



Preparation, Characterization and *In vitro* Toxicity Study of Antiparasitic Drugs Loaded onto Functionalized MWCNTs



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Abstract


This work focuses on the preparation of immobilized ivermectin (IVM) and essential oil carvacrol (Cav), as models of antiparasitic drugs, on functionalized multi-walled carbon nanotubes (MWCNTs) using sol-gel technique. Fourier transform infrared spectroscopy (FTIR), transmission electron microscope (TEM) and particle size distribution analysis were used to characterize the prepared materials. In addition, the *in vitro* cytotoxic effect was investigated against normal fibroblast cell line (BHK-21) using SRB assay. Also, toxicity to *Rhipicephalus annulatus* female ticks was also performed *in vitro*. The FTIR and TEM results showed that the drugs loaded on the functionalized MWCNTs were successfully prepared through covalent bonding with a particle size range of around 407 and 268 nm in the case of IVM and Cav, respectively. The cytotoxic activity showed that the drugs loaded had low cytotoxic effects of about 4.5 and 4.4 % relative to the free IVM (8.5 %) and Cav (7.8 %), respectively, at 100 µg/mL concentration. In addition, the loaded drugs displayed high ticks mortality at about 100 % compared to the free IVM (23.3 %) and Cav (26.7 %), respectively at 250 µg/mL concentration after 72 h of exposure.

Keywords: Ivermectin; Carvacrol; MWCNTs; sol-gel technique; *in vitro* toxicity; SRB assay; *Rhipicephalus annulatus* ticks.

Introduction

Carbon nanotubes (CNTs) have been used for the drug delivery system as therapeutics in pharmacy and medicine since the early 21st century. They adsorb or conjugate with a wide range of therapeutic molecules (drugs, proteins, antibodies, DNA, enzymes and so on) [1]. These have been shown to be an excellent tool for drug delivery by penetrating directly into the cells and holding the drug intact during transport within the body without metabolism [2]. Several studies have shown that these molecules are transmitted more effectively and safely into cells when bound to CNTs than with conventional methods [3–5]. This exciting breakthrough has opened up an entirely different direction for drug formulations from traditional techniques used in the pharmaceutical industry before and radically changed anterior pharmacological principles. CNTs have thus

become the focus of attention of scientists in a wide variety of disciplines within a very short time. These may be important antioxidants for potential health-protective effects and prevention of ailments [6]. It is told, however, that all these medicinal findings are in an experimental stage and still not being applied in people. Our group has recently contributed to the development of different novel functionalized CNT techniques as delivery systems and also to the study on the interactions between CNTs and natural bioactive compounds or drugs [7–15]. Parasites are a class of pathogens that are more harmful to humans and animals than bacteria and that cause chronic diseases in general. They always have distinct growth stages from one generation to the next for survival. They can also reside in host cells and set up reservoirs from which reinfection occurs, often leading to long-term and repeated infections. Such properties have contributed to

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substantial difficulties in the treatment of parasite infections [16]. Chemically antiparasitic medications are used mainly for the treatment of parasite diseases. Many of them however have poor bioavailability due to their insolubility and limited half-life. In addition, repeated treatment may cause animal stress, farmer labor intensity, and drug resistance [17]. In order to avoid these limits, innovative methods are needed to improve the efficacy of antiparasitic drugs. Nanoparticles also attracted attention for antiparasitic drug delivery, with the rapid development of nanomedicine. They are physically or chemically loaded into the nanoparticles by means of adsorption, encapsulation and conjugation. These nanoparticles can be administered by oral, skin, pulmonary, intravenous and other routes according to disease treatment and drug properties requirements [18-20]. Nanoparticles have presently shown broad prospects for development in the application of antiparasitic drug delivery. Hard ticks are the most common cattle ectoparasites distributed all over the globe. Animals are important vectors for the transmission of diseases to humans around the world. They also cause significant economic losses for the livestock sector. Due to the serious economic and health-related damage caused by ticks, their control is very important [21]. Chemical management of these parasites is achieved primarily by acaricides. The cattle tick *Rhipicephalus* (formerly *Boophilus*) *annulatus* is the cattle's principal tick species [22]. Also, *R. Annul* protozoan disease such as *Babesia bigemina* and bacterial diseases such *Borrelia theileri* [23]. In addition, trials were performed to test alternatives against ticks to chemical acaricides that attack animals in Egypt to prevent acaricidal problems. *R. Annulatus* population control has been implemented by applying several classes of acaricides including derivatives of macrocyclic lactone (ML) such as ivermectin (IVM) [24, 25]. It is composed of 23-dihydroavermectin-B1a (80%) and 23-dihydroavermectin-B1b (20%) and it is generally used for endo- and ecto-parasite control. Several pharmacokinetic studies have been published since 1981, when the IVM was marketed [26]. The widespread use of IVM for tick control has disadvantages like the advent of cattle's resistance ticks, helminths and their long withdrawal time. On the other hand, a large number of essential oils-bearing plants have been studied over the centuries as sources of therapeutics for the treatment of different parasites [27]. In previous research, geraniol, eugenol, carvacrol and 1,8-cineol have shown strong acaricidal activity [28, 29], but pure compounds have not been tested against *in*

vitro ticks to our knowledge. In this study, the preparation and characterization of the immobilized IVM and Cav on functionalized MWCNTs was investigated using sol-gel technique. In addition, SRB assay was used to conduct cytotoxic *in vitro* activity against normal fibroblast (BHK) cell line. Also, toxicity *in vitro* to *R. annulatus* female ticks were also studied and evaluated.

Experimental

Materials

Multi-walled carbon nanotubes (MWCNTs), carbon content 95%, diameters 6–9 nm × 5 μm, and tetra ethyl orthosilicate (TEOS) were obtained by Sigma-Aldrich. Normal fibroblast (BHK) cell line was collected and prepared for SRB cytotoxicity assay by National Cancer Institute, Cairo University. Phosphate buffer saline sterile at pH 7.4 obtained from Bio-West. Both ivermectin (IVM) and carvacrol (Cav) (Fig 1) were used as drug models and obtained by Sigma. All chemicals and other reagents will be used without further purification.

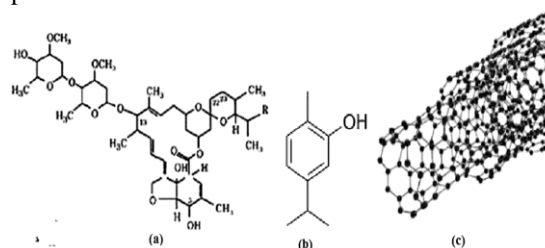


Fig 1. The chemical structures of (a) IVM, (b) Cav and (c) MWCNTs.

Oxidation and purification of MWCNTs

Oxidized MWCNTs (Ox-MWCNTs) have been made available by using olive oil to handle them. In a 500 mL flask, 1g of pristine MWCNTs was distributed in blended 30% nitric acid and olive oil with a ratio (3:2 v /v). The flask was then refluxed with continuous stirring at 1100°C for 2 h to create ox-MWCNTs. The resulting material was collected under vacuum through filtration and then thoroughly washed with 500 mL of chloroform to remove the remaining oil [30]. The substance collected was treated with ultra-pure water, until the filtrate was neutralized (pH 7.0). The collected solid was vacuum-dried for 12 h at 70°C and kept for further investigation.

Functionalization of ox-MWCNTs using sol-gel technique

Functionalization of ox-MWCNTs using sol-gel technique by hydrolysis and polycondensation of tetraethyl orthosilicate as a source of SiO₂ containing HCL as a catalyst [31, 32] in the presence of different drug models (IVM or Cav) was employed as follows: The silicate solutions were stirred for 1 h with the molar ratio TEOS: C₂H₅OH: H₂O: HCl (1:6:8:0.6). After 2 h reflux, 100 mg of ox-MWCNTs and 2 mg of IVM or Cav were added and sonicated, accompanied by addition of benzoin methyl ether (2,0 mmol). In the sol-gel cycle, ox-MWCNTs and drug moieties were crosslinked, followed by a radical UV photoinitiated carboxylic group reaction. The sol was cast into plastic molds and held for 3 days at room temperature. The materials collected have been preserved for further study.

Characterization

FTIR spectra were recorded using a jasco-400 Model FTIR spectrophotometer. The UV- spectra were taken using double beam Agilent model. The TEM morphologies were carried out with a JEOL transmission electron microscope JEM-1230. The Particle size distribution analysis was recorded with a Malvern Zetasizer 3000 HAS using dynamic light scattering technique (DLS).

In vitro cytotoxicity using sulpho-rhodamine-B stain (SRB) assay

Potential cytotoxicity of the prepared nanomaterials was tested using the method Sehkan *et al.*[33] as follows: standard Baby Hamster Kidney fibroblasts (BHK-21) cell line was placed separately in the 96-multiwell plate (10 cells / well) for 24 hours prior to treatment with the prepared materials to allow the cells to be attached to the plate wall. Different concentrations of the materials obtained under study (0, 10, 25, 50 and 100) µg/mL were prepared for each individual dosage. Monolayer cells were incubated with the formulated materials for 48 h at 37°C in a 5 % carbon dioxide atmosphere. 48 h cells with sulpho-rhodamine-B stain are cleaned and stained upon correction. Excess stain sprayed with acetic acid and replenished with Tris-EDTA buffer. The frequency of colour has been measured in an ELISA reader. The relation between the surviving fraction (number of the viable cells) and the prepared concentrations of the formulation was plotted in order to obtain the survival curves of the cell line used.

In vitro toxicity using *R. Annulatus* female ticks

Field population of *R. annulatus*, were collected from Monofeya province, Egypt. Adult engorged female ticks were collected and hatched larvae were then used in the experiment. Adult immersion test (AIT) was performed by application of the prepared acaricides on engorged *R. annulatus* female ticks. The study contained different concentrations (100, 150, 200, 250 and 300 µg/mL). Each concentration or control procedure was repeated 3 times and there were 10 females in the replicates. Treatment was applied by dipping females for 2 min at each concentration and transferred to filter paper afterwards. The females are divided into plastic cups with cones (female/cup). The females are incubated at room temperature and the mortality rate was observed for three days. Mortality (%) based on brown-black ticks and the lethal concentration (LC₅₀) values for each concentration were calculated [34].

Statistical analysis

The data will be expressed as mean ±SD. Differences between non-treated and treated cells with the prepared nanomaterials were analyzed using an unpaired t-test.

Results and discussion

Characterization of the prepared materials

Oxidation of MWCNTs was usually done, as reported previously [35], in a mixture of sulfuric and nitric acids which introduced some polar groups into the side walls of the MWCNTs. On the other hand, using of olive oil, demonstrated excellent stability of ox-MWCNTs in the aqueous media [36].

Figs 2 and 3 show FT-IR spectra of the immobilized IVM and Cav onto the functionalized MWCNTs, respectively, in comparison with the spectra of the free IVM or Cav and ox-MWCNTs. It was found that the ox-MWCNTs had small characteristic peaks corresponding to the reacted carbonyl groups, at 1634 and 1362 cm⁻¹. While the immobilized IVM and Cav spectra showed characteristic peaks (str) corresponding to the CH, CH₂ and CH₃ alkyl chains of IVM and Cav, at 2866, 2964 and 3035 cm⁻¹, respectively. Also all other characteristic peaks corresponding to the various functional groups such as aromatic C=C (str), C-O (str), C-C (str) and CH aromatic were observed and changed with changes in their intensities. In addition, the big band appeared at a high intensity at 3445 cm⁻¹ corresponds to the stretching vibration of-OH (str) groups suggesting TEOS esterification of the carboxyl groups. Moreover, Si-O-Si, Si-C and C-O-C were given the characteristic peaks at 1183, 806 and 448 cm⁻¹, respectively [37]. This may be due to the presence of IVM or Cav drug moieties

during sol-gel process which led to Si-O-Si interactions enhancement. It can be assumed that the presence of IVM or Cav during the sol-gel cycle, which acts as a catalyst, intensified the reaction in the presence of ox-MWCNTs.

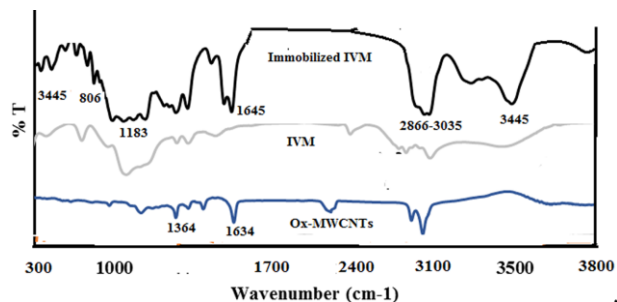


Fig 2. FTIR spectra of immobilized IVM in comparison with ox-MWCNTs and free IVM.

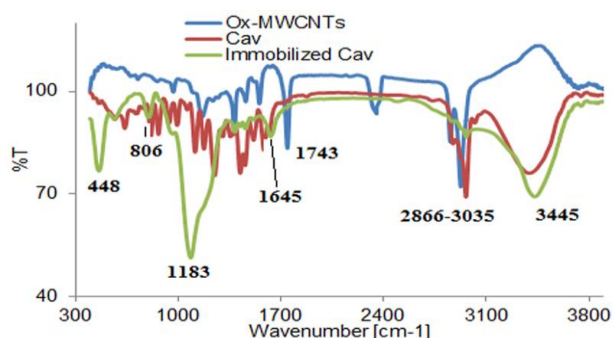


Fig 3. FTIR spectra of immobilized Cav in comparison with ox-MWCNTs and free Cav.

Fig 4 shows TEM images of the immobilized IVM and Cav onto the functionalized MWCNTs relative to the ox-MWCNTs. It can be noticed that there are no MWCNTs structural damaged occurred after drug immobilization materials in comparison with the ox-MWCNTs. This is proved that using of the olive oil was more suitable than that the conventional strong acids which caused severe structural damage to the nanotubes structure through tubes scission. Also, the loaded samples revealed that the tubular shapes were covered by the dark colour zones indicating the presence of the drug molecules.

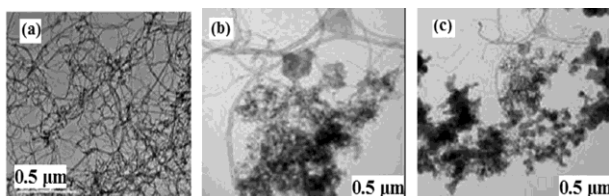


Fig 4. TEM images of (a) immobilized IVM, (b) immobilized Cav and (c) oxidized MWCNTs.

Fig 5 and Table 1 show the particle size distribution analysis of the prepared materials by correlation with intensity using DLS technique. It can be noticed that the particle size of the immobilized IVM and Cav onto the functionalized MWCNTs were about 268 ± 31.9 and 407 ± 48.9 nm, respectively, relative to that in the case of ox-MWCNTs and free drugs (495 ± 63.1 and (IVM 230 ± 16.0 and Cav 268 ± 16.0 nm), respectively). This may be due to the covalent attachment of both drugs to the side walls of the ox-MWCNTs. It can be concluded that immobilization of IVM and Cav onto the functionalized MWCNTs using sol-gel was taken place *via* covalent rather than adsorption technique. This was in accordance with our previous study that reported about the effect of ox-MWCNTs on the particle size of the immobilized L-asparaginase enzyme [38].

TABLE 1. Particle size distribution analysis of the prepared materials using DLS technique.

Sample	Particle size (nm)
Ox-MWCNTs	495 ± 63.1
IVM	230 ± 16.0
Immobilized IVM	268 ± 31.9
Cav	268 ± 16.0
Immobilized Cav	407 ± 48.9

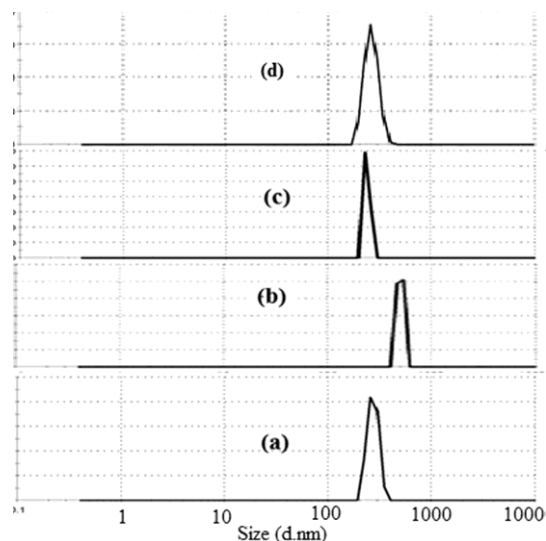


Fig 5. The particle size distribution analysis of (a) immobilized IVM, (b) free IVM, (c) oxidized MWCNTs and (d) immobilized Cav using DLS technique.

In vitro cytotoxic study using SRB assay

The normal fibroblast cell line (BHK) was selected in order to investigate the potential safety of the prepared antiparasitic drugs loaded onto the functionalized MWCNTs on the healthy cells. However, further studies may be required to

examine the mode of action of the different formulations using various cell lines.

Fig 6 and Table 2 show *in vitro* cytotoxic surviving fraction profiles and the dead cells (%) of the treated BHK cell line with the immobilized IVM and Cav onto the functionalized MWCNTs using SRB assay relative to the ox-MWCNTs, TEOS and the free drugs. It was found that the surviving fractions decreased when the concentration of the immobilized IVM increased and thus the dead cells (%) increased. This may be due to the amount of the drug released into the culture medium. In the case of the immobilized Cav, the same activity was observed compared to the free drug. On the other hand, it was observed that the immobilized IVM and Cav had low cytotoxicity (about 4.5 and 4.4 %, respectively) against BHK-21 cell line at concentration up to 100 µg/mL in comparison with that in case of the free drugs (about 8.5 and 7.8 %, respectively) and with the other materials (ox-MWCNTs 3.4 % and TEOS 6.9 %). These results confirmed that the ox-MWCNTs had no significant effects on the toxicity of the free drugs against BHK-21 cell line. Moreover, the *in vitro* cytotoxicity of both IVM and Cav could be retarded after loading onto the functionalized MWCNTs.

Table 2. In vitro cytotoxicity of the prepared materials at different concentrations against BHK cell line using SRB assay

Conc. µg/mL	% Dead cell			
	12.5	25	50	100
Sample code				
Ox-MWCNTs	1.7	2.4	2.4	3.4
TEOS	1.7	2.7	3.4	6.9
IVM	2.6	5.3	6.3	8.5
MWCNTs/IVM	1.9	2.9	3.7	4.5
Cav	2.1	6.3	7.3	7.8
MWCNTs/CAV	1.5	2.1	3.5	4.4

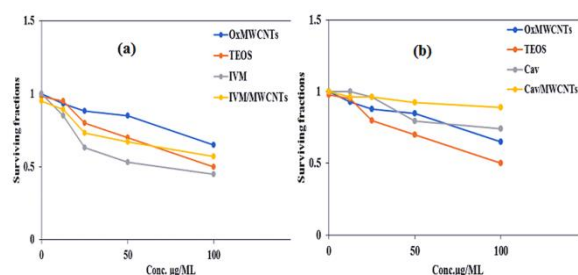


Fig 6. Surviving fraction curves of BHK cell line after treatment with (a) the immobilized IVM and (b) Cav in comparison with the free drugs, TEOS and ox-MWCNTs using SRB assay at different concentrations.

In vitro toxicity study using *R. Annulatus* female ticks

The adulticidal activity of the immobilized IVM and Cav at different concentrations (50, 100, 150, 200, 250 and 300 µg/mL) induced a significant ($P < 0.001$) lethal effect on adult female ticks. The larvicidal efficacy of the prepared materials resulted in a complete larval mortality (100 %) within 72 h of exposure at concentration about 250 µg/mL. While, at low concentration (50 µg/mL), the mortality was about $16.7 \pm 3.3e$ and $26.7 \pm 3.3d$ %, in case of the immobilized IVM and Cav, respectively (Table 3). In other words, the *in vitro* experiments showed a considerable reduction in the ticks survival after using ox-MWCNTs. This was in accordance with the previous study by Arafa *et al.* [39] which reported that the novel formulations of deltamethrin (deltamethrin-ZnO NPs and deltamethrin-Ag NPs) against *R. annulatus* were induced a significant ($P \leq 0.05$) lethal effect on adult ticks compared to deltamethrin-Ag NPs at the same concentrations.

According to the previous study reported about LC_{50} of *R. annulatus* ticks in Egypt [40] as well as a history of acaricide failure to ticks, *R. annulatus* developed resistance to the IVM more than that in case of Cav. On the other hand, Fig. 7 shows LC_{50} of *R. annulatus* female ticks treated with the immobilized IVM and Cav. It can be noticed that the immobilized IVM showed high activity against ticks relative to that in case of the immobilized Cav. This may be due to the different modes of drug action. In case of IVM, high affinity to glutamate-gated chloride channels (Glu-Cl) was found in the muscles and nerves. Consequently, the blocking of these channels resulted in a sluggish and irreversible conductance of the membrane leading to the death of the parasite [41, 42]. While, in case of Cav, the hydrophobic nature of the oil might be exerted mechanical effects on the parasite and blocking the spiracles leading to the death by water stress [43].

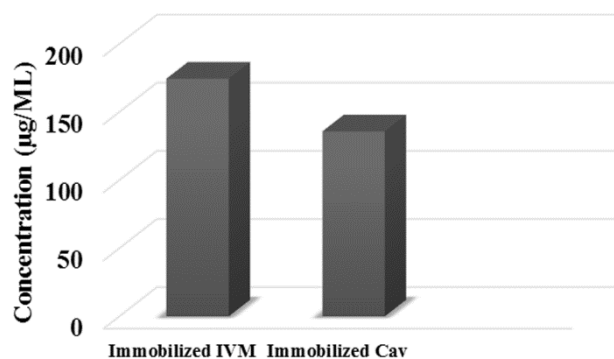


Fig 7. LC_{50} of *R. annulatus* female ticks treated with the immobilized IVM and Cav.

It can be concluded that the immobilized IVM and Cav onto functionalized MWCNTs using sol gel technique Improved drug activity and reduced cytotoxicity with sustained release performance in comparison with the other drug delivery systems [44-46], as shown in Table 4. In respect of the

originality, the manuscript provide for the first time data about immobilization of ivermectin (IVM) and carvacrol (Cav) on MWCNTs, and their physico-chemical and in vitro toxicity characteristics using normal fibroblast cell and *R. annulatus* female ticks.

Table 3. Mortality (%) (Mean±SE) of *R. annulatus* treated with the prepared materials.

Conc. µg/mL	MWCNTs/ IVM			MWCNTs/Cav		
	12 h	48 h	72 h	12 h	48 h	72 h
50	0.0±0.0c	0.0±0.0e	16.7±3.3e	0.0±0.0b	10.0±0.0c	26.7±3.3d
100	0.0±0.0c	3.3±3.3e	26.7±3.3d	0.0±0.0b	10.0±0.0c	40.0±0.0c
150	10.0±0.0b	13.3±3.3d	36.7±3.3c	0.0±0.0b	10.0±0.0c	46.7±3.3c
200	10.0±0.0b	16.7±3.3cd	40.0±0.0bc	3.3±3.3b	20.0±0.0b	56.7±3.3b
250	16.7±3.3a	36.7±3.3b	100±0.0a	10.0±0.0a	30.0±0.0a	100.0±0.0a
300	20.0±0.0a	50.0±0.0a	100±0.0a	10.0±0.0a	30.0±0.0a	100.0±0.0a
control drug	0.0±0.0c	0.0±0.0e	10.0±0.0e	0.0±0.0b	0.0±0.0d	10.0±0.0e
F	49.000	47.057	217.964	11.357	90.143	187.107

Table 4. Comparison between the different antiparasitic drug delivery systems and the present work.

Drug delivery system	Drug	Parasite	Advantages	The used Technology	Effect	Ref
Functionalized MWCNTs	Ivermectin Carvacrol	<i>R. annulatus</i> females	Low toxicity/ Good biocompatibility and sustained release performance	Sol-gel technique	Improved drug activity and reduced cytotoxicity	Present work
Liposomes	Avermectin	Swine fever	Targeting excellent safety	Rapid evaporation method	Significantly improved cure rate	[44]
Solid lipid nanoparticles	Ivermectin	/	Low toxicity and sustained release performance	Ultrasonic crushing method	Slow release and enhanced transdermal effect	[45]
Nano-suspension	Ivermectin	/	Simple preparation with high drug loading	High pressure homogenization	Enhancing dissolution rate by 4 times	[46]

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Conflict of Interest

There is no conflict of interest

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