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An Electrochemical Biosensor for the Sensitive Detection of Hepatitis C Virus in Unpurified Polymerase Chain Reaction Amplified Real Samples based on Peptide Nucleic Acid and Double-stranded DNA Hybridization

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Abstract- Several studies show that acute infections with hepatitis C virus (HCV) frequently progresses to chronic diseases, eventually can lead to liver cirrhosis and hepatocellular carcinoma. Thus, development of simple and reliable HCV detection methods is in demand. The present paper describes electrochemical detection of polymerase chain reaction (PCR)-amplified Core/E1 encoding cDNA corresponding to hepatitis C virus (573 bp size) directly in double stranded form without any purification, pre-treatment and the need for denaturation of the target. The biosensor relies on the hybridization between self-assembled cysteine conjugated 20-mer peptide nucleic acid (PNA) oligomer probe and complementary ds-PCR products to form PNA-ds-PCR hybrid. The extent of hybridization between the probe and target sequences was determined by using differential pulse voltammetric signal of methylene blue (MB) as the hybridization indicator. In order to improve biosensing performance, the effect of various factors was investigated. The selectivity of the sensor was assessed with two different non-complementary PCR products (ds-$PCR_{non\text{-}COM}$). Diagnostic performance of the biosensor is described and the detection limit is found to be 1.58 ppm. The reliability of the electrochemical biosensing results was verified by electrophoresis of the PCR products.

Keywords- DNA biosensor, Electrochemical methods, Hepatitis C virus, Polymerase chain reaction, peptide nucleic acid

1. INTRODUCTION

Specific DNA sequence detection techniques have become a significant set of tools for many important applications. They have broad applications in various fields including molecular biology, clinical diagnostics, agricultural researches, forensic identifications, and for the detection of pathogens.

However, main problem in detection of DNA hybridization at physiological levels is that the amount of DNA in the test samples is usually very lower than the detection limit of general analytical techniques. Hence, nucleic-acid amplification by the polymerase chain reaction (PCR) is an essential step to increase the amount of the target sequence.

The PCR products are usually identified by gel electrophoresis using primers that are sequence specific for the chosen region of DNA and ethidium bromide as staining agent [1,2]. Although it is simple and effective for the detection of PCR products, however, don't provide information about the sequence of the amplified target DNA and ethidium bromide is a carcinogenic chemical. Therefore, analysis of PCR products (or genomic DNA) is in the main focus of nucleic acid-based biosensor researches. In these biosensors various transduction strategies have been reported which are based on the hybridization of PCR amplicons to surface-attached DNA probe (or DNA analogs) and identifying the binding reaction.

Electrochemical transducers have received considerable interest in developing DNA biosensors because they are highly sensitive, inexpensive, easy-to-use, portable and compatible with microfabrication technologies [3]. In these devices hybridization can be detected directly, or by electroactive indicators. The specificity of DNA hybridization devices depends primarily on the selected probe and secondarily upon the hybridization condition. Peptide nucleic acid (PNA) is a nucleic acid analog in which the negatively charged sugar phosphate backbone is replaced with a polyamide backbone consisting of N-(2-aminoethyl) glycine units. PNA hybridizes with complementary nucleic acids with remarkable high affinity and specificity compared with natural nucleic acids [4-6]. PNA-based hybridization (duplex formation) biosensors have been constructed based on the various detection methods [7-16]. Alternatively, PNA can bind sequence specifically to double-stranded DNA (ds-DNA) in analogy to triplex-forming oligonucleotides with Watson–Crick base pairing and Hoogsteen hydrogen binding [4-6]. Some groups have reported biosensors for detection of DNA sequences on ds-oligonucleotide chains, but a few of these publications are performed for detection of DNA hybridization in PCR-amplified real sample [17-27]. In order to improve the selectivity and performance of the electrochemical detection method this work dealt with the purification of PCR products using the Clean-up QIA quick purification kit (QIAGEN, Germany) before detection [27]. PCR product is in the double stranded DNA form and direct detection of this DNA without any purification or pre-treatment, eventually

eliminates the need for denaturation step, leads to reduction in the assay time and contamination risk.

Hepatitis C virus (HCV), a small enveloped single-stranded RNA virus that belongs to the Flaviridae family is considered to be responsible at present for the majority of post-transfusion non-A/non-B hepatitis cases [28]. HCV genome contains some 10,000 of nucleotides that becomes transmitted through blood and blood products as clotting factors; the infection by HCV is associated with organ transplantation, blood transfusions, renal dialysis and intravenous drug abuse. One of the most important features of HCV genome is its high degree of genetic variability. Core/E1 region of HCV genome is one of the most conserved regions of the HCV genome that has no sequence diversity among various genotypes. The conserved nature of this region signifies its functional importance in the viral life cycle and consequently indicates significance of its detection and discrimination for diagnosing or confirming active infections.

Here, we report for the first time, the sequence-specific hybridization of self-assembled PNA probe and double stranded PCR-amplified samples (ds-PCR_{COM}) without any purification or pre-treatment of samples. It is also intended to apply the method for detection of Core/E1 encoding cDNA corresponding to hepatitis C virus 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 and the effect of some experimental parameters on the performance of the developed biosensor was described.

2. EXPERIMENTAL

2.1. Chemicals

A cysteine conjugated 20-mer PNA oligomer (Cys-O-O-ATG TAC CCC ATG AGG TCG GC) was purchased from PANAGENE and used as the probe. This sequence corresponds to a consensus sequence at core/E1 region of HCV genome and consequently is used as a universal segment for detection of all HCV genotypes. Accordingly, this segment is used to investigate the presence of HCV in the PCR products.

The PNA probe was dissolved in 0.1% trifluoroacetic acid and kept frozen at -20 °C. More diluted solutions of the PNA probe were prepared using 0.01 M Tris buffer solution (pH 7).

DNA oligonucleotides were supplied by Eurofins MWG Operon. Taq DNA polymerase and deoxynucleotide triphosphates (dNTPs) were purchased from Fermentas, Lithuania. Other chemicals were of analytical reagent grade from Merck. Distilled, deionized and sterilized water was used in all solution preparation. All DNA solutions were kept frozen at -20 °C and all the experiments were performed at room temperature in an electrochemical cell.

2.2. PCR amplification of complementary fragments

PCR was carried out to amplify the HCV core gene fragment. The PCR reaction mixture contained 0.1 µg of synthesized cDNA, 0.1 mM dNTPs, 1.5 mM $MgCl_2$, 20 pmol of each of the forward and reverse primers (Hcor F 5`- GAG CTC ATA TG A GCA CGA ATC CTA AAC -3` and Hcor R 5`- GGA TCC GGC TGA CGC GGG CAC AGT C- 3`), and 1.25 units of Taq DNA polymerase in 50 µL of final volume. PCR reaction was carried out within 30 cycles; denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, and elongation at 72 °C for 40 seconds [29]. The PCR product was submitted to electrophoresis using 1.5% agarose gel, stained by ethidium bromide, and visualized under a UV transilluminator.

2.3. Preparation of the working electrode and probe immobilization

Gold disk electrode (7 mm^2 area) was polished with wet alumina slurry for at least 10 min and rinsed repeatedly with water. For complete removal of self-assembled monolayers on AuE the polished electrode was dipped in 1:1 EtOH–H_2O solution containing 0.5 M $NaBH_4$ for 10 min [30]. The electrode subsequently electrochemically cleaned in 0.05 M H_2SO_4 solution by potential scanning between -0.3 and 1.5 V until a reproducible cyclic voltammogram was obtained, indicating that the electrode surface was clean. The electrode was then rinsed with copious amount of water, and placed upside-down in a disposable glass tube as a humidity chamber. A 6 µL droplet of the 5 µM PNA probe was deposited onto the pre-treated gold electrode for self-assembly. This probe self-assembled AuE was rinsed with water and the unmodified regions of the electrode were then blocked with 1 mM 6-mercapto-1-hexanol (MCH) solution for 30 min. The post-treatment with MCH displaces non-specifically adsorbed PNA molecules and fills pinholes within the PNA monolayer. Evaluation of the PNA self-assembled monolayer (SAM) formation was performed using the electrochemical signal of Fe $(CN)_6^{3-}$/ Fe $(CN)_6^{4-}$ redox system as described in previous work.

2.4. DNA hybridization and labeling of the hybrid

PCR products were used for hybridization with and without denaturation. For PNA/ds-PCR hybridization PCR product was diluted to desired concentration and a 6 µl droplet of target ds-PCR in phosphate buffer was deposited onto the electrode in the humidity chamber for 3 hours, then the electrode was rinsed in Tris buffer by temperature 55 ºC for 5 minute as optimized in previous work. The same protocol was applied for the interaction of the probe with non-complementary ds-PCR products.

For PNA/ss-PCR hybridization, PCR product was diluted to desired concentration and denatured by heating in a water bath at 95 ºC for 5 min and immediately immersed in an ice bath for 2 min. Then, a 6 µl droplet of denatured PCR sample solution was pipetted directly

onto the electrode surface. The hybridization was allowed to proceed as same as ds-PCR product hybridization. The same protocol was applied for the interaction of the probe with non-complementary ss-PCR samples.

MB was interacted with the probe or the hybrid, by dipping the modified electrode surface in a stirring 20 mM Tris–HCl buffer (pH 7.0) solution, containing 20 µM MB with 20 mM NaCl, for 5 min without applying any potential. After accumulation of MB, the electrode was rinsed with 20 mM Tris–HCl buffer (pH 7.0) solution for 10 s.

2.5. Electrochemical measurements

The reduction signal of the accumulated MB was measured by using differential pulse voltammetry (DPV) in 20 mM Tris–HCl buffer (pH 7.0) solution containing 20 mM NaCl and scanning the electrode potential between -0.10 and -0.50 V at a pulse amplitude of 25 mV. The raw data were treated utilizing Savitzky and Golay filter (level 2) of the GPES software, followed by the GPES software moving average baseline correction using a 'peak width' of 0.01 between non-hybridized and hybridized status.

2.6. Instrumentation

All electrochemical experiments were done on an AUTOLAB PGSTAT 30 electrochemical analysis system and GPES 4.7 software package (Eco Chemie the Netherlands) with a conventional three electrode test cell. The utilized three-electrode system was consisted of a polycrystalline gold working electrode (Bioanalytical Systems, UK) with a 3 mm diameter, a saturated calomel electrode (SCE) as the reference electrode and a platinum wire as the auxiliary electrode. Supporting electrolyte was deoxygenated by purging nitrogen gas for 10 min prior to measurements, and the cell was blanketed with nitrogen gas for duration of the measurements.

3. RESULTS AND DISCUSSION

3.1. Preliminary investigation

The present study aimed to detect target DNA sequences directly in ds-PCR products. The detection principle was based on the hybridization between the PNA and the target double stranded PCR product.

Detection of the hybridization was accomplished by monitoring difference between the DPV response of MB accumulated on the PNA-SAM modified AuE before and after hybridization with targets. Fig. 1 shows the differential pulse voltammograms (DPVs) for the reduction signal of MB following self-assembly of the PNA probe onto the AuE (curve a) and after hybridization of the probe with single-stranded (ss) complementary PCR product (curve b), with the double-stranded (ds) complementary PCR product (curve c) and with the

non-complementary PCR products (curve d, e) as the target DNA. As seen in Fig. 1 significant increase in the MB signal was occurred following hybridization of the PNA probe with the target ss-PCR and ds-PCR product (Fig. 1b, 1c), which indicates that hybridization took place on the electrode surface. Moreover, signal increase in the case of ds-PCR is greater than signal increase in the case of ss-PCR which addressed to the hybridization of the PNA probe with the ds-PCR resulting in PNA/ds-PCR formation with which more MB molecules are interacted than PNA/ss-PCR and proves formation of PNA-ds-DNA complex on the electrode surface. On the other hand, the reduction signals of MB almost remained unchanged in the case of the non-complementary PCR products. This result represented that no remarkable interaction between the non-complementary PCR products and self-assembled probe exists. In continue, for improving biosensor sensitivity and selectivity for detection of ds-PCR product, effect of some experimental variables such as hybridization conditions of probe modified AuE affecting performance of the sensor were studied as below.

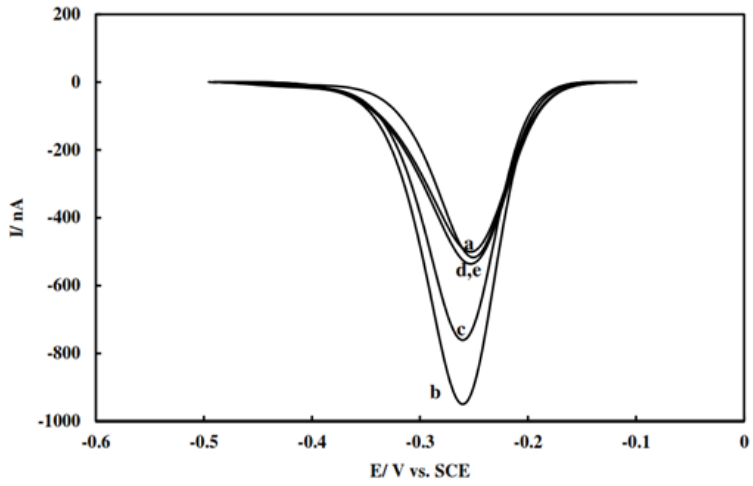

Fig. 1. Differential pulse voltammograms of accumulated MB on the PNA probe modified AuE: (a) before hybridization and after hybridization with 200 ppm (b) complementary ss-PCR, (c) complementary ds-PCR, (d) non-complementary ss-PCR and (e) non-complementary ds-PCR. Electrode conditions: A 6 µL droplet of 2.5 µM PNA probe solution onto the pre-treated AuE for 2h, Hybridization condition: A 6 µL droplet of 200 ppm target solution with pH 7 onto the probe modified AuE for 3 h

3.2. Effect of ionic strength

The effect of ionic strength on the response of the sensor to the complementary and non-complementary ds-PCR products was studied and compared. For this purpose, hybridization carried out in buffers by various NaCl concentrations. The obtained results (Fig. 2) showed that by increasing the NaCl concentration up to 20 mM, ΔI (difference between peak current

related to complementary and non- complementary ds-PCR products) increased and consequently the sensitivity and selectivity of the sensor was improved. But at NaCl concentrations higher than 20 mM ΔI decreased maybe because of nonspecific adsorption of target to the probe sequence. Thus 20 mM was chosen as the optimal NaCl concentration for further experiments.

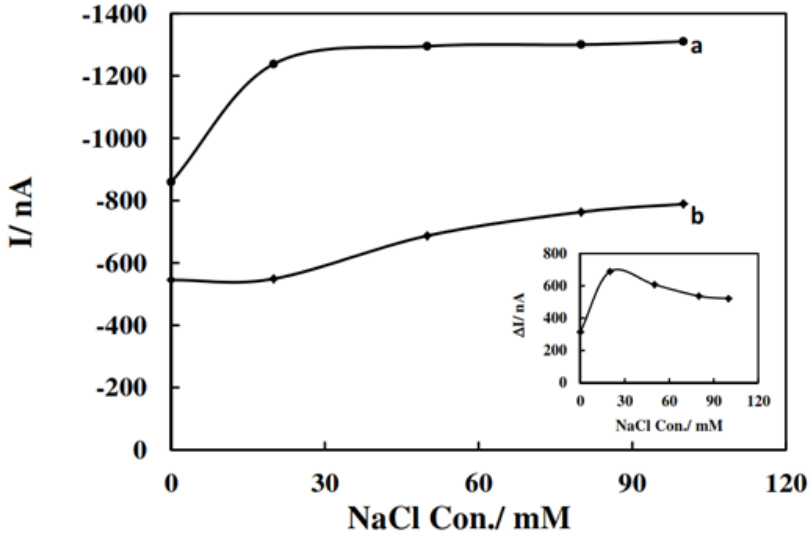

Fig. 2. Plot of MB peak current on the PNA probe modified AuE versus NaCl concentration of hybridization buffer after hybridization with (a) 200 ppm ds-PCR_{COM} and (b) 200 ppm ds-$PCR_{non\text{-}COM}$. Inset of Fig. 2: Variation of the difference between the MB reduction signal of the PNA probe modified AuE after hybridization with ds-PCR_{COM} and ds-$PCR_{non\text{-}COM}$ (ΔI) versus NaCl concentration; other experimental conditions as in Fig. 1

3.3. Effect of pH

Effect of hybridization buffer pH on the hybridization efficiency has been studied. For this purpose, hybridization was carried out in 20 mM phosphate buffer containing 20 mM NaCl with various pH values of 5, 7 and 9. Figure 3 represents a histogram illustrating the MB reduction signal of probe SAM modified AuE after hybridization with ds-PCR_{COM} (a) as well as after interaction with ds-$PCR_{non\text{-}COM}$ (b) at different pH values. As seen in Fig. 3, by increasing pH value, the reduction signal of MB remained almost unchanged for probe modified electrode after hybridization with ds-PCR $_{non\text{-}COM}$, but it decreased after hybridization with ds-PCR_{COM}. This shows that in pH 5 maximum hybridization efficiency is achieved. Since the triplex formation is pH sensitive with an acidic pH (5.0) being required to form the Hoogsteen pairing bonds, we anticipated that raising pH may affect the stability of the triplex. Therefore we used pH 5 as optimal pH for hybridization experiments.

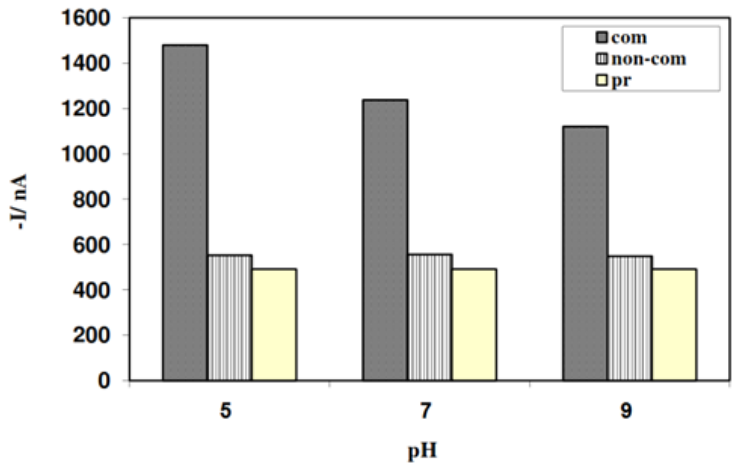

Fig. 3. histograms related to various MB peak current on the PNA probe modified AuE (a) before hybridization and after hybridization with (b) 200 ppm ds-PCR_{COM} and (c) 200 ppm ds-$PCR_{non\text{-}COM}$ as a function of hybridization solution pH; other experimental conditions as in Fig. 1

3.4. Effect of probe concentration, hybridization temprature and hybridization time

There are many reports about the effect of thiolated PNA probe density on the hybridization efficiency and hybridization kinetics [31,32]. Probe density has a key role on the efficiency of target capture as well as on the kinetics of the target/probe hybridization reaction. In most cases, high probe densities are desired in order to increase the signal strength and thus to improve the detection limits. However, binding efficiency may be decreased at higher probe densities. The probe density can be controlled by altering the probe concentration on the gold surface during the probe film fabrication. Effect of PNA probe density on the hybridization efficiency has been studied. For this purpose, we prepared electrodes using various concentrations of the probe to obtain an optimal PNA probe density for ds-PCR product hybridization. The resulting MB peak currents versus probe concentrations are plotted in Fig. 4A and showed that by increasing the probe concentration up to 0.5 μM, MB DPV peak current is increased; but at higher PNA probe coverage (probe concentration greater than 2.5 μM), a decrease in the MB reduction signal was observed. We attribute this effect to the decrease in the number of triplexes formed on the electrode surface due to the steric and electrostatic hindrances arising from the more tightly packed PNA monolayer at higher surface coverage as described earlier.

The influence of hybridization temperature on the hybridization efficiency was also studied. For this reason hybridization was done in a humidity chamber (with fixed temperature) for first 30 minute and then cooled gradually to room temperature for 2.5 hours. Results (Fig. 4B) showed that by increasing hybridization temperature up to 55 ºC, the amount of MB reduction signal increased and then leveled off for higher temperatures. Thus we selected 55 ºC as optimum temperature for hybridization.

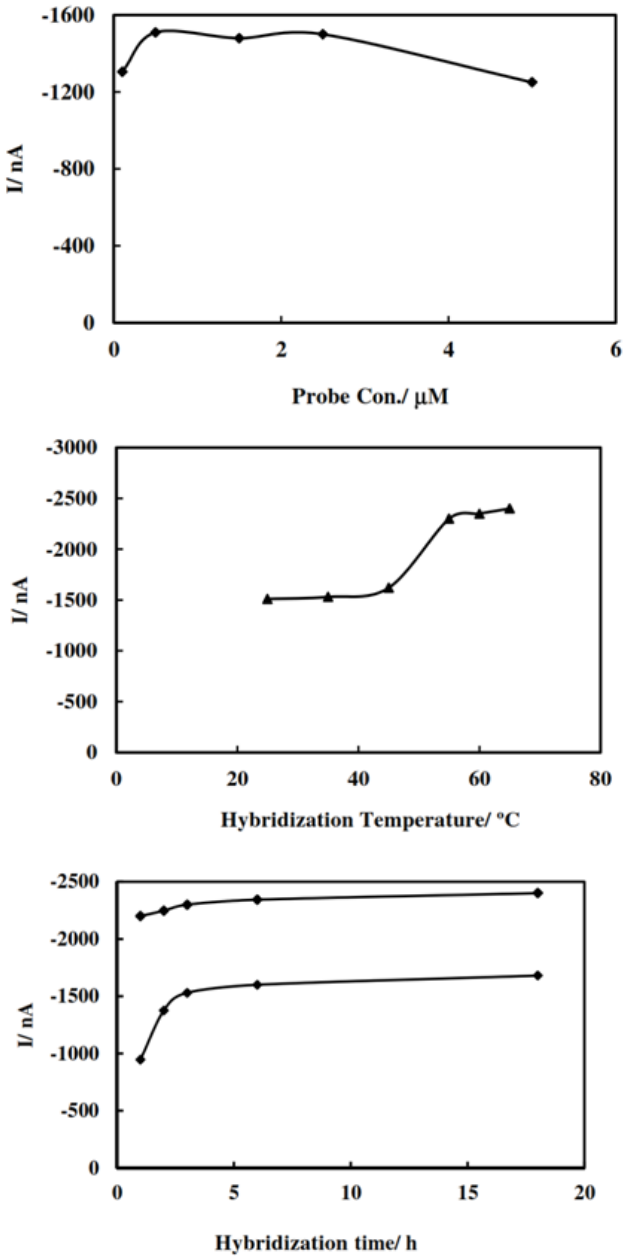

Fig. 4. (A) Plot of MB peak current on the PNA probe modified AuE using various probe concentrations after hybridization with ds-PCR_{COM} product versus probe solution concentration; Hybridization condition: A 6 µL droplet of 200 ppm target solution with pH 5 onto the probe modified AuE for 3h; **(B)** Plot of MB peak current on the PNA probe modified AuE after hybridization with 200 ppm ds-PCR_{COM} at various temperatures versus hybridization solution temperature; Hybridization condition: A 6 µL droplet of 200 ppm target solution with pH 5 onto the probe modified AuE for 3h (30 minute at given temperature and 2.5 hour at room temperature); **(C)** Plot of MB peak current on the PNA probe modified AuE after hybridization with 200 ppm ds-PCR_{COM} product at various hybridization time versus hybridization time; Electrode conditions: A 6 µL droplet of 0.5 µM PNA probe solution onto the pre-treated AuE for 2 h, Hybridization condition: A 6 µL droplet of 200 ppm target solution with pH 5 onto the probe modified AuE

The influence of hybridization time on the hybridization efficiency was also studied and results (Fig. 4C) showed that for hybridization at room temperature optimum time needed to get maximum signal is 3 hour, but by using temperature program for hybridization (first 30 minute in 55 ºC and other at room temperature), maximum hybridization efficiency can be achieved in 1 hour. Thus by using this kind of hybridization we can reduce the assay time and increase sensitivity of the biosensor.

3.5. Selectivity of the method in optimized condition

Some experiments have been carried out with non-complementary PCR products to assess whether the suggested DNA sensor responds selectively to the target DNA. For this purpose, PNA-SAM modified AuE were subjected with complementary ds-PCR product, and non-complementary one in the optimized condition.

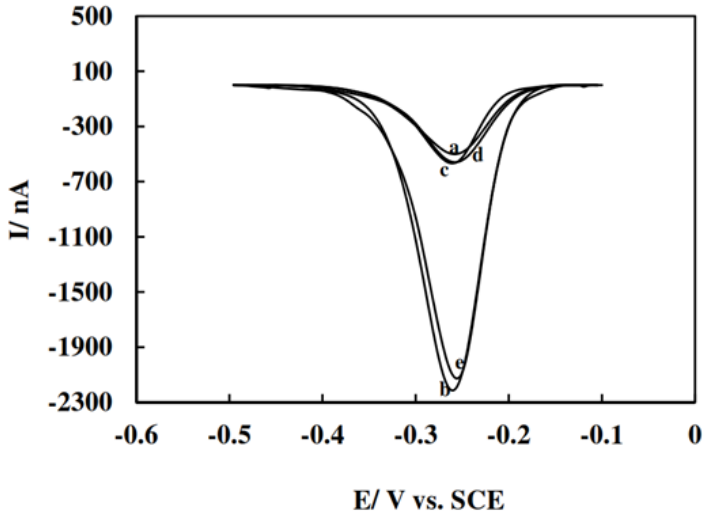

Fig. 5. Differential pulse voltammograms of accumulated MB on the PNA probe modified AuE: (a) before hybridization and after hybridization with 200 ppm (b) complementary ds-PCR product, and after interaction with negative PCR products (c) p53, (d) 16S rRNA and (e) a mixture of complementary and non-complementary ds-PCR products. Electrode conditions: A 6 µL droplet of 0.5 µM PNA probe solution onto the pre-treated AuE for 2h, Hybridization condition: A 6 µL droplet of 200 ppm target solution with pH 5 onto the probe modified AuE for 1h (30 min at 55 ºC and 30 min at room temperature)

As seen in Fig. 5, interaction between ds-PCR $_{\text{non-COM}}$ products and PNA-SAM modified AuE, did not lead to a significant increase in the MB reduction signal due to the absence of significant hybridization between the probe and non-complementary DNA (compare curves c, d with curve a). As shown in Fig. 5 the MB reduction signals for the hybridized ds-

PCR_{COM} alone (curve b) and in the presence of a mixture of ds-PCR_{COM} and ds-$PCR_{non\text{-}COM}$ is almost the same (compare curve b with curve e).

This result indicates that only the complementary ds-PCR product could form a PNA/ds-PCR structure and thus, the proposed biosensor can detect complementary PCR product with high selectivity.

3.6. Diagnostic performance

The difference between the signals of the MB accumulated on the PNA-SAM modified AuE in the presence and absence of the complementary ds-PCR product (ΔI) is increased with increasing concentration of the target up to about 400 ppm (Fig. 6). At this concentration of target ds-PCR product, the maximum capacity of the probe available on the electrode surface is involved in the hybridization. The calibration curve is linear between 5 and 200 ppm with a correlation coefficient of 0.999 for the complementary ds-PCR.

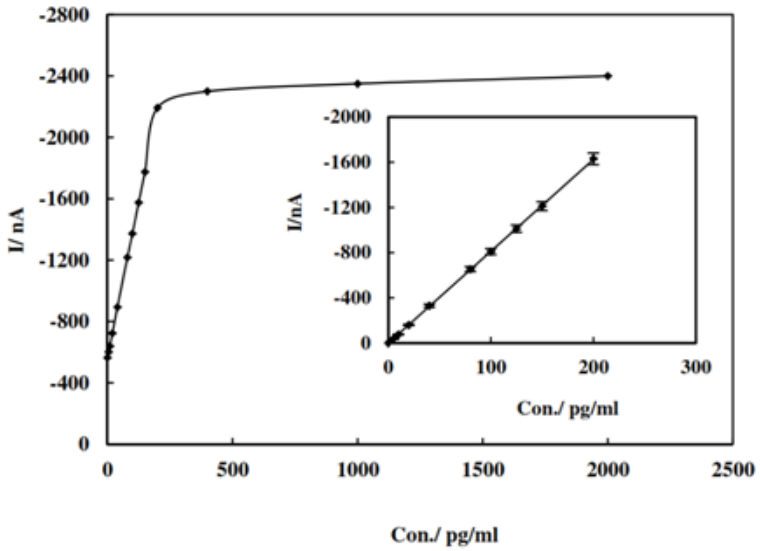

Fig. 6. Variation of the difference between the MB reduction signal of the PNA probe modified AuE in the presence and absence of complementary ds-PCR product (ΔI) versus target concentration. Inset of Fig. 5: related calibration graph at concentration range 5-200 ppm. Electrode conditions: as in Fig. 4

The calculated detection limit by means of equation: $y_{lod}=y_B+3S_{y/x}$ and regression equation: $\Delta I=-8.134\ C+1.801$ was about 1.58 ppm, where, y_B is signal of the blank (here intercept of calibration graph) and $S_{y/x}$ is standard deviation of blank (here standard deviation of the calibration graph). The relative standard deviation in three independently probe modified electrodes measured in the presence of 200 ppm complementary ds-PCR was 3.19%, indicating the reproducibility of the direct detection of the complementary ds-PCR by this procedure.

3.7. Gel electrophoresis of the PCR samples

Gel electrophoresis of the PCR samples were 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 573 bp size indicating the presence of core/E1 encoding cDNA amplificant in the PCR sample (Fig. 7b). The presence of a DNA band in each bacterial PCR amplificants revealed the amplification and presence of p53 fragments in corresponding PCR samples (Fig. 7c). 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.

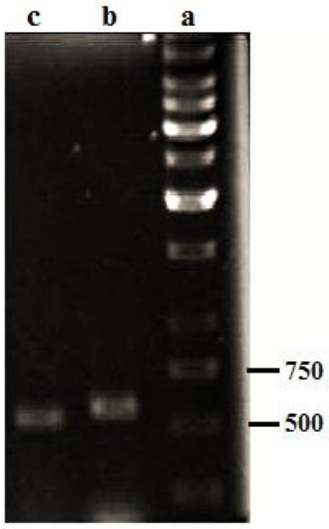

Fig. 7. Agarose gel electrophoresis of PCR-amplified real samples related to the (a) 1 kb DNA ladder as the DNA size marker, (b) complementary 573 base pair HCV core/E1 encoding cDNA, and (c) non-complementary PCR sample corresponding to p53

4. CONCLUSION

A gold electrode modified with a mixed self-assembled monolayer of the PNA probe and MCH can be employed for detection of core/E1 encoding cDNA corresponding to hepatitis C virus in double-stranded PCR products. In this strategy, MB peak current increases after hybridization of the self-assembled PNA with complementary ds-PCR product and remain unchanged following interaction with non-complementary one. Results showed that hybridization event depend on pH and ionic strength and initial temperature of the hybridization solution. In the optimized condition LOD of the biosensor is 1.58 ppm. The present strategy could be employed for development of further DNA detection techniques based on PNA-ds-DNA hybrid formation.

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