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Investigation of the effect of Aflatoxin B1 and Aflatoxin G1 on DNA Hybridization by Using Electrochemical DNA Biosensor

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Abstract. Biomolecular detection has become a current issue together with the increase in electrochemical sensor studies. The effect of Aflatoxin B1 and Aflatoxin G1 on DNA hybridization was analyzed with electrochemically by using pencil graphite electrode. For this purposes; the changes in guanine currents were monitored before and after hybridization occurred between DNA complementary base sequences using differential pulse voltammetry (DPV). In the first step of this work the capture probe was immobilized by wet adsorption onto the surface of PGE optimizing concentration and immobilization time and then hybridization event was performed between capture and its target probe. In the second step the hybridization experiments were repeated using inosine modified capture probe instead of capture probe which used in the first step. As differently from the first step the inosine modified probe was interacted with 2 µg/mL and 4 µg/mL of AFB1 for 10 min before allowing hybridization and the same protocol was repeated for AFG1. The decrease in guanine current after hybridization was the reference for the genotoxic effects of aflatoxins. The decrease in guanine oxidations in the cases of 2 µg/mL and 4 µg/mL AFG1 and 2 µg/mL and 4 µg/mL AFB1 concentrations were found to be 18% and 26 % for AFG1; and 50% and 61% for AFB1, respectively. AFB1 showed the maximum genotoxic effect to DNA hybridization.

***Keywords*:**Aflatoxin B1, Aflatoxin G1, Pencil Graphite Electrode, Hybridization, Differential Pulse Voltammetry

Aflatoksin B1 ve Aflatoksin G1'in DNA Hibridizasyonuna Etkisinin Elektrokimyasal DNA Biyosensörü ile İncelenmesi

Özet. Biyomoleküler analizlerde yeni teknolojilerin gelişmesi, elektrokimyasal sensör alanındaki çalışmaların da katılmasıyla güncel bir konu olmuştur. Bu çalışmada Aflatoksin B1'in (AFB1) ve Aflatoksin G1'in (AFG1) DNA hibridizasyonu üzerindeki etkisi, kalem grafit elektrot (PGE) kullanılarak elektrokimyasal yöntemlerle analiz edildi. Bu amaçla; guanin oksidasyon akımlarındaki değişiklikler, diferansiyel puls voltametrisi (DPV) ile birbirlerini eşleniği olan DNA baz sekansları arasında hibridizasyon oluşmadan önce ve sonra izlendi. Çalışmanın ilk aşamasında, yakalama probu ıslak adsorpsiyonla PGE yüzeyine immobilize edilirken prob derişimi ve adsorpsiyon süresi optimize edildi ve sonra, hedef prop ile hibritleşmeye tabi tutulurken hedef proba ait konsantrasyon ve hibridizasyon süresi optimize edildi. İkinci aşamada, hibridizasyon deneyleri, birinci aşamada kullanılan yakalama probu yerine inosin modifiye edilmiş yakalama probu kullanılarak tekrarlandı. Ancak, ilk aşamadan farklı bir şekilde inosin modifiye edilmiş prob, hibridizasyona tabi tutulmadan önce 10 dakika süre ile 2 µg / mL ve 4 µg / mL AFB1 ile etkileştirildi ve aynı protokol, aynı koşullarda AFG1 için de tekrarlandı. Hibridizasyondan sonra guanin oksidasyon akımındaki azalma, aflatoksinlerin DNA sekanslarının hibridizasyonuna etkisinin bir sonucu olup, AFB1 ve AFGl'in DNA üzerine genotoksik etkisinin bir göstergesidir. Guanin oksidasyon sinyalindeki azalmalar 2 µg/mL ve 4 µg/mL AFG1 için % 18 ve % 26; 2

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µg/mL ve 4 µg/mL AFB1 için % 50 ve % 61 olarak bulundu. Sonuç olarak AFB1'in DNA üzerindeki genotoksik etkisi AFG1'e göre çok daha fazla olarak tespit edildi.

***Anahtar Kelimeler*:** Aflatoksin B1, Aflatoksin G1, Kalem Grafit Elektrot, Hibridizasyon, Diferansiyel Puls Voltametrisi

1. INTRODUCTION

The biosensors in which DNA molecule is used for recognition surface is defined as DNA biosensors or genosensors [1]. DNA biosensors have been frequently used for diagnosis of hereditary and infectious diseases [2, 3], for detection of the microorganisms which trigger food [4] and environment [5] and biological weapons [6]. Particularly DNA biosensors which are used in health field are interesting. Efforts for detection of certain diseases from biological materials like blood, serum, tissue, cell etc. have gained importance in recent years and these studies have begun to be used for detection of bacterial, viral, parasitic and fungal diseases and the mutations [2, 3] which lead to many hereditary diseases.

Hybridization-based DNA biosensors work through binding of the probe which has a short base sequence (20-40 bases) that corresponds to the target sequence to the electrode surface [7, 8]. Hybrid is formed by applying the sensor to the analyte solution which contains the target sequence. This formation converts to an observable signal through the converting system which is found in electrochemical or optic systems.

Hybridization detection of the genes with certain sequences has been performed based on the changes in the oxidation signal (non-indicator method) or the changes in reduction or oxidation signal of a hybridization indicator which interacts with at least one of these bases or an inter-catalyzer hybridization indicator (indicator method) [9, 10]. In recent years, hybridization detection has been carried out by using oxidation signals of DNA bases, guanine and adenine, without using an indicator in electrochemical DNA biosensors and progressed rapidly. Studies are carried out for diagnosis of some infectious and hereditary diseases with non-indicator DNA hybridization method [11, 12]. Nucleic acid recognition-based electrochemical DNA biosensors is a more rapid, inexpensive and easy method than the routine analysis methods in diagnosis of hereditary and infectious diseases [13].

The toxic substances which are synthesized from the secondary metabolisms of molds are defined as “mycotoxin”. Mycotoxins are the substances which have various chemical and biological activities on the contrary to the bacterial toxins which are mainly in protein structure and which have antigenic properties. Mycotoxins are accepted as very important natural toxins due to being available and producing toxins by being formed in many food and feedstuff. Aflatoxins which are the most studied mycotoxins were discovered in 1960 and understood to have “hepatotoxic” and “hepatocarcinogen” effects [14]. Aflatoxins are the substances which have the greatest potential to cause hepatocellular cancer and evaluated in the context of Group I carcinogens by The International Agency for Research on Cancer (IARC) [15-17]. Aflatoxins have many significant toxic effects like immune-supression, protein metabolism impairment besides leading to hepatocellular cancer [18]. Aflatoxins are produced by some strains of Aspergillus flavus and by almost all strains of Aspergillus parasiticus [19, 20].

In the present study, the effect of interaction between DNA and aflatoxin (B1 and G1) on the hybridization was investigated by using differential pulse voltammetry (DPV). An inosine modified probe (inosine was used instead of guanine in the sequence of main probe) was immobilized onto the surface of PGE by using wet adsorption (probe concentration and immobilization time were optimized) and hybridization with target probe was performed under optimum conditions. AFB1 and AFG1 were interacted with main probe before hybridization and the changes in guanine oxidation

current signals were monitored before and after hybridization by using differential pulse voltammetry (DPV) technique. To the best of our knowledge, this is the first report on that the effect of interaction between DNA and aflatoxin on the hybridization of DNA.

2. MATERIAL AND METHOD

2.1. Devices

The guanine oxidation peak current was monitored by using DPV technic with an potentiostat/galvanostat analysis system (AUTOLAB PGSTAT 302 N) and GPES 4.8 software. A 7 mL voltammetric cell was used to electrochemical determinations carried out under ambient conditions (23 ± 2 °C). The triple electrode system composed of disposable PGE (as working electrode), platinum wire (as counter electrode) and silver/silver chloride (as reference electrode) was used to perform electrochemical measurements. A Rotring T 0,5 pencil model was the holder of graphite bars. A thin metallic wire was used to provided electrical contact between conductive parts of the pencil.

2.2. Chemical Substances

The oligonucleotides (probes and its complementary) were supplied from IONTEK (İstanbul, Turkey). Concentrated (1000 µg/mL) stock solutions of the lyophilize nucleotide were prepared in Tris-EDTA buffer containing of 10 mM Tris–HCl and 1 mM EDTA (pH: 8.0) and stored as frozen. The experiment solutions were prepared by diluting the concentrated stock solution with 0.5 M acetate buffer (contained 20 mM NaCl; pH 4.8).

0.05 M PBS (phosphate buffer solution which contains 20 mM NaCl; pH 7.4) was used to prepare diluted target solution. All the other chemical reagents used in experiments were in analytical purity grade and supplied from Sigma-Aldrich (Germany). In every case deionized water was used to prepare all solutions.

The sequences of the oligonucleotides (probes and its complementary) were as below:

5’ – NH2-(CH2)6-GCTCCCCTACTGCTGGCGAAAAG-3’: Capture probe

5′-NH2(CH2)6-ICTCCCCTACTICTIICIAAAAI-3′:Probe substituted with inosine

5′-CTTTTCGCCAGCAGTAGGGGAGC-3′:Target probe

AFB1 and AFG1 stock solution were prepared so as to contain 1000 µg/ml in 99.8% methanol (MeOH) solution.

2.3. Guideline for AFB1 and AFG1

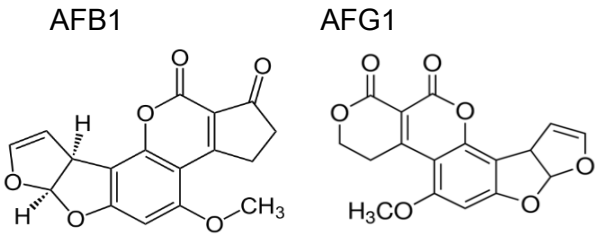

Figure 1. Structural formula of AFB1 ($C_{17}H_{12}O_6$) and AFG1 ($C_{17}H_{12}O_7$).

2.4. Method

AUTOLAB PGSTAT 302 N (Eco Chemie, Netherland) was used as potentiostat device and GPES was used as software program in the present study which was conducted by using differential pulse voltammetry. As triple electrode system, pencil graphite electrode (PGE) was used as working electrode, Ag/AgCl as reference electrode and platin wire was used as counter electrode. All electrodes were bond to the system with metallic connector and measurement solution was adjusted in 7 mL volume.

Activation of PGE:

All in voltammetric measurements the renewable PGE was used to electrochemical determination of DNA hybridization. Graphite ends of the PGE (Rotring T 0,5 pencil, Tombo HB model and 0.5 mm graphite bar) which was 6 cm was cut as 3 cm [21, 22]. 1,5 cm of this 3 cm was marked and

inserted to the voltammetric cell so as the part of 1 cm to be dipped into the solution.

The working electrodes were pretreated in ABS by applying a potential of +1.4 V for 0,5 min. The oxidation peak currents of guanine were monitored with DPV after baseline fitting.

Immobilization of the capture probe and inosine modified probe:

The pencil bars activated by electrochemically were dipped into the vials containing 120 µL capture probe solution at different concentrations ranged between 8- 48 µg/mL in ABS for 30 min. Thus the single stranded capture probe was attached to PGE's surface by adsorption. In order to remove unbounded probe parts the electrodes were immersed in ABS for 3 s. After optimization of probe concentration as 24 µg/mL, optimum adsorption time also optimized as 40 min. In the second step inosine modified probe sequence was immobilized on to surface of PGE at the same conditions.

Hybridization and Aflatoxin interaction:

The hyridization experiments between inosine modified probe and its target were carried out following of inosine modified probe immobilized wit wet adsorption. For this purposes the PGE's which probe modified on their surface were immersed into the vials containing 120 µL target probe solution at different concentrations ranged between 10 - 60 µg/mL in PBS for 30 min. In order to remove unbounded probe parts the electrodes were immersed in PBS for 3 s. After optimization of hybridized target probe concentration as 40 µg/mL, optimum hybridization time also optimized as 50 min. In another step, inosine probe modified PGE were immersed in 2 µg/mL AFB1 and 4 µg/mL AFB1 solutions for 10 min in order to analyze interaction effect on hybridization. This step was repeated for AFG1.

Voltammetric measurement:

The guanine oxidation peak current was determined by using DPV in the potential range of + 0.8 V - + 1.4 V in 0.5 M ABS (pulse amplitude and scan rate were 50 mV and 50 mV/s respectively). All experiments were repeated for at least 5 times.

3. RESULTS AND DISCUSSION

In this work, the DNA biosensor (without labeling) based on DPV measurement of the hybridization occured between capture probes (one is interacted with aflatoxins another one is without interacted) and target. This study was aimed to develop a label-free DNA biosensor to analyze the effect on hybridization of interaction between aflatoxins and DNA by using disposable sensor (PGE). The detection of hybridization was monitored according to guanine oxidation peak current occurred at + 1.0 V. The critical event that the probe modified inosine didn't give any oxidation signal before hybridization.

When the capture probe was immobilized by wet adsorption, its concentration (Fig. 2) and the immobilization time (Fig. 3) were optimized according to the guanine oxidation current. According to the optimization experiments, capture probe concentration was found as 24 µg/mL and the immobilization time as 40 min.

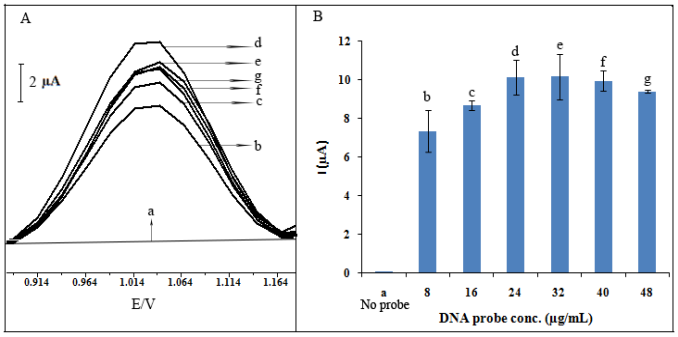

Fig. 2. (A) Voltamograms and (B) Histograms related to guanine oxidation currents according to different capture probe concentrations (immobilization time was constant as 30 min).

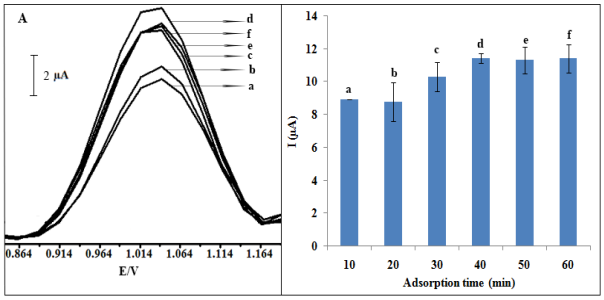

Fig. 3.(A) Voltammograms and (B) Histograms related to guanine oxidation currents according to different capture probe immobilization time (for constant concentration of 24 µg/mL)

The following experiments were related to optimizing the hybridization between capture probe and its complementary target in different hybridization times range of 15 and 60 min (Fig. 4) and the value of the optimum hybridization time was found as 50 min and in different target concentration range of 15 and 60 µg/mL (Fig. 5) and the value of the optimum target concentration was found as 40 µg/mL.

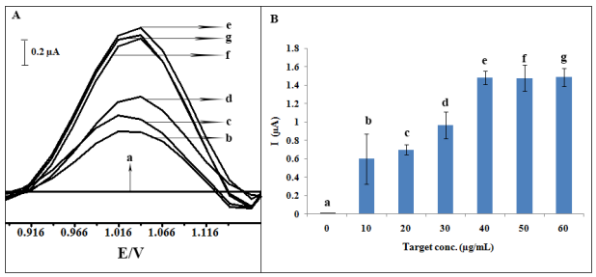

Fig. 4.(A) Voltammograms and (B) Histograms related to guanine oxidation currents according to different concentrations of the target at constant hybridization time of 30 min.

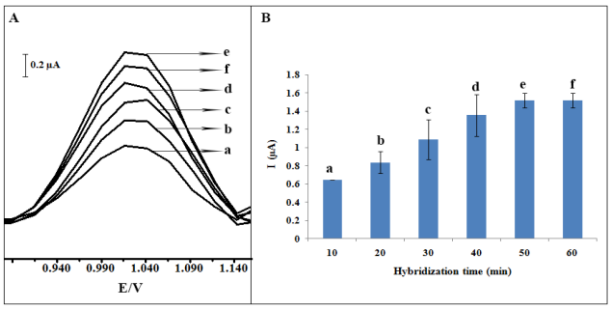

Fig. 5 (A) Voltammograms and (B) Histograms related to guanine oxidation currents according to different hybridization times at constant target concentrations (40 µg/mL).

In the optimization experiments, 50 min hybridization time and 40 ppm complementary target concentration were chosen according to the guanine oxidation signal with a better reproducibility and these values were used for further hybridization studies.

The effects of 2 µg/mL and 4 µg/mL AFB1 and AFG1 (at the same concentrations) on DNA hybridization were analyzed through the changes in oxidation signals of guanine. The obtained voltammograms and histograms were given in Fig. 6 and Fig. 7, respectively.

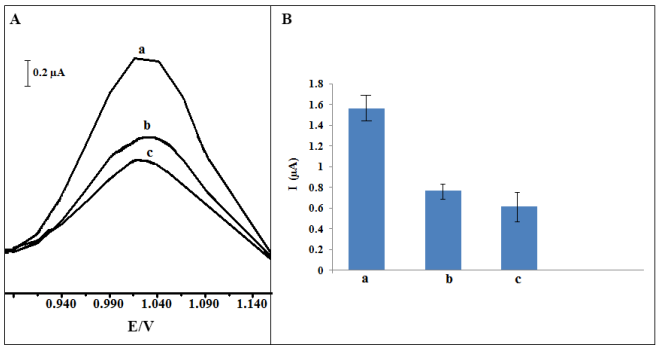

Figure 6 (A) Voltammograms and (B) Histograms related to guanine oxidation currents according to hybridization [without AFB1 interaction (a), interaction with 2 µg/mL AFB1 (b), interaction with 4 µg/mL AFB1 (c)] with target probe.

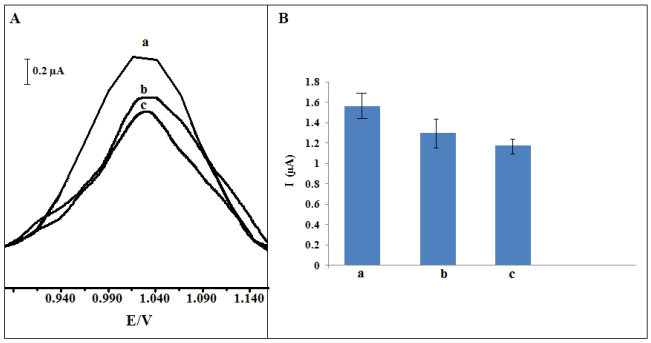

Figure 7 (A) Voltammograms and (B) Histograms related to guanine oxidation currents according to hybridization [without AFB1 interaction (a), interaction with 2 µg/mL AFB1 (b), interaction with 4 µg/mL AFB1 (c)] with target probe.

Figure 6 shows that the effect of interaction between AFB1 and DNA on hybridization of inosine modified probe with its target according to oxidation signal obtained before interaction with AFB1 (a) and after interaction with 2 µg/mL (b) and 4 µg/mL (c) AFB1. When increased of AFB1 concentration to 4 µg/mL the guanine oxidation current decreased more. So AFB1 was concluded to prevent DNA hybridization with proportion to the concentration of AFB1. The guanine oxidation current was found to decrease 50 % after interaction with AFB1 and this reduction was found to be about 60% when the amount of AFB1 was increased to 4 µg/mL.

The effect of interaction between AFG1 and DNA on hybridization of inosine modified probe with its target according to oxidation signal obtained before interaction with AFG1 (a) and after interaction with 2 µg/mL (b) and 4 µg/mL (c) AFG1 (Figure 7). When increased of AFG1 concentration to 4 µg/mL the guanine oxidation current decreased more. It was concluded that AFG1 also prevented DNA hybridization with proportion to its concentration. The guanine oxidation current was found to decrease 18 % after interaction with AFG1 and this reduction was found to be about 26 % when the amount of AFG1 was increased to 4 µg/mL.

4. CONSCLUSION

In our study conducted for assessment of sensor technologies-based DNA analyses, activation of pencil graphite electrodes (PGE) and the effect of AFB1 and AFG1 on DNA hybridization on electrode surface were analyzed with electrochemical method. The effect of AFB1 and AFG1 on DNA hybridization was analyzed through the changes in guanine oxidation signal by using DPV with hybridization method using PGE as a working electrode without any labeling. The biosensor consisting of the immobilization of probe which doesn't contain guanine (modified with inosine) onto the surface of PGE was utilized successfully to evaluate of interactions between DNA and Aflatoxins. The duplex formed between inosine modified probe and its target was detected in connection with DPV by measuring the guanine oxidation peak current. In the first step, capture probe modified with inosine was immobilized by wet adsorption onto the pretreated PGE surface. The hybridization occurred between the modified probe and its target on the PGE's surface was then determined according to the guanine oxidation current [23, 24]. Before hybridization experiments, the inosine modified probe was interacted with AFB1 and AFG1 at the same concentrations and the same time. It was seen that the aflatoxins had genotoxic effect on the hybridization of DNA. It's well known that the aflatoxins have very much genotoxic properties among the mycotoxins and also known that these carcinogenic toxins damage DNA [25]. The aflatoxins contained furan ring when covalently binds to DNA form an epoxide. Studies related to in vitro reaction of synthetic AFB1 –8,9-epoxide with DNA showed that the adduct formation proceed by a pre-covalent intercalation complex between double-stranded DNA and AFB1–exo-8,9-epoxide isomer [26]. Our results in this study also showed that the genotoxic effect could be easily understood and compared each other by utilized hybridization event and AFB1has much more genotoxic effect than AFG1.

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