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Fabrication of a novel impedimetric biosensor for label free detection of DNA damage induced by doxorubicin

Mohammad Mahdi Zangeneh [a,b], Hasan Norouzi [c], Majid Mahmoudi [c], Hector C. Goicoechea [d], Ali R. Jalalvand [c,*]

[a] Department of Clinical Sciences, Faculty of Veterinary Medicine, Razi University, Kermanshah, Iran
[b] Biotechnology and Medicinal Plants Research Center, Ilam University of Medical Sciences, Ilam, Iran
[c] Research Center of Oils and Fats, Kermanshah University of Medical Sciences, Kermanshah, Iran
[d] Laboratorio de Desarrollo Analítico y Quimiometría (LADAQ), Cátedra de Química Analítica I, Universidad Nacional del Litoral, Ciudad Universitaria, CC 242, S3000ZAA Santa Fe, Argentina

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ABSTRACT

In this work, a novel impedimetric biosensor has been fabricated for detection of DNA damage induced by doxorubicin (DX). Cytochrome P450 reductase (CPR) is required for electron transfer from nicotinamide adenine dinucleotide phosphate (NADPH) to cytochrome P450 (CP450) which causes DX to undergo a one-electron reduction of the *p*-quinone residue to form the semiquinone radical resulting in the generation of free hydroxyl radical which causes DNA damage. After modification of bare glassy carbon electrode (GCE) with multiwalled carbon nanotubes (MWCNTs) and chitosan (Ch), CPR and CP450 were co-immobilized onto the surface of Ch/MWCNTs/GCE by cross-linking CPR, CP450 and Ch through addition of glutaraldehyde. Then, the DNA was assembled onto the surface of CPRCP450/Ch/MWCNTs/GCE to fabricate the biosensor (DNA/CPRCP450/Ch/MWCNTs/GCE). Modifications applied to the bare GCE to fabricate the biosensor were characterized by CV, EIS and SEM. The DNA/CPRCP450/Ch/MWCNTs/GCE was treated in the damaging solution (DX + NADPH) which caused a significant DNA damage and the exposed DNA bases reduced the electrostatic repulsion of the negatively charged redox probe leading to Faradaic impedance changes. Performance of the biosensor for detection of DNA damage in the presence of Spinach extract was also examined and finally, an indirect impedimetric method was developed for determination of DX.

1. Introduction

DNA is an important functional biomacromolecule and detection of its damage is important because of its critical role in mutagenesis, carcinogenesis and aging [1]. Reactive oxygen species (ROS), ionizing radiation and chemicals are some endogenous and exogenous which are able to DNA damage [1,2]. If the damaged DNA cannot be repaired duly, the induced gene mutation can cause cancer and tumor in DNA replication process [3]. Therefore, rapid detection of DNA damage is important from clinical point of view.

Doxorubicin (DX) with the trade name of adriamycin is a chemotherapy medication used to treatment of a variety of human cancers [4–8]. The main anticancer action of DX is related to involve DNA damage through topoisomerase II inhibition and free radical generation [6,7]. Many previous studies have shown that the quinone residue of DX can undergo one-electron reduction at the *p*-quinone residue mediated by nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome P450 reductase (CPR)-cytochrome P450 (CP450) where CPR causes one-electron transfer from NADPH to CP450 [9]. The semiquinone radical generated by CPR can react with O_2 to generate $O_2^{\bullet -}$ which is dismutated to H_2O_2 [9]. The semiquinone radical reacts with H_2O_2 to generate •OH, resulting in DNA damage (Scheme 1).

There are many analytical methods such as capillary liquid chromatography-mass spectrometry/mass spectrometry [4], fluorescence [5], ^{32}P-postlabeling [6], capillary zone electrophoresis [7] and photoelectrochemical method [8] which can be applied to detect DNA damage. Although, these methods are accurate but, generally are performed at centralized laboratories which require long assay time and high cost. Therefore, developing new methods which are rapid and low-cost for detection of DNA damage are highly demanded. Among the existed methods, electrochemical methods offer an attractive alternative approach for simple, inexpensive and sensitive detection of DNA damage [10–21].

The antioxidants molecules are able to remove the excess ROS in the body which make a balance between produced ROS and antioxidant defense system. If overproduction of ROS or insufficient antioxidant defense disturbs this balance, its consequence will increase the ROS which ends up in oxidative stress [22]. Therefore, an essential way to prevent the DNA damage is using antioxidants. Spinach with the term

* Corresponding author at: Research Center of Oils and Fats, Kermanshah University of Medical Sciences, Kermanshah, Iran
E-mail addresses: ali.jalalvand1984@gmail.com, alireza.jalalvand@kums.ac.ir (A.R. Jalalvand).

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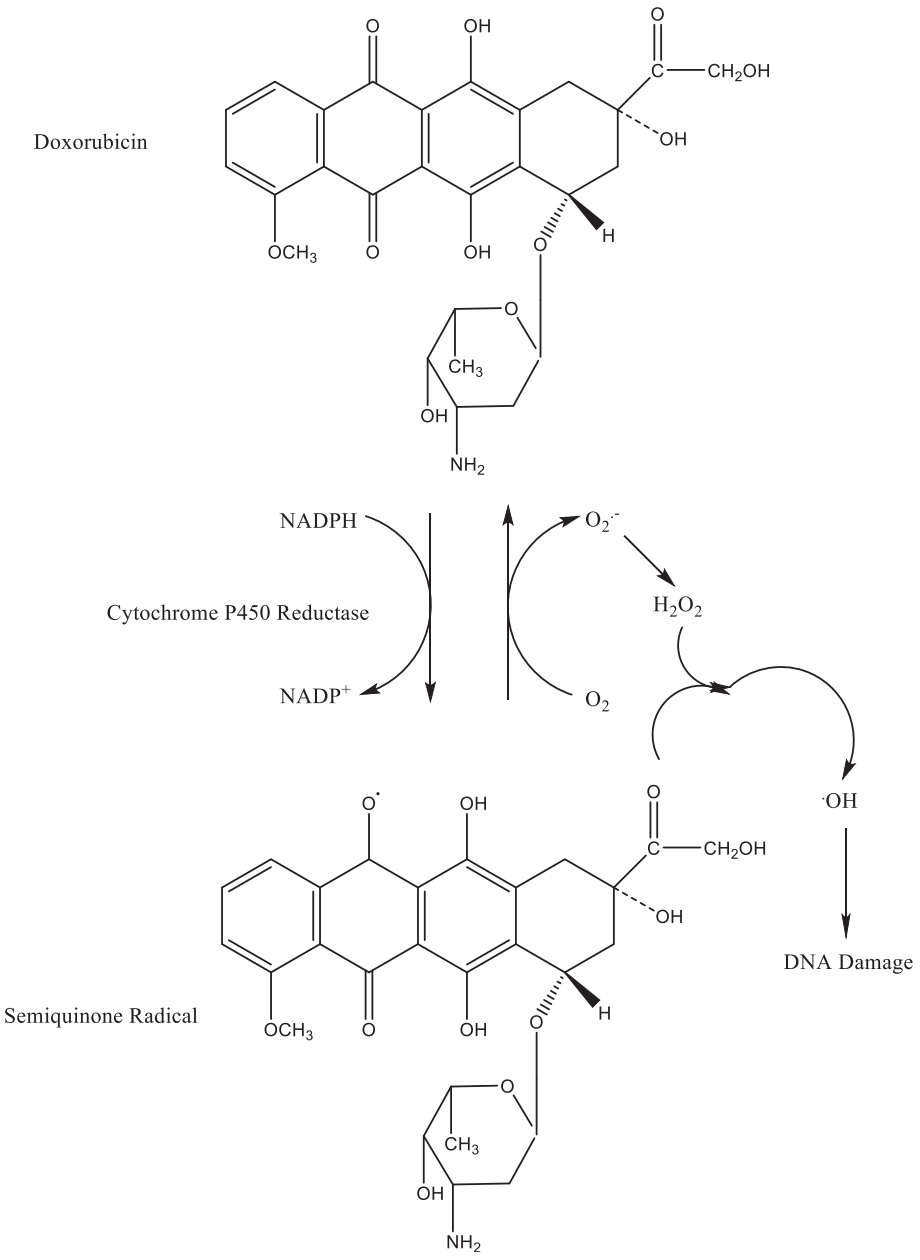


Scheme 1. The proposed mechanisms for DNA damage induced by doxorubicin [9].

of *Spinacea oleracea* is an important vegetable from beet group. The previous studies have reported the presence of a series of powerful natural antioxidants such as glucuronic acid derivatives of flavonoids and three additional fractions as *trans* and *cis* isomers of *p*-coumaric acid and others as meso-tartarate derivatives of *p*-coumaric acid in the extract of Spinach leaves [23]. Therefore, in the present study, spinach effects in preventing the DNA damage induced by DX will be investigated.

In fabricating electrochemical sensors and biosensors, the bare glassy carbon electrode (GCE) is usually modified with different materials with the aim of improving its selectivity and sensitivity and sometimes electrochemical methods are combined with chemometric methods [24–47]. In this work, to achieve this goal, we have modified the bare GCE to fabricate a novel biosensor for detection of DNA damage induced by DX. Carbon nanotubes (CNTs) because of their excellent electrical and mechanical properties such as high surface area, high electron transfer rate, high stability and minimization of the surface fouling have received a lot of attention for constructing electrochemical sensors and biosensors [47]. Chitosan (Ch) as a natural biopolymer is biocompatible, water permeable and nontoxic which has high mechanical strength and excellent film forming ability [45]. Immobilization of the enzyme onto the biosensor surface could be performed by entrapment, adsorption cross-linking and covalent attachment [47]. Among the mentioned approaches, cross-linking approach is preferable because of the limitation of physical adsorption by some problems such as enzyme leaching from the biosensor surface [47]. Therefore, CPR and CP450 were simultaneously co-immobilized onto the electrode surface by cross-linking enzymes and Ch through addition of glutaraldehyde. Finally, the DNA was assembled onto the surface of CPRCP450/Ch/MWCNTs/GCE to fabricate the biosensor (DNA/CPRCP450/Ch/MWCNTs/GCE).

In this project, we are going to develop a novel biosensor for detection of DNA damage induced by DX and testing its potential as an analytical tool for indirect quantitative analysis of DX. However, DX is a chemotherapy medication used to treatment of a variety of human cancers and its main anticancer action is related to involve DNA damage through topoisomerase II inhibition and free radical generation but, in

second section of our study, we are going to examine the performance of the developed biosensor to monitor the DNA damage induced by DX but in the presence of Spinach extract which its antioxidant effects have been proven by the previous studies. In this section of our study, we have to main goals including examination of the performance of the developed biosensor for detection of DNA damage even in the presence of an antioxidant and confirming the antioxidant effects of the Spinach extract by a novel biosensor. Finally, the biosensor will be used to indirect quantitative analysis of DX.

2. Experimental

2.1. Chemicals and solution

The DNA, DX, CPR, CP450, glutaraldehyde, 1,1-diphenyl-2-picrylhydrazyl, Ch, sodium hydroxide, methanol and dimethylformamide (DMF) were purchased from Sigma. The MWCNTs were purchased from Ionic Liquid Technologies. The other reagents were purchased from legal sources and used as received without any further purification. Doubly distilled water (DDW) was used to prepare all the solutions. A Tris-HCl buffer solution (TBS, 0.1 M) of pH 7.0 was prepared in DDW and kept in a refrigerator. To prepare MWCNTs solution, 6.0 mg MWCNTs was added to 2.0 mL DMF and ultrasonicated for 30 min. To prepare the Ch solution, 10 mg Ch was added to 1.0 mL acetic acid and ultrasonicated for 60 min. A stock solution of DX (0.1 M) was prepared in the TBS (0.1 M, pH 7.0) and working solutions were prepared by applying appropriate dilutions to the stock solution. Solutions of CPR and CP450 were prepared in the TBS (0.1 M, pH 7.0) with a concentration of 50 U mL^{-1}. A DNA solution with a concentration of 2.0 mg/ mL was prepared in the TBS (0.1 M, pH 7.0). The electrochemical probe, $[Fe(CN)_6]^{3-/4-}$, with a concentration of 5 mM was prepared in the TBS (0.1 M, pH 7.0) containing 0.1 M KCl. Stock solution of the spinach extract with a concentration of 100 ppm was prepared in the TBS (0.1 M, pH 7.0). All the solutions were covered and kept in the refrigerator until analysis time.

2.2. Instruments

All the electrochemical experiments were performed by an Autolab PGSTAT302N-high performance controlled by the NOVA 2.1.2 software. The Autolab instrument was equipped by an electrochemical cell where a bare or modified GCE, an Ag/AgCl electrode and a Pt wire were acted as working, reference and counter electrode, respectively. Electrochemical impedance spectroscopy (EIS) was carried out using the same three-electrode configuration mentioned above in 5 mM $[Fe(CN)_6]^{3-/4-}$ solution containing 0.1 M KCl adjusted by the TBS (0.1 M, pH 7.0) in a frequency range from 0.1 Hz to 100 kHz. The SEM images were captured by a KYKYEM 3200 scanning electron microscope. An ELMEIRON pH-meter (CP-411) was used to pH adjustments.

2.3. Preparation of the biosensor

Prior to the modification of the bare GCE, it was well polished on a silky pad rinsed in an alumina slurry and then rinsed with DDW and immersed into a beaker containing ethanol and ultrasonicated for 15 min. Finally, the GCE was rinsed with DDW. At the first step of the modification process, 10 μL MWCNTs was dropped onto the surface of GCE and left to be dried at room temperature. Then, 8 μL Ch was dropped onto the surface of MWCNTs/GCE and left to cover the electrode surface. The electrode was then immersed in 0.25% glutaraldehyde solution for 2 h. Then, 5 μL CPR 50 U mL^{-1} was mixed with 5 μL CP450 50 U mL^{-1} and dropped onto the electrode surface. Finally, 10 μL DNA (2.0 mg/ mL) was dropped onto the surface of the electrode and left to be dried at room temperature. The preparation procedure is schematically illustrated in Scheme 2.

2.4. Preparation of the spinach extract

Spinach extract was prepared according to Ref. [48]. Spinach leaves were collected from an agricultural land in Kermanshah, Iran. Spinach leaves were washed and cut into small pieces and dried in the shade. Dried small pieces of Spinach were ground into a fine powder using a homogenizer. Then, 300 g of the obtained powder was dissolved in 3000 mL ethanol 70% and put in Soxhlet extractor for 8 h. The collected extract was filtered and evaporated into a glass container. The remained dried extract was poured into a glass container and kept in a refrigerator until analysis time.

2.5. Determination of 1,1-diphenyl-2-picrylhydrazyl (DPPH•) radicals scavenging activity

DPPH• is stable free radical at room temperature and accepts an electron/hydrogen radical to become a stable diamagnetic molecule. The reduction capability of DPPH• is determined by the decrease in its absorbance at 5 l7 nm, induced by antioxidants. The decrease in absorbance of DPPH• is caused by antioxidants, because of the reaction between antioxidant molecules and radicals, progresses, which results in the scavenging of the radical by hydrogen donation. Hence, DPPH• is usually used as a substrate to evaluate the antioxidative activity. To assess radical scavenging activities of Spinach leaves extract, different concentrations of the extract including 0.5, 1, 2.5, 5, 7.5 and 10 mg/L were taken in separate test tubes and the volume was adjusted to 100 μL with methanol and 5 mL of 0.1 mM methanolic solution of DPPH• was added to these tubes and shaken vigorously. The tubes were allowed to stand for 20 min at 27 °C. The control was prepared as above without any extract, and methanol was used for the baseline correction (blank). Changes in the absorbance of the samples were measured at 517 nm. Radical scavenging activity was expressed as the inhibition concentration (IC_{50}), i.e., the concentration of extract necessary to decrease the initial concentration of DPPH• by 50% (IC_{50}) under the specified experimental condition. This was obtained by interpolation and using linear regression analysis which was calculated to be 5 mg/L.

$$\text{IC}\ (\%) = \left(\frac{A_{\text{Sample}} - A_{\text{Blank}}}{A_{\text{Control}}}\right) \times 100 \quad (1)$$

3. Results and discussion

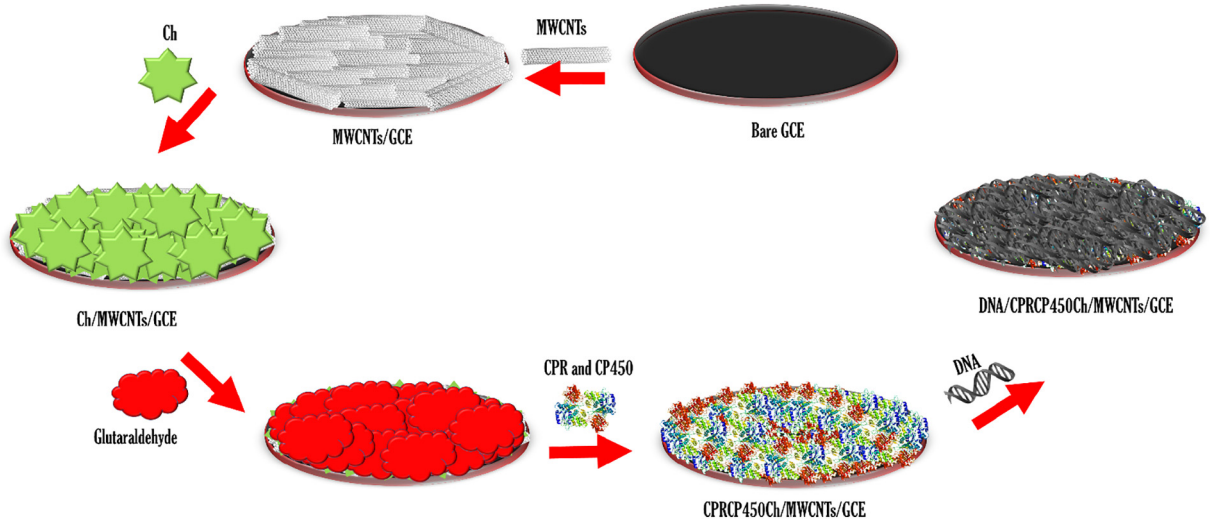
3.1. Characterization of the prepared biosensor

When a bare GCE is modified to fabricate a novel biosensor, different methods are usually applied to characterize the modifications. Therefore, we are going to use electrochemical and microscopic methods to characterize the modifications applied to the bare GCE to fabricate the proposed biosensor in this study. The results of the characterizations will be discussed in next sections.

3.1.1. Electrochemical characterization

Electrochemical methods such as EIS and cyclic voltammetry (CV) are well-known methods which have been frequently used to monitor the modifications applied to the bare electrodes to fabricate electrochemical sensors and biosensors. Therefore, in this section, we are going to use them for characterizing the modification steps applied to the bare GCE to fabricate the proposed biosensor.

The EIS measurements were performed in an electrochemical cell where a bare or modified GCE, an Ag/AgCl and a Pt wire were acted as working, reference and counter electrode, respectively. These three electrodes were immersed into the electrochemical probe, $[Fe(CN)_6]^{3-/4-}$, with a concentration of 5 mM prepared in the TBS (0.1 M, pH 7.0) containing 0.1 M KCl and then, the EIS curves were recorded



Scheme 2. Schematic representation of the fabrication procedure of the proposed biosensor in this study.

and fitted by fit and simulation command provided by the Nova software. An EIS curve has a semicircle portion and its diameter reflects the charge transfer resistance (R_{ct}). By the modification of the bare GCE with different layers, different R_{ct} values will be obtained which can be used to justify the impact of the modifications. The EIS curves of the bare GCE (curve a), MWCNTs/GCE (curve b), Ch/MWCNTs/GCE (curve c), CPRCP450/Ch/MWCNTs/GCE (curve d) and DNA/CPRCP450/Ch/MWCNTs/GCE (curve e) are showing in Fig. 1A. As can be seen, the bare GCE showed a semicircle with a R_{ct} value of 1920 Ω and after drop-casting of MWCNTs onto its surface, its R_{ct} value was decreased (389 Ω) which confirmed faster and more facile electron transfer was occurred at the surface of MWCNTs/GCE. By the presence of Ch at the surface of MWCNTs/GCE, the R_{ct} value was increased (1108 Ω) which confirmed slower charge transfer was occurred at the surface of Ch/MWCNTs/GCE. Observing higher R_{ct} value at the surface of Ch/MWCNTs/GCE than MWCNTs/GCE is related to the presence of Ch which can block the electrode surface in some extent. After co-immobilization of CPR and CP450 at the surface of Ch/MWCNTs/GCE, the R_{ct} value was decreased (757 Ω) which may be related to the electrostatic attraction between positively charged surface of the enzymes at pH 7 and negatively charged electrochemical probe [38]. The final modification step of the biosensor which included drop-casting of DNA at the electrode surface showed an increase in R_{ct} value which was related to the repulsion of the electrochemical probe carrying negative charge with negatively charged DNA.

The CV is another important electrochemical method which has been applied to monitor the modifications. Fig. 1B shows the CVs of different electrodes including GCE (curve a), MWCNTs/GCE (curve b), Ch/MWCNTs/GCE (curve c), CPRCP450/Ch/MWCNTs/GCE (curve d) and DNA/CPRCP450/Ch/MWCNTs/GCE (curve e) in the electrochemical probe. As can be seen, the bare GCE showed a well-defined CV and after modifying it by MWCNTs, its peak currents were increased manifesting the great role of MWCNTs in fastening the rate of charge transfer at the electrode surface. After drop-casting of Ch onto the surface of MWCNTs/GCE, the rate of charge transfer at the electrode surface was decreased as can be clearly observed from the peak currents and peak separation ($\Delta E = E_c - E_a$). The presence of the enzymes at the electrode surface caused an electrostatic attraction between the enzymes and the electrochemical probe and therefore, reinforcing the electrochemical response of the CPRCP450/Ch/MWCNTs/GCE. Finally, the presence of DNA at the electrode surface caused an electrostatic repulsion between DNA and the electrochemical probe and therefore, a weaker response was observed for DNA/CPRCP450/Ch/MWCNTs/GCE.

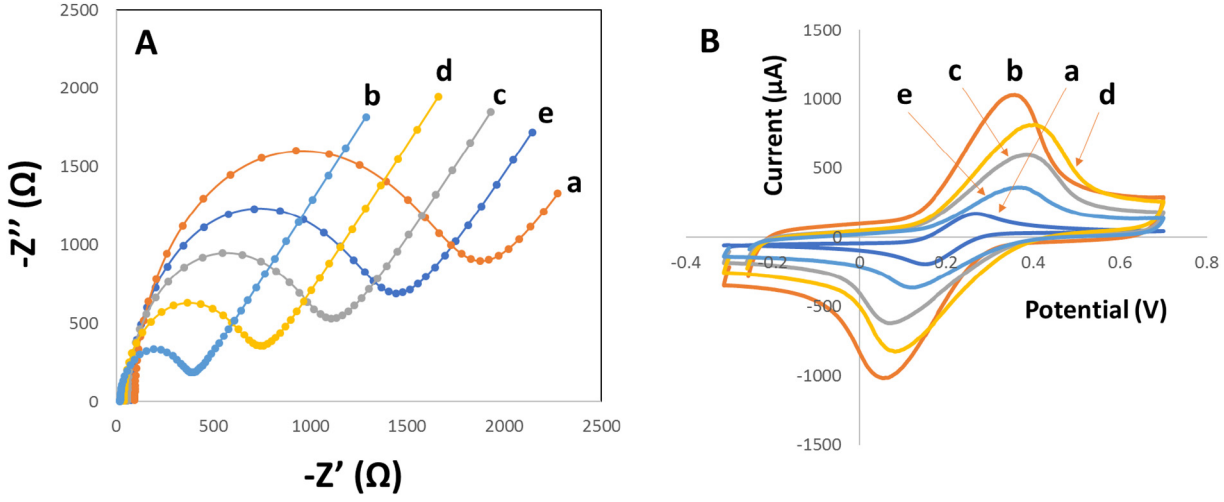


Fig. 1. (A) and (B) EISs and CVs of (a) GCE, (b) MWCNTs/GCE, (c) Ch/MWCNTs/GCE, (d) CPRCP450/Ch/MWCNTs/GCE and (e) DNA/CPRCP450/Ch/MWCNTs/GCE immersed into the electrochemical probe solution, $[Fe(CN)_6]^{3-/4-}$, with a concentration of 5 mM prepared in the TBS (0.1 M, pH 7.0) containing 0.1 M KCl. Scan rate of CVs is 50 mV s^{-1}.

3.1.2. Morphological characterization by SEM

SEM is a powerful method which can obtain suitable information about the surface of the modified electrodes. Therefore, it was applied to the monitoring of the modifications applied to the fabrication of the biosensor and the results are shown in Fig. 2. The first step of the modification caused presence of MWCNTs as can be seen in Fig. 2A. The tubes of MWCNTs have been twined around each other and formed a layer at the electrode surface. The SEM image captured from the surface of Ch/MWCNTs/GCE is shown in Fig. 2B which shows that Ch has formed a new layer which has covered the MWCNTs. Fig. 2C shows that after co-immobilization of CPR and CP450 onto the surface of Ch/MWCNTs/GCE, another new layer is formed on the surface of the previous layer. Our records related to the DNA/CPRCP450/Ch/MWCNTs/GCE showed that DNA has formed a dark layer on the surface of the biosensor which can be clearly observed in Fig. 2D.

3.2. Optimization of the factors affecting the biosensor response

In order to obtain a satisfied response for the biosensor, the effects of some factors have been investigated. It was found that the amounts of MWCNTs, Ch, CPR, CP450, DNA, NADPH and incubation time affected the EIS response of the biosensor. To optimize the EIS response, the performance of the biosensors fabricated with different concentrations of MWCNTs, Ch, CPR, CP450, DNA, NADPH was studied under different incubation times with the same concentration of the target DX in the damaging solution DX (0.1×10^{-6} M) + NADPH (varying). To investigate the effect of these parameters, one parameter was changed while the other parameters were kept constant. The effects of the parameters were investigated to achieve the maximum value for R_{ct} as the optimal response. As can be seen in Fig. 3, the R_{ct} value was affected by MWCNTs, Ch, CPR, CP450, DNA, NADPH and incubation time. According to the maximum value for R_{ct} as the desired response, the optimal values of MWCNTs, Ch, CPR, CP450, DNA, NADPH and incubation time were chosen to be 6 mg/2 mL, 10 mg/mL, 50 U mL^{-1}, 2 mg/mL, 2 mM and 8 min, respectively. Thus, these optimized values were used in the standard procedures.

3.3. Impedimetric detection of the DNA damage

Monitoring of the DNA damage was performed via the developed DNA biosensor based on EIS approach. Any alteration in the interfacial properties between the electrode and the electrolyte created by the DNA damage can cause a change in the EIS response of the biosensor therefore, the DNA damage can be monitored by the biosensor response. To investigate the DNA damage caused by DX, the EIS data were recorded by the immersion of DNA/CPRCP450/Ch/MWCNTs/GCE into the damaging solution DX (0.1×10^{-6} M) + NADPH (2.0×10^{-3} M) for 8 min (Fig. 4, curve a) and then, the biosensor was immersed into the electrochemical probe. The EIS response of the biosensor showed a semicircle with a diameter of 1424 Ω, after increasing concentration of the DX in the damaging solution (5.0×10^{-6} M), an obvious decrease (Fig. 4, curve b) was observed in R_{ct}, 923 Ω. Our next trying in increasing concentration of DX to 1.0×10^{-5} M showed more decrease in the R_{ct} (615 Ω) Fig. 4, curve c. The main anticancer action of DX is related to involve DNA damage through topoisomerase II inhibition and free radical generation and the quinone residue of DX can undergo one-electron reduction at the *p*-quinone residue mediated by NADPH-CPR-CP450 where CPR causes one-electron transfer from NADPH to CP450. The semiquinone radical generated by CPR can react with O_2 to generate $O_2^{\bullet -}$ which is dismutated to H_2O_2. The semiquinone radical reacts with H_2O_2 to generate •OH, resulting in DNA damage and the exposed DNA bases reduced the electrostatic repulsion of the negatively charged redox probe leading to decreasing the R_{ct}.

The results mentioned above confirmed that the biosensor can be used in monitoring the DNA damage induced by DX. But, in our next studies, the biosensor will be used to investigate the protective effects of the Spinach extract on DNA against damage.

3.4. Evaluation of the protective effect of spinach extract on DNA against damage

In this section, we have examined the performance of the developed biosensor for detection of DNA dame induced by DX in the presence of

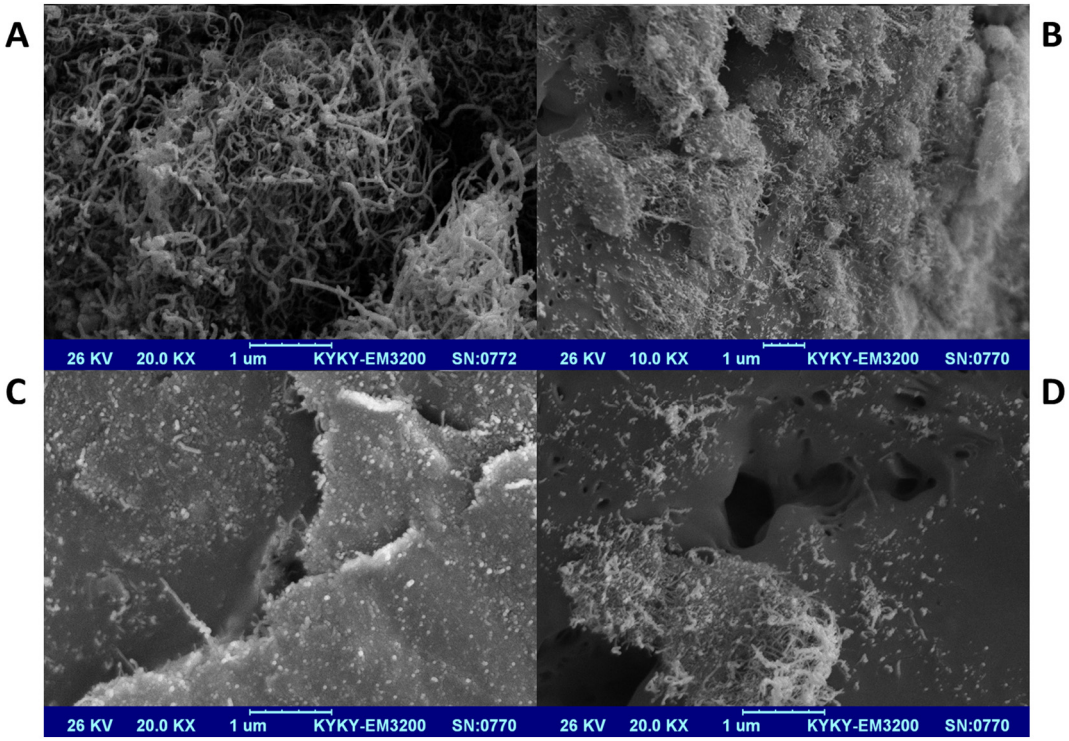


Fig. 2. SEM images of (A) MWCNTs/GCE, (B) Ch/MWCNTs/GCE, (C) CPRCP450/Ch/MWCNTs/GCE and (D) DNA/CPRCP450/Ch/MWCNTs/GCE.

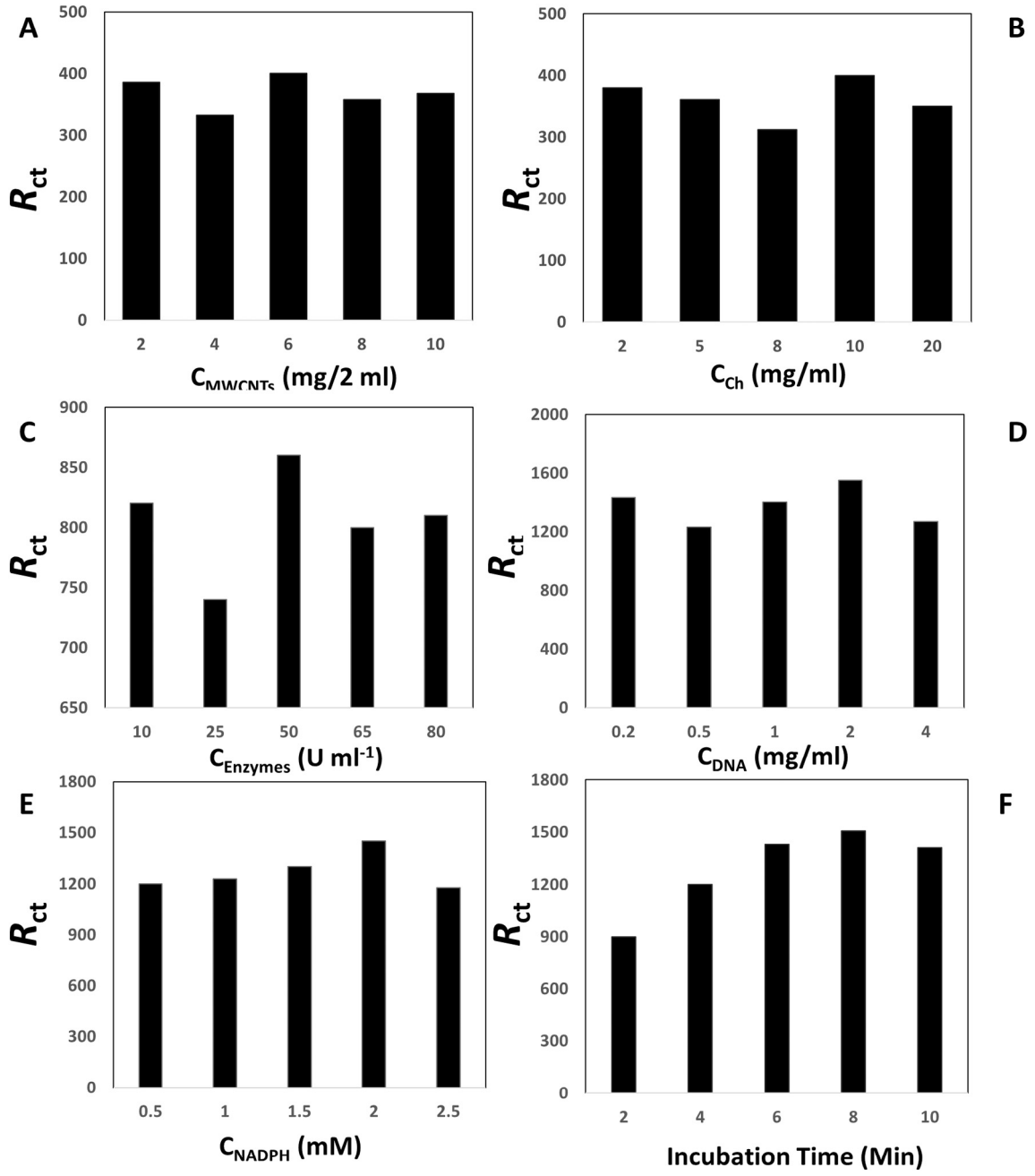


Fig. 3. The effects of different variables on impedimetric response of DNA/CPRCP450/Ch/MWCNTs/GCE to DX (0.1×10^{-6} M).

Spinach extract and the EIS results are showing in Fig. 4 (curves d and e). As can be seen, after incubation of the biosensor in the damaging solution DX (0.1×10^{-6} M) + NADPH (2.0×10^{-3} M) but in the presences of 5 ppm Spinach extract and immersing into the electrochemical probe, its EIS response didn't show any significant change in comparison with its EIS response in the absence of Spinach extract (curve a). A same result was observed in the presence of increasing concentration of the Spinach extract (10 ppm, curve e). These results illustrate that the Spinach extract can prevent DNA damage due to its antioxidant activity [23].

3.5. EIS as an indirect method for quantitative analysis of DX

As demonstrated before, the EIS response of the DNA/CPRCP450/Ch/MWCNTs/GCE was affected by the concentration of DX; this possibly occurred because the exposed DNA bases reduced the electrostatic repulsion of the negatively charged probe leading to Faradaic impedance changes. Therefore, the EIS method could be used as an indirect method for the determination of DX. To achieve this goal, the DNA/CPRCP450/Ch/MWCNTs/GCE was immersed for 8 min into several damaging solutions containing different concentrations of DX and then, the pretreated biosensor was immersed into the electrochemical probe and its EIS responses were recorded. Subsequently, the relationship between $\Delta R_{ct}/R^0_{ct}$ values ($\Delta R_{ct} = R^0_{ct} - R_{ct}$, R^0_{ct}: the diameter of the semicircle of the EIS curve of DNA/CPRCP450/Ch/MWCNTs/GCE in the absence of DX (Fig. 5A, curve a), R_{ct}: the diameter of the semicircle of the EIS curve of DNA/CPRCP450/Ch/MWCNTs/GCE in the presence of DX (Fig. 5, curves b-n)) and the concentrations of synthetic DX samples was studied (Fig. 5B). To obtain a linear calibration graph, the $\Delta R_{ct}/R^0_{ct}$ values were regressed on log (C_{DX}) values as shown in Fig. 5C. As can be seen, there is a linear relationship between $\Delta R_{ct}/R^0_{ct}$ and the concentration of DX over a concentration range of 0.1–150 μM (R^2 0.996). The limit of detection (LOD) of this method was calculated to be 0.05 μM

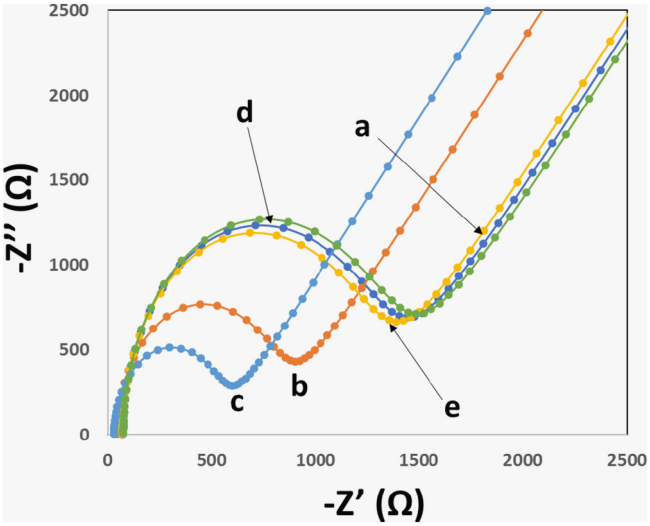


Fig. 4. The EIS responses of the DNA/CPRCP450/Ch/MWCNTs/GCE in 5 mM $[Fe(CN)_6]^{3-/4-}$ after immersing into the damaging solutions (NADPH (2.0×10^{-3} M) + DX (varying)) and incubation for 8 min. Curves a, b and c have been recorded upon 0.1×10^{-6} M, 5.0×10^{-6} M and 5.0×10^{-6} M DX, respectively. Curves d and e are the same as curve a but in the presence of 5 and 10 ppm spinach extract, respectively.

according to IUPAC recommendations ($3S_b/b$, where S_b is the standard deviation ($n = 5$) of the blanks, and b is the slope value of the respective calibration graph). Therefore, we can conclude that the proposed biosensor can be used for indirect determination of DX.

3.6. Stability, and reproducibility

Stability and reproducibility are two important characteristic parameters of a biosensor which must be examined and determined prior to suggesting it for practical purposes. Therefore, this section of our study was devoted to the investigation of the stability and reproducibility of the developed biosensor. The stability of the DNA/CPRCP450/Ch/MWCNTs/GCE was investigated by immersing it into the damaging solution DX (0.1×10^{-6} M) + NADPH (2.0×10^{-3} M) for 8 min and then, the EIS analysis was performed in the electrochemical probe. The results showed that two weeks later, the response of the biosensor retained 96% of its initial value. Therefore, we can conclude that the biosensor response was stable. Furthermore, the reproducibility of the biosensor was examined by recording the EIS responses of six individual biosensors in the electrochemical probe after immersing into the damaging solution DX (0.1×10^{-6} M) + NADPH (2.0×10^{-3} M) for 8 min and a relative standard deviation of 3.34% was obtained which confirmed that the biosensor had a reproducible response.

3.7. Comparison with the previous works

A comparison between previously published works in the literature and this work has been listed in Table 1. Our new biosensor studied label free detection of the DNA damage induced by DX using the EIS technique. EIS has some advantages compared to other electrochemical methods, including ease of signal quantification, high sensitivity and nondestructive. This biosensor has the ability to detect both the DNA damage and the antioxidant effect of spinach extract in preventing the DNA damage. Moreover, this method is simple, fast, without electroactive indicator requirement, and can be used at biological pH for damage induction and detection.

4. Conclusions

In this study, we have fabricated a novel impedimetric biosensor for label free detection of DNA damage induced by DX. The main anticancer

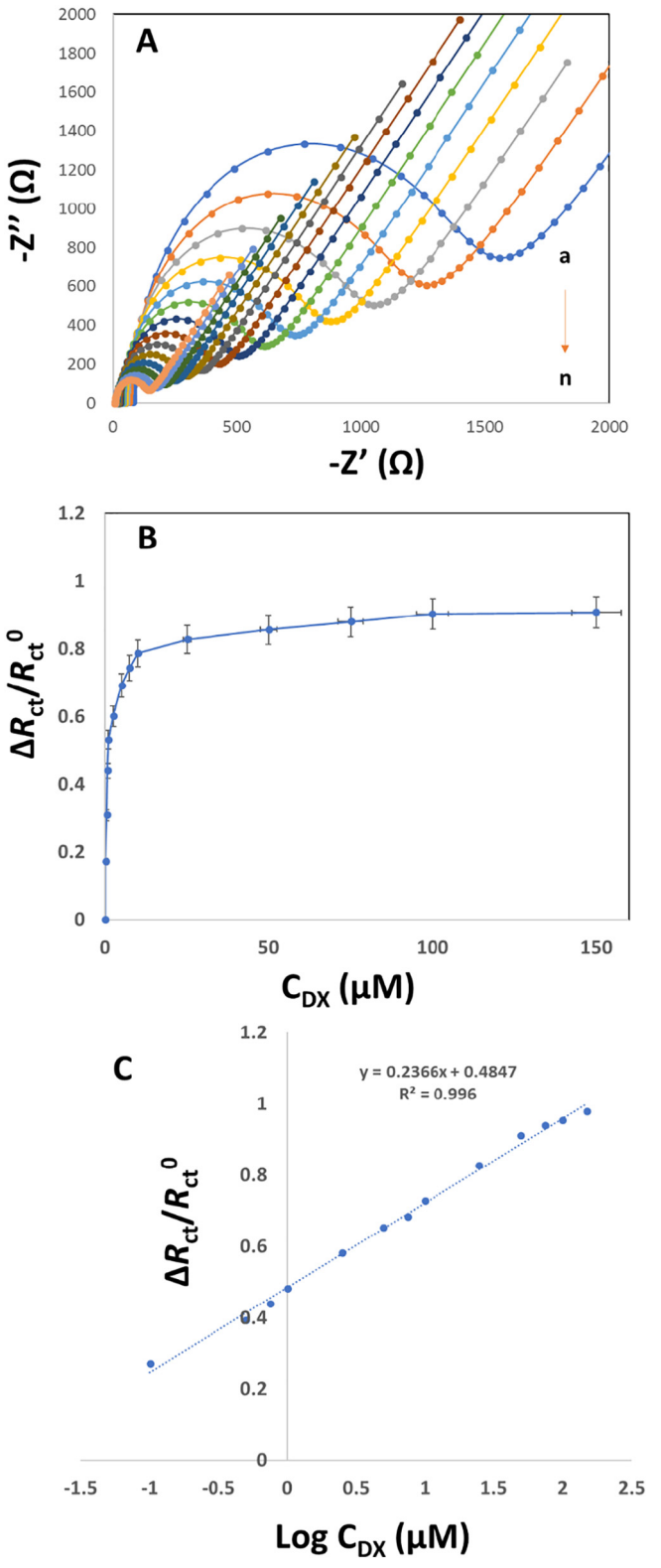


Fig. 5. (A) EIS responses of the DNA/CPRCP450/Ch/MWCNTs/GCE against variation in concentration of DX, (B) and (C) calibration graphs for $\Delta R_{ct}/R_{ct}^0$ vs. concentration of DX and $\Delta R_{ct}/R_{ct}^0$ vs. log (concentration of DX), respectively.

action of DX is related to involve DNA damage through topoisomerase II inhibition and free radical generation and the quinone residue of DX can undergo one-electron reduction at the *p*-quinone residue mediated by NADPH-CPR-CP450 where CPR causes one-electron transfer from NADPH to CP450. The semiquinone radical generated by CPR can react

Table 1
A comparison study between different electrochemical approaches for detection of DNA damage.

Sensor	Method	Damaging reagent	DNA attachment method	Long term stability	Ref.
CS/ds-DNA/Ag-PGL/GCE	LSV	Fe^{2+}/H_2O_2	Adsorption	15 days	[49]
DNA–XOD/GCE	SWV	Xanthine/$FeSO_4$	Adsorption	7 days	[50]
DNA–XOD/GCE	SWV	$FeSO_4$/glucose	Adsorption	–	[51]
dsDNA/GCE	SWV	Fe^{2+}/EDTA/H_2O_2	Adsorption	–	[52]
$(MWCNTs/PDDA/ds\text{-}DNA)_2$/PGE	DPV	Cr(VI)/GSH/H_2O_2	Layer by layer	–	[53]
(PDDA/dsDNA)3/GCE	CV	V_2O_5nanobelts/HCl/H_2O_2	Layer by layer	30 days	[54]
DNA/GCE	SWV	Fe^{2+}/H_2O_2	Adsorption	–	[15]
DNA/AuNPs/SPGE	EIS	$CuSO_4/H_2O_2$/AA	Covalent attachment	21 days	[55]
DNA/CPRCP450/Ch/MWCNTs/GCE	EIS	DX + NADPH	Cross linking	14 Days	This work

PDDA: poly (diallyldimethylammoniumchloride); PGE: pencil graphite electrode; GSH: glutathione (γ-L-glutamyl-L-cysteinyl-glycine); MB: methylene blue; CS: chitosan; PGL: poly l-glutamic acid; LSV: linear sweep voltammetry; SWV: square wave voltammetry; DPV: differential pulse voltammetry.

with O_2 to generate $O_2^{\bullet -}$ which is dismutated to H_2O_2. The semiquinone radical reacts with H_2O_2 to generate •OH, resulting in DNA damage and the exposed DNA bases reduced the electrostatic repulsion of the negatively charged redox probe leading to decreasing the R_{ct}. The biosensor was impedimetrically able to detect the DNA damage induced by DX which could also be used as an indirect method for determination of DX over a concentration range of 0.1–150 μM (R^2 0.996) with a LOD of 0.05 μM. The results showed that the biosensor response was stable and reproducible. The biosensor was also able to confirm the antioxidant activity of the Spinach extract against DNA damage. Preparation and detection protocols were simple and cheap which required a short time and can be easily adapted for the detection of the DNA damage induced by different damaging systems. Therefore, other researchers from different scientific fields can connected to electrochemists to expand their studies focused on DNA damage because, the electrochemists are able to set up cheap, portable and fast systems for detection of DNA damage which enable the other scientists to have better studies on DNA damage. The results obtained in this study confirmed the results of the previous studies which had claimed the main anticancer action of DX was related to involve DNA damage. Our studies which were related to the further examination of the performance of the biosensor showed that the Spinach extract due to its antioxidant effects can prevent the DNA damage. Finally, we showed that the biosensor can be applied to impedimetric determination of DX.

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