Int. J. Electrochem. Sci., 10 **(2015)** 7707 - 7719

International Journal of ELECTROCHEMICAL SCIENCE
www.electrochemsci.org

Characterization of Multi-Walled Carbon Nanotubes Double-Functionalization with Cytostatic Drug Etoposide and Phosphorothioate Oligodeoxynucleotides

Zbynek Heger[1,2], Amitava Moulick[1,2], Hoai Viet Nguyen[1,2], Monika Kremplova[1,2], Pavel Kopel[1,2], David Hynek[1,2], Tomas Eckschlager[3], Marie Stiborova[4], Ondrej Zitka[1,2], Vojtech Adam[1,2], Rene Kizek[12,]*

[1] Laboratory of Metallomics and Nanotechnologies, Department of Chemistry and Biochemistry, Mendel University in Brno, Zemedelska 1, CZ-613 00 Brno, Czech Republic - European Union
[2] Central European Institute of Technology, Brno University of Technology, Technicka 3058/10, CZ-616 00 Brno, Czech Republic - European Union
[3] Department of Paediatric Haematology and Oncology, 2nd Faculty of Medicine, Charles University, and University Hospital Motol, V Uvalu 84, CZ-150 06 Prague 5, Czech Republic - European Union
[4] Department of Biochemistry, Faculty of Science, Charles University, Albertov 2030, CZ-128 40 Prague 2, Czech Republic - European Union
[*]E-mail: kizek@sci.muni.cz

Received: 20 May 2015 / *Accepted:* 26 June 2015 / *Published:* 28 July 2015

Carbon nanomaterials possess unique structural and physicochemical properties, and thus they are broadly utilized as carriers for a variety of therapeutic agents in nanomedicinal applications. Herein we present an electrochemical characterization of binding capability of acidic oxidized multi-walled carbon nanotubes (*o*MWCNTs) modified with poly(ethylene glycol) towards common cytostatic drug etoposide (or VP-16). The comparison of willingness of *o*MWCNTs and MWCNTs-PEG to form complexes with etoposide revealed significantly higher capacity in PEGylated variant of carbon nanotubes (the 15 mM etoposide loading capacity was approximately 46.4% in 2 mg of MWCNT-PEG or 28.1% in equal amount of *o*MWCNT). To obtain a multifunctional nanotransporter we employed the phosphorothioate oligodeoxynucleotide (PODN), which could further extend the possible biological effects of MWCNT-PEG-Etoposide complex. By using square wave voltammetry, the binding capacity of 2 mg of MWCNT-PEG-Etoposide (15 mM) towards PODNs was determined to be 4.5 µM. Such characterized multifunctional complex can be further employed for biological testing on chemoresistant tumors, such as non-small-cell lung cancer, to enhance the treatment efficiency.

Keywords: Antisense oligodeoxynucleotides; Carbon nanomaterials; Electrochemistry; Nanotechnology; Poly(ethylene) glycol

1. INTRODUCTION

Nanomedicine, as a continuously evolving discipline, is still looking for a structure with perfect properties usable as a multifunctional transporter [1]. By enabling controlled spatial and temporal delivery of traditional drugs, novel nanodrugs hold the promise to improve dramatically the treatment efficiency. A great potential is nowadays attributed to various carbon materials like graphene, fullerenes, single-walled or multi-walled carbon nanotubes (MWCNT). MWCNTs possess unique physicochemical and structural properties such as a high aspect ratio and surface area, tunable surface chemistry, ultrahigh drug loading capacity via π–π stacking interactions, which make these nanocarriers an attractive probe for multifarious biomedical applications including targeted drug delivery [2,3].

Over the past decade, various approaches of MWCNTs functionalization have been exploited to develop a multifunctional carbon-based platform for nanomedicinal applications. Acidic oxidation of MWCNTs with nitric acid generates covalently bound functional groups [4]. Based on these oxygen-containing moieties, the subsequent organic or inorganic modification on the CNTs surface has been reported, which demonstrated an improvement in the solubility of CNTs and broaden their application [5-7]. One of the most popular modifications of MWCNTs is tethering with biofunctional spacer - poly(ethylene glycol) (PEG), particularly due to its biocompatibility and capability to extend MWCNT modification possibilities and their blood circulation time [8]. Although the mechanism of increased circulation time is still under discussion, it was demonstrated that PEGylation leads to reduced binding of opsonin in the blood serum, which reduce the macrophage recruitment and MWCNTs removal [9]. Hence, PEGylated MWCNTs have been used as delivery systems for a variety of anticancer agents including cytostatic [10-12], or antisense oligonucleotides [13]. Moreover, MWCNT-PEG offers a possibility of multi-functionalization [14], which is important for development on innovative nanomaterials for next-generation theranostic nanomedicine (combining therapeutical and diagnostic functions).

Etoposide (Eto) or VP-16 is a semi-synthetic derivative of podophyllotoxin, a non-alkaloid toxin lignan extracted from the mandrake plant (*Podophyllum peltatum* L.) [15]. Eto is commonly used for the treatment of a variety of malignancies, including small-cell lung cancer and other solid tumors [16]. Among the most common side effects for patients taking etoposide belongs the low white blood cell and low platelet counts, hair loss, peripheral neuropathy and slight risk of developing secondary leukemia [17]. The side effects and their severity depend on how much of the drug is administered, thus the suitable nanotransporter could decrease the etoposide exposition in unwanted bodily sites and provide a more effective delivery of the drug to tumor cells. To our knowledge, currently there are no reports about utilization of MWCNT-PEG for carrying etoposide.

Antisense oligonucleotides are powerful tools for the *in vivo* regulation of gene expression. Their hybridization to the target sequence is proposed to interfere with RNA intermediary metabolism and/or to induce RNA degradation, resulting in a decreased expression of the target gene [18]. The development of this new promising class of therapeutic agents has been however delayed, due to the difficulties in oligonucleotides delivery to cells and their limited stability, caused by degradation by ubiquitous nucleases [19]. Hence, the phosphorothioate modification in which a non-bridging oxygen

atom in the phosphate backbone is replaced by sulfur has been developed with the aim of the stability increase. This led to the introduction of few novel phosphorothioate oligonucleotides for therapy (or chemosensitivization) of small cell lung cancer and other malignancies (G3139 or Isis 3521 into clinical trials [20,21]. For further enhancement of the cellular uptake of antisense oligonucleotides, carbon nanomaterials can be employed [22].

Thus, the present study is focused on the preparation and characterization of acid oxidized MWCNTs (*o*MWCNTs), double functionalized with anticancer drug etoposide and phosphorothioate oligodeoxynucleotide (PODN). Our current objective is therefore dual. First, we want to compare the binding efficiency of *o*MWCNT and MWCNT-PEG towards etoposide to optimize the ideal applied concentration of drug, which can be carried out by carbon nanoparticles. The second aim was to evaluate the binding capacity of MWCNT-PEG-Eto towards PODN to obtain multifunctional nanotransporter - characterized in detail, suitable for further biological applications.

2. EXPERIMENTAL PART

2.1. Chemicals

All reagents for syntheses, phosphorothioate oligodeoxynucleotides, standards, and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) in ACS purity, unless noted otherwise.

2.2. Preparation of multiwall carbon nanotubes and PEGylation

2 mg of MWCNTs (Sigma Aldrich, St. Louis, MO, USA) was taken in an Eppendorf tube and subsequently 1 mL of 68% HNO_3 (Sigma Aldrich, MO, USA) in aqueous solution (*w/w*) was added for its oxidation. The mixture was heated using a thermo-mixer (Eppendorf, Hamburg, Germany) for 1 h at 80 °C and 800 rpm. Further, the sample was sonicated using an ultrasonic bath (Bandelin, Berlin, Germany) for 15 min and centrifuged at 25 000 rpm at 20 °C for 10 min using a table top centrifuge machine (Eppendorf, Hamburg, Germany). The supernatant was discarded and the MWCNTs were washed 6-7 times by centrifugation (25 000 rpm at 20 °C for 10 min) with MiliQ water until the pH became 7. Finally, the volume was made up to 2 mL of MiliQ water.

To prepare PEGylated MWCNT 1 mL of crude PEG solution (40%, *w/w*) was mixed with 1 mL of MWCNT and resulting mixture was 20 min at 25 °C. The solution was further centrifuged (25 000 rpm at 20 °C for 10 min) and the supernatant was discarded to remove unbound PEG. The PEGylated MWCNT was re-dissolved in 1 mL of H_2O. Purified MWCNTs-PEG solution was stored at 4 ^oC.

2.3. Modification of oMWCNT and MWCNT-PEG with etoposide and oligodeoxynucleotides

A 30 mM Stock solution of etoposide was prepared using dimethyl sulfoxide and H_2O. The stock solution was serially diluted to make required concentrations. Etoposide solutions were added to either MWCNT-PEG or *o*MWCNT in a ratio of 1:1 *v/v*. Then they were mixed for 1 h at 25 °C. The final concentrations of etoposide in the samples were 15; 12; 8; 4; 1 and 0 mM.

For further modification of MWCNT-PEG complex, the PODNs (antisense to Bcl-2 encoding mRNA) with following sequence 5'-TCTCCCAGCGTGCGCCAT-3' was utilized. The modification of oligodeoxynucleotides was performed by using its aqueous solution (final concentrations 0; 1; 3; 5; 10 μM) for direct mixing with MWCNT-PEG with etoposide (mixed in ratio 1:1 *w/w*) in thermo-mixer for 24 h at 25 °C and 800 rpm. The resulting solution was centrifuged (25 000 rpm at 20 °C for 10 min) and the supernatant was discarded to remove unbound PODNs. For re-dissolution MiliQ water was utilized.

2.4. Characterization of oMWCNT and MWCNT-PEG

Firstly, the morphology of *o*MWCNT and MWCNT-PEG was examined using scanning electron microscope MIRA3 LMU (Tescan, a.s., Brno, Czech Republic). An accelerating voltage of 15 kV and beam current about 1 nA were used with satisfactory results regarding to maximum throughput.

The behavior of relative current response of MWCNT before and after PEGylation was investigated using scanning electrochemical microscope (SECM) Model 920D (CH instruments, Inc., Austin, TX, USA), consisting of 10 mm measuring platinum disc probe electrode with potential of 0.2 V. The measurement was performed in Teflon cell with volume of 1.5 mL under the following setting: amperometric mode, vertical scan was carried out in the area 800×800 μm with rate of 30 $\mu m.s^{-1}$. The same parameters were employed for determination of relative current response changes after modification of *o*MWCNT and MWCNT-PEG with etoposide.

Particles hydrodynamic diameter and zeta potential were determined by dynamic light scattering (DLS) on Particle Size Analyzer (Zetasizer Nano ZS90, Malvern instruments, Malvern, UK) using 1 $mg.mL^{-1}$ of pristine or modified MWCNTs. For this purpose, particles were dispersed in MiliQ water and incubated at 25 °C for 15 min prior the measurement.

The success of PEGylation of MWCNT was confirmed using UV-Vis spectrophotometer SPECORD 210 (Analytik Jena, Jena, Germany) with carousel heated to 37 °C by a flow thermostat Julabo F25 (Julabo, Seelbach, Germany).

2.5. Determination of fluorescence

Fluorescence analyses were performed using multifunctional microplate reader Tecan Infinite 200 PRO (TECAN, Maennedorf, Switzerland). Samples (50 μL) were applied into UV-transparent 96 well microplate with flat bottom Costar® (Corning, NY, USA). All measurements were performed at 25°C controlled by Tecan Infinite 200 PRO (TECAN, Switzerland) with the λ_{exc} 250 nm.

2.6. Differential pulse voltammetry

Differential pulse voltammetry (DPV) for detection of etoposide and the complexes of etoposide with MWCNT and MWCNT-PEG was performed by using glassy carbon electrode (GCE) modified by spreading of 10 μL of solution of these complexes over the electrode, which was dried prior the measurements. Such modified GCE was further employed for measuring in supporting electrolyte - Britton-Robinson buffer (0.04 M, pH 6). After each measurement, modified GCE was manually polished with aqueous slurry of alumina powder (Φ: 0.3 μm) on a damp smooth polishing cloth (BAS velvet polishing pad). Parameters for DPV analysis are initial potential 0 V; end potential 1 V; modulation amplitude 0.1 V; modulation time 0.004s; interval time 0.1 s.

2.7. Square wave voltammetry

MWCNT-PEG-Eto complex was incubated with solution of PODNs (0; 1; 3; 5; 10 μM) and after decantation; supernatant was removed and utilized for measurements. Determination of PODNs in supernatant by square wave voltammetry was performed with 797 VA Computrace instrument (Metrohm, Herissau, Switzerland), using a standard cell with three electrodes. A hanging mercury drop electrode with a drop area of 0.4 mm^2 was used as the working electrode. An Ag/AgCl/3M KCl electrode was the reference and platinum electrode was auxiliary. The analyzed samples were deoxygenated prior to measurements by purging with argon (99.999%) for 120 s. 0.2 M acetate buffer (p*H* 5.0) was utilized as a supporting electrolyte. The supporting electrolyte was exchanged after each analysis. The parameters of the measurement were as follows: initial potential -0.1 V, end potential -1.6 V, deposition time 240 s, pulse amplitude 0.02 V, voltage step 0,006 V, sweep rate 0,2975 V/s, frequency 50 Hz, volume of injected sample: 10 μL, volume of measurement cell 2 mL (10 μL of sample + 1990 μL of acetate buffer).

3. RESULTS AND DISCUSSION

3.1. Preparation of oMWCNTs

MWCNTs are usually functionalized by harsh oxidative processes, such as refluxing in concentrated acids, generating defects, which can serve as anchor moieties for further modification [23]. Thus, we utilized protocol using concentrated HNO_3 for treatment of pristine MWCNTs. The protocol of acidic oxidation is schematically depicted in Fig. 1 together with subsequent PEGylation of introduced COOH moieties, resulted from oxidation [24].

3.2. Characterization of MWCNTs PEGylation

As it is well known, carbon nanotubes are one representative nanomaterial with heterostructure. The characterization of the carbon nanotubes is thus hard to be determined,

particularly due to their complicated surface chemistry induced by different modification processes [25]. Scanning electron microscopy (SEM) is widely used to characterize morphology as well as individual size of carbon nanotubes [26,27].

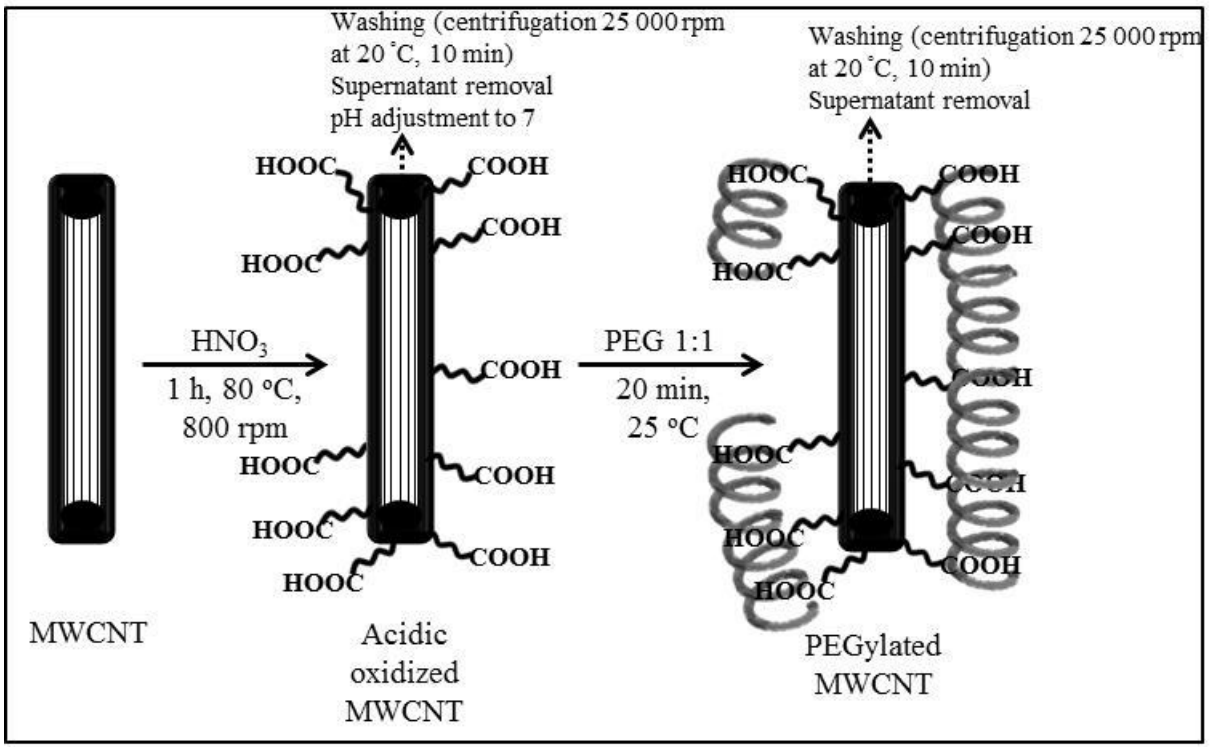

Figure 1. Overall schematic workflow of multistep preparation of acidic oxidized MWCNT-PEG.

Fig. 2A shows SEM micrograph for the *o*MWCNTs (scale bar 500 nm), exhibiting the appearance of loose curly nanotubes tens of nanometers wide, seemingly having a broad length distribution. The electroactivity of the *o*MWCNTs was documented by SECM (3D scan depicted in Fig. 2B). It is obvious that MWCNTs layer on GCE oxidized willingly, which resulted in a shift of positive relative current response to positive values (approx. 14.8 nA). Such *o*MWCNTs were further employed for modification with PEG, working as a biofunctional spacer for further modifications [8].

The aqueous solution containing MWCNT-PEG presented itself as monodisperse and stable. In order to establish the hydrodynamic diameter of the MWCNTs-PEG, DLS technique was employed (Fig. 2C). DLS measurements of MWCNTs-PEG colloid in aqueous media indicated that nanoparticles bundles exhibit hydrodynamic diameter ranging from 610 - 1106 nm. Although the DLS mainly provides information about sphere-shaped particles that have the same translational diffusion coefficient [28], thus, the data obtained did not correspond to a single dimension (length or diameter) of the nanomaterial, but rather to a combined value of both dimensions. Zeta potential analyses showed values around -20.05 ± 0.3 mV (mean of three different batches), which points at good repulsive forces, pushing particles apart and therefore they do not tend to aggregate. Fig. 2D illustrates SEM micrograph showing the surface morphological structure of the MWCNT-PEG exhibiting the change

in the structure caused by polymerization. PEGylation of MWCNTs further affected also the relative current response of its layer, determined by SECM (Fig. 2E), where shift to reduction, expressed as relative current response has been determined (approx. -7.5 nA). Finally, to confirm the success of MWCNT PEGylation, UV-Vis spectra were recorded (Fig. 2F). The most intense absorbed wavelength for PEG and MWCNT-PEG was located at approx. 198 nm, which is in correspondence with [29]. Previously, it was described that PEG binds to CNTs through hydrophobic interactions between the lipophilic moieties and the graphitic sidewalls, leaving the PEG chains projecting from the sidewalls, suitable for subsequent covalent or non-covalent modifications [8]. Based on above-mentioned data, MWCNT-PEG solution was suitable for further modifications with etoposide.

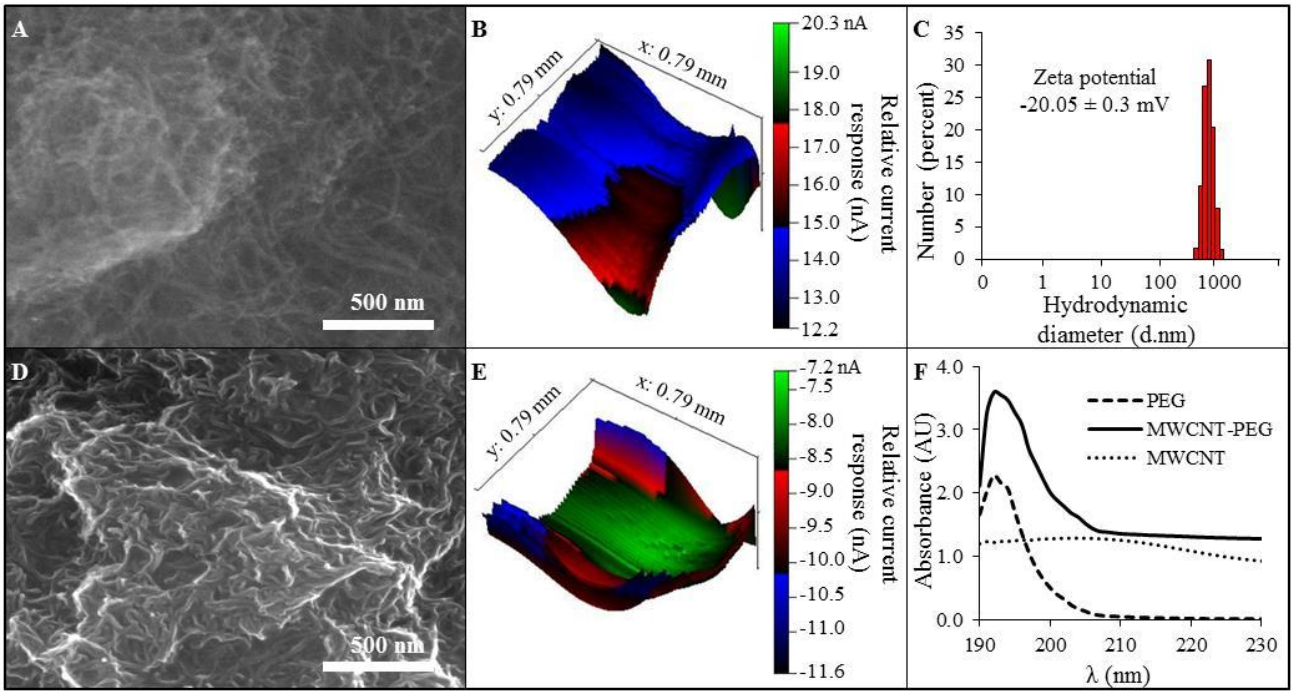

Figure 2. Characterization of *o*MWCNT and its surface modification with PEG. (A) SEM micrograph (length of scale bar is 500 nm) of *o*MWCNT and (B) their SECM 3D scan of relative current response. (**C**) Hydrodynamic diameter of MWCNT after modification with PEG and (**D**) SEM micrograph of MWCNT modified with PEG (length of scale bar is 500 nm) and (**E**) its corresponding SECM 3D scan of relative current response. (**F**) Absorption attributes of MWCNT, PEG and MWCNT-PEG, showing succesfull modification and suitability for further modification.

3.3. Comparison of oMWCNT/MWCNT-PEG modification with etoposide

Etoposide exerts its antineoplastic effect by forming a ternary complex with topoisomerase II and DNA, leading to DNA breaks and cell death [30]. However, the administration of etoposide is limited by its lipophilicity. Furthermore, the vehicles required to increase its aqueous solubility are often associated with adverse side effects [31]. Etoposide is thus a good candidate for development of drug delivery platform. The delivery vehicle has the ability to concentrate only inside the targeted

bodily site. The use of MWCNTs is a very promising approach because these materials can fulfill all the requirements for an ideal oncologic agent. Generally, surface modification of systemic drug carriers by PEG is one of the preferred ways to decrease opsonization by reducing interactions with blood proteins due to its hydrophilicity and steric repulsion effects [32]. Although PEGylation offers beneficial attributes as is mentioned above, it is important to determine its influence on binding of etoposide. Therefore, we prepared two aliquots of MWCNTs - first were only acidic oxidized without addition of PEG and second MWCNTs were fully PEGylated. Both aliquots were modified with etoposide as is described in section *2.3. Modification of oMWCNT and MWCNT-PEG with etoposide and oligodeoxynucleotides* and employed to evaluate their capability to bind etoposide.

Firstly, the interaction between *o*MWCNTs and etoposide was analyzed using three different batches of complexes. Fluorescence determination of drug (λ_{exc} = 250 nm, λ_{em} = 320 nm) revealed that the highest concentration of etoposide reached fluorescence of approx. 61 000 a.u. (Fig. 3A). In Fig. 3B are shown data obtained using DPV, which demonstrate that increasing applied concentration of drug led to linear elevation of etoposide peak height (96.9 μA in case of etoposide in applied concentration 15 mM) determined to be bound in *o*MWCNTs (corresponding to binding recovery of 28.1% in 15 mM etoposide regarding to calibration curve inset in Fig. 3B).

Table 1. The values of recovery and determined concentration of etoposide after conjugation with both - *o*WMCNT and MWCNT-PEG.

MWCNTs functionalization	Applied concentration of etoposide (mM)	Binding recovery (%)	Determined concentration of etoposide (mM)
***o*MWCNT**	1.0	19.4	0.2
***o*MWCNT**	4.0	22.2	0.9
***o*MWCNT**	8.0	26.1	2.1
***o*MWCNT**	12.0	28.8	3.5
***o*MWCNT**	15.0	28.1	4.2
MWCNT-PEG	1.0	65.1	0.7
MWCNT-PEG	4.0	62.9	2.5
MWCNT-PEG	8.0	54.7	4.4
MWCNT-PEG	12.0	54.8	6.6
MWCNT-PEG	15.0	46.4	7.0

The electrochemical behavior of layer composed of *o*MWCNT-Etoposide (15 mM) was further analysed using SECM and 3D scan of relative current response is documented in Fig. 3C, where one spot exhibiting relative current response about -25 nA probably belongs to *o*MWCNTs agglomerate which remained unmodified by etoposide. In case of MWCNT-PEG, fluorescence measurements revealed significantly higher fluorescence of drug (approx. 201 000 a.u. for 15 mM applied etoposide) (Fig. 3D). The DPV results revealed the etoposide binding trend in agreement with its fluorescence (Fig. 3E). In case of the highest applied concentration of etoposide (15 mM), the determined peak

height was about 162.4 µA (corresponding to 46.4% recovery). Using lower applied concentration (12 mM), relatively similar result (153.2 µA, recovery 54.8%) was obtained, which points at probable saturation plateau of MWCNT-PEG complex. Interestingly, in the lowest applied concentrations, higher recoveries were observed. This phenomenon is connected with relatively short incubation time (1 h) and it could be expected that longer time will led to fully saturation of MWCNT-PEG with etoposide. Recovery values and determined concentrations of etoposide are summarized in Tab. 1.

Finally, SECM 3D scan of MWCNT-PEG-Etoposide layer (15 mM) was analysed. As it is shown in Fig. 3F, relative current response was turned to positive values (approx. 7.0 nA), which is probably caused by presence of relatively high amount of bound drug that significantly influences the electrochemical behavior of entire complex.

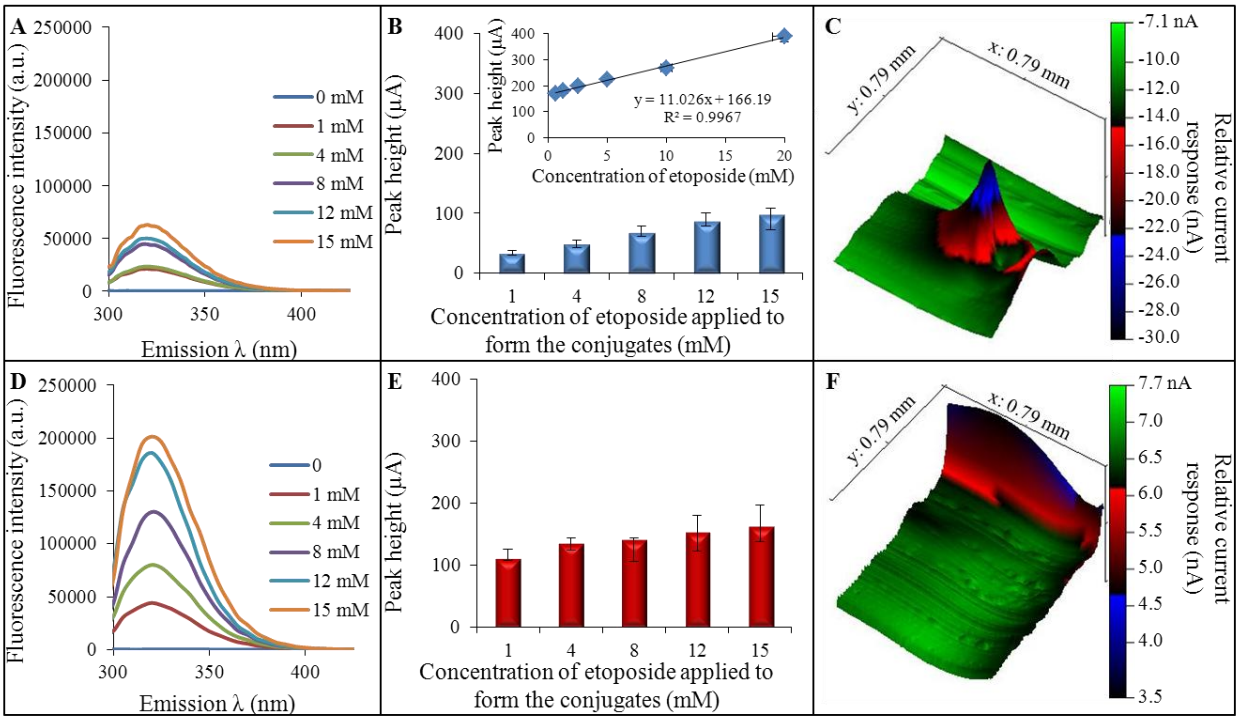

Figure 3. Efficiency of *o*MWCNT conjugation with etoposide. (**A**) Fluorescence emission spectra of etoposide (concentrations 0 - 15 mM) complexed to *o*MWCNT (λ_{exc} = 250 nm). (**B**) Dependence of etoposide applied for concentration (0 - 15 mM) on its electrochemical response (DPV). Calibration curve of etoposide in linear dynamic range 0.6 - 20 mM is shown in inset. (**C**) SECM 3D scan of relative current response of *o*MWCNT after modification with etoposide. Efficiency of MWCNT-PEG modification with etoposide described as (**D**) Fluorescence emission spectra of etoposide (concentrations 0 - 15 mM) modified to MWCNT-PEG (λ_{exc} = 250 nm). (**E**) Dependence of etoposide applied for concentration (0 - 15 mM) on its electrochemical response (DPV) and (**F**) SECM 3D scan of relative current response of MWCNT-PEG after modification with etoposide is shown. Values are means of three independent replicates (*n* = 3). Vertical bars indicate standard error.

PEGylated CNTs have been previously used as the delivery system for a variety of drugs including anticancer agents [10,11]. PEGylation allows MWCNTs for surprisingly high degrees of π-

stacking of aromatic molecules, including cytostatics. Thus they can be simply mixed together with PEGylated CNTs to form non-covalent bond through π- π stacking, as was shown in case of paclitaxel [33], doxorubicin [34], irinotecan [35], and others. In our case, we employed etoposide, which non-covalently binds MWCNT-PEG with superior recovery, and thus its application could be simply improved to enhance its insufficient aqueous solubility and reduce the undesired side effects [31].

3.4 Characterization of binding capability of MWCNT-PEG-Eto towards PODNs

To further extend the possible biological applications of MWCNT-PEG-Eto, phosphorothioate oligodeoxynucleotide with antisense sequence complementary to mRNA encoding Bcl-2 was utilized. Bcl-2 family proteins are tightly connected with apoptotic resistance in a number of cancers, including non-small cell lung cancer (NSCLC), which is one of the most aggressive cancers as per as the mortality and occurrence [36]. PODNs are designed as nuclease-resistant with exceptional biological effects; hence they are recently broadly utilized as agents in antisense therapy [37], allowing down-regulation of expression of target protein (Bcl-2 in our case), and thus enhancing the susceptibility of cancer cells on presence of common cytostatics.

For identification of binding ability of PODNs to MWCNT-PEG-Eto, square wave voltammetric technique was employed. As a results, molecule of DNA exhibits typical CA peak (potential about -1.39 V) [38]. Calibration curve of PODN (0.08 - 20.0 μM) is illustrated in Fig. 4A, together with inset, showing its square wave voltammograms.

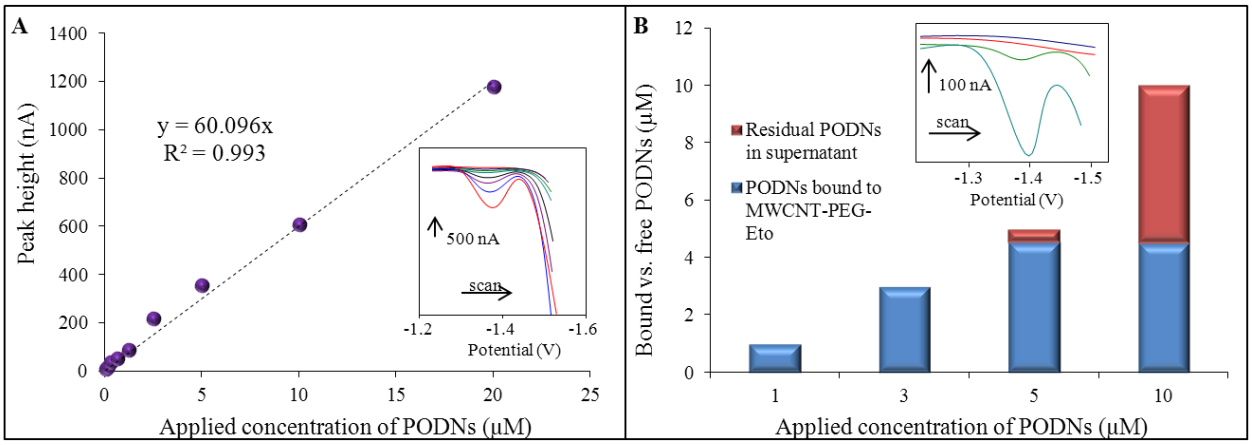

Figure 4. Electrochemical evaluation of loading efficiency of MWCNT-PEG-Eto (variant with the highest applied concentration of etoposide - 15 mM). (**A**) Calibration curve of PODNs (0.08 - 20.0 μM) with inset with corresponding square wave voltammograms. (**B**) Expression of electrochemical determination of residual PODNs in supernatant after forming a complex with MWCNT-PEG-Eto and subsequent decantation.

To evaluate whether PODNs are able to form a multifunctional complex with MWCNT-PEG-Eto, we employed the variant with the highest determined concentration of etoposide (15 mM). We hypothesized that nucleic acids can bind complex either through a non-covalent π-π stacking between

the unoccupied sites on a surface of MWCNT-PEG-Eto and the backbone of oligodeoxynucleotide as was described in [39] or through direct etoposide/nucleic acid interactions. In Fig. 4B can be seen the PODNs concentrations (red bar), determined in discarded supernatant after incubation and decantation, following the protocol in *Experimental section*. It is obvious that application of 1 and 3 μM PODNs led to maximum saturation of MWCNT-PEG-Eto, thus no CA peak was identified in supernatant. In case of higher applied concentrations - 5 and 10 μM, PODNs presence in supernatant was observed (0.47 μM or 5.50 μM, respectively). This fact reveals a binding capacity of MWCNT-PEG-Eto towards PODNs to be 4.50 μM. According to recent literature, such concentrations could be sufficient dose for down-regulation of target protein [19,40,41], however the biological effects after formation of the complex have to be subjected to an extensive testing. Overall, it can be stated that MWCNT-PEG-Eto-PODN complex could be helpful as novel multifunctional agent in combinatorial therapy of cancer, where simultaneous inhibition of chemoresistance and cytotoxicity are required.

4. CONCLUSION

Etoposide/PODNs loaded PEGylated MWCNTs were prepared with exceptional drug and antisense nucleic acid loading capacities. The complex can be prepared by a simple multi-step process, however, further biological studies have to be carried out to show the effects on proliferation, target protein expressions of cancer cells and determine the complex behavior in circulatory system. Overall, it can be stated that electrochemistry offers broad possibilities in characterization of nano-, microscaled technologies and their interaction with molecules, enhancing their usefulness. The suggested, prepared and characterized MWCNT-PEG-Eto-PODN could be helpful in the future as multifunctional agent in combinatorial therapy, utilizing simultaneous dampening of chemoresistance and cytotoxic effects.

ACKNOWLEDGEMENTSFinancial support from League against cancer Prague (project 18257/2014-981) is highly acknowledged. The authors wish to express their thanks to Michal Zurek for perfect technical assistance.

CONFLICTS OF INTEREST
The authors declare no conflicts of interest.

References

1. Z. Heger, S. Skalickova, O. Zitka, V. Adam, R. Kizek, *Nanomedicine* 9 (2014) 2233.
2. V. S. Thakare, M. Das, A. K. Jain, S. Patil, S. Jain, *Nanomedicine* 5 (2010) 1277.
3. A. Bianco, K. Kostarelos, M. Prato, *Expert Opin. Drug Deliv*. 5 (2008) 331.
4. Z. W. Wang, M. D. Shirley, S. T. Meikle, R. L. D. Whitby, S. V. Mikhalovsky, *Carbon* 47 (2009) 73.

5. J. Chen, M. A. Hamon, H. Hu, Y. S. Chen, A. M. Rao, P. C. Eklund, R. C. Haddon, *Science* 282 (1998) 95.
6. Z. F. Chen, S. Nagase, A. Hirsch, R. C. Haddon, W. Thiel, P. V. Schleyer, *Angew. Chem.-Int. Edit.* 43 (2004) 1552.
7. K. Balasubramanian, M. Burghard, *Small* 1 (2005) 180.
8. M. Bottini, N. Rosato, N. Bottini, *Biomacromolecules* 12 (2011) 3381.
9. E. B. Malarkey, V. Parpura, *Neurodegener. Dis.* 4 (2007) 292.
10. R. P. Feazell, N. Nakayama-Ratchford, H. Dai, S. J. Lippard, *J. Am. Chem. Soc.* 129 (2007) 8438.
11. S. Dhar, Z. Liu, J. Thomale, H. J. Dai, S. J. Lippard, *J. Am. Chem. Soc.* 130 (2008) 11467.
12. Z. Liu, K. Chen, C. Davis, S. Sherlock, Q. Z. Cao, X. Y. Chen, H. J. Dai, *Cancer Res*. 68 (2008) 6652.
13. N. W. S. Kam, Z. Liu, H. J. Dai, *J. Am. Chem. Soc*. 127 (2005) 12492.
14. S. Marchesan, M. Prato, *ACS Med. Chem. Lett*. 4 (2013) 147.
15. S. K. Pang, N. Y. Zheng, C. A. Felix, J. Scavuzzo, R. Boston, I. A. Blair, *J. Mass Spectrom.* 36 (2001) 771.
16. T. Groh, J. Hrabeta, J. Poljakova, T. Eckschlager, M. Stiborova, *Neuroendocrinol. Lett.* 33 (2012) 16.
17. M. V. Relling, Y. Yanishevski, J. Nemec, W. E. Evans, J. M. Boyett, F. G. Behm, C. H. Pui, *Leukemia* 12 (1998) 346.
18. D. W. Leaman, *Expert. Opin. Drug Discov*. 3 (2008) 997.
19. Z. Heger, J. Gumulec, N. Cernei, K. Tmejova, P. Kopel, J. Balvan, M. Masarik, O. Zitka, M. Beklova, V. Adam, R. Kizek, *Oncol. Rep*. 33 (2015) 921.
20. K. Li, J. Zhang, *Curr. Opin. Invest. Drugs* 2 (2001) 1454.
21. B. Jansen, V. Wacheck, E. Heere-Ress, H. Schlagbauer-Wadl, C. Hoeller, T. Lucas, M. Hoermann, U. Hollenstein, K. Wolff, H. Pehamberger, *Lancet* 356 (2000) 1728.
22. B. D. Dolash, D. E. Bergstrom, Progress and Road Blocks in the Development of Carbon Nanotubes as Cellular Carriers for Nucleic Acids, Pan Stanford Publishing Pte Ltd, Singapore, 2010,
23. L. Li, Y. C. Xing, *J. Phys. Chem*. C 111 (2007) 2803.
24. M. Piran, V. Kotlyar, D. D. Medina, C. Pirlot, D. Goldman, J. P. Lellouche, *J. Mater. Chem*. 19 (2009) 631.
25. X. L. Cheng, J. Zhong, J. Meng, M. Yang, F. M. Jia, Z. Xu, H. Kong, H. Y. Xu, *J. Nanomater.* (2011) 1.
26. L. Song, L. Ci, L. Lv, Z. P. Zhou, X. Q. Yan, D. F. Liu, H. J. Yuan, Y. Gao, J. X. Wang, L. F. Liu, X. W. Zhao, Z. X. Zhang, X. Y. Dou, W. Y. Zhou, G. Wang, C. Y. Wang, S. S. Xie, *Adv. Mater.* 16 (2004) 1529.
27. Y. B. Wang, Z. Iqbal, S. Mitra, *J. Am. Chem. Soc*. 128 (2006) 95.
28. Y. K. Moon, J. Lee, J. K. Lee, T. K. Kim, S. H. Kim, *Langmuir* 25 (2009) 1739.
29. H. Xu, T. Zhu, R. P. Yu, *Spectrosc. Spectr. Anal*. 27 (2007) 1381.
30. K. R. Hande, *Eur. J. Cancer* 34 (1998) 1514.
31. P. J. Odwyer, R. B. Weiss, *Cancer Treat. Rep*. 68 (1984) 959.
32. F. Danhier, N. Lecouturier, B. Vroman, C. Jerome, J. Marchand-Brynaert, O. Feron, V. Preat, *J. Control. Release* 133 (2009) 11.
33. J. M. Berlin, A. D. Leonard, T. T. Pham, D. Sano, D. C. Marcano, S. Y. Yan, S. Fiorentino, Z. L. Milas, D. V. Kosynkin, B. K. Price, R. M. Lucente-Schultz, X. X. Wen, M. G. Raso, S. L. Craig, H. T. Tran, J. N. Myers, J. M. Tour, *ACS Nano* 4 (2010) 4621.
34. Y. J. Wang, H. Xu, H. Z. Liu, Y. Wang, J. Sun, Z. G. He, J. Biomed. Nanotechnol. 8 (2012) 450.
35. M. Zhou, Z. Peng, S. Q. Liao, P. W. Li, S. D. Li, *Drug Deliv*. 21 (2014) 101.
36. A. Chatterjee, D. Chattopadhyay, G. Chakrabarti, *Cell. Signal.* 27 (2015) 189.
37. C. A. Stein, *Chem. Biol*. 3 (1996) 319.

38. K. Tmejova, L. Krejcova, D. Hynek, V. Adam, P. Babula, L. Trnkova, M. Stiborova, T. Eckschlager, R. Kizek, *Anti-Cancer Agents Med. Chem*. 14 (2014) 331.
39. G. Liu, M. Swierczewska, S. Lee, X. Y. Chen, *Nano Today* 5 (2010) 524.
40. S. Lledo, R. Alfonso, S. F. Alino, *Rev. Esp. Enferm. Dig*. 97 (2005) 472.
41. Y. Jin, S. J. Liu, B. Yu, S. Golan, C. G. Koh, J. T. Yang, L. Huynh, X. J. Yang, J. X. Pang, N. Muthusamy, K. K. Chan, J. C. Byrd, Y. Talmon, L. J. Lee, R. J. Lee, G. Marcucci, *Mol. Pharm*. 7 (2010) 196.