Available online at www.sciencedirect.com
ScienceDirect
Bioelectrochemistry 70 (2007) 245–249
Bioelectrochemistry
www.elsevier.com/locate/bioelechem

Short communication

Electrochemical detection of short sequences related to the hepatitis B virus using MB on chitosan-modified CPE

Guo Mandong *, Li Yanqing, Guo Hongxia, Wu Xiaoqin, Fan Lifang

Department of Chemistry, Shanxi Normal University, Linfen 041004, Shanxi, China

Received 9 May 2006; received in revised form 27 July 2006; accepted 15 September 2006
Available online 22 September 2006

Abstract

A novel electrochemical DNA biosensor based on methylene blue (MB) and chitosan-modified carbon paste electrode (CCPE) for short DNA sequences and polymerase chain reaction (PCR) amplified real samples related to the hepatitis B virus (HBV) hybridization detection is presented. Differential pulse voltammetry (DPV) was used to investigate the surface coverage and hybridization event. The decrease in the peak current of MB, an electroactive label, was observed upon hybridization of probe with the target. Numerous factors affecting the target hybridization and indicator binding reaction are optimized to maximize the sensitivity.

Keywords: DNA biosensor; Hybridization; Chitosan; MB; HBV

1. Introduction

DNA hybridization biosensor holds an enormous potential for pharmaceutical, clinical and forensic application [1]. The enormous information generated in the Human Genome Project has generated tremendous demands for innovative analytical tools capable of delivering the genetic information in a faster, simpler, and cheaper manner at the sample source. In this context, DNA electrochemical biosensors with the nucleic acids directly immobilized on an electrode surface for hybridization offer innovative routes [2]. Electrochemical biosensors offer great profits for their high sensitivity, small dimensions, low-cost/low-volume, and compatibility with microfabrication technology [3,4]. Many protocols have been proposed for electrochemical monitoring of DNA hybridization. Oligonucleotides labeled with enzymes such as alkaline phosphatase [5] or electroactive tags such as ferrocene or anthraquinone [6,7] or nanoparticle [8] have been used in hybridization detection analysis.

Persistent infection with hepatitis B virus (HBV) is a major health problem worldwide and may lead to chronic hepatitis, cirrhosis and primary liver cancer [9]. There has been considerable interest in developing reliable methods for detecting and quantifying the HBV.

Chitosan oligomer [10] is a kind of β-1,4-linked glucosamine oligomer; It is a natural cationic polymer and can form a stable complex with the polyanionic phosphodiester backbones of DNA, either native or denatured. DNA electrochemical biosensor based on cationic polymer chitosan to immobilized ssDNA, which have been developed [11,12]. The immobilization of target ssDNA on glassy carbon electrode (GCE) was fabricating according to the literature by formation of a stable electrostatic complex with the cationic polymer chitosan [10]. Similar approach with target DNA immobilization at the electrode was recently used by Li et al. [11]. The main advantages of using chitosan were that chitosan could form a tight complex with DNA, which made the immobilization very stable. Compared with the DNA immobilization methods using self-assembly monolayer (SAM) and biotin, the use of chitosan for DNA immobilization did not need the mercapto-DNA biotin-DNA, which could greatly reduce the detection cost.

This report describes the detection of specific DNA sequences related to the HBV by using an electrochemical DNA biosensor

* Corresponding author. Department of Chemistry, Shanxi Normal University, Gongyuan Street No. 1, Linfen city, Shanxi province, China. Tel.: +86 357 2051382; fax: +86 357 2051000.
E-mail address: guomd@dns.sxtu.edu.cn (G. Mandong).

1567-5394/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.bioelechem.2006.09.002

and MB as a hybridization indicator. In recent year, detection of specific DNA sequences related to the HBV is performed by using different electrochemical method [12,13], but the detection of HBV on chitosan-modified carbon paste electrode (CCPE) is reported for the first time. The scheme, in our paper, is a simple DNA immobilization strategy that provides a well-defined recognition interface. Oligonucleotide probe could easily and fast be attached the chitosan film at the electrode. The results showed that the probe ssDNA immobilized on the chitosan film modified electrode could be successfully used for discriminating between a complementary target sequence and a noncomplementary strand.

2. Experimental

2.1. Apparatus

Differential pulse voltammetry (DPV) was performed with a LK98II Microcomputer-based Electrochemical System(LANLIKE, Tianjin, China).The three-electrode system consisted of a chitosan-modified carbon paste electrode as the working electrode, the Ag/AgCl (saturated KCl) reference electrode and a platinum wire as the auxiliary electrode. A magnetic stirrer provided the convective transport.

2.2. Chemicals

All stock solutions were prepared with double distilled-water. MB was obtained from Beijing Chemical Reagent Company and used without further purification. Chitosan was obtained from China chemical Reagent Company.20 mmol l^{-1} Tris–HCl buffer (pH 7.00), 0.3 mol l^{-1} PBS (0.3 mol l^{-1} NaCl + 10 mmol L^{-1} salium phosphate buffer, pH 7.00) and TE (10 mmol l^{-1} Tris–HCl, 1 mmol l^{-1} EDTA, pH 8.00) were used. Other reagents were commercially available and were all of analytical reagent grade. The synthetic oligonucleotide were purchased from Sheng-gong Bioengineering (Shanghai, China), and their base sequences are as follows:

Probe 5′-GAG GAG TTG GGG GAG CAC ATT-3′
Complementary target 5′-AAT GTG CTC CCC CAA CTC CTC-3′
One-mismatch target 5′-AAT GTG GTC CCC CAA CTC CTC-3′
Noncomplementary target 5′-AAC GTG TGA ATG ACC CAG TAC-3′

The oligonucleotide stock solution (100 mg l^{-1}) was prepared with TE (10 mmol l^{-1} Tris–HCl, 1 mmol L^{-1} EDTA, pH 8.00) and kept frozen. All experiment were conducted at room temperature (25 °C).

2.3. Procedure

2.3.1. PCR amplification of DNA from blood samples

DNA was extracted from venous blood by applying a salting out method [14]. PxE Thermal cycler (Thermo Electron corporation Molecular Biology) using oligonucleotide primers 5′-GAG GAG TTG GGG GAG CAC ATT-3′and 5′-GGG TCA ATG TCC ATG CCC TAA-3′ in 0.2 thin-walled polypropylene tubes. The amplification mixture, in a final of 100 μL, containing 10.0 μL 10 × PCR buffer, 6.0 μL 25 mM $MgCl_2$, 6 μL 4 × 5 mM dNTPs, 3.0 μL of each primer, and 10.0 μL of 1×10^7 copies of HBV DNA as template. Before the amplification cycles, the amplification mixture was predenatured at 100 °C for 5 min and then was supplemented with 25 units Taq polymerase, The thermal program comprised an initial denaturation at 94 °C for 90 s followed an initial denaturation at 94 °C for 25 s and at 60 °C for 60 s. After the cycle step, an extension step was performed at 70 °C for 10 min. Finally the mixture was kept at 4 °C before use. Maximum ramp rates were used throughout. The fragments were separated by agarose gel electrophoresis to obtain 100 μL PCR product. The amplification product without further purification product without further purification gave an A_{260}/A_{280} ratio of 1.82, thus the DNA was pure enough.

2.3.2. Preparation of chitosan-modified CPE (CCPE)

The carbon paste was prepared in the usual way by hand-mixing graphite powder to nujol oil. The ratio of graphite powder to nujol oil was 75:25. Then, a portion of the carbon paste was mixed with chitosan, so that the final quantity was 5% (w/w). The mixture was tightly packed into a glass tube (3 mm id.). A copper wire inserted into the carbon paste provided the electrical contact. The surface was polished on a weighing paper to a smoothed finish. Then, the CCPE was immersed into 2% acetate and take it out at once, and then it was naturally dried.

2.3.3. Probe ssDNA immobilization on CCPE

The immobilization of probe ssDNA on CCPE was fabricating according to the literature by formation of a stable electrostatic complex with the cationic polymer chitosan [11].The pretreatment CCPE was rinsed with double-distilled water then immersed in 500 μl TE solution containing 1.5×10^{-7} mol L^{-1} probes ssDNA. The solution was stirred at room temperature (25 °C) for 30 min. After that, the electrode was rinsed three times with PBS (0.3 mol l^{-1} NaCL + 10 mmol L^{-1} salium phosphate buffer, pH 7.00) before hybridization.

2.3.4. Hybridization and indicator binding to the hybrid

Hybridization reaction was conducted by immersing the DNA probe captured CCPE into a stirred hybridization solution (0.3 mol l^{-1} PBS, pH 7.00) containing the target DNA at room temperature (25 °C) for 15 min, the electrode was washed 10 s with 20 mmol L^{-1} Tris–HCl buffer (pH 7.00) and remove the non-hybridized DNA. Thus, a hybrid-modified CCPE was obtained. After that, the electrode was immersed into the stirred 20 mmol l^{-1} MB with 20 mmol l^{-1} NaCl for 5 min without applying any potential. After accumulation of MB, the electrode was rinsed with 20 mmol l^{-1} Tris–HCl buffer (pH 7.00) 5 s.

2.3.5. Electrochemical detection

The electrochemical investigation was carried out in a 10 ml electrochemical cell with the hybridized CCPE as the working

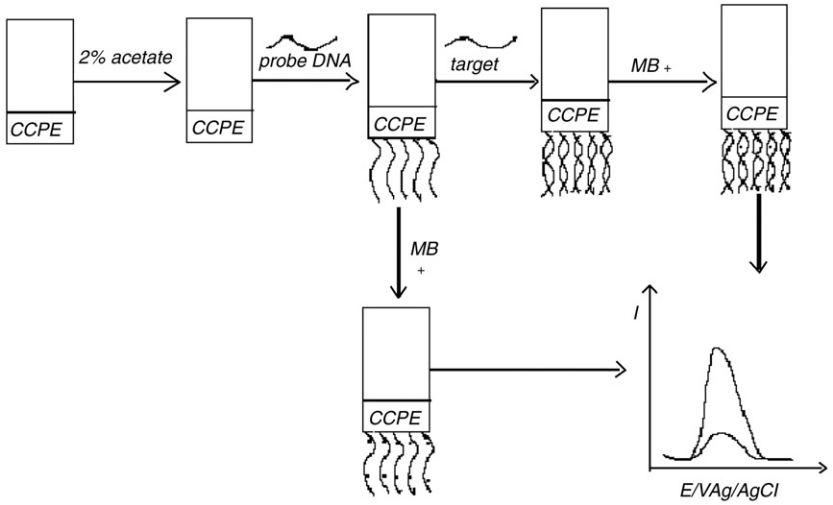

Fig. 1. Steps involved in the fabrication of the probe DNA-modified electrode and the detection of a target sequence.

electrode, the Ag/AgCl reference electrode and a platinum wire as the auxiliary electrode by DPV with amplitude of 10 mV at 20 mV/s scan rate. The reduction signal of the accumulated MB was obtained in the MB free 20 mmol l^{-1} Tris–HCl buffer (pH 7.00).

3. Results and discussion

Principle of the electrochemical DNA detection assay based on MB indicator consisted of four steps: immobilization of probe DNA on the CCPE, hybridization with target, MB accumulation, and electrochemical DPV detection of ssDNA or dsDNA by using the reduction signals of MB. The whole process is described in Fig. 1.

The effect of experimental parameters including probe concentration, immobilization time of probe and hybridization time were also explored for optimum analytical performance.

Optimization studies were carried out to obtain reproducible signals with high sensitivity. Probe concentration greatly affected the binding of the short DNA sequences related to the hepatitis B virus onto the electrode surface. It was observed that the reduction signals of MB increased almost linearly until 1.5×10^{-7} mol L^{-1} probe ssDNA and then reached a plateau, indicating that full surface coverage by the short DNA of HBV (Fig. 2) by Chronopotentiometric. Thus, 1.5×10^{-7} mol L^{-1} probe ssDNA was used for further experiments. The change trend of probe immobilization time also was similar to probe concentration (Fig. 3) by DPV. Thus, 30 min was used for further experiments to immobilize the probe.

The time of hybridization for DNA probe with complementary target DNA is displayed in Fig. 4. The response for the reduction of MB after hybridization with the increasing of time decreased up to 15 min indicating all the available immobilized probe on the CCPE surface have become involved in hybridization. After 15 min, the reduction signal of MB started

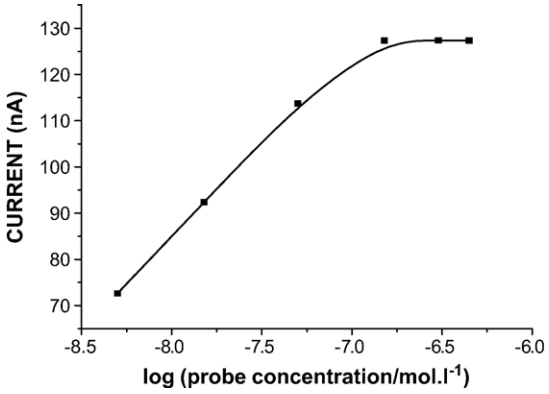

Fig. 2. Probe concentration effect onto the reduction signal of MB. Probe immobilization was allowed to proceed for 5.0×10^{-9} mol l^{-1}, 1.5×10^{-8} mol l^{-1}, 5.0×10^{-8} mol l^{-1}, 1.5×10^{-7} mol l^{-1}, 3.0×10^{-7} mol l^{-1}, 4.5×10^{-7} mol l^{-1} at a stirred TE buffer solution; the CCPE as working electrode, the Ag/AgCl reference electrode and a platinum wire as the auxiliary electrode. Measurement: Chronopotentiometric Probe immobilization signals of MB, the stripping were performed in a quiescent 20 mmol l^{-1} Tris–HCl buffer (pH 7.00) using an applied oxidative current of 10 nA.

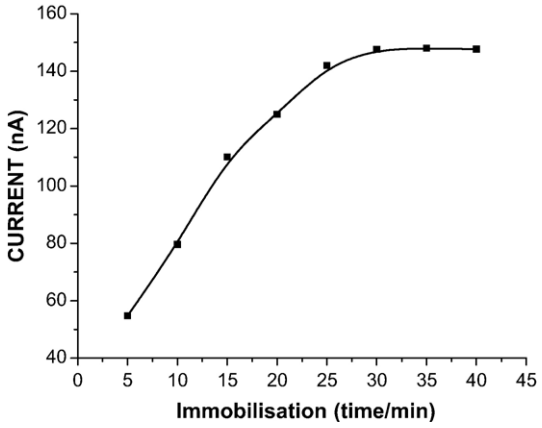

Fig. 3. Probe immobilization time effect onto the reduction signal of MB. Probe immobilization was allowed to proceed for 5, 10, 15, 20, 25, 30, 35,40 min in a stirred TE buffer solution containing 1.0×10^{-7} mol l^{-1} of DNA probe. Measurement: Differential pulse voltammogram with amplitude of 10 mV at 20 mV/s scan rate.

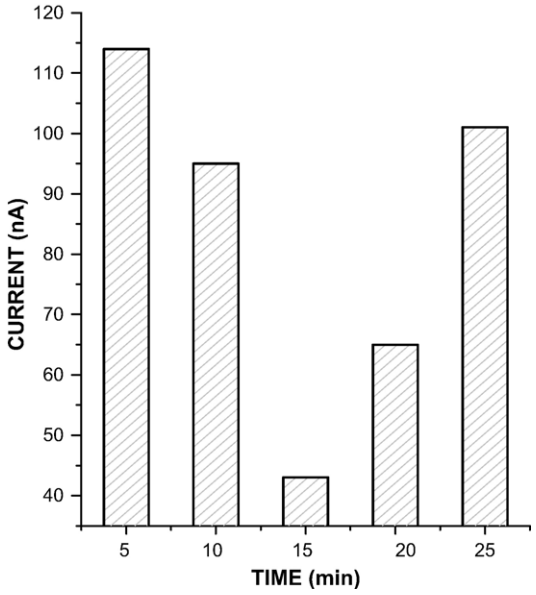

Fig. 4. Hybridization time effect onto the reduction signal of MB. Probe immobilization (1.5×10^{-7} mol l^{-1} in a stirred TE buffer solution 30 min), hybridization times tested: 5,10,15,20,25 min. Other condition as Fig. 3.

to increase, as the hybridization time increased. This increase was attributed to the CCPE surface following events: single stranded target DNA from the sample non-specifically accumulated on the electrode surface and could not be removed with the washing step. The guanine bases from the excess single-stranded target oligonucleotide made the signals of MB increase. This situation proves that the hybridization time is directly affecting the MB signal. For the subsequent experiments, 15 min was used as the optimum hybridization time.

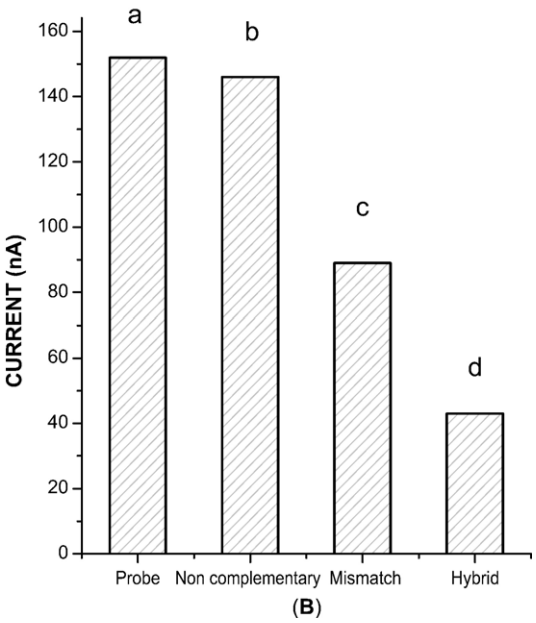

Fig. 5. Bar chart of DPV responses using the reduction signal of MB for: (a) Probe-modified CCPE; (b) After exposure to a non-complementary target; (c) After exposure to a target sequence containing a single-base pair mismatch; (d) After exposure to a complementary target. Other conditions as Fig. 3.

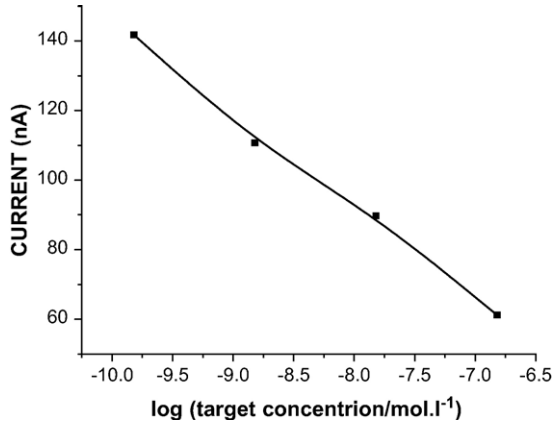

Fig. 6. DPV using 20 μM MB as the redox indicator for the resulting logarithmic standard plot of different target concentration from 0 to 1.5×10^{-9} mol/L. Other conditions as Fig. 3.

Fig. 5 shows the DPV for the reduction signals of MB at ssDNA probe immobilized electrode (Fig. 5a) and after hybridization with complementary DNA sequence (Fig. 5d), and the one-single mismatch DNA sequence (Fig. 5c), and the non-complementary sequence (Fig. 5b). The highest MB reduction signal was obtained with the ssDNA probe on the CCPE surface (Fig. 5a), because MB has a strong affinity for the free guanine bases and hence the greatest amount of MB accumulation occurs at this surface. A significant decrease in the voltammetric reduction signal of MB was observed when incubating with the complementary target sequence (Fig. 5d), which was because the interaction of MB and guanine residues of the probe was prevented by duplex formation on CCPE surface. The hybrid formed with the single-base mismatch (Fig. 5c) containing oligonucleotide result unbound two guanine bases. These accessible guanine bases slightly increased the voltammetric signal obtained with the hybrid-modified CCPE surface. This difference indicates that the complete hybridization was not accomplished. The peak current value hardly decreased when it exposed the ssDNA CCPE to the non-complementary oligonucleotide in the control experiment (Fig. 5b), which indicated that no change occurred at the CCPE surface and hence hybridization was not achieved. The results

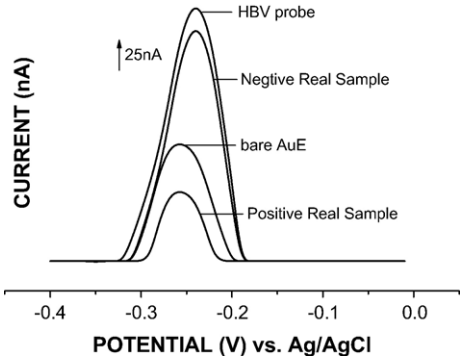

Fig. 7. DPV responses using the reduction signal of MB for: HBV probe modified CCPE; HBV probe modified CCPE after hybridization with PCR amplified negative real sample; bare CCPE surface; HBV probe modified CCPE after hybridization with PCR amplified positive real sample. Other conditions as Fig. 3.

demonstrate that only a complementary sequence could form a double-stranded DNA with the DNA probe and give a significant decreasing signal. No changes in the signal were observed for non-complementary sequence, which shows the high selectivity of the hybridization detection.

The sensitivity of the electrochemical hybridization assay was investigated by varying the target oligonucleotide concentration according to the procedure described. The average reduction currents of MB were linear with the logarithmic value of the complementary. Oligonucleotides concentration ranged from 1.5×10^{-9} mol l^{-1} to 1.5×10^{-7} mol l^{-1} (show in Fig. 6). The regression equation was $y=191.22\ \log x-24.76$ (x was the concentration of target DNA, pmol l^{-1}; y was the DPV peak current of MB, nA), and regression coefficient (r) of the linear curve was 0.9962.A detection limit of 3.0×10^{-10} mol l^{-1} of oligonucleotides can be estimated.

During the optimization studies, it was observed that constituents of the PCR (primer and polymerase) did not show any voltammetric signals in the range used in the absence of MB. Even in the presence of MB, the constituents of PCR did not increase the nonspecific adsorption effect of MB at the CCPE surface.

Fig. 7 represents the MB signals obtained, when the probe for HBV was immobilization and hybridized with real samples in the hybridization step. The MB signals from the HBV probe modified CCPE was higher than the CCPE surface. The obvious decrease in the magnitude of the MB signals obtained with PCR positive real samples. The decrease in the voltammetric signals showed that hybridization at the CCPE surface occurred and MB could not interact with the bound guanine base of the hybrid. The decrease in the reduction signal of MB was attributed to the steric inhibition of the reducible groups of MB because of the formation of hybrid at the CCPE surface. If the blood sample, from which virus DNA was extracted, were a negative real sample, the amplified PCR product would not contain a target sequence complementary to the specific HBV probe. The interaction between these negative real samples and the immobilization probe, did not lead to the hybridization and so the magnitude of the MB signal was nearly as high as the probe signal (Fig. 7).

In this study, electrochemical DNA biosensor, which offers fast and reliable results for HBV analysis, is described. The biosensor is able to detect the complementary sequence by using MB as the hybridization indicator. It was able to distinguish between full-matched (target), mismatched and noncomplementary at the CCPE surface. DNA sequence with a detection limit 3.0×10^{-10} mol l^{-1} of short target sequence was obtained. This method provides a highly sensitivity detection of DNA of 1×10^{4} copies (1.7×10^{-20} mol) of original genomic HBV DNA by combining a PCR procedure. This method is one of the most sensitivity at present. Huang-Xian Ju et al. [15] have gained the similar results by CV and XPS techniques.

References

[1] J. Wang, From DNA biosensors to gene chips, Nucleic Acids Res. 28 (2000) 3011–3016.

[2] S.R. Mikkelssen, Electrochemical biosensors for DNA sequence detection, Electroanalysis 8 (1996) 15–19.

[3] D.W. Pang, H.D. Abruna, Micromethod for the investigation of the interactions between DNA and redox-active molecules, Anal. Chem. 70 (1998) 3162–3169.

[4] H.H. Thorp, Cutting out the middleman: DNA biosensors based on electrochemical oxidation, Trends Biotechnol. 16 (1998) 117–121.

[5] A.K. Singh Alfonta, I. Willner, Liposomes labeled with biotin and horseradish peroxidase: a probe for the enhanced amplification of antigen–antibody or oligonucleotide–DNA sensing processes by the precipitation of an insoluble product on electrodes, Anal. Chem. 73 (2001) 91–102.

[6] H. Korri-Youssoufi, B. Makrouf, Electrochemical biosensing of DNA hybridization by ferrocenyl groups functionalized polypyrrole, Anal. Chim. Acta 469 (2002) 85–92.

[7] M. Fojta, R. Doffkova, E. Palecek, Determination of traces of RNA in submicrogram amounts of single- or double-stranded DNAs by means of nucleic acid-modified electrodes, Electroanalysis 8 (1996) 420–426.

[8] H. Cai, Y.Q. Wang, P.G. He, Y.Z. Fang, Electrochemical detection of DNA hybridization based on silver-enhanced gold nanoparticle label, Anal. Chim. Acta 469 (2002) 165–172.

[9] R.P. Bealey, Hepatitis B virus — the major etiology of hepatocellular carcinoma, Cancer 61 (1988) 1942–1956.

[10] C. Xu, H. Cai, P.G. He, Y.Z. Fang, Electrochemical detection of sequence-specific DNA using a DNA probe labeled with aminoferrocene and chitosan modified electrode immobilized with ssDNA, Analyst 126 (2001) 62–65.

[11] L. Li, M. Wang, S. Dong, E. Wang, Immobilization target ssDNA on GCE by formation of a stable electrostatic complex with the cationic polymer chitosan, Anal. Sci. 13 (1997) 305–310.

[12] Y.K. Ye, J.H. Zhao, F. Yan, Y.L. Zhu, H.X. Ju, Electrochemical behavior and detection of hepatitis B virus DNA PCR production at gold electrode, Biosens. Bioelectron. 18 (2003) 1501–1508.

[13] A. Erdem, K. Kerman, B. Meric, U.S. Akarca, M. Ozsoz, Novel hybridization indicator methylene blue for the electrochemical detection of short DNA sequences related to the hepatitis B virus, Anal. Chim. Acta 422 (2000) 139–149.

[14] S.A. Miller, D.D. Dykes, H.F. Polesky, A simple salting out procedure for extracting DNA from human nucleated cells, Nucleic Acids Res. 16 (1988) 1215.

[15] H.X. Ju, Y.K. Ye, J.H. Zhao, Y.L. Zhu, Hybridization biosensor using di (2,2′-bipyridine) osmium (III) as electrochemical indicator for detection of polymerase chain reaction product of hepatitis B virus DNA, Anal. Biochem. 313 (2003) 255–261.