Talanta 149 (2016) 244–249

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Voltammetric detection of sequence-selective DNA hybridization related to *Toxoplasma gondii* in PCR amplicons

Gultekin Gokce [a,b,*], Arzum Erdem [b,**], Cagdas Ceylan [a], Muslum Akgöz [c]

[a] Cumhuriyet University, Education Faculty, Department of Elementary Science, 58140 Sivas, Turkey
[b] Department of Analytical Chemistry, Faculty of Pharmacy, Ege University, Bornova, 35100 Izmir, Turkey
[c] TUBITAK Ulusal Metroloji Enstitusu, Bioanalysis Laboratory, Gebze, 41470 Kocaeli, Turkey

ARTICLE INFO

Article history:
Received 18 August 2015
Received in revised form
22 November 2015
Accepted 26 November 2015
Available online 28 November 2015

Keywords:
Toxoplasma gondii
DNA hybridization
Pencil graphite electrode
Guanine signal

ABSTRACT

This work describes the single-use electrochemical DNA biosensor technology developed for voltammetric detection of sequence selective DNA hybridization related to important human and veterinary pathogen; *Toxoplasma gondii*. In the principle of electrochemical label-free detection assay, the duplex of DNA hybrid formation was detected by measuring guanine oxidation signal occured in the presence of DNA hybridization. The biosensor design consisted of the immobilization of an inosine-modified (guanine-free) probe onto the surface of pencil graphite electrode (PGE), and the detection of the duplex formation in connection with the differential pulse voltammetry(DPV) by measuring the guanine signal. *Toxoplasma gondii* capture probe was firstly immobilized onto the surface of the activated PGE by wet adsorption. The extent of hybridization at PGE surface between the probe and the target was then determined by measuring the guanine signal observed at +1.0 V. The electrochemical monitoring of optimum DNA hybridization has been performed in the target concentration of 40 μg/mL in 50 min of hybridization time. The specificity of the electrochemical biosensor was then tested using non-complementary, or mismatch short DNA sequences. Under the optimum conditions, the guanine oxidation signal indicating full hybridization was measured in various target concentration from 0.5 to 25 μg/mL and a detection limit was found to be 1.78 μg/mL. This single-use biosensor platform was successfully applied for the voltammetric detection of DNA hybridization related to *Toxoplasma gondii* in PCR amplicons.

1. Introduction

In recent years, an increasing attention has been received for development of electrochemical DNA hybridization biosensors that could rapidly and accurately recognize and detect the pathogens in environmental and clinical samples [1–4]. Electrochemical nucleic acid (NA)-based biosensors, also known as "DNA biosensors" (or genosensors), are devices that combine of an NA (usually a short synthetic oligonucleotide) as the biological recognition component and an electrode as physicochemical transducer. Due to their short assay time, low cost, small dimensions as well as compatibility with microfabrication technology DNA biosensors hold a great promise for the detection of hybridization event [5–7] polymorphisms [8–9], mutations [10–12] and interaction between drug and DNA [13–15]. These biosensing devices have been designed in such a way that they can monitor sequence-specific hybridization processes based on the oxidation signal of guanine and adenine or by DNA intercalators; such as $Co(phen)_3^{3+}$ [16], which form complexes with the nitrogenous bases of DNA or by detecting the oxidation signal at some metal tags; such as gold and silver nanoparticles [17–20] in the presence of DNA hybridization.

Electrochemical DNA biosensors for label-free detection of DNA hybridization [21–33] have been increasingly developed after the presentation of a label-free electrochemical DNA biosensor protocol by Wang et al. [21], which involved the immobilization of inosine-substituted (guanine-free) probe onto CPE. Using inosine bases, a base which is not naturally present in DNA but can act as base-pair of cytosine, at the capture probe instead of guanine bases eliminated the need for the indicator addition and shortened the assay time in the monitoring of the duplex formation between capture DNA probe and target DNA. In the another work, Wang et al. shown that label-free hybridization detection at pencil graphite electrodes in connection with magnetic separation minimized the non-specific binding effects [22]. Another simple method for detection of single-base mismatch was introduced by

* Corresponding author at: Cumhuriyet University, Education Faculty, Department of Elementary Science, 58140 Sivas, Turkey
** Corresponding author.
E-mail addresses: ggokce2000@gmail.com (G. Gokce), arzum.erdem@ege.edu.tr, arzume@hotmail.com (A. Erdem).

http://dx.doi.org/10.1016/j.talanta.2015.11.071
0039-9140/

Palecek and coworkers [23] using DNA hybridization biosensors. In the paper of Mascini et al. [26], a label-free electrochemical genosensor was described for the analysis of real samples of total genomic DNA extracts of mammalian species (bovine) via hybridization detection. Erdem et al. [27] reported a biosensor in combination with genomagnetic assay for the electrochemical detection of a specific sequence for *Salmonella* spp. by using graphite-epoxy composite and magneto- graphite-epoxy composite electrodes as electrochemical transducers. This assay was performed by the capture and hybridization of DNA target with a biotinylated inosine-substituted probe onto streptavidin-coated magnetic beads and the electrochemical detection based on the signal coming from guanine oxidation of DNA target. In another label-free assay, M. H. Pournaghi-Azar et al. [28] detected the human interleukine-2 gene using a non-inosine substituted probe immobilized on the pencil graphite electrode and then, the guanine oxidation signal was monitored using differential pulse voltammetry technique. The detection of human papilloma virus using a label-free electrochemical DNA sensor was carried out by treating a captured double stranded DNA duplex with acid and directly measuring the guanine bases released using square wave voltammetry [29]. Carboxylic acid functionalized single-walled carbon nanotubes modified graphite sensors were developed by Caliskan et al. [30] for electrochemical monitoring of direct DNA hybridization related to specific sequence of Hepatitis B virus employing label-free assay. Zhu et al. [31] demonstrated a label-free hybridization assay based on poly (amidoamine)/multi-walled carbon nanotubes–cobalt phthalocyanine nanocomposite modified glassy carbon electrode for the electrochemical detection of DNA sequence related to Avian Influenza Virus genotype. In the paper of Souza et al. [32], a label-free electrochemical genosensor was described for the analysis of specific oligonucleotide sequence of dengue virus type 1 on pencil graphite electrode. Bollo et al. [33] reported on the analytical performance of glassy carbon (GCE) electrodes modified with a dispersion of multiwall carbon nanotubes (CNT) in chitosan (CHIT) for the quantification of DNA.

The protozoan *T. gondii* is one of the most common infectious pathogen causing toxoplasmosis. The pathogen is more effective on patients with the immune system impaired or weakened such as those undergoing chemotherapy, organ transplants and/or infected with human immunodeficiency virus (HIV) [34–36]. In addition, toxoplasmic infection during pregnancy may lead to numerous detrimental consequences for the fetus and newborn [37]. The presence of viable *T. gondii* can be detected with injection of body fluids into experimental animals or tissue culture. Antibody levels can be measured from body fluids or the presence of protozoa can be detected directly by means of the conventional molecular biological methods [38]. The serological method is important diagnostic tool used for the detection of toxoplasmosis even though its disadvantage such as it does not show the *T. gondii* in the active phase of the infection [39]. PCR-based methods [40–42] have also been developed used for the detection of *T. gondii* from various clinical samples. Recently, more sensitive methods such as piezoelectric immunoagglutination assay using gold nanoparticles [43], or silica nanoparticles [44] have been developed for the analysis of toxoplasmosis. However, the major disadvantage of these methods is that are time consuming, cost-expensive, labor intensive and sometimes are not providing quantitative data. Because of these reasons, the development of novel, simpler, sensitive, selective and faster methods for the identification and detection of *Toxoplasma gondii* has a key importance for diagnosis of the toxoplasmosis. To the best of our knowledge, no electrochemical label-free DNA biosensor selective to*T. gondii* has been reported yet in the literature. This study presents a novel DNA electrochemical biosensor for label-free detection of sequence-selective DNA hybridization related to the *T. gondii* based on the oxidation signal of guanine after hybridization between inosine-modified probe and its synthetic complementary target, and PCR amplified target sequences.

2. Experimental

2.1. Apparatus

The oxidation signal of guanine was investigated by using DPV with an AUTOLAB PGSTAT 302 N (integrated with FRA2.220 module) electrochemical analysis system and GPES 4.8 software package (Eco Chemie, The Netherlands). Electrochemical measurements were carried out in a 10 mL voltammetric cell at room temperature (25 °C). The three-electrode system consisted of the pencil graphite electrode (PGE; each electrode was used as disposable) as the working electrode, the reference electrode (Ag/AgCl/3 M KCl) and a platinum wire as the auxiliary electrode. A Noki pencil model 2000 (Japan) was used as a holder for graphite leads (Tombo, Japan). Electrical contact with the lead was obtained by soldering a metallic wire to the metallic part.

2.2. Reagents

The synthetic oligonucleotides (probe and its complementary; target, single-base mismatch – MM and noncomplementary – NC) were provided from IONTEK (İstanbul, Turkey) as lyophilized powder and their base sequences are as below:

***T. Gondii* capture probe (22-mer sequence):**

5′ – NH_2-$(CH_2)_6$- GCTCCCCTCTGCTGGCGAAAAG-3'

***T. Gondii* capture Inosine substituted probe (22-mer sequence, I=Inosine):**

5′-$NH_2(CH_2)_6$-ICTCCCCTCTICTIICIAAAAI-3′

Target (22-mer sequence):

5′-CTTTTCGCCAGCAGAGGGGAGC-3′

Single base mismatch; MM (22-mer sequence):

5′-CTTTTCGCCA**A**CAGAGGGGAGC-3′

Noncomplementary; NC (22-mer sequence):

5′-TCCGCTAATGTTCTGATTATCA-3′

22-base target was a complementary sequence of probe and MM was a mutant type of the target with one base changed as indicated in bold.

The oligonucleotide stock solutions (1000 μg/mL) were prepared with Tris-EDTA buffer solution (10 mM Tris–HCl, 1 mM EDTA, pH: 8.00; TE) and kept frozen. More diluted probe solutions were prepared in 0.5 M acetate buffer solution containing 20 mM NaCl (pH 4.80; ABS). More diluted target/NC/MM solutions were prepared in 0.05 M phosphate buffer solution containing 20 mM NaCl (pH 7.40, PBS). Other chemicals were in analytical reagent grade and they were supplied from Merck. All stock solutions were prepared using deionized water.

The probe system with DNA length of 129 base pair was designed and tested for PCR assays. The 3' overlapping sequence of *T Gondii* B1 gene annealed and double stranded template was created by extension of 3' end with PCR. This method uses PCR to obtain double stranded template DNA that was used in hybridization experiments. The extension was performed in 100-μL reaction mixtures containing the following: 5 μL of template (100 pmol/μL), 40 μL ddH_2O and 50 μL PCR 2X Master Mix (Fermentas). First step, denaturation at 95 °C for 4 min; 30 cycles of amplification, 30 s, 95 °C, and annealing, 30 s, 60 °C and extension, 30 s, 72 °C, the final extension step continued for an additional 5 min. to obtain full length product. The product was diluted 20 times and quantified by DNA gel electrophoresis in a 2% agarose gel stained with ethidium bromide and visualized under ultraviolet light. Amount of DNA marker (100 bp ladder, Fermentas,

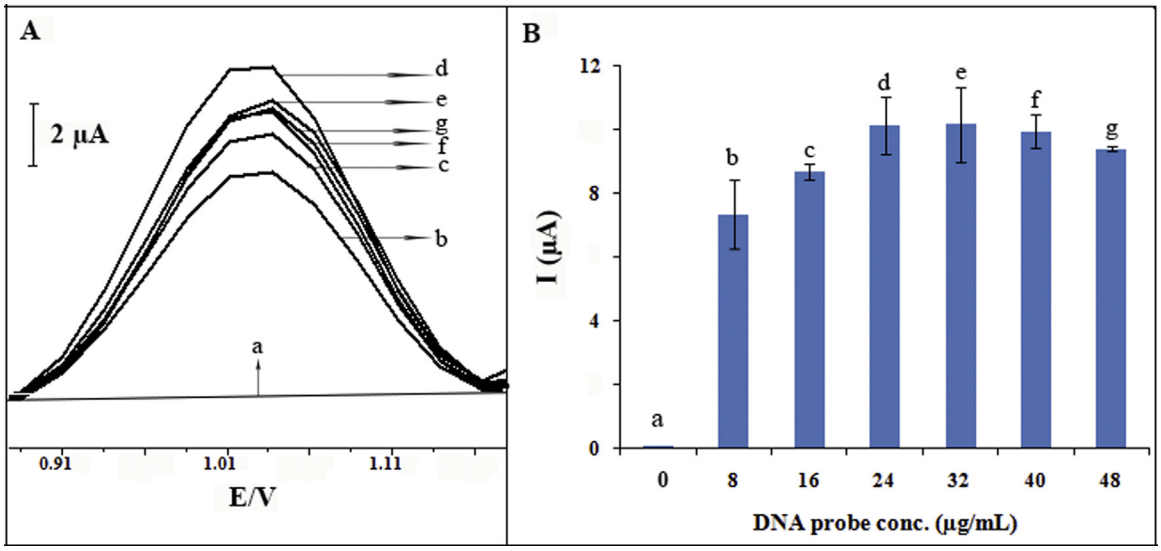

Fig. 1. (A) Voltammograms and (B) Histograms based on guanine oxidation signals observed in different probe concentrations at constant immobilization time; (a) is control signal, (b, c, d, e, f, g) are peak currents of guanine correspond to concentration of DNA probes.

DNA GeneRuler™ molecular weight marker) was used in the quantification after measuring PCR product and DNA Marker bands with ImageJ software (http://rsbweb.nih.gov/ij/). PCR amplification was performed in a DNA Thermal Cycler (Veriti® Thermal Cycler).

2.3. Procedure

2.3.1. Electrode preparation

The renewable PGE described by Wang et al. [33] was used in voltammetric measurements for the electrochemical detection of DNA hybridization. A Noki pencil Model 2000 (Japan) was used as a holder for the graphite lead. Electrical contact with the lead was obtained by soldering a metallic wire to the metallic part. The pencil was hold vertically with 12 mm of the lead extruded outside (10 mm of which was immersed in the solution). In all cases, the PGE transducer was pretreated by applying +1.40 V for 30 s in sodium acetate buffer solution (ABS, 0.5 M, pH 4.8) containing 20 mM NaCl without stirring. The guanine oxidation signal was detected using DPV. The guanine oxidation peak height after baseline fitting was used as the analytical signal.

2.3.2. Capture probe immobilization

The electrochemically pre-treated pencil leads were immersed into the vials containing 110 μL of the inosine-substituted DNA solutions of 20 μg/mL in ABS (0.5 M, pH 4.8) for half-hour allowing single-strand DNA probe attachment by wet adsorption. The electrodes were then immersed in ABS for 2 s to remove unbound DNA. Thus, a probe-modified PGE was obtained.

2.3.3. Hybridization

After probe immobilization by wet adsorption, the hybridization with both synthetic targets and PCR products were carried out immersing the probe-attached PGE into the PBS solution (0.05 M, pH 7.40) containing target oligonucleotide of 40 μg/mL for 50 min. The electrodes were then immersed in PBS for 2 s to remove unbound DNA. The same protocol was applied for the hybridization process between probe and other sequences; NC or MM, and also applied PCR products for testing the selectivity of biosensor.

For using PCR products, 20 times diluted PCR samples with PBS solution were used before they were thermally denatured [25].

All the experiments were performed at room temperature in an electrochemical cell.

2.3.4. Voltammetric transduction

The oxidation signal of guanine was measured by using differential pulse voltammetry (DPV) in blank ABS by scanning from +0.80 to +1.40 V at the pulse amplitude as 50 mV and the scan rate as 30 mV/s. The raw data were treated using the Savitzky and Golay filter (level 2) of the General Purpose electrochemical software (GPES) of Eco Chemie (The Netherlands) with a moving average baseline correction, using a “peak width” of 0.04.

3. Results and discussion

The label free genosensor relies on the DPV transduction of the hybridization reaction between the capture probe and the two types of target DNA sequences, which of one is synthetic and one is present in the PCR amplified amplicons. In this study, a label-free genomagnetic assay was developed for the sequence-specific detection of *T. gondii*, by using disposable sensor, PGE, in a target concentration of 40 μg/mL in 50 min of hybridization time. The detection of hybridization is accomplished with the appearance of the guanine oxidation signal [45], whereas no guanine signal is obtained from inosine-substituted probes (before hybridization). The increase at the magnitude of the guanine oxidation peak thus shows that there is a full hybridization between probe and its target at the electrode surface.

The effect of probe concentration on immobilization efficiency (Fig. 1) as well as that of immobilization time (Fig. 2) have been investigated using the magnitude of guanine signal. According to the optimization assays, the optimum concentration of *Toxoplasma gondii* capture probe was determined as 24 μg/mL and the optimum immobilization time as 40 min.

The following experiment was focused on optimizing the time of hybridization process between the *T. gondii* probe and its target varying from 15 min to 60 min (Fig. S1). The highest guanine oxidation signal was obtained in 50 min hybridization time with a better reproducibility. In addition, as the non-specific adsorption of the oligonucleotide sequences at the electrode surface in the case of longer hybridization times could cause the loss of selectivity of the biosensor system, and thus, an optimum hybridization time of 50 min was employed for further hybridization studies.

Then, the effect of target concentration from 15 μg/mL to 60 μg/mL upon to biosensor response was studied, and 40 μg/mL

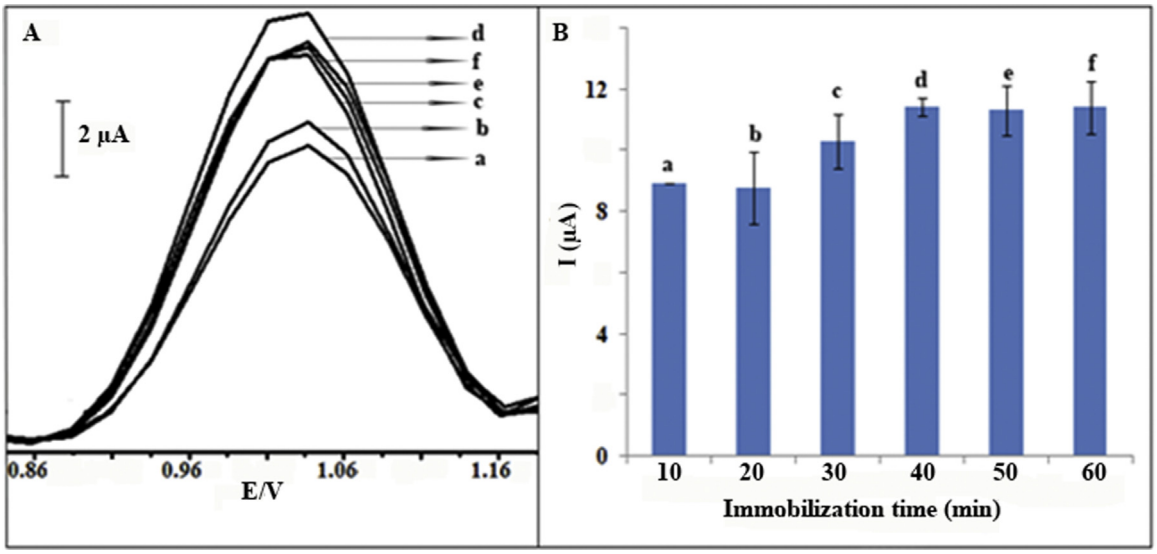

Fig. 2. (A) Voltammograms and (B) Histograms based on guanine oxidation signals observed in different probe immobilization time at constant probe concentration; (a, b, c, d, e, f) are peak currents of guanine correspond to immobilization time of DNA probes.

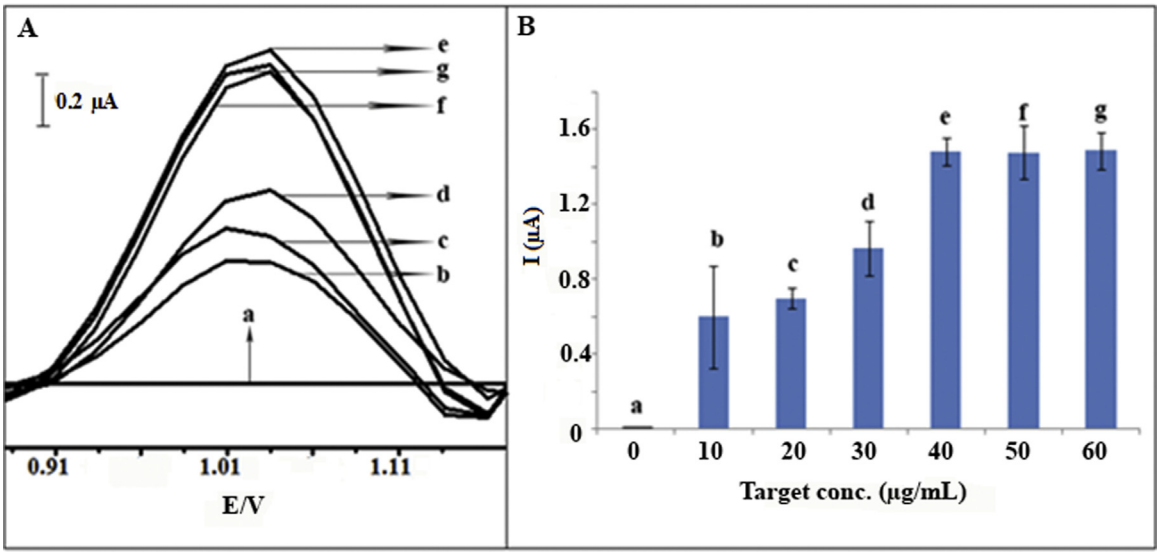

Fig. 3. (A) Voltammograms and (B) Histograms based on guanine oxidation signals observed in different target concentrations at constant hybridization time; (a) is control signal, (b, c, d, e, f, g) are peak currents of guanine correspond to concentration of target DNA probes.

was found as optimum target concentration due to observing the highest response with a good reproducibility in the case of full hybridization (Fig. 3).

There was an increase at the guanine oxidation signal in the presence of hybridization while increasing the concentration of target varying from 0.5 to 25 μg/mL (Fig. S2). The detection limit [46] was calculated by means of equation: $DL = y_B + 3\,s_B$ and regression equation: $y = 0.046x + 0.0121$ with correlation coefficient of 0.998. Where yB is signal of the blank (here intercept of regression line, 0.0121) and s_B is standard deviation of blank (here standard deviation of the regression line, 0.0273). According to this regression equation, the detection limit was found to be 1,78 μg/mL. This detection limit was found as lower one comparison to the ones of earlier reports related to other label-free (guanine-based) electrochemical DNA hybridization biosensors [47,48], or indicator based assay based on dendrimer modified electrodes [49]. Moreover, the detection protocol is herein resulting in a shorter time without using any extra nanomaterial or biopolymer modification of electrode surface [48,49] or any extra electroactive indicator for monitoring DNA hybridization [49].

Next, our sequence-specific hybridization protocol was carried out to assess whether the inosine-substituted *T. gondii* probe-modified PGE could recognize selectively target contrast to other oligonucleotides (NC or MM). The highest guanine oxidation signal with a better reproducibility was observed in the presence of full-match *T.gongii* DNA hybridization between probe and its target (Fig. 4). It was found that the hybridization of probe with one base single-mismatch (MM), or non complementary target (NC) did not lead to any significant increase at the guanine oxidation signal due to absence of entire hybridization.

The detection of PCR amplified real samples by using an electrochemical DNA biosensor relies on the differential puls voltammetric transduction of the hybridization reaction between the *T. gondii* capture probe and target DNA sequences which are available in the PCR amplified real samples. The results obtained in the presence of PCR samples were shown in Fig. 5. The appearance of the guanine signal obtained with a positive sample which contains the target *T.gondii* DNA confirmed the hybridization in PCR samples. No voltammetric signals were obtained when only probe (without any target) and the PCR blank solution used as control

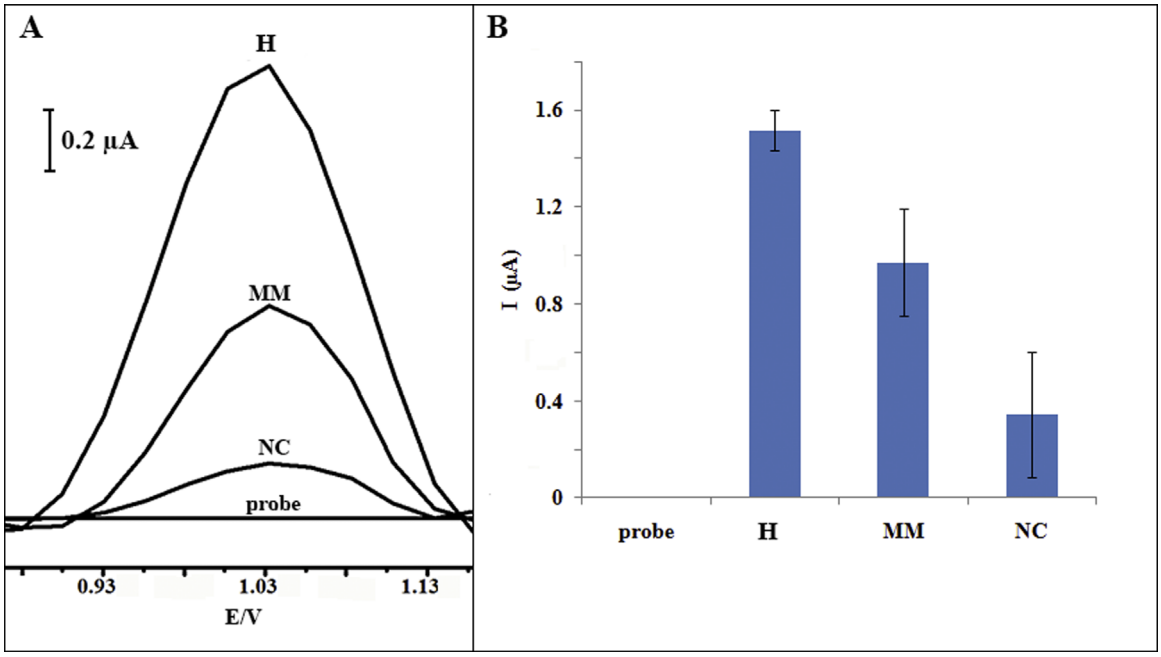

Fig. 4. (A) Differential pulse voltammograms and (B) Histogram presenting the oxidation signals of guanine measured in ABS by using 24 μg/ml capture probe modified PGE after hybridization with synthetic targets (40 μg/mL of full-match target, 40 μg/mL of MM, 40 μg/mL of NC) and only probe before the hybridization event (H). Hybridization time was 50 min for all targets and DPV measurements in ABS were performed by scanning between +0.2 V and 1.25 V at the pulse amplitude as 50 mV and the scan rate as 50 mV/s.

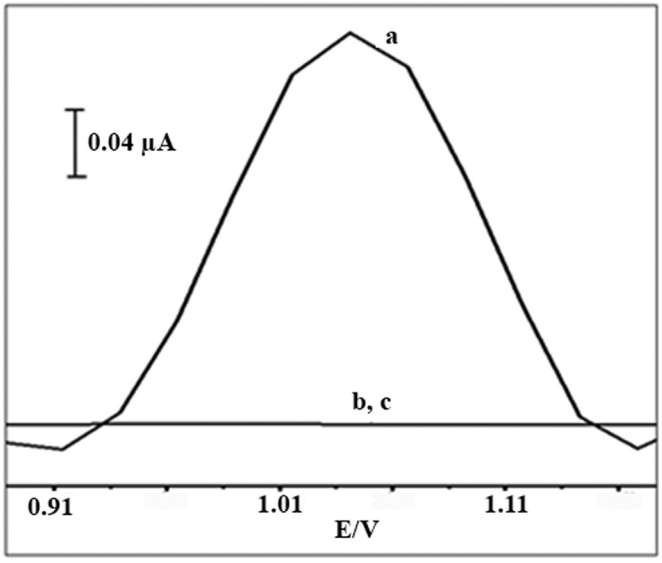

Fig. 5. Differential pulse voltammograms for the oxidation signals of guanine in ABS at 24 μg/mL capture probe modified PGE after hybridization with (a) thermally denaturated *T.gondii* PCR amplicon (hybridization time of 10 min), (b) PCR blank solution, (c) only probe without any target during hybridization. Other experimental conditions were as cited in Fig. 4.

sample during hybridization. It was observed that the hybridization time between capture probe and PCR amplicons was occured effectively in 10 min (not shown). The results obtained from hybridization experiments carried out PCR amplicons shown that hybridization time may not be same as the one obtained using synthetic short target oligonucleotides due to the higher steric hindrance of the amplified nucleic acid sequences resulting in a lower hybridization efficiency compare to densely packed DNA probes [50].

4. Conclusion

In our study, a novel label-free disposable electrochemical biosensor has been demonstrated successfully for the detection of *T. gondii* sequences in DNA samples amplified by PCR. This indicator-free biosensor was able to distinguish between full-matched (target), mismatched and non-complementary DNA sequences, with a detection limit of 1,78 μg/mL of target sequence. Using label-free detection protocol could eliminate the need for external indicators, such as carcinogenic antitumor drugs, metal complexes and organic dyes; thus, this method could offer important advantages, such as simple design, small dimensions, low cost, and low power requirements and provides more rapid detection. Future work in this laboratory will focus on the design of novel electrochemical microarray based protocols for detection of different target sequences from amplicons related to inherited diseases.

Acknowledgment

The authors greatly acknowledge the financial support from Cumhuriyet University (project no: EGT 022).

Appendix A. Supplementary material

Supplementary material associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2015.11.071.

References

[1] J. Wang, G. Rivas, C. Parrado, X. Cai, M.N. Flair, Talanta 44 (1997) 2003–2010.
[2] F. Jelen, A. Erdem, E. Palecek, Bioelectrochemistry 55 (2002) 165–167.

[3] M.L. Del Giallo, D.O. Ariksoysal, G. Marrazza, M. Macsini, M. Ozsoz, Anal. Lett. 38 (2005) 2509–2523.
[4] R. Miranda-Castro, N. Santos-Álvarez, M.J. Lobo-Castanon, A.J. Miranda-Ordieres, P. Tunon-Blanco, Biosens. Bioelectron. 24 (2009) 2390–2396.
[5] J. Wang, M. Musameh, Anal .Chem. 75 (2003) 2075–2079.
[6] S. Tombelli, M. Macsini, C. Sacco, A.P.F. Turner, Anal. Chim. Acta 418 (2000) 1–9.
[7] M. Mascini, I. Palchetti, G. Marrazza, Fresen. J. Anal. Chem. 369 (2001) 15–22.
[8] A.A. Gorodetsky, M.C. Buzzeo, J.K. Barton, Bioconjugate Chem. 19 (2008) 2285–2296.
[9] S. Schwonbeck, A.K. Griep, N.G. Eichelmann, E.E. Förster, W. Meinl, H. Glatt, F. F. Bier, Biosens. Bioelectron. 20 (2004) 956–966.
[10] J. Wang, D.K. Xu, A. Erdem, R. Polsky, M.A. Salazar, Talanta 56 (2002) 931–938.
[11] L. Xiaoteng, H. I-Ming, Analyst 134 (2009) 1957–1964.
[12] F. Berti, C. Eisenkolbl, D. Minocci, P. Nieri, A.M. Rossi, M. Mascini, G. Marrazza, J. Electroanal. Chem. 656 (2011) 55–60.
[13] S. Rauf, J.J. Gooding, K. Akhtar, M.A. Ghauri, M. Rahman, M.A. Anwar, A. M. Khalid, J. Pharm. Biomed. 37 (2005) 205–217.
[14] S. Furlanetto, M. Mascini, Bioanalysis 3 (2011) 269–270.
[15] M. Ravera, G. Bagni, M. Mascini, J.C. Dabrowiak, D. Osella, J. Inorg .Biochem. 101 (2007) 1023–1027.
[16] J. Labuda, M. Buckova, L. Heilerova, A. Caniova-Ziakova, E.J. Mattusch, R. Wennrich, Sensors 2 (2002) 1–10.
[17] M.T. Castaneda, S. Alegret, A. Merkoci, Electroanalysis 19 (2007) 743–753.
[18] A.M. Mohammed, R.A. Rahim, I.J. Ibraheem, F.K. Loong, H. Hisham, U. Hashim, Y. Al-Douri, J. Nanomater. 2014 (2014) 1–7.
[19] H. Li, Z.Y. Sun, W.Y. Zhong, N. Hao, D.K. Xu, H.Y. Chen, Anal. Chem. 82 (2010) 5477–5483.
[20] J. Wang, D.K. Xu, R. Polsky, J. Am. Chem. Soc. 124 (2002) 4208–4209.
[21] J. Wang, G. Rivas, J.R. Fernandes, J.L.L. Paz, M. Jiang, R. Waymire, Anal. Chim. Acta 375 (1998) 197–203.
[22] J. Wang, A.-N. Kawde, A. Erdem, M. Salazar, Analyst 126 (2001) 2020–2024.
[23] E. Palecek, M. Trefulka, M. Fojta, Electrochem. Commun. 11 (2009) 359–362.
[24] K. Kerman, Y. Morita, Y. Takamura, E. Tamiya, Electrochem. Commun. 5 (2003) 887–891.
[25] D.O. Ariksoysal, H. Karadeniz, A. Erdem, A. Sengonul, A.A. Sayiner, M. Ozsoz, Anal. Chem. 77 (2005) 4908–4917.
[26] M. Macsini, M. Del Carlo, M. Minunni, B. Chen, D. Compagnone, Bioelectrochemistry 67 (2005) 163–169.
[27] A. Erdem, M.I. Pividori, A. Lermo, A. Bonanni, M. Del Valle, S. Alegret, Sens. Actuators B Chem. 114 (2006) 591–598.
[28] M.H. Pournaghi-Azar, M.S. Hejazi, E. Alipoura, Electroanalysis 19 (2007) 466–472.
[29] N. Zari, A. Amine, M.M. Ennaji, Anal. Lett. 42 (2009) 519–535.
[30] A. Caliskan, A. Erdem, H. Karadeniz, Electroanalysis 21 (2009) 2116–2124.
[31] X. Zhu, S. Ai, Q. Chen, H. Yin, J. Xu, Electrochem. Commun. 11 (2009) 1543–1546.
[32] E. Souza, G. Nascimento, N. Santana, D. Ferreira, M. Lima, E. Natividae, D. Martins, J. Lima-Filho, Sensors 11 (2011) 5616–5629.
[33] S. Bollo, N.F. Ferreyra, G.A. Rivas, Electroanalysis 19 (2007) 833–840.
[34] R. Gazzinelli, Y. Xu, S. Hieny, A. Cheever, A. Sher, J. Immunol. 149 (1992) 175–180.
[35] R. Gazzinelli, E.Y. Denkers, A. Sher, Infect. Agents. Dis. 2 (1993) 139–149.
[36] F. Bertoli, M. Espino, J.R. Arosemena, J.L. Fishback, J.K. Frenkel, Arch. Pathol. Lab. Med. 119 (1995) 214–224.
[37] P. Hohlfeld, F. Daffos, J.-M. Costa, P. Thulliez, F. Forestier, M. Vidaud, N. Engl. J. Med. 331 (1994) 695–699.
[38] J.G. Montoya, O. Liesenfeld, Lancet 363 (2004) 1965–1976.
[39] K. Hedman, M. Lappalainen, I. Seppala, O. Makela, J. Infect. Dis. 159 (1989) 736–739.
[40] C.D. Jones, N. Okhravi, P. Adamson, S. Tasker, S. Lightman, Ophthalmol. 41 (2000) 634–644.
[41] P.Y.C. Lee, J. Mangan, R.E. Holliman, P.D. Butcher, J. Clin. Pathol. 52 (1999) 61–64.
[42] M. Pujol-Rique, F. Derouin, A.G. Quintanilla, M.E. Valls, J.M. Miro, M.T. Jimenez de Anta, J. Med. Microbiol. 48 (1999) 857–862.
[43] H. Wang, C. Lei, J. Li, Z. Wu, G. Shen, R. Yu, Biosens. Bioelectron. 19 (2004) 701–709.
[44] H. Wang, J. Li, Y. Ding, C. Lei, G. Shen, R. Yu, Anal. Chim. Acta 501 (2004) 37–43.
[45] J. Wang, Anal. Chim. Acta 469 (2002) 63–71.
[46] J.N. Miller, J.C. Miller, Statistics and Chemometrics for Analytical Chemistry, 5th ed., Pearson Education, Essex, UK (2005), p. 121–123.
[47] J. Wang, A.N. Kawde, M. Musameh, Analyst 128 (2003) 912–916.
[48] A. Erdem, M. Muti, F. Mese, E. Eksin, Colloid Surface B (114), 261– 268.
[49] F. Mese, G. Congur, A. Erdem, J. Electroanal. Chem. 719 (2014) 92–97.
[50] F. Lucarelli, G. Marrazza, I. Palchetti, S. Cesaretti, M. Mascini, Anal. Chim. Acta 469 (2002) 93–99.