Analytical Biochemistry 399 (2010) 118–124

Contents lists available at ScienceDirect

Analytical Biochemistry

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

Electrochemical detection of short sequences of hepatitis C 3a virus using a peptide nucleic acid-assembled gold electrode

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ARTICLE INFO

Article history:
Received 9 October 2009
Received in revised form 15 November 2009
Accepted 16 November 2009
Available online 18 November 2009

Keywords:
Hepatitis C 3a virus
DNA biosensor
Methylene blue
Differential pulse voltammetry

ABSTRACT

Development of an electrochemical DNA biosensor, using a gold electrode modified with a self-assembled monolayer composed of a peptide nucleic acid (PNA) probe and 6-mercapto-1-hexanol, is described. The sensor relies on covalent attachment of the14-mer PNA probe related to the hepatitis C virus genotype 3a (pHCV3a) core/E1 region on the electrode. Covalently self-assembled PNA could selectively hybridize with a complementary sequence in solution to form double-stranded PNA-DNA on the surface. The increase of peak current of methylene blue (MB), upon hybridization of the self-assembled probe with the target DNA in the solution, was observed and used to detect the target DNA sequence. Some hybridization experiments with noncomplementary oligonucleotides were carried out to assess whether the suggested DNA sensor responds selectively to the target. Diagnostic performance of the biosensor is described and the detection limit was found to be 5.7×10^{-11} M with a relative standard deviation of 1.4% in phosphate buffer solution, pH 7.0. This sensor exhibits high reproducibility and could be used for detection of the target DNA for seven times after the regeneration process.

Surface-based biosensors for detection of DNA hybridization biosensors are rapidly developing devices and considered as important tools due to their numerous applications ranging from decentralized clinical testing to environmental monitoring, food safety, rapid detection of infectious microorganisms, and forensic investigations. During the past decade, a variety of techniques have been developed for the detection of DNA hybridization, including fluorescence techniques [1], quartz-crystal microbalance [2,3], electrochemiluminescence [4], surface plasmon resonance spectroscopy [5–7], and electrochemical methods [8–10]. Among these, electrochemical methods have been attracting a great deal of attention because of their cost effectiveness, simplicity of the process, small dimensions, fast response time, and compatibility with microfabrication technology. Such devices rely on the immobilization of a single-stranded oligonucleotide on the electrode surface to recognize its complementary sequence with a high specificity. Several methods have been developed for immobilizing of the ssDNA probe onto electrode surfaces, including direct adsorption [11,12], avidin–biotin system [13], self-assembled methods [14,15], and covalent binding [16–18]. Self-assembled methods are an effective way for DNA immobilization on a gold electrode with the advantages of simplicity, high level of orientation, and versatility [19]. Over the last two decades, electrochemical DNA biosensors were mainly based on DNA self-assembled monolayers (SAMs)[1] [20,21]. Recently, there has been an increasing interest for SAMs of peptide nucleic acid (PNA)-modified electrodes for construction of DNA sensors [22–25]. PNA, which was first described by Nielsen et al. in 1991, is a nucleic acid analog in which the sugar phosphate backbone is replaced with a polyamide backbone consisting of *N*-(2-aminoethyl) glycine units. PNAs hybridize with complementary nucleic acids according to the Watson-Crick rules for base pairing with remarkable high affinity and specificity, essentially because of their uncharged and flexible polyamide backbone. PNA chains are resistant to nuclease and protease activities. Because of these interesting characteristics, PNA has been employed in a number of DNA detection schemes [26–28].

Hepatitis C virus (HCV) is the major causative agent of parentally transmitted and sporadic blood-borne non-A, non-B hepatitis infection [29]. According to the World Health Organization (WHO), HCV infection currently affects approximately 170 million people worldwide [30]. The majority of acute HCV infection frequently progresses to chronic diseases, which eventually can lead to liver cirrhosis and hepatocellular carcinoma. There is no

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[1] *Abbreviations used*: HCV, hepatitis C virus; MB, methylene blue; MCH, 6-mercapto-1-hexanol; PNA, peptide nucleic acid; SAMs, self-assembled monolayers.

0003-2697/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2009.11.019

comprehensively effective therapy for chronic HCV infection and in most cases current treatments call for major improvements. Despite the fact that hepatitis C is a major public health threat, no therapeutic or prophylactic vaccine has been developed for HCV, yet. HCV isolates are classified into six major genotypes (from 1 to 6) with multiple subtypes (a,b,c, ...), which display remarkably various responses to the treatments. Subtype 3a is considered a risky subtype and is distributed globally [31,32]. Accordingly, development of simple and reliable HCV genotype 3a (HCV-3a) detection methods is in demand.

Some reports have already been published on electrochemical genosensing of hepatitis B virus [33–36], but, despite considerable interest in developing simple and reliable methods for detection and quantification of HCV, there are few works for HCV detection based on electrochemical techniques [37–39]. Electrochemical DNA hybridization detection methods are divided into major two direct and indirect strategies and accordingly different forms of electrochemical DNA biosensors have been developed by now [40–44]. Methylene blue (MB), which is an organic dye and belongs to the phenothiazine family, displays a very good voltammetric behavior. The interaction of methylene blue with oligonucleotides has been widely studied with various methods [45–49]. It was also employed for development of DNA hybridization detection devices [50–53].

In the present work, we utilized MB as an electrochemical indicator for the detection of hepatitis C virus genotype 3a core/E1 corresponding DNA oligonucleotide using a proper guanine-free PNA probe covalently attached on a gold electrode. It is shown that as the utilized uncharged PNA probe is a guanine-free oligonucleotide, MB has no remarkable tendency toward PNA single-stranded probe. Whereas interaction with guanine bases, electrostatic interaction, and intercalation binding coexist the PNA-DNA hybrid-modified electrode. Therefore the peak currents of MB further increased after hybridization of the self-assembled PNA with the target DNA which could be used as a platform for the development of electrochemical methods for detection of HCV3a.

Experimental

Chemicals

In this study the cysteine-conjugated 14-mer PNA oligomer called pHCV3a complementary to the universal sequence of HCV3a core/E1 region was used as the probe and its complementary DNA oligonucleotide (cHCV3a) corresponding to the consensus sequence of HCV3a core/E1 region was used as target DNA. NC1 and NC2 oligonucleotides corresponding to two different nucleotide polymorphic types of human uridine diphosphate glucuronosyltransferase 1A9 (UGT1A9) gene promoter region and HCV1a DNA oligonucleotide corresponding to the consensus sequence of HCV1a core protein gene were used as the noncomplementary DNA samples. PNA probe was purchased from PANAGENE and the DNA oligonucleotides were supplied as lyophilized powder from Eurofins MWG Operon Company, with the following sequences:

- PNA probe (pHCV3): HS-Cys-O-O-CACACATATCACCC,

where HS-Cys represents the anchoring cysteine group and O is 8-amino-3,6-dioxaoctanoic acid (AEEA) linker and of the PNA chain. This probe contains a carboxamide ($CONH_2$) group at c-terminal.

- Complementary DNA target (cHCV3a): 5′-GGGTGATATGTGTG-3′.
- Noncomplementary DNAs:
- HCV1a: 5′-TAATGAGGGCTGCGGGTGGG-3′
- NC1: 5′-AGTTTAGTAGCAG-3′
- NC2: 5′-AGTTTAGAAGCAG-3′

The cysteine-tethered PNA oligonucleotide was dissolved in 0.1% trifluoroacetic acid and kept frozen at −20 °C. More diluted solutions of the PNA probe were prepared using 0.5 M acetate buffer solution (pH 4.8).

Stock solutions of the DNA oligonucleotides (500 μg/ml) were prepared with TE buffer solution (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and kept frozen. More diluted solutions of the oligonucleotides were prepared using 0.2 M phosphate buffer solution (pH 7.2). Other chemicals were of analytical reagent grade. The distilled, deionized and sterilized water was used in all solution preparation. All the experiments were performed at room temperature in an electrochemical cell.

Apparatus

All electrochemical experiments were performed in a 10-ml volume cell, connected to an UTOLAB PGSTAT 30 electrochemical analysis system and GPES 4.7 software package (Eco Chemie, The Netherlands). The utilized three-electrode system 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.

Preparation of the working electrode

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 [54]. The electrode was treated with cycling of the potential between −0.3 and 1.5 V with a scan rate of 0.1 V s^{-1} until the cyclic voltammogram did not change, indicating that the electrode surface was clean. The electrode was then washed with water and placed upside down in a humidity chamber. A 6-μl droplet of 5 μM PNA probe aqueous solution was deposited onto the gold electrode and kept at room temperature for self-assembly. For treatment of the probe self-assembled AuE with 6-mercapto-1-hexanol (MCH), the electrode was rinsed with water and then dipped in 1 mM aqueous MCH at room temperature for 30 min. MCH deposition is important both for competitive removal of nonspecific probe adsorption and for filling "pinholes" in the PNA monolayer. Before use, the electrode was washed repeatedly with water.

Evaluation of PNA self-assembled monolayer formation

The extent of probe *self-assembly* was monitored by the influence of the probe on the electrochemical signal of the $Fe(CN)_6^{3-}$/$Fe(CN)_6^{4-}$ system on the pretreated Au electrode based on the decline in the height of the $Fe(CN)_6^{3-}$/$Fe(CN)_6^{4-}$ redox peaks. For this purpose, the electrode was scanned between −0.5 and 0.6 V in 10 mM $K_3Fe(CN)_6$ + 10 mM $K_4Fe(CN)_6$ solution [55].

Probe–DNA hybridization

Three types of hybridization were tested in this research. These types include drop hybridization, non-preheated solution hybridization, and preheated solution hybridization. Drop hybridization was carried out by dropping 6 μl of DNA solution on the PNA self-assembled AuE for 150 min. Then the electrode was rinsed with PBS. Non-preheated solution hybridization was conducted by immersing the PNA self-assembled AuE in 0.2 M phosphate buf-

fer (pH 7.0) containing the target DNA with stirring for 150 min. In the preheated solution hybridization PNA self-assembled probe was hybridized with the target DNA by dipping the modified AuE in 0.2 M phosphate buffer (pH 7.0) containing target DNA with stirring for 3 min at 85 °C. Then the solution was left at room temperature in order to cool down gradually. Thus, a hybrid-modified Au electrode, PNA-DNA/AuE, was obtained. Hybridization of the probe with noncomplementary DNA sequence and a mixture solution of complementary and noncomplementary DNA sequences was carried out following the same method as described above.

Labeling of the hybrid

MB was interacted with the probe or the hybrid by immersing the electrode into the stirring 20 mM Tris–HCl buffer (pH 7.0) 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) for 10 s.

Voltammetric 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) 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 using the 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 nonhybridized and hybridized statue.

Results and discussion

Optimization of operational conditions

Some experimental variables such as the probe SAM formation conditions (i.e., SAM formation time and probe concentration) and hybridization conditions, affecting the performance of the sensor, were studied and special emphasis was given to the optimization of such experimental variables.

Probe SAM formation time and concentration

The important factors affecting the SAM formation on the electrode are probe SAM formation time and probe concentration. The extent of the SAM formation on the electrode surface was evaluated by means of cyclic voltammetric measurement in $K_3Fe(CN)_6/K_4Fe(CN)_6$ redox solution. Indeed as the SAM formation is extended the redox signal of $K_3Fe(CN)_6/K_4Fe(CN)_6$ is decreased. The results obtained from the voltammetric measurements revealed that the reduction signal of $K_3Fe(CN)_6$ decreased as the SAM formation time increased to about 2 h and remained constant between 2 and 16 h (Fig. 1, curve a). Similarly, the influence of the probe concentration was studied. The cyclic voltammograms showed that the reduction signal of $K_3Fe(CN)_6$ decreased as the probe concentration increased up to 25 μM and leveled off between 25 and 75 μM, due to full coverage of the electrode surface (Fig. 1, curve b). Therefore 2 h and 25 μM are suggested as a suitable time and concentration for the probe self-assembly, respectively.

Monitoring of the hybridization

Detection of the hybridization was accomplished using reduction of the accumulated MB, where the electroactivity of this label leads to discrimination of the PNA-DNA hybrid from the probe. Generally, three kinds of binding including electrostatic, affinity of MB to DNA guanine bases, and intercalative binding take place between small molecules and oligonucleotides. The binding property depends on both the nature of the small molecules and the experimental conditions, such as ionic strength, concentration of the small molecules and DNA, and sequence of the oligonucleotide. The PNA probe used in the present study has no guanine base and is neutral in terms of electric charge. The DP voltammograms for the probe-modified AuE before and after hybridization with 1 μM complementary oligonucleotide confirmed that a significant increase in the MB signal from 200 ± 6.7 nA (Fig. 2, curve a) to 600 ± 17.6 nA (Fig. 2, curve b) was observed following hybridization of the PNA probe with the target DNA. Thus MB has no considerable electrostatic tendency toward PNA probe and only a small signal mainly related to the MB penetrated to the electrode surface is observed. Whereas the effects of electrostatic, interaction with guanine bases, and intercalation interactions coexist in the hybrid-modified electrode. Consequently, the peak current of MB further increases after hybridization of the self-assembled PNA with the target DNA. The enhancement of the peak current indicates that more MB molecules interact with the SAM PNA-DNA hybrid. Additionally, the MB peak obtained from PNA-DNA/AuE was found at potential ca. 15 mV more negative than that of PNA probe/AuE and this might be related to the different mechanisms of the interaction between electroactive indicators and nucleic acid chains in the case of single- and double-stranded PNA self-assembled on the electrode surface. This difference is reflected in the shift of methylene blue peak potential. Such observation was in agreement with the results of the investigation carried out by Tani et al. for DNA self-assembled probes [56]. This change in the potentials and magnitude of the voltammetric signal represents the extent of the hybridization on the electrode surface. Having observed the ability of the electrode in detection of the target DNA, a preliminary investigation concerning the hybridization of the probe with a noncomplementary oligonucleotide was considered. For this purpose a solution of NC1 noncomplementary oligonucleotide was subjected and results represented (Fig. 2, curve c) that no remarkable interaction between noncomplementary DNA and self-assembled probe exists.

Effect of the hybridization type

Three hybridization procedures including drop hybridization, non-preheated solution hybridization, preheated solution hybridization were applied on the PNA-AuE and then the DPV signal of MB was measured in deoxygenated Tris–HCl experimental solutions. The results depicted in Fig. 3 indicate that the previous heating of the hybridization solution had a significant effect on the hybridization efficiency and almost 100% increase was observed in the MB signal of the preheated solution hybridization compared to the other methods. Therefore, the preheated solution hybridization method was suggested for future experiments.

Effect of experimental variables

Probe density is a controlling factor for the efficiency of target capture as well as for 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 decrease at higher probe densities. The probe density can be controlled by varying the probe concentration in the solution during the probe film fabrication. The influence of the probe solution concentration on the hybridization efficiency was studied and results are shown in Fig. 4, curve a. As seen in Fig. 4a, by increasing the probe concentration up to 5 μM, MB reduction signal is increased after hybridization, but at

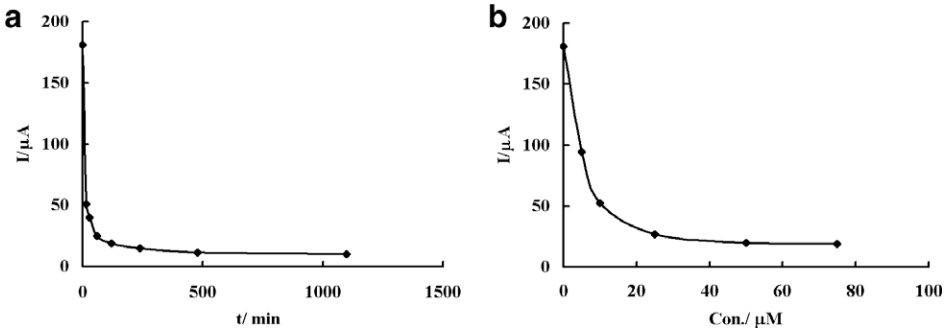

Fig. 1. Variation of cathodic peak current of voltammograms of PNA probe-modified AuE in 10 mM $K_3Fe(CN)_6$–10 mM $K_4Fe(CN)_6$ solution versus (a) pHCV3a SAM formation time (b) pHCV3a concentration.

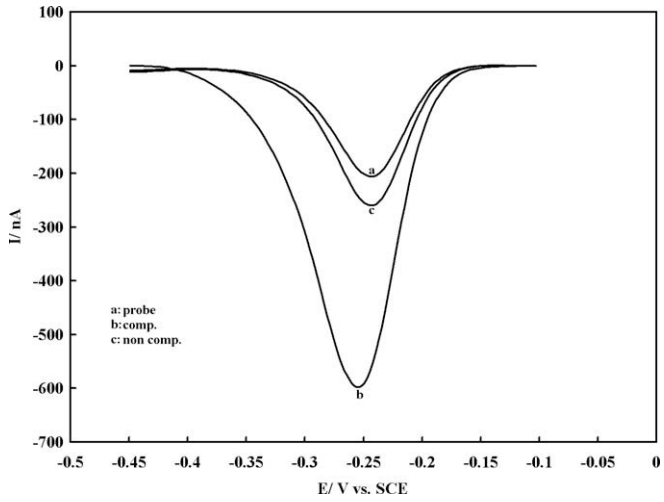

Fig. 2. Differential pulse voltammograms of accumulated MB on the 25 μM PNA probe-modified AuE: (a) before hybridization and (b) after hybridization with 1 μM cHCV3a target oligonucleotide; (c) after hybridization with 1 μM NC1 noncomplementary oligonucleotide. Experimental conditions were as described in the text.

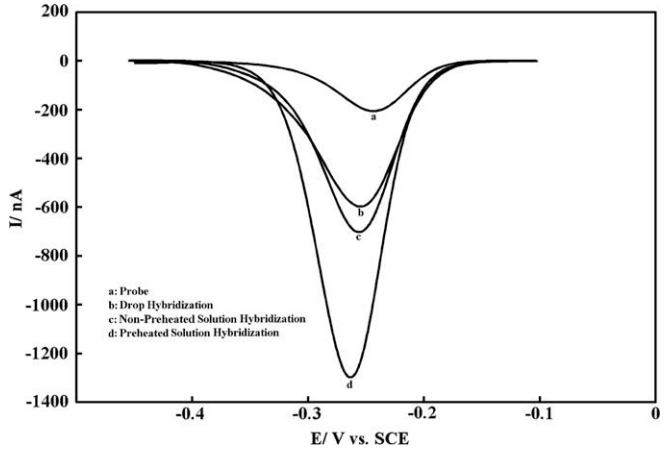

Fig. 3. Differential pulse voltammograms of accumulated MB on the 25 μM PNA probe-modified AuE: (a) before hybridization and after hybridization using (b) drop hybridization, (c) non-preheated solution hybridization, and (d) preheated solution hybridization methods with 1 μM target oligonucleotide.

higher PNA probe coverage (probe concentration >5 μM), a decrease in the MB reduction signal was observed. We attribute this effect to a decrease in the number of duplexes formed on the electrode surface due to the steric and electrostatic hindrances arising from the more tightly packed PNA monolayer for higher surface coverage as described earlier [57].

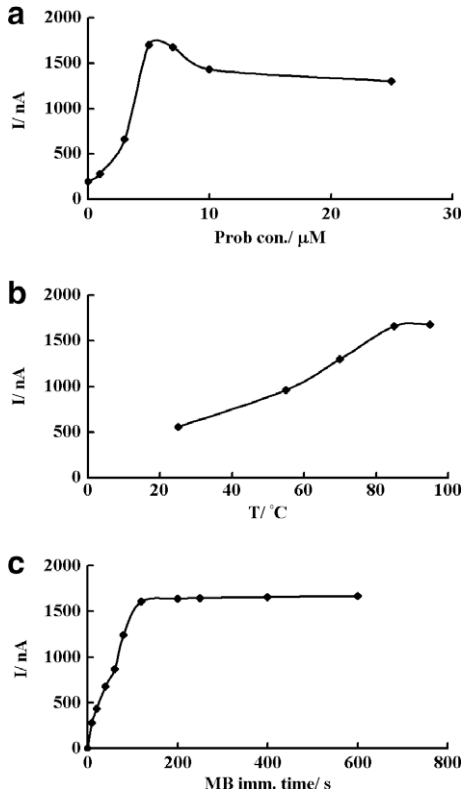

Fig. 4. Variation of the MB peak current versus (a) probe solution concentration, (b) hybridization temperature, and (c) MB accumulation time after hybridization with 1 μM target oligonucleotide. For curves a and b MB immobilization time was 5 min.

In order to investigate the influence of the temperature of the target solution on the hybridization extent, the PNA self-assembled AuE was immersed in 0.2 M phosphate buffer (pH 7.4) of temperatures from 50 to 90 °C, containing 1 μM complementary oligonucleotide for a few minutes and then cooled gradually to room temperature. The results (Fig. 4, curve b) showed that in 85–90 °C, maximum efficiency of hybridization can be obtained.

The influence of MB accumulation time was also explored for optimum analytical performance. Thus, accumulation time was varied between 30 s and 10 min (Fig. 4, curve c). The results obtained from the voltammetric measurements revealed that the reduction signal of MB was elevated as the accumulation time increased up to 2 min and remained constant between 2 and 10 min. Thus 2 min was chosen as accumulation time for further measurements.

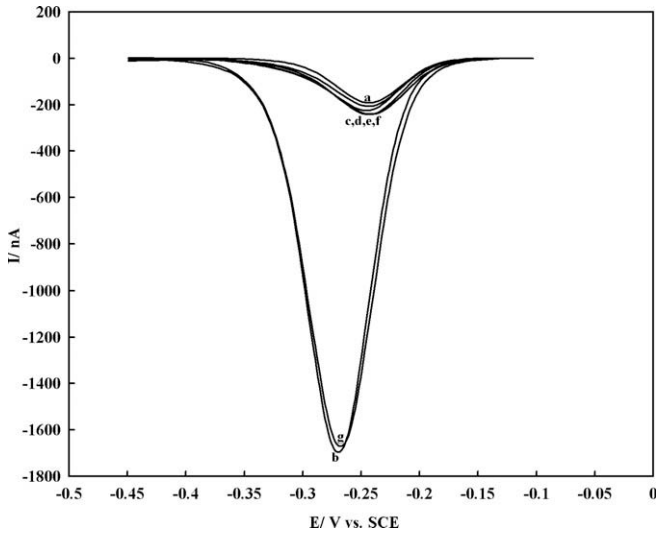

Fig. 5. Differential pulse voltammograms of accumulated MB on the 5 μM PNA probe-modified AuE: (a) before hybridization and after hybridization with (b) 1 μM cHCV3a target oligonucleotide, 1 μM noncomplementary oligonucleotides (c) HCV1a; (d) NC1; (e) NC2; (f) mixed solution of HCV1a, NC1, and NC2; and (g) mixed solution of 1 μM complementary cHCV3a and 1 μM noncomplementary HCV1a, NC1, and NC2 oligonucleotides.

Reproducibility of the sensor

Reproducibility of the sensor surface is very important for rapid and accurate detection in automatic systems. The electrode surface was washed with 10 mM Tris–HCl and 1 mM EDTA buffer solution (pH 8.0) at 97 °C for 10 min in order to remove all hybridized strands. Results showed that after rehybridization of the probe with the target DNA and accumulation of MB, the redox response of the MB is successfully recovered. The present sensor surface showed good performance after regeneration up to seven times.

Selectivity of the sensor

A hybridization experiment with some noncomplementary oligonucleotides was carried out to assess whether the suggested DNA sensor responds selectively to the target DNA. For this purpose, three different oligonucleotides, NC1, NC2, and HCV1a containing 4, 4, and 11 free guanine bases, respectively, and a mixture of them were subjected. Fig. 5 shows the DPV related to 1 μM solutions of various oligonucleotides, noted above. As seen in Fig. 5, the interaction between these noncomplementary oligonucleotides and the self-assembled probe did not lead to a significant increase in the MB reduction signal due to the absence of entire hybridization between the probe and the solution of noncomplementary DNAs and the signals were nearly equal to those of the probe (curves c, d, e, and f). The selectivity of the biosensor was also accomplished in samples containing both complementary and noncomplementary sequences. Fig. 5, curve g, also shows the DP voltammograms for probe-modified AuE after hybridization with 1 μM cHCV3a in a mixture solution of cHCV3a and the three noncomplementary oligonucleotides of the same concentrations. Curve g implies that the presence of the noncomplementary sequences in the mixture solutions has a negligible effect on the hybridization between the self-assembled probe and the cHCV3a target DNA.

Diagnostic performance of the sensor

The MB reduction signal on the probe-modified Au electrode in the presence of target is increased with increasing the target concentration (Fig. 6). As seen in inset A the difference between the MB reduction signal of the probe-modified AuE in the presence and absence of the target sample (ΔI) is increased with increasing the complementary target concentration and leveled off at ca. 60 nM. Thus, in this concentration of target, the maximum capacity of the probe available on the electrode surface is involved in the hybridization event. The calibration graph is linear between 1

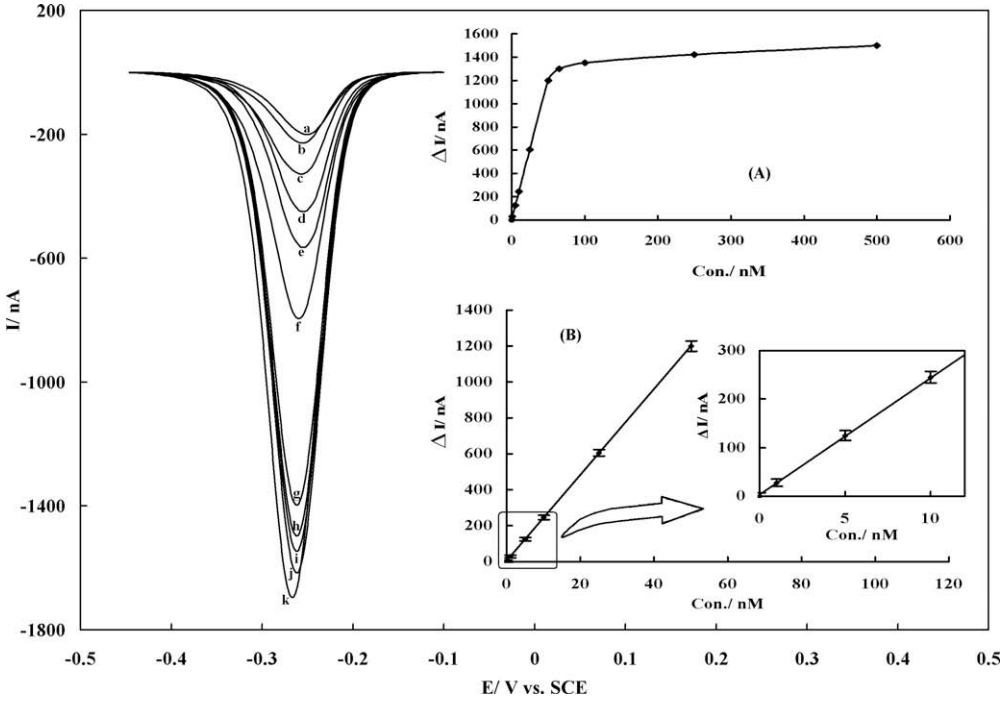

Fig. 6. Differential pulse voltammograms of accumulated MB on the 5 μM PNA probe-modified AuE (a) before hybridization and after hybridization with cHCV3a target oligonucleotide of different concentrations: (b) 1, (c) 5, (d) 10, (e) 15, (f) 25, (g) 50, (h) 65, (i) 100, (j) 250, and (k) 500 nM. Inset: (A) Variation of the difference between the MB reduction signal of the PNA probe-modified AuE in the presence and absence of the target sample (ΔI) versus target concentration; (B) related calibration graph at concentration range 1–50 nM.

and 50 nM with a correlation coefficient of 0.999 (inset B). The detection limit calculated by means of the equation y_{LOD} $(\Delta I) = y_B + 3S_{y/x}$ and regression equation y $(\Delta I) = 0.61 - 24.53x$ (nM) was about 5.7×10^{-11} M. Where, y_B is signal of the blank (here intercept of calibration graph), and $S_{y/x}$ standard deviation of blank (here standard deviation of the calibration graph). Such detection limit compares favorably with those reported electrochemical hybridization detections [20]. The relative standard deviation over three independently probe-modified electrodes measured at 1 μM cHCV3a was 1.4%, indicating a remarkable reproducibility of the detection method.

Conclusion

The gold electrode modified with a SAM composed of the PNA probe and MCH was prepared and employed for the detection of the complementary oligonucleotide related to hepatitis C virus genotype 3a core/E1 region. As the PNA probe contains no guanine and 7 cytosine bases, MB has no considerable tendency toward the PNA single-stranded probe, while the effects of electrostatic, interaction with guanine, and intercalation bindings coexist in the hybrid-modified electrode. Thus, the peak current of MB further increases after hybridization of the immobilized PNA with the target DNA and provides a possibility of hybridization monitoring of the PNA probe with the target DNA, cHCV3a. The proposed DNA biosensor in this work could be utilized for detection of PCR products of hepatitis C virus genome without purification and denaturation of the PCR samples. Reports from our laboratory are in progress toward this direction.

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