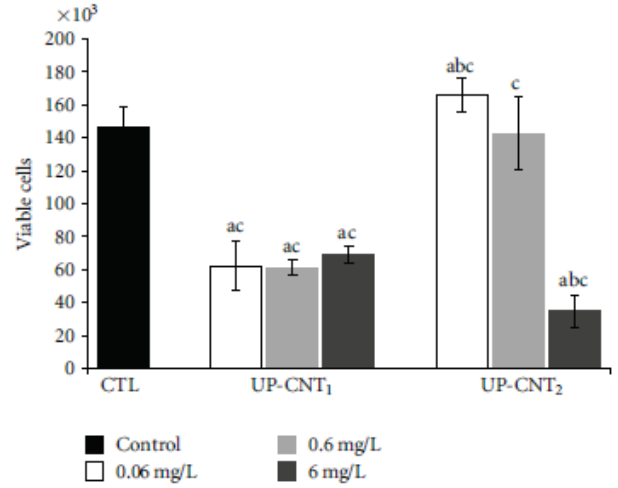

FIGURE 3: Viability of MOs that interacted with UP-CNTs at different concentration at 24 h. Each bar represents mean ± SD of two experiments done in triplicates ($n = 6$). a, $P < 0.01$ denotes significant differences between mean values measured in the indicated group as compared to control without stimulus (CTL); b, $P < 0.01$ denotes differences between mean values for CNTs at different concentration; c, $P < 0.01$ denotes differences between mean values for a particular concentration among different CNTs.

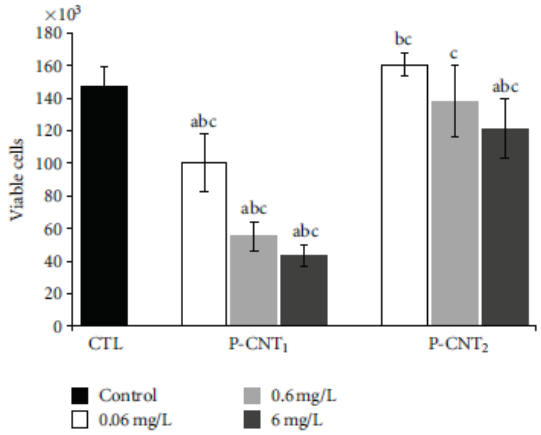

FIGURE 4: Viability of MOs that interacted with P-CNTs at different concentration at 24 h. Each bar represents mean ± SD of two experiments done in triplicates ($n = 6$). a, $P < 0.01$ denotes significant differences between mean values measured in the indicated group as compared to control without stimulus (CTL); b, $P < 0.01$ denotes differences between mean values for CNTs at different concentration; c, $P < 0.01$ denotes differences between mean values for a particular concentration among different CNTs.

Some authors have reported that CNTs interact with MTT and avoid salt metabolism, blocking formazan formation, a colored compound that is detected at 590nm [30, 31]. In

this study we incubated NP-CNTs and/or P-CNTS with MTT, and no interference or nonspecific reduction of MTT was detected (data not shown).

Several works have reported that the purification process eliminates residues present on the CNTs superface and that additionally introduces certain charged groups on their surface, allowing higher stability in aqueous solution and decreasing the toxic effect [10, 12].Nevertheless, the purification procedure could also contribute to CNT toxicity. Many studies have found that P-CNTs or acid-treated CNTs had greater toxic effect than UP-CNTs [31, 32]. Some authors attribute this behavior to the COOH groups, which may be causing a stress on cells [10, 12]. On the contrary, works such as reported by Pulskamp et al. [30] showed that P-CNTs are less toxic than UP-CNT. These contradictory results could be related with the purification methodology used in those studies, since acid treatment modifies chemical and structural CNTs characteristics. Indeed, there are scarce cytoxicity studies in which purification and structural nanoparticle characteristics are considered and that is probably one reason why it has been hard to identify the origin of toxicity. In our work, we found that the toxic effect of CNTs was dependent of length and COOH content. P-CNTs$_2$ whose length was <1 μm and COOH percentage was 7% were less toxic compared with P-CNTs$_1$ (length >100 μmand 2.76% of COOH). The relationship of COOH groups with the toxicity of CNTs on cellular cultures is not clear yet, since in most of the studies COOH groups are not quantified. However, the effect of O content has been reported in some toxicity studies, where a greater toxicity of P-CNTs was found with low content of O [27, 30], and P-CNTs with O content above 20% decreased notably the cytotoxicity [31, 33]. Considering that the O content is associated with the COOH groups in the P-CNTs, our

results agree with those in which P-CNTs have high O content. However the COOH quantification is desirable to make a direct toxicity comparison.

In addition to chemical and structural properties, the aggregation differences between CNTs could be related with cytotoxicity variations [33–35]. In our study, treatment with 3 : 1 H_2SO_4/HNO_3 and sonication for 48 h allowed to obtain CNTs shorter and with a higher content of COOH groups, which were more soluble in aqueous medium. Moreover, increase of COOH groups in the surface of the CNTs is indispensable to favour nanoparticle functionalization with drugs or peptides, a relevant process for nanocarriers or nanovaccines production.

Conclusion

Results obtained in this work demonstrated that purification methodology is a key event for P-CNTs production for their use in nanobiotechnology; acid treatment with 3 : 1 H_2SO_4/HNO_3 and sonication was highly effective to remove Fe and permits us to obtain P-CNTs with low cytotoxicity on MOs of J774A cell line. Cytotoxic effect was related to the length and COOHcontent of P-CNTs. This finding is of great importance for generation of nanobiotechnological products such as nanocarriers or nanovaccines, in which CNTs exhibit minimal toxicity and high expectations in the future.

Acknowledgments

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