In vitro cytotoxicity assessment of pristine and carboxyl-functionalized MWCNTs

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Abstract

The wide use of carbon nanotubes (CNTs) in consumer products, i.e. composites, coatings, food packaging, etc, raise concerns about the adverse effects that CNTs can induce in humans and environment. Yet, there is no global consensus regarding risks that CNTs may pose, while controversial evidence exists also on the toxic effects associated with chemical surface modification, a prerequisite for their incorporation in different matrices. Moreover, there is limited information available about the underlying mechanisms, especially when cells' interactions with the nanomaterial is assessed by imaging techniques. The present study aims at evaluating the *in vitro* cytotoxicity of pristine and oxygen functionalized multi-walled CNTs (MWCNTs) by assessing cell viability and apoptosis in combination with scanning electron microscopy (SEM) observations of stabilised cells. Direct observation of adenocarcinoma human epithelial cells (A549) was performed after incubation with 12.5, 50 and 100 μg/ml MWCNTs, for 0.5, 1 and 3h, simulating a real exposure scenario during an accident, taking into account industrial safety issues during the production and use of the nanomaterial. Functionalized MWCNTs induced higher time- and dose-dependent toxic effects as compared to pristine. The SEM observations revealed the damaging effect on the cell membrane, offering insights about the toxic mechanism that takes place.

Keywords: CNTs, Cytotoxicity, Exposure, Functionalisation, Nanosafety, SEM

1. Introduction

Nanotechnology has been identified as a key enabling technology (KET) providing the basis for further innovation, leading to materials with a new level of performance. Specifically, carbon-based engineered nanomaterials such as carbon nanotubes (CNTs) especially when functionalized, have attracted the interest of scientific community due to their peculiar properties. Indeed, when included in a matrix, they can act as an additional, multifunctional reinforcement for composites, due to their high thermal and electrical conductivity, electromagnetic interference, shielding ability, flexibility and transparency as well as low coefficient of thermal expansion, in addition to their superior mechanical properties [1].

However, since the intrinsic properties of nanomaterials may also result in a distinctive health hazard profile [2], their development must be complemented with a scientifically sound identification of their potential toxicity to minimize risks and lead to a sustainable implementation of nanotechnology [3]. Owing to the multiplicity of types and forms of MWCNTs, many safety aspects of these materials are still incomplete and the information describing the relative health risk assessment is lacking, especially regarding the interactions of CNTs with cellular membranes and how they affect cell behavior. However, different physico-chemical determinants, besides their length-to-diameter ratio, have been shown to correlate with acute or long-term toxicity [4, 5]. Acute and/or chronic toxicity should be assessed at the route of entry, namely pulmonary, gastro-intestinal, dermal or intra-ocular [6]. CNTs toxicity has been examined in several cell lines and organisms [7] and the similarities between CNTs and asbestos fibers have been proven by numerous experimental studies [8, 9]. Therefore, the inhalation of CNTs has been identified as the prime health concern during handling and manufacturing processes, since it may accumulate in the lungs cavity and result in severe inflammation [10, 11]. Several toxicity mechanisms have been proposed for CNTs, including interruption of trans-membrane electron transfer, disruption/penetration of the cell membrane,

oxidation of cell components, physical interference with specialized functions, phagocyte activation, altered gene expression, impairment of DNA and mitotic spindle, and indirect effects caused by the dissolution of some contaminants, such as heavy metal releasing ions or reactive oxygen species generation (ROS) [12-14].

Nevertheless, the nature of CNTs toxic effects can be tuned by factors such as their size, length, diameter, type of chemical functionalization, purity and surface chemistry. Indeed, as it has been proven by Poland et al. [5], CNTs length and diameter can severely altered CNTs toxicity, revealing that long multi-walled CNTs (MWCNTs) present greater propensity to produce significant inflammation to cells compared to short of tangled MWCNTs. The CNTs length in many cases, may exceed the size of macrophages and thus cannot be fully engulfed, leading to severe distortion of cells morphology and incidents of chronic inflammation [15]. To explain the potential toxicity of airborne MWCNTs, the adenocarcinoma cell line A549, has been utilised due to the similarities of these cells with the primary pulmonary alveolar cells II (ATII). It has been demonstrated that the interaction of CNTs with this type of cells led to the damage of cell membrane, allowing to identifying some determinants responsible of severe pathological conditions [16].

Functionalisation (e.g. surface oxidation) is the process through which the surface of CNTs becomes active, in order to intentionally improve the interaction with their environment, including biological systems; however, it has also been reported to determine the type and severity of inflammation. A relevant research study by Vittorio et al., has demonstrated that acidic treatment of MWCNTs negatively affects cell viability in a dose-dependent way [17]. Same results aroused from Bottini et al., where surface oxidation of CNTs initiated cell apoptosis in contrast of pristine ones with hydrophobic nature which appeared to lesser affect T cell lines [18].

It has been proved [19] that it is crucial to visualize the interaction of MWCNTs with cells, since it may negatively affect cell morphology and induce cell monolayer damage, as it is schematically depicted in Figure 1, which cannot be easily proved with the typical cytotoxicity tests. Indeed, Rotoli et al. [19] demonstrated that distinctive, contact-mediated cytotoxic effects of MWCNTs to Calu-3 cells, were only detectable when utilizing imaging techniques (confocal microscopy), while the biochemical assays used at the cell population level, showed that cell viability was not altered.

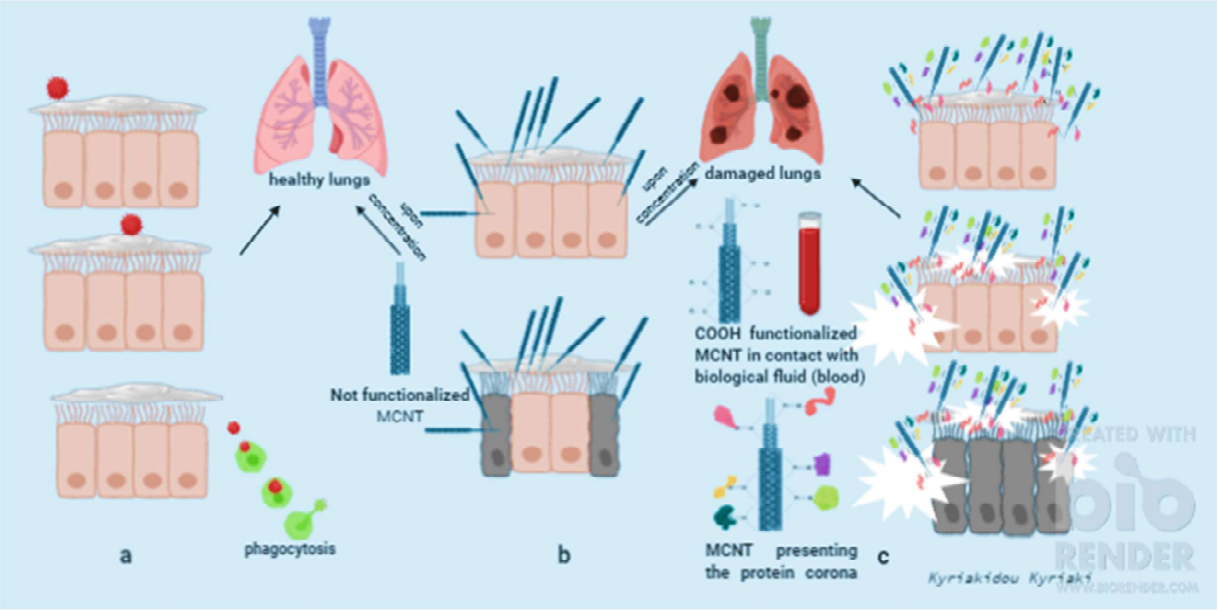

Figure 1. Schematic representation of cell uptake for: (a) round-shape nanoparticles that can be engulfed, defragmented and cleared by the cells; (b) needle-shaped MWCNTs that interact with plasma membrane causing cell distortion and (c) carboxylic-functionalized MWCNTs where cell deformation is occurring, since CNTs penetrate and destroy cell membrane's integrity, causing apoptosis.

In this context, in the present study the evaluation of the potential cytotoxic effect of MWCNTs either in their pristine (i.e., not functionalized) form or after their functionalization has been carried out by assessing viability and cytotoxity in a cell testing system. The experiments have been performed using adenocarcinoma human epithelial cells (A549), to assess the potential risk from acute inhalation exposure. The distinctiveness of the protocol that was followed, lies

in the fact that the upstream damaging mechanisms of the first hours of direct contact of MWCNTs with the A549 cells monolayer, were studied and visualized through Scanning Electron Microscopy analysis.

2. Materials and Methods

2.1 CNTs growth and functionalization

The CNTs used in this study have been developed in-house, in the Research Unit of Advanced, Composite, Nanomaterials and Nanotechnology (R-NanoLab), by the Chemical Vapour Deposition (CVD) method, through the supported catalyst approach. By this method, MWCNTs can be grown on the appropriate substrate. The carbon source chosen for this reaction was acetylene (C_2H_2) and as carrier/inert gas Ar was used, in a volumetric ratio of 1:20, respectively. The catalyst was based on iron and specifically, iron oxide (Fe_2O_3) particles embedded on zeolite, synthesized in house through the precipitation method. In order to have uniform growth of the MWCNTs, the catalyst particles were sieved and then deposited on three Si wafers, inside the active zone of the furnace (main body of the reactor). In total, 1.123g of catalyst was used. After a 20min purging with Ar, the reactor was heated up to 700^{o}C. Afterwards, the acetylene flow was adjusted to 60ml/min for four hours, and after completing the reaction, the system was left to cool to room temperature overnight. In total, 10.137g were produced, which were further purified, functionalized and examined.

In order to assess the toxic effect of the as-produced MWCNTs, catalyst residues need to be removed. Thus, MWCNTs undergone a step by step purification procedure, with mild conditions, in order to obtain a total pure material, suitable for biological characterization. MWCNTs were then mechanically grinded in an agate mortal, to break any agglomerates and to achieve the finest powder. Afterwards, a thermal treatment took place at 400^{o}C for 1h, in atmosphere, in order to remove any amorphous carbon residues. To remove the catalyst

particles, Soxhlet extraction was implemented, with 5M HCl acid solution for 2 hours. The purified nanomaterial was left to dry after washing with water to neutralize and remove residues of the acid. The purification procedure led to a decrease of the total mass of the MWCNTs, up to 0.767g.

Finally, the functionalization procedure of the half quantity of the produced MWCNTs took place. For this, a 6M acid mixture of HNO_3:H_2SO_4 in volume ratio of 1:3 was used. The solid/liquid ratio was 0.15gCNTs/10ml acidic solution. The reaction was carried out at 80^{o}C, under intense steering and lasted 48hrs. To separate the functionalized MWCNTs from the acid solution, Buchner filtration was implemented. Washing with 6L of water was necessary, in order to remove the acids. The filtration was completed only when the pH reached 7. As a final step, the sample was rinsed with ethanol and left to dry in an oven at 70^{o}C. The powder was then grinded and proceeded for the toxicity testing, together with the reference sample.

2.2 CNTs characterization

The morphologies of the MWCNTs used in this study, were examined by scanning electron microscopy (SEM) using a NovaTM NanoSEM 230 (FEI company, Hillsboro, OR, USA) microscope with a W (tungsten) filament, with all images acquired in secondary electron mode using an operating voltage of 15 kV. Energy Dispersive X-ray Spectroscopy Analysis (EDS) was performed using a Hitachi High Technologies Corporation SEM equipped with a QUANTAX 70 EDS system. Raman spectra were collected with a Renishaw in Via reflex Raman microscope. Measurements were carried out using a solid state 532 nm laser (green) as excitation source, at room temperature. The power of the laser was 1mW with a spot diameter of 1μm. Spectra analysis was carried out through MATLAB software. XPS was conducted using a Thermo Scientific K-Alpha XPS system (Thermo Fisher Scientific, UK) equipped with a micro-focused, monochromatic Al Ka X-ray source (1486.6 eV) with an energy resolution of 0.8 eV and pass energy of 15 eV.

2.3 Cell cultures

A549 lung adenocarcinoma cells were cultured using RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycinin standard conditions (37°C, 5% CO_2. A549 cells were seeded in 24 multi well-plates at a density of 20.000cells/cm^2. 24 hours after seeding the medium was removed and 2 ml of a medium containing either not functionalized or functionalized MWCNTs were added at the experimental groups and incubated for 30 min, 1h and 3h. In order to achieve an optimal dispersion of the MWCNTs in the cell culture media prior to the addition on the cultures all the solutions have been sonicated. Three different concentrations of MWCNTs suspensions have been evaluated: 12.5 μg/ml, 50 μg/ml and 100 μg/ml, that were selected according to the study of Kim, et al., 2016 [20]. A screening test was first conducted to determine the treatment concentrations for the cytotoxity testing. Since MWCNTs were able to be dispersed only below 0.5% (w/v), the concentration of 100 μg/mL was selected for the highest treatment and afterwards the dilutions to 50 μg/ml and 12.5 μg/ml took place. Cells were also seeded on the tissue culture plastic as control samples (TCP). All the experiments were performed using cells between the 4^{th} and the 5^{th} passage, in triplicate in three independent runs.

2.4 Optical Microscope Monitoring

Cell cultures have been monitored in a Zeiss Axiover 40 CFL optical microscope after seeding to detect the state of the cells and any possible bacterial infection.

2.5 MTT Proliferation assay

Cell proliferation was assessed using the MTT photometric methods. Briefly, cells in phenol-free media were incubated with 5μg/ml solution of the MTT reagent for 2 h. Following, they were visualized into a bright field inverted microscope for verification of purple formazan crystals. The media was removed and the insoluble formazan crystals were solubilized into

isopropanol acidified with 1% HCl. The absorbance was then measured at 570 nm and 690nm using a spectrophotometer.

2.6 LDH Toxicity Assay

Pierce Lactate dehydrogenase (LDH) Cytotoxicity Assay kit (Thermo Scientific, USA) was selected to evaluate MWCNTs mediated cytotoxicity. LDH is a glycolytic enzyme (it catalyzes the reversible conversion of lactate in the presence of NAD+ to pyruvate and NADH + H+) found in almost every tissue including lungs. Usually, the reduction of intracellular LDH and its release into the extracellular medium is a sensitive indicator of non-reversible cell death because of damage to cell membrane. In detail, when A549 cells are incubated with MWCNTS, the plasma membrane damage releases LDH into the cell culture media. The amount of released LDH can be quantified by a coupled enzymatic reaction in which LDH catalyzes the conversion of lactate to pyruvate via NAD+ reduction to NADH. Diaphorase, then uses NADH to reduce a tetrazolium salt (INT) to a red formazan product that can be measured at 490nm. The level of formazan formation is directly proportional to the amount of LDH released into the medium. 50µL of the supernatants of cell culture medium of all the experimental groups, were collected for LDH activity measurement assay using a UV–visible spectrophotometer at the 440 nm absorbance [21].

2.7 Live/Dead cells staining and quantification

In order to detect cell viability, the experimental graft powder materials were double stained with Fluorescein diacetate and Propidium Iodide stains (FDA/PI). FDA/PI solution was added to stain live and dead cells, respectively, and solution was allowed to stand at room temperature for 5 min in the dark.

2.8 Statistical analysis

Statistical analysis was performed using the Graph Pad prism software using One Way Anova Test. P-values of <0.05 were considered statistically significant.

2.9 Scanning Electron Microscopy (SEM) (Cell morphology observation)

Scanning Electron Microscopy (SEM) has been employed to monitor A549 cells' morphology and structure after being in contact with MWCNTs for 30, 60 and 180 min. Prior to SEM analysis of cells' morphology on the culture surface, cells were fixed and stabilized with 2% glutaraldehyde in a sodium cacodylate 1M solution. Fixed cells were incubated in a solution of osmium tetroxide in 1% sodium cacodylate for 60min at 4°C and then dehydrated in ascending concentrations of ethanol up to 100%. The samples were then mounted on SEM stubs, sputter coated with Au and observed under SEM microscope at 5 kV.

3. Results & Discussion

3.1 Characterisation of MWCNTs

The surface morphology of the as-produced MWCNTs (pristine) was initially evaluated through SEM. From Figure 2 SEM micrographs it can be remarked that the produced MWCNTs are entangled, while they are thin, with a diameter range between 20nm to 50nm. This is a narrow range distribution, proving the homogeneity of the nanomaterial. However, in our preparations, agglomerates of amorphous carbon are also evident, in the form of "sponge-like" structures, indicating the need of the purification steps which were executed prior to functionalisation.

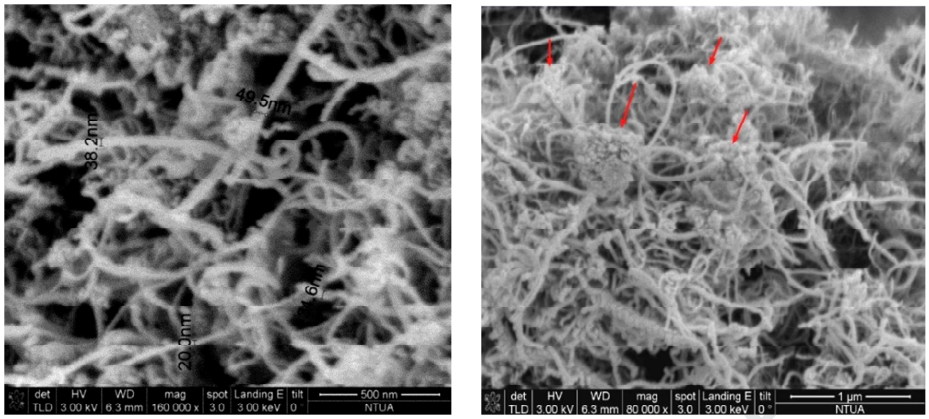

Figure 2: SEM images of pristine MWCNTs.

To prove the effectiveness of the purification procedure EDS analysis took place. As it may be seen from Figure 3, the weight percentage of Fe has been reduced to 1.27% from 3.08% in the sample. Additionally, due to the thermal oxidation, oxygen has been increased to 8% wt.

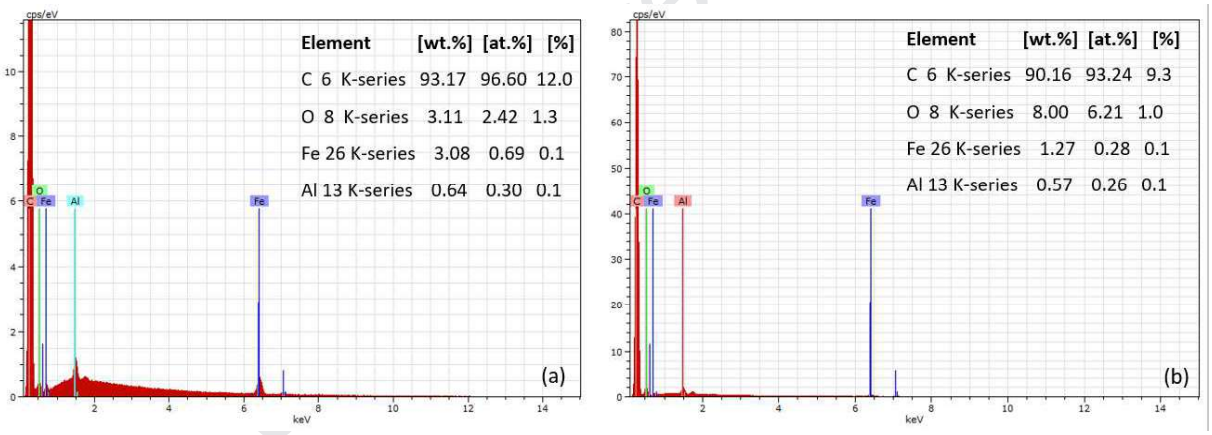

Figure 3: EDS analysis of MWCNTs prior (a) and after (b) their purification.

In Figure 4, the Raman spectra of the pristine (black line) and functionalized (red line) CNTs are depicted. In these spectra, the two main graphite peaks can be observed: the first one at 1580 cm^{-1} that is assigned to the in-plane vibration of the C–C bond (G band) and the second one at ~1350 cm^{-1} (D band) due to the presence of disorder in CNTs. Additionally, the Raman spectra also exhibit a band at ~2700 cm^{-1} that can be attributed to the overtone of the D band (2D band).

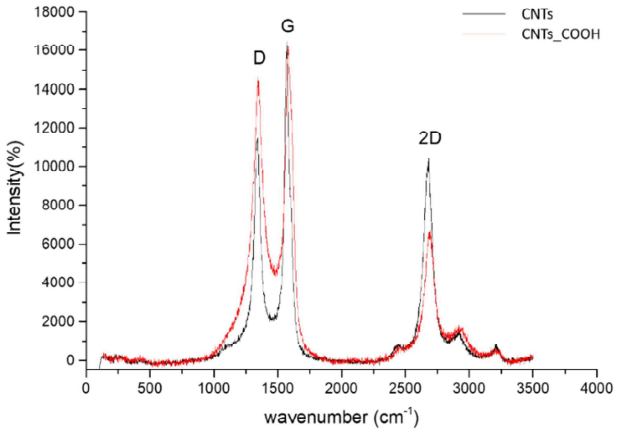

Figure 4: Raman spectra of pristine and functionalized MWCNTs.

Prior to the functionalisation, the G and D peaks are separated and individually standing, however it is evident, that after this procedure, the two peaks become consolidated on their base. Moreover, D peak has increased its height, thereby indicating that defects have been embedded on the MWCNTs surface, as a result of the functionalization process (both the thermal and chemical oxidation). In addition, 2D peak has decreased. Nevertheless, G peak remains the same, which means that the structure of the MWCNTs has not been altered after the treatment. The I_D/I_G ratio which is calculated from the intensities of D and G band receptively, indicates the quality of the MWCNTs. For the pristine sample, it has been calculated 0.67 (<1), while for the chemically functionalized ones1.09 (>1), thus confirming the addition of oxygen groups.

In order to quantify the oxygen groups' attachment on the MWCNTs surface, XPS analysis was exploited. Even though in the functionalized sample, a decrease of total atomic % of oxygen is observed, an increase in the oxygen peak is also evident, attributed to an incrase of C-O bonds. An increase on the atomic % of C (increase of C-C bonds) is also recored for this sample (Figures 5, 6).

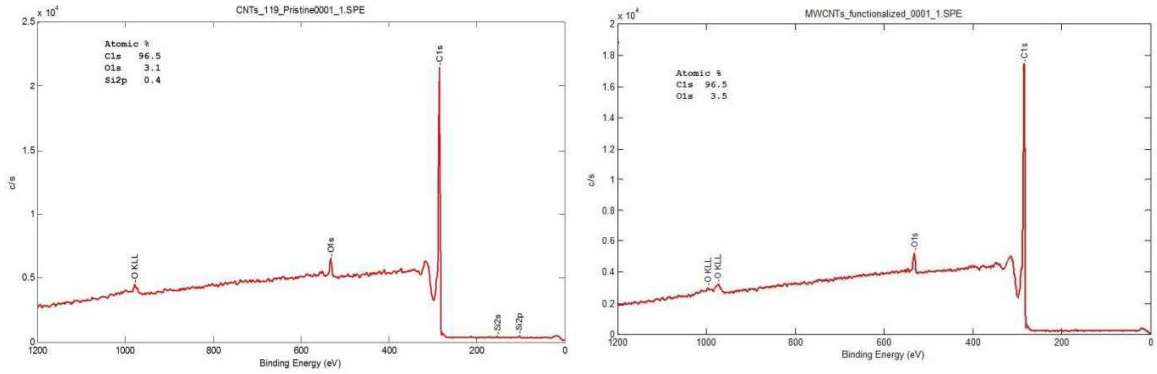

Figure 5: XPS spectra of pristine (left) and functionalized (right) MWCNTs.

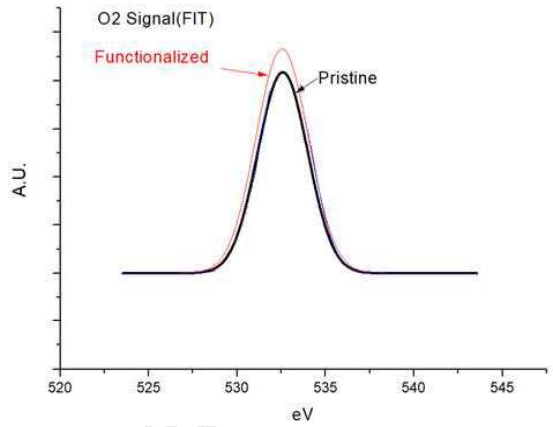

Figure 6: Overlapping of fit curves for oxygen peaks.

3.2 MTT and LDH Assays

Due to the unique chemical properties of CNTs promoting interactions with the 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide and as it is reported by a Belyanskaya, et al. [22], it was decided to perform both MTT and LDH assay. Despite the fact that several concerns have been raised in the literature [23, 24], regarding MTT's applicability for studying toxicity of CNTs, a fully controlled protocol was followed in the present study to avoid any unwanted interferences. Indeed, MTT and LDH assays diagrams (Figure 7 and 8) revealed a time-dependent, but not dose-dependent toxicity of the MWCNTs to the adenocarcinoma cells. However, there were no significant statistical differences between absorbance values for MTT test recorded from cells that remained in contact both for pristine and functionalized MWCNTs for shorter time (30 min and 1h), whereas after 3h incubation with pristine MWCNTs, a dose-dependent (and statistically significant) trend in the loss of

viability ($p<0.05$) as compared to untreated cells, was evident. This effect was marked at concentration of 100 μg/m ($p=0.002$).

Figure 7. Graphic representation of the MTT assay for all the groups for 30 min, 1h and 3h of culture.

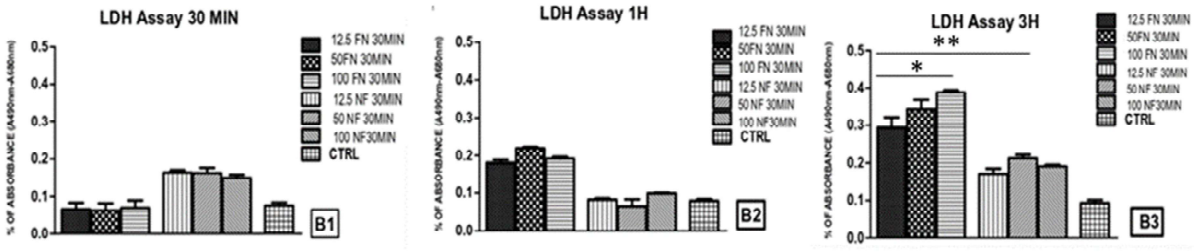

Figure 8. Graphic representation of LDH activity for all the groups for 30 min, 1h and 3h of culture.

Membrane lysis, as revealed by LDH test showed very acute and statistically significant effects on cells from functionalized MWCNTs, whereas an increase in LDH was observed with pristine MWCNTs, after 3h of incubation, revealing a dose-related trend. These results clearly demonstrate that functionalized MWCNTs are highly cytotoxic (LDH assay) even at the early stages of contact with cells, namely upon acute exposure, while pristine MWCNTs require further contact with cells to demonstrate their cytotoxic effects. This has been evidenced also in the study of Fountoucidou et al, where it appeared that toxic effects in low concentrations may be evident after long-term exposure [25].

The early bioreactivity exhibited in the case of the functionalized MWCNTs as well as their higher cytotoxicity, compared with pristine MWCNTs, may be attributed to: i) the higher surface reactivity of functionalized MWCNTs that leads to induction of their toxicity on the population of cells with which they come into contact; ii) the fact that pristine MWCNTs

require a more extended contact with cells to exert their toxic effects; iii) the tendency of A549 cells to grow as non-confluent cells; thus the likelihood of being targeted by MWCNTs depends on the dispersibility (single tubes vs tangled) and the density of MWCNTs, affecting their sedimentation rate, and subsequently the possibility to come into contact with cells. The above variability is not considered in common assays, that are designed to assess late effects on both the viability and vitality.

Nevertheless, both from the MTT and LDH assay results, indications on the physiological impact of the concentration of MWCNTs on the alveolar epithelial cells could be extracted. Functionalized MWCNTs induced a remarkable damage in terms of cytotoxicity after 3h of cultivation only at the concentration of 100 μg/ml, while the impact of both pristine and functionalized MWCNTs at the lower concentrations after 30 min and 1h of exposure, followed almost the same pattern. As it has been reported by Zhou et al. [26], the threshold dose of MWCNTs, either pristine or functionalized ones, is still ambiguous, thus instead of the concentrations and the time points that have been used in the present study, any other combination could be employed with similar results. Indeed, the results of toxicological studies on CNTs may vary, since they are influenced by several factors such as CNTs dimension, dispersion in the cell culture media and cell line used [27].

3.3 Live/Dead cells staining and quantification

The results obtained by the double staining (FDA/PI) process of the cells, where contradictory compared to the results of viability and toxicity assays, in accordance with similar studies [19]. Fluorescence microscope images indicate a toxic behavior of MWCNTs, since population of viable cells is decreased as the time in contact with MWCNTs is increased. However, in all end-points examined (data non-shown), there are only a few apoptotic cells detected, possibly due to agglomerates existing in the medium.

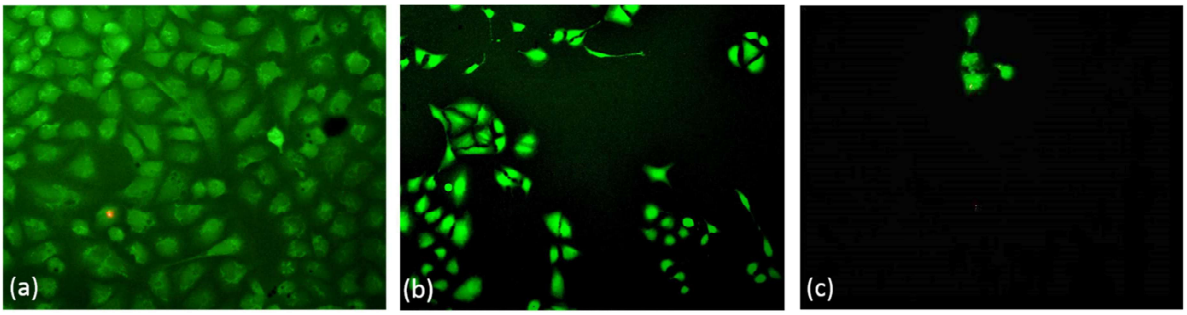

Figure 9. Indicative pictures of fluorescent microscope showing cells double stained with FDA/PI, in contact with a suspension of 100 μg/ml of functionalized MWCNTs for: (a) for 30min, (b) for 1h and (c) for 3 h.

These results may be also attributed to the fact that the apoptosis rate of the A549 cells that are in contact with 100 μg/ml MWNCTs, could be affected by the mitochondrial damage caused upon exposure to MWCNTs, which is in line with the MTT assay results. Although, the proliferative rate of these cells is obviously perturbed, A549 cells did not undergo apoptosis seeking to maintain their proliferative rate in an effort to repair the MWCNTs induced damage [28, 29].

It is noteworthy to mention that with this method, the results acquired are indicative since only a specific spot of the surface culture is monitored and additionally the acquired information refer to the presence of either viable or dead cells. In this context and in an effort to identify the possible toxic effect of MWCTNs to cells morphology, optical observation (through SEM) of cells is employed.

3.4 Cell morphology observation

As already mentioned, cell attachment on the culture surface and cell morphology at three end-points was assessed through SEM analysis. SEM micrographs presented in Figure 10, demonstrate the typical morphology of the A549 cells, seeded on the cell culture plate without any contact with MWCNTs, corresponding to the control (TCP) sample. Specifically, in Figure

10b, the typical morphology of the microvilli in the surface of the lung adenocarcinoma cells can be clearly observed.

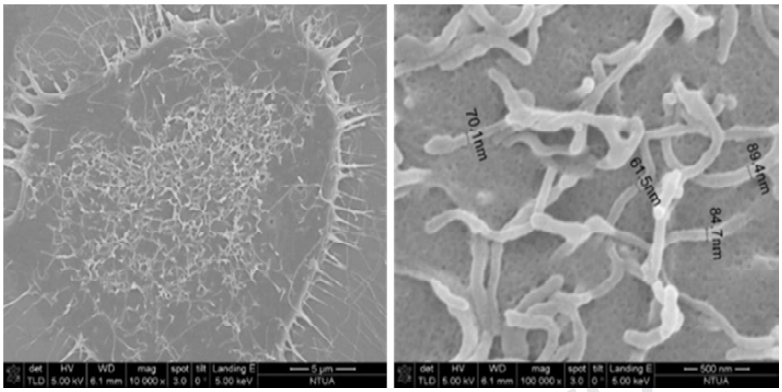

Figure 10. SEM micrographs of control samples (cells seeded on the cell cultures treated plastic). Panel A: cell morphology of a A549 alveolar cell; Panel B: typical morphology of the microvilli in the surface of the alveolar A549 cell.

Figure 11 includes the SEM micrographs of the A549 cells that have remained in contact for 30 min, with 12.5 μg/ml, 50 μg/ml and 100 μg/ml respectively of both non-functionalized MWCNTs and carboxyl-functionalized ones. For this time interval, the differences that have been spotted on the cells' morphology, can be mainly attributed to the increased number of functionalized groups, rather than the increasing dose of the of MWCNTs.

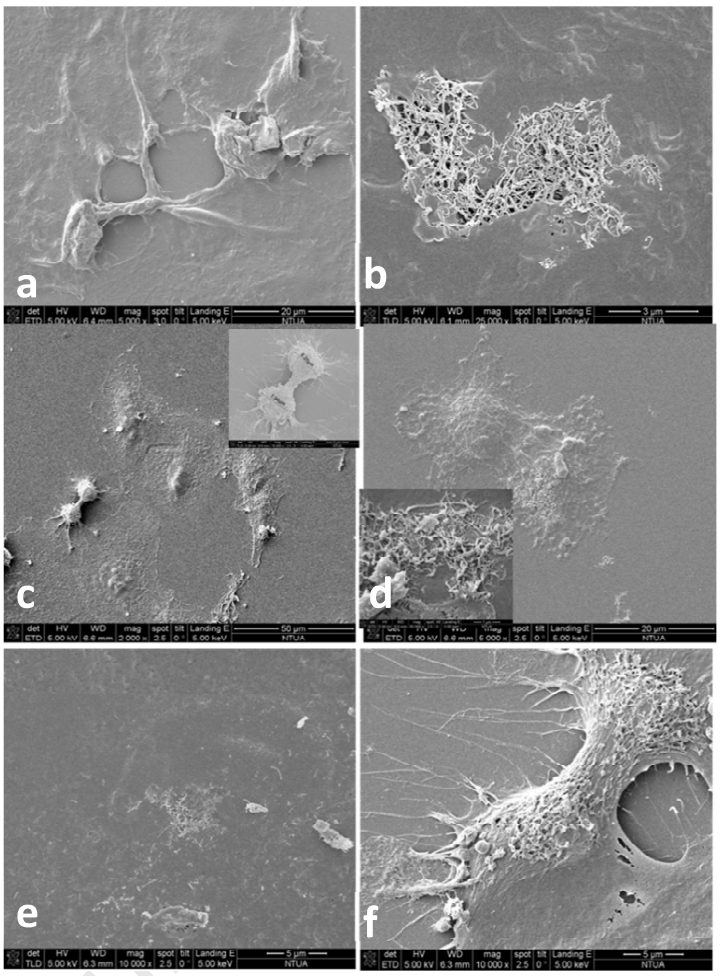

Figure 11. SEM micrographs showing cells morphology in contact for 30 min with a suspension of 12.5 μg/ml of (a) not functionalized MWCNTs and (b) functionalized MWCNTs; a suspension of 50 μg/ml of (c) not functionalized MWCNTs and (d) functionalized MWCNTs; a suspension of 100 μg/ml of (e) not functionalized MWCNTs and (f) functionalized MWCNTs.

By increasing the time of contact and the concentration of MWCNTs from 12.5 μg/ml to 100 μg/ml, it can be observed that a firm contact between MWCNTs and the cells' plasma membrane is established. By increasing contact time, initially, the number of microvilli after 1h in contact is decreased (data non-shown) and after 3h microvilli are totally absent (Figure 12). SEM micrographs also revealed that the interactions of cultured cells with MWCNTs, both in the case of functionalized and pristine ones, led to a negative effect on their morphology. Indeed, cells' morphology appeared altered and distorted compared to the morphology of the cells in the

control sample (Figure 10), thus indicating that the presence of MWCNTs negatively affect cell viability, since cells morphology is strongly correlated with cell functions (proliferation, differentiation).

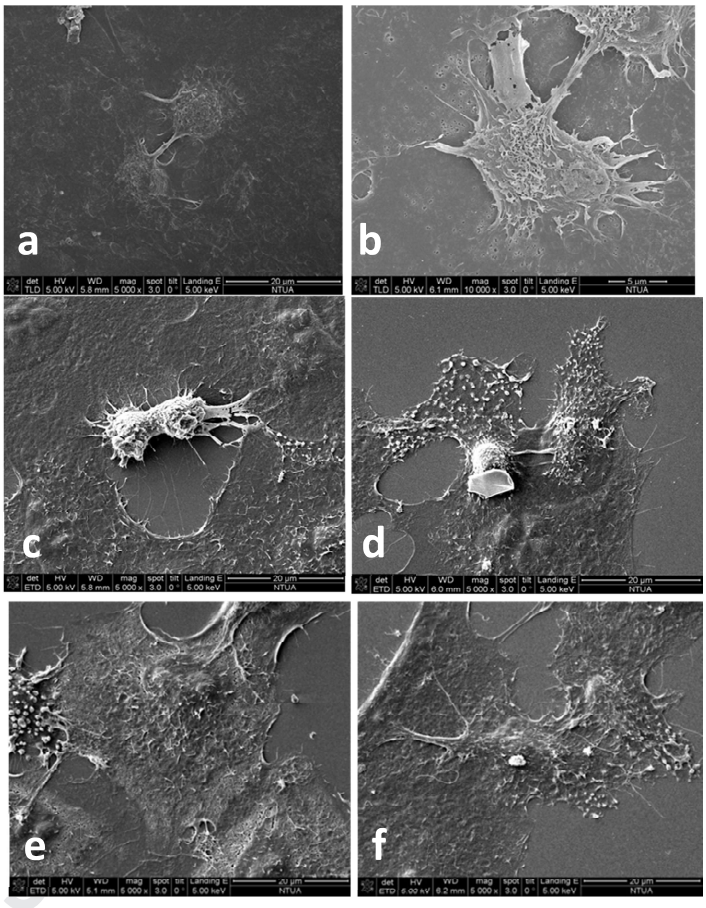

Figure 12. SEM micrographs showing cells morphology in contact for 3h with a suspension of 12.5 μg/ml of (a) not functionalized MWCNTs and (b) functionalized MWCNTs; a suspension of 50 μg/ml of (c) not functionalized MWCNTs and (d) functionalized MWCNTs a suspension of 100 μg/ml of (e) not functionalized MWCNTs and (f) functionalized MWCNTs.

From Figure 12, it can be clearly seen that MWCNTs established unyielding contacts with the cellular membrane and as the time that cells remained in contact with MWCNTs increased, the cellular membrane started to break down, suggesting that cells have initiated their apoptotic process. In the case of not functionalized MWCNTs this effect was mild, which can be attributed to the absence of the carboxylic groups (present in functionalized MWCNTs). Finally, a

reduction in cell population, both for the case of functionalized and not MWCNTs, has been reported, as empty spaces among the cultured cells can be spotted in SEM micrographs (Figure 12).

The described investigation of the upstream damaging mechanisms, led to the conclusion that the functionalization with carboxylic groups can increase the bioreactivity of MWCNTs towards A549 alveolar cells since their early contact with cell wall. SEM observation of cells morphology clearly reveals the negative effect of MWCNTs on cell viability, mainly arising from the established unyielding contacts between MWCNTs and the cell surface. Especially, in the case of functionalized MWCNTs, where the nanomaterial is strongly integrated in the cellular membrane, further toxicity can be predicted on this morphological basis [30].

Indeed, when nanomaterials (including CNTs) enter into the human body, they come into contact with biological fluids and due to their high surface free energy, they absorb proteins and other biological constituents thus forming a nanomaterial-protein complex, also known as protein corona. The fate and the possible consequences of unintentional exposure to nanomaterials depend on the type and the binding efficiency with proteins, defining the biological entity of MWCNTs [31, 32]. Typically, proteins with high affinity to bind with MWCNTs are potential targets after exposure, resulting in disruption of their conformation and functions [33].

The results of this study, reveal that the strong physical interaction of MWCNTs with cells' membrane, suggests a direct link between MWCNTs binding affinity and cytotoxicity and indicate that epithelial functions, such as control of surfactant production and secretion, could be affected [34]. It is thus concluded that the physico-chemicals characteristics of MWCNTs are key determinants of their interactions with cells and subsequently determine the level of their cytotoxic behavior [35].

4. Conclusions

The results of this experimental study confirm previous studies and show a time- and dose-depended toxicity of MWCNTs with significant difference between pristine and functionalized MWCNTs, thus suggesting that harmful side effects to human health may occur upon acute exposure. It can be estimated that exposure to MWCNTs will not have acute toxic effects to human organism, nevertheless prolonged and repeated exposure may cause undesired health implications.

The results obtained by SEM revealed the damaging effect on the cell plasma membrane. By increasing the time in contact and the dose of functionalized MWCNTs, the surface of the cells showed overt morphological changes with potential functional consequences. Specifically, it has been spotted that MWCNTs have been totally engulfed by the cells.

Although there are several studies performed in order to test the bioreactivity and toxicity of CNTs, conflicting results have been obtained. The research regarding the carbon-based nanomaterials lacks of international standard protocols, claiming the need to generate technical guidelines regarding the characteristics of the fibers and cell types as well as organisms a priority. Defined standard CNT structures should be proposed as golden standards, considering the impurities continent and their chemical and structural characteristics (layers number, shape, length, and agglomeration).

Identification of potential hazards, arising from the use of MWCNTs, will favor the use of this type of nanomaterials, by illuminating the uncertainties that currently characterize them. Subsequently, “safe” manufacturing routes will be established, in order to utilize MWCNTs without affecting the reputation of the final products, thus facilitating their market penetration.

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