J. Iran. Chem. Soc., Vol. 5, No. 3, September 2008, pp. 476-483.

JOURNAL OF THE
Iranian
Chemical Society

Electrochemical Detection of Human Papilloma Virus (HPV) Target DNA Using MB on Pencil Graphite Electrode

R.E. Sabzi[a,b,*], B. Sehatnia[a], M.H. Pournaghi-Azar[c] and M.S. Hejazi[d,e,*]

[a]*Department of Chemistry, Faculty of Science, Urmia University, Urmia, Iran*

[b]*Institute of Biotechnology, Urmia University, Urmia, Iran*

[c]*Faculty of Chemistry, University of Tabriz, Tabriz, Iran*

[d]*Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran*

[e]*Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran*

(Received 20 October 2007, Accepted 23 November 2007)

The present paper describes the use of methylene blue (MB) as an electroactive label on a pencil graphite (lead) electrode (PGE) to provide a well-defined recognition interface for the detection of HPV target DNA. In order to construct the sensor, a 20-mer single strand oligonucleotide probe related to human papilloma virus (HPV) major capsid protein L1 gene was immobilized on the PGE electrode. Hybridization event between the probe and its complementary sequence was studied by measurement of MB signal accumulated on the PGE using square wave voltammetry (SWV) method. Some hybridization experiments with non-complementary oligonucleotides were carried out to examine the selectively of the sensor to the target DNA from other DNAs related to Hepatitis C virus (HCV), fungi, and bacterial genes. Moreover, some factors affecting the function of sensor including electrode activation and probe immobilization condition were also investigated. The data showed that the constructed electrode detects the target DNA with detection limit of 1.2 ng μl^{-1} and discriminates it from various DNAs originated from a wide variety of organisms.

Keywords: Human papilloma virus, Methylene blue, Pencil graphite electrode, Biosensor, Square wave voltammetry

INTRODUCTION

Electrochemically biosensing of DNA targets for the rapid and inexpensive diagnosis of genetic diseases, forensic analysis and other applications is rapidly developing. A large number of genetic information from disease inducing microorganisms are now available and are employed for development of various diagnostic strategies such as DNA biosensor based on electrochemical protocols. Electrochemical biosensing techniques are on great demand due to their high sensitivity, small dimensions, low-cost/low-volume, and compatibility with microfabrication technology [1]. Many strategies have been developed for electrochemical monitoring of DNA hybridization. DNA electrochemical biosensors containing immobilized probes on an electrode for hybridization offer innovative routes [2]. Electroactivity of nucleic acids is due to the electroactivity of DNA bases composing nucleic acid chains [3]. Purine nucleobases have attracted scientists' attention more than pyrimidine bases [4,5]. Due to electroactivity characteristics, purins are considerable bases for determination of nucleic acid concentration using

*Corresponding author. E-mail: rezasabzi@yahoo.com and saeidhejazi@tbzmed.ac.ir

square wave voltammetry (SWV) [6].

DNA immobilization on the transducer has a key role in the performance of the DNA biosensors. Because of wide potential window of carbon electrodes, different carbon-based surfaces have been used as electrochemical transducers for DNA hybridization biosensors. Carbon paste [7], pencil lead [8] and screen printed electrodes [9] have been widely used by various groups to fabricate DNA biosensors.

Methylene blue (MB) has been widely used as an electrochemical intercalator to monitor the DNA hybridization reaction, as ssDNA and dsDNA have different affinity for MB [10,11,12,13,14,15]. Recently we used methylene blue as an electrochemical intercalator to monitor the interleukin-2 DNA hybridization reaction [16]. MB binds specifically to the guanine bases in ssDNA [17] and a lower current signal is observed following DNA hybridization due to less MB that could incorporate to dsDNA. The reason is due to the inaccessibility of the guanine bases in dsDNA. In this paper we describe an electrochemical DNA biosensor based on the employment of sense strand of HPV major capsid protein L1 gene as a probe, MB as an electroactive label and pencil graphite electrode (PGE) as a renewable transducer. Additionally some experimental factors including PGE activation and probe immobilization as well as specificity of the sensor using complementary and various non-complementary DNA segments from human genome, hepatitis C virus, fungi and bacterial cells for the hybridization event were studied.

HPV (human papilloma virus) is the common wart virus causing various kinds of warts (genital warts, plantar warts and flat warts) as well as cervical dysplasia, vaginal dysplasia, and cervical cancer. More than 100 types of HPV are known. Most types of this virus are relatively harmless, like the ones causing common warts on hands and feet. In most people, the virus is cleared by the body's immune response. HPV is contagious even when warts and dysplasia are not present. Some HPV types have a greater association with cervical dysplasia and cancer. This group of HPV includes types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. All of the HPV types are contagious. HPV can lie dormant in human body for an unknown period of years. However, most individuals develop immunity against the virus, after which time the virus is no longer contagious. It is well established that HIV-positive people are more likely to be infected with HPV than HIV-negative people [18]. Due to the key role of HPV in induction of some serious diseases, development of sensitive and rapid detection methods are in demand. Accordingly, this study aimed to develop a simple and sensitive pencil graphite based biosensor for HPV detection.

EXPRIMENTAL

Materials and Methods

A 20-mer oligonucleotide corresponding to sense strand of major capsid protein L1 gene of HPV called HPVp (5´GTA TCT ACC ACA GTA ACA AA 3´) was employed as HPV DNA probe and its complementary strand called HPVc (5´ TTT GTT ACT GTG GTA GAT AC 3´) was used as target oligonucleotide. Some non-complementary oligonucleotids corresponding to various genomes including human, hepatitis C virus, fungi and bacterial cells genome were used to test the selectivity of the purposed electrode. These oligonucleotids include hIL-2 (5´GGA GGA AGT GCT AAA TTT AG 3´) and chIL-2 (5´ CTA AAT TTA GCA CTT CCT CC 3´) corresponding to sense and antisense strands of human interleukine-2 gene. Lb16s (5´ TAC CTT GTT AGG ACT TCA CC 3´) corresponding to Lactobacillus bacterial cells 16s rDNA consensus sequence, HCV (5´GGA GGT CTC GTA GAC CGT GC 3´) corresponding to hepatitis C virus 5´ untranslated region (5´ UTR), 18sr (5´ ATG TAT TAG CTC TAG AAT TA 3´) a 20 nucleotide residues from fungi 18s rDNA were used as non-complementary oligonucleotides, respectively. All of the oligonucleotides were supplied by MWG-BIOTECH (Germany).

Pencil lead (type H) from Fiber Castell (Malaysia) commonly composed of natural graphite; a polymeric binder and clay with different percentages, and a diameter of 2.0 mm was used as pencil graphite electrode. MB was of analytical grade and purchased from Merck. The stock solution of the oligonucleotids was prepared with TE buffer solution (10 mM Tris-HCl, 1 mM EDTA, pH 8.00) and kept frozen. More diluted solutions were prepared using 0.5 M acetate buffer (pH 4.8) containing 20 mM NaCl. All solutions were prepared using deionized and sterilized water. The experimental conditions for electrochemical analysis were: the three-electrode system consisted of a pencil lead graphite electrode

(PGE), an Ag/AgCl (with saturated KCl solution) reference electrode and a platinum wire as counter electrode.

All measurements were done at the room temperature. Experimental conditions for square wave voltammetry were frequency of 200 Hz, amplitude of 25 mV and step potential of 5 mV. Electrochemical experiments were performed using an AUTOLAB PGSTAT 30 electrochemical analysis system equipped with GPES 4.7 software.

Modification of the Working Electrode

Tightly covered pencil lead with Teflon band, was fixed vertically so that when it is immersed in a solution, the contact was only achieved *via* cross section of the lead. The electrode surface was polished on the smooth sandpaper No. 2500 to a smoothed finish before immersing in acetate buffer solution consisting 20 mM NaCl for activation.

Electrochemical Activation of the PGE

The activation of both non-polished and polished PGEs was carried out potentiostatically (using chronoamperometric technique) in acetate buffer solution (pH = 4.8) containing 20 mM NaCl without stirring. In order to obtain an optimum value for activation, the potential of electrode was kept at a fixed value situated between -2.00 and 2.00 V/ref. and for different time durations. Figure 1 shows the variation of MB peak current of a polished, probe-modified and MB accumulated PGE as a function of applied activation potentials for 450 s. As it is seen, the MB signal increases with increasing the activation potential until it reaches a maximum value at 1.5 V/ref. While, a sharp decrease appears at peak current for more positive potentials. So, we used 1.5 V as the optimum potential for activation. The effect of activation time on the probe immobilization was also evaluated by measurement of MB signal, following the activation of PGE at 1.5 V as the optimum potential for different time durations. The results shown in Fig. 2 indicate that the highest peak was obtained with a moderate activation time of 450 s. In other words, the activation of electrode for shorter or longer times than about 450 s is not suitable for probe immobilization, and subsequent adsorption of MB on the electrode. So, 450 s was used subsequently as optimum time for activation process.

Probe Immobilization on the PGE

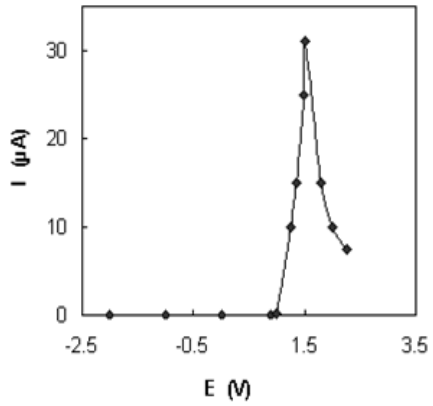

Fig. 1. Variation of SW voltammetric signal (I) of the immobilized HPVp on the activated PGE *vs.* activation potentials (E) for 450 s in 0.5 M acetate buffer solution (pH 4.8) without stirring. Probe immobilization potential was -0.5 V/ref.

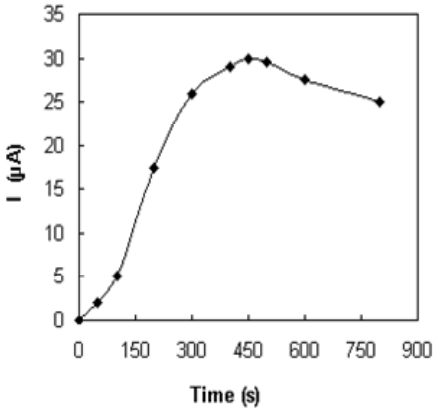

Fig. 2. Variation of SW voltammetric signal (I) of HPVp *vs.* activation time. Activation was achieved at 1.5 V/ref in 0.5 M acetate buffer solution (pH 4.8) without stirring.

The probe immobilization was achieved on both activated and non-activated PGE by applying a fixed potential situated between -0.80 and 0.80 V/ref. to the electrode for 5 min in acetate buffer solution containing 10 ppm HPV and 20 mM NaCl with 200 rpm stirring.

The influence of the imposed potential on the

immobilization of the probe was studied according to the SWV response of the adsorbed MB on probe immobilized electrode. To achieve this, immobilization of HPV probe on the electrode was performed at different imposed potentials ranging between -0.8 and 0.8 V/ref. and for a fixed time of 300 s. The results showed that the immobilization of probe at negative potentials favored the accumulation of MB on the probe-modified PGE and maximum MB signal was observed at -0.5 V/ref. (Fig. 3). Therefore a potential of -0.5 V/ref. was selected for following experiments.

It has reported that larger MB signal obtained for probe-modified PGE at -0.5 V does not arise only from an electrostatic interaction between positively charged MB and electrode surface, but arises from simultaneous binding of MB on the negatively charged electrode and to guanine bases of the probe present on the PGE surface [16]. Our finding confirmed this hypothesis.

The effect of probe immobilization time on the activated PGE was also investigated. The results revealed that the MB reduction signal elevated as the probe immobilization time increased to about 300 s and then slightly decreased with an increase of the time (see Fig. 4). This decrease maybe attributed to the massive accumulation of probe on the electrode and leading to less availability of guanine bases bound to MB.

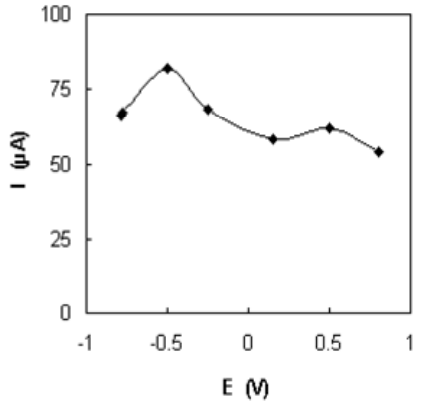

Fig. 3. Effect of immobilization potential on the SW voltammetry response (I) of MB accumulated on the probe-modified activated PGE. Probe immobilization conditions: 300 s in stirring 0.50 M acetate buffer solution containing 10 ppm HPV and 20 mM NaCl.

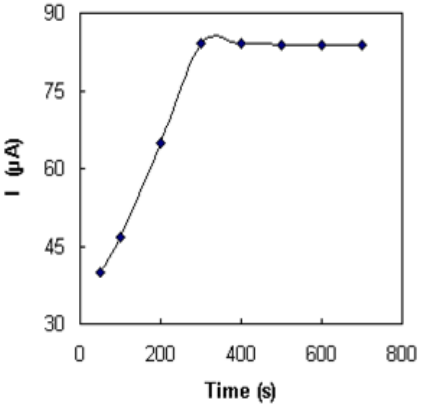

Fig. 4. Variations of SW voltammetric signal (I) of the immobilized HPV on the activated PGE *vs*. activation time. Probe immobilization was carried out at -0.5V/ref in stirring 0.50 M acetate buffer solution containing 10 ppm HPV and 20 mM NaCl.

Hybridization

The hybridization experiments are usually carried out in phosphate, citrate, acetate or Tris-HCl buffers. The hybridization was performed by immersing the probe-modified electrode into the acetate buffer solution containing a known amount of complementary oligonucleotide and 20 mM NaCl for 3 min, while the electrode potential was held at 0.5 V/ref., for which the probe exhibits the highest signal. Then the electrode was rinsed with sterilized and deionized water for 10 s. The same protocol was applied for the hybridization of probe with non-complementary sequences.

Accumulation of MB on the PGE

MB was accumulated on the probe-modified PGE by immersing the modified electrode into the 20 mM Tris-HCl buffer containing 25 µM MB and 20 mM NaCl for 5 min with 200 rpm stirring without applying any potential to the electrode. Then electrode was rinsed in Tris-HCl buffer solution for 10 s. The same method was used for the accumulation of MB on the bare or probe-unmodified electrode. The SW voltammetry was then used to examine the accumulation of MB on the electrodes.

RESULTS AND DISCUSSION

Preliminary Investigation

Early works demonstrated the utility of pencil electrodes and DNA probe for adsorptive stripping potentiometric measurements of trace nucleic acids [8] and, in this work, we used this procedure for detection of oligonucleotides corresponding to human IL-2 gene. The effective adsorptive pre-concentration of DNA onto graphite surface was thus combined with the response of accumulated MB on it.

Some pretreatments are found to be essential for DNA adsorption on the electrodes. In this study, at first, the influence of the pretreatment of PGE at 1.5 V was investigated. Figure 5 shows the square wave voltammograms of accumulated MB on the (a) non-activated bare PGE, (b) probe-modified non-activated PGE, (c) activated bare PGE and (d) probe-modified activated PGE. As seen from Fig. 5, no significant difference was observed between signals of non-activated bare electrode and probe-modified non-activated electrode and activated bare electrode. This was while the signal of the activated probe modified PGE was significantly higher than the signals of other three electrodes. This observation indicates that the adsorption of HPV occurs predominantly at the activated electrode and MB is better accumulated on this electrode compared to other three ones.

Hybridization Detection

Detection of the target DNA is monitored on the SWV responses of MB accumulated on the probe modified PGE in the absence and presence of target. Typically, there is a low MB reduction signal on the bare-activated PGE (curve a) as shown in Fig. 6. It shows the SW voltammogerams for accumulated MB at probe-modified activated PGE before hybridization (curve e) and after hybridization in 20 ng μl^{-1} solutions of complementary (curve b), and non-complementary oligonucleotids including hIL-2 and chIL-2 (curves c and d).

The same experiment was done with other non-complementary oligonucleotides including Lb16s, HCV and 18sr. As it is shown in Fig. 7, there is not a remarkable decrease in the voltammetric signals of non-complementary oligonucleotids compared to the complementary DNA and also these signals are close to the probe signal.

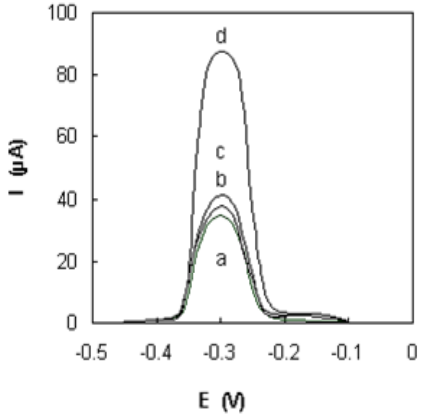

Fig. 5. SW voltammograms (I) of accumulated MB at (a) non-activated bare PGE (b) probe-modified non-activated PGE (c) activated bare PGE and (d) activated probe-modified PGE. Voltammetric condition: Scanning potential steps, 5 mV. SW potential amplitude, 25 mV. Supporting electrolyte, 20 mM Tris-HCl buffer solution containing 20 mM NaCl.

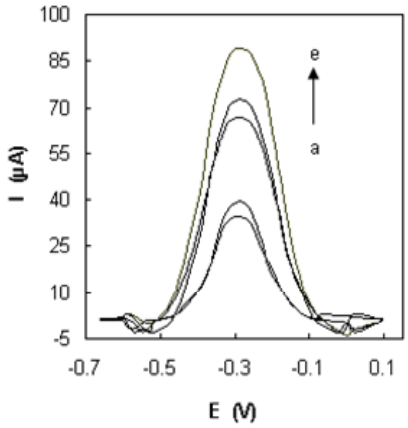

Fig. 6. Square wave voltammograms (I) of accumulated MB at (a) an activated bare electrode, (b) the same electrode after hybridization with complementary, (c, d) the same electrode after hybridization with 20 ng μl^{-1} of hIL-2 and chIL-2 as non-complementary sequences, respectively and (e) probe-modified activated PGE.

The highest MB signal reduction was observed with the probe-modified activated PGE because MB has a strong affinity for free guanine bases present in ssDNA and hence the

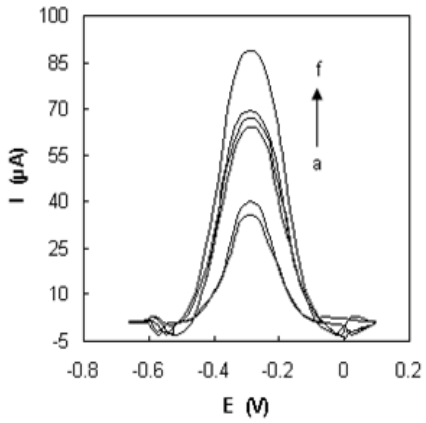

Fig. 7. Square wave voltammograms (I) of accumulated MB at (a) an activated bare electrode, (b) the same electrode after hybridization with complementary, (c, d, e) the same electrode after hybridization with 18sr, HCV and Lb16s as non-complementary sequences, respectively. Solution concentration for targets was 20 ng μl^{-1}, and (f) probe-modified activated PGE.

greatest amount of MB accumulated on the PGE surface. This was while a significant decrease in the MB signal was observed following hybridization of complementary oligonucleotids with the probe. This maybe attributed to less MB accumulation on the ds-DNA caused by the inaccessibility of MB to the guanine bases [19] or maybe due to a steric inhibition of the reducible groups of MB packed between the bulky double helix of the DNA hybrids [20]. However, a slight decrease was observed for the MB signal using Lb16s, HCV and 18sr, which may be attributed to the small and irregular hybridization of non-complementary oligonucleotids with the probe sequences.

The results indicate that only the complementary sequence could form a complete double-strand DNA on the probe, causing a significant decrease in the accumulation of MB.

Analytical Performance of the Sensor

In order to determine the detection limit of the electrode, target DNA was conducted in the presence of various concentrations of the DNA. The results revealed that the difference between MB signal of the probe in the presence and absence of the target HPV is increased with increasing concentration of the target DNA and is leveled off at 15 ng μl^{-1} (Fig. 8).

As seen in the inset of Fig. 8, there is a liner relationship between ΔI and target concentration up to 10 ng μl^{-1} with a correlation coefficient of 0.998. The regression equation is ΔI (μA) = 3.4542C (μM) + 0.827. The detection limit, estimated as three times of the ratio between the blank signal and the sensitivity, is 1.2 ng μl^{-1}.

The relative standard deviation over three independently modified probe electrodes measured at 15 ng μl^{-1} of target was 2.8% indicating a remarkable reproducibility of the detection method.

It should be noted that comparison of ΔI of the complementary target DNA with that of non-complementary DNAs like HCV related that the ΔI of non-complementary DNAs were considerably low indicating that this DNA sensor can distinguish target DNA from others successfully.

CONCLUSIONS

Due to important advantages such as being easy to use and portable, which are crucial properties of devices for DNA chip technology, and considering that this technique has not been utilized for detection of HPV yet, according to our knowledge, we employed PGE electrode and MB for detection of HPV target DNA for the first time. The optimum conditions for immobilization of HPV probe and detection of DNAs on the pretreated PGE surfaces were investigated. At these conditions, the electrode could detect the HPVc target DNA properly. The sensitivity of the electrode was approved by five non-complementary DNAs corresponding to human genome, HCV, fungi and bacterial cells genome. Sensitivity experiments showed that the electrode could discriminate HPV DNA from other organisms DNAs. Having confirmed the sensitivity and specificity of the developed electrode in detection of HPV DNA, further experiments in our laboratories are currently in progress towards discrimination of HPV universal region PCR product from other organisms various and consensus segments PCR products. In the future work, we will attempt to discriminate the HPV universal region from mixed oligousenucleo HCV universal region, bacterial 16S rDNA and fungi 5/18s rDNA region using DNA biosensors.

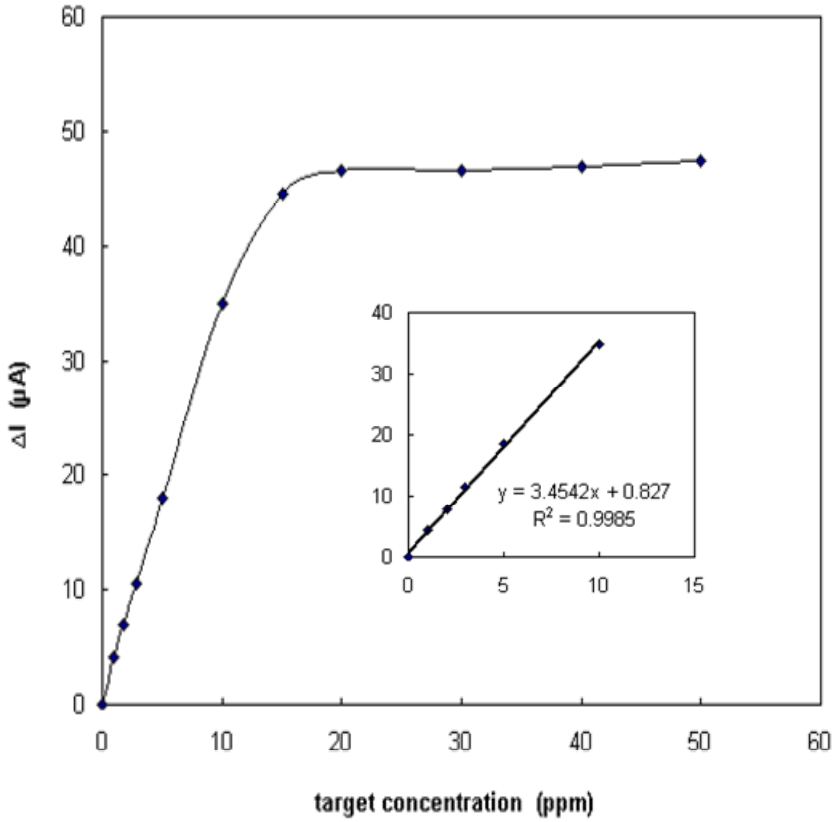

Fig. 8. Plot of ΔI (difference between the SWV signal of MB on the probe-modified PGE in the presence and absence of target) *vs*. target concentration. Inset related to the calibration curve at concentration range up to 10 ng μl^{-1}.

ACKNOWLEDGMENTS

The authors hereby would like to thank Dr. Esmaeel Alipour for his technical supports in this research.

REFERENCES

[1] D.W. Pang, H.D. Abruna, Anal. Chem. 70 (1998) 3162.
[2] J. Wang, Nucleic Acids Res. 28 (2000) 3011.
[3] E. Palecek, Talanta 56 (2002) 809.
[4] H.S. Wang, H.X. Ju, H.Y. Chen, Anal. Chim. Acta 461 (2002) 243.
[5] A.M. Oliveira-Brett, J.A.P. Piedade, L.A. Silva, V.C. Diculescu, Anal. Biochem. 332 (2004) 329.
[6] J.M. Zen, M.R. Chang, G. Ilangovan, Analyst 124 (1997) 679.
[7] M. Ligaj, J. Jasnoweska, W.G. Musial, M. Filipiak, Electrochim. Acta 51 (2006) 5193.
[8] M.S. Hejazi, E. Alipour, M.H. Pournaghi-Azar, Talanta 71 (2007) 1734.
[9] F. Davis, A.V. Nabok, S.J. Higson, Biosens. Bioelectron. 20 (2005) 1531.
[10] B. Meric, K. Kerman, D. Ozkan, P. Kara, S. Erensoy, U.S. Akarca, M. Mascini, M. Ozsoz, Talanta 56 (2002) 837.
[11] P. Kara, K. Kerman, D. Ozkan, B. Meric, A. Erdem, Z. Ozkan, M. Ozsoz, Electrochem. Commun. 4 (2002) 705.
[12] A. Erdem, K. Kerman, B. Meric, U.S. Akarca, M. Ozsoz, Anal. Chim. Acta 422 (2000) 139.

[13] A. Erdem, K. Kerman, B. Meric, M. Ozsoz, Electroanalysis 13 (2001) 219.
[14] A. Tani, A.J. Thomson, J.N. Butt, Analyst 126 (2001) 1726.
[15] W. Yang, M. Ozsoz, D.B. Hibbert, J.J. Gooding, Electroanalysis 14 (2002) 1299.
[16] M.H. Pournaghi-Azar, M.S. Hejazi, E. Alipour, Anal. Chim. Acta 570 (2006) 144.
[17] I.V. Yang, P.A. Ropp, H. Holden Thorp, Anal. Chem. 74 (2002) 347.
[18] D. Dell'atti, M. Zavaglia, S. Tombelli, G. Bertacca, A.O. Cavazzana, G. Bevilacqua, M. Minunni, M. Mascini, Clin. Chimi. Acta 175 (2007) 3061.
[19] N. Zhua, A. Zhang, Q. Wang, P. He, Y. Fang, Anal. Chim. Acta 510 (2004) 163.
[20] B. Meric, K. Kerman, D. Ozkan, P. Kara, S. Erensoy, U.S. Akarca, M. Mascini, M. Ozsoz, Talanta 56 (2002) 837.