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Electrochemical monitoring of the interaction between mitomycin C and DNA at chitosan–carbon nanotube composite modified electrodes

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Abstract: Single-walled carbon nanotube (CNT) and chitosan composite (chitosan*CNT) based sensors were developed as DNA biosensors, and then they were applied for electrochemical investigation of the interaction between the anticancer drug mitomycin C (MC) and DNA. The oxidation signals of MC and guanine were monitored before and after the interaction process by differential pulse voltammetry (DPV). The DPV results were in good agreement with those of electrochemical impedance spectroscopy (EIS). Analytical parameters such as DNA concentration, MC concentration, and MC interaction time with DNA were optimized. The detection limits were 6.85 μg/mL for DNA and 11.01 μg/mL for MC.

Key words: Chitosan, single-walled carbon nanotubes, mitomycin C, DNA interactions, disposable graphite electrode, electrochemical impedance spectroscopy, differential pulse voltammetry

1. Introduction

Electrochemical DNA biosensors attract attention due to their speediness, reproducibility, and reliability. Because of their remarkable characteristics, electrochemical DNA biosensors enable us to perform research in many areas such as clinical diagnosis, environmental monitoring, and drug investigation. Recently, the modification of the sensors' surfaces with various nanomaterials has received a lot of attention. Since carbon nanotubes (CNTs) were discovered in 1991, they have been tested in many fields because of their unique features.[1] CNTs have good electronic properties and electric conductivity; thus, naturally they have also become popular in the field of electrochemical sensors technology.[2] By surface modification with CNTs or CNT–polymer composite structures, DNA biosensors have presented an advanced sensitivity and, in some cases, selectivity.[3–7]

Chitosan, a linear β-1,4-linked polysaccharide, is a biological cationic macromolecule with primary amines.[2] It is obtained by the partial de-acetylation of chitin, a major component of the shells of crustaceans such as crab, shrimp, and crawfish. Chitosan has distinct chemical and biological properties, because it has reactive amino and hydroxyl groups in its linear polyglucosamine high molar mass chains.[8,9] In addition, it is biocompatible, biodegradable, nontoxic, and natural, and is a high mechanical strength biopolymer with an excellent film-forming ability and is also a very good matrix for enzyme and/or biomacromolecule immobilization.[10] For instance, Xu et al. investigated electrochemically DNA hybridization at a chitosan modified platinum electrode and thus they declared that ssDNA immobilized on the chitosan modified electrode can hybridize efficiently with its complementary sequences.[11]

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Chitosan has also been tested for the development of CNT based electrochemical biosensors.[8,9] The electrodeposition of chitosan and CNTs was performed and this modified electrode was then used for biosensors by Luo et al.[12] Due to the increasing problems related to environmental pollution and industrial wastes in parallel with developing technology, new diseases are emerging with each passing day. Electrochemical sensor techniques may offer a new outlook for investigation of these diseases and development of new drugs. Electrochemical drug studies in the literature have particularly focused on the drug–DNA interaction, especially for the interaction of anticancer drugs with nucleic acids.[13] Electrochemistry provides an easy, simple, rapid, low-cost way for preparation of desired electrodes and measurement. Different electrochemical techniques (cyclic voltammetry (CV), differential pulse voltammetry (DPV), square wave voltammetry, electrochemical impedance spectroscopy etc.) have been used for monitoring the interaction of anticancer drugs with DNA.[14−17] The interaction between the anticancer drug epirubicin and DNA using differential pulse voltammetry and cyclic voltammetry at a carbon paste electrode was studied by Erdem et al.[18] Electrochemical investigation of the interaction between titanocene dichloride and single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) was performed by CV technique by Ravera et al.[19] The DNA–danthron interaction was explored electrochemically using a pyrolytic (PG) disk electrode.[20] The investigation of *cis*-diamminedichloroplatinum(II) (*cis*-DDP) and the chemotherapeutic agent *cis*-bis(3-aminoflavone)dichloroplatinum(II) (*cis*-BAFDP) electrochemically with calf thymus dsDNA by DPV with a disposable pencil graphite electrode (PGE) at the surface was done by Erdem et al.[21]

Mitomycin C (MC) is an antitumor agent and an anticancer and antibiotic drug against a broad spectrum of solid tumors. MC was isolated from *Streptomyces caespitosus* and used in treating several cancers including gastric cancer, anal and colon cancer, breast cancer, nonsmall cell lung cancer, head and neck cancer, small bladder papillomas, pancreatic cancer, and cervical cancer.[22] Since MC is an alkylating agent that binds to DNA, causing cross-linking and inhibition of DNA synthesis, there have been some studies on the electrochemical monitoring of MC–DNA interaction.[6,17,22−27] After interaction with DNA, MC was reduced and then 2 N-alkylations followed this reductive activation. Both alkylations are sequence specific for a guanine nucleoside in the sequence 5'-CpG-3'.[23] The interaction between MC and DNA in a novel drug-delivery system was studied using a PGE.[17] The interaction of MC with different types of DNA immobilized onto the surfaces of carbon electrodes was also investigated.[25] Perez et al. studied the interactions of surface-confined DNA with electroreduced MC in comparison with acid-activated MC using a hanging mercury drop electrode (HMDE).[26]

In our study, the single-walled CNT chitosan polymeric composite nanostructure (chitosan*CNT) modified PGE was developed for electrochemical monitoring of the interaction between an anticancer drug, MC, and DNA. The changes in guanine and MC oxidation signals were detected before and after the interaction process by using DPV. The surface morphologies of unmodified PGEs and modified ones were explored using scanning electron microscopy (SEM). CV and electrochemical impedance spectroscopy (EIS) techniques were used for characterization of unmodified and modified electrodes with chitosan and chitosan*CNT modified PGEs. Concerning the results of studies on the interaction process, EIS results were in good agreement with those of DPV. The effects of different experimental parameters, such as DNA concentration, MC concentration, and MC interaction time with DNA were also studied to find the optimum analytical performance upon electrochemical detection of the surface confined interaction process between MC and DNA.

2. Results and discussion

Firstly, the surface morphologies of unmodified PGEs (Figures 1a–1c), ones modified using chitosan (Figures 1d–1f), and ones modified using CNT (Figures 1g–1i) and chitosan*CNT (Figures 1j–1l) were explored using SEM analysis at different magnitudes: 10 μm, 2 μm, and 500 mm (shown in Figure 1). After chitosan modification of the PGE surface, different surface morphology was more distinctive, particularly at 500-nm magnitude (in Figures 1b and 1e). In addition, it can be seen from Figures 1j–1l that the electrode surface was covered more homogeneously by the chitosan*CNT mixture.

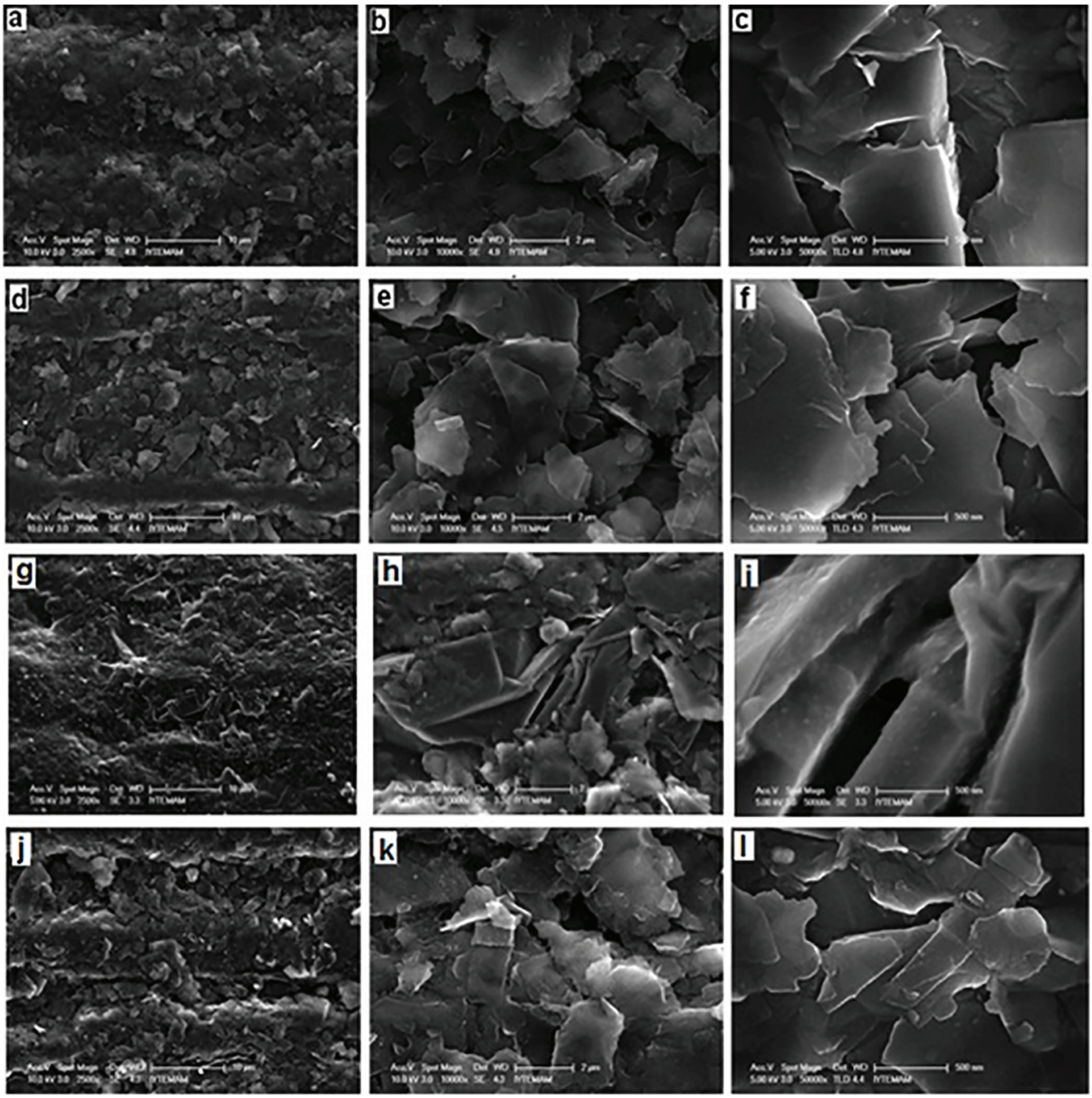


Figure 1. SEM images of bare PGE (a, b, c), chitosan PGEs (d, e, f), CNT PGEs (g, h, i) and chitosan*CNT PGEs (j, k, l). Identical acceleration voltage between 5.0 and 10.0 kV with resolution of various magnitudes: 10 μm, 2 μm, and 500 mm.

The cyclic voltammograms of the unmodified PGE, chitosan PGE, CNT PGE, and chitosan*CNT PGE were performed in 2 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ (1:1) containing 0.1 M KCl in order to explore their electrochemical behavior (shown in Figure 2). The average anodic peak current (*Ia*) of the bare PGE (Figure 2a) was 41.23 ± 6.71 μA (RSD %, 16.27%, n = 3). After chitosan modification of the PGE surface, there was an increase in anodic peak current (i.e. 118.10 ± 5.07 μA) (RSD %, 4.29%, n = 3) (Figure 2b). Similarly, after CNT modification of the PGE surface, an increase was obtained and the average *Ia* was 111.26 ± 5.02 μA (RSD %, 4.51%, n = 3) (Figure 2c). However, the highest increase in *Ia*, 2.20-fold, was observed with the chitosan*CNT modified PGE (shown in Figure 2d). The average *Ia* of the chitosan*CNT PGE was 131.63 ± 6.63 μA (RSD %, 5.04%, n = 3). These results show that the role of the chitosan*CNT modification is to accelerate electron transfer, similar to the earlier study reported by Shieh et al.[8]

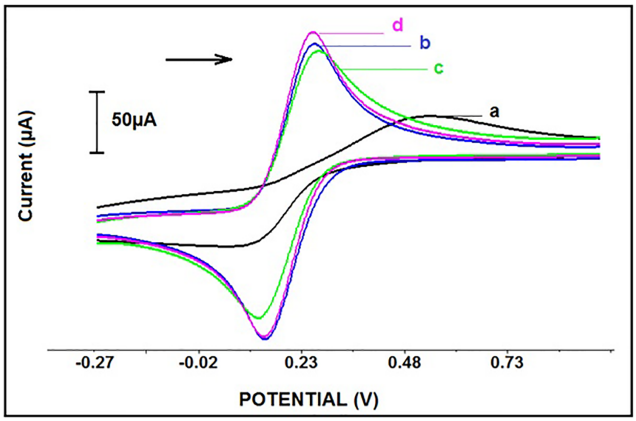


Figure 2. Cyclic voltammograms of (a) unmodified (bare) PGE, (b) chitosan PGE, (c) CNT PGE, (d) chitosan*CNT PGE in $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$.

Next, the electrochemical performances of the PGEs (Figures 3A–3a, 3B–3a), chitosan PGEs (Figures 3A–3b, 3B–3b), and chitosan*CNT PGEs (Figures 3A–3c, 3B–3c) were compared according to the magnitudes of MC and guanine signals. In the absence of an interaction process, the oxidation signals of MC (Figure 3A) and guanine (Figure 3B) were separately measured respectively at +0.83 V and +1.04 V. As can be clearly observed, the response obtained by the unmodified PGE is slightly higher than that observed by using the chitosan modified one, i.e. meaning that chitosan may prevent the conductivity of the modified electrode, similar to the report by Ghica et al.[9] They reported that chitosan has a relatively poor conductivity and this has the effect of partially blocking the electrode. However, slight increases were obtained in the response by the chitosan*CNT PGE related to MC and guanine oxidation signals, respectively, about 33.99% and 32.55%, in contrast to the responses at the unmodified PGE. As the greatest enhancement in both MC and guanine oxidation signals was recorded by using chitosan*CNT PGE, further experiments on electrochemical monitoring of MC and DNA were performed at these sensor platforms.

The changes in guanine oxidation signals were also monitored at different dsDNA concentrations varying between 10 and 60 μg/mL at the surface of chitosan*CNT modified PGEs. A sharp increase in response was obtained up to 50 μg/mL (Figure 4). However, the response gradually decreased after 50 μg/mL concentration level of DNA. The DNA concentration of 50 μg/mL was chosen as the optimum concentration for development of DNA modified chitosan*CNT electrodes. Based on 3 repetitive measurements using 50 μg/mL DNA modified

chitosan*CNT PGEs, the RSD% (n=3) was calculated as 5.27%. The resulting calibration plot is shown in the inset in Figure 4.

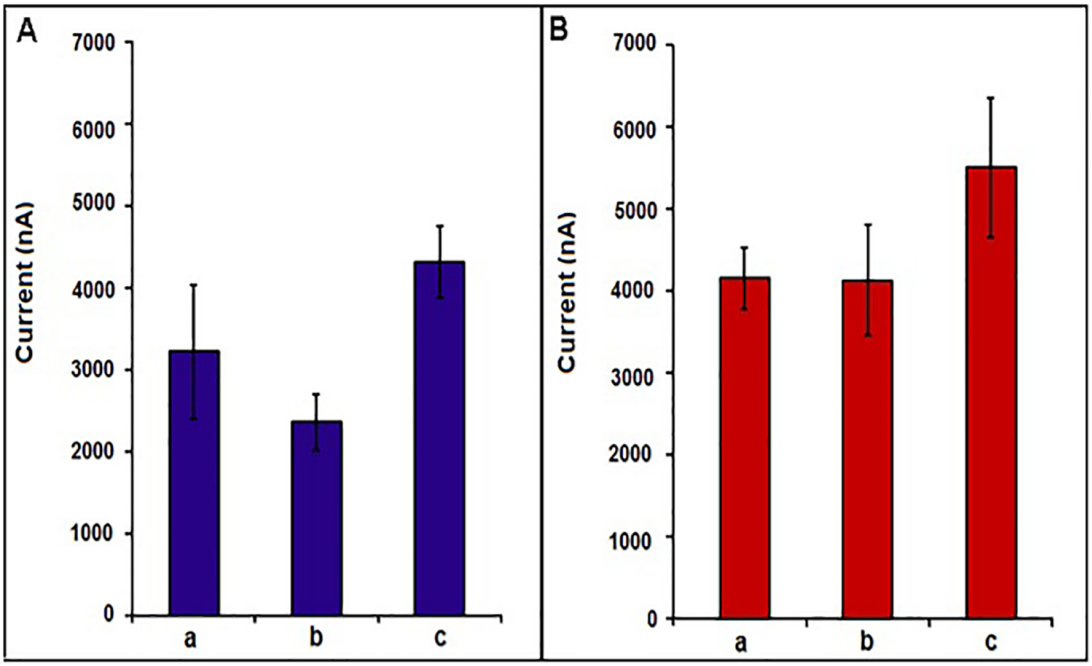


Figure 3. Histograms representing the MC (A) and guanine (B) oxidation signals observed by using bare PGE (a), chitosan PGEs (b), and chitosan*CNT PGEs (c) in the presence of 40 μg/mL MC immobilized for 7.5 min and 50 μg/mL dsDNA immobilized for 1 h.

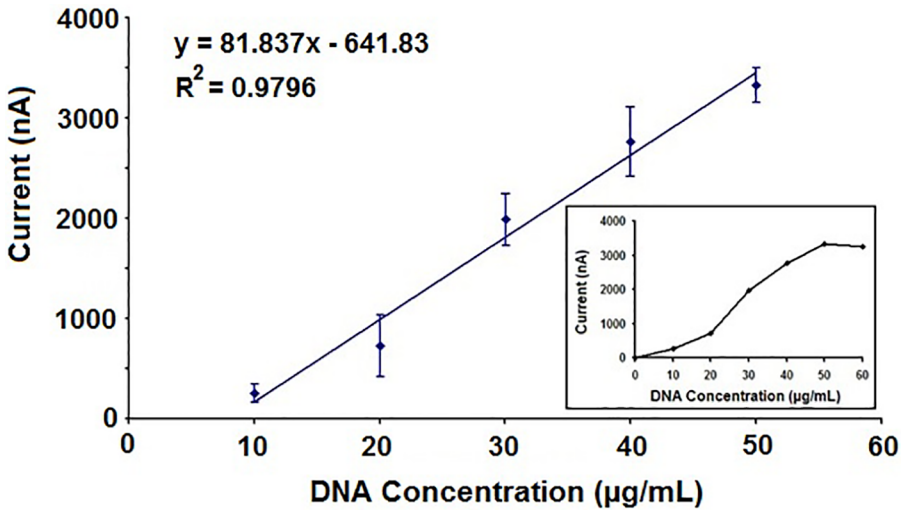


Figure 4. Calibration plot presenting the changes in guanine oxidation signal measured in the presence of various concentration levels of dsDNA from 10 to 50 μg/mL and inset figure representing the line graph based on the guanine oxidation signal obtained in the presence various DNA concentrations between 0 and 60 μg/mL by using chitosan*CNT PGEs.

The detection limit (DL) was calculated as explained in the literature as a regression equation and the definition $y = yB + 3SB$ (yB is the signal of the blank solution and SB is the standard deviation of the blank

solution).[28] According to this procedure, the DL was estimated as 6.85 μg/mL dsDNA concentration.

The changes in MC oxidation signal were also monitored at different MC concentrations from 5 to 50 μg/mL. There was a gradual increase obtained up to 40 μg/mL (Figure 5). Thus, 40 μg/mL was chosen as the optimum MC concentration at the surface of chitosan*CNT modified PGEs.

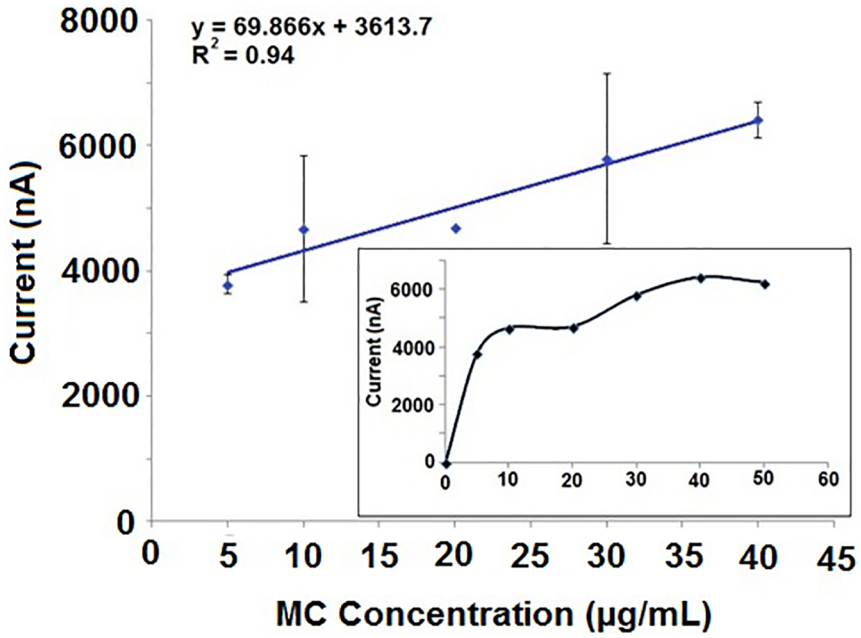


Figure 5. Calibration plot presenting the changes in guanine oxidation signal measured in the presence of various concentration levels of MC from 5 to 40 μg/mL and inset figure representing the line graph based on the guanine oxidation signal obtained in the presence various MC concentrations between 0 and 50 μg/mL by using chitosan*CNT PGEs.

The resulting calibration plot is shown in Figure 5. The DL was calculated as explained in the literature and also above.[28] According to this procedure, the DL was estimated as 11.01 μg/mL MC concentration.

Disposable chitosan*CNT PGEs were also used herein for electrochemical monitoring of anticancer drug–DNA interactions, and consequently MC was chosen as a target compound. MC is an alkylating agent and this alkylation process is related to the sequence specific to guanine nucleoside in the sequence 5'-CpG-3' (shown in Figure 6 in the scheme). Similar to the results in earlier reports,[6,17,23,24,27] it was aimed to monitor a decrease in guanine oxidation signal in the case of the interaction of MC with DNA.

In Figure 6, the representative voltammograms and histograms show the changes in MC and guanine signals observed before and after the surface confined interaction process at chitosan*CNT PGEs in the presence of 40 μg/mL MC and 50 μg/mL of dsDNA for different interaction times: 7.5 min (Figures 6A and 6B), 15 min (Figures 6C and 6D), and 30 min (Figures 6E and 6F). The representative DPVs (Figures 6A, 6C, and 6D) show the oxidation signals of MC and guanine measured in the same voltammetric scale, correspondingly, at +0.83 V and +1.04 V. In each experiment performed at different interaction times, there was a decrease obtained in MC and guanine signals after the interaction process. The highest decrease was obtained at 7.5 min interaction time with a decrease of about 96.08% for MC and 76.10% for guanine. Similar to these results, the decrease for 15 and 30 min for MC was 91.80% and 56.17% and for guanine was 71.64% and 70.00%, respectively.

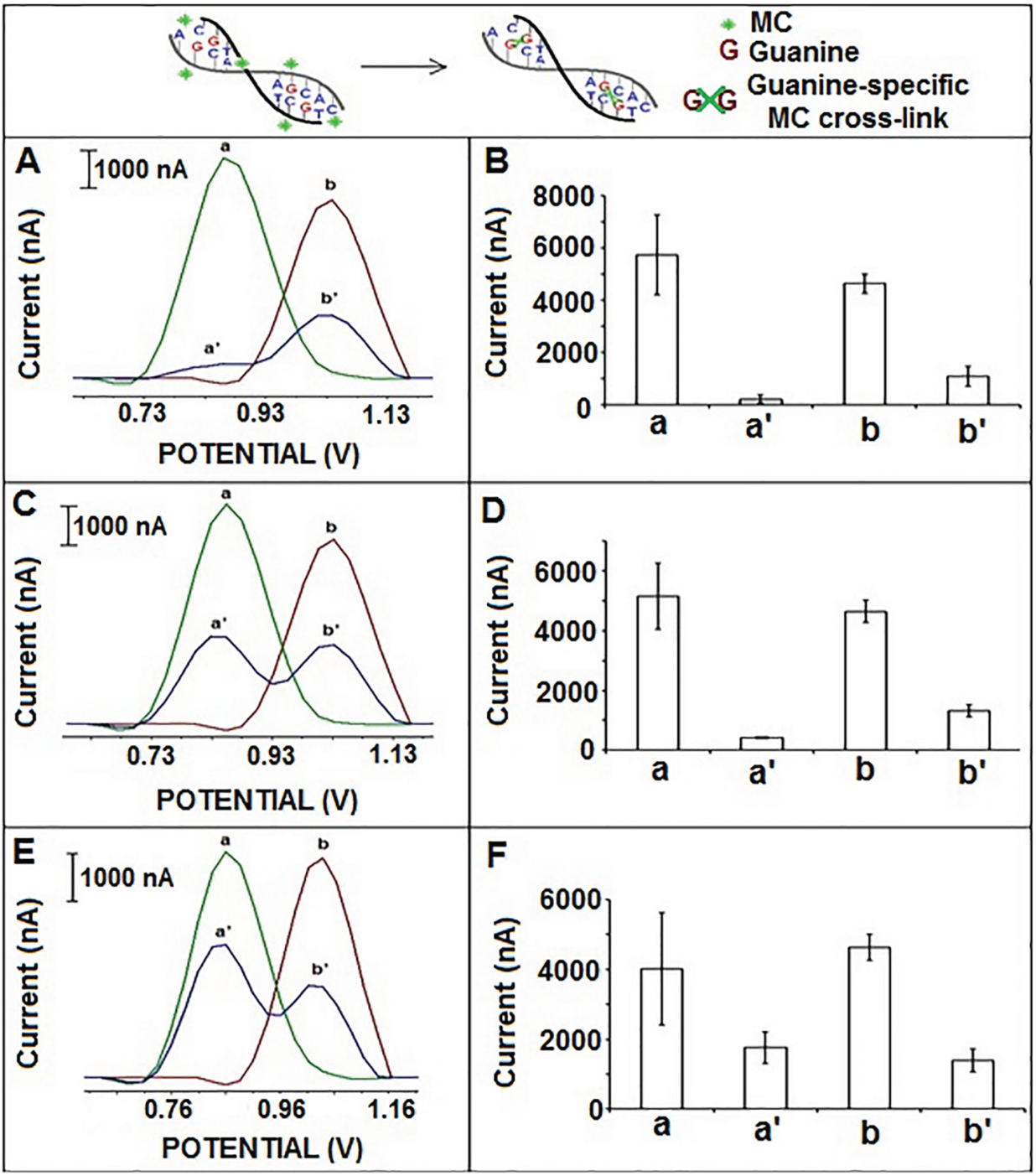


Figure 6. The representative interaction between MC and dsDNA by the alkylations and MC binding to dsDNA with cross-links. (A), (C), (E) DPVs and (B), (D), (F) Histograms representing MC and guanine oxidation signals observed before and after the surface confined interaction between 40 μg/mL MC and 50 μg/mL dsDNA using chitosan*CNT-PGEs. (A) and (B) for 7.5 min interaction time/(C) and (D) for 15 min interaction time/(E) and (F) for 30 min interaction time; the oxidation signal of MC: (a) before interaction, (a') after interaction with dsDNA; and the oxidation signal of guanine: (b) before interaction, (b') after interaction of MC with dsDNA.

In our study, the changes in guanine signal were used as the transduction signal for monitoring of DNA's interaction with MC. As a result of MC's interaction with dsDNA, a decrease in guanine signal was obtained (Figure 6). The value of the percentage of guanine peak height change (S%) was calculated herein according to the literature,[29] which is the ratio of the guanine peak height after the interaction (S_s) and the guanine peak height before the interaction (S_b) as in Eq. (1):

$$S\% = (S_S/S_b) \times 100 \quad (1)$$

Thus, the DPV signal of the sensor in the absence of the analyte served as a "blank" or 100%. Conventionally, if a sample had S > 85%, it was considered nontoxic. If S% was between 50 and 85, it was considered moderately toxic and if S < 50%, it was considered toxic.[29] Concerning the equation given above, the values of S% were calculated for 7.5, 15, and 30 min, and found respectively as 23.9, 28.4, and 30.0. Concerning all these calculated S% values, MC can be considered a toxic chemical.

The partition coefficient of MC on the surface of the chitosan*CNT modified electrode was estimated[25] from the results presented in Figure 6 by the following equation:

$$MC_{bound}/MC_{free} = |(i_{bound} - i_{free})/i_{free}| \quad (2)$$

i_{free} is the oxidation peak current of MC obtained before and i_{bound} the oxidation peak current of MC obtained after interaction with dsDNA. The partition coefficient of 40 μg/mL MC was 0.96, 0.92, and 0.56 for interaction times of 7.5, 15, and 30 min, respectively.

EIS was used to investigate the effect of modification of the surface of disposable graphite electrodes with chitosan and chitosan*CNT. Firstly, the average R_{ct} value of unmodified PGEs was about 50.55 Ω (Figures 7B–7a). After the modification of the PGE surface with chitosan the average R_{ct} value was 5.07 Ω (Figures 7B–7b). Moreover, the average R_{ct} value was 0.00 Ω after chitosan*CNT immobilization onto PGE surfaces (Figures 7B–7c). The changes in the Rct values were strong proof that chitosan and chitosan*CNT could have been immobilized onto the surfaces of PGEs. A further increase in the R_{ct} values was obtained after dsDNA immobilization onto the surface of chitosan*CNT PGEs as a result of the enhanced resistance to the charge transfer at the electrode surface, since the negatively charged phosphate backbone of dsDNA prevented redox couple $[Fe(CN)_6]^{3-/4-}$ from reaching the electrode surface, leading to larger R_{ct} values (Figures 7B–7d).

EIS was also used in order to investigate the surface confined interaction of MC with DNA at chitosan*CNT PGEs. After the interaction of MC with the double helix form of DNA at the electrode surface the negative charge at the electrode surface should decrease by reducing the resistance to charge transfer. These results were also consistent with the DPV results shown in Figure 6A.

In conclusion, chitosan*CNT modified electrochemical disposable graphite sensors were tested for the first time herein for electrochemical monitoring of the anticancer drug MC and nucleic acids. The detection limit (S/N = 3) was 6.85 μg/mL for dsDNA and 11.01 μg/mL for MC.

The electrochemical characterization of these unmodified/modified sensors was performed by DPV and EIS techniques.

There have been some studies in the literature about applications of chitosan modified or chitosan CNTs composite based electrodes (graphite-epoxy composite electrode, indium tin oxide electrode etc.) for monitoring of glucose and DNA.[9,30] With respect to the time length of their detection protocol and transducer type, these sensors are time consuming and expensive in terms of preparation in contrast to the ones presented in our

study. Disposable chitosan*CNT-PGEs have also presented many advantages, being easy to use, sensitive, cost effective, and with good repeatability in comparison to other conventional electrodes, e.g., carbon paste electrode, glassy carbon electrode, and gold electrode.

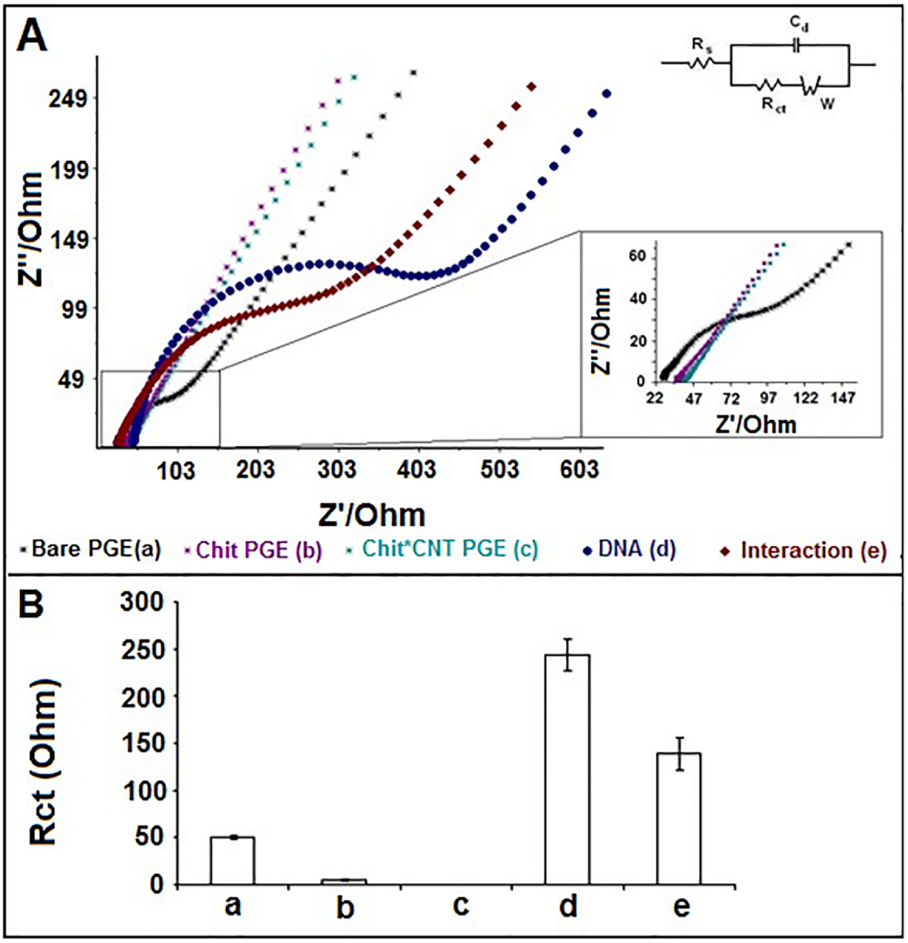


Figure 7. (A) Nyquist diagrams recorded at bare PGE (a), chitosan modified PGE (b), chitosan and CNT modified PGE (c), and 50 μg/mL dsDNA immobilized chitosan*CNT PGEs before (d) and after (e) interaction with 40 μg/mL MC. Supporting electrolyte solution is 2.5 mmol/L $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ (1:1) containing 0.1 mol/L KCl. Inset shows the equivalent circuit model used to fit the impedance data, the parameters of which are listed in the text; R_S is the solution resistance. The constant phase element C_d is then related to the space charge capacitance at the DNA/electrolyte interface. R_{ct} is related to the charge transfer resistance at the DNA/electrolyte interface. The constant phase element W is the Warburg impedance due to mass transfer to the electrode surface. (B) Histograms representing the average R_{ct} values (n = 4) measured at bare PGE (a), chitosan PGEs (b), chitosan*CNT PGEs and 50 μg/mL dsDNA immobilized on chitosan*CNT PGEs before (d) and after (e) interaction with 40 μg/mL MC.

3. Experimental

3.1. Apparatus and chemicals

All experimental measurements were carried by using AUTOLAB – PGSTAT 302 electrochemical analysis system supplied with a FRA 2.0 module for impedance measurements and GPES 4.9 software package (Eco

Chemie, the Netherlands). For electrochemical measurements, DPV, CV, and EIS were used. The 3-electrode system consisted of a PGE, a Ag/AgCl/KCl reference electrode (BASi, Model RE-5B, West Lafayette, IN, USA), and a platinum wire as the auxiliary electrode. The EIS measurements were performed in a Faraday cage (Eco Chemie, the Netherlands).

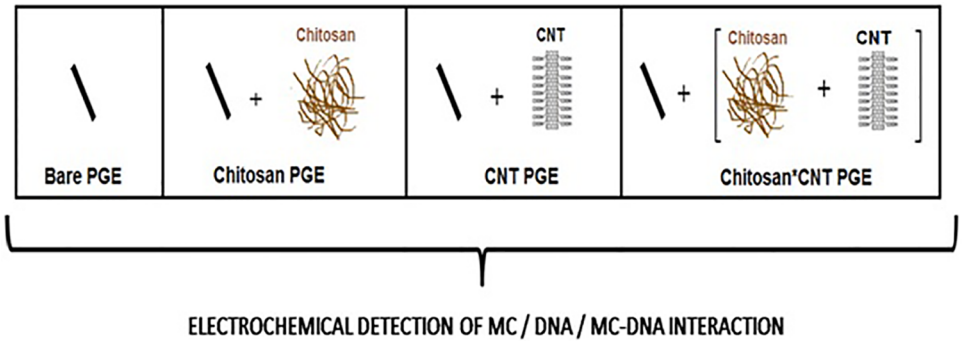
The calf thymus dsDNA was purchased as lyophilized powder from Sigma. The stock solutions of dsDNA were prepared as 1 mg/mL concentration with Tris–EDTA buffer solution (10 mM Tris–HCl, 1 mM EDTA, TE, pH 8.00) and kept frozen (1000 mg/L). More dilute solutions of dsDNA were prepared with 0.50 M acetate buffer solution containing 20 mM NaCl pH 4.80 (ABS). MC was purchased from Sigma. The stock solution of MC (1000 μg/mL) was prepared in ultrapure water and more diluted solution was prepared in 20 mM Tris–HCl buffer solution pH 7.00 (TBS).

Carboxylic acid (80%–90%) functionalized single-walled CNTs (diameter 4–5 nm; length 500–1500 nm bundles) were purchased from Aldrich.

Chitosan and other chemicals were of analytical reagent grade and they were supplied by Sigma and Merck. All stock solutions were prepared using ultrapure and deionized water.

3.2. Procedure

All the experiments were conducted at room temperature. A freshly prepared chitosan/CNT/chitosan*CNT modified electrode was used in each electrochemical detection cycle. The preparation of chitosan/chitosan*CNT solution and chitosan/chitosan*CNT modified PGEs was as described below and is represented in the Scheme.



Scheme. Experimental scheme for the modification of PGEs with chitosan/CNT/chitosan*CNT.

First 5.0 mg/mL chitosan was suspended in 1% acetic acid solution, followed by sonication for 1 h at room temperature. Next 3.0 mg/mL CNT was added to this chitosan solution, again followed by sonication for 1 h at room temperature.

PGEs were pretreated by applying +1.40 V for 30 s in ABS. Each pretreated PGE was immersed in vials containing 110 μL of chitosan, CNT, or chitosan*CNT solution for 1 h.[31]

3.3. Microscopic characterization of bare PGEs, and chitosan, CNT, and chitosan*CNT modified PGEs by SEM

The microscopic characterization of bare PGEs, and chitosan modified, CNT modified and chitosan*CNT modified PGEs was conducted by Quanta 250 FEI scanning electron microscope (Tokyo, Japan) with required

acceleration voltage between 5.0 and 10.0 kV with the resolution at various magnitudes: 10 μm, 2 μm, and 500 mm.

3.4. DNA and MC immobilization onto the surface of chitosan and chitosan*CNT modified PGEs

Each unmodified/modified PGE was immersed in a vial containing 110 μL of 50 μg/mL DNA solution for 1 h. Each electrode was then rinsed with ABS for 3 s before voltammetric transduction.

Each modified PGE was immersed in a vial containing 110 μL of 40 μg/mL MC solution for 1 h. Each electrode was then rinsed with TBS for 3 s before voltammetric transduction.

3.5. Interaction of MC with DNA at the surface of chitosan*CNT modified PGEs

First 50 μg/mL dsDNA immobilized PGEs were immersed in vials containing 110 μL of 40 μg/mL MC for interaction for 7.5 min. Each electrode was then rinsed with TBS for 3 s before voltammetric transduction.

3.6. Voltammetric transduction

CV measurements were obtained with a step potential of 25 mV, a scan rate of 100 mV/s, forward scan +0.4 to +1.2 V, reverse scan +1.2 to +0.4 V, in a redox probe containing 2 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) mixture prepared in 0.1 M KCl.

DPV measurements were used for monitoring the MC and guanine oxidation signal before/after interaction. DPVs were performed in ABS with a 3-electrode system by scanning from +0.20 to +1.40 V at the pulse amplitude 50 mV and the scan rate 50 mV/s.

3.7. EIS measurements

The surfaces of unmodified PGEs, and chitosan and chitosan*CNT modified PGEs were characterized by EIS according to the procedure given below. Chitosan*CNT PGEs were also used to investigate the effect of interaction of MC with DNA by EIS measurement under the same conditions.

EIS measurements were performed in the presence of 2.5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) mixture as a redox probe prepared in 0.1 M KCl. The impedance was measured in the frequency range from 10^5 Hz to 10^{-1} Hz in a potential of open-circuit value of +0.23 V versus Ag/AgCl with a sinusoidal signal of 10 mV. The frequency interval was divided into 98 logarithmically equidistant measure points. The respective semicircle diameter corresponds to the charge-transfer resistance, Rct, the values of which are calculated using the fitting program AUTOLAB 302 (FRA, version 4.9 Eco Chemie, the Netherlands).

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