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ORIGINAL PAPER

Direct detection and discrimination of double-stranded oligonucleotide corresponding to hepatitis C virus genotype 3a using an electrochemical DNA biosensor based on peptide nucleic acid and double-stranded DNA hybridization

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Abstract Development of an electrochemical DNA biosensor for the direct detection and discrimination of double-stranded oligonucleotide (dsDNA) corresponding to hepatitis C virus genotype 3a, without its denaturation, using a gold electrode is described. The electrochemical DNA sensor relies on the modification of the gold electrode with 6-mercapto-1-hexanol and a self-assembled monolayer of 14-mer peptide nucleic acid probe, related to the hepatitis C virus genotype 3a core/E1 region. The increase of differential pulse voltammetric responses of methylene blue, upon hybridization of the self-assembled probe with the target ds-DNA to form a triplex is the principle behind the detection and discrimination. Some hybridization experiments with non-complementary oligonucleotides were carried out to assess whether the developed DNA sensor responds selectively to the ds-DNA target. Diagnostic performance of the biosensor is described and the detection limit was found to be 1.8×10^{-12} M in phosphate buffer solution, pH 7.0. The relative standard deviation of measurements of 100 pM of target ds-DNA performed with three independent probe-modified electrodes was 3.1%, indicating a remarkable reproducibility of the detection method.

Keywords DNA biosensor · Hepatitis C · PNA probe · Triplex formation · Gold electrode

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Introduction

The use of nucleic acid recognition layers in biosensor design represents a new and exciting area in analytical chemistry. The specificity of DNA hybridization devices (i.e., their ability to respond to the target sequence in the presence of non-complementary strands) depends primarily on the selected probe and secondarily upon the hybridization. Peptide nucleic acid (PNA) is an extremely good structural mimic of DNA (or RNA) in which the negatively charged sugar–phosphate backbone is replaced with a neutral, acyclic, and achiral pseudopeptide backbone composed of repeating *N*-(2-aminoethyl)glycine units linked by amide bonds. The nucleobases are attached to the backbone through methylene carbonyl linkages. PNA may be used in many of the same applications as traditional synthetic DNA or DNA analogs. However PNA chains exhibit superior hybridization properties towards complementary ss-DNA (duplex formation), as well as improved chemical and enzymatic stability, faster hybridization, tighter binding—especially at low ionic strength, and greater specificity compared with natural nucleic acids [1–6]. Furthermore PNA oligomers are able to form very stable duplex structures

with Watson–Crick complementary DNA, RNA, or PNA chains. PNA-based hybridization (duplex formation) biosensors have been constructed based on the quartz-crystal microbalance [7], surface plasmon resonance spectroscopy [8, 9], ion-selective field effect transistors [10, 11], and electrochemical methods [12–18]. Among these, electrochemical detection has significant advantages and is used for converting the hybridization event into an analytical signal [19, 20]. The benefits of using PNA probes in electrochemical DNA biosensors for sequence-specific detection of hybridization have been reported [7, 21–23]. The detection with PNA-based biosensors relies on the electrochemical signal of redox-active nucleobases or changes in the electrochemical properties of electrode/solution interfaces caused by a hybridization event [24–26] (electro-active species are usually used as reporters).

One of the merits of PNA oligonucleotide is that 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 during which the natural backbone of PNA improves the triplex formation capability of the PNA [2, 4, 6].

In spite of extensive attention paid to DNA detection based on various procedures, there are a few studies about direct detection of DNA sequences on ds-oligonucleotide chains. Kuhn et al. used molecular beacons for the detection of a chosen target sequence directly in ds-DNA with the aid of PNA "openers" by using a fluorescent method [27]. Baker and colleagues described a biosensor for detection of only double-stranded oligonucleotide chains based on fluorescence resonance energy transfer (FRET) utilizing cationic conjugated polymer (CCP) donors and dye-labeled PNA acceptors [28]. There is no report on direct detection of ds-DNA based on electrochemical methods. Recently, we have developed electrochemical DNA biosensors based on short DNA and PNA probes with a high selectivity and sensitivity [29–37]. In these studies, the target DNA samples were utilized in the form of single-stranded DNA or were denatured into single-stranded structure for detection purposes. Direct detection of target DNA in ds-DNA form based on triplex structure formation between PNA probe and the ds-DNA eliminates the need for a denaturation step and simplifies the detection procedure. This eventually leads to reduction in the assay time and expense as well as contamination risk, such that the method could be a basic platform for development of further DNA detection strategies. Here we demonstrate an electrochemical approach for the direct detection of ds-DNA related to hepatitis C virus genotype 3a using a proper guanine-free PNA probe covalently attached to a gold disk electrode and methylene blue (MB) as an electro-active intercalator.

Experimental

Chemicals

In this study the cysteine-conjugated 14-mer PNA oligomer called pHCV3a, which is complementary to the universal sequence of HCV3a core/E1 region, was used as the probe. The target complementary double-stranded oligonucleotide was prepared by annealing a pair of complementary DNA oligonucleotides called diHCV3a and dcHCV3a, where diHCV3a is identical to the probe and dcHCV3a is complementary to the probe.

UGT oligonucleotide corresponding to human uridine diphosphate glucuronosyltransferase 1A9 (UGT1A9) gene promoter region with its complementary (cUGT) and HCV1a oligonucleotide corresponding to the consensus sequence of HCV1a core protein gene with its complementary (cHCV1a) were used for preparation of non-complementary double-stranded oligonucleotides.

PNA probe was purchased from PANAGENE and the DNA oligonucleotides were supplied as lyophilized powder from Eurofins MWG Operon, with the following sequences:

- PNA probe (pHCV3a): Cys-O-O-CACACATAT CACCC where HS-Cys represents the anchoring cysteine group and O is the 8-amino-3,6-dioxaoctanoic acid (AEEA) linker of the PNA chain. This probe contains a carboxamide ($CONH_2$) group at the C-terminal.
- Complementary ds-DNA:
  ds-HCV3a:

```
5`-GGGTGATATGTGTG-3`
   | | | | | | | | | | | | | |
3`-CCCACTATACACAC-5`
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  This ds-DNA was prepared by annealing of diHCV3a (5′-CACACATATCACCC-3′) and dcHCV3a (5′-GGGTGATATGTGTG-3′) oligonucleotides pair.
- Non-complementary ds-DNAs:
  ds-HCV1a:

```
5`-TAATGAGGGCTGCGGGTGGG-3`
   | | | | | | | | | | | | | | | | | | | |
3`-ATTACTCCCGACGCCCACCC-5`
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  This ds-DNA was prepared by annealing of HCV1a (5′-TAATGAGGGCTGCGGGTGGG) and cHCV1a (5′-CCCACCCGCAGCCCTCATTA-3′) oligonucleotides pair.
  and
  ds-UGT:

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5`-AGTTTAGTAGCAG-3`
   | | | | | | | | | | | | | |
3`-TCAAATCATCGTC-5`
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  This ds-DNA was prepared by annealing of UGT (5′-AGTTTAGTAGCAG-3′) and cUGT (5′-CTGCTAC TAAACT-3′) oligonucleotides pair.

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 by using 0.5 M acetate buffer solution (pH 4.8).

The 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 by 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.

Preparation of double-stranded oligonucleotides

To prepare double-stranded oligonucleotides, equal concentrations (50 μM) of the complementary oligonucleotide pairs were effectively mixed in a microtube. The mixed solution was heated at 95 °C in a water bath for 10 min and then was allowed to cool down gradually to room temperature.

Apparatus

All electrochemical experiments were performed in a 10-ml-volume cell, connected to an AUTOLAB 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 with nitrogen gas for 10 min prior to measurements, and the cell was blanketed with nitrogen gas for the duration of the measurements.

Procedures

Preparation of the working electrode

Gold disk electrode (AuE, 7 mm^2 area) was polished with wet alumina slurry for at least 10 min and rinsed repeatedly with water. For complete removal of fouling layer (self-assembled monolayers) on the AuE, the polished surface was dipped in 1:1 EtOH–H_2O solution containing 0.5 M $NaBH_4$ for 10 min [36, 38]. The electrode was treated by cycling of the potential between −0.3 and 1.5 V at 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 pre-treated gold electrode for self-assembly. This probe self-assembled AuE was rinsed with water and then dipped in 1 mM aqueous solution of 6-mercapto-1-hexanol (MCH) at room temperature for 30 min. Evaluation of the PNA self-assembled monolayer formation was performed by using the electrochemical signal of the $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ redox system.

Probe-DNA hybridization

Two types of hybridization were tested in this research, namely drop hybridization and pre-heated electrode drop hybridization. Drop hybridization was carried out by dropping 6 μl of target ds-DNA onto the PNA self-assembled monolayer (PNA-SAM) AuE for 20 h in a humidity chamber, and then the electrode was rinsed with PBS. In the pre-heated electrode drop hybridization the PNA self-assembled AuE was immersed in Tris–EDTA buffer at 85 °C for 5 min and then a 6-μl droplet of target ds-DNA in PBS was deposited onto the PNA self-assembled AuE in a humidity chamber. Hybridization of the probe with non-complementary ds-DNA sequences was carried out following the same method as described above.

Labeling of the hybrid

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

Voltammetric measurements

The reduction signal of the accumulated MB was measured by using differential pulse voltammetry (DPV) in the 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 by 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 non-hybridized and hybridized status.

Results and discussion

Detection of target DNA as ds-oligonucleotide

Detection of ds-DNA target is accomplished by monitoring of the difference occurring between the DP voltammetric

responses of MB accumulated on the PNA-SAM AuE, in the absence and presence of target. The DP voltammograms for the probe-modified AuE before and after hybridization with 1 μM complementary ds-oligonucleotide confirmed that a significant increase in the MB signal from −200±6.7 nA to −630±19.3 nA was observed (not shown). The enhancement of the peak current is attributed to the hybridization of the PNA probe with the ds-oligonucleotide and indicates that more MB molecules interact with the PNA-SAM/ds-DNA hybrid.

Effect of some experimental variables

SAM formation time and probe concentration are important factors affecting the SAM formation on the electrode. The effect of these parameters was studied by 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 showed that 2 h and 25 μM are a suitable time and concentration for the probe self-assembly, respectively.

The two hybridization procedures, namely drop hybridization and pre-heated electrode drop hybridization, were applied to the PNA-modified AuE. The results depicted in Fig. 1 indicate that the pre-heating of the electrode had a significant effect on the hybridization efficiency, because a 70% increase was observed in the MB signal, compared with the other method. Therefore, the pre-heated electrode drop hybridization method is recommended for future experiments. Note that the probe-modified AuE subjected to the heating procedure showed no significant change in the MB signal when compared with the non-heated probe-modified electrode. This indicates that the MB signal on the probe-modified electrode does not depend on the electrode heating and also no SAM peeling off occurred.

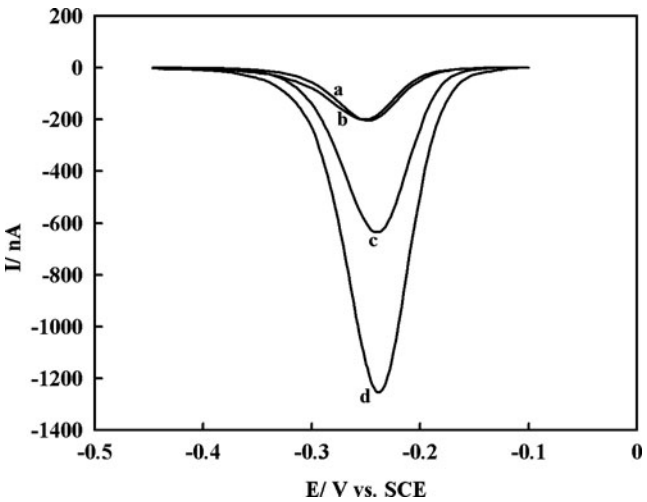

Fig. 1 Differential pulse voltammograms of accumulated MB on the PNA probe-modified AuE: before hybridization **a** without heating or **b** after 10 min heating at 85 °C; and after hybridization using **c** non-heated electrode drop hybridization or **d** pre-heated electrode drop hybridization methods with 5 μM ds-HCV3a target oligonucleotide solution. Electrode conditions: a 6-μl droplet of 25 μM PNA solution dropped onto the pre-treated AuE

There are many reports about the effect of thiolated PNA probe density on hybridization efficiency and kinetics [39–41]. Probe density has a key role in the function of biosensor. 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. We used various concentrations of the probe in order to obtain an optimal PNA probe density for DNA hybridization. The resulting MB DP voltammograms are shown in Fig. 2. As can be seen in the inset in Fig. 2, by increasing the probe concentration up to 5 μM, MB DP voltammetric signal is increased; but at higher PNA probe coverage (probe concentration greater than 7 μM), a decrease in the MB reduction signal was observed. We attribute this effect to a 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 [36, 39–41].

The influence of the hybridization time on the hybridization efficiency was also studied and results are shown in Fig. 3. As displayed in the inset in Fig. 3, by increasing the hybridization time up to 20 h, MB reduction signal increases and then levels off for more hybridization times. Therefore 20 h is suggested as the optimum time for the hybridization; however, as with other sensing procedures, the hybridization time may be reduced to 4–5 h, because under these conditions about 65–70% of maximum hybridization is achieved and is enough for the present biosensor to function correctly, while the selectivity and sensitivity of determination remain nearly unchanged.

Sensor performance

Selectivity

Several hybridization experiments were carried out to assess whether the developed DNA sensor responds selectively to the target ds-DNA. For this purpose, 1 nM of complementary ds-oligonucleotides (ds-HCV3a), two different non-complementary ds-oligonucleotides—ds-UGT and ds-HCV1a, and a mixture of them and a mixture of complementary dsHCV3a and two non-complementary ds-oligonucleotides were used. As seen in Fig. 4, the interaction between the non-complementary oligonucleo-

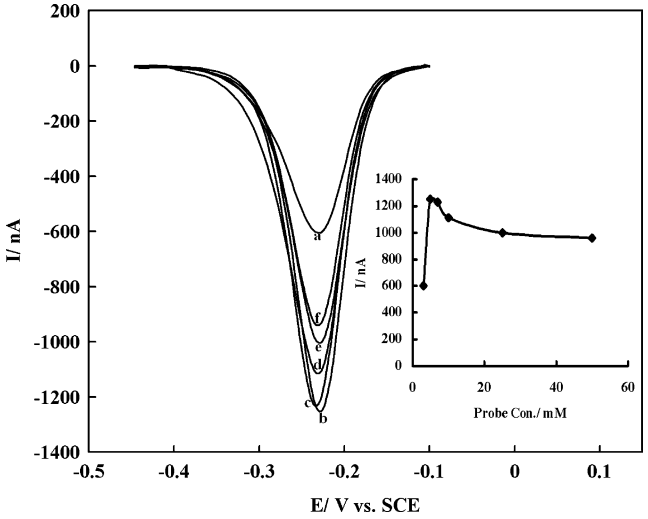

Fig. 2 Differential pulse voltammograms of accumulated MB on PNA probe-modified AuE using various probe concentrations, namely **a** 3, **b** 5, **c** 7, **d** 10, **e** 25, and **f** 50 μM, after hybridization with 5 μM ds-HCV3a target oligonucleotide solution. *Inset* variation of the MB peak current versus probe solution concentration

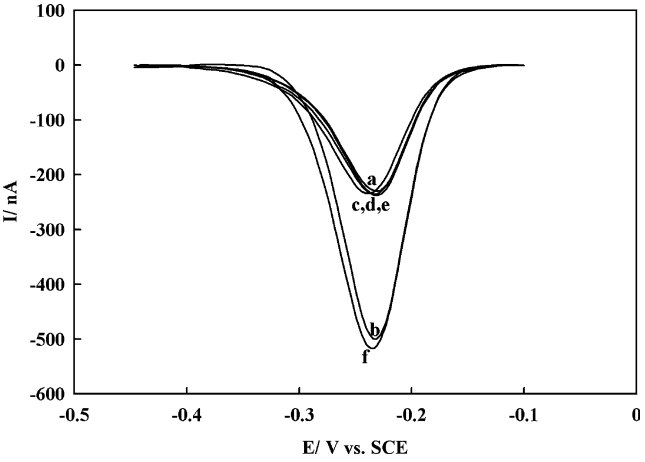

Fig. 4 Differential pulse voltammograms of accumulated MB on the PNA probe-modified AuE after hybridization with: **a** blank buffer solution; 1 nM of **b** ds-HCV3a complementary oligonucleotides and **c** ds-UGT; **d** ds-HCV1a; **e** mixed solution of ds-UGT and ds-HCV1a as ds-non-complementary oligonucleotides; and **f** after hybridization with ds-HCV3a in a mixture solution of ds-HCV3a and two non-complementary oligonucleotides each with same concentration of 1 nM. Electrode conditions: as in Fig. 3

tides and self-assembled probe did not lead to a significant increase in the MB reduction signal (curves c, d, and e) due to the absence of significant hybridization between the probe and the non-complementary ds-DNAs (compare curves c, d, and e with curve a). As shown also in Fig. 4 the MB signals for the hybridized complementary ds-DNA alone (curve b) and in the presence of two non-complementary ds-DNA (curve f) are almost the same (compare curve b with curve f). This result indicates that only the complementary ds-DNA could form a triplex on the probe self-assembled AuE. Thus the biosensor can detect fully matched ds-DNA in a mixed solution of complementary and non-complementary ds-oligonucleotides, and high selectivity of this new strategy (ds-DNA complementary detection) is confirmed.

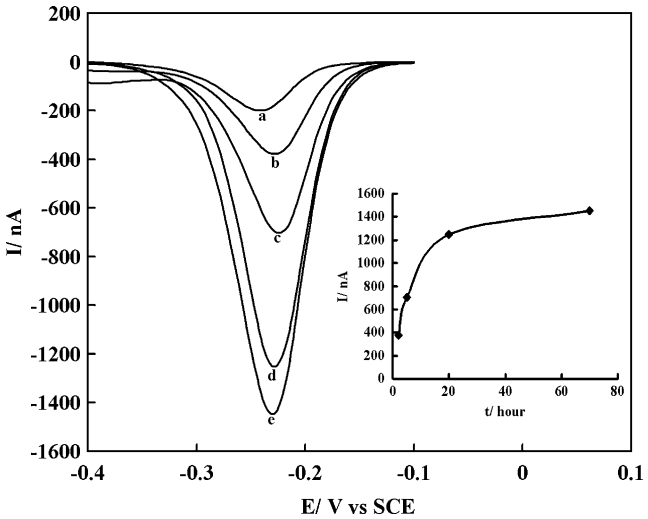

Fig. 3 Differential pulse voltammograms of accumulated MB on the PNA probe-modified AuE before (**a**) and after hybridization with 5 μM ds-HCV3a target oligonucleotide at various hybridization times: **b** 2, **c** 5, **d** 20, and **e** 72 h. *Inset* variation of the MB peak current versus hybridization time. Electrode conditions: a 6-μl droplet of 5 μM PNA solution was dropped onto the pre-treated AuE

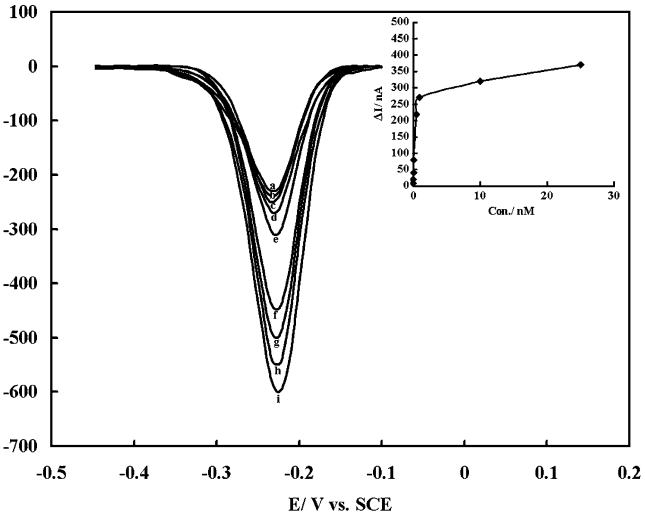

Fig. 5 Differential pulse voltammograms of accumulated MB on the PNA probe-modified AuE after hybridization with ds-HCV3a target oligonucleotide of different concentrations: **a** 0, **b** 10, **c** 25, **d** 50, **e** 100, **f** 500, **g** 1,000, **h** 10,000, **i** 25,000 pM. *Inset* variation of the difference between the MB reduction signals of the PNA probe-modified AuE in the presence and absence of ds-HCV3a target sample (ΔI) versus target ds-DNA concentration. Electrode conditions: as in Fig. 4

Sensitivity

The MB reduction signal on the probe-modified Au electrode in the presence of ds-HCV3a target is increased with increasing the ds-target concentration (Fig. 5). As seen in the inset in Fig. 5 the difference between the MB reduction signal of the probe-modified AuE in the presence and in the absence of ds-target sample (ΔI) increases with increasing ds-target concentration and levels off at ca. 1 nM. Thus, in the presence of this concentration of the ds-target, the maximum capacity of the probe available on the electrode surface is involved in the hybridization event. The calibration graph is linear between 10 and 100 pM with regression equation $y\ \Delta I = 30.6 + 0.79\,X\,(pM)$ and correlation coefficient of 0.999. The detection limit, calculated by means of the following equation: $y_{LOD}\ \Delta I = y_B + 3S_{y/x}$ and the regression equation was about 1.8×10^{-12} M, where y_B is the signal of the blank (here intercept of calibration graph) and $S_{y/x}$ is the standard deviation of the blank (here standard deviation of the calibration graph). The relative standard deviation of measurements of 100 pM of ds-HCV3a performed with three independent probe-modified electrodes was 3.1%, 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 direct detection of ds-oligonucleotide corresponding to HCV3a via triplex formation. The enhancement of the peak current arising from the hybridization of the PNA probe with the target HCV3a ds-oligonucleotide indicates that more MB molecules intercalate within the triple-helix of PNA–ds-DNA. This provides the possibility of monitoring the hybridization of the PNA probe with the target ds-DNA. The present strategy could be considered as a fundamental platform for the development of further DNA detection techniques.

References

1. Nielsen PE, Egholm M, Berg RH, Buchardt O (1991) Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. Science 254:1497–1500
2. Egholm M, Buchardt O, Nielsen PE, Berg RH (1992) Peptide nucleic acids (PNA). oligonucleotide analogs with an achiral peptide backbone. J Am Chem Soc 114:1895–1898
3. Egholm M, Buchardt O, Christensen L, Behrens C, Freier SM, Driver DA, Berg RH, Kim SK, Norden B, Nielsen PE (1993) PNA hybridizes to complementary oligonucleotides obeying the Watson–Crick hydrogen-bonding rules. Nature 365:566–568
4. Mateo-Martí E, Briones C, Pradier CM, Martín-Gago JA (2007) A DNA biosensor based on peptide nucleic acids on gold surfaces. Biosens Bioelectron 22:1926–1932
5. Demidov VV, Potaman VN, Frank-Kamenetskii MD, Egholm M, Buchard O, Sönnichsen SH, Nielsen PE (1994) Stability of peptide nucleic acids in human serum and cellular extracts. Biochem Pharmacol 48:1310–1313
6. Nielsen PE, Egholm M (1999) Peptide nucleic acids: protocols and applications. Horizon Bioscience, Wymondham, UK
7. Wang J, Nielsen PE, Jiang M, Cai X, Fernandes JR, Grant DH, Ozsoz M, Beglieter A, Mowat M (1997) Mismatch-sensitive hybridization detection by peptide nucleic acids immobilized on a quartz crystal microbalance. Anal Chem 69:5200–5202
8. Endo T, Kerman K, Nagatani N, Takamura Y, Tamiya E (2005) Label-free detection of peptide nucleic acid-DNA hybridization using localized surface plasmon resonance based optical biosensor. Anal Chem 77:6976–6984
9. Liu J, Tiefenauer L, Tian S, Nielsen PE, Knoll W (2006) PNA-DNA hybridization study using labeled streptavidin by voltammetry and surface plasmon fluorescence spectroscopy. Anal Chem 78:470–476
10. Uno T, Tabata H, Kawi T (2007) Peptide nucleic acid-modified ion-sensitive field-effect transistor-based biosensor for direct detection of DNA hybridization. Anal Chem 79:52–59
11. Ohtake T (2006) Development of high sensitive DNA sensor by using probe PNA with IS-FET electrode. Electrochem 74:114–117
12. Wang J, Palecek E, Nielsen PE, Rivas G, Cai X, Shiraishi H, Dontha N, Luo D, Farias PAM (1996) Peptide nucleic acid probes for sequence-specific DNA biosensors. J Am Chem Soc 118:7667–7670
13. Aoki H, Buhlmann P, Umezawa Y (2000) Electrochemical detection of a one-base mismatch in an oligonucleotide using ion-channel sensors with self-assembled PNA monolayers. Electroanalysis 12:1272–1276
14. Sugawara M, Hirano A, Buhlmann P, Umezawa Y (2002) Design and application of ion-channel sensors based on biological and artificial receptors. Bull Chem Soc Jpn 75:187–201
15. Kerman K, Ozkan D, Kara P, Erdem A, Meric B, Nielsen PE, Ozsoz M (2003) Label-free bioelectronic detection of point mutation by using peptide nucleic acid probes. Electroanalysis 15:667–670
16. Umezawa Y, Aoki H (2004) Ion channel sensors based on artificial receptors. Anal Chem 76:320A–326A
17. Kerman K, Vestergaard M, Nagatani N, Takamura Y, Tamiya E (2006) Electrochemical genosensor based on peptide nucleic acid-mediated PCR and asymmetric PCR techniques: electrostatic interactions with a metal cation. Anal Chem 78:2182–2189
18. Aoki H, Tao H (2007) Label- and marker-free gene detection based on hybridization-induced conformational flexibility changes in a ferrocene–PNA conjugate probe. Analyst 132:784–791
19. Bard AJ, Faulkner LR (2001) In: Electrochemical methods: fundamentals and applications, 2nd edn. Wiley, New York
20. Palecek E, Fojta M (2001) Detecting DNA hybridization and damage. Anal Chem 73:74A–83A
21. Wang J, Rivas G, Cai X, Chicharro M, Parrado C, Dontha N, Begleiter A, Mowat M, Palecek E, Nielsen PE (1997) Detection of point mutation in the *p*53 gene using a peptide nucleic acid biosensor. Anal Chim Acta 344:111–118
22. Tomschik M, Jelen F, Havran L, Trnkova L, Nielsen PE, Palecek E (1999) Reduction and oxidation of peptide nucleic acid and DNA at mercury and carbon electrodes. J Electroanal Chem 476:71–80
23. Wittung-Stafshede P, Rodahl M, Kasemo B, Nielsen PE, Norden B (2000) Detection of point mutations in DNA by PNA-based quartz-crystal biosensor. Coll Surf A 174:269–273
24. Wang J (2002) Electrochemical nucleic acid biosensors. Anal Chim Acta 469:63–71

25. De-los-Santos-Allvarez P, Lobo-Castanon MJ, Miranda-Ordieres AJ, Tunon-Blanco P (2004) Current strategies for electrochemical detection of DNA with solid electrodes. Anal Bioanal Chem 378:104–118
26. Kerman K, Kobayashi M, Tamiya E (2004) Recent trends in electrochemical DNA biosensor technology. Meas Sci Technol 15: R1–R11
27. Kuhn H, Demidov VV, Coull JM, Fiandaca MJ, Gildea BD, Frank-Kamenetskii MD (2002) Hybridization of DNA and PNA molecular beacons to single-stranded and double-stranded DNA targets. J Am Chem Soc 124:1097–1103
28. Baker ES, Hong JW, Gaylord BS, Bazan GC, Bowerset MT (2006) PNA/dsDNA complexes: site specific binding and dsDNA biosensor applications. J Am Chem Soc 128:8484–8492
29. Pournaghi-Azar MH, Hejazi MS, Alipour E (2006) Developing an electrochemical deoxyribonucleic acid (DNA) biosensor on the basis of human interleukine-2 gene using an electroactive label. Anal Chim Acta 570:144–150
30. Sabzi RE, Sehatnia B, Pournaghi-Azar MH, Hejazi MS (2008) Electrochemical detection of human papilloma virus (HPV) target DNA using MB on pencil graphite electrode. J Iran Chem Soc 5:476–483
31. Hejazi MS, Alipour E, Pournaghi-Azar MH (2007) Immobilization and voltammetric detection of human interleukine-2 gene on the pencil graphite electrode. Talanta 71:1734–1740
32. Pournaghi-Azar MH, Hejazi MS, Alipour E (2007) Detection of human interleukine-2 gene using a label-free electrochemical DNA hybridization biosensor on the basis of a non-inosine substituted probe. Electroanalysis 19:466–472
33. Hejazi MS, Pournaghi-Azar MH, Alipour E, Karimi F (2008) Construction, electrochemically biosensing and discrimination of recombinant plasmid (pEThIL-2) on the basis of interleukine-2 DNA insert. Biosens Bioelectron 23:1588–1594
34. Pournaghi-Azar MH, Alipour E, Zununi S, Foruhandeh H, Hejazi MS (2008) Direct and rapid electrochemical biosensing of the human interleukin-2 DNA in unpurified polymerase chain reaction (PCR)-amplified real samples. Biosens Bioelectron 24:524–530
35. Pournaghi-Azar MH, Ahour F, Hejazi MS (2009) Differential pulse voltammetric detection of hepatitis C virus 1a oligonucleotide chain by a label-free electrochemical DNA hybridization biosensor using consensus sequence of hepatitis C virus 1a probe on the pencil graphite electrode. Electroanalysis 21:1822–1828
36. Hejazi MS, Pournaghi-Azar MH, Ahour F (2009) Electrochemical detection of short sequences of hepatitis C 3a virus using a peptide nucleic acid-assembled gold electrode. Anal Biochem 399:118–124
37. Hejazi MS, Raoof J-B, Ojani R, Golabi SM, Hamidi Asl E (2010) Brilliant cresyl blue as electroactive indicator in electrochemical DNA oligonucleotide sensors. Bioelectrochem 78:141–146
38. Yuan M, Zhan S, Zhou X, Liu Y, Feng L, Lin Y, Zhang Z, Hu J (2008) A method for removing self-assembled monolayers on gold. Langmuir 24:8707–8710
39. Herne TM, Tarlov MJ (1997) Characterization of DNA probes immobilized on gold surfaces. J Am Chem Soc 119:8916–8920
40. Peterson AW, Heaton RJ, Georgiadis RM (2001) The effect of surface probe density on DNA hybridization. Nucleic Acids Res 29:5163–5168
41. Steel AB, Levicky RL, Herne TM, Tarlov M (2000) Immobilization of nucleic acids at solid surfaces: effect of oligonucleotide length on layer assembly. Biophys J 79:975–981