In vitro Assessment of Functionalized Multiwalled Carbon Nanotubes with Oxaliplatin on Human Cancerous Cells

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Functionalization of multiwalled carbon nanotubes with carboxyl group (–COOH) and oxaliplatin (OX) was investigated using FTIR spectroscopy, high performance liquid cromatography (HPLC), transmission electron microscopy (TEM), inductively coupled plasma mass spectrometry (ICP-MS). Encapsulation efficiency was computed and in vitro behavior regarding MDA-MB-231 cells was studied.

Keywords: carbon nanotubes, oxaliplatin, encapsulation, FTIR, TEM

Carbon nanoarchitecture as nanotubes are nano-scale systems capable of delivering anti-cancer agents [1]. The influence of different types of carbon nanotubes on the synthesis and properties of nanocomposite have been investigated for both, bio and non biointeraction [2]. In addition, these carbon nanoparticles are protected from degradation cancer drugs, increasing the payload of these drugs allows control of anticancer drug release kinetics and significantly improves the solubility of insoluble [3-6]. Biomolecules can be covalently grafted or non-covalently adsorbed on the nanotube surface. In addition, the inner core of CNTs can be exploited to encapsulate drugs or nanoparticles [7-9]. Oxaliplatin was discovered in 1976 [10]. The compound features a square planar platinum (II) center. In contrast to cisplatin and carboplatin, oxaliplatin features the bidentate ligand 1,2-diamino-cyclohexane in place of the two monodentate ammine ligands. It also features a bidentate oxalate group. According to in vivo studies, oxaliplatin fights carcinoma of the colon through non-targeted cytotoxic effects. Like other platinum compounds, its cytotoxicity is thought to result from inhibition of DNA synthesis in cells. In particular, oxaliplatin forms both inter- and intra-strand cross links in DNA, which prevent DNA replication and transcription, causing cell death. Loading and release of chemo-therapeutic agent within multi-wall carbon nanotubes is a topical subject worthy of investigation and in this approach [11], this paper proposes grafting of new antitumor drug oxaliplatin, trying to achieve a comparison based on previous experience [12-14]. The comparison includes in vitro behavior (regarding MDA-MB-231 cells) of the new compound as a novelty as well. The cells response includes viability, superoxide anion detection and Western blot experiments.

Experiment part

Materials and methods

Multi-walled Carbon Nanotubes (MWCNTs) were purchased from DropSens having more than 90% carbon basis and D x L 10-15 nm x 0.1-10 μm, produced by Catalytic Chemical Vapor Deposition (CCVD). Oxidation was made using a mixture of 98% sulfuric acid (Merck). The ethylenediamine (EDA) modifier agent was supplied by Fluka. We have been using saline solution, glucose, oxaliplatin, and all reagents were not further purified. Functionalization of MWCNTs to MWCNTs-COOH was performed according to previous paper [15]. 0.1 mg MWCNT-COOH were ultrasonically prepared with thionyl chloride$SOCl_2$ (50 mL) for 30 min at room temperature. The suspension obtained was refluxed under magnetic stirring at room temperature for 48 h and then filtered. The filtrate was washed with tetrahydrofuran (THF) and dried at room temperature for 20 min. MWCNTs-$SOCl_2$ were immersed in ethylenediamineat room temperature for 10 h. The mixture was washed with etrahydrofuran and filtered. The filtrate was dried at 80°C for 10 h. After this, drugs were added as follows: oxaliplatin (5 mg) in solution (0.5 mL saline solution with 0.5 mL glucose) was dispersed in MWCTs, MWCNT-COOH and in MWCNT-NH_2 and then the samples were sonicated for 48 h at 50°C and filtered**.** Structural IR bands identification has been performed with an Perkin Elmer 100 equipment.

Methods of characterization

For HPLC tests, the column used was C18-reversed phase HPLC, Perkin Elmer SCIEX U.S.A. (5 μm, 150mm x 4.1 mm), which is based on rigid spherical styrene-divinylbenzene copolymer. The solvent mixture consisted of phosphate buffer *p*H 9.6 (10 mM)/acetonitrile (Fisher Scientific) at a ratio of 55:45 containing 3 mg % tetrabutylammonium perchlorate (Sigma Aldrich).The flow rate was 1.0 ml/min. The detection wavelength was 240nm. Nano-sized particles were investigated using TEM analysis with a microscope Philips EM-410, 60kV microscope. ICP-MS analysis was carried out using ELAN DRC-e Inductively Coupled Plasma Mass Spectrometer from Perkin Elmer. The detection limit was 0.001$\mu g.g^{-1}$. The encapsulations efficiency (EE) of platinium-based antitumor drugs into carbon nanotubes were evaluated using equation (1) according to literature [16]. Liu at al. [17] measured the encapsulation efficiency using a similar expression: Encapsulation efficiency

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$$\text{efficiency}\% = \frac{Mtotal\ drug - M\ free\ drug}{Mtotal\ drug} \quad (1)$$

Cell culture and treatment

The human breast cancer cell line MDA-MB-231 was purchased from ATCC (Cat. No. HTB-26™) and maintained in Dulbecco's Modified Eagle's Medium (DMEM; Cat. No. 31600-083), supplemented with 10% fetal bovine serum and 1% antimycotic solution. Cells were cultured in 75 cm^2 flasks and incubated at 37°C in a 5% CO_2 – 95% air atmosphere. Culture medium was changed every 2 days and cells were sub-cultured before reaching confluence by harvesting using a trypsin-EDTA solution. The epithelial cancerous cells were then exposed for 24 h to different concentrations suspensions of MWCNT-COOH and MWCNT-NH_2 functionalized or not with oxaliplatin. Unexposed cells have been used as controls.

Cell viability assay

The MTT test [18] was used in order to evaluate the effects of carbon nanotubes and oxaliplatin drug on cell viability. To evaluate cytotoxicity of each suspension, MDA-MB-231 cells were seeded into 96-well plates at a density of 4 x 10^4 cells/cm^2 and left to incubate for 24-48 h in order to obtain exponential growth, after whichdifferent doses of aminated and carboxylated MWCNT (0.06, 0.15, 0.3, 0.6 μg/mL), simple oxaliplatin drug (0.1, 0.25, 0.5, 1μg/mL) and functionalized MWCNT(-COOH/-NH2)-OX (0.06+0.1, 0.15+0.25, 0.3+0.5, 0.6+1μg/mL) were added to the culture medium. After 24 h incubation time all medium was removed and cells were washed with phosphate buffer saline (PBS). To determine the colorimetric response after exposure, 100μL of 1 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution were carefully pipetted over cells and left for 2 h for the formazan crystal formation. These crystals in turn were solubilized by pouring 100 μL of isopropanol 100% in each plate well. Lastly the optical density was read using a multiplate reader (TECAN GENios, Grödic, Germany) and cell viability was expressed as a percentage considering that control cells are 100% viable.

Superoxide anion detection

The method used for determining the amount of superoxide anion, formed in MDA-MB-231 cancerous cells after exposure to carbon nanotubes and chemotherapeutic drug oxaliplatin. The method of Huang and Li (2014) [19] consists in the reduction of nitrobluetetrazolium (NBT) dye and gradual formation of NBT-formazan deposits that the superoxide anion is involved in. For this assay MDA-MB-231 cells were seeded in plates (24-well plates, 5x10^4cells/cm^2density), incubated to adhere overnight and then exposed 24 h to the highest and lowest doses used for cell viability detection(MWCNT(-COOH/-NH2): 0.06 and 0.6 μg/mL, OX: 0.1 and 1 μg/mL and the MWCNT(-COOH/-NH_2)-OX hybrid: 0.06+0.1 and 0.6+1μg/mL). Cell culture medium was removed and replaced by a volume of 100 μL of NBT dye and incubated at room temperature for one hour. The reaction was stopped using a volume of 20 μL solution of 2 N HCl and the formazan was dissolved by adding 200 μL of DMSO. Finally the absorbance was read at 570 nm using a microplate reader (TECAN GENios, Grödic, Germany).

Western Blot analysis

Western blot analysis was performed by seeding MDA-MB-231 cells on 75 cm^2 flasks and treating themwith the same concentrations used for the superoxide anion detection of functionalized carbon nanotubes, oxaliplatin and a combination of both, followed by incubation for 24 h. The total cell lysates were prepared by sonication on ice (UP50H Ultrasonic Processor). For the determination of protein concentrations (mg/ml), necessary for calculating the amount of protein to be loaded in the gels, Bradford's method was used [20] with bovine serum albumin (BSA) as standard. Protein samples (30 μg) were loaded on 10% SDS-PAGE gels, runfor 2 h and transferred to a PVDF membrane by wet transfer in Tris-Glycine buffer. The Western Breeze Chromogenic Immunodetection Kit was used for protein detection, with specific mouse monoclonal primary antibodies for Hsp60, Hsp90, Mdm2, Bcl2, beclin 1, p53 (Santa Cruz, Biotechnology) and anti-mouse secondary antibody conjugated with alkaline phosphatase.Protein band staining was done with a chromogen substrate (BCIP/NBT solution), visualized with the ChemiDoc Imaging System (BioRad) and protein expression was quantified with Image Lab software. β-actin was used as reference protein.

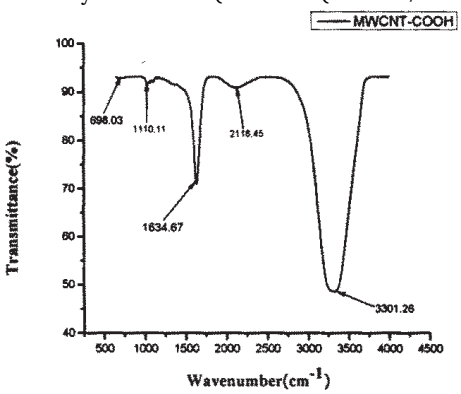
Statistical analysis

Data were expressed as mean value ± standard deviation of three independent experiments. The results were considered significant only if the *p* value was less than 0.05 and significant differences between untreated control cells and exposed cells were analyzed by Student's t-test using Microsoft Excel software.

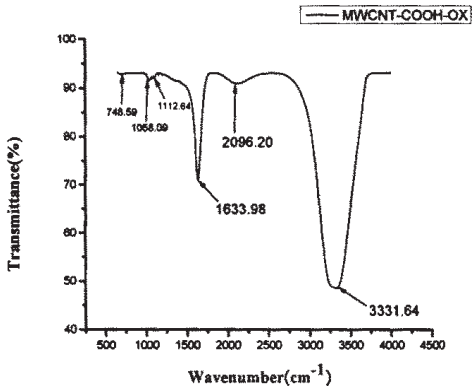
Results and discussions

Structural IR analysis

FTIR spectroscopy put in evidence the presence of functional groups on the surface of MWCNT-COOH and MWCNT-NH_2. As we can see, the bands allocated to groups: -OH, CH_2 and amino in the four samples are almost identical. For MWCNT-COOH-OX functionalized sample bands for functional groups appears in a range from: 3332 to 2096 cm^{-1} from OX range grouping: 749 to 521 cm^{-1}as shown figure 1a,b.



a) MWCNT - COOH

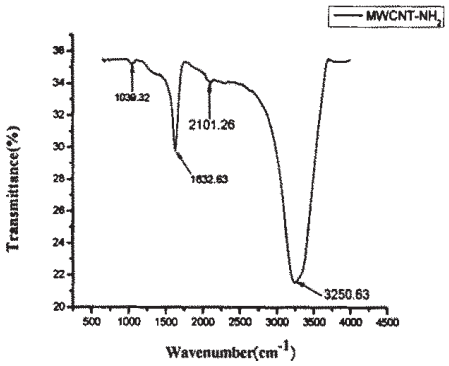


b) MWCNT-COOH-OX

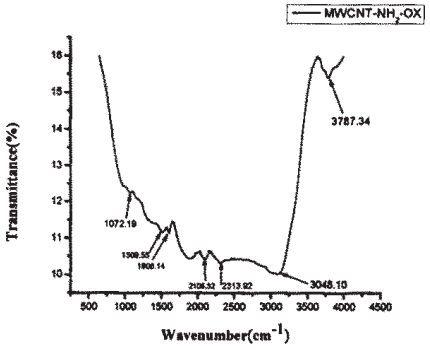
Fig 1. FTIR spectra MWCNT-COOH-OX

Table 1
THE BANDS ALLOCATING FOR THE FUNCTIONAL GROUPS OF CNTs

	MWCNT-COOH-OX	MWCNT-COOH
Corespondence	**Wave number, cm^{-1}**	**Wave number cm^{-1}**
-OH	3332	3301
-CH2	2096	2116
C=C	1113	1110
C=O	1634	1635
OX	749	----------



a) MWCNT - NH_2



b) MWCNT-NH_2-OX

Fig. 2 FTIR spectra MWCNT-NH_2-OX

As can be seen from figure 1 and table 1 the drug attributed characteristic functional groups are almost identical.

For sample MWCNT-NH_2-OX the band that characterize multiwalled carbon nanotubes functionalized with amino groups appears in the range 3787 to 2106 cm^{-1} and for OX this peak appear at 655 cm^{-1} as we can see in figures 2.

As the can be seen from figures 2 and table 2 the two drugs factions samples attributed characteristic functional groups are almost identical.

Characterization of functionalized MWCNT

HPLC analysis was used to identify compounds based on platinum, oxaliplatin. Chromatographic separation was performed on a column Zorbax® SB-C18 (100x4,5 mm id, stationary phase particles 3.5 um), under isocratic using a mobile phase of 55:45 (v/v) mixture of ACN in a flow rate of 2.5 mL / min. The detection for oxaliplatin was performed at 215 nm. The samples for the functionalization with the two drugs were prepared identically. The better results were obtained for samples that contain oxaliplatin in

Table 2
THE BANDS ALLOCATING FOR THE FUNCTIONAL GROUPS OF CNTs

	MWCNT-NH_2	MWCNT-NH_2-OX
Corespondence	Wave number, cm^{-1}	Wave number, cm^{-1}
-OH	3301.26	3331.64
-CH2	2116.45	2096.20
C=C	1634.67	1633.95
O=O	1029-	1058-
C–N stretching vibration	1110.11	1158.14
N–H distortion	-	1112.64
NH_2 stretching	-	748.59

Table 3
THE AMOUNT OF DRUG FROM OXALIPLATIN ENCAPSULATED

Sample	**Concentrations OX (µg/mL)**
MWCNT-COOH-OX	9.02
MWCNT-NH_2-OX	8.23

carboxylated multiwalled carbon nanotubes. For oxaliplatin samples embedded in multiwalled carbon nanotubes functionalized with carboxyl group we obtained a concentration of 8.23 µg/mL OX and in exchange for the amino group functionalized samples the concentration was 9.02µg /mL OX. The results are summarized in the table 3.

After HPLC analysis and calculation encapsulation efficiency of equation (1), it was found that OX in two samples is 87% respectively 96%, the results are summarized in figure 3.

Nanosized particle morphologies are investigated using TEM. From this analysis, it can be seen in figures 3 and 4, the surface of MWCNT-COOH and MWCNT-NH_2 encapsulated with oxaliplatin. In figure 3, on the surface of MWCNT-COOH functionalized with oxaliplatin, because of the big molecule of this drug, OX is practically grafted on the multiwalled carbon nanotubes surface. In figure 4 are shown morphologies of aminated functionalized multiwalled carbon nanotubes prepared with oxaliplatin. Oxaliplatin grains are in a range of 3-5 nm and 10 nm in length. Surface is not homogeneous and the encapsulation of two drugs could not be detected.

For ICP-MS analysis all samples (typically 1mL) were digested in 100 mL concentrated nitric acid ULTRAPURE, Fa. Merck). Acid digestion was done in a well determined volume of HNO_3 65%; after digestion, the samples were diluted 100 times and liquid fractions were analyzed. Platinum (20 mg/L) as internal standard was applied to analyze solutions in ICP-MS.

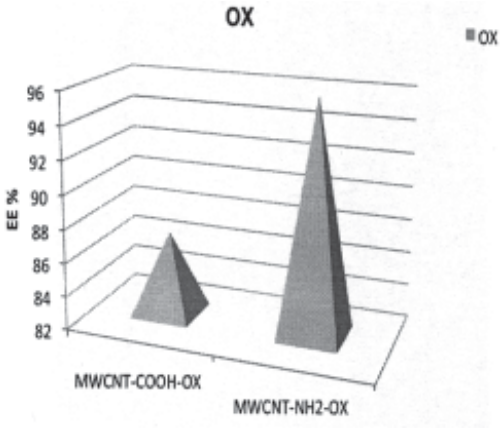


Fig. 3 The amount of drug from oxaliplatin encapsulated

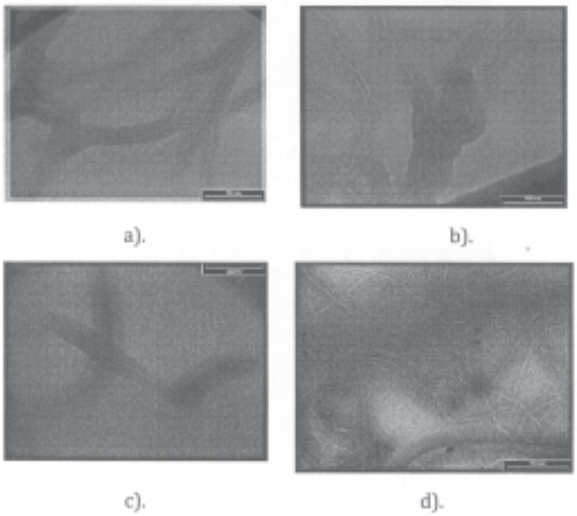


Fig. 4 TEM morphologies for MWCNT-COOH-OX

From table 4 we can see that the drugs begin to release after 2.5 h 1% for OX. After 72 h , the drugs release in a proportion of approximatively 21% OX . This low release rate is probably due to the fact that the drugs covalently bonded to the surface of multiwalled carbon nanotubes and breaking the barrier of C-N and C-C bonds it will take some time. Not all the bonds will be favorable breaking as we know that covalent bond is a very stable one.

Analysis of cell viability and morphology

The cytotoxicity of carbon nanotubes and oxaliplatin was evaluated by 24 h exposure to cancerous cells and cell viability was assessed by the MTT assay. An overall analysis of the data (fig. 6 and 7) showed that the decrease of cell viability was inversely proportional with exposure doses. An exception is in the case of MWCNT-COOH where no significant changes of cell viability were observed after exposure. A significant impact on cell viability was observed in the case of both aminated and carboxylated MWCNTs functionalized with OX. The combination of MWCNT-NH_2 and OX has the most pronounced effects on

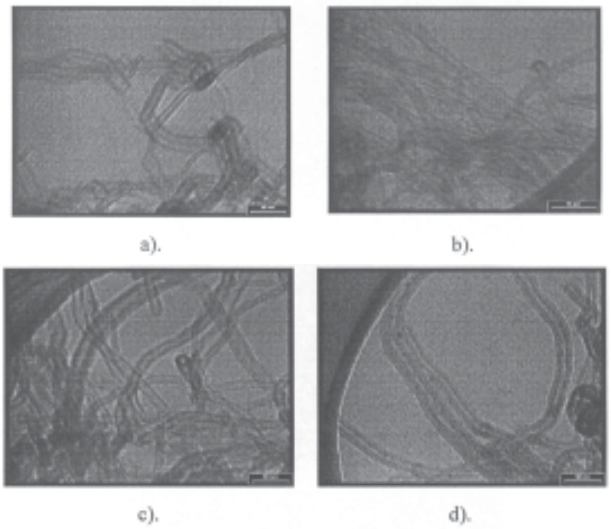


Fig. 5 TEM morphologies for MWCNT-NH_2-OX

cell viability reducing it to 60.8, 48, 32.6 and 20.5% for 0.06+0.1, 0.15+0.25, 0.3+0.5, respectively 0.6+1 μg/mL doses. Also, in the case of MWCNT-COOH-OX the viability decrease significantly in a dose dependent manner by 78.8% and 72.5 to 46.9 and 37.9%.

MWCNT-NH_2 showed higher toxicity than carboxylated MWCNT, but comparable with simple oxaliplatin which reduced viability for the first dose to 82.7 and to 60% for the higher dose (0.6 μg/mL), compared to control. Cell morphology of exposed cancerous cell line MDA-MB-231 (fig. 7) was analyzed by optical microscopy for the lowest and highest doses. The results confirmed the MTT assay evaluations. MWCNT(-COOH/-NH_2)-OX generated significant cell death and a change in cell morphology as shown in figure 7. For the more toxic dose, cells showed lower adherence to plate, changed size and form and finally died. Also, MWCNT formed clusters of various sizes, dispersed randomly across the MDA-MB-231 cell monolayer.

Evaluation of superoxide anion production

In order to detect the formation of superoxide anion in the adenocarcinoma derived epithelial cell line MDA-MB-231 exposed to OX, MWCNT-COOH and MWCNT-NH_2 in combination and without OX, NBT assay was used. The total amount of superoxide anion formed was detected by quantifying the NBT-formazan complexes, which were found to increase in a dose dependent manner. These results were correlated with data obtained in the MTT assay and cell morphology imaging. Statistical analysis showed that the highest increase in superoxide level was noticed for carbon nanotube-oxaliplatin hybrids. In the case of MWCNT-COOH functionalized with OX, superoxide anion formation increased up to 190.4% for the lower dose and 384.1% for the higher one. In the same time, MWCNT-NH_2-OX at higher dose generated a raise by 443.6% of this reactive oxygen species compared to control. The high dose of OX increased the concentration of superoxide by

Sample	Platinum ion release (mg/mL) 2.5 hours	Platinum ion release (mg/mL) 72 hours
OX	5.00	5.00
OX-COOH	0.06	1.07
OX-NH_2	0.04	1.05

Table 4
THE AMOUNT OF PLATINUM ION RELEASE FROM OXALIPLATIN AFTER 72 h

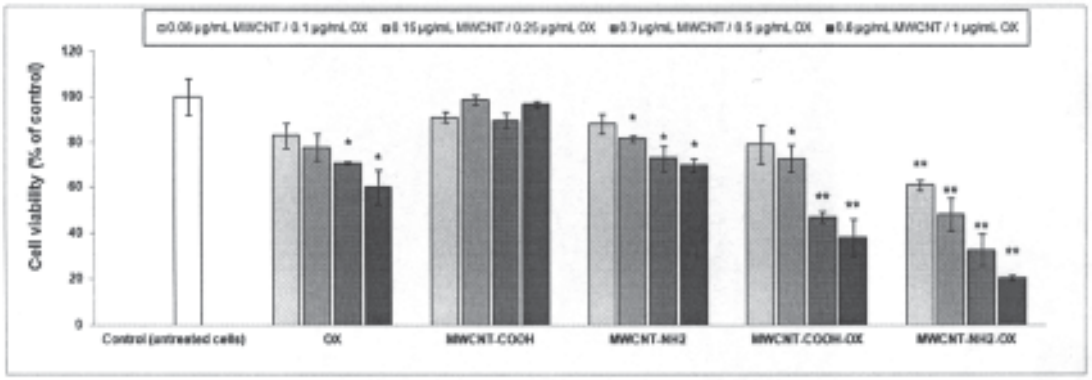


Fig. 6. Cell viability assessed after 24 h exposure to carbon nanotubes (MWCNT-COOH and MWCNT-NH_2) and OX. Values were calculated as means ± SD (n = 3) and expressed as% from controls. *P <0.05, **P < 0.01, ***P < 0.001 vs. control

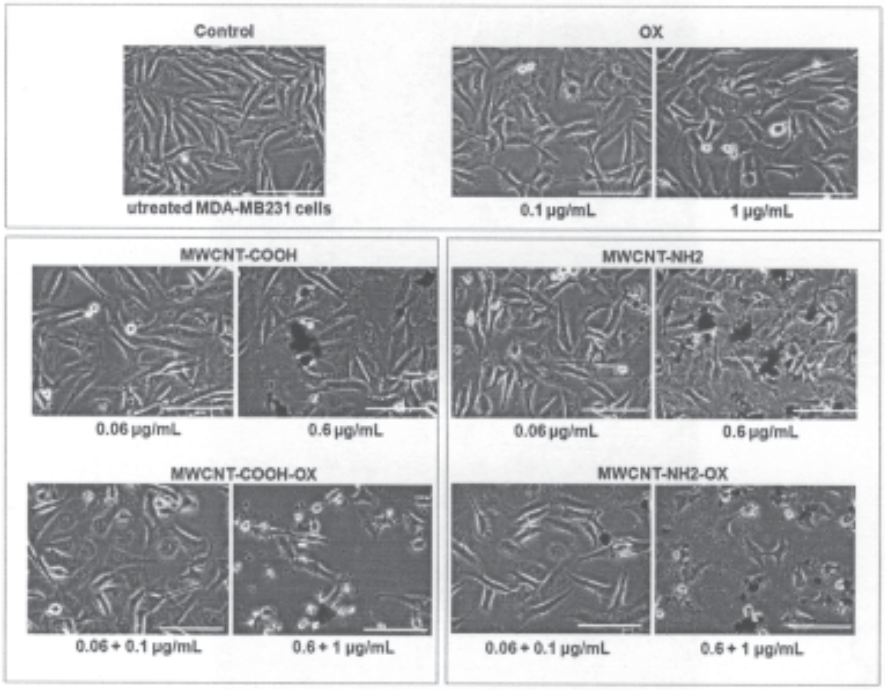


Fig. 7. MDA-MB-231 cells after 24 h of exposure to functionalized carbon nanotubes and OX. Untreated cells were used as control. Images were acquired by a bright field inverted microscope (Olympus IX71), objective 16X, using a CCD video camera COLOR VIEW. The scale bar is the same for all images and corresponds to 40 µm

79% compared to control, at a level similar to that registered for the high dose of MWCNT-COOH (fig. 8).

MWCNT could enter cells by direct penetration or endocytosis and generate morphological and functional changes. They also interact with NADPH oxidases [21] present on the plasma membrane, but also on mitochondria and endoplasmic reticulum [22] generating superoxide anion. This decreased survival rate of cancer cells.

Protein expression analysis

Protein expressions of Hsp60, Hsp90, p53, Beclin1, MDM2 and Bcl-2 were analyzed by Western blot analysis. The results presented in figure 9 and table 5 show the response of treated cells. The two heat shock proteins Hsp60 and Hsp90, are essential for cell growth and survival in a stressful environment, p53 has a key role in tumor suppression in normal cells but it is greatly repressed in cancer cells, and Beclin1, MDM2 and Bcl-2 proteins that are markers for autophagy. The data evaluation for the two heat shock proteins Hsp60 and Hsp90 resulted in a decrease of protein expression in a dose dependent manner. An oxidative cleavage of Hsp90 generating fragments of lower molecular weights, as a response to reactive oxygen species generation was noticed. Proteolysis was more

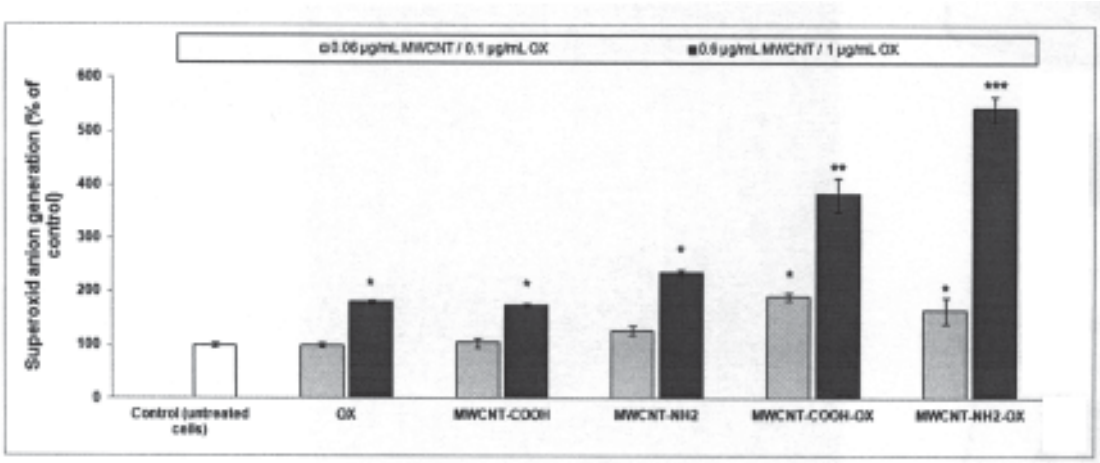


Fig. 8. Superoxide anion generation after 24 h exposure to carbon nanotubes (MWCNT-COOH and MWCNT-NH2) and OX. Values were calculated as means ± SD (n = 3) and expressed as % from controls. *P <0.05, **P < 0.01, ***P < 0.001 vs. control

Protein	Control	OX		MWCNT-COOH		MWCNT-NH_2		MWCNT-COOH-OX		MWCNT-NH_2-OX	
		d 1	d 2	d 1	d 2	d 1	d 2	d 1	d 2	d 1	d 2
Hsp60	100%	71.51± 2.14**	71.05± 1.26**	84.93± 1.42**	7.60± 0.09***	79.51± 4.38**	6.96± 0.89***	68.95± 1.50**	4.56± 2.50***	21.38± 2.13***	1.11± 0.16***
Hsp90		89.45± 15.86	82.91± 4.75	96.26± 1.40	68.99± 3.78**	107.09± 5.89	80.53± 3.76*	95.90± 2.47	61.92± 3.99**	101.16± 1.70	65.01± 2.65**
p53		85.28± 2.14**	82.27± 2.54**	74.72± 3.21***	6.20± 3.08***	60.71± 3.04**	11.68± 2.28**	54.91± 1.15**	9.60± 5.22**	35.75± 1.27***	2.27± 0.75***
MDM2		54.64± 2.76**	54.37± 0.98**	60.13± 3.68**	55.21± 3.82**	56.33± 2.49**	46.27± 1.88***	52.66± 0.43**	41.72± 1.48***	47.00± 8.70***	22.39± 1.09***
Beclin1		117.71± 8.92*	115.25± 4.61*	121.75± 0.15**	112.13± 2.51*	122.34± 4.88**	149.08± 4.67***	143.86± 6.49***	152.30± 10.7**	150.26± 5.00***	194.15± 8.87***
Bcl-2		84.56± 5.67	93.63± 5.37	126.69± 4.03**	114.02± 6.55*	218.45± 3.41***	81.12± 9.55*	217.24± 10.5***	95.91± 4.44	213.63± 7.74***	71.06± 3.11**

Table 5
QUANTIFICATION OF Hsp60, Hsp90, p53, MDM2, BECLIN1, BCL-2 PROTEIN EXPRESSION IN MDA-MB-231 CELLS AFTER 24 h EXPOSURE TO CARBON NANOTUBES AND OX. SAMPLE VALUES WERE REPRESENTED AS % FROM CONTROL ± SD (n = 3).*P <0.05, **P < 0.01, ***P < 0.001 vs. CONTROL

*d 1- dose 1 = 0.06 μg/mL MWCNT / 0.1 μg/mL CBDCA

d 2 - dose 2 = 0.6 μg/mL MWCNT / 1 μg/mL CBDCA

pronounced for samples containing the amino group (MWCNT-NH_2 and MWCNT-NH_2-OX) compared to their carboxylated counterparts. For Hsp60 band intensity was less pronounced for the higher dose used. The MWCNT-COOH/MWCNT-NH_2-OX complexes generated the lowest protein expression. For the higher dose of MWCNT-COOH-OX the expression of Hsp60 was diminished by about 95% whereas for that of MWCNT-NH2-OX by almost 99%. Similar results could be found in the case of p53 tumor suppressor protein, the amount of protein loss being of 35.7% for the low dose and 2.27% for the high dose compared to control. Simple OX generated the lowest significant alterations for Hsp60, Hsp90 and p53 protein expressions for the both doses used (0.1 and 1 μg/mL). The evaluation of data obtained for anti-apoptotic Bcl-2, autophagy indicator Beclin1 and MDM2 protein, clearly expressed the appearance of an autophagy process, more pronounced for MWCNT-NH_2-OX and MWCNT-COOH-OX, but also for MWCNT-NH_2. This could be confirmed by band densitometry according to Beclin1 expression increased by 52 and 94% for the higher doses of MWCNT-COOH-OX respectively MWCNT-NH_2-OX compared to control. These data were further verified by determining the expression of MDM2 and Bcl-2 proteins, both having roles in autophagy. Similarly simple OX exposure had little significant on all these proteins. Hsp60 and Hsp90 are utilized by tumor cells to evade the pathways of tumor suppression. The decrease of Hsp60 and Hsp90 expression suggests the failure of cytoprotection mechanism of cancer cells. The p53 protein is a client of Hsp90 [23] the degraded Hsp90 could not stabilize p53 and as a result, Mdm2 dependent degradation probably occurred [24] .The fact that Mdm2 decreased significantly, suggests that some isoforms, involved in cellular proliferation were inactivated, whereas those

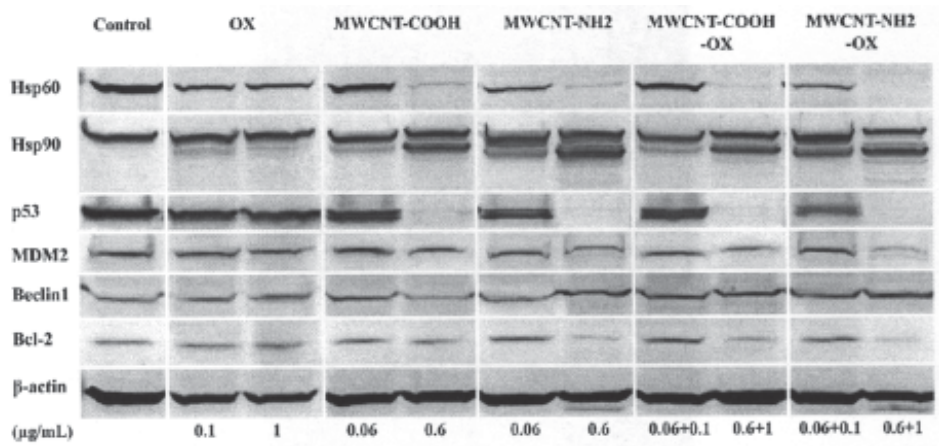


Fig. 9 . Western blot analysis of Hsp60, Hsp90, p53, MDM2, Beclin1, Bcl-2 protein expression in MDA-MB-231 cells after 24 h exposure to carbon nanotubes and oxaliplatin. b-actin was used as reference protein

involved in the proteasomal proteolysis of p53 remained functional and as a result, the inhibition of Mdm2 along with that of p53 might lead to autophagy by MDA-MB-231 cells. This hypothesis is sustained by the increase of Beclin1 expression, that is an indicator of an increased number of autophagic vacuoles [25] and the down regulation of Bcl-2 (an autophagy inhibitor) in the cell treated with the highest dose.

Conclusions

In this study, we referred drug used in cancer therapy oxaliplatin that have been encapsulated in MWCNTs functionalized with both, -COOH and -NH_2 groups. After 72 h the release of platinum ions is 21%. The biological studies revealed that MWCNT-NH_2-OX induced MDA-MB-231 cells death in a more significant way than MWCNT-COOH-OX, probably by inducing autophagy. As a result, this formulation could be used for OX delivery in tumor cells. Further study on the influence of the process parameters of the starting material on OX release is required.

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