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Full Paper

Application of Ethyl Green as Electroactive Indicator in Genosensors and Investigation of its Interaction with DNA

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Abstract- A new electrochemical DNA hybridization biosensor based on carbon paste electrode (CPE) and ethyl green (EG) is described. The interaction of EG and DNA is investigated in the solution and at the surface of the electrode. The results of electrochemical and spectroscopic studies indicate electrostatic interaction mode between DNA and EG. Hybridization event between the probe and its complementary sequence is investigated by square wave voltammetry of EG accumulated on the CPE. EG displays different signals in the interaction to ssDNA and dsDNA and variation in the EG signal represents the extent of hybridization at the electrode surface. The effects of some experimental variables on the performance of the biosensor are studied and optimized conditions are suggested. The selectivity of the biosensor is studied using some noncomplementary oligonucleotides. Under optimized experimental conditions, limit of detection is calculated 2.0×10^{-10} M.

Keywords- DNA Biosensor, Ethyl Green, Hybridization Detection, Square Wave Voltammetry, Electrostatic Interaction

1. INTRODUCTION

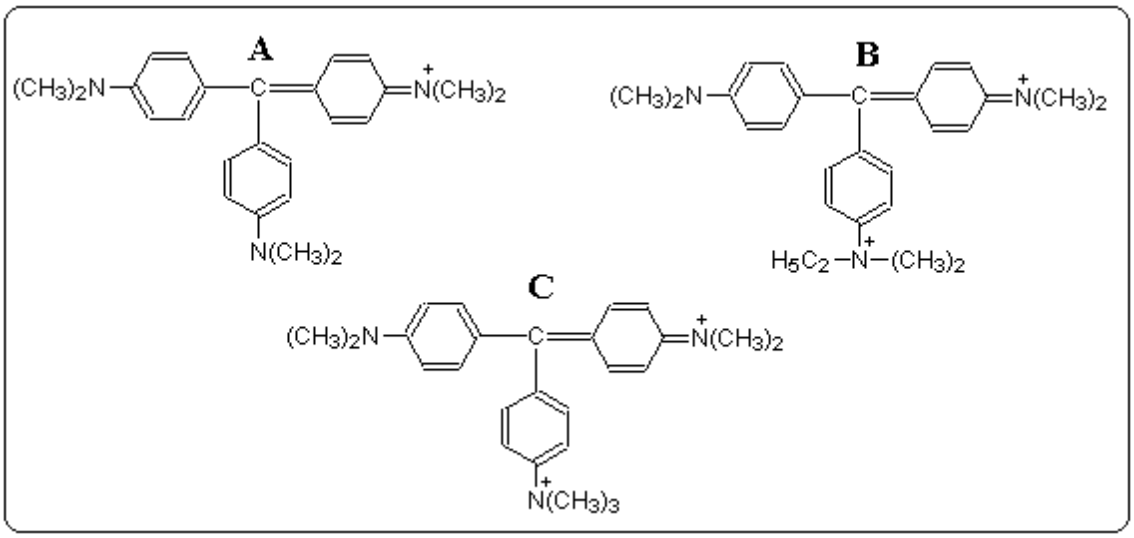
Genosensors or DNA hybridization biosensors are analytical devices for detection of specific DNA target sequences upon hybridization of the target with complementary probe immobilized on the solid substrate [1]. Several methods for studying of DNA hybridization or its interactions with small molecules such as fluorescence, surface Plasmon resonance, quartz crystal microbalance and electrochemistry were used. Among these methods, electrochemical transducers are more attractive for interfacing DNA detection at the molecular level and converting the hybridization event into an analytical signal [2]. In fact high sensitivity, low cost and minimal power requirements of DNA biosensors has caused this device is developed in this field and other fields such as electrochemical clinical diagnosis, environmental monitoring and drug analysis [3].

Electrochemical DNA biosensors rely on the conversion of the DNA base pair recognition events into a useful electrical signal. Recognition of electrochemical DNA hybridization is accomplished via two methods including direct and indirect. In direct DNA hybridization detection that carry out without any label, the signal induces owing to the inherent oxidation of adenine and guanine bases in DNA strands directly [4-7], whereas in indirect method the oxidation or reduction signal of an electroactive indicator causes detection of hybridization event. Therefore, some electroactive compounds such as some anticancer agents like epirubicin [8] and echinomycin [9,10], several metal complexes such as ruthenium bipyridine [11,12], cobalt phenanthroline [13,14] and cobalt bipyridine [15,16], and organic dyes such as methylene blue [17-25], nile blue [26] and brilliant cresyl blue [27] have been used as electroactive indicators for the detection of DNA hybridization.

Electrochemical indicators for detection of hybridization have usually small molecular weight and different affinities for ssDNA relative to dsDNA. Binding of small molecules to DNA are commonly performed in three modes of interaction involving electrostatic interaction with the negative charged sugar-phosphate structure, groove binding and intercalation of planar aromatic ring systems between base pairs [28]. It is obvious that grooves are created only within the double helix DNA. As a result, the affinity of groove-binders is greater in dsDNA than ssDNA, whereas, electrostatic binders have the lower affinity to dsDNA than ssDNA. Intercalators commonly have much higher duplex affinity and binding stability. Therefore the electrochemical signal changes of labels after probe-target DNA hybridization are dependent on the mode of DNA-label interaction. If the interaction mode is electrostatic, after hybridization, the electrochemical signal of label at probe-modified electrode largely decreases, it looks like the strict hindrance between DNA and big cationic molecules (electrostatic binders) causes decreased signal of indicators after hybridization of ssDNA and dsDNA. dsDNA occupy a large area of electrode surface than ssDNA, therefore the electrostatic binder labels could accumulate more at the probe modified

surface than at the hybrid modified electrode surface, whereas, if the interaction mode is intercalating or groove bonding, after hybridization, the signal of label largely increases [28].

Ethyl green is a dye from triphenylmethane class and is made from crystal violet by the addition of an ethyl group. It's very similar to methyl green (MG), which has a methyl group as the seventh group. The difference in the formula weights for EG probably comes from chloride or bromine salt. In many cases, ethyl green is likely the dye actually provided when methyl green is purchased [29,30]. The structures of these three dyes from triphenylmethan class are shown in scheme 1. According to results of some research methyl green defined as a DNA major-groove binding drug and can be considered as having pseudo-C_{2v} symmetry [31,32]. While ethyl green doesn't have this symmetry due to ethyl group, our electrochemical and spectroscopic results indicate the dominant interaction mode of DNA and ethyl green (EG) which is electrostatic binding. Researchers have reported results of biophysical studies on the MG-DNA complex and have concluded that the binding forces are predominantly electrostatic [33].



Scheme 1. Molecular structures of A) crystal violet, B) ethyl green and C) methyl green

According to our knowledge, using the EG dye for detection of DNA hybridization has not been reported yet. In the present work, a new electrochemical biosensor is purposed for recognition of DNA hybridization on the carbon paste electrode. CPE has great popularity, mainly because of favorable characteristics such as low cost, wide electrochemically accessible potential window and renewable surface. A 15-mer oligonucleotides corresponding to p53 gene tumor suppressor oligonucleotide as the probe, its complementary oligonucleotide as target DNA and ethyl green as electroactive label were used. Square wave voltammetry, differential pulse voltammetry and cyclic voltammetry were applied as

electrochemical techniques in this study. Also, the influence of some experimental parameters was investigated and optimized.

2. EXPERIMENTAL

2.1. Materials

A 15–mer oligonucleotide as probe and its complementary oligonucleotide as target DNA and non-complementary oligonucleotides were prepared by MWG-BIOTECH, Germany. The sequences of the applied oligonucleotides were listed below:

Probe DNA: 5'-TGG GGA TGG AG AACT -3'
Complementary DNA: 5'-AGT TCT CCA TCC CCA-3'
Non-complementary DNAs:
NC_1: 5'-CTA AAT TTA GCA CTT CCT CC-3'
NC_2: 5'-TGG GGA TGC AGA ACT-3'
NC_3: 5'-AGT TCT GCA TCC CCA-3'
NC_4: 5'-GGA GGA AGT GCT AAA TTTAG-3'

EG, potassium ferricyanide $K_3Fe(CN)_6$ and potassium ferrocyanide $K_4Fe(CN)_6$ were purchased from Fluka. The stock solution of oligonucleotides (10^{-2} M) was prepared with a TE buffer solution (10.0 mM Tris-HCl, 1.0 mM EDTA, pH 8.00). This solution was kept in a freezer. Acetate buffer solution 0.5 M (pH 4.80) comprising 20.0 mM of NaCl was used to prepare the more diluted solutions of nucleotides. Water used for the preparation of all solutions was deionized and distilled. All other reagents were of analytical reagent grade. All the electrochemical measurements were carried out at room temperature.

2.2. Apparatus

Electrochemical measurements were performed by using the AUTOLAB PGSTAT 30 electrochemical analysis system and GPES 4.9 software package and FRA software (Eco Chemie, The Netherlands). The three-electrode system composing a bare CPE as working electrode, a platinum wire as the auxiliary electrode and a saturated calomel electrode (SCE) as the reference electrode was used for the electrochemical experiments. The UV-Vis absorption measurements were performed on a computing double beam UV spectrophotometer (CECIL 5000, elegant technology) using a quartz cell with 1.0 cm path.

2.3. Procedure

2.3.1. Preparation of Working Electrode

The carbon paste electrode (CPE) was prepared as follows: adequate amounts of graphite powder and paraffin oil were mixed at 70/30 mass ratio and hand - mixed thoroughly using a

pestle and mortar. Amount of the resulting mixture was packed into the bottom of a glass tube (ID: 2.4 mm). Electrical contact was established via a copper wire lead was fitted into the clean and plain tube. The resulting paste electrode surface was smoothed on weighing paper and washed with distilled and deionized water.

2.3.2. Electrochemical Activation of CPE

Pretreatment of the electrode surface is necessary for adsorption of DNA on the electrode. For this aim, the polished electrode was pretreated at optimized potential of 1.80 V *vs.* SCE for 5 min. Pretreatment was carried out in 0.50 M acetate buffer solution (pH 4.80) containing 20 mM of NaCl without stirring.

2.3.3. Probe Immobilization on the CPE

After activation of CPE surface, the electrode was dipped in 0.5 M acetate buffer solution (pH 4.8) containing a 1.0 μM probe and 20.0 mM NaCl at +0.5 V imposed potential to the electrode with stirring for 5 min. The electrode was then washed with deionized and sterilized water.

2.3.4. Hybridization of Probe with Target Sequence

The hybridization was performed by immersing the probe modified CPE in the stirred hybridization solution (0.5 M acetate buffer at pH 4.8) containing 10^{-6} M of the target DNA and 20.0 mM of NaCl for 5 min while the electrode potential was held at 0.5 V *vs.* SCE. The electrode was then rinsed with sterilized and deionized water. This procedure was applied for the hybridization of the probe with non-complementary sequences.

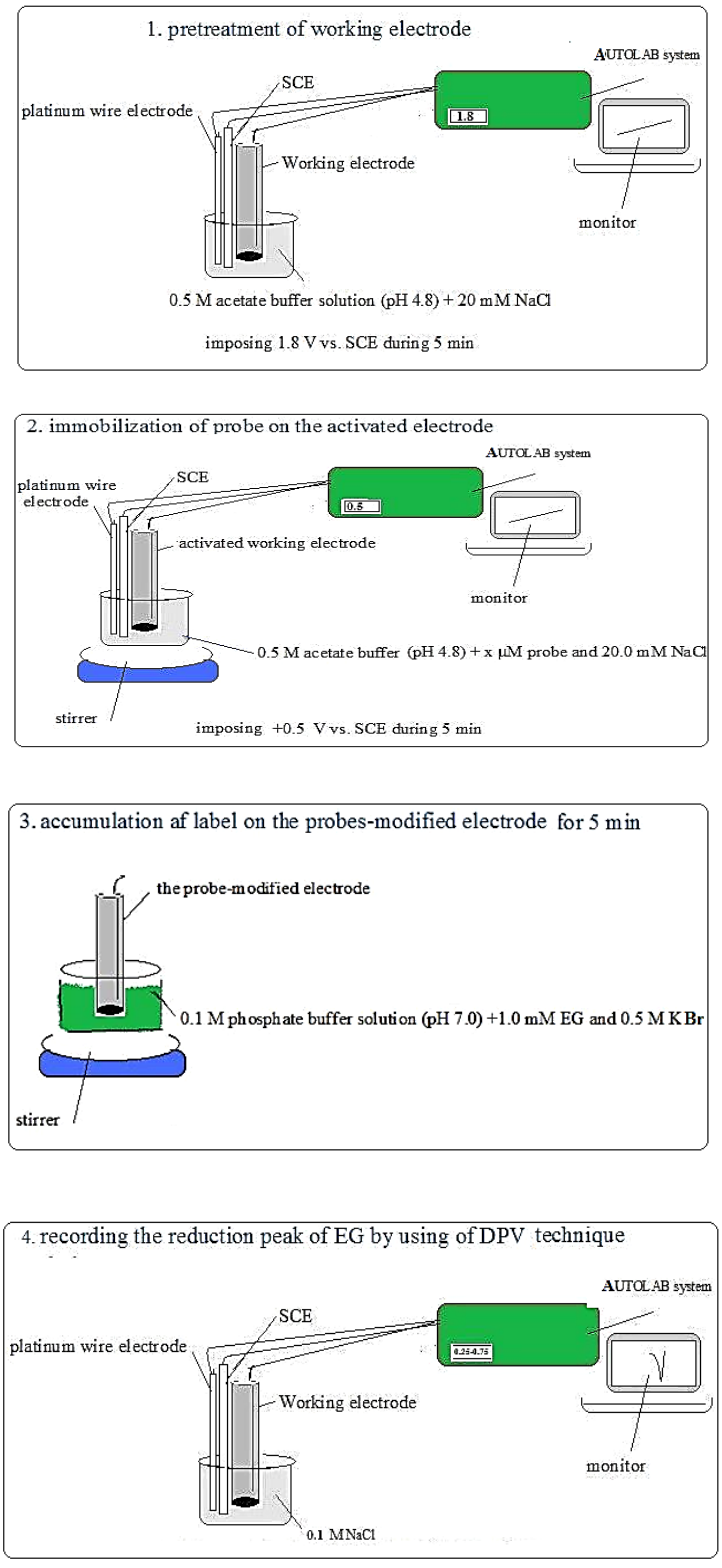
2.3.5. Accumulation of Indicator on the CPE

EG was accumulated on the probe following immobilization of the probe on the surface of CPE by immersing the electrode in a 0.1 M phosphate buffer solution (pH 7.0) containing 1.0 mM EG and 0.5 M KBr for 5 min with stirring at an open circuit potential. The electrode was then washed with sterilized and deionized water. This strategy was used for accumulation of EG following the hybridization of the probe with complementary or non-complementary sequence. The general scheme of all electrode modification before the measurements is shown in scheme 2.

2.3.6. Voltammetric Studies

In cyclic voltammetry measurement, the potential was scanned between 0.25 and 0.75 V *vs.* SCE at scan rate of 0.1 Vs^{-1}. The square wave voltammetry was employed with a frequency of 200 HZ and a step potential of 0.01 V. Differential pulse voltammetry was employed with the modulation amplitude of 0.01245 and the step potential of 0.01005 V *vs.* SCE. The preliminary data were treated by the Savitzky and Golay filter (level 2) of the GPES software, followed by GPES software moving an average baseline correction, by

means of a peak width of 0.01. Any time before using the electrode renewing the CPE surface by polishing or cutting must be carried out. The precise and baseline values of current peaks were read by the peak search icon in the GPES 4.9 software.



Scheme 2. The general scheme of all electrode modification before the measurements

3. RESULT AND DISCUSSION

3.1. Investigation of the Effect of Supporting Electrolyte on the Electrochemical Behavior of EG

In order to electrochemical study of the label some parameters were investigated. As preliminary study, cyclic voltammograms of 1.0 mM EG in 0.1 M phosphate buffer solution containing 0.5 M of various supporting electrolytes i.e., NaCl, KI, NaBr and KBr were recorded in the potential range between 0.25 to 0.75 V *vs*. SCE on the CPE at scan rate of 0.1 Vs^{-1}. As can be seen in Fig. 1, the highest peak current of the EG occurred when 0.5 M KBr was employed as the supporting electrolyte. Hence the 1.0 mM EG in the 0.1 M phosphate buffer solution containing 0.5 M KBr was chosen as the optimized solution for the electrochemical investigation in the subsequent experiment.

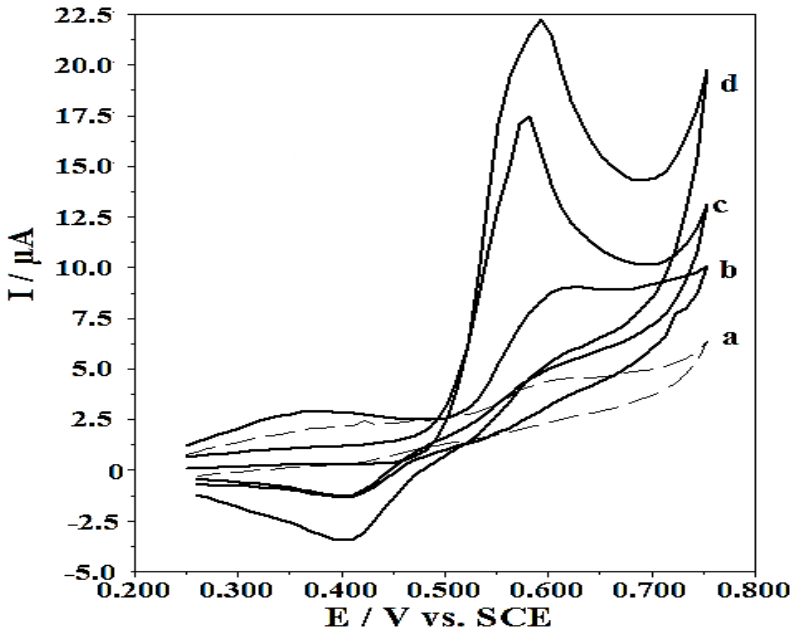


Fig. 1. Cyclic voltammograms of 1.0 mM EG in 0.1 M of phosphate buffer solution (pH 7.0) containing 0.5 M a) NaCl, b) NaBr, c) KI and d) KBr at the surface of CPE at scan rate of 0.1 Vs^{-1} in the potential range 0.25 to 0.75 V *vs*. SC

3.2. Cyclic Voltammetric Study of Electroactive Label

For electrochemical investigations of label's behavior, the cyclic voltammograms of 1.0 mM EG in 0.1 M phosphate buffer solution containing 0.5 M KBr were recorded in the potential range 0.25 to 0.75 V *vs*. SCE on the CPE at different low and high scan rates, from 0.005 to 0.1 Vs^{-1} (Fig. 2A) and 0.2 to 0.5 Vs^{-1} (Fig. 2B). As can be seen, in this condition at

the scan rate of 0.1 Vs^{-1} the EG has one oxidation peak (E_{pa}=0.592 V *vs*. SCE) and one reduction peak (E_{pc}=0.411 V *vs*. SCE). The separation of the anodic and cathodic peak potentials, ΔE_p=0.181 V, indicated quasireversible electrochemical behavior. The formal potential, $E^{\circ'}$ (or voltammetric $E_{1/2}$), taken as the average of E_{pc} and E_{pa} was 0.501 V *vs*. SCE. Because of the cyclic voltammograms shapes affect by the scan rate, the anodic and cathodic peak currents depend linearly to the square root of the scan rate over the whole examined scan rate (Fig. 2C). These results imply diffusion-controlled processes for the electrochemical behavior of the EG on the CPE [34]. As can be seen in Fig. 2C, ΔEp increase at the higher scan rates (i.e. ΔEp=0.156 V *vs*. SCE at scan rate=0.005 Vs^{-1}, while ΔE_p=0.181 V *vs*. SCE at scan rate=0.1 Vs^{-1} and ΔE_p=0.191 V *vs*. SCE at scan rate=0.5 Vs^{-1}). This result confirms quasireversible electrochemical behavior of the EG solution on CPE and indicates the kinetics of the EG electron transfer at high scan rates is slower than low scan rates.

3.3. DNA Binding Studies

The interaction of EG and DNA in the solution was studied by cyclic voltammetry, differential pulse voltammetry and UV-Vis adsorption spectrophotometry. The results indicated that the dominant interaction mode of EG with DNA was electrostatic binding under the selected condition.

3.3.1. Spectroscopic Studies

Absorption spectroscopy has been widely used in DNA-binding studies. In fact, results of spectroscopic studies can give the information concerning the mode and the binding intensity of small molecules to DNA [35-37]. The EG is a cationic organic dye from the triphenylmethane family. Fig. 3 shows the UV-Vis spectra of EG (curve a), EG-ssDNA (curve b) and EG-dsDNA (curve c). In order to prepare dsDNA the equal amounts of two single stranded oligonucleotides were mixed in a vial and put in a water bath. The solution was heated at 100 ºC for 10 min. Then, it was gradually cooled at room temperature. As can be seen in Fig. 3, after mixing DNA with EG, the maximum of the absorption shifts from 633.75 nm to 634.75 and 635.25 nm and the absorbance decreases from 0.072 to 0.064 and 0.059 for ssDNA and dsDNA, respectively. According to the finding of some researchers [38-45], intercalation binding of a low molecular weight compound with DNA usually results in a shift of the maximum absorbance to higher wave lengths (red shift effect or bathochromic effect) and decreasing in absorbance (hypochromism effect). But the magnitude of hypochromism and red shift observed for EG (11% and 1 nm for EG-ssDNA and 14% and 1.5 nm for dsDNA) are lower than those observed for typical classical intercalators [46-48]. Therefore, the EG interaction mode with DNA is partly intercalation. Because of EG is a cationic dye, electrostatic attraction could also exist.

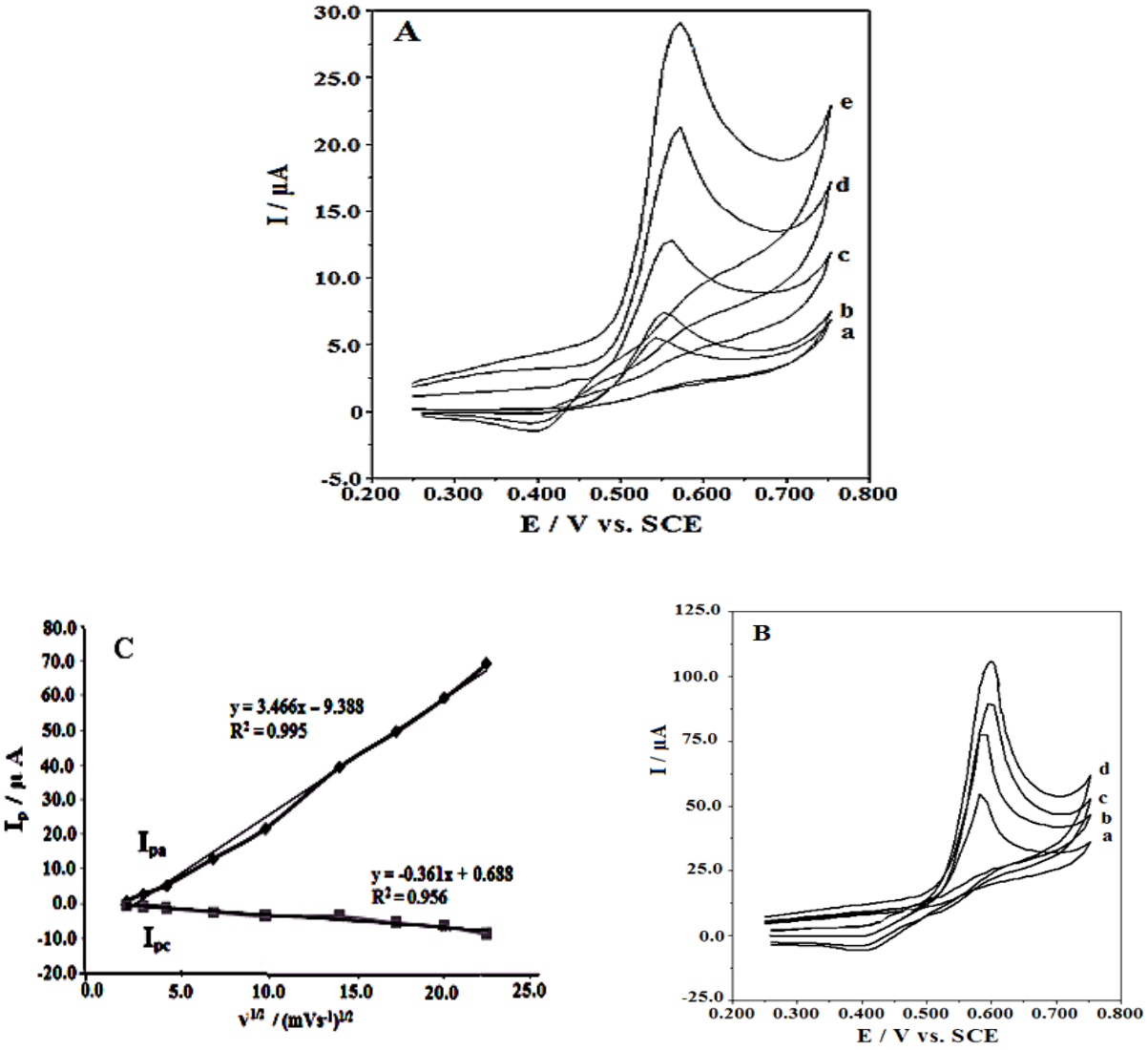


Fig. 2. Cyclic voltammograms of 1.0 mM EG in 0.1 M of phosphate buffer solution (pH 7.0) containing 0.5 M KBr at the surface of CPE at scan rate of **(A)** 0.005 (a), 0.02 (b), 0.05 (c), 0.1 Vs^{-1} (d) and **(B)** 0.2 (a), 0.3 (b), 0.4 (c), 0.5 Vs^{-1} (d) in the potential range 0.25 to 0.75 V vs. SCE, **(C)** Plots of peak current of EG *vs.* square root of scan rate calculated from the cyclic voltammograms shown in B and C

3.3.2. Electrochemical Studies

There are two basic ways to investigate the DNA-molecules interaction; 1) An interaction study on the electrode surface that should be performed by using either a DNA modified electrode or one modified by molecules. If a DNA modified electrode was to be used, it would then be immersed into the molecules solution and after some given time of interaction, the changes in the intrinsic signals of DNA bases would be observed. If a small molecules modified electrode was to be used, the molecules would be then immobilized on the electrode surface and the changes of the electrochemical signals of the small molecules observed. 2)

An interaction study in a solution where molecules and DNA would be put in the same solution and after some given time for interaction, the changes in the electrochemical signals of the molecule-DNA complex would be recorded and compared with the electrochemical signals of DNA or molecule alone in the solution [49].

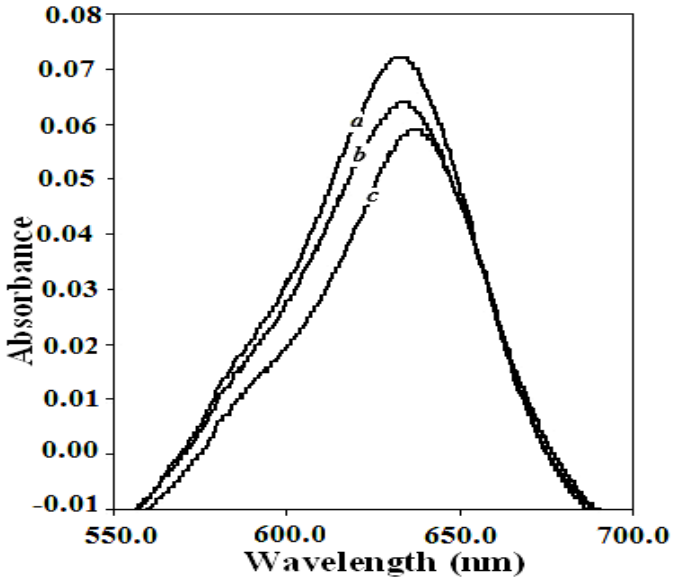


Fig. 3. Absorption spectra of EG solution in the absence (curve a) and the presence of ssDNA (curve b) and dsDNA (curve c). Concentrations of EG and DNA were 8.0×10^{-6} M and 2.0×10^{-6} M, respectively

3.3.2.1. Cyclic Voltammetric Investigation of EG and DNA

The cyclic voltammetric data of the interaction of EG with ssDNA (probe) in solution which are listed in Table 1. Fig. 4 shows the cyclic voltammograms of 800 µL of 1.0 mM EG at pH 7.0 phosphate buffer solution containing 0.5 M KBr in the absence and presence of 5 µL of 1.0 mM ssDNA on the CPE at the scan rate of 0.1 Vs^{-1}. As can be seen, EG has a pair of redox peaks on the bare CPE, an oxidation peak at the potential of 0.592 V *vs.* SCE and a reduction peak at the potential of 0.411 V *vs.* SCE. ΔEp was calculated as 0.181 V (dash line of Fig. 4 and Table 1). The addition of 5 µL of 1 mM probe into 800 µL EG solution resulted in decrease of peak current by 53.93% and 29.68% for oxidation and reduction peaks respectively, with the negative shift of the $E_{1/2}$ by 0.011 V (solid line of Fig. 1 and Table 1). It was the typical electrostatic binding mode of DNA interaction [50-52]. ΔE_p was calculated as 0.187 V, which was bigger than EG solution alone (0.181 V), indicating that reversibility of electron-transfer process was reduced after addition of DNA. Also, the peak currents decreased with the increase in concentration of DNA in EG solution and the $E_{1/2}$ shifted to

less positive potentials; which are the characteristic of the electrostatic attraction. Because the diffusion coefficient of EG became much smaller after addition of DNA, the EG peak current is decreases [38].

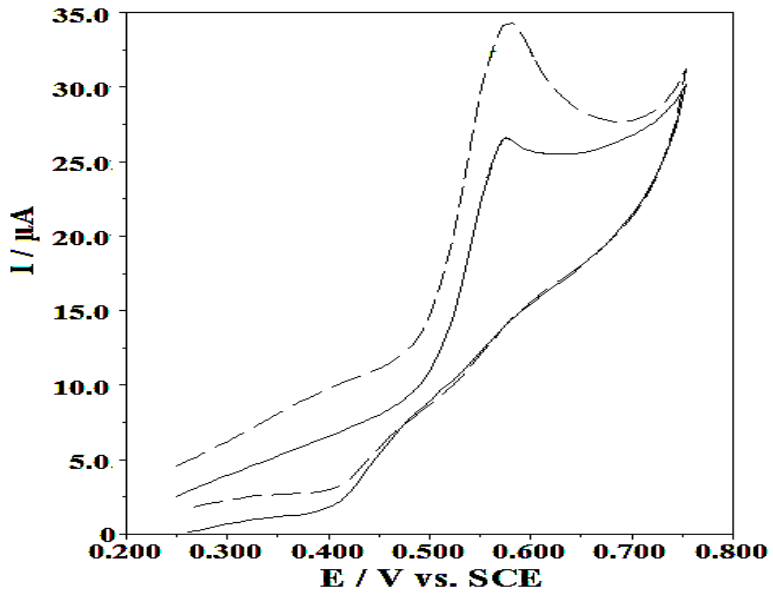


Fig. 4. Cyclic voltammograms of 10^{-3} M EG solution in 0.1 M of phosphate buffer solution (pH 7.0) containing 0.5 M KBr in the absence (dashed line) and presence (solid line) of 10^{-3} M probe at the surface of CPE at scan rate of 0.1 Vs^{-1} *vs.* SCE

3.3.2.2. Differential Pulse Voltammetric Investigation of EG and DNA

To obtain further information about interaction of EG and DNA, differential pulse voltammetry was also used. Fig.5 shows differential pulse voltammograms of 1.0 mM EG in pH 7.0 phosphate buffer solution containing 0.5 M KBr in the absence (carve a) and presence of various concentrations of ssDNA (carve b, c and d). Fig. 5A and 5B illustrate cathodic and anodic DPV, respectively. In the absence of probe, Fig. 5A carve a, EG has two peaks at potentials of E1=0.547 and E2=0.438 V *vs.* SCE. Also, Fig. 5B carve a shows two peaks in potentials of E1=0.433 and E2=0.542 V *vs.* SCE. As seen, in both cathodic and anodic DPV, after addition of various concentrations of probe, the current of the electrochemical signals of EG decreased and the position of E2 shifted to less positive potential. The negative potential shifting indicates an electrostatic interaction mode between DNA and electroactive label [38,50]. Because the technique of DPV is more sensitive than CV, only one oxidation and one reduction peak can be seen for EG by cyclic voltammetry. While, DPV method shows two peaks for oxidation and two peaks for reduction.

Table 1. Voltammetric behavior of 1.0 mM EG at pH 7.0 phosphate buffer solution containing 0.5 M KBr in the absence and presence of various concentrations of ssDNA at the surface of CPE at the scan rate of 0.1 Vs^{-1}. ($[DNA]_f$ and $[EG]_f$ are the final concentration of DNA and EG in solution)

V_{DNA}/mL	$[DNA]_f$/µM	$[EG]_f$/µM	E_{pa}/v	E_{pc}/V	$E_{1/2}$/V	I_{pa}/µA	I_{pc}/ µA
0.0	0.0	1000	0.592	0.411	0.501	19.56	-3.20
5.0	6.2	993	0.583	0.396	0.490	9.01	-2.25
10.0	12.3	987	0.574	0.402	0.488	8.54	-2.20
15.0	18.4	981	0.572	0.401	0.486	6.61	-1.66
20.0	24.4	975	0.572	0.391	0.481	6.46	-1.56

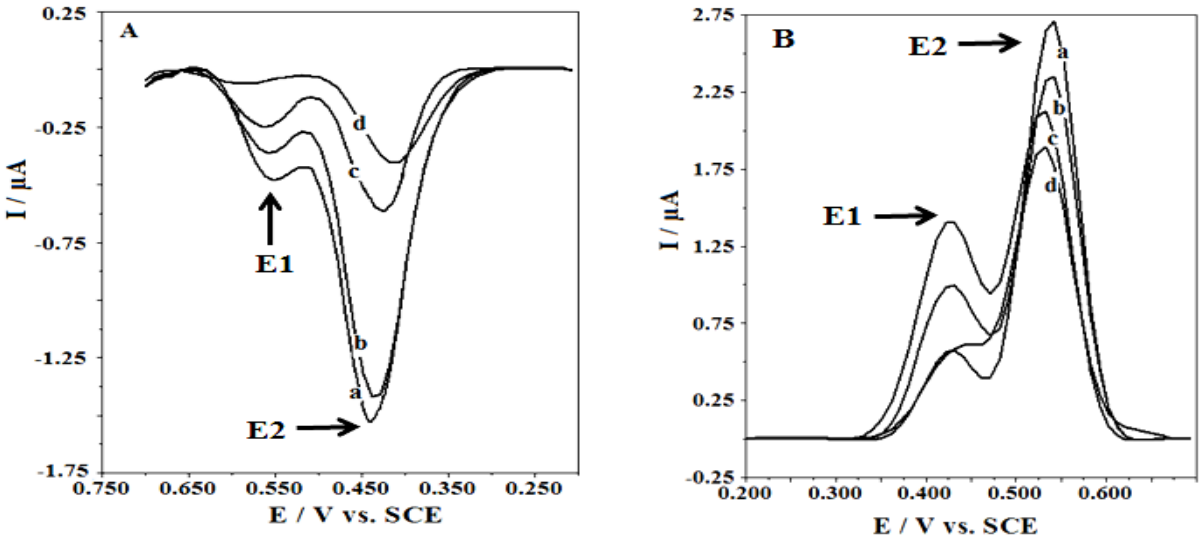


Fig. 5. Differential pulse voltammograms of 10^{-3} M EG solution in 0.1 M of phosphate buffer solution (pH 7.0) containing 0.5 M KBr in the absence (a) and the presence of 5 (b), 15 (c) and 20 µL (d) of 10^{-3} M probe in solution; **(A)** Cathodic DPV (0.75 to 0.20 V *vs*. SCE) and **(B)** Anodic DPV (0.20 to 0.75 V *vs*. SCE)

3.4. Accumulation of Label at the Surface of CPE

As mentioned in section of 3.2.2, there are two methods for investigation of interaction between DNA and label. Fig. 4 and Fig. 5 illustrated the study of their interaction in solution. On the other hand, the immobilization of probe on the surface of working electrode is an important parameter in electrochemical DNA biosensors. This factor affects on the response and the performance of genosensors. For these reasons, the interaction between DNA and

indicator was studied at the surface of carbon paste electrode. Fig.6 displays square wave voltammograms of accumulated EG on the bare CPE (a) and single stranded DNA immobilized CPE in 0.1 M phosphate buffer solution pH 7.0. Because the reduction peak current of accumulated EG on the probe-modified CPE (40.12 µA) is much higher than the bare CPE (5.15 µA), strong affinity of EG to DNA can be deduced.

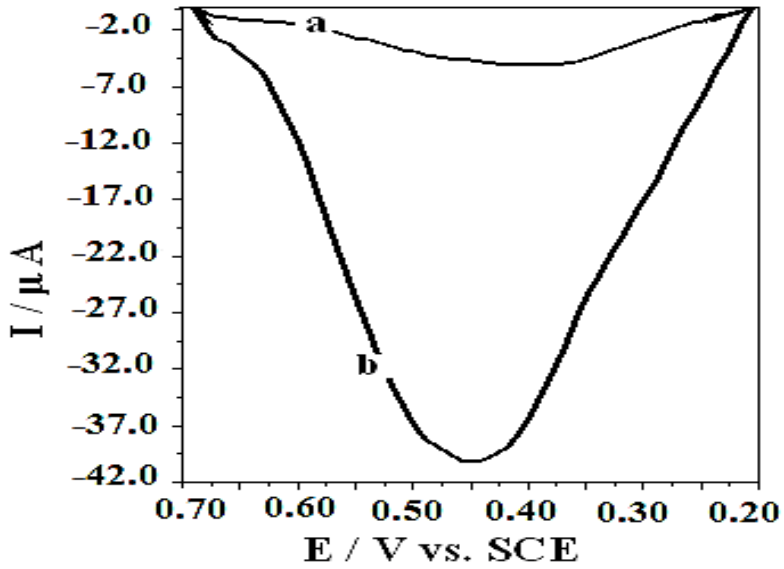


Fig. 6. Square wave voltammograms of 10^{-3} M accumulated EG at the bare (a) and probe immobilized CPE (b) in 0.1 M phosphate buffer solution (pH 7.0). SWV frequency: 200 HZ, SWV step Potential: 0.01 V

3.5. Optimization of Analytical Conditions

To achieve the best result, the experimental variables were examined and optimized. The SWV signal of accumulated EG on the probe-modified CPE in 0.1 M phosphate buffer solution pH 7.0 was chosen in order to study the influence of different parameters.

3.5.1. Influence of Pretreatment Potential

In order to find an optimum pretreatment potential, the polished CPE was activated at different potentials within a voltage range (i.e., from 1.5 to 2.5 V *vs.* SCE) in 0.5 M acetate buffer solution (pH 4.8) comprising 20 mM of NaCl without stirring [20]. The results show that the highest signal was observed at potential of 1.8 V *vs.* SCE (data were not shown). Hence, 1.8 V *vs.* SCE was selected as the optimized potential for CPE activation.

3.5.2. Influence of Pretreatment Time

To optimize the CPE pretreatment time, the activation was carried out at +1.8 V *vs.* SCE for various times with identical conditions. The SWV response increased with activation time and reached the maximum at 5 min and then kept a constant value (data were not shown). Therefore, 5 min was chosen as the best pretreatment time in following experiment.

3.5.3. Influence of Potential on the EG Accumulation

EG was accumulated on the electrode surface with / without applying any potential to the CPE. The ranges of this applied potential was between -0.5 and +0.5 V *vs.* SCE. The results indicated the highest signal at open circuit. Hence the accumulation of this electroactive label was performed without imposing any potential.

3.5.4. Influence of Time on the EG Accumulation

To obtain the optimized time on the accumulation of label, EG was accumulated on the bare CPE without imposing any potential to CPE, during various times with identical conditions. The results showed 5 min was adequate time for this purpose (data were not shown). Hence the accumulation of EG was performed without imposing any potential during 5 min, while solution was stirred.

3.5.5. The Effects of Kind and Concentration of Supporting Electrolyte on the Accumulation of EG

In order to study the influence of the kind of supporting electrolyte on the accumulation of the label, several salts i.e., KNO_3, KI and KBr, with concentration of 0.5 M were used. Fig. 7A shows the SWV signals of accumulated EG in phosphate buffer solution pH 7.0. As seen, the highest accumulation of EG occurred when 0.5 M KBr was used as supporting electrolyte in accumulation step. Hence, KBr was chosen for this purpose in the subsequent experiments.

For investigation the influence of supporting electrolyte concentration on the accumulation of EG, various concentrations of KBr were used in the range of 0.0 to 1.0 M. The results of the experiments are shown in Fig. 7B. As can be seen the best concentration of supporting electrolyte was 0.5 M.

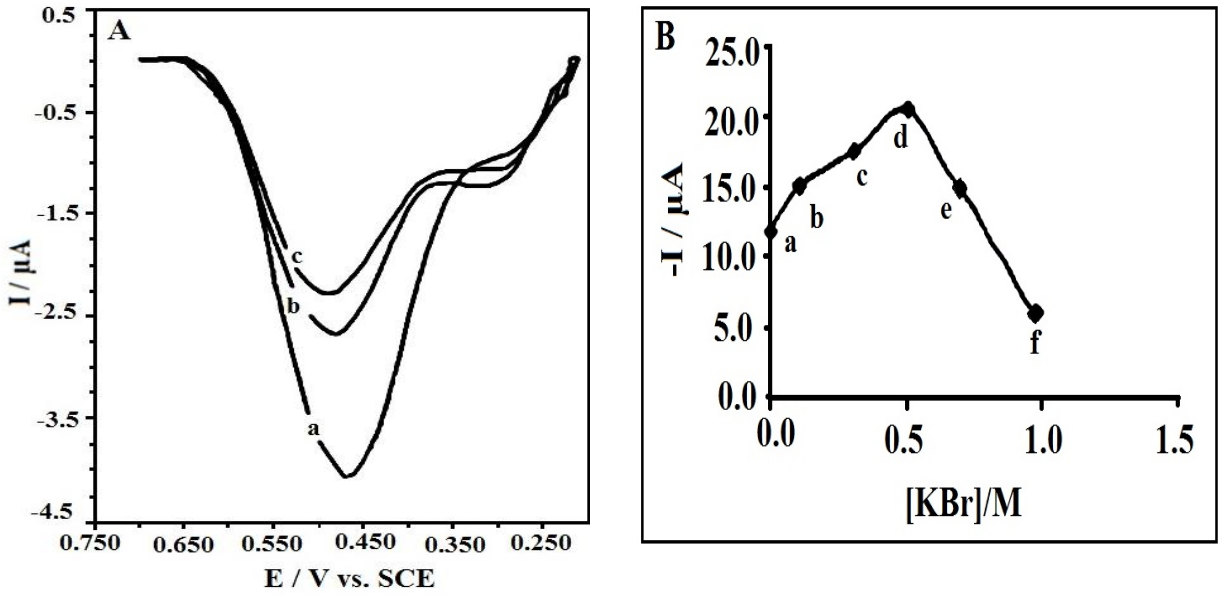


Fig. 7. (A) Square wave voltammograms of 10^{-3} M accumulated EG at the CPE in 0.1 M of phosphate buffer solution (pH 7.0). Accumulation solution of EG containing 0.5 M KBr (a), KNO_3 (b) and KI (c), **(B)** Influence of supporting electrolyte concentration on SWV reduction current peak of accumulated EG. Concentration of KBr: 0.0 M (a), 0.1 M (b), 0.3 M (c), 0.5 M (d), 0.7 M (e), 0.9 M (f) and 1.0 M (g)

3.5.6. Influence of Imposed Potential for Immobilization of Probe

The influence of the imposed potential to the electrode during the immobilization of probe, as one of the key factors was investigated using SWV responses of accumulated EG. The voltammetric measurement was performed following immobilization of probe on the CPE at various applied potentials ranging from -0.80 to 0.80 V *vs.* SCE. The results showed the highest voltammetric signal at -0.50 *vs.* SCE. Hence the potential of -0.50 V *vs.* SCE was chosen for the immobilization of probe on the CPE surface.

3.5.7. Effect of the Kind of Supporting Electrolyte in SWV Measurement

In order to investigate the influence of the supporting electrolyte at the stage of SWV measurement several salts i.e. KBr, KCl, NaBr and NaCl 0.1 M were used (Fig. 8). As can be seen, the highest current peak of the accumulated label took place in 0.1 M NaCl. Hence 0.1 M NaCl selected as the supporting electrolyte at the stage of SWV measurement in the rest of experiments.

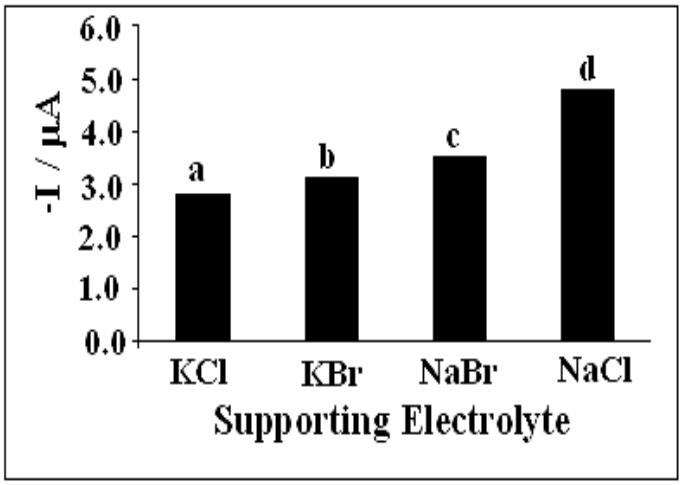


Fig. 8. Influence of supporting electrolyte on SWV reduction current peak of 10^{-3} M accumulated EG at the CPE in 0.1 M of various supporting electrolytes (a) KCl, (b) KBr, (c) NaBr and (d) NaCl

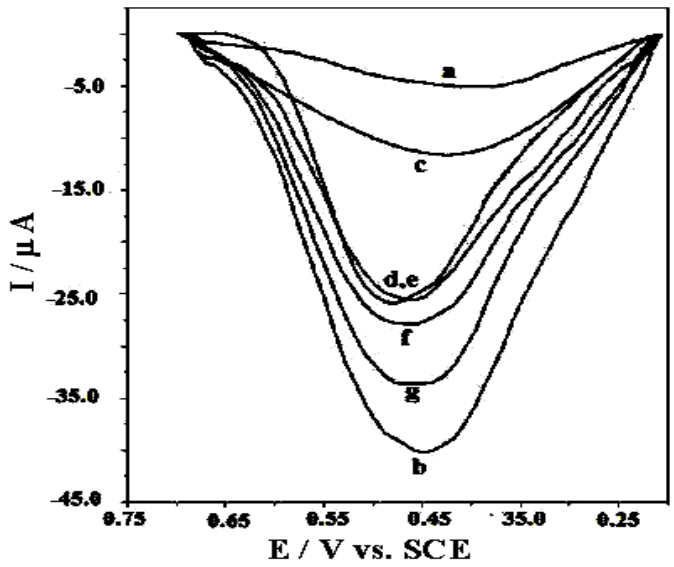


Fig. 9. Square wave voltammograms of 10^{-3} M accumulated EG at bare CPE(a), probe immobilized CPE (b), after hybridization with complementary target sequence (c) and some non-complementary sequences (d) NC_1 ,(e) NC_2 ,(f) NC_3, (g) NC_4

3.6. Recognition of DNA Hybridization with Electrochemical Method

The hybridization of probe with a complementary target sequence was investigated by square wave voltammetric responses of accumulated EG on the CPE surface (Fig. 9). As seen, EG has a low reduction signal on the bare CPE (curve a), while this signal highly

increases after modification of CPE with the probe (curve b). This result indicates that EG accumulated finely on the ssDNA modified electrode by the interaction with DNA. After hybridization of probe with a complementary DNA target, the cathodic peak current of EG at the probe modified electrode decreased greatly (curve c). This result confirms that the dominant interaction mode of DNA and EG is electrostatic binding [28] and is compatible with our obtained results in the section of 3.3.

3.7. Selectivity Study

In order to study of selectivity, the effects of some non-complementary oligonucleotides were investigated. Figure 9 depicts the SWV responses of the accumulated EG on bare CPE and probe-modified CPE (curve b) after hybridization with complementary (carve c) and some non-complementary sequences (curves d, e, f and g). As seen, the interaction between these noncomplementary oligonucleotides and immobilized probe did not lead to a significant decrease in EG signal, due to negligible hybridization. These results further confirmed that this genosensor selectively responds to the target DNA and EG can be acted as able indicator in DNA electrochemical biosensors.

3.8. Detection Limit of probe immobilization

Fig. 10 A illustrates the SWV signals of accumulated EG obtained after immobilization of increasing levels of ssDNA (0.0–4.0 µM) on the surface of CPE. The variation of the voltammetric response *vs.* probe concentration is shown in Fig.10B. The calibration graph is shown in inset of this Fig. There is a linear dependence between SWV signal and ssDNA concentration between 0.03 and 1.0 nM. The detection limit estimated as three times of the ratio between the blank signal and the sensitivity, is 2.0×10^{-10} M.

3.9. Diagnostic performance of the biosensor

The EG reduction signal on the probe-modified CPE in the presence of target is increased with increasing the target concentration (Fig. 11). As seen in inset A the difference between the EG reduction signal of the probe-modified CPE in the presence and absence of the target sample (ΔI) is increased with increasing the complementary target concentration and leveled off at ca.1.2 µM. Thus, in this concentration of target, the maximum capacity of the probe available on the electrode surface is involved in the hybridization event. The calibration graph is linear between 0 and 1 µM with a correlation coefficient of 0.994 (inset B). The detection limit calculated by means of the equation y LOD (ΔI)=yB+3Sy/x and regression equation y (ΔI)=33.14x+1.095 (µM) was about 2 nM. Similar experiments were carried out in the presence of non-complementary DNA (NC_1, NC_2,..). When non-complementary oligonucleotides were used, ΔI was fond very low and close to 9 and remained almost unchanged. With regard to these findings can conclude that the present DNA biosensor

successfully distinguishes complementary oligonucleotides from non-complementary oligonucleotides and confirms the high selectivity of this biosensor.

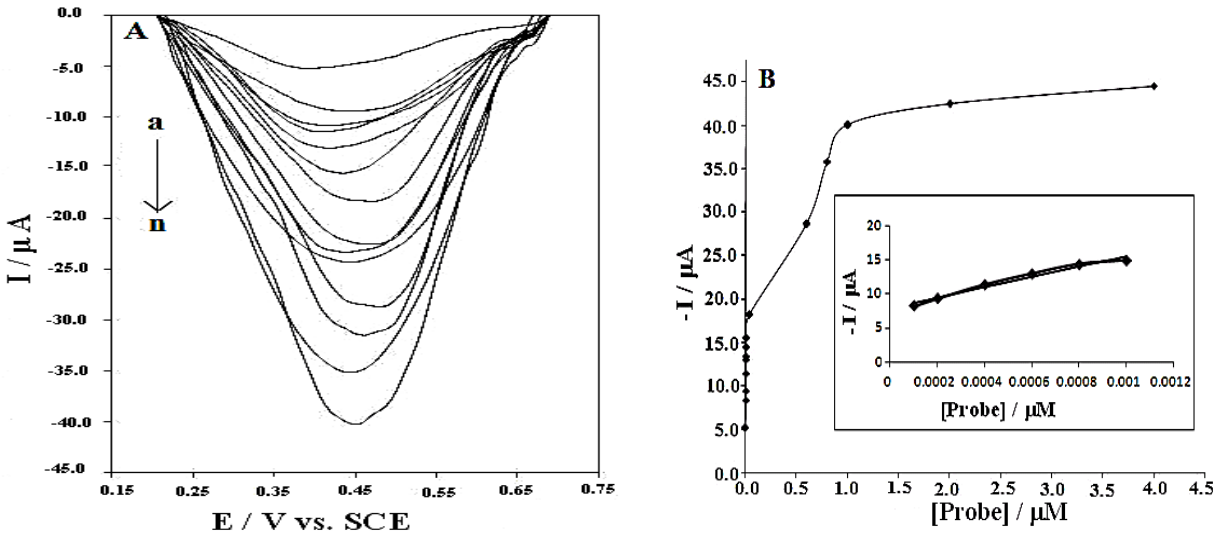


Fig. 10. Square wave voltammograms of 10^{-3} M accumulated EG at different probe concentrations immobilized at CPE (a) 0.00, (b) 0.0001, (c) 0.0002, (d) 0.0004, (e) 0.0006, (f) 0.004, (g) 0.006, (h) 0.04, (i) 0.2, (j) 0.4, (k) 0.6, (l) 0.8, (m) 0.9 and (n) 1.0 µM in 0.1 M phosphate buffer (PH 7.0) with NaCl 0.1 M (B) variation of SWV responses *vs*. probe concentration, *inset*: related calibration plot *vs*. probe concentration in the range of 0.2–1.0 nM

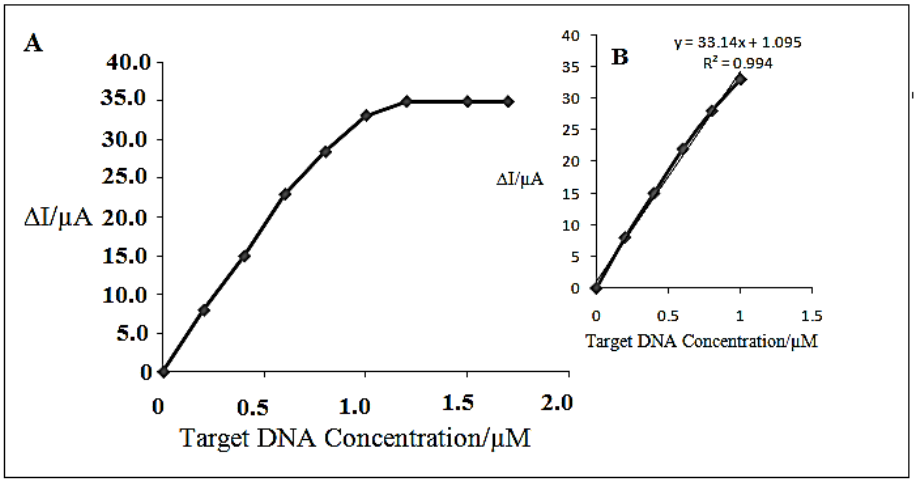


Fig. 11. Plot of ΔI (difference between the SWV signals of EG on the probe modified CPE in the presence and absence of target) *vs*. target concentration; (B) Related calibration graph at concentration range 0–1 µM. Experimental and solution conditions as mentioned in Fig. 10

4. CONCLUSION

The results in this paper illustrate the ability of EG as a new electroactive label for developing a novel DNA biosensor by carbon paste electrode. EG is a cationic organic dye from triphenylmethane family that has affinity to DNA. The spectroscopic and electrochemical studies on the interaction of DNA and EG indicated the electrostatic interaction as dominant interaction mode. In order to achieve the optimized conditions several experiments were designed. The results of these studies corresponded to 0.5 M KBr as optimized kind and concentration of supporting electrolyte in the stage of EG accumulation on the CPE surface, NaCl as supporting electrolyte in the SWV measurement, the imposed potentials of +1.8 and -0.5 V *vs.* SCE for the activation of CPE and the probe immobilization, respectively. The hybridization between probe and DNA oligomers was determined by changes in SWV signal of the accumulated indicator. Effective discrimination against non complementary DNA was obtained. Finally, the results demonstrated the genosensor could be used selectively and sensitively.

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