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Electrochemical Detection of Specific DNA Sequences From PCR Amplicons on Carbon and Mercury Electrodes Using Meldola's Blue as an Intercalator

Kağan KERMAN[1], Dilşat ÖZKAN[1], Pınar KARA[1], Hakan KARADENİZ[1], Zeynep ÖZKAN[1], Arzum ERDEM[1], Frantisek JELEN[2], Mehmet ÖZSÖZ[1]*

[1]*Department of Analytical Chemistry, Faculty of Pharmacy, Ege University, 35100 Bornova, İzmir-TURKEY*
e-mail: ozsozs@pharm.ege.edu.tr
[2]*Institute of Biophysics, Academy of Sciences of the Czech Republic, Královopolská 135, 612 65 Brno, CZECH REPUBLIC*

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The electrochemical parameters for 7–dimethyl–amino–1,2– benzophenoxazinium Meldola's Blue, (MDB) on binding to DNA at both a hanging mercury drop electrode (HMDE) and carbon paste electrode (CPE) are described. MDB, which interacts with immobilized calf thymus DNA, was detected using double stranded DNA modified HMDE or CPE (dsDNA modified HMDE or CPE), bare HMDE or CPE and single stranded DNA modified HMDE or CPE (ssDNA modified HMDE or CPE) in combination with adsorptive transfer stripping voltammetry (AdTSV) techniques and decreased peak currents were observed. The discrimination of dsDNA and ssDNA and detection of hybridization between synthetic oligonucleotides were determined from changes in the voltammetric peak of MDB. With the help of the planar phenoxazine ring, MDB was found to be intercalating between the base pairs of dsDNA. Several factors affecting the DNA immobilization, hybridization and indicator accumulation were investigated. The partition coefficient was also obtained from the signal of MDB with a dsDNA modified glassy carbon electrode (GCE). Specific DNA sequences from PCR amplicons were detected based on changes in the MDB reduction signal at the CPE. These results demonstrated that MDB could be used as an electroactive hybridization label for DNA biosensors.

Key Words: DNA, Biosensor, Meldola's Blue, Intercalator, PCR, Hybridization.

Introduction

Various tools for DNA diagnostics and other areas of biotechnology can be realized based on novel methods for detecting DNA, RNA, and various genetic substructures[1–5]. DNA biosensors based on nucleic acid recognition processes are being developed towards the goal of rapid and inexpensive testing of genetic and infectious diseases. Electrochemical transducers are powerful tools for converting the hybridization event

*Corresponding author

into an analytical signal [6]. Nowadays, they are used in many studies for detecting the DNA hybridization event, due to their high sensitivity, small dimensions, low cost, and compatibility with microfabrication technology [7–9].

The strong affinity of DNA for mercury surfaces has led to the development of adsorptive transfer stripping voltammetric (AdTSV) procedures, first described by Palecek et al.[10] This procedure is performed with the immersion of HMDE in the DNA solution for a short period of time. The electrode can then be washed and dipped into the blank buffer solution to measure the electrochemical signals of the immobilized DNA at the HMDE surface. A small drop of a DNA solution (4-20 μL) is enough for AdTSV procedures, whereas 1 – 5 mL of DNA is often used in conventional voltammetric analysis. The strong adsorptive immobilization of DNA at the HMDE provides two fold enhancement of the sensitivity compared to pulse polarographic procedures[11]. Cai et al.[12] described the adsorptive stripping potentiometry of DNA at electrochemically pretreated carbon paste electrode (CPE), hanging mercury drop electrode (HMDE)[13] and carbon fiber microelectrodes[14], and thus opened the possibility of measuring nanogram amounts of DNA depending on the oxidation signal of guanine.

Electrochemical techniques that are well suited for detecting hybridization and DNA damage events have recently been reported. Hybridization was detected by redox-active metal complexes that associated selectively and reversibly with double stranded immobilized DNA[15,16]. Marrazza et al[17] showed that daunomycin (DM) could be used as an electrochemical hybridization indicator for detecting Apolipoprotein E (APO E) poylmorphisms, using DNA fragments amplified by PCR. Erdem et al.[18,19] reported that methylene blue was used as a promising indicator for the electrochemical detection of mismatched bases in oligonucleotides.

MDB has been used as an effective electron acceptor for enzymes in various enzyme based biosensor configurations. Phenoxazine derivatives and structurally related phenothiazines are known to shuttle electrons between the reduced flavin adenine dinucleotide of glucose oxidase and the CPE surface. The use of these compounds as electron transfer mediators formed the basis for a patent[20]. MDB in particularly has been widely utilized for designing dehydrogenase based biosensors, because of its ability to mediate the oxidation of dihydronicotinamide adenine dinucleotide (NADH). The low redox potential of MDB circumvented the problems linked to the detection of NADH[21]. Sensors based on NAD^+ - dependent dehydrogenases have thus been designed, including glucose probes using glucose dehydrogenase[21,22]. Biosensors for D-lactate and acetaldehyde were developed, based on screen-printed electrodes and NAD(+)-dependent dehydrogenases[23]. A detailed study was recently reported by Munteanu et al.[24] on the electrocatalytic oxidation of P-nicotinamide adenine dinucleotide at 3 different carbon paste electrodes modified with redox mediators commonly used in bioelectrochemistry, such as MDB, methylene green and riboflavin, adsorbed on zirconium phosphate.

Methylene blue (MB) is a well-described intercalator[18,19,25–28]. This study aimed to find out if MDB could be used as a hybridization indicator. MDB has recently been reported as an intercalator[29]. Femtosecond electron-transfer reactions of MDB and other similar organic dyes with DNA were observed by Reid et al[29]. In this work an HMDE and CPE were used in combination with adsorptive transfer stripping voltammetry techniques (AdTSV) to obtain information about the interaction of MDB, which intercalates into dsDNA in significant quantities at the electrode surface. Changes in the MDB signal resulting from the indicator interactions with dsDNA and ssDNA were observed. The advantages of the method are discussed and it is compared with these methods reported previously.

Experimental

Apparatus

The cyclic voltammetry (CV) measurements were carried out by using an AUTOLAB electrochemical analysis system and GPES 4.8 software package (Eco Chemie, The Netherlands) in connection with Metrohm VA – Stand 663 (Switzerland). HMDE mode was used with an electrode area of 0.40 mm^2. The 3 electrode system involved the HMDE, Ag/AgCl/3 M KCl as the reference electrode and platinum wire as the auxiliary electrode. 0.30 M ammonium formate, 50 mM phosphate buffer solution (pH 6.91) was the buffer solution.

The differential pulse voltammetry (DPV) measurements were obtained by using an AUTOLAB electrochemical analysis system and GPES 4.8 software package (Eco Chemie, The Netherlands). The 3-electrode system, consisted of the in-house made CPE or a glassy carbon electrode (GCE, Bioanalytical Systems, USA) as the working electrode, the reference electrode (Ag/AgCl) and a platinum wire as the auxiliary electrode. The body of the CPE was a glass tube (3 mm i.d.) tightly packed with carbon paste. The electrical contact was provided by a copper wire inserted into the carbon paste. This paste was prepared in the usual way by hand-mixing graphite powder (Fisher) and mineral oil (Acheson 38) in a 70:30 mass ratio. The surface was polished on a weighing paper to a smoothed finish before use. The convective transport was provided by a magnetic stirrer.

Chemicals

The 17-base synthetic oligonucleotides were purchased from Synthegen, LLC (Houston, Texas, USA); their base sequences are as below:

Probe I:
5′-TCA-AAT-CAG-GTT-GCT-TA- 3′
Target I:
5′-TAA-GCA-ACC-TGA-TTT-GA- 3′
Two - bases mismatch:
5′- TAA-GCA-A**GG**-TGA-TTT-GA- 3′
noncomplementary:
5′- AAC-GTG-TGA-ATG-ACC-CA- 3′

The 23-mer synthetic oligonucleotides of the PCR capture probe and its complementary target were purchased (as lyophilized powder) from Interactiva Thermo Hybaid (Erlangen, Germany); their base sequences are below :

Probe II:
5′ – AAT ACC TIT ATT CCT CIC CTI TC – 3′
Target II:
5′ – GAC AGG CGA GGA ATA CAG GTA TT – 3′

Probes I and II are complementary to targets I and II; the 2-base mismatch is a mutant of target I with 2-bases changed, as indicated by the underlines. All oligonucleotide stock solutions of the 17-base oligomers (100 mg/L) were prepared with TE solution (10 mM Tris-HCl, 1 mM EDTA, pH 8.00) and kept frozen.

Double-stranded calf thymus DNA (dsDNA, activated and lyophilized) and single-stranded calf thymus DNA (ssDNA, activated and lyophilized) were purchased from Sigma. All DNA stock solutions 100

mg/L were prepared with TE solution (10 mM Tris - HCl, 1 mM EDTA, pH 8.00) and kept frozen. More dilute solutions of DNA were prepared with 0.50 M acetate buffer (pH 4.80) for DPV analysis and with 0.3 M ammonium formate, 50 mM phosphate buffer solution (pH 6.91) for CV analysis. Meldola's Blue (MDB) was purchased from Sigma. The stock solutions of MDB (1 mM) were prepared with distilled water. Other chemicals were of analytical reagent grade. Distilled and deionized water was used in all solutions. All the experiments were performed at room temperature, 25.0 ± 0.5 °C. All buffer solutions contained 20 mM NaCl to provide ionic strength.

Procedure

A) Detection of interaction between DNA and MDB:

Cyclic Voltammetry (CV):

DNA immobilization : The dsDNA or ssDNA modified HMDE was prepared by immersing the bare HMDE into a 20 μL drop containing 10 ppm dsDNA or ssDNA in 0.30 M ammonium formate, 50 mM phosphate buffer solution (pH 6.91, AFP) for 2 min without applying any potential.

MDB accumulation : The DNA modified electrode was subsequently washed with AFP and transferred into blank AFP, which contained various concentrations of MDB. The MDB solution was stirred at 200 rpm for 5 min and MDB was accumulated onto the dsDNA or ssDNA modified HMDE without applying any potential.

Voltammetric transduction : The DNA modifed electrode was subsequently washed with the blank AFP and transferred into the blank AFP for voltammetric measurement. The cyclic voltammograms at the HMDE were collected at a 500 mV/s scan rate. The cyclic voltammograms at the CPE were taken at various scan rates. The raw data were treated using the Savitzky and Golay filter (level 4) of the GPES software. All CV measurements at the HMDE involved a fresh mercury surface. For the measurements performed with the bare HMDE, no DNA was adsorbed onto the electrode surface.

Differential Pulse Voltammetry (DPV):

DPV measurements required each immobilization/detection cycle at a fresh carbon paste surface.

CPE pretreatment: The CPE was activated by applying +1.70 V for 1 min in 0.05 M phosphate buffer solution (pH 7.40, PBS) without stirring.

GCE pretreatment: The GCE was polished with 6 and 1 μm alumina slurries, sonicated in water and oxidized at +0.50 V for 1 min in PBS. After the oxidation step, the GCE was rinsed with water for 10 s.

DNA immobilization: dsDNA or ssDNA was immobilized on a pretreated CPE by applying a potential of + 0.50 V for 5 min in 10 ppm dsDNA or ssDNA containing 0.50 M acetate buffer solution (pH 4.80, ABS) at 200 rpm stirring. The electrode was then rinsed with ABS for 10 s.

MDB accumulation: MDB was accumulated onto the surface hybrid by immersing the electrode into the stirred 20 mM Tris-HCl buffer (pH 7.00, TBS) containing 20 μM MDB for 5 min. while holding the potential at +0.50 V. After accumulation of MDB, the electrode was rinsed with TBS for 5 s.

Voltammetric transduction: The electrode was then transferred into the blank TBS for the voltammetric measurement. Differential pulse voltammograms were collected at 5 mV/s. The raw data were treated using the Savitzky and Golay filter (level 2) of the GPES software, followed by moving average baseline correction with a "peak width" of 0.01 V. For the measurements performed with the bare CPE, no DNA was adsorbed onto the electrode surface.

B) Gene sequence detection

The hybridization detection from synthetic oligonucleotides and PCR amplicons was transduced by means of DPV. The MDB reduction peak height after baseline fitting was used as the analytical signal. The procedure of hybridization detection of the PCR amplified amplicons and the synthetic 17-mer oligonucleotides consisted of the following steps;

Probe immobilization: The CPE was pretreated by applying +1.70 V for 1 min in blank PBS. The probe was subsequently immobilized onto the pretreated CPE by applying at a potential of +0.50 V for 5 min in the ABS containing 10 μg/mL probe at a 200 rpm stirring rate. After immobilization, the probe modified CPE was washed with blank ABS.

Hybridization with synthetic target: The probe modified CPE was inverted and about 10 μL of TBS containing 15 μg/mL DNA target was pipetted onto the surface. The hybridization was allowed to proceed for 6 min. The non-specific adsorption effect was partly eliminated with the following washing step. The hybrid modified CPE was dipped into 1% sodium dodecylsulphate dissolved in TBS (TBS-SDS) for 3 s. and then immediately dipped into blank TBS for 3 s. Hybridization with only two-bases mismatch containing the DNA target and non-complementary DNA sequence was monitored following the same method, as described above.

Hybridization with DNA fragments obtained from PCR amplification: The amplicon obtained from the PCR amplification was diluted with TBS; the diluted sample was then placed in a vial and denatured by heating in a water bath at 95 °C for 6 min and subsequent freezing in an ice bath for 2 min. After the immobilization of the probe, the CPE was inverted and about 10 μL of the denatured sample was pipetted directly onto the probe modified electrode surface. The hybridization was allowed to proceed for 6 min. The hybrid modified CPE was then dipped into TBS-SDS for 3 s. and then immediately dipped into blank TBS for 3 s.

MDB accumulation: MDB was accumulated onto the surface hybrid by immersing the electrode into the stirred 20 mM Tris-HCl buffer (pH 7.00, TBS) containing 20 μM MDB for 5 min. while holding the potential at +0.50 V. After accumulation of MDB, the electrode was rinsed with TBS for 5 s.

Voltammetric transduction: The reduction signal of MDB was measured by using DPV in blank TBS. The raw data were treated using the Savitzky and Golay filter (level 2) of GPES with moving average baseline correction, using a peak width of 0.01 V.

The amplicons were characterized by the method performed previously[30] with 1.5% agarose gel electrophoresis in the Dept. of Medicinal Biology of the Faculty of Medicine, Ege University. Thus, the results of electrochemical biosensor were confirmed by those obtained from the conventional method.

Results and Discussion

The voltammetric signals of MDB from the dsDNA modified, bare and ssDNA modified CPEs are displayed in Figure 1. Due to the lack of intercalation sites at the ssDNA modified electrode (Figure 1-a), the MDB signal was low in comparison with the one obtained from the dsDNA modified electrode (Figure 1-c). MDB displayed a reduction signal at –0.55 V with the HMDE (not shown) and at ~ -0.20 V with the CPE (Figure 1-b). The increase on signals at dsDNA modified electrodes was attributed to the intercalation of the planar phenoxazine ring between the dsDNA helix (see Scheme 1) as recently described by Reid et al[29]. When the aromatic ring intercalated into DNA, the electrochemically active center of MDB was not enveloped by the bulky DNA molecule and was avaliable for redox activity. No shift in the peak potential was observed and so

it was found that MDB behaved as the former intercalating molecules such as tris (1,10-phenanthroline)cobalt (III) perchlorate[12−16]. MDB displayed a low signal when interacted with ssDNA, because ssDNA did not provide the duplex form necessary for the intercalation process. Thus, MDB could not accumulate at the ssDNA modified electrode as much as in the case of the one at the dsDNA modified electrode. The clearly observed difference in the AdTSV responses of MDB obtained from dsDNA and ssDNA modified HMDEs and CPEs showed that MDB could be utilized as an indicator for the detection of hybridization.

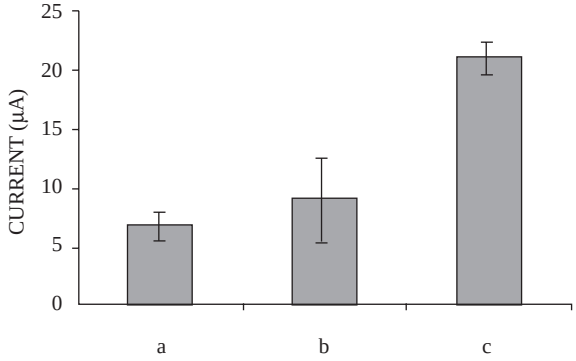
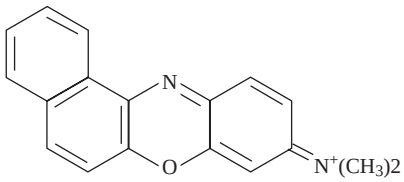


Figure 1. Histograms with error bars of 20 μM MDB at **(a)** ssDNA modified CPE, **(b)** bare CPE and **(c)** dsDNA modified CPE, *CPE pretreatment*, 1 min at +1.70 V in PBS; *DNA immobilization*, 5 min at +0.50 V in strirred 5 ppm DNA containing ABS; *MDB binding* 5 min with no potential in TBS containing 20 μM MDB; *Measurement* of accumulated MDB using DPV at 5 mV/s in blank TBS.

MB, which is a phenothiazine dye, was also found to have an interaction with guanine bases[31−34]. The electrochemical signals of MB at the ssDNA modified CPE were higher than those obtained from the dsDNA modified CPE[18,19]. These signals were attributed to the interaction between guanine and MB. Such an interaction was not observed in the case of MDB. The only structural difference was that MDB lacked one free amino group, which might have played an important role in the binding of the molecule to guanine bases. MB has 2 free amino groups at both sides of the aromatic planar ring, which might have caused the binding of MB to the guanine bases. A series of 3 repetitive measurements of the interaction of MDB with dsDNA and ssDNA modified CPEs resulted in reproducible results with a relative standard deviation (RSD) of 10.12% and 9.74%, respectively. A series of 3 repetitive measurements of the reduction signal of MDB at bare CPE resulted in reproducible results with a RSD of 8.62%.



Scheme. Chemical structure of Meldola's Blue (MDB).

Figure 2 displays the calibration plot of the MDB current signals obtained from the AdTSV analysis at the HMDE, with increasing concentrations of MDB. The intercalator accumulated into the base pairs of the double helix at the dsDNA modified HMDE and gave the highest response at each concentration level (Figure 2-a). The lack of the double helix form of ssDNA resulted in the low MDB signals from the ssDNA modified HMDE (Figure 2-c). MDB signals at the bare HMDE was shown in Figure 2-b. It was

noteworthy that voltammetric measurements with CPE required 20 μM MDB, whereas the HMDE required 50 nM MDB for an optimum signal. Data obtained from all of the modified electrodes during the AdTSV analysis showed saturation behavior with peak currents tending to plateau at concentrations above 100 nM MDB.

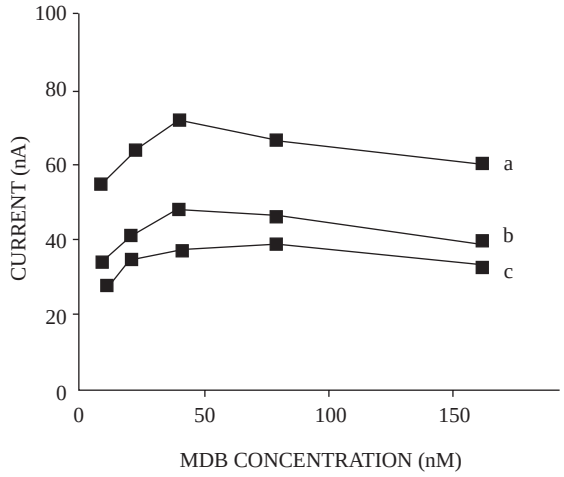


Figure 2. Calibration plots of peak current against MDB concentration, obtained with AdTSV at **(a)** dsDNA modified HMDE, **(b)** bare HMDE and **(c)** ssDNA modified HMDE in blank AFP. *DNA immobilization*; the dsDNA or ssDNA modified HMDE was prepared by immersing the bare HMDE into a 20 μL drop of AFP containing 10 ppm dsDNA or ssDNA for 2 min. *MDB binding*; the electrode was subsequently washed with the blank AFP and transferred into the AFP, which contained various concentrations of MDB, for 5 min with 200 rpm stirring. *Measurement*; the electrode was subsequently washed with the blank AFP and transferred into the blank AFP for voltammetric measurement at 500 mV/s.

According to the reference method proposed by Millan et al.[35], calibration data (not shown) obtained at the dsDNA modifed and bare GCE were used to estimate the partition coefficient of MDB in the microenvironment near the GCE surface as in the equation below:

$$\text{MDB}_{\text{bound}} \,/\, \text{MDB}_{\text{free}} = (\, i_{\text{bound}} - i_{\text{free}})/i_{\text{free}}$$

where MDB $_{bound}$ and MDB $_{free}$ are the concentrations of free and bound MDB complexes, respectively, i_{bound} is the voltammetric peak current obtained at the dsDNA modifed GCE and i_{free} is the current obtained from the bare GCE. According to the equation reported by Millan et al. [35], the partition coefficient of MDB was found to be 0.86 by using the voltammetric peak currents obtained under the same conditions at dsDNA modifed and bare GCEs at various MDB concentrations.

Figure 3 shows the differential pulse voltammograms for the detection of hybridization by using MDB at the CPE. The smallest signal was observed for the indicator at the probe I modified CPE (Figure 3-e). An increase in the MDB signal was observed upon repeating this experiment in the presence of target I (Figure 3-a) due to the intercalation of MDB between the double helix of the hybrid. A series of 3 repetitive measurements for the hybridization of the probe with the DNA target resulted in reproducible results with a RSD of 8.5%. The MDB response for the hybridization of the probe with the 2-bases mismatched oligonucleotide at the CPE was also detected by MDB (Figure 3-b). In the presence of an oligonucleotide containing a 2-bases mismatch, which was nearly at the end of this sequence, the difference between the

signal of probe I - target I hybridization (Figure 3-a) and that probe I- mismatch hybridization (Figure 3-b) could be observed, as shown in the voltammograms. The hybrid with the 2-bases mismatch containing oligonucleotide resulted in a partially formed hybrid and thus a decrease in the MDB hybrid signal was observed. This difference indicated that complete hybridization was not accomplished. Only the 20μM MDB signal is shown in (Figure 3-c) A series of 3 repetitive measurements of hybridization of the probe with the 2-bases mismatch resulted in reproducible results with a RSD of 8.34%. Control experiments were performed to assess whether MDB gave signals selectively, via hybridization, to the target. The response to the exposure of the probe I-modified CPE (Figure 3-e) to the noncomplementary oligonucleotide (Figure 3-d). The voltammetric signal obtained from the probe I -modified CPE when associated with a noncomplementary oligonucleotide was found to be as high as the signal of the probe. The effect of increasing the noncomplementary sequence on the reduction signal of MDB was also tested (not shown). The peak height did not change with noncomplementary concentration at first (up to 5 ppm), but then very slightly decreased above 15 ppm. This study showed that no hybridization occurred on the CPE surface because the nonspecifically adsorbed oligonucleotides were successfully removed by the washing step; as described in the Experimental section. A series of 3 repetitive measurements of hybridization of the probe with the noncomplementary oligonucleotide resulted in reproducible results and there was no decrease in the response. The RSD was 9.72%.

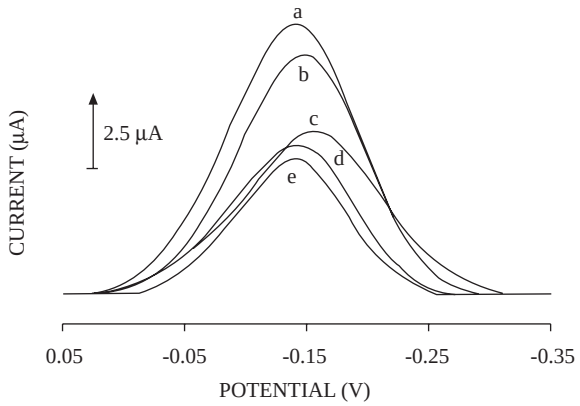


Figure 3. Differential pulse voltammograms for the probe I modified CPE, **(a)** after hybridization with target I, **(b)** after hybridization with 2-bases mismatch, **(c)** bare electrode signal was obtained with MDB, **(d)** after hybridization with non-complementary (NC) sequence, **(e)** probe I modified CPE after the interaction with MDB. *CPE pretreatment*, 1 min at +1.70 V in PBS; *Probe immobilization*, 5 min at +0.50 V in stirred 10 ppm probe I containing ABS; *Hybridization*, 5 min at +0.50 V in stirred 15 ppm target II containing TBS; *MDB binding*, 5 min at +0.50 V in TBS containing 20 μM MDB; *Measurement* of accumulated MDB in blank TBS by using DPV at 5 mV/s.

Figure 4 displays the effect of increasing the target concentration on the MDB signal. The peak height (i_{pc}) increased rapidly with the target concentration at first (up to 15 ppm) and then started to level off above 15 ppm. Such a curvature reflected the saturation of the intercalation sites on the CPE surface. Increasing the target concentration caused a fouling of the surface and thus less MDB signal could be obtained. The hybridization signal along with the corresponding noise level indicated a detection limit of 17.32 ng/mL (S/N = 3) in connection with the 5 min hybridization time. Further lowering of the detection limit is expected in connection with longer hybridization time or immobilization periods.

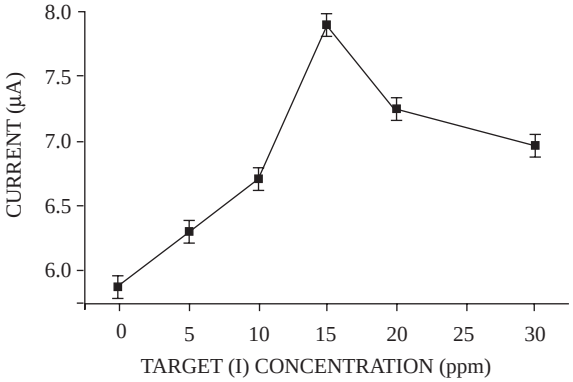


Figure 4. Calibration plot for the dependence of MDB reduction signal upon increasing concentration of target I. Other conditions are as in Figure 3.

The lowest MDB signal was observed from the probe II modified CPE (Figure 5-a). When hybridization occurred on the CPE surface by using synthetic oligonucleotides as probe II and target II, the MDB signal was increased (Figure 5-c). Figure 5 also represents the hybridization detection studies with PCR amplicons. The increase on the MDB signal obtained with a positive amplicon, containing the target DNA sequence, confirmed hybridization. When probe II was immobilized on the CPE surface, the high MDB reduction signal (Figure 5–d) showed that the patient had homozygous DNA. The decrease in the voltammetric signal (Figure 5–e) to ca. half of its intensity showed that the patient had a heterozygous mutation. The 6 subsequent experiments for the detection of hybridization between probe II and the target DNA from ten amplicons gave reproducible results as shown in the histograms of Figure 5. The MDB signal obtained from the probe II modified CPE after hybridization with homozygous amplicons gave a RSD of 8.34%. The MDB signal obtained from the hybridization of probe II with the heterozygous amplicons a RSD of 7.92%.

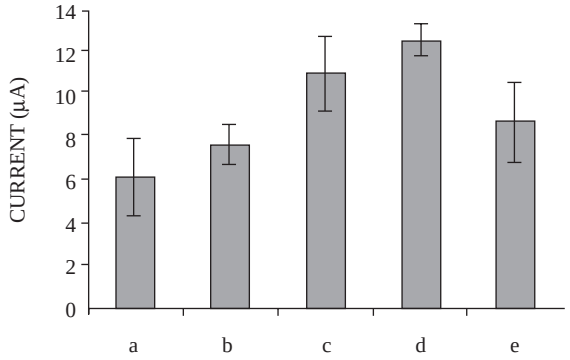


Figure 5. Histograms for the MDB signals collected in 3 repetitive DPV measurements of the detection of a specific DNA sequence from PCR amplicons with error bars: (a) probe II modified CPE, (b) bare electrode, (c) after hybridization by using synthetic probe II and target II, (d) after the hybridization with probe II and denatured homozygous PCR amplicons, (e) after the hybridization with probe II and denatured heterozygous PCR amplicons.

Conclusion

This is the first report demonstrating that MDB is a novel intercalator for detecting DNA hybridization in connection with electrochemistry. The detection of hybridization was accomplished using MDB, where inter-

calation of the planar aromatic ring into the immobilized dsDNA led to significantly increased voltammetric signals. Future work in this laboratory will focus on employing MDB as an electrochemical hybridization indicator for the detection of different DNA sequences from PCR amplified real samples. The authors hope that this and similar reports will accelerate the adaptation of electrochemical devices in decentralized DNA diagnostic tests.

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