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Direct and rapid electrochemical biosensing of the human interleukin-2 DNA in unpurified polymerase chain reaction (PCR)-amplified real samples

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ABSTRACT

Electrochemical detection of polymerase chain reaction (PCR)-amplified human interleukin-2 (IL-2) coding DNA sample (399 bp size) without any purification and pre-treatment is described. To achieve this goal, a sensor was made by immobilization of a 20-mer oligonucleotide (chIL-2) as the probe on the pencil graphite electrode (PGE). This probe is related to the antisense strand of human interleukin-2 gene. The results showed that the electrode could effectively sense the PCR product of human interleukin-2 DNA by anodic differential pulse voltammetry (ADPV) based on guanine oxidation signal. In order to inhibit PCR components interfering effects and improve biosensing performance, various factors were investigated. We found that the desorption of non-specifically adsorbed components of the unpurified PCR samples from PGE surface is easily achieved by washing of the electrode in washing solution for about 300 s. The effectiveness of this procedure was confirmed using purified PCR samples. The selectivity of the sensor was assessed with negative control PCR sample and seven different non-complementary PCR products corresponding to 16S rDNA (bigger than 1500 bp) of various bacterial genuses. Diagnostic performance of the biosensor is described and the detection limit is found to be 69 pM. The reliability of the electrochemical biosensing results was verified by electrophoresis of the PCR products.

1. Introduction

DNA diagnosis has enormous applications in various fields including molecular biology, clinical diagnostics, agriculture, forensic science, and for the detection of pathogens. Main problem in detection of DNA hybridization at physiological levels is that the amount of DNA to be detected is in the femtomolar or attomolar ranges, very lower than the detection limit of general analytical techniques. There are two alternatives to solve this problem: to amplify the sample or to amplify the signal. Obviously, it is difficult to detect DNA at attomolar level by only amplifying the signal. Thus the amplification of sample by the polymerase chain reaction (PCR) is commonly required for this purpose.

The PCR products are usually identified by gel electrophoresis and ethidium bromide staining (Vollenhofer et al., 1999; Permingeat et al., 2002). Capillary electrophoresis has also been used for faster and automatable separation and detection of PCR products (Garcia-Canas et al., 2002; Obeid et al., 2004). Electrophoretic methods, however, do not provide information about the sequence of the DNA fragments. As a result, non-specific amplification products of similar size may lead to erroneous interpretation. Additionally, in some diagnostic tests further procedures are required to be performed on PCR products so that without these complementary experiments diagnosis will not be achieved. Several authors have reported the coupling of biosensors with a DNA amplification method (e.g. PCR) to obtain reliable measurements of clinical interests. For this purpose, various transduction strategies have been reported. For instances, the mass change on the surface of the transducer can be detected with a piezoelectric device (quartz crystal microbalance) (Mannelli et al., 2003; Minunni et al., 2001; Willner et al., 2002; Patolsky et al., 2000). Alternatively, the surface plasmon resonance (SPR) biosensor detects the change of the refractive index in the vicinity of the sensor's surface as a result of hybrid formation (Mariotti et al., 2002; Wang et al., 2004; Feriotto et al., 2002; Giakoumaki et al., 2003; Liu et al., 2005). However, the electrochemical transducers have attracted more attention

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than other methods because they are highly sensitive, inexpensive, easy-to-use, portable and compatible with microfabrication technologies. DNA hybridization detection using electrochemical methods are divided into indirect and direct strategies. Incorporation of an electroactive label makes up the principle of indirect DNA hybridization detection methods. The basis of direct DNA hybridization detection strategy lies on direct transduction of signal induced from the oxidation of guanine or adenine moieties in DNA strands that is also called label-free detection method.

Label-free detection strategy seems to be simple, less time consuming and more applicable. Electrochemical label-free biosensing of DNA based on guanine moiety oxidation signal of probe (Yang et al., 2001) or target using inosine substituted probe (Wang et al., 2003) are reported. We have developed a label-free DNA hybridization biosensor using guanine moiety oxidation signal of both probe (non-inosine substituted) and target DNA (Pournaghi-Azar et al., 2007). The presence of only one guanine and seven cytosine bases in the utilized probe, allowed: (a) producing an electrochemically detectable signal for direct optimizing of the probe immobilization conditions and (b) obtaining a significant enhanced electrochemical signal following hybridization with target DNA possessing multiple guanine bases.

Electrochemical methods were used for monitoring the polymerase chain reaction (PCR)-amplified real samples of lysteria monocytogen, monitoring guanine signal after electrochemical enrichment on plastic composite electrodes (Schulein et al., 2002). The PCR samples were tested to detect factor V leiden mutation with the help of guanine oxidation signal on CPE (Ozkan et al., 2002). A label-free electrochemical assay based on guanine oxidation signal to measure telomerase activity is also reported (Eskiocak et al., 2007). A novel electrochemical DNA biosensor for detection of the common functional polymorphism of catechol-*O*-methyl transferase gene in PCR amplicons is described (Ozkan-Ariksoysal et al., 2008).

Some groups (Steichen et al., 2007; Suye et al., 2005; Kobayashi et al., 2004; Kara et al., 2003, 2004; Ozkan et al., 2002; Meric et al., 2002) have reported electrochemical biosensors for detection of DNA hybridization in PCR-amplified real samples to obtain reliable measurement of clinical interests. In order to improve the selectivity and performance of the electrochemical detection methods some works dealt with the purification of PCR products using the Clean-up DNA Purification kits (e.g. Microcon® PCR kits) or polyacrylamide gel by a classical electroelution procedure (Carpini et al., 2004; Djellouli et al., 2007; Berganza et al., 2007).

Following our published paper about interleukin-2 DNA (an immune system cytokine secreted by activated T cells and promotes immune response against bacteria and viruses) detection studies (Pournaghi-Azar et al., 2006, 2007), and its use in biosensing and discrimination of recombinant plasmid on the basis of interleukin-2 DNA insert (Hejazi et al., 2008), here we describe the electrochemical label-free detection of DNA hybridization event in PCR-amplified samples without any purification or pre-treatment of the samples. It is also intended to apply the method for detection of human interleukin-2 DNA and its discrimination from non-complementary unpurified PCR-amplified samples. To achieve this goal, anodic differential pulse voltammetry (ADPV) was used as the monitoring procedure.

2. Materials and methods

2.1. Materials

The pencil graphite was obtained as pencil lead; composed of natural graphite, a polymeric binder and clay; from Rotring Co. Ltd., Germany (R 505210 N) of type H. All leads had a diameter of 2.0 mm and were used as received. A 20-mer oligonucleotide called chIL-2 (5′-CTA AAT TTA GCA CTT CCT CC-3′) corresponding to antisense strand of human IL-2 encoding DNA was used as the probe. This oligonucleotide was supplied (as lyophilized powder) from MWG-Biotech.

The stock solution of the oligonucleotide (100 μg/ml) was prepared in TE buffer solution (10 mM Tris–HCl, 1 mM EDTA, pH 8.00) and kept frozen. More diluted solutions of the oligonucleotide were prepared using 0.50 M acetate buffer (pH 4.80) solution containing 20 mM NaCl.

2.2. Bacterial strains and plasmid

Lactobacillus rhamnosus strain LQ108, Idiomarina sp., Halomonas salina GSP2, Marinobacter sp., Salicola marensis 7Mb1, Halomonas taeanensis and Basillus halophila were used for amplification of 16S rRNA encoding gene of 16S rDNA. Plasmid pEThIL-2 (Hejazi et al., 2008); a prokaryotic expression vector; was used as the template DNA for amplification of human IL-2 encoding DNA.

2.3. Media and enzyme

MH medium (60.7 g NaCl, 15 g $MgCl_2{\cdot}6H_2O$, 7.4 g $MgSO_4{\cdot}7H_2O$, 0.27 g $CaCl_2$, 1.5 g KCl, 45 mg $NaHCO_3$, 19 mg NaBr, 5 g proteose peptone No.3, 10 g yeast extract, 1 g glucose in 1000 ml distilled water; pH 7.2) was used to grow *Halomonas salina GSP2* and *Marinobacter* sp. cells. Halomonas medium (80 g NaCl, 20 g $MgSO_4{\cdot}7H_2O$, 7.5 g casamino acids (with vitamins), 5 g proteose peptone No. 3, 1 g yeast extract, 3 g Na_3-citrate, 0.5 g K_2HPO_4, 0.05 g $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$ in 1000 ml distilled water; pH 7.5) was used as culture medium for *Idiomarina* sp., *Salicola marensis 7Mb1*, *Halomonas taeanensis* and *Basillus halophila*. *Lactobacillus rhamnosus* strain LQ108 cells were grown in MRS medium (10 g peptone, 8 g beef extract, 4 g yeast extract, 20 g glucose, 2 g triammonium citrate, 5 g sodium acetate, 0.2 g magnesium sulphate, 0.05 g manganese sulphate, 2 g Di-potassium phosphate in 1000 ml distilled water; pH 6.2 ± 0.2 at 25 °C). *Taq* DNA polymerase and PCR reagents were purchased from Fermentas company.

2.4. Genomic DNA extraction

The protocol described by Corbin and co-workers (1994) was used for genomic DNA extraction with some modifications. Briefly, a single colony was cultured in 50 ml liquid MRS medium over night in shaker incubator at 25 °C. Following separation of bacterial cells from the medium by centrifugation for 3 min at 5000 rpm, the bacterial cells were pulverized in liquid nitrogen, suspended in a solution I (10 mM Tris (pH 7.4), 1 mM EDTA, 0.5% sodium dodecyl sulphate (SDS), 0.1 mg/ml of proteinase K) and lysed by incubation at 37 °C for 1 h. Then the solution II containing 0.8 M NaCl and 1% CTAB was added to the lysates, and incubated at 65 °C for 20 min and extracted with equal volume of chloroform-isoamylalcohol (24:1). DNA was precipitated from the aqueous phase with 0.6 volume of isopropanol and finally purified using ethanol 70%.

2.5. PCR amplification of 16S rRNA and IL-2 encoding DNA fragments

Plasmid pEThIL-2 was used as the template DNA for IL-2 DNA amplification using polymerase chain reaction (PCR) technique with Taq DNA polymerase in the presence of forward (5′-GCA CCT ACT TCA AGT TCT ACA-3′) and reverse (5′-TTA AGT CAG TGT TGA GAT GAT G-3′) primers. PCR amplifications were carried out on a

gradient master cycler (eppendorf). PCR reactions contained about 100 ng template DNA, 0.1 μM of each primer, 1 mM of deoxynucleoside triphosphates (dNTPs), Taq DNA polymerase buffer with final concentration of 1×, and 1 Taq DNA polymerase in total volume of 50 μl. Program was composed of a first step of denaturation at 95 °C for 5 min, 35 amplification cycles and final extension at 72 °C for 10 min. Each of the amplification cycles consisted of 40 s of DNA denaturation at 94 °C, 40 s of primer annealing at 58 °C, and 40 s of extension at 72 °C. The product of IL-2 PCR amplification was expected to be a 399 bp DNA fragment.

16S rDNA amplification of *Halomonas salina GSP2*, *Marinobacter* sp.; *Idiomarina* sp., *Salicola marensis 7Mb1*, *Halomonas taeanensis* and *Basillus halophila* cells were conducted directly on bacterial cells grown on the media, without genomic DNA extraction step. 16S rDNA amplification of *Lactobacillus rhamnosus* was conducted on the extracted genomic DNA. The PCR reaction mixtures (50 μl) contained 50 pmol each of forward (16F27: 5′-AGA GTT TGA TC(AC) TGG CTC AG-3′) (Karlson et al., 1993) and reverse (16SR: 5′-TAC CTT GTT AGG ACT TCA CC-3′) (designed in this study) primers, four dNTPs with 0.2 mM of each, 2.0 mM $MgCl_2$, 0.5 ng/μl *Lactobacillus rhamnosus* genomic DNA as the template DNA and 1.5 U Taq DNA polymerase.

Amplification of 16S rDNA was carried out for 35 cycles, each cycle consisting of DNA denaturation at 95 °C for 1 min, annealing at 55 °C for 50 s, extension at 72 °C for 85 s. The cycles were preceded by an initial denaturation at 95 °C for 5 min and followed by a final extension at 72 °C for 10 min.

The PCR products were analyzed using electrophoresis on 1% agarose gel, marked using 1 kb DNA ladder as the size marker, stained with ethidium bromide and imaged at UV exposure. All DNA manipulation procedures were conducted based on common molecular biology protocols (Sambrook et al., 2001).

Following PCR amplification, the PCR samples were diluted using 10 mM Tris–HCl buffer solutions (pH 7.0) containing 20 mM NaCl (hybridization buffer). As the PCR products are very sensitive to freeze–thaw, the samples were aliquot to small volumes and stored at −20 °C. Other chemicals were of analytical reagent grade. The distilled, deionized and sterilized water was used in all solution preparation. Each measurement consisted of immobilization, hybridization and detection cycles carried out on a fresh PGE surface. All the experiments were performed at room temperature in an electrochemical cell.

2.6. Electrochemical measurements

Electrochemical experiments were performed using AUTOLAB PGSTAT 30 electrochemical analysis system and GPES 4.9 software package (Eco Chemie, The Netherlands). The utilized three-electrode system was composed of a PGE (surface area of 0.037 cm^2) as the working electrode, a saturated calomel electrode (SCE) as the reference electrode and a platinum wire as the auxiliary electrode.

2.6.1. Preparation and electrochemical pre-treatment of the pencil graphite electrode (PGE)

The body of pencil lead was tightly coated with Teflon band and electrical contact with the lead was made via a copper wire connected to the metallic holder of the working electrode. The pencil lead was fixed vertically and immersed in the solution to which the lead was connected only via cross-section of the electrode. The surface was polished on a weighing paper to a smoothed finish before each use. The electrochemical pre-treatment of the surface of polished PGEs was carried out at optimized potential of 1.80 V for 10 min in 0.50 M acetate buffer solution (pH 4.80) containing 20 mM NaCl without stirring.

2.6.2. Probe immobilization on the PGE

Immobilization of the probe was conducted following electrochemical pre-treatment of the PGE. To achieve this, the electrode was exposed to 0.50 V for 5 min in 0.50 M acetate buffer solution (pH 4.80) containing 1 μM chIL-2 and 20 mM NaCl with 200 rpm stirring. The electrode was then rinsed with 0.50 M acetate buffer solution (pH 4.80) containing 20 mM NaCl.

2.6.3. DNA Hybridization

IL-2 PCR product was diluted to 10 ng/μl and denatured by heating in a water bath at 95 °C for 5 min and immediately immersed in an ice bath for 2 min. After immobilization of the probe, the PGE was inverted upside down and about 2.5 μl of the diluted PCR sample solution was pipetted directly onto the electrode surface. The hybridization was allowed to proceed for 10 min at room temperature, then the electrode was rinsed for 300 s with 20 mM Tris–HCl buffer solution (pH 7.0) containing 20 mM NaCl (washing solution) with 200 rpm stirring. The same protocol was applied for the interaction of the probe with dNTPs, blank and non-complementary PCR samples.

2.6.4. Voltammetric measurements

The electrochemical behavior of the electrode surface was monitored using anodic differential pulse voltammetry (ADPV) in 20 mM Tris–HCl buffer (pH 7.00) solution and scanning of the electrode potential was conducted between 0.70 and 1.10 V at a pulse amplitude of 25 mV. The raw data were treated by means of the Savitzky and Golay filter (level 2) of the GPES software, followed by the GPES software moving average baseline correction utilizing a 'peak width' of 0.01. Measurements were carried out in triplicate following renewing the PGE surface by cutting and polishing of the electrode.

3. Results and discussion

3.1. Preliminary investigation

For primary investigation, an aliquot of the PCR sample was simply diluted in the hybridization buffer solution and then hybridization reaction conducted as described in Section 2. Fig. 1 shows the ADP voltammograms of the probe-modified PGE (pretreated electrochemically for 300 s) before (curve a) and after (curve b) hybridization with IL-2 PCR product as the target DNA. As seen in Fig. 1, a significant increase in the guanine signal was observed following hybridization of the target DNA with the probe (curve b). The observed increase in voltammetric signal represents the extent of the hybridization events on the electrode. However, the interaction between PCR sample and immobilized probe gives rise to appearance of an undesired oxidation signal near to the guanine oxidation peak. This signal may be attributed to non-specific adsorption of the unpurified PCR sample components such as primers, DNA polymerase and dNTPs on some free sites on the PGE. Lucarelli et al. (2002) reported that raw PCR samples have significant fouling effect on the graphite electrodes and thus purification of PCR samples with specific kits is a necessary step before hybridization experiments. In order to confirm the effect of PCR sample purification on improvement of the voltammetric signal, we conducted a parallel sensing experiment using a PCR sample purified by PCR Clean-up DNA purification kit (QIAGEN). As seen in Fig. 1 (curve c), the undesired voltammetric signal was effectively removed in purified complementary PCR sample. However, the purification of PCR samples is an additional and time-consuming step in electrochemically biosensing process. In order to overcome the fouling problem in unpurified PCR samples as well as to improve

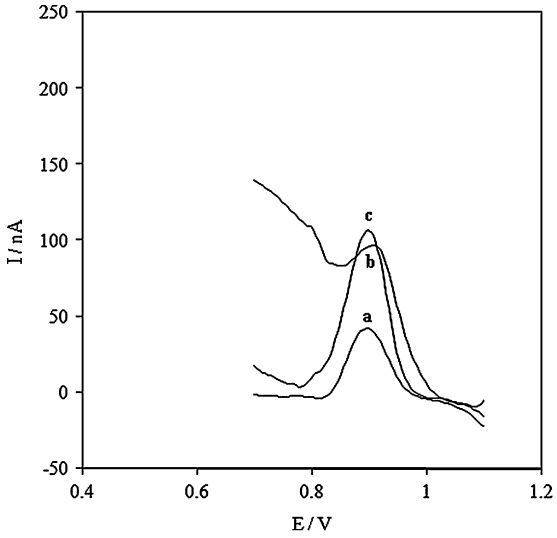

Fig. 1. ADPVs of chIL-2-modified PGE before hybridization (a) and after hybridization with: complementary unpurified PCR sample (b) and complementary purified PCR sample (c). Pre-treatment time of the electrode: 300 s, drop volume: 2.5 μl, and hybridization time: 20 min. Other experimental conditions were as described in Section 2.

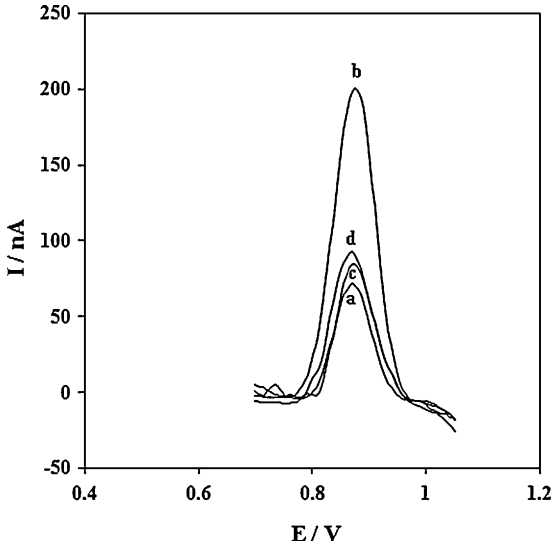

Fig. 2. ADPVs of chIL-2-modified PGE before hybridization (a) and after hybridization with: complementary unpurified PCR sample (b); after interaction with dNTPs solution (c); after interaction with blank PCR solution (d). Pre-treatment time of the electrode: 600 s, concentration of dNTPs solution: 0.2 mM. Other experimental conditions were as cited in Fig. 1.

the response of the sensor, further optimizations of some experimental conditions are required.

3.2. Effect of electrochemically pre-treatment time of PGE

We investigated the effect of electrochemical pre-treatment time of the electrode in order to find an appropriate time which not only is suitable for effective immobilization of the probe on the electrode surface, but also produces such an electrode surface that decreases its direct interaction with constituents of PCR sample. In the previous work we had found that the pre-treatment of the electrode at 1.8 V for 5–10 min provides an optimum condition in which the biosensor performs properly (Pournaghi-Azar et al., 2007). Accordingly, 600 s was chosen as proper time for PGE pre-treatment in this study. Fig. 2 shows the ADP voltammograms of the probe-modified pre-treated PGE for 600 s at 1.8 V before (curve a), after (curve b) hybridization with the complementary unpurified PCR sample, after interaction with dNTPs solution (curve c) and after interaction with unpurified blank PCR solution (curve d). As seen in Fig. 2 curve b, with longer pre-treatment time (i.e., 600 s) the guanine oxidation signal is significantly increased desirably for all cases and signal related to the impurities of the PCR sample disappeared (see Fig. 2, curve b). Indeed, the pre-treatment of the electrode for 600 s yields more positively charged sites on the electrode surface. This favors more strongly the adsorption of the negatively charged probe, and consequently prevents the further accumulation of PCR sample impurities on pre-occupied sites. These observations indicate that the purification of PCR sample is not necessary in the present conditions. On the other hand as illustrated in Figs. 1 and 2, ΔI_p (difference between peak currents of a and b curves) remarkably increased (from 70.2 ± 4.4 nA for 300 s pre-treatment time to 124.76 ± 4.0 nA for 600 s pre-treatment time) which is considered as an improvement in sensing of target sample. Whereas, as seen in Fig. 2, the interaction between immobilized probe and dNTPs (curve c) or unpurified blank PCR sample (curve d) did not lead to great ΔI_p (difference between peak currents of a and c or d curves) values (8.80 ± 2.5 nA and 9.0 ± 5.53 nA, respectively). Therefore, 600 s was suggested as optimum time for the electrochemical pre-treatment of the PGE in following studies where unpurified PCR samples were used.

3.3. Effect of hybridization time

In order to study the effect of hybridization time on the hybridization efficiency upon immobilization of the probe, the hybridization was conducted for various durations from 1 up to 30 min at room temperature as described in Section 2. Fig. 3A shows the variations of the I_c/I_b (where I_c is guanine oxidation signal of immobilized probe after hybridization with complementary PCR sample and I_b is guanine oxidation signal of the probe after interaction with blank PCR solution) versus hybridization times. As seen in Fig. 3A, the value of I_c/I_b enhanced as the hybridization time prolonged up to 10 min. This enhancement could be attributed to the more accumulation of target on the electrode. After 10 min, the oxidation signal started to decrease slightly as the hybridization time increased. This may be due to the fouling of the electrode surface by some constituents of the PCR sample.

3.4. Effect of imposed PCR sample drop volume on the hybridization efficiency

Having optimized the hybridization time, the effect of the imposed PCR sample drop volume on the hybridization efficiency was investigated. Fig. 3B shows the variations of the I_c/I_b (where I_c is guanine oxidation signal of immobilized probe after hybridization with complementary PCR sample and I_b is guanine oxidation signal of the probe after interaction with blank PCR solution) versus PCR drop volumes. As seen in Fig. 3B, the I_c/I_b ratio increased as the volume of the PCR drop increased up to 2.5 μl. This enhancement may be attributed to more coverage of electrode surface with the drop which increases the amount of the samples hybridizing with the probe. Note that, the drop volumes less than 2.5 μl could not cover electrode surface completely. After 2.5 μl, the oxidation signal started diminishing in spite of increasing the drop volume. Thus the drop volumes greater than 2.5 μl were inconvenient for achieving the hybridization and stabilization of the double strand DNA on the electrode surface in 10 min. On the basis of the results

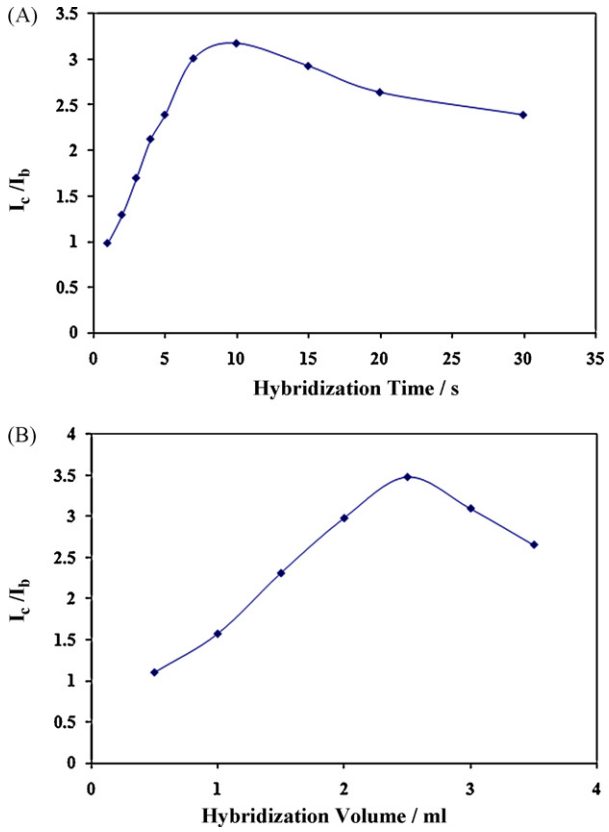

Fig. 3. (A) Variations of the ratio I_c (guanine oxidation signal of immobilized probe after hybridization with complementary PCR sample) to I_b (guanine oxidation signal of the probe after interaction with blank PCR solution) versus hybridization times. (B) Variations of the ratio I_c (guanine oxidation signal of immobilized probe after hybridization with complementary PCR sample) to I_b (guanine oxidation signal of the probe after interaction with blank PCR solution) versus PCR drop volumes. Hybridization time: 10 min. Other experimental conditions: as cited in Fig. 2.

obtained, 2.5 μl was chosen as the optimum drop volume for the subsequent experiments.

3.5. Selectivity study

Some hybridization experiments with non-complementary PCR samples were carried out to assess the selectivity of the suggested DNA sensor. For this purpose, seven different non-complementary PCR samples corresponding to 16S rDNA of bacterial samples (*Lactobacillus rhamnosus* strain LQ108, *Idiomarina* sp., H*alomonas salina GSP2*, *Marinobacter* sp., *Salicola marensis 7Mb1*, *Halomonas taeanensis* and *Basillus halophila*) were subjected. The size of the PCR products was more than 1500 bp which is about fourfold of the IL-2 PCR product size. Fig. 4 shows typical ADP voltammograms for probe-modified PGE before hybridization (curve a) and after (curve b) hybridization with complementary PCR sample and after interaction with negative real samples (curves c–i) at optimum conditions. As illustrated in Fig. 4, a significant increase in the guanine oxidation signal observed following hybridization of the complementary PCR sample with the probe (from 81.21 ± 4.47 nA to 274.4 ± 10.27 nA). Whereas, the interaction between negative real samples and immobilized probe did not lead to a significant increase in the guanine oxidation signal (from 81.21 ± 4.47 nA to 84.10 ± 5.50 nA). This effect is attributed to the lack of hybridization event and easy removal of the non-specifically bound negative real samples during the washing process. The PCR blank solution was also used as the negative control to examine the effect of the PCR

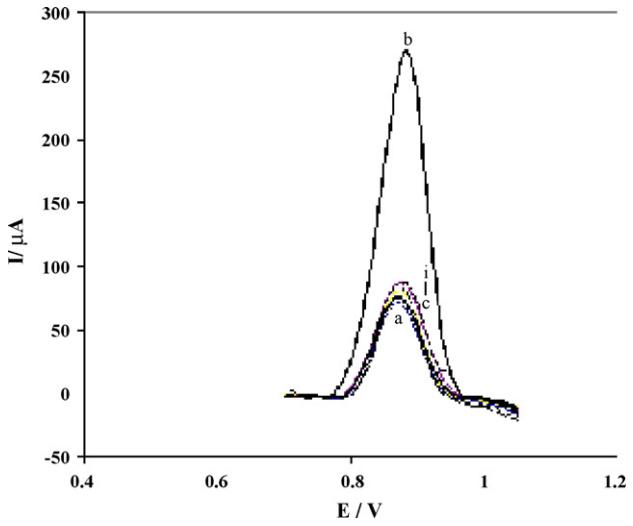

Fig. 4. ADPVs of probe-modified PGE before hybridization (a) and after hybridization with complementary PCR sample (b) and after interaction with negative PCR samples: *Lactobacillus rhamnosus* strain LQ108 (c), *Idiomarina* sp. *yjo238* (d), *Halomonas salina GSP2* (e), *Marinobacter* sp. *yoj35* (f), *Salicola marensis 7Mb1* (g), *Halomonas taeanensis* (h) and *Basillus halophila* (i) at optimum conditions. All PCR sample concentrations were 10 ng/μl.

components such as primers and dNTPs on the oxidation signal. The signal observed for the PCR blank solution on the probe-modified PGE showed a guanine oxidation signal (86.31 ± 4.72 nA) slightly greater than that of the probe-modified PGE (81.21 ± 4.47 nA). These observations indicate that in the established experimental conditions, only complementary PCR sample could form a stable double strand DNA on the probe-modified PGE and consequently the selectivity of the biosensor is approved.

3.6. Diagnostic performance of the sensor

The difference between the oxidation signal of the probe-modified PGE in the presence and absence of the complementary PCR sample (ΔI) is increased with increasing the complementary PCR concentration and leveled off at ca. 20 nM (Fig. 5). Thus, at this concentration of target, the maximum capacity of the probe avail-

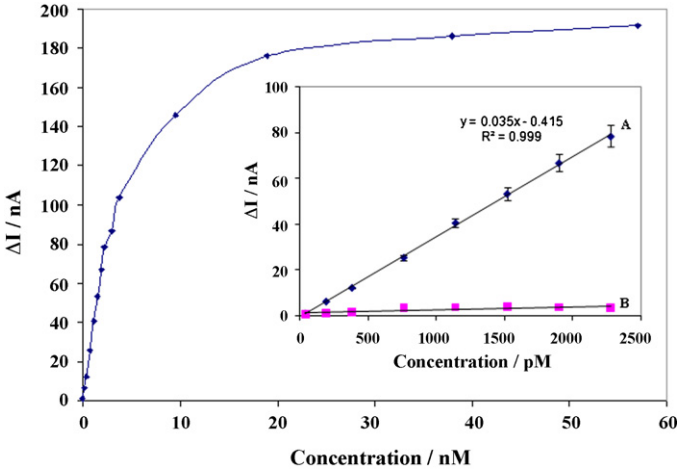

Fig. 5. Plot of ΔI (difference between the guanine oxidation signals of probe-modified PGE in the presence and in the absence of complementary PCR sample) versus concentration of complementary PCR samples. Inset: related calibration graph at concentration range 100–2300 pM for complementary PCR sample (A) and for non-complementary PCR sample (*Salicola marensis 7Mb1*) (B).

able on the electrode surface is involved in hybridization event. As seen in inset A of Fig. 5, the calibration graph is linear between 100 pM and 2300 pM with correlation coefficient of 0.999 for complementary PCR sample. The relative standard deviation of three independently probe-modified electrodes measured at 1500 pM of complementary PCR sample was 5.3% indicating a remarkable reproducibility of the detection method. The detection limit of the method, estimated as three times of the ratio between the blank signal and the sensitivity, that was about 69 pM. Such detection limit is comparable with those reported for other label free (guanine-based) electrochemical hybridization detections (e.g. Lucarelli et al., 2002; Xu et al., 2001). The similar experiments were carried out in the presence of non-complementary PCR sample (*salicola marensis 7Mb1*). As seen in inset B of Fig. 5, the increase in oxidation signal of probe-modified PGE after interaction with non-complementary PCR sample was negligible. These findings clearly confirm that the present DNA biosensor successfully distinguishes the complementary PCR product and discriminates it from non-complementary PCR samples.

3.7. Stability of the sensor

The effect of various factors on the stability and response of the sensor, including the exposing time of the electrode to air and supporting electrolyte, were studied. The obtained results showed that after 24 h storage the stability and current response of the probe-modified electrode before and after hybridization or interaction with PCR samples did not vary. Furthermore, exposing of the modified electrode to electrolyte solution (20 mM Tris-HCl buffer pH 7.00) before and after hybridization, for relatively long times (at least 6 h) did not significantly alter the performance of sensor (results are not shown).

3.8. Gel electrophoresis of the PCR samples

Gel electrophoresis of the PCR samples was performed in comparison with 1 kb DNA ladder as the DNA size marker, in order to evaluate the presence of the amplificants in PCR samples and reliability of the electrochemical detection results. Electrophoresis of the samples showed a DNA band with 399 bp size relating to IL-2 DNA indicating the presence of IL-2 DNA amplificant in the PCR sample (Fig. 6A). The presence of a DNA band in each bacterial PCR amplificants revealed the amplification and presence of 16S rDNA fragments in corresponding PCR samples (Fig. 6B). Accordingly, the results obtained from the gel electrophoresis confirmed the reliability of the present biosensing strategy to detect and discriminate the complementary target in unpurified PCR real sample.

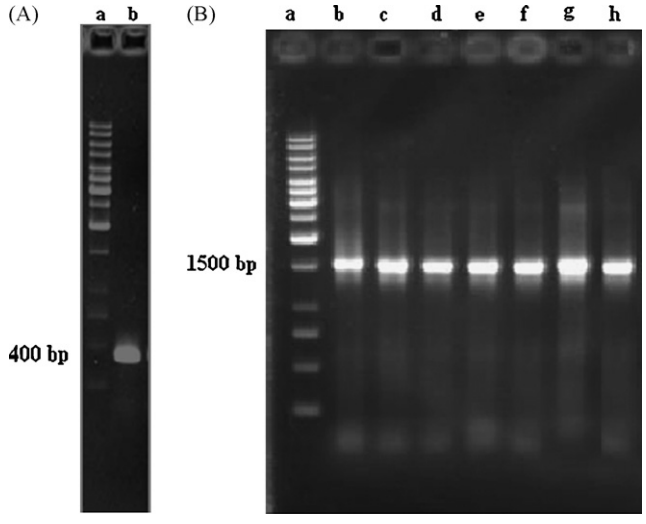

Fig. 6. Gel electrophoresis of PCR-amplified real samples related to the—(A) hIL-2 DNA: 1 kb DNA ladder as the DNA size marker (a) and hIL-2 DNA (b); (B) seven different non-complementary PCR samples corresponding to 16S rDNA: 1 kb DNA ladder as the DNA size marker (a), *Lactobacillus rhamnosus* strain LQ108 (b), *Idiomarina* sp. *yjo238* (c), *Halomonas salina GSP2* (d), *Marinobacter* sp. *yoj35* (e), *Salicola marensis 7Mb1* (f), *Halomonas taeanensis* (g) and *Basillus halophila* (h).

4. Conclusion

Direct electrochemical sensing of the human IL-2 DNA in PCR-amplified real samples without any purification can be achieved via a label-free DNA hybridization biosensor using corresponding probe (chIL-2) on the pencil graphite electrode. Optimization of the experimental conditions such as electrode pre-treatment, hybridization times and PCR sample drop volume imposed on the electrode surface, resulted in enforcement and improvement of the hybridization events and consequently improved diagnostic performance. Moreover, desorption of the "non-specifically adsorbed PCR components" as well as the adsorbed "non-complementary DNA fragments" from the pre-treated electrode surface is easily achieved by washing of the electrode. Accordingly, the sensitive detection and selective discrimination of the target IL-2 DNA in unpurified PCR sample can be directly achieved.

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