

Carbon nanotube dispersion for *in-vitro* applications

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Abstract

Carbon nanotubes (CNTs) have gained popularity due to their many characteristics which make them the promising material for biomedical applications. A major challenge when working with CNTs is the difficulty to distribute them evenly throughout liquids. This research is a comparative analysis of the dispersion of multi wall CNTs (MWCNTs) in different liquid media (distilled water, ultrapure water of type I [Milli-Q], ethanol, dimethyl sulfoxide [DMSO] and cell culture medium [RPMI-1640]) by using two different sonication techniques (probe-type sonication and ultrasonic bath sonication) as well as determining the effect of sterilization in the presence and absence of fluid before dispersion. The results indicate that the best method to achieve a CNTs dispersion is to use a probe-type sonicator as well as to sterilize the CNTs samples in the presence of fluid.

Keywords: Carbon Nanotube; Dispersion; Ultrasound.

Dispersión de nanotubos de carbono para aplicaciones *in-vitro*

Resumen

Los nanotubos de carbono (CNTs) han atraído un enorme interés debido a sus muchas características que los convierten en un material prometedor para aplicaciones biomedicas. Un desafío importante cuando se trabaja con CNTs es la dificultad de dispersarlos homogéneamente en un fluido. Esta investigación es un análisis comparativo de la dispersión de nanotubos de carbono de pared múltiple (MWCNTs) en diferentes fluidos (agua destilada, agua ultrapura de tipo I [Milli-Q], etanol, dimetilsulfóxido [DMSO] y medio de cultivo celular [RPMI-1640]). Utilizando dos diferentes técnicas de sonicación (sonicación con lanza ultrasónica y sonicación ultrasónica de baño), así como la determinación del efecto de la esterilización en presencia y ausencia de fluido antes de la dispersión. Los resultados indican que el mejor método de dispersión de los CNTs es usando un sonicador tipo lanza y esterilizarlos en presencia de fluido.

Palabras clave: Nanotubos de Carbono; Dispersión; Ultrasonido.

1. Introduction

Sumio Iijima accidentally discovered CNTs in 1991 [1]; ever since his discovery new approaches for CNTs synthesis and applications have been explored. Their implementation in many areas has augmented due in part to their physico-chemical characteristics which make them a novel material with a wide range of applications [2,3]. The possibility of dispersing CNTs in fluids makes them promising candidates for their implementation in different systems due to their many properties. However, effective dispersants and mechanical mixing methods are necessary to produce

uniform dispersions and stable over long periods of time. Factors that affect the rheology of nanotube dispersions include the chemistry of the continuous phase (the base fluid), nanoparticle loading throughout the fluid volume, the average aspect ratio and surface chemistry of the nanoparticles (mechanical mixing methods and chemical treatments) and the dispersants employed [4].

Temperature influences the rheological properties of the base fluid, but can also affect the stability of the CNTs dispersions be it by changes in the dispersant conformation in the liquid or by changes in the interactions between the dispersant and the solid surface of the CNTs. Therefore, the

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rheological properties of the CNTs dispersions are crucial for their application in different systems [5]. CNTs tend to form bundles and aggregates in the dry form as well as when they are suspended in a liquid, CNT agglomeration can be attributed to their morphology, high aspect ratio, van der Waal's forces, π - π stacking and hydrophobic interaction between the tubes; such molecular forces are influenced by the chirality and surface curvature of the CNTs hence increasing the dispersion difficulty [6,7]. Agglomeration of CNTs interfere with some of the CNTs properties such as size distribution, surface to volume ratio as well as the surface reactivity, which could become an obstacle in the application of CNTs in the biological field. Uniform and reproducible dispersions of CNTs in liquid phases could be challenging to accomplish [8-12] but are also crucial when it comes to biological applications of nanoparticles, failure to disperse CNTs could account for inaccurate *in vitro* interpretation of their effects [13,14].

The use of CNTs in the field of medicine and other industries has gained popularity due to their physicochemical properties. However, exposure to nano-engineered materials is of great concern due to the potential harm they pose on the environment and the human body. Several researchers have emphasized the importance of studying the adverse effects of nanomaterials on the environment and biological tissues. Extensive reviews have provided a better explanation of the many factors behind the nanotoxicology of engineered nanomaterials [15,16]. Physicochemical properties of nanoparticles such as size, chemical composition and surface structure exhibit adverse biological effects at the cellular level. Biological alterations such as genotoxicity and immunogenic changes are attributed to engineered nanoparticles; therefore, new approaches such as computer simulation are currently being explored to decipher and help understand the interaction of CNTs with the cell [17].

This work evaluated the dispersion of CNTs in several fluids (distilled water, ultrapure water of type I [Milli-Q], ethanol, dimethyl sulfoxide (DMSO) and cell culture medium [RPMI-1640]) at concentrations ranging from 0.01 to 0.1 mg/mL. Pristine and Nitrogen doped (N-doped) CNTs were used to compare the dispersion state with respect to each fluid tested, in addition turbidity tests were performed to the different dispersions in order to determine the amount of CNTs suspended in the fluid.

2. Materials and methods

2.1. Carbon nanotube synthesis

Carbon nanotubes employed in this research were synthesized by chemical vapor deposition (CVD) in the Synthesis and Special Processes Laboratory at Universidad Pontificia Bolivariana. The catalyst used was a mixture of $\text{Co}_2\text{O}_4 + \text{Fe}_2\text{O}_3$ (40-10%) [18]. Both pristine and N-doped CNTs tested had an average wall number between one and ten. The purification process of the CNTs samples was done by microfiltration with 10% hydrofluoric acid and hydrochloric acid [1M] resulting in a purity >95%. Characterization was achieved by micro-Raman with a confocal Horiba Jobin Yvon, high spectra resolution

analytical Raman microscope Model LabRAM HR at a confocal distance of 800 mm, Laser spot size of 1 to 300 mm, CCD detector with a resolution of 1024x256 pixels, optimized spectral range of 400-1100 nm and diffraction gratings of 1800 and 600 lines/m. The scanning electron microscope (SEM) used was a JEOL JSM-6490 LV as well as a FEI Nova NanoSEM 200 to verify the vibrational features and structure of the CNTs.

2.2. Carbon nanotube dispersion in different fluids

CNTs were dispersed in distilled water, ultrapure water of type I (Milli-Q) it was obtained through Synergy Water Purification System de Millipore Sigma, ethanol (absolute for analysis EMSURE®), dimethyl-sulfoxide (DMSO) for analysis EMSURE® with a purity of $\geq 99.9\%$ and cell culture medium (RPMI-1640 with L-glutamine). The dispersion process was achieved in the following manner: 1mg of the CNTs sample (either pristine or N-doped) was mixed with 10mL of fluid, dispersion was achieved by using a 130-watt Cole-Parmer ultrasonic processor at amplitude of 50% during 2 minutes and 30 second pulsations. Sonication time, frequency and choice of equipment was determined based on the results reported by Hilding et al. [19]. Serial dilutions ranging from 0.01mg/mL to 0.1mg/mL with a dilution factor of 0.01 were prepared from the stock dispersion (0.1mg/mL) of the pristine and the N-doped CNTs samples. Turbidity measurements were taken with a Multiskan-GO Microplate Spectrophotometer from Thermo Scientific, in order to determine an optimal base fluid for dispersing the CNTs. Turbidity measurements were taken at 692nm for all fluids tested, after determining a no absorbance reading for the cell culture medium RPMI-1640.

2.3. Probe-type vs ultrasonic bath sonicator

After determining the most suitable liquid for the dispersion of CNTs based on the results obtained from the turbidity tests, the effectiveness and efficiency of two sonication techniques (probe-type and ultrasonic bath) were evaluated. The Cole Parmer Ultrasonic Processor Model CPX 130, 20 kHz ultrasonic frequency was used to sonicate the samples by immersing the probe in the sample and sonicating at an amplitude of 50% during 2 minutes and 30 second. The Elmasonic Ultrasonic Bath Model E30H, 37 kHz ultrasonic frequency was used to sonicate the samples which were placed in a sealed container and sonicated during 2 minutes. Turbidity measurements were performed with a Multiskan-GO Microplate Spectrophotometer from Thermo Scientific at 692nm.

2.4. CNT sterilization in the presence and absence of fluid

Pristine and N-doped CNTs were autoclaved in the presence and absence of cell culture medium RPMI-1640 prior to dispersing them either by probe-type or ultrasonic bath sonication. The CNTs samples were autoclaved in a Yamato Scientific America Series SM52, 1mg of CNTs was placed in a glass bottle and covered with a rubber stopper, while in a different glass bottle 1mg of CNTs was mixed with

10 mL cell culture medium RPMI-1640. The samples were sterilized during 15 minutes at 121°C, and let to cool off during 24 hours, then 10 mL of RPMI-1640 were added to the sample autoclaved in the absence of liquid. Dispersion was achieved using the probe-type sonicator and ultrasonic bath sonicator for comparison reasons. The stock dispersions were used to prepare serial dilutions ranging from 0.01-0.1mg/mL, turbidity measures were taken at 692nm during five different moments with a time interval of 10 minutes over a 40-minute period.

3. Results and discussion

3.1. CNTs synthesis

Fig. 1 shows SEM micrographs of CNTs. The high productivity obtained through the use of the catalyst can be observed qualitatively. Fig. 1b shows the carbon nanotubes after the purification process grown from the catalyst mentioned in section 2.1.

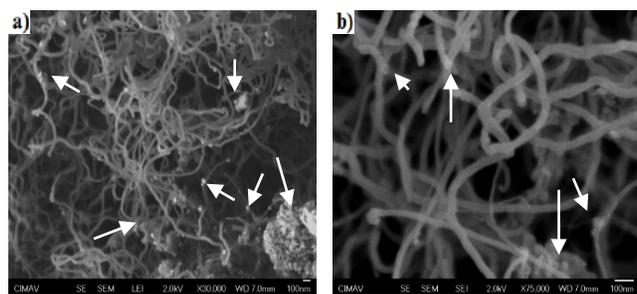


Figure 1. SEM micrographs of CNTs a) prior to purification, and b) after purification.

Source: The authors

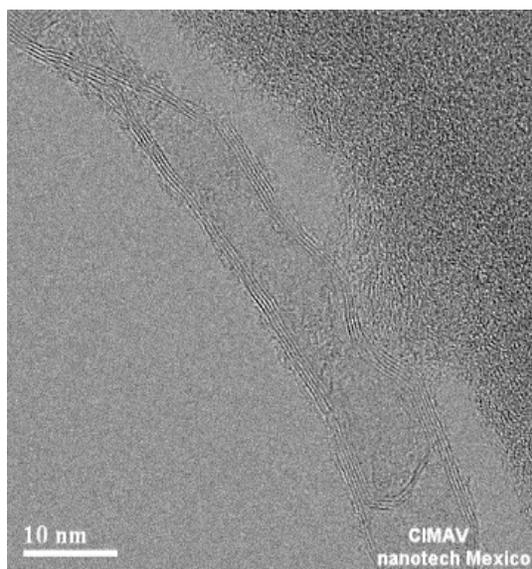


Figure 2. TEM micrograph of multi-walled CNTs grown from Co. Average wall number ranging between 1 and 10 walls.

Source: The authors

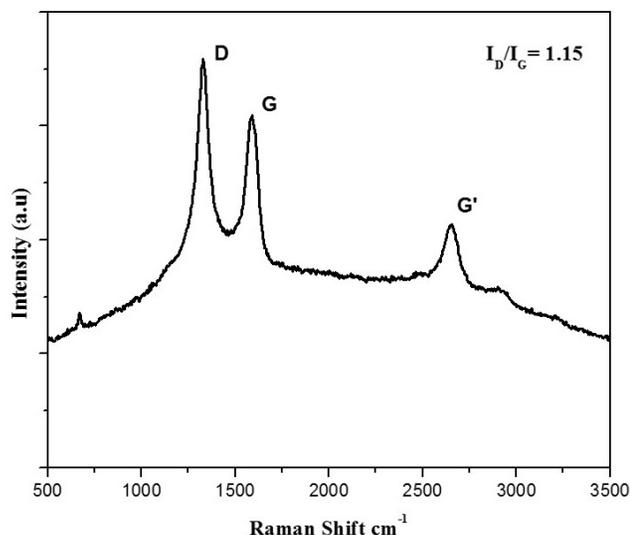


Figure 3. Raman spectroscopy of purified CNTs.

Source: The authors

The TEM micrograph corresponding to Fig. 2 shows CNTs from a different perspective, the micrograph shows evidence of the number of walls obtained by the synthesis method chosen. The average number of walls observed is between 1 and 10. The inner diameter is about 6.5 nm and the outer diameter corresponds to 8 nm; it may also be noted that the nanotube is closed tip.

The Raman spectra depicted in Fig. 3 shows bands D, G and G' respectively, as well as the ratio of band D and G (I_D/I_G) which equals to 1.15. Such band ratio indicates that once the catalyzer is eliminated from the CNTs structure, the walls are shifted generating an increase in the CNTs crystallinity [15].

3.2. CNTs dispersion: Probe-type vs ultrasonic bath sonicator

Figs. 4 and 5 account for turbidity measurements of both N-doped and pristine CNTs after being dispersed with a probe-type ultrasonic processor (Fig. 4) and an ultrasonic bath sonicator (Fig. 5). The measurements were taken during five different times with a time interval of ten minutes in order to determine which of the two sonication techniques was best at breaking up the CNTs agglomerates and allowing the CNTs to remain suspended in the fluid. As it can be observed in Fig. 5 both types of CNTs were more or less uniformly dispersed when the probe-type sonicator was used. In contrast, the turbidity measurements obtained from the samples sonicated with the ultrasonic bath (Fig. 6) indicate that the CNTs were not as uniformly dispersed and remained agglomerated.

3.3. CNTs sterilization in the presence and absence of fluid

The information shown in Fig. 6 accounts for turbidity measures of ten concentrations of pristine (Fig. 6a) and N-doped (Fig. 6b) CNTs. The readings were taken in order to

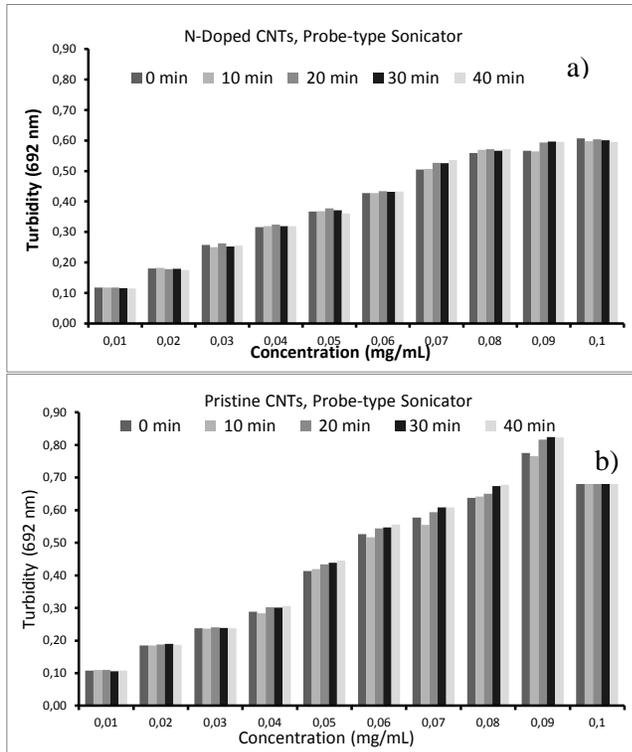


Figure 4. CNTs dispersed with a probe-type sonicator in RPMI-1640. a) N-Doped CNTs and b) Pristine CNTs. Source: The authors

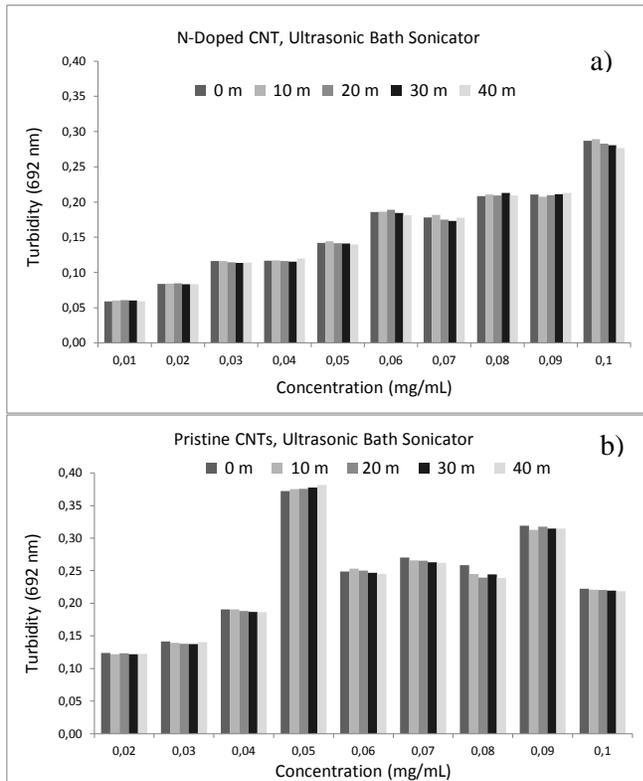


Figure 5. CNTs dispersed in RPMI-1640 with an ultrasonic bath sonicator a) N-Doped CNTs and b) Pristine CNTs. Source: The authors

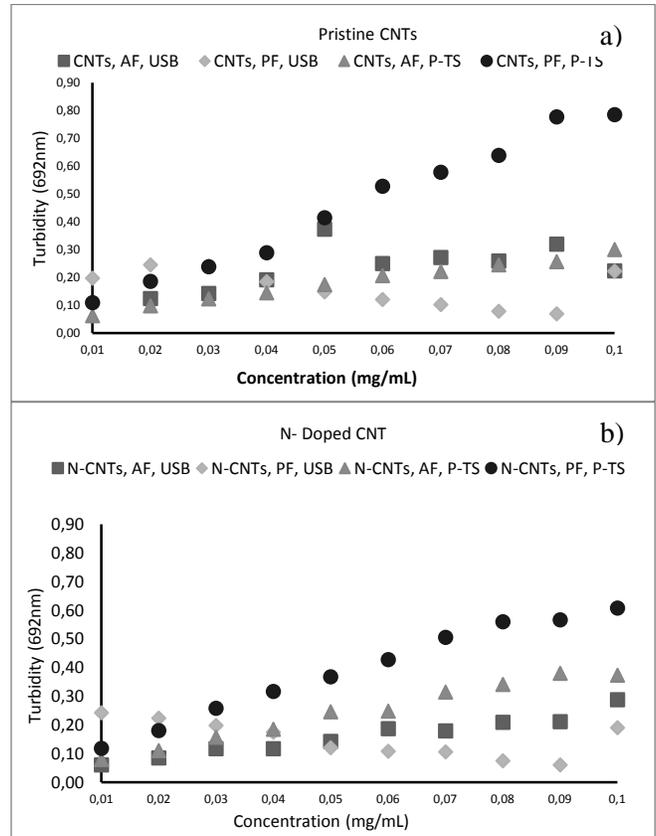


Figure 6. Turbidity measurements of a) pristine and b) N-doped CNTs dispersions. Prior to dispersing the CNTs with either ultrasonic bath (USB) or probe-type sonicator (P-TS), the samples were autoclaved in the presence (PF) and absence of fluid (AF). Source: The authors

determine if the sterilization process in combination with the dispersion technique caused any effect in the ability of the CNTs to remain suspended in the liquid. As it can be seen in both Figures, CNTs that were sterilized in the presence of fluid (RPMI-1640) and dispersed with the probe-type sonicator yield higher turbidity measurements for all the ten concentrations tested. Overall N-doped CNTs performed better than pristine CNTs when sterilized with fluid and dispersed with the probe-type sonicator.

3.4. Turbidity measurements of CNTs in five different fluids

Turbidity measurements of both types of CNTs were taken at 692nm after dispersing the CNTs samples in five different fluids. Fig. 7 illustrates the measurements obtained for each of the fluids tested; as it can be observed the cell culture medium RPMI-1640 yield the highest turbidity measurements for all ten concentrations when compared to the other fluids. A possible explanation for this behavior would be that the nitrogen atoms present in the doped CNTs have an affinity for the components of the cell culture medium, therefore facilitating their dispersion; such observation indicates that the RPMI-1640 was the optimal fluid to disperse the CNTs, since a substantial amount of CNTs remained suspended in the fluid.

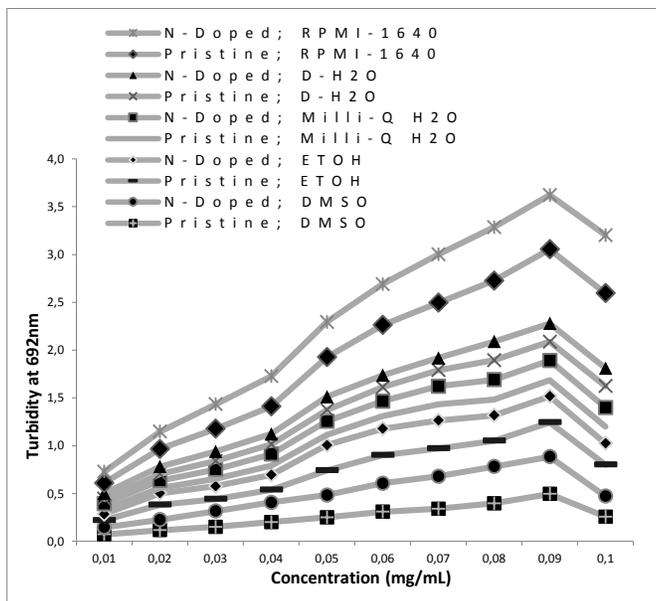


Figure 7. Turbidity measurements of ten concentrations of pristine and N-Doped CNTs in RPMI-1640, ethanol (ETOH), DMSO, distilled (D) and Milli-Q water.
Source: The authors

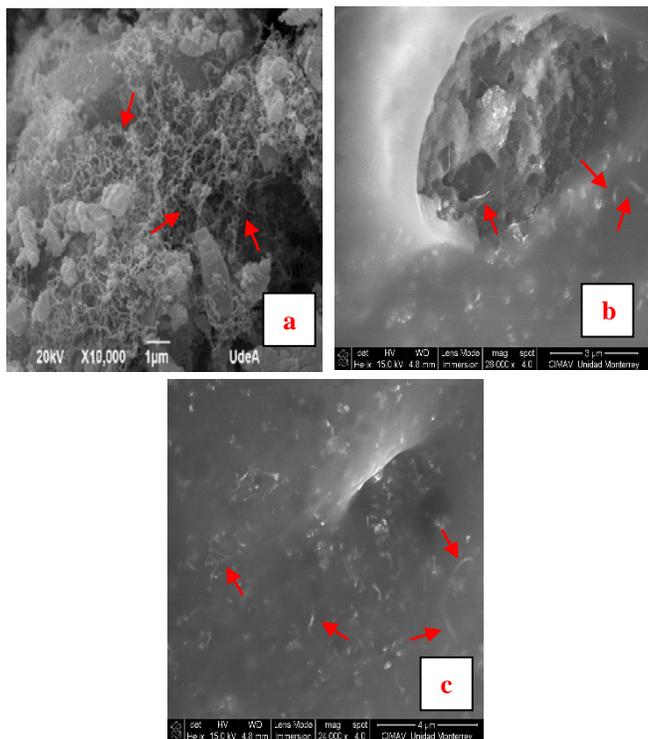


Figure 8. SEM micrographs of a) N-doped CNTs grown from Cobalt before dispersion, b) and c) N-doped CNTs [0.1mg/mL] dispersed in RPMI-1640, with an average size of less than 1µm, image magnified at 28000X and 24000X respectively.
Source: The Authors.

Fig. 8 shows the appearance of the carbon nanotubes obtained after each process. Fig. 8a shows a SEM micrograph of intact N-doped CNTs before being sonicated, it is

important to note that CNTs appear clumped and tangled (see arrows). Figs. 8b and 8c show the dispersed N-doped CNTs [0.01mg/mL] samples after being autoclaved and sonicated during 2 minutes it can be seen that the carbon nanotubes used are dispersed in the cell culture medium (see arrows).

The data obtained was consistent with the objective of this research, since the application of CNTs for in-vitro testing has gained popularity in the last few years. Some authors have dispersed CNTs in aqueous solutions [16-20], but the dispersion is slightly different since tenso-active agents are employed to stabilize the solution and inhibit the agglomeration of CNTs. Murdock et al. [21] also prepared CNTs dispersions prior to in vitro exposure of cells, the authors used water as the dispersion liquid as well as cell culture media and serum. It is worth to mention that RPMI-1640 supplemented with 5% fetal bovine serum (FBS) was one of the first dispersion liquids used to sonicate CNTs samples employed in this research, but the attempt resulted in failure due to excessive foaming of the dispersion liquid caused by the probe-type sonicator. In this research, the dispersion of the CNTs samples was finally achieved in RPMI-1640 without FBS.

It is important to note that factors that could potentially affect the rheology of the CNTs dispersions include the chemistry of the continuous phase (base fluid), the amount of CNTs in the total volume of fluid, the aspect ratio and the average surface chemistry of the CNTs (mechanical dispersion methods and chemical treatments).

Another parameter that must be kept in mind when dispersing CNTs is the sonication technique chosen to achieve the separation of CNT agglomerates; however, separation methods could compromise the length and number of walls of the CNTs, which results in CNTs fragmentation as well as layer shedding. Authors such as Hilding et al. [19] have investigated the sonication effects on the CNTs morphology and have reported that such dispersion methods could alter the length and diameter of the CNTs structure. The data obtained in this research are congruent with the data reported in the literature [19] where a probe-type sonicator is employed to successfully disperse CNTs. A possible explanation for such result could be due to the uniform distribution of the sound waves compared to the ultrasonic bath, also the probe-type sonicator excels the ultrasonic bath by a factor of 1000 (1000x higher energy input per volume) [19], therefore it is possible to achieve a homogenous dispersion with this sonication technique. Another important factor that influences the CNT dispersion is temperature, which has an effect on the rheological properties of the base fluid. Temperature could also affect the stability of the CNTs dispersion by changing the dispersant conformation of the liquid or by changing the interactions between the dispersant and the solid surface of the CNTs. Therefore, the rheological properties of CNTs dispersions are quite important for their application in biological systems [5]. Our data shows a better dispersion of CNTs when the CNTs samples were sterilized in the presence of the base fluid (RPMI-1640). This behavior could be explained due to the thermal stability conferred by the CNTs to the base fluid [22].

4. Conclusions

This study evaluated the dispersion capacity of carbon nanotubes using different fluids and two dispersion

techniques. It was possible to obtain carbon nanotubes by means of the CVD method, as well as their purification to obtain that they were dispersed in the different fluids. The results obtained indicate that the best dispersion of the carbon nanotubes for in vitro applications is the cell culture medium (RPMI-1640), using the probe-type sonicator.

In order to achieve a successful dispersion there needs to be full control over the most important sonication parameters to ensure the linear scalability of the process and completely reproducible results. The possibility of achieving a favorable CNT dispersion makes N-doped CNTs promising candidates for in-vitro testing. Moreover, when CNTs were dispersed using a bath sonicator, their dispersion state did not last over long periods of time although time was not a determinant factor to achieve a successful dispersion.

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