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Synthesis, characterization and controlled toxicity of a novel hybrid material based on cisplatin and docetaxel

Research Article

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Abstract: This paper is focused on the synthesis and characterization of a novel hybrid material based on cisplatin and docetaxel-loaded functionalized simultanously carbon nanotubes able to be used in cancer therapy as drug delivery system with controlled toxicity. This material was physico-chemically investigated by determining the structure, as evidenced by Fourier transform infrared (FTIR) spectroscopy, transmission electronmicroscopy (TEM) and its stability was studied with the aid of thermogravimetric analysis (TGA). The amount of platinum ions released into the solution of simulated body fluid (SBF) was highlighted by coupled plasma mass spectrometry (ICP-MS). Toxicology experiments were performed with MDA-MB 231 breast cancer epithelial cells. The performance of the new drug delivery hybrid material was compared with functionalised carbon nanotubes with therapeutic agents functionalized with a single therapeutic agent.

Keywords: *Carbon nanotubes • TGA • Functionalization • Drug • Cell viability*

1. Introduction

Breast cancer seems to be the most common cancer in women all over the world in the last decade [1]. Being so agressive for both incidence and mortality, research concerning breast cancer therapy has been widely developed in the last decade [1,2]. The research was focused mainly on the common types of treatment for different forms of cancer, such as surgery, radiotherapy and chemotherapy, but has also involved less common therapies, such as thermotherapy, immunotherapy, and targeted therapy [3]. The treatment modalities were able to partially alleviate the burden of this terible disease, but much more needs to be done in complementary fields, including materials as therapeutic agents, new systems for drug delievery, *etc*. [4,5] and this fact enhances research in the field. The therapeutic agents can be divided into various categories: alkylating agents, antibiotics which attack nucleic acids, platinum derivative agents as cisplatin, carboplatin or oxiplatin, mitotic inhibitors, antimetabolites, camptothecin type agents, and biological response modifiers. In this idea targeting specific carriers [6] to achieve higher therapeutic efficacy is gaining more importance in the pharmaceutical field and specifically, the nanohybrid compounds with potential sinergetic effects is posed to edge over chemotherapy [7].

An important part of efficacy agents such as the alkylating agents, the tumor targeting antibiotics, and the platinum compounds, is that they act by generating cellular damage by initiating free radical formation, the altered redox status and the presence of reactive oxygen

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species (ROS) being common biochemical aspects in cancer cells [8]. Oxidative stress is generated by an imbalance favouring prooxidants and/or disfavouring antioxidants, potentially leading to cellular damage and its presence in cancer cells could have important treatment implications [2,9] and allow the development of novel therapeutic strategies [10].

In recent years, lots of nanomaterials were developed and some of the most promising and exciting applications involve their use in cancer therapy. The nanoscale systems have proved to be efficient carriers for drugs to specific tissues or cell populations due to decreased deleterious side effects. In this context, carbon nanotubes (CNTs) are considered possible components of delivery systems [4,11], due to their transporting capabilities and their physicochemical properties. Over the past decade, such properties resulted in the use of CNTs in many other important bioapplications, such as antibacterial studies [12] and biosensors [13].

CNTs are formed by thin sheets of benzene ring carbons rolled up into the shape of a seamless tubular structure. They belong to the family of fullerenes, the third allotropic form of carbon, along with graphite and diamond [14] and present properties such as ultralight weight, high mechanical strength, as well as high electrical and thermal conductivity [15]. According to their structure, CNTs can be single-walled (SWCNTs) and multi-walled (MWCNTs). The appropriate functionalization of SWCNTs and MWCNTs allows a better biocompatibility and dispersability [8,16]. There are two main ways to increase their water miscibility; covalent and noncovalent functionalizations. In the search for new cancer drugs, both approaches have been widely used, and functionalized nanotubes have been loaded with various molecules to generate new hybrid materials as drug delivery systems [11,17].

Double functionalization via oxidation and introduction of an anticancer agent was firstly tested in nanotube cancer therapy with cisplatin [12], but soon after, carboplatin and doxorubicin were also investigated [13,14]. In our previous paper [18], our goal was to introduce docetaxel in SWCNTs and MWCNTs, and to estimate encapsulation efficiency, which was better for MWCNT-COOH. According to known data, both types of CNTs had positive impacts on cell proliferation and differentiation [2,3,19].

It has been shown that two therapeutic compounds, cisplatin and docetaxel, carried by MWCNT-COOH, had positive impacts on cell proliferation and differentiation [2]. However, their effect on cell death and potential usage in cancer treatment are still absent. The present paper is focused on the synthesis, characterization and controlled toxicity of such a drug delivery system simultaneously containing cisplatin and docetaxel loaded on functionalized carbon nanotubes (MWCNT-COOH).

The aim of this paper was to investigate the *in vitro* response of a cancer breast cell line, MDA-MB 231, to exposure to functionalized CNTs and citostatic dopped functionalized CNTs.

2. Experimental procedure

2.1. Synthesis

Multi-walled Carbon Nanotubes (MWCNTs) were purchased from Sigma Aldrich having more than 90% carbon basis and D×L 10-15 nm × 0.1-10 μm, produced by Catalytic Chemical Vapor Deposition (CCVD). Functionalization via oxidation was achieved by their dispersing (3.0 g) in 8% sulfuric acid and ultrasonication at 50°C for 48 hours, to afford MWCNT-COOH [20]. The drugs (cisplatin and docetaxel) and 7% NaCl solution were purchased from the pharmaceutical markets. Cisplatin (CDDP, 20 mg) was dispersed in saline solution (5 mL) and then MWCNT-COOH (0.5 mg) was added. Docetaxel (DOX, 20 mg) was dispersed in saline solution (10 mL) and then MWCNT-COOH (0.5 mg) was added. Subsequently, both suspensions were ultrasonicated for 48 hours at 50°C and filtered.

2.2. Characterization methods

For structural characterization FT-IR spectra of functionalized MWCNTs with cisplatin and docetaxel were obtained with a Perkin Elmer, Spectrum 100 in the 400-4500 cm^{-1} range with 4 cm^{-1} resolution and 32 scans.

Nano-sized particles were investigated using transmission electronic microscopy (TEM) analysis [21,22] with a microscope (Philips EM-410, 60 kV).

Inductively coupled mass spectrometry (ICP-MS), (ELAN DRC-e Perkin Elmer SCIEX U.S.A. with the detection limit of 0.001 μg g^{-1}) was used for platinum release determinations over the time course of the experiment.

The stability of the hybrid materials was evaluated using thermogravimetric analysis (TGA) curves (Q 500 TA Instrument), under a nitrogen atmosphere using a heating rate of 10ºC/min from room temperature to 850ºC.

2.3. Cell culture and treatment

MDA-MB 231 epithelial cells (a cell line derived from a human breast adenocarcinoma (ATCC, Catalog No. HTB-26) (Rockville, MD)) were cultured in Dulbecco's

Modified Eagle Medium (DMEM medium) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, California, USA) and 1% antimycotic solution (Sigma, St. Louis, Missouri, USA) and replaced every 3 days. The exposure to doses of 0.5, 2.5, 5 and 10 μg mL^{-1} of MWCNT-COOH, CDDP dopped MWCNT-COOH, DOX dopped MWCNT-COOH and CDDP-DOX dopped MWCNT-COOH of these cells were done for 24 and 72 hours at 37°C in the presence of 5% CO_2. UV exposure for 30 minutes was utilized as a sterilization method for MWCNTs suspensions. Unexposed MDA-MB 231 cells were used as controls.

2.4. Cell viability

Cell viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric technique [23-26] in a 96 well plate. After treatment, the medium from each well was removed and the cells were washed once with 100 μl of phosphate buffer saline (PBS)/well. Then 50 μL of 1 mg mL^{-1} of MTT solution was added in each well and the reulting solutions were incubated for 2 h at 37°C.

After the MTT solution was removed from each well, a volume of 50 μL isopropanol was added in order to solubilize formazan crystals and the optical density of each well was determined at 595 nm using a Tecan multiplate reader (Tecan GENios, Grödic, Germany). The quantity of formazan is directly proportional to the number of living cells in culture. The absorbance for unexposed MDA-MB231 cells to MWCNTs was taken to represent the 100% viability.

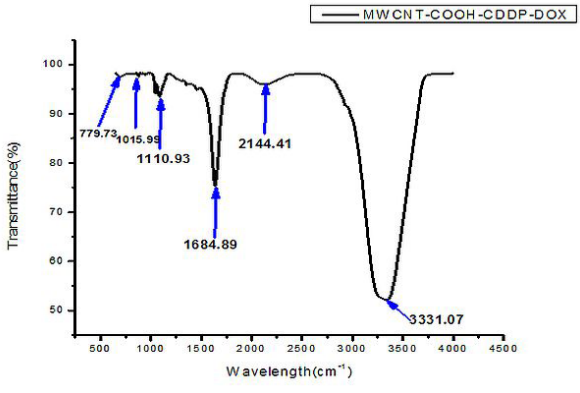


Figure 1. Infrared spectra of novel hybrid material MWCNT-CDDP-DOX.

2.5. Statistical analysis

For the statistical analysis of the MTT assays [27], one-way ANOVA with Bonferroni's multiple comparison tests was performed. All values are expressed as mean value±standard deviation of three independent experiments.

3. Results and discussion

3.1. FT-IR measurements

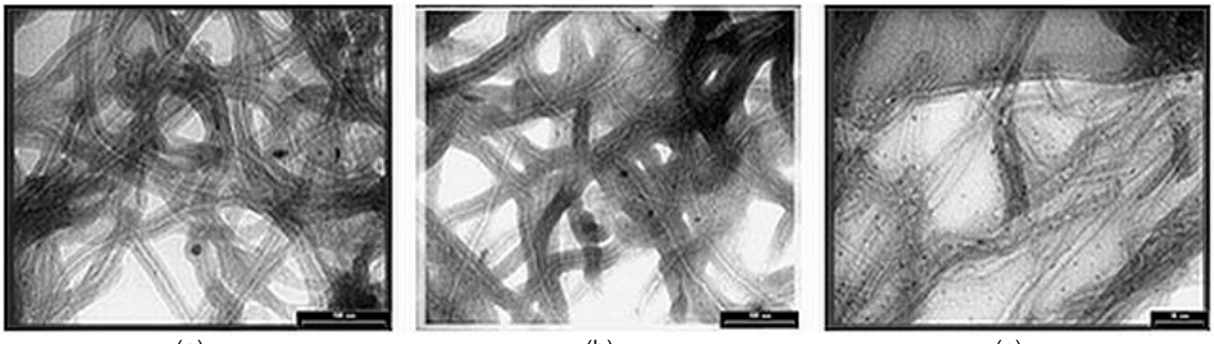
FT-IR spectroscopy is a very useful tool to show the presence of functional groups on the surface of MWCNTs, the bands allocated to groups: -CH, -C=C, -C=O, DOX and CDDP bands functionalized samples to control samples range between 3317.23 to 779 cm^{-1}. (ForMWCNTs-COOH range: 1098.86 to 597.43 cm^{-1} for CDDP range: 925.99 to 825.98 cm^{-1} for DOX) as shown in Fig. 1 and Table 1, where a comparison of the structure of functionalised carbon nanotubes with the therapeutic agents cisplatin and docetaxel is presented. It should be noted that the band at 1,745 cm^{-1} is attributed to the carbonyl stretching vibration in the carboxyl groups. The broad absorbance band about 1,300 cm^{-1} is due to O–H bending deformation in –COOH, whereas the enhanced and broaden peak at 3,131 cm^{-1} is assigned to O–H stretching vibration in the carboxyl groups.

3.2. TEM measurements

In Fig. 2, TEM images of nanosized particle morphologies for functionalized MWCNT, *i.e.*, MWCNT-COOH-CDDP (Fig. 2a), MWCNT-COOH-DOX (Fig. 2b), MWCNT-COOH -CDDP-DOX (Fig. 2c) are presented and the new hybrid components encapsulation are evidenced.

Table 1. The comparison of the structure of functionalised carbon nanotubes with various therapeutic agents.

	MWCNTs-COOH-CDDP	MWCNTs-COOH-DOX	MWCNTs-COOH-CDDP-DOX
Corespondence	Wavenumber, cm^{-1}	Wavenumber, cm^{-1}	Wavenumber,cm^{-1}
-OH	3340.97	3325.47	3331.07
-CH2	2142.17	2869.99	2144.41
C=O	1644.93	1697.67	1684.89
O-C	1125	1072	1110.93
CDDP	723	-	779.73
DOX	730.99		



(a) (b) (c)

Figure 2. TEM morphologies for: a) MWCNT-COOH-CDDP, b) MWCNT-COOH-DOX, c) MWCNT-COOH-CDDP-DOX.

The grains of cisplatin are in a range of 4-6 nm in width and 100 nm in length and those of docetaxel are about of 4-6 nm in width and 50 nm in length . The surface is quite homogenous.

3.3. ICP-MS measurements

The samples were introduced in SBF (from simulated body fluid) and the suspensions were analyzed using an *in-situ* nebulizer/vapor generator sample introduction system. The conditions were selected in order to maximize the platinum ion signal. The generated vapor was then transported to the ICP-MS for platinum determination.

According to Fig. 3, a range of platinum ion release could be established, the highest value for platinum ions release being observed for the CDDP sample and the lowest one registered for MWCNTs-COOH-CDDP-DOX sample. The amounts of platinum ions release varied between 26.16 and 152.2 μg Pt per 100 μg MWCNTs according to ICP-MS determinations for all hybrid materials.

3.4. TGA measurements

The presence of drug molecules (DOX, CDDP) within host carbon nanotubes (MWCNT) was also demonstrated by thermogravimetric analysis.

Fig. 4 shows the TGA curves of oxidized MWCNT and drug dopped oxidized MWCNT. It is apparent that the weight loss for oxidized MWCNT (MWCNT-COOH) and modified MWCNT is different. In comparison with MWCNT-COOH, the modified MWCNTs with drug molecules (DOX, CDDP) exhibit an increase of weight loss (27% for MWCNT-COOH-CDDP, 71% for MWCNT-COOH-CDDP-DOX and 76% for MWCNT-COOH-DOX) which could be assigned to the degradation of organic molecules (drugs) attached onto the MWCNTs surface or trapped in the nanotubes. The TGA results showed that the thermostability of modified MWCNT depends on the drug type. Thus, the thermostability of the system based

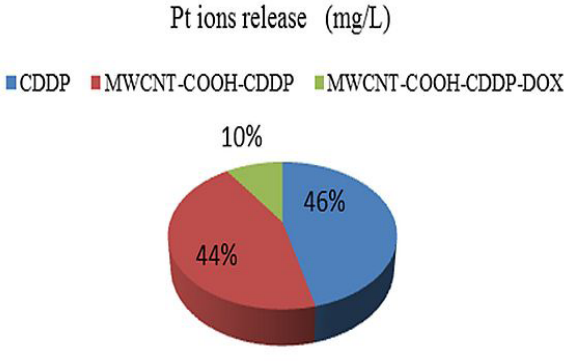


Figure 3. Pt ions release from CDDP, MWCNT-COOH-CDDP, MWCNT-COOH-CDDP-DOX.

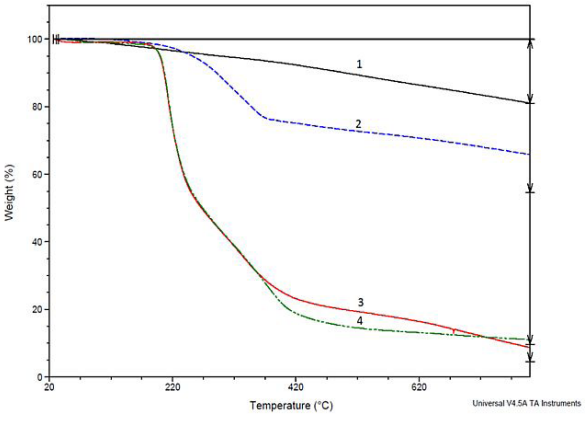


Figure 4. TGA curves of 1: MWCNT-COOH, 2-MWCNT-COOH-CDDP, 3-MWCNT-COOH-DOX, 4-MWCNT-COOH-CDDP-DOX.

on MWCNT-COOH-CDDP is higher in comparison with the MWCNT-COOH-DOX and MWCNT-COOH-CDDP-DOX.

3.5. Analysis of cell viability

The MDA-MB231 cell line was used in order to evaluate the effects of the new composites (Fig. 5).

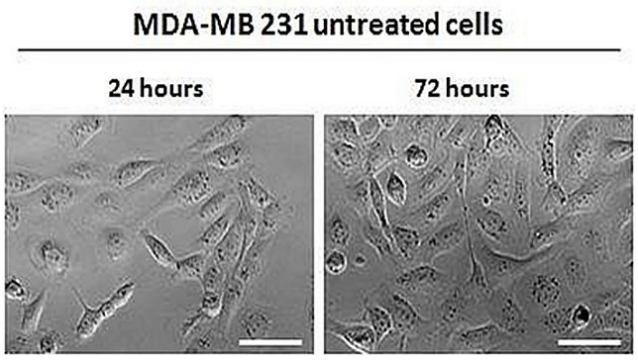


Figure 5. Fields with control cells correspondig to 24 and 72 hours intervals. Images were acquired by a bright field inverted microscope (Olympus IX7), objective 10X, using a CCD video camera COLORVIEW. The scale bar is the same for all images and corresponds to 25 μm.

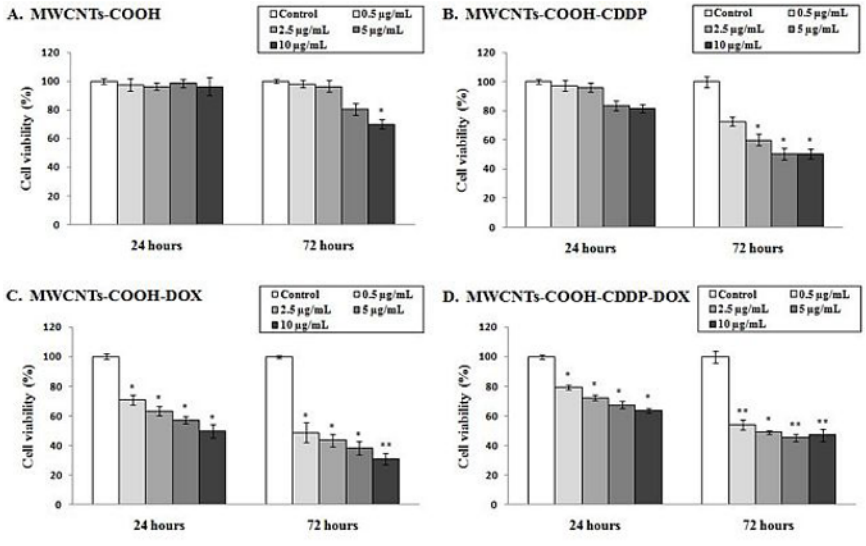


Figure 6. Cell viability. MTT test for MDA-MB 231 cells exposed to (A) MWCNTs-COOH, (B) MWCNTs-COOH-CDDP, (C) MWCNTs-COOH-DOX, (D) MWCNTs-COOH-CDDP-DOX. Values are calculated as means ± SD (n = 3) and expressed as % from controls. *P <0.05, **P < 0.01 vs. control.

The therapeutic efficacy of the drug loaded MWCNTs complexes was evaluated by an *in vitro* cell viability assay. After 24 hours of exposure, a significant loss in viability, in a dose-dependent manner, was observed only in MWCNT-COOH-DOX and MWCNT-COOH-CDDP-DOX treated cells. At the highest dose, this parameter was diminished by 50% in MWCNT-COOH-DOX exposed cells and by 38% in those treated with MWCNT-COOH-CDDP-DOX, whereas MWCNT-COOH and MWCNT-COOH-CDDP did not induce significant cytotoxic effects compared with control.

After 72 hours of treatment, the level of cell viability was significantly reduced in breast cancer cells exposed to MWCNT-COOH-CDDP, MWCNT-COOH-DOX, and MWCNT-COOH-CDDP-DOX. Our results showed that MWCNT-COOH were less toxic in comparison with the other composite nanomaterials for the tumoral cells, a significant decrease of viability by about 30% being registered only for the dose of 10 µg mL^{-1}. In MDA-MB 231 cells, the level of cell viability decreased significantly after exposure to MWCNT-COOH-CDDP at doses of 2.5, 5, 10 µg mL^{-1} by 40%, respectively 50%, compared to control. MWCNT-COOH-DOX decreased the celular viability by 52% for the lowest dose and by 70% for the highest one. Exposure to 0.5, 2.5, 5, 10 µg mL^{-1} MWCNT-COOH-CDDP-DOX decreased MDA-MB 231 cell viability by 46%, 52%, 55% and 53%, respectively (Fig. 6).

Fig. 7 shows that exposed breast cancer cells suffered severe changes in their morpholgical aspect, leading to a necrotic fate. The CNT suspensions presented a low dispersibility in the culture medium, however these results indicate that a stable dispersion is of minor importance for the therapeutic success of the system in cell culture studies.

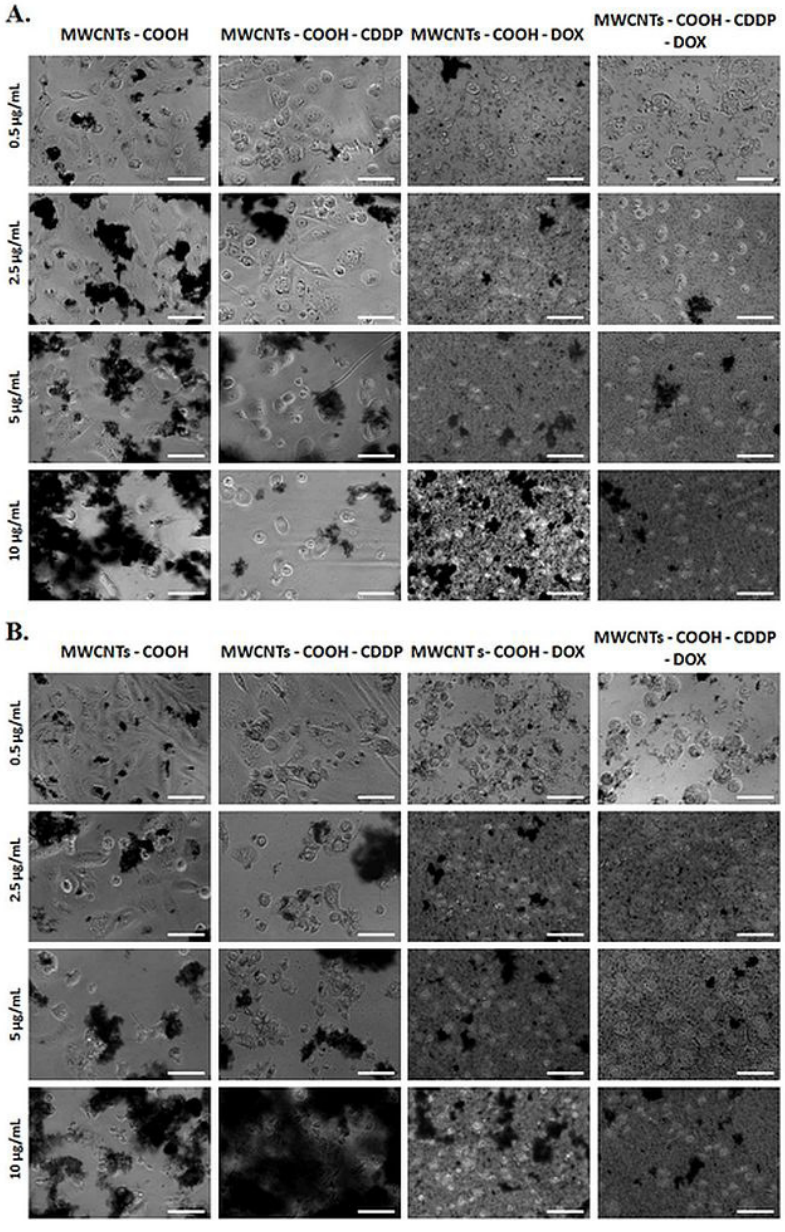


Figure 7. MDA-MB 231 cells exposed to four doses (0.5, 2.5, 5, 10 μg mL^{-1}) of MWCNTs-COOH, MWCNTs-COOH-CDDP, MWCNTs-COOH-DOX and MWCNTs-COOH-CDDP-DOX for 24 (A) and 72 hours (B). Images were acquired by a bright field inverted microscope (Olympus IX7), objective 10X, using a CCD video camera COLORVIEW. The scale bar is the same for all images and corresponds to 25 μm.

According to our data, the eficacy of MWCNT-COOH-CDDP and MWCNT-COOH-CDDP-DOX treatments on MDA-MB 231 cells were similar and slightly lower compared to MWCNT-COOH-DOX.

Previous studies revealed that CNTs induce generation of ROS, decrease the mitochondrial membrane potential and arrest the cell cycle, leading to cell death in different *in vitro* models [28-31]. The type of cell death was considered mainly apoptosis and/or necrosis. It seems that these events occured after physical penetration of the cells by CNTs, and oxidative stress generation via formation of ROS. In contrast, other studies reported that these nanotubes were nontoxic [32]. In the current experiments, MWCNT-COOH showed low toxicity only at the highest dose and time exposure.

On the other hand, acording to Wilson *et al.* [33], incubation of cells with docetaxel leads to the formation of abnormal bundles of microtubules and results in the arrest of cells in the G2/M phase of the cell cycle.

Studies on toxicity of docetaxel also demonstrated its capacity to produce ROS by activating NADPH oxidases and generating mitocondria dysfunction, which in turn triggered activation of caspase-3, leading to apoptosis [34,35].

The other bioactive agent, CDDP is well-known as a DNA-damaging agent and it appeared that apoptosis plays a central role in CDDP-induced death of cancer cells [36].

In our oppinion, the lower cell viability registered for MWCNT-COOH-DOX treatment is due to the cooperative contribution to ROS generation of MWCNT-COOH and DOX. But in the case of MWCNT-COOH-CDDP-DOX, probably the level of DOX is lower in the composite and the effect on cells was similar with that of MWCNT-COOH-CDDP.

4. Conclusions

This study demonstrates the simultaneous encapsulation of docetaxel and cisplatin in MWCNTs with surface functionalized MWCNT-COOH and FT-IR measurements have indicated the structure of the new hybrid material. The cell experiments were performed with the MDA-MB 231 breast cancer epithelial cell line. Promising results were obtained in comparing the efficiency of the new composites after 24 and 72 hours and for various concentrations. *In vivo* studies are essential in order to further validate the efficiency of the reported systems.

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