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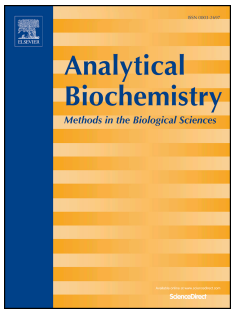

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ELECTROCHEMICAL DETECTION OF INTERACTION BETWEEN CAPSAICIN AND NUCLEIC ACIDS IN COMPARISON TO AGAROSE GEL ELECTROPHORESIS

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Short title: DETECTION OF CAPSAICIN-DNA INTERACTION

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Abstract

In this study, the biomolecular interaction occured between nucleic acids and Capsaicin (CPS), the active compound in chilli peppers, which has been reported to have anti-carcinogenic properties, was investigated for the first time herein using disposable electrochemical biosensor. It is aimed to perform the surface-confined interaction between CPS and different types of nucleic acids and under this aim, the experimental conditions were optimized; such as, the concentration of CPS and DNA, DNA immobilizaton time and interaction time etc. The detection limit (DL) of DNA was estimated based on guanine oxidation signal in the linear concentration range of DNA from 1 to 5 µg/mL, and it was found to be 0.62 µg/mL. The effect of time-dependent manner from 1 min to 30 min on the interaction of CPS with nucleic acids was explored upon to the changes at guanine signal coming from double stranded DNA and cDNA as well as PCR samples.The interaction of CPS with double stranded DNA was also determined by agarose gel electrophoresis.

Keywords: Capsaicin; DNA interaction; electrochemical DNA biosensor; differential pulse voltammetry; pencil graphite electrode; agarose gel electrophoresis

Abbreviations used: CPS, Capsaicin; cDNA, complemetary DNA; dsDNA, Double stranded DNA; PCR, Polymerase chain reaction; PBS, Phosphate buffer saline; ABS, Acetate buffer solution; DPV, Differential pulse voltammetry; PGE, Pencil graphite electrode; FLR-GCE, Fullerene modified glassy carbon electrode; DL, Detection limit

1.Introduction

There has been an increasing interest in electrochemical devices for DNA biosensing since they allow a lot of advantages like simple, fast, and sensitive analysis with using of inexpensive equipment. Electrochemical DNA biosensensor technologies represent another important subject which is the development of fast and accurate methods of DNA damage detection, especially caused by anticancer drugs or hazardous compounds. And also the ways in which drugs interact with DNA, with the goal of understanding the toxic as well as chemotherapeutic effects of many molecules. The interaction of matters with DNA is important topic for studies in drug discovery and pharmaceutical development processes. The mostly known intercalation and groove binding are the two most common modes by which small molecules bind directly and selectively to double-stranded DNA (dsDNA) [1,2]. The binding interactions, whichare the topic of several electrochemical studies, result in structural change of both DNA and matter or drug molecules to accomodate complex formation [3-6].

The observation of the pre and post electrochemical signals of DNA or matter interaction provides good evidence for the interaction mechanism to be elucidated. Intercalation, which is an enthalpically driven process, results from deinsertion of a planar aromatic ring system between dsDNA base pairs with accompanying unwinding and lengthening of the DNA helix [7-11].

Capsaicin which is a homovanillic acid derivative (8-methyl-N-vanillyl-6nonenamide) that has been used in food additives and various pharmaceutical products. Thus, there is a need for development of conventional methods for montioring CPS and its interaction with (bio) molecules [12-27].

Under various names such as chilli pepper, red pepper, paprika etc. has a great deal of attentions as a chemo-preventive agent against cancer [12]. In this regard there is many studies that show the anticancer activity of capsaicin. For example leukemia cells [13], multiple myeloma cells [14], cutaneous cell carcinoma [15] , tongue cancer cells [16] , gastric cancer cells [17], pancreatic cancer cells [18], small cell lung cancer [19], breast cancer cells [20] and prostate cancer cells [21] etc. are some of the studies which explain the effect of Capsaicin on cancer cells.

Genotoxicity of Capsaicin (CPS) is another interesting research topic. Toth et al., in 1984, show the genotoxicity of CPS in a study. CPS was found to be mutagenic in Salmonella typhimurium strain TA 98 in the presence of Aroclor induced rat liver S9 fraction [22], whereas another study in which S9 from phenobarbital-induced rats was used for metabolic activation was negative [28]. And also Richeux et al. in 1999, show a nice results of the comet assay and DNA fragmentation assay that capsaicin is able to induce DNA damage in human neuroblastoma cells. They get a result as; DNA damage increased with the concentration of capsaicin, thus indicating that capsaicin is able to induce DNA single strand breaks [29].

Qais et al. [30] investigated the spectroscopic detection of interaction between CPS and calf thymus DNA using UV-vis absorbance spectra and fluourescence spectra thatthat indicates the formation of complex between CPS and DNA. It was concluded that the interaction mechanism of CPS with calf thymus DNA would be reported as non-intercalative groove binding.

To understand the interactions between capsaicin and DNA, one of the preferred technique in recent years are electrochemically biosensors. Principle of operation of these type biosensors are basically, devices that integrates an nucleic acid as the biological recognition element and an electrode as the electrochemical signal transducer [31]. More generally, chemical sensors are devices which transform (bio)chemical stimulus from an analyte in relation into analytically useful information. As we mentioned before this type measurments detect the

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analytes more rapidly, sensitively and selectively [31-33]. The recent studies present increasing advantages of such sensors day by day. There are several publications about the easy use and low cost of electrochemical biosensors [34-37]. For future development in chip technologies, electrochemical biosensors are preffered very much because of the easy manipulation and application possibilities [38,39].

The aim of our study is voltammetric detection of the interaction between CPS and double stranded DNA, cDNA from total RNA isolated from Huh7 human hepatocellular carcinoma cell line and PCR samples. The surface confined interaction of CPS with different type of nucleic acids was investigated by disposable pencil graphite electrode (PGE) in combination with differential pulse voltammetry (DPV) for the first time in the literature. Firstly, the experimental conditions, such as; CPS and DNA concentration, DNA immobilization time onto the surface of PGE and interaction time were optimized in order to find the optimum analytical performance upon electrochemical detection of the interaction occured between CPS and DNA. The interaction process was evaluated based on the changes at oxidation signals of CPS and guanine oxidation signal of dsDNA in a time-dependent manner as well as the study performed on cDNA and PCR samples while measuring the changes at guanine signal. The electrophoretic studies were also performed in our study to confirm the interaction of CPS with PCR samples.

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2.Experimental

2.1. Apparatus

For differential pulse voltammetry (DPV) measurements were carried by using AUTO-LAB PGSTAT 30 electrochemical analysis system supplied with GPES 4.9 software package (Eco Chemie, The Netherlands). All measurements were performed in the Faraday cage (Eco Chemie, The Netherlands).The three electrode system consisted of the PGE, an Ag/AgCl/3M KCl reference electrode (BAS, Model RE-5B, W. La- fayette, USA) and a platinum wire as the auxiliary electrode. As a holder for the graphite lead (Tombow 0.5 HB, Japan) a Rotring Pencil model (Germany) was used. Electrical contact with the lead was obtained by soldering a metallic wire to the metallic part. During each measurement, the pencil lead was held vertically with 10 mm of which was immersed into the solution.

2.2. Chemicals

CPS was purchased from Sigma-Aldrich (Gemany). Stock solution of CPS (1000 µg/mL) was prepared in ethanol and kept in dark. Diluted solutions were prepared in 0.05 M phosphate buffer saline containing 20 mM NaCl (PBS, pH 7.40). 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. More dilute solutions of dsDNA were prepared with 0.50 M acetate buffer solution containing 20 mM NaCl (ABS, pH 4.80).

All stock solutions of buffers were prepared with ultrapure water.

Other chemicals and equipments were obtained from Sigma- Aldrich (Germany) and Merck (Germany).

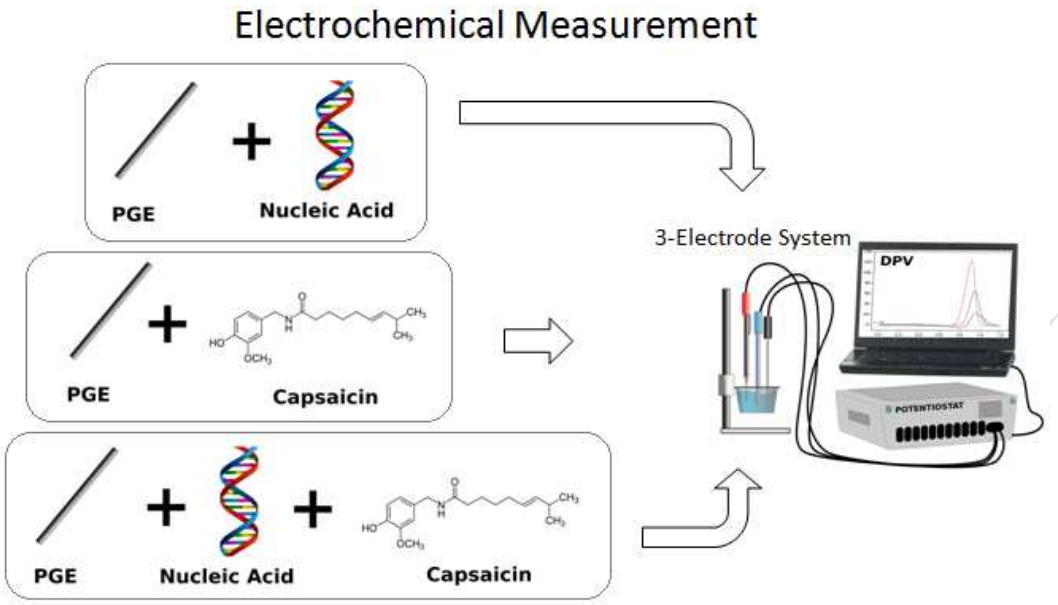
2.3. Preparation of cDNA and PCR samples

Total RNA was isolated from Huh7 human hepatocellular carcinoma cell line using High Pure RNA Isolation Kit (Roche Applied Science) according to manufacturer's instructions. RNA concentrations were determined by measuring the absorbance at 260 nm in a spectrophotometer (Nanovette, Beckman Coulter). cDNA was synthesized from total RNA using Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science). Polymerase chain reaction (PCR) was performed using Taq PCR kit (New England and Biolabs) and thermal cycler (Techne).

18S ribosomal 5 (RNA 18S5) spesific primer sequences used in PCR are 5'- CGA CGA CCC ATT CGA ACG TCT-3' and 5'-G CTA TTG GAG CTG GAA TTA CCG-3' to generate 312 bp product. GC content of PCR product is 54%. 18S rRNA plasmid cDNA (NR_003286.2) was obtained commercially from Thermo Scientific. The length and the GC content of cDNA used in this study are 1869 bp and 56%, respectively.

2.4. Procedure

The experimental procedure on electrochemical detection of biointeraction of CPS with nucleic acids (presented in Scheme 1) was comprised of three steps: (*i*) CPS immobilization and its detection at the surface of PGEs, (*ii*) the immobilization of the nucleic acids and its detection cycle, and (*iii*) electrochemical investigation of the surface confined interaction between CPS and nucleic acids (e.g, dsDNA, cDNA and PCR samples) at the surface of PGEs. All experiments were carried out at room temperature.



Scheme 1. Experimental presentation for the immobilization of CPS and nucleic acids at the surface of PGEs, and electrochemical detection of interaction between CPS and nucleic acids at the surface of PGEs.

2.5. Preparation of the PGEs

PGEs were pretreated by applying a potential of +1.40 V for 30 s in ABS (pH 4.80). These pretreated electrodes were used for further experiments.

2.6.CPS immobilization on to the surface of PGEs and electrochemical detection of CPS

Each pretreated electrodes were dipped into 100 µL of required amount of CPS solution and kept during required time for immobilization. After rinsing immobilized electrodes with ABS (pH 4.80) for 5 s, measurements were performed.

2.7.Surface confined interaction of CPS with nucleic acids at the surface of PGEs

The PGEs were immersed into the vials containing 100 µL of 80 µg/mL dsDNA solution in ABS (pH 4.8) for 4 h Each of the electrodes were then rinsed with ABS (pH 4.8) for 5 s before interaction with CPS. Nucleic acids immobilized electrodes were immersed into the vials containing 100 µL of 0.5 µg/mL CPS solution and allowed to interact. Then, each electrode was rinsed with ABS for 5 s before voltammetric transduction.

2.8. Voltammetric transduction

DPV measurements were performed for detection of CPS and guanineoxidation signal in ABS (pH 4.80) by scanning between0.00 V and +1.40 V at the pulse amplitude; 50 mVand the scan rate; 50 mV/s.

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2.9. Gel Electrophoresis

Nondenaturing agarose (2%) gels were prepared in TAE buffer. The samples (PCR samples/ capsaicin samples/ capsaicin-incubated PCR samples, with 0.25 % bromophenol blue in water, threated with different interaction time as 30, 15, 5 and 1 min, which were loaded into the wells. Then, electrophoresis was carried out in TAE buffer for 1 h at 100 V. Finally, 2 % ethidium bromide (EtBr) stained DNA was visualized and photographed.

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3. Results and Discussion

The effect of DNA concentration upon to biosensor response was studied in the presence of different dsDNA concentration varying from 20 to 100 µg/mL by applying 30 min immobilization time (Fig.1). There was a gradual increase at guanine signal measured at the potential of + 1.019 V in DNA concentration range of 20 - 80 µg/mL and the highest guanine signal was measured as 2503.2 ± 293.4 nA (RSD%, 11.7 %, n=5). A decrease at signal was recorded at 100 µg/mL DNA concentration. Therefore, 80 µg/mL was chosen as optimum for dsDNA concentration (Fig 1.)

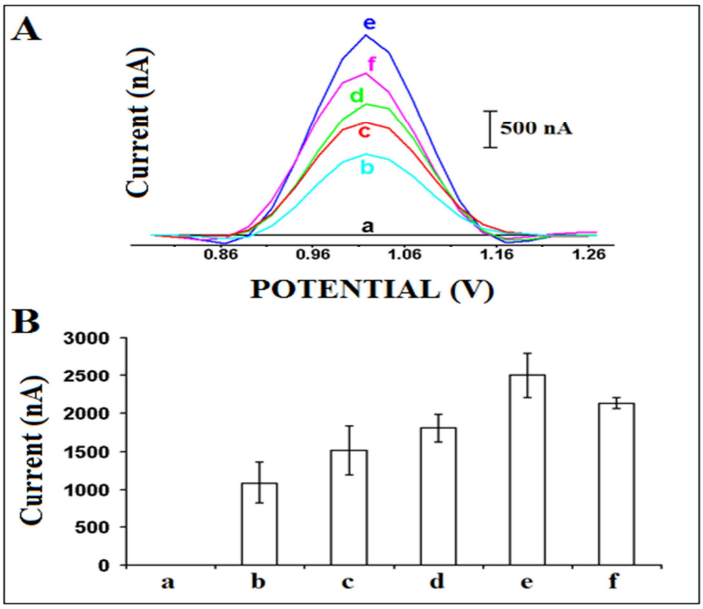

Figure 1. Voltammograms (A) and histograms (B) representing the oxidating signals of dsDNA depending on different concentration range. Control experiment with PGE in ABS (a), the guanine oxidation signal of 20 µg/mL(b), 40 µg/mL (c), 60 µg/mL (d), 80 µg/mL (e),100 µg/mL (f) dsDNA after 30 min DNA immobilization time.

Next, the effect of DNA immobilization time upon to biosensor response was investigated. For this purpose, 80 µg/mL dsDNA was immobilized onto PGE by applying different immobilization times varying from 15 min to 6 h (Fig. 2). In the presence of 4 h duration of DNA immobilization time, the highest guanine signal was measured (i.e, 5145.0 ± 509.1 nA (RSD%, 9.9 %, n=2) and shown in Fig. 2-h). When DNA immobilization performed for 6 h, a decrease at guanine signal was recorded in comparison to the result observed for 4 h duration of DNA immobilization time (Fig. 2-i).

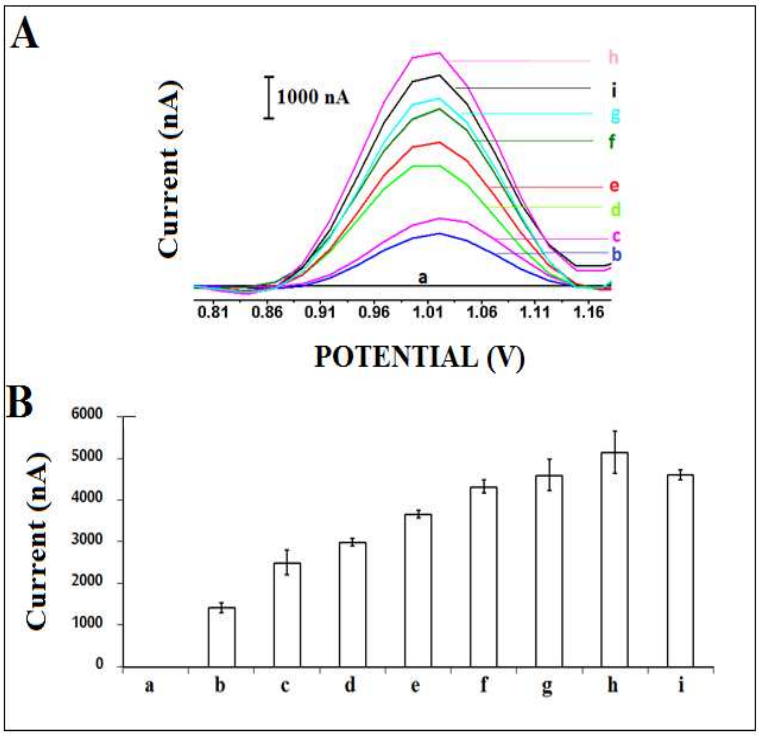

Figure 2. Voltammograms (A) and histograms (B) representing the guanine oxidation signal of 80 µg/mL dsDNA obtained by DPV. Control experiment with PGE (a), the guanine oxidation signal after immobilization of dsDNA onto the PGE surface during 15 (b), 30 (c), 60 (d), 90 (e), 120 (f), 180 (g), 240 (h) and 360 min (i).

The guanine oxidation signal was measured in different dsDNA concentration varying from 1 to 5 µg/mL. The resulting calibration plot is shown in Figure S1. According to the method described by Miller and Miller [40], the detection limit (DL) was calculated in the linear concentration range from 2 to 5 µg/mL and found to be 0.62 µg/mL. This DL value is lower than the ones obtained by modified graphite electrodes; such as, poly(methyl methacrylate)–graphite microcomposite electrode (i.e, 1.0 µg/mL) [41] and chitosan-SWCNT composite modified PGE (i.e, 6.85 µg/mL) [42].

Next, the effect of interaction time of 0.5 µg/mL CPS and 80 µg/mL dsDNA was studied during different interaction times varying from 1, 5, 15 and 30 min (Fig. 3). The oxidation signal of CPS and guanine were measured at the same voltammetric scale before and after interaction process at the potential of + 0.510 V and + 1.019 V, respectively. Then, the changes at both signals of CPS and guanine were evaluated in terms of interaction process and these results were given in Table 1.

Based on the three repetitive measurements, the oxidation signal of guanine was measured as 4971.4 ± 489.2 nA (RSD %, 9.8 %, n=12) before DNA interaction with CPS. When the interaction time was applied as 30 min, the average signals of CPS and guanine were measured respectively as 551.8 ± 193.5 nA (RSD %, 35.1%, n=9) and 4350.3 ± 321.6 nA (RSD %, 7.4 %, n=6), and accordingly, there was a highest decrease (i.e, 12.5 %) observed at guanine signal in 30 min interaction time.

As it has been previously reported by Richeux et al. [29] and Lewinskaa et al. [43], CPS is able to induce DNA strand breaks in a concentration dependent manner.

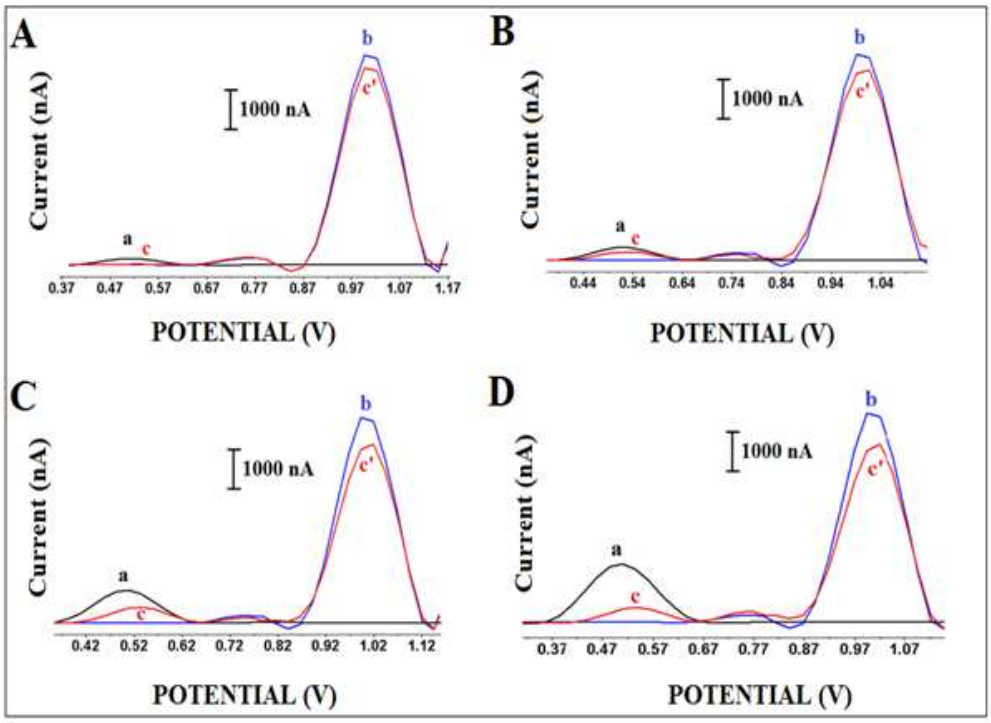

Figure 3. All voltammograms represents the results between 80µg/mLdsDNA and 0.5 µg/mL CPS interactions on different times. (a) CPS oxidation signal before interaction, (b) guanine oxidation signal before interaction, (c) CPS signal after interaction, (c') guanine signal after interaction process that performed onto the PGE surface during (A) 1 min, (B) 5 min, (C) 15 min, (D) 30 min.

According to our results, there was an increase at the decrease ratio % obtained at guanine signal when surface-confined interaction of CPS with DNA was performed in a time-dependent manner from 1 min to 30 min. Similarly to earlier reports [29,43], this phenomania could be possibly explained herein that more CPS interaction with DNA was occured due to increase at interaction time of CPS and accordingly, it may be resulted with a more DNA damage.

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Table 1. The oxidation signals of CPS and guanine before and after different interaction times and the decrease ratios after interaction process between CPS and dsDNA.

Interaction Time	CPS signal Before interaction (nA)	CPS signal After interaction (nA)	Decrease % at CPS Signal	Guanine Signal After Interaction (nA)	Decrease % at Guanine Signal
1 min.	144.8 ± 37.5	48.0 ± 15.3	66.6 %	4762.0 ± 590.1	4.2 %
5 min.	349.9 ± 75.9	162.0 ± 71.9	53.7 %	4627.8 ± 450.9	6.9 %
15 min.	956.8 ± 257.2	452.4 ± 85.0	52.6 %	4489.0 ± 525.4	9.7 %
30 min.	1573.3 ± 424.4	551.8 ± 193.5	64.9 %	4350.3 ± 321.6	12.5 %

In order to see the interaction of CPS with cDNA, a batch of experiments was performed. Then the interaction between 0.5 µg/mL CPS and 80 µg/mL cDNA was investigated by applying 5 min interaction time. Before and after interaction process, the average guanine oxidation signal was respectively found to be 7161.5 ± 135.0 nA (RSD %, 1.8 %, n=2) and 5896.5 ± 665.3 nA (RSD %, 11.2 %, n=2). A decrease about 17.6 % was recorded at guanine signal after surface confined interaction of CPS with cDNA. Additionally there was 58.2 % decrease obtained at CPS signal.

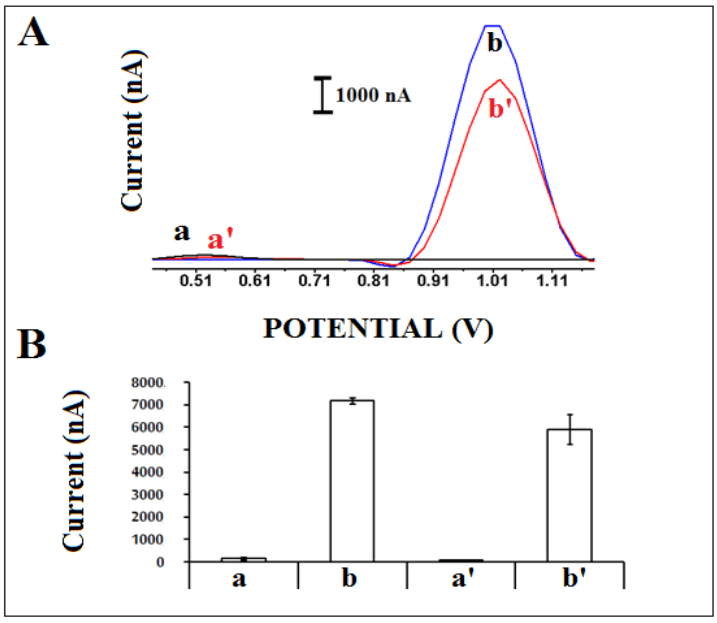

Figure 4. Voltammogram (A) and histogram (B) representing the guanine oxidation signals of 80µg/mL cDNA and 0.5 µg/mL CPS oxidation signal before and after 5 minute interaction. The oxidation signal of CPS before (a), after (a') interaction. The guanine oxidation signal before (b), after (b') interaction.

The interaction between 0.5 µg/mL CPS and 80 µg/mL PCR sample was also investigated in different interaction time varying from 1, 5, 15 and 30 min (Fig. 5). The oxidation signal of CPS and guanine were measured before

and after interaction process and then, the changes at the signals were evaluated in terms of interaction process and given in Table 2. As seen from table 2, there was a gradual decrease at guanine oxidation signal when interaction time increased similar to decrease observed at CPS signal (shown in Table S1).

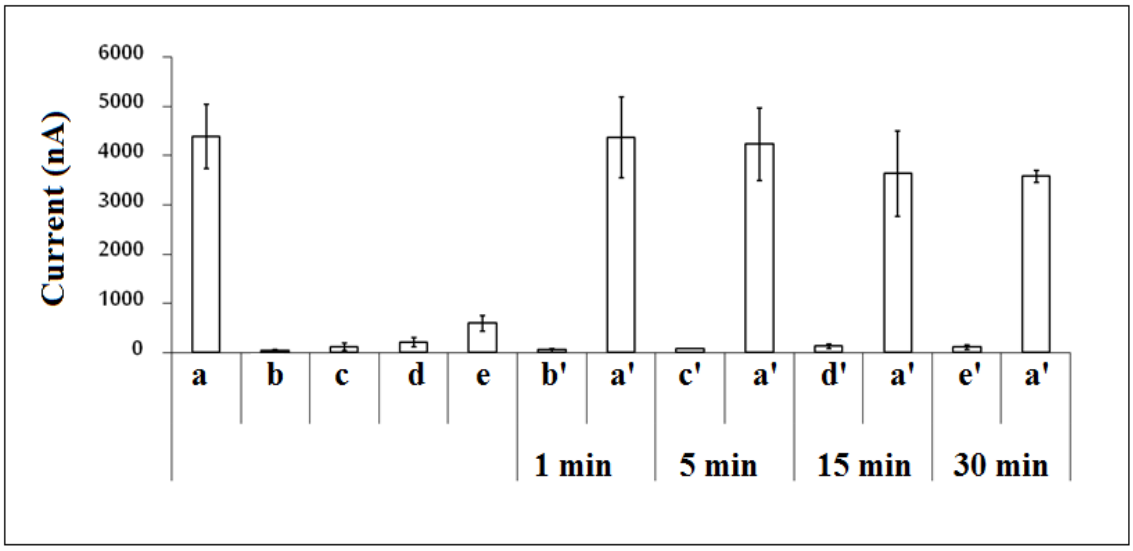

Figure 5. Histograms representing the average oxidation signals of guanine and CPS before and after interaction between 0.5 µg/mL CPS and 80 µg/mL PCR sample. Guanine oxidation signal (a) before, (a') after interaction with CPS during 1, 5, 15 and 30 min. CPS oxidation signal (b) before, (b') after 1 min interaction, (c) before, (c') after 5 min interaction, (d) before, (d') after 15 min interaction, (e) before, (e') after interaction during 30 min. onto the surface of PGE (n=3).

In order to confirm the interaction between CPS and nucleic acids by using a well-known method like gel electrophoresis, a batch of experiments was performed. When the interaction time of CPS with DNA was increased from 1 min to 30 min, a decrease at ethidium bromide intensity was observed in comparison to the one of PCR samples (Fig. 6). This decrease indicates that ethidium bromide is removed from DNA binding sites due to a possible binding of CPS to DNA as similiarly reported in earlier work [30].

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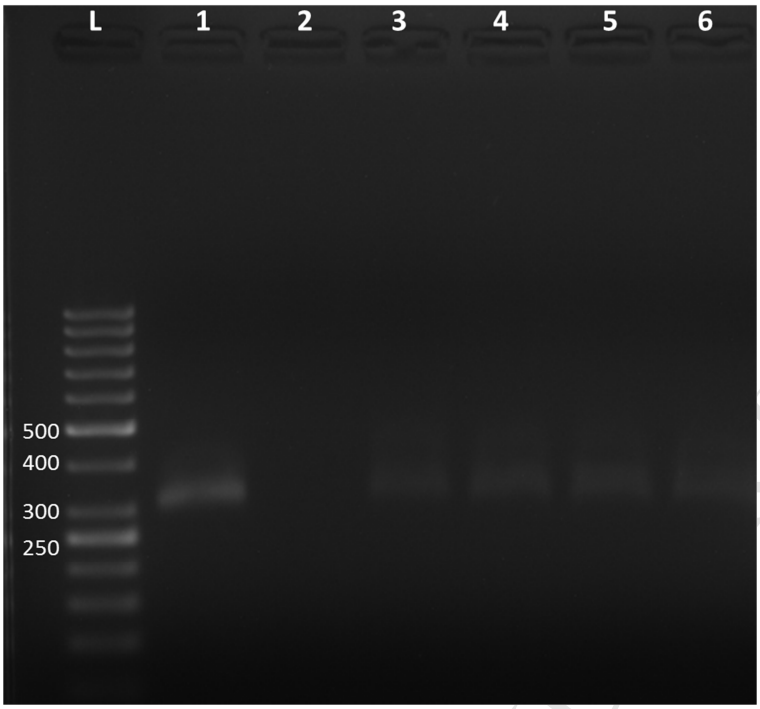

Fig. 6. Nondenaturing agarose (2%) gel electrophoresis of 80 µg/mL PCR sample without capsaicin (312 bp, lane 1), 0.5 ug/mL capsaicin (lane 2) and 0.5 µg/mL capsaicin-incubated PCR samples (80 µg/mL) during 30, 15, 5 and 1 min (lane 3, 4, 5 and 6, respectively). L: 50 bp DNA ladder.

Table 2. The oxidation signals of guanine before and after different interaction times and the decrease ratios after interaction process between CPS and PCR samples.

Interaction Time	Guanine Signal After Interaction (nA)	Decrease % at Guanine Signal
1 min.	4366.0 ± 816.0	0.4 %
5 min.	4229.6 ± 736.3	3.5 %
15 min.	3626.3 ± 858.2	17.2 %
30 min.	3570.6 ± 115.8	18.5 %

The value of the percentage at guanine peak height change **(S%)** was calculated herein according to the literature, [44] 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 following Eq. (1):

$$S\% = (S_S/S_b)\times 100 \qquad \text{Eq.(1)}$$

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* [44].

Table 3. S %values calculated according to the results obtained before/after CPS interaction with dsDNA and PCR samples in different interaction times varying 1 to 30 min.

Interaction time (min)	**S %**	
	dsDNA	**PCR samples**
1	95.7	99.5
5	93.0	96.5
15	90.2	82.7
30	87.5	81.4

It is known that the dsDNA has 1000 base pair with 41.9 mole G-C %, while PCR sample has 312 base pair with 54 mole G-C %. According to interaction time study performed with CPS and dsDNA (containing 41.9 mole G-C %) or PCR samples (containing 54 mole G-C %), guanine oxidation signal was decreased. There was an increase at the decrease ratio % when the interaction time of CPS with DNA was increased from 1 min to 30 min. In parallel to these results, a decrease was also recorded at S % value. Similiar to the previous studies on the genotoxicity of CPS [29,43], possible DNA damage could be occured in the case of a longer interaction time between CPS and DNA (or, in a higher CPS concentration) since CPS is able to induce DNA strand breaks in a concentration dependent manner resulting with a possible DNA damage.

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4. Conclusion

Through this work, the single-use graphite electrodes; PGEs were used for the first time herein in order to detect voltammetrically the interaction of Capsaicin with nucleic acids based on the changes at the oxidation signal of guanine. The voltammetric detection of surface confined interaction of CPS with calf thymus double stranded DNA as well as cDNA and PCR samples was explored regarding to the changes at guanine signal in the presence of various interaction times. An increase at the decrease ratio % was obtained when the interaction time of CPS with DNA was increased from 1 min to 30 min. Moreover, S % values [44] were calculated for the studies using dsDNA and PCR samples, and a decrease was recorded similiarly at S % value.

Moreover, the interaction of CPS with double stranded DNA was also determined by agarose gel electrophoresis. Thus, our voltammetric results were found in a good agreement with the electrophoretic ones. A decrease at ethidium bromide intensity was recorded in contrast to the one obtained by PCR samples while increasing the incubation time of CPS with PCR samples because of a possible binding of CPS to DNA as in reported in a earlier work [30]. It was concluded that the electrochemical monitoring of the interaction between CPS and nucleic acids by PGE is easy to handle, cost-effective and more rapid assay in contrast to the electrophoretic assay.

In comparison to the results of earlier studies related to electrochemial detection of interaction between DNA and small molecules or drugs, which were performed by using nanomaterials modified electrodes; such as, fullerene modified glassy carbon electrode (FLR-GCE) [45], SWCNT-Chitosan polymeric composite nanostructure modified PGE [42], our assay was developed in this present study by single-use PGE resulted with a sensitive and cost effective monitoring of interaction between CPS and DNA without any surface modification or chemical treatment applied to the electrode [46-48]. In the terms of preparation, nanomaterials modified electrodes are expensive and time consuming in contrast to the PGEs presented in our study.

The development of electrochemical biosensors for monitoring of nucleic acid interactions have a key importance in order to develop novel biotechnological tools to the point of care diagnosis. Such types of investigations could yield for probing the mechanism of the interaction between drugs/toxins/chemicals and DNA. Therefore, they can provide a knowledge to clarify the effects of matters on nucleic acids, so that they provide new opportunities for effective treatment on cancer.

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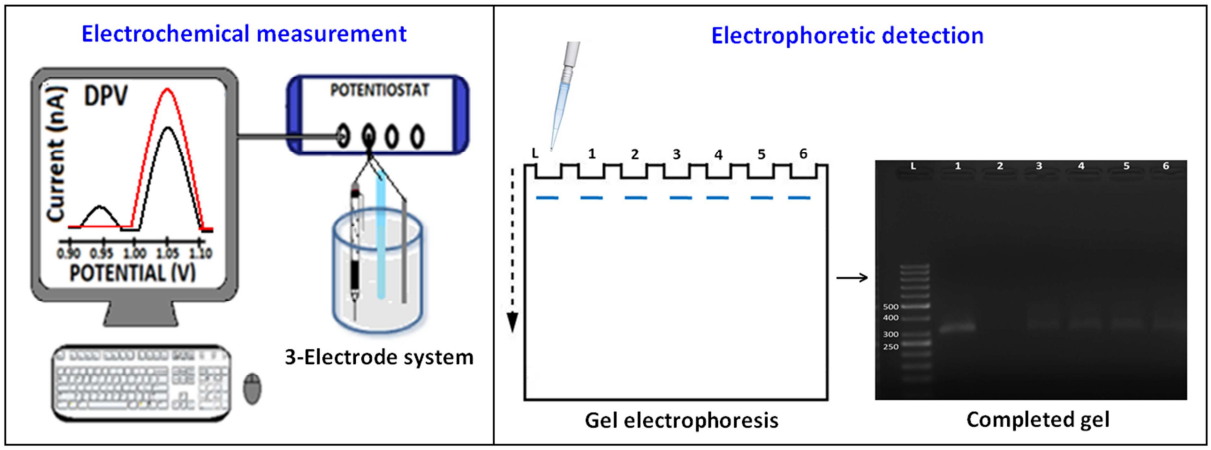
Graphical abstract



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