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Brilliant cresyl blue as electroactive indicator in electrochemical DNA oligonucleotide sensors

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ABSTRACT

A new electrochemical DNA biosensor is presented based on carbon past electrode (CPE) for immobilization and detection of short DNA sequences with brilliant cresyl blue (BCB) as electroactive label. The interaction of BCB with DNA is electrochemically detected and BCB displays different signals in the interaction to ssDNA and dsDNA and variation in the BCB signal represents the extent of hybridization at the electrode surface. The effect of solution pH on electrochemical behavior of BCB was investigated. Additionally, the effect of solution pH on BCB accumulation on the CPE was studied. Furthermore, experiments showed that the solution pH could influence the differential pulse voltammetry (DPV) signal of BCB accumulated on the electrode and the highest BCB signal was obtained in pH 7.00. The effect of electrochemical pretreatment of CPE on the ability of electrode in probe adsorption, BCB accumulation and conditions of probe immobilization including potential and time was investigated and optimum conditions were suggested. The peak currents of BCB were linearly related to the concentration of the target oligonucleotide sequence in the range of 1.0×10^{-8} to 5.0×10^{-6} M. The detection limit of this approach was 9.00 nM. The selectivity of the biosensor was studied using noncomplementary oligonucleotide.

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1. Introduction

The use of DNA recognition layers represents an exciting development in analytical chemistry. Among the various sensing devices developed so far, electrochemical biosensors have received a great deal of attention due to their high sensitivity and rapid speed of detection. In addition, electrochemical techniques are ideally suitable for miniaturization and have the potential to simplify nucleic acid analysis using low cost electronics [1].

The other important feature about DNA is its interaction with biological compound and drugs that can lead to advances in pharmacology and diagnosis basis of many diseases [2,3].

Electrochemical detection of DNA hybridization is classified into two direct and indirect methods. Signal transduction induced directly from oxidation of guanine or adenine moieties in DNA strands (label-free detection) makes the principle of DNA hybridization detection in direct strategy [4–7], while indirect DNA hybridization detection method is based on incorporation of an electroactive label [8–11]. Electroactive indicators include anticancer agents [12], organic dyes [13] and metal complexes [14].

Brilliant cresyl blue (BCB), one of the organic dyes, was commonly used in various biological studies such as usefulness of BCB staining as an auxiliary method of screening for α-thalassemia [15], supplementation with cysteamine during maturation and embryo culture on embryo development of prepubertal goat oocytes selected by the BCB test [16], developmental competence of heifer oocytes selected using the BCB test [17], developmental competence of prepubertal goat oocytes selected with BCB and matured with cysteamine supplementation [18], and selection of prepubertal goat oocytes using the BCB test [19]. BCB belongs to quinine-imide dyes with planar structure and it is positively charged in neutral solutions. BCB was also used to determine heparin [20], oxalate [21], nitrite [22], protein [23], oxalic acid [24] and hydrazine [25] through its photochemical property. BCB could bind with DNA by electrostatic attraction, which was used for determination of DNA in spectroscopic method such as molecular spectroscopy study of the reaction of nucleic acids with BCB [26]. BCB as a new red region fluorescent probe for determination of nucleic acids [27] and binding behavior of BCB to calf thymus DNA was studied by spectrophotometric and voltammetric methods [28].

Recently, the use of BCB for detection of DNA hybridization has been reported by resonance light scattering method [29]. However, to our knowledge, application of BCB as an electrochemical indicator has never been devoted to fabrication of DNA hybridization sensors by electrochemical method and carbon paste electrode.

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We have already reported detection of IL-2 corresponding oligonucleotides using a non-inosine substituted probe on pencil graphite electrode (PGE) by direct and indirect methods [10,30,31]. In the indirect method we used methylene blue as the electroactive label. The present study aims to elucidate the electrochemical behavior of BCB as an electroactive label for electrochemical DNA biosensors. To take full advantage of this label, it has also been attempted to employ BCB for the development of electrochemical based DNA biosensors. To achieve this, CPE was employed as a low cost, easy preparation and renewable electrode and hIL-2 oligonucleotide was utilized as the probe. Differential pulse voltammetry was used as an electrochemical method and the effect of some experimental factors was investigated. The specificity of the sensor was monitored using complementary and noncomplementary DNA chains for the hybridization event.

2. Experimental

2.1. Chemicals

Brilliant cresyl blue was of analytical grade and was purchased from Merk. A 20-mer oligonucleotide corresponding to antisense strand of human IL-2 gene (hIL-2) was used as the probe and its complementary (chIL-2) oligonucleotide corresponding to sense strand of human IL-2 was used as target DNA. 16SR, YF270 and HgbBF oligonucleotides were used as noncomplementary oligonucleotides. All of the oligonucleotides were supplied as lyophilized powder by MWG-Biotech Company, with the following sequences:

Probe DNA (hIL-2): 5′-GGA GGA AGT GCT AAA TTT AG-3′
Complementary DNA (chIL-2): 5′-CTA AAT TTA GCA CTT CCT CC-3′
Noncomplementary DNAs:
16SR: 5′-TAC CTT GTT AGG ACT TCA CC-3′
YF270: 5′-TGT AAA TTC TGT GAG TAT GAG-3′
HgbBF: 5′-TCA TTG AGT ACG GCT TGAC-3′.

The stock solutions of the oligonucleotides (100 μM) were prepared with TE buffer solution (10 mM Tris–HCl, 1 mM EDTA, pH 8.00) and kept frozen. More diluted solutions of the oligonucleotides were prepared using 0.50 M acetate buffer solution (pH 4.80) containing 20 mM NaCl. The stock solution of BCB (1 mM) was prepared using distilled and sterilized water. Other chemicals were of analytical grade. The distilled, deionized and sterilized water was used in all solution preparations. Each measurement consisted of the immobilization/detection cycle carried out on a fresh CPE surface. All the experiments were performed at room temperature in an electrochemical cell.

2.2. Apparatus

Electrochemical experiments were performed using AUTOLAB PGSTAT 30 electrochemical analysis system and GPES 4.9 software package (Eco Chemie, Netherlands). The utilized three-electrode system was composed of a CPE (surface area of 0.015 cm^2) as the working electrode, a saturated calomel electrode (SCE) as the reference electrode and a platinum wire as the auxiliary electrode. A computing double beam UV spectrophotometer (CECIL 5000, elegant technology) with quartz cell was used to measure the absorbance.

2.3. Procedure

2.3.1. Preparation of the working electrode

The carbon paste electrode was prepared in the usual way by hand mixing graphite powder with paraffin oil in a ratio of 70:30 (w/w). A portion of the resulting paste was then inserted in the bottom of a glass tube. The electrical connection was implemented by a copper wire lead fitted into the glass tube. The surface of the resulting paste electrodes was smoothed on a weighing paper and rinsed carefully with distilled water.

2.3.2. Electrochemical activation of the CPE

Pretreatment of the polished electrode was done at optimized potential of +0.20 V vs. SCE for 5 min in 0.50 M acetate buffer solution (pH 4.80) containing 20 mM of NaCl without stirring. All experiments were done on activated CPE.

2.3.3. Immobilization of probe on the CPE

Following activation, the working electrode was immersed in 0.50 M acetate buffer solution (pH 4.80) containing 1 μM probe and 20 mM of NaCl by applying −0.50 V potential vs. SCE for 5 min into the stirred solution for immobilization of probe on the CPE. Then, the electrode was rinsed with sterilized and deionized water.

2.3.4. Hybridization

The hybridization was performed by immersing the probe modified CPE into a stirred hybridization solution (0.5 M acetate buffer pH 4.8) containing 1 μM of target oligonucleotide and 20 mM of NaCl, for 5 min, while the working electrode potential was held at +0.50 V vs. SCE. The electrode was washed with sterilized and deionized water to remove the non-hybridized DNA. For hybridization of probe with noncomplementary sequences, the same protocol was applied.

2.3.5. BCB accumulation on the CPE

Following immobilization of the single stranded DNA (ssDNA) probe on CPE, BCB was accumulated on the probe by dipping the electrode into BCB (1 mM) for 5 min with stirring and without applying any potential to the working electrode. Once BCB was accumulated, the electrode was rinsed with sterilized and deionized water. The same strategy was performed for the accumulation of BCB on the bare electrode and probe modified electrodes following hybridization of the probe with complementary or noncomplementary oligonucleotides.

2.3.6. Voltammetric measurements

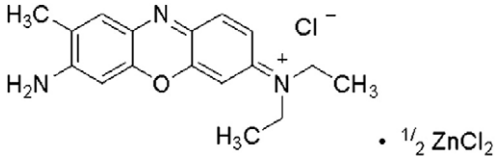
In cyclic voltammetric experiments, the electrode potential was scanned in −0.80 to +0.80 V vs. SCE in degassed BCB solution (1 mM) at scan rate of potential 100 mV s^{-1}. Electrochemical investigation was carried out using DPV in 0.1 M of phosphate buffer (pH 7.00) solution and scanning the electrode potential between −0.10 and −0.40 V vs. SCE at pulse amplitude of 50 mV.

The raw data were treated using the Savitzky and Golay filter (level 2) of GPES software, followed by the GPES software moving average baseline correction using a “peak width” of 0.01. Repetitive measurements were carried out following renewing the electrode surface by cutting and polishing of the electrode.

3. Results and discussion

3.1. Spectral characteristics

BCB is a cationic strong fluorescent dye and its structure is shown in Scheme 1. UV–Vis spectra of BCB, BCB-ssDNA and BCB-dsDNA are shown in Fig. 1. dsDNA was prepared by mixing equal amounts of two



Scheme 1. Structural formula of brilliant cresyl blue.

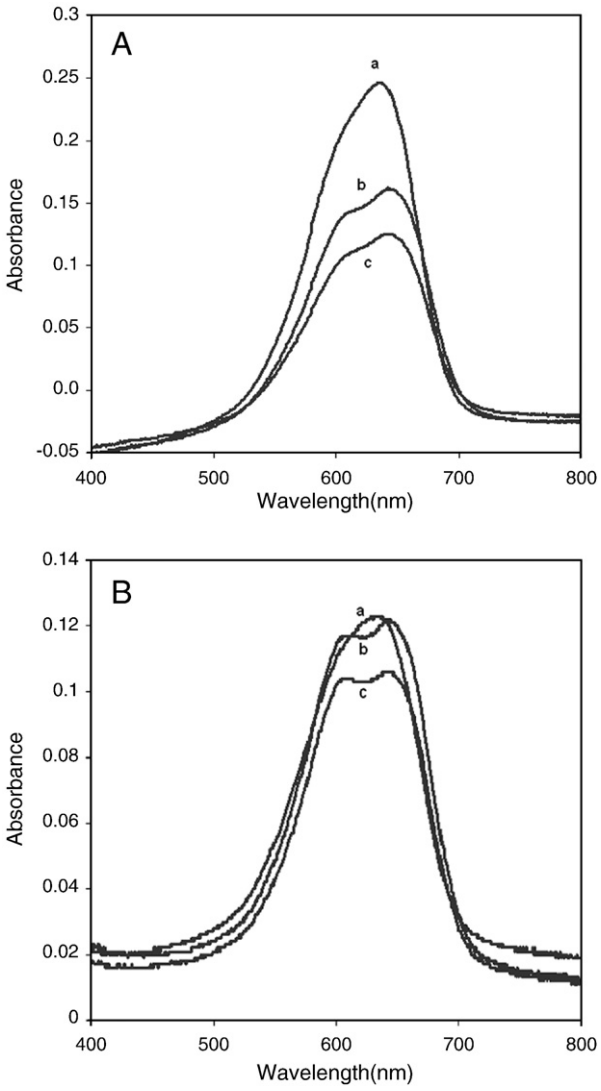


Fig. 1. The absorption spectra of BCB solution in the absence (curve a) and presence of ssDNA (curve b) and dsDNA (curve c). (A) Concentration of BCB: 8.0×10^{-6} M and DNA: 2.0×10^{-6} M. (B) Concentration of BCB and DNA: 2.0×10^{-6} M.

single stranded oligonucleotides, heating the solution at 95 °C for 10 min and cooling the solution at room temperature. The concentration of BCB in Fig. 1A is four times as much as the concentration of DNA, but in Fig. 1B, the concentration of BCB and DNA is equal. It displays a maximum absorption at 637 nm for BCB (Fig. 1, curve a). Obvious change in BCB spectrum was observed in the presence of hIL-2 as ssDNA (Fig. 1, curve b) and in the presence of hybrid of hIL-chIL-2 as dsDNA (Fig. 1, curve c). These results indicate that BCB interacts strongly with DNA [27]. The interaction between BCB and DNA relies on interaction between positively charged molecule of BCB and negative phosphate backbone of DNA.

3.2. Cyclic voltammetric behavior of BCB

The cyclic voltammogram of 1.0 mM BCB solution at the surface of CPE is showed in Fig. 2. Nitrogen gas was purged through the working compartment for 5 min for removing O_2 in solution before electrochemical experiment. It can be seen that an oxidation peak and two reduction peaks were obtained at $E_{pa}=+0.032$ and $E_{pc}=-0.263$, -0.480 V vs. SCE, respectively.

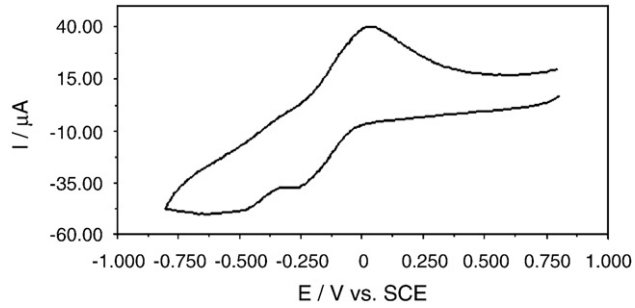


Fig. 2. Cyclic voltammogram of 1.00 mM BCB solution at the surface of CPE at scan rate of 100 mV s^{-1} vs. SCE.

3.3. Electrochemical study on the interaction between BCB and DNA

To study the interaction of BCB with DNA, CPE was immersed in BCB solution containing hIL-2 oligonucleotide as a primary investigation. Fig. 3 displays the cyclic voltammograms of 5.0×10^{-4} M BCB solution in the absence and presence of 5.0×10^{-6} M of hIL-2 DNA. As shown in this figure, the anodic and cathodic current peaks were increased significantly, after addition of hIL-2 oligonucleotide.

In order to have more information about interaction between indicator and oligonucleotides on the surface of working electrode, cyclic voltammetric measurements were performed in phosphate buffer 0.1 M solution (pH 7.00) containing 5.0 mM $K_4Fe(CN)_6/K_3Fe(CN)_6$ as a redox couple. Fig. 4 (curves a and b) shows the cyclic voltammograms of $Fe(CN)_6^{3-/4-}$ redox couple at bare activated CPE and hIL-2 immobilized CPE, respectively. As shown, the anodic and cathodic peak current decreased after immobilization of probe. The reduction in picks current is attributed to the adsorption of negatively charged DNA on the electrode surface that decreases the electron transfer of $[Fe(CN)_6]^{3-/4-}$ marker ion due to electrostatic repulsion.

Fig. 4 (curves c and d) represents the cyclic voltammogram of marker ion on the bare CPE and probe immobilized CPE after accumulation of BCB, respectively. It is noticeable that the anodic and cathodic peak currents of redox couple increase after immobilization of the probe on the electrode. Increasing of pick current can be the result of more accumulation of positively charged BCB onto the probe immobilized CPE. Consequently, the electron transfer rate of $[Fe(CN)_6]^{3-/4-}$ marker ions was increased at the surface of working electrode in buffer solution. These results indicate that after accumulation of BCB on CPE surface, positively charged molecules of brilliant cresyl blue bind with negatively charged probe.

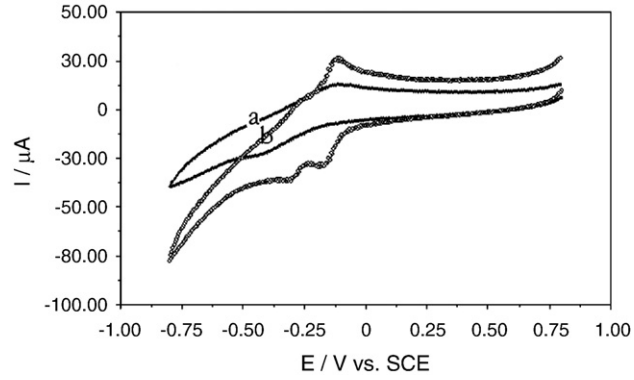


Fig. 3. Cyclic voltammograms of 5.0×10^{-4} M BCB solution in the absence (a) and presence of 5.0×10^{-6} M of hIL-2 DNA (b) at the surface of CPE at scan rate of 100 mV s^{-1} vs. SCE.

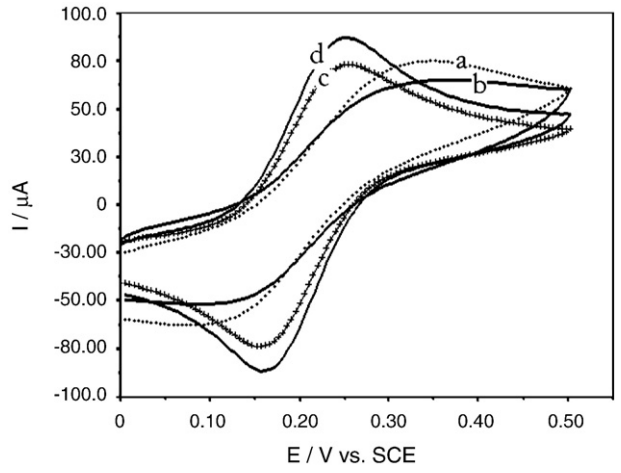


Fig. 4. Cyclic voltammograms of 5.0 mM $[Fe(CN)_6]^{3-/4-}$ (1:1) in phosphate buffer (pH 7.00) at the surface of bare activated CPE (a), hIL-2 immobilized onto activated CPE (b), BCB accumulated onto bare activated CPE (c) and BCB accumulated onto hIL-2 immobilized CPE (d). Electrochemical activation potential was +2.0 V vs. SCE for 5 min and oligonucleotide concentration in accumulation solution was 1.00 µM. Other conditions for electrode activation such as BCB accumulation and voltammetric measurements were as described in Section 2.3.

3.4. Preconcentration of BCB at CPE

In order to further investigate the accumulation and interaction of BCB with DNA on CPE, differential pulse voltammetry was selected as simple and sensitive electrochemical technique. Fig. 5a and b illustrates the differential pulse voltammograms of accumulated BCB at the bare activated CPE and hIL-2 modified activated CPE, respectively. BCB was preconcentrated on the electrode as described in Section 2.3.5. As shown in this figure, reduction signal of BCB at the bare activated CPE (6.5 µA) is significantly lower than the probe modified electrode (33 µA). This observation clearly confirmed that BCB has strong affinity for ssDNA and a considerable amount of BCB accumulates on the probe modified CPE surface, so that interaction of BCB with ssDNA could be electrochemically detected.

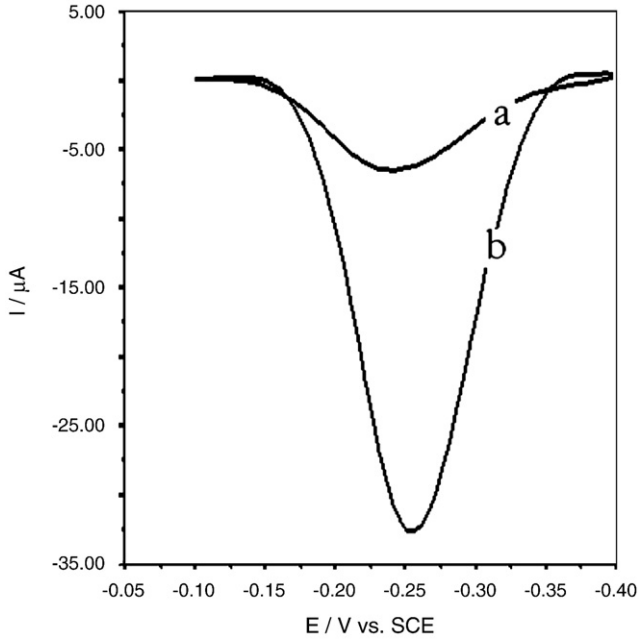


Fig. 5. Differential pulse voltammograms of BCB accumulated onto bare activated CPE (a) and hIL-2 immobilized CPE (b) in 0.1 M phosphate buffer (pH 7.00).

3.5. Optimization of the experimental variable parameters

3.5.1. Effect of pH on behavior of BCB

For the investigation of the effect of pH on the BCB electrochemical behavior, various solutions of BCB (1.0 mM) with different pHs were prepared. Fig. 6 shows the cyclic voltammograms obtained for BCB in phosphate buffer solutions (0.1 M) with various pHs ranging from 2.00 to 10.00 values. The shift of the anodic and cathodic peak potentials towards negative potentials by increasing the solution pH, demonstrates pH dependency of BCB electrochemical behavior and electrostatic binding for interaction mode of label to DNA [32].

Parallel to the shift in the peak potentials, the peak currents also gradually increase with increasing solution pH. This variation is rapid for pHs from 2.00 to 7.00, while the peak current and potential is independent at pHs 8.00, 9.00 and 10.00.

In order to study the effect of the solution pH on the accumulation of BCB on the electrode, bare CPE was immersed in different solutions with various pHs and then DPV signal was evaluated in 0.1 M phosphate buffer solution (pH 7.00). Interestingly, the results of this experiment indicate that the pH of accumulation BCB solution does not have any effect on the final DPV signal. Consequently, the accumulation process of BCB is independent of pH.

The effect of solution pH in the stage of voltammetric measurements was also investigated. The result showed that the highest amount of DPV signal is obtained in pH 7.00 buffer solutions. Therefore phosphate buffer solution with pH 7.00 is selected for this study.

3.5.2. Effect of potential on the accumulation of BCB

In order to study the influence of imposed potential on preconcentration of electrochemical indicator, BCB was accumulated on the bare CPE, with/without applying any potential to the electrode. The applied potential ranged between −0.50 and +0.50 V vs. SCE. Highest accumulation of BCB took place when electrochemical cell was open circuit. Then preconcentration of electrochemical indicator was conducted without applying any potential for 5 min in stirring solution.

3.5.3. Effect of immersing time on the accumulation of BCB

In order to obtain the optimum accumulation time, preconcentration of BCB was conducted for different time durations. The results obtained from the voltammetric measurements revealed that the BCB reduction signal elevated as the accumulation time increased from zero to about 300 s and remained constant between 300 and 700 s. Therefore 300 s was suggested as optimum time for the accumulation of electroactive indicator on the activated CPE.

3.5.4. Effects of pretreatment potential and time

The electrochemical pretreatment is usually required to activate the surface of the electrode and electrochemical pretreatment is commonly conducted either at negative or positive potential [33]. In order to find the optimum activation potential, the electrochemical

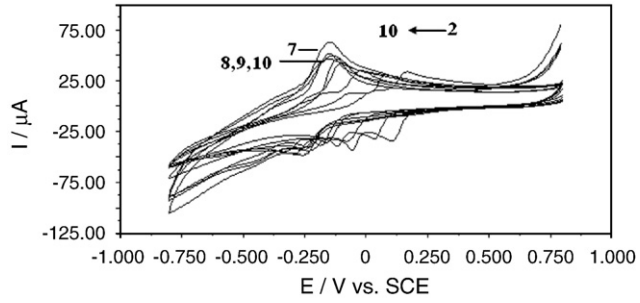


Fig. 6. Cyclic voltammograms of 1.00 mM BCB in various phosphate buffer solution pH 2.00 to 10.00.

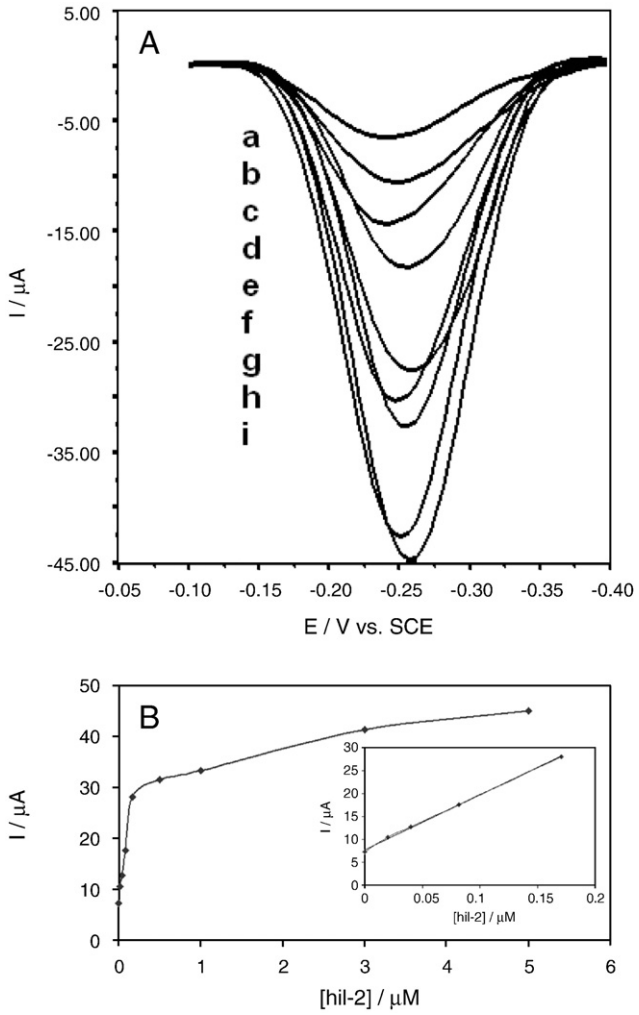


Fig. 7. (A) Differential pulse voltammograms of different hIL-2 concentrations: (a) 0.00, (b) 0.02, (c) 0.04, (d) 0.08, (e) 0.10, (f) 0.50, (g) 1.00, (h) 3.00, and (i) 5.00 µM in 0.1 M phosphate buffer (pH 7.00) at CPE. (B) Variation of DPV signals vs. hIL-2 concentrations ($y = 119.45 \times + 7.72$, $R^2 = 0.9982$). *Inset*: related calibration plot at hIL-2 concentration range (0.01–0.20 µM).

activation of the surface of polished CPE was carried out at various potentials between −0.20 and +2.50 V vs. SCE in 0.50 M acetate buffer solution (pH 4.80) containing 20 mM of NaCl without stirring. Results showed that the highest signal is obtained at potential of +0.20 V vs. SCE. Accordingly, this potential was addressed as the optimum potential for proposed electrode.

Furthermore, in order to optimize the activation time of the electrode, the activation was conducted at +2.00 V vs. SCE for different time durations in the same solution conditions. The results of experiments showed that hIL-2 signal was increased with increasing the activation time and nearly leveled off after 10 min. Therefore moderate time (5 min) was selected for activation of the CPE surface in order to accumulate the considerable amount of hIL-2.

3.5.5. Effect of immobilization potential of probe

One of the important factors affecting the immobilization of probe on the electrode is the imposed potential to the electrode during the accumulation of ssDNA. The influence of the imposed potential was investigated on the basis of DPV response of BCB. The voltammetric measurement was performed following immobilization of hIL-2 on the CPE at different potentials ranging between −0.80 and +0.80 vs. SCE. The results showed that imposing a negative potential to the CPE, favored the accumulation of hIL-2 DNA on the electrode and maximum voltammetric signal was observed at −0.50 V vs. SCE. Therefore a potential of −0.50 V was selected for most subsequent works.

3.6. Detection limit of probe immobilization

Fig. 7A shows the DPV signals obtained after immobilization of increasing levels of hIL-2 (0.01–5 µM). Well defined peaks were obtained over a flat background. The variation of the voltammetric response versus hIL-2 concentration is shown in Fig. 7B. The linear range was obtained at 0.01–0.20 µM (inset). The probe detection limit of the BCB based developed electrode was calculated about 9.00 nM.

3.7. Electrochemical detection of hybridization

DNA hybridization of probe with chIL-2 as a complementary target sequence was studied using accumulated BCB as electroactive indicator on the surface of probe modified activated CPE in phosphate buffer solution (pH 7.00) with DPV method (Fig. 8). The results showed that the signal of accumulated BCB in complementary dsDNA hybrid was less than that of ssDNA. The highest BCB reduction signal (33 µA) was observed with the hIL-2 modified activated CPE (curve b). This was while, a significant decrease in BCB (9.2 µA) was observed following hybridization of complementary target oligonucleotide (chIL-2) with the probe (curve c). This may be attributed to less BCB accumulation on the dsDNA because of inaccessibility of BCB to the guanine bases [34–36] or may by due to a steric inhabitation of the reducible groups of BCB packed between the bulky double helix of the DNA hybrids [37]. It is concluded that BCB displays various signals in the presence of ssDNA and dsDNA and the decrease in the BCB signal represents the extent of the hybridization at the electrode surface.

3.8. Selectivity study

In order to study the selectivity of the proposed biosensor based on BCB electroactivity, the probe modified electrode was treated with some noncomplementary oligonucleotides including 16SR, YF270 and

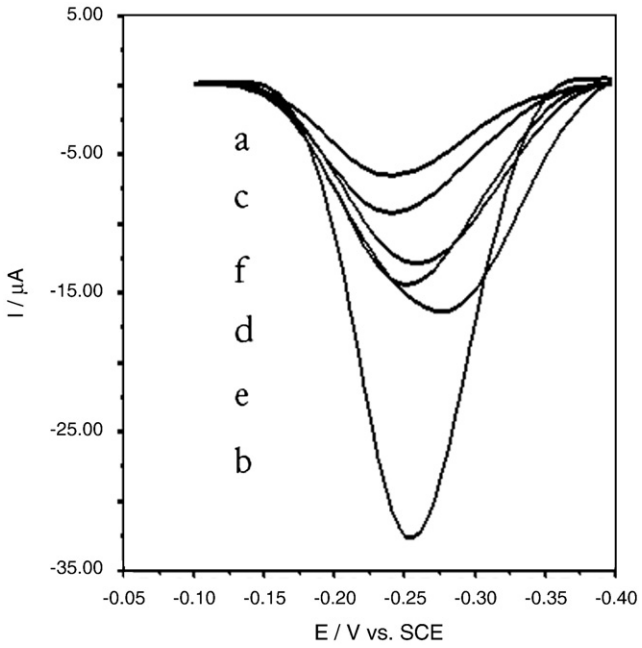


Fig. 8. Differential pulse voltammograms of accumulated BCB at (a) bare activated CPE, (b) probe modified activated CPE, after hybridization with (c) chIL-2 as complementary target sequence, and some noncomplementary sequences (d) 16SR, (e) YF270 and (f) HgbBF. The $t_{calculated}$ values for (c) and (f), (c) and (d) and (c) and (e) are 6.36, 2.84 and 15.29, respectively. The values of $t_{critical}$ are 2.13 and 3.75 for $p = 0.05$ and $p = 0.01$, respectively.

HgbBF (Fig. 8: curves d–f). As seen in Fig. 8, the interaction between these noncomplementary oligonucleotides and immobilized probe did not lead to a significant decrease in BCB signal, due to negligible hybridization. These results further confirmed that this DNA sensor selectively responds to the target DNA and that BCB signal could be addressed to the hybridization extent of the probe with the interacted DNA. We employed the *t*-test for the evaluation of difference among the complementary and the noncomplementary oligonucleotides.

Therefore, we calculated the values of $t_{calculated}$ for the difference between the signals of c–f, c–d and c–e of Fig. 8 for the claim that the difference among the complementary and the noncomplementary oligonucleotides is logical. The comparison of $t_{calculated}$ and $t_{critical}$ values (in probability levels: $p = 0.05$ and 0.01) shows that the values of $t_{calculated}$ are greater than the $t_{critical}$ at confidence levels 95% and 99% [38]. So, there are logical differences between differential pulse voltammograms of accumulated BCB at CPE after hybridization with (c) chIL-2 as complementary target sequence, and some noncomplementary sequences (d) 16SR, (e) YF270 and (f) HgbBF.

4. Conclusions

Employment of BCB as an electroactive indicator for electrochemical detection of DNA hybridization was investigated using carbon paste electrode. Carbon paste electrodes possess advantages of ease of preparation and easy renewable surface. BCB is positively charged in neutral solutions suggesting that it could bind with nucleic acids by electrostatic attraction and its planar molecular structure was expected to facilitate the intercalation of BCB into the interior of the DNA helix. The experiments support this conjecture and it was demonstrated that under neutral conditions addition of nucleic acids to BCB solution changed the absorption spectra. The experimental results showed the optimum values of +2.0 V and 300 S for CPE activation, −0.5 V and 300 S for the probe immobilization for this DNA sensor. The proposed biosensor selectively responded to the complementary target DNA.

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