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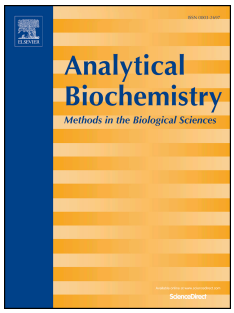

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Electrochemical Label free and sensitive nanobiosensing of DNA hybridization by graphene oxide modified pencil graphite electrode

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Abstract

Based on the strong interaction between single-stranded DNA (ss-DNA) and graphene material, we have constructed a novel label-free electrochemical biosensor for rapid and facile detection of short sequences ss-DNA molecules related to hepatitis C virus 1a using graphene oxide modified pencil graphite electrode. The sensing mechanism is based on the superior adsorption of single-stranded DNA to GO over double stranded DNA (ds-DNA). The intrinsic guanine oxidation signal measured by differential pulse voltammetry (DPV) has been used for duplex DNA formation detection. The probe ss-DNA adsorbs onto the surface of GO via the π– π* stacking interactions leading to a strong background guanine oxidation signal. In the presence of complementary target, formation of helix which has weak binding ability to GO induced ds-DNA to release from the electrode surface and significant variation in differential pulse voltammetric response of guanine bases. The results indicated that the oxidation peak current was proportional to the concentration of complementary strand in the range of 0.1 nM to 0.5 μM with a detection limit of 4.3×10^{-11} M. The simple fabricated electrochemical biosensor has high sensitivity, good selectivity, and could be applied as a new platform for a range of target molecules in future.

Keywords: Graphene oxide; DNA biosensor; Label free; Conformational transformation

1. Introduction

In recent years, sensitive, effective and rapid detection of specific biomolecules have attracted much attention due to their potential roles in medical diagnosis, genetic screening, biological engineering, food quality analysis and environmental protection. DNA sequence detections have various applications such as detection of target genes, discrimination and classification of various organisms and also detection of genetic based disorders. Various DNA biosensors have been developed including fluorescence techniques [1-2], surface plasma resonance spectroscopy [3-6], quartz-crystal microbalance [7, 8], electrochemiluminescence [9], electrochemical [10-15] and so on. Electrochemical detection has attracted a great deal of attention in the development of biosensors because of low background, simplicity operation, fast response time, high sensitivity, miniaturization, cost effectiveness, and etc. New kind of carbon materials carbon nanotube (CNT), graphene (GN) and graphene oxide GO based DNA sensors have attracted considerable attention in recent years due to a number of the outstanding electronic, thermal and mechanical properties and good chemical stability [16-22].

Various electrochemical DNA biosensors based on graphene or its derivatives have been developed [23-27]. Although these approaches have high sensitivity, hybridization indicators or labeled DNA probes are usually needed. Most convenient electrical readout technique is electrochemical impedance spectroscopy (EIS) by using electrochemical redox indicator like $[Ru(phen)_3]^{2+}$, $[Fe(CN)_6]^{3-/4-}$, which has been shown to be well suited for hybridization detection [28-31]. To overcome probe labeling or indicator usage, increasing researches have been made to develop label-free electrochemical DNA biosensor. 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 and seems to be a simple, less time consuming and more applicable strategy in comparison with the others.

The GN modified carbon electrodes were used for the sensitive determination of hybridization based on guanine oxidation signal [32]. Their strategy is similar to unmodified electrodes and both ss-DNA and ds-DNA adsorb on the GN modified electrode surface. Researches showed that GN and GO has superior binding to ss-DNA over rigid ds-DNA and has been used for the fabrication of biosensors for detecting nucleic acids [30, 33, 34], proteins [35, 36], and small molecules [37-39]. Recently, we developed an electrochemical aptasensor for the determination of thrombin based on decrease of guanine oxidation signal after interaction between nucleotides and thrombin [40].

In this work, we have proposed a platform for the fabrication of electrochemical biosensors by using GO as electrode modifier and short sequence oligonucleotides related to hepatitis C virus selected as model oligonucleotides. Unlike the graphene, oxidized form of graphene, GO, has negative surface charge which result in desorption of probe DNA from electrode surface after hybridization with its target DNA. As shown in scheme 1, the probe ss-DNA can be easily immobilized on the surface of GO/PGE due to the π-π* hydrophobic physical adsorption and van der Waals attraction between the purine/pyrimidine ring structure in the nucleobases and the hexagonal cells of GO and target molecules will alter the structure of probe ss-DNA and leads to desorption of formed duplex from the surface of GO and decreasing of guanine oxidation signal. Consequently, electrochemical response obtained at the electrode will be changed, thus the target can be detected. By this approach, more practical biosensor was obtained because of the simple electrode preparation and further signal change due to the ds-DNA desorption from electrode surface. Besides, there is no need for inosine substituted probe as used in previous work. This strategy was demonstrated as a convenient, sensitive and selective detection platform for a range of target analytes.

2. Experimental

2.1. Materials

The pencil graphite was obtained as pencil lead from Rotring Co. LTD, Germany (R 505210 N) of type H. All leads had a diameter of 2.0 mm and were used as received. Oligonucleotides were purchased as lyophilized powder from MWG-Biotech Company. The sequence of probe DNA (PHCV1a) is 5`-TAATGAGGGCTGCGGGTGGG -3`. The sequence of complementary DNA (HCV1a) is 5`-CCCACCCGCAGCCCTCATTA -3`. The sequences of non-complementary DNAs are as 5`-GTGGGTGATATGTGTGG -3`; 5`-TCCACCGCTTCTTGTCCTGCT -3` and 5`-GTGTTATCTCCTAGGTTGGC -3` named HCV2, R2X8B and F4X7B respectively. The sequence of three base mismatched DNA is 5`-CCCGAACGCAGCCCTCATTA -3`. The stock solutions of the oligonucleotides (500µg/ml) were prepared with TE buffer solution (10 mM Tris–HCl, 1 mM EDTA, pH 7.00) and kept frozen. More diluted solutions of the oligonucleotides were prepared by diluting stock solution with phosphate buffer solution pH 5 (PBS, 0.1 M NaH_2PO_4/Na_2HPO_4, 0.10 M NaCl, pH 5). All chemicals were of analytical reagent grade. Distilled, deionized and sterilized water was used in all solution preparation. All DNA solutions were kept frozen at -20 ˚C and 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 4.7 software package (Eco Chemie. The Netherlands). The utilized three-electrode system was composed of a PGE (surface area of 0.0314 cm^2) as the working electrode, a saturated calomel electrode (SCE) as the reference electrode and a platinum wire as the auxiliary electrode.

GO was synthesized from natural graphite powder by the modified Hummers method according to the previous report [40, 41].

2.3. Procedure

2.3.1. Preparation of the Working electrode

The body of pencil lead was tightly coated with Teflon band. Electrical contact with the lead was achieved by soldering a copper wire to the metallic holder of the working electrode. The pencil lead was fixed vertically and immersed in the solution in which the contact was only achieved via cross section of the electrode. The surface was polished on a weighing paper to a smoothed finish then sonicated in nitric acid and washed with doubly distilled water in turn. 1.0 mg graphene oxide was dispersed in 1 mL H_2O by sonication for 20 min to form a homogenous mixture. 3 µL of this mixture (1.0 $mgmL^{-1}$ GO in H_2O) was dropped on the surface of PGE and dried at room temperature before each use. The prepared electrode can be storage for at least one month at room temperature which is usable in this duration.

2.3.2. Probe DNA immobilization

To immobilize the probe DNA (PHCV1a) onto the surface of the GO modified electrode, the electrode should be immersed into a 500 µL PBS (0.1 M NaH_2PO_4/Na_2HPO_4, 0.10 M NaCl, pH 5) containing 6 µM PHCV1a for at least 20 min at room temperature, followed by thoroughly rinsing with PBS before each experiment.

2.3.3. Hybridization

Probe-target interaction (hybridization) was performed by dipping the probe-modified electrode into 1 mL PBS (pH 7.00) containing certain concentration of target DNA for 30 min at room temperature. Then, the electrode was rinsed in a stirred PBS solution for optimum time to remove any unbound or weakly bound substances.

2.3.4. Preparation of double-stranded oligonucleotides

In order to prepare ds-oligonucleotides, the desired pair oligonucleotides (PHCV1a and HCV1a) were effectively mixed in a micro tube with the same concentrations (50 µM). The

mixed solution was heated at 95 ºC in a water bath for 10 min and then was allowed to cool down gradually at room temperature [42, 43]

2.3.5. Voltammetric measurements of hybridization

Electrochemical measurements performed using AUTOLAB PGSTAT 30 electrochemical analysis system and GPES 4.9 software package (Eco Chemie. The Netherlands).

The electrochemical behavior of the electrode surface was studied using anodic differential pulse voltammetry (ADPV) in 20 mM PBS solution (pH 7.00) and scanning the electrode potential between 0.50 and 1.2 V at pulse amplitude of 25 mV. The raw data were treated using the Savitzky and Golay filter (level 2) of the 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 PGE surface by cutting and polishing of the electrode.

3. Results and discussion

3.1. Preliminary investigation

The morphology of the PGE and Go/PGE was characterized using SEM (Fig. 1A, B) and results represent that the prepared graphene oxide shows the flake-like shape and layer–layer structure of graphene oxide edges.

In order to check whether our proposal can work or not, a series of experiments were carried out. Firstly, we have examined whether the ss-DNA or ds-DNA can be immobilized onto the GO modified electrode by using guanine oxidation signal. For this purpose, we used PHCV1a, HCV1a and ds-HCV1a for adsorption on the surface of GO/PGE and then electrochemical behavior of the modified electrodes was investigated. As seen in Fig. 1B, the guanine oxidation signal appears in the presence of PHCV1a and HCV1a immobilized electrodes at about 0.95 V and the peak height for HCV1a adsorbed GO/PGE is very smaller than PHCV1a adsorbed electrode. The smaller signal is due to the low guanine content in the

HCV1a compared to PHCV1a. In the case of ds-HCV1a, there is a negligible peak at 0.95 V which may be due to that ds-DNA cannot be adsorbed onto the GO/PGE surface.

3.2. Optimization of Probe Immobilization Conditions

Some experimental variables such as probe concentration, probe immobilization time, probe immobilization buffer pH and NaCl concentration affecting the immobilization of probe at the sensor surface were studied, and special emphasis was given to the optimization of such experimental variables.

As immobilization of probe ss-DNA onto the GO modified electrode surface is a time and concentration-dependent process, thus the effects of these parameters were studied.

For this purpose, Different amounts of probe were immobilized on the GO modified PGE and DPV response of modified electrodes studied. As shown in Fig. 2A, by increasing probe concentration up to about 6 µM signal increase and then remains unchanged for higher concentrations may be due to the full coverage of the electrode surface. Therefore 6 µM is proposed as a suitable concentration for probe immobilization at electrode surface. The effect of probe immobilization time on the GO modified PGE was also studied. The results obtained from the voltammetric measurements (fig. 2B) revealed that the guanine oxidation signal elevated as the immobilization time increased to about 20 min and remained constant between 20 and 60 min. Therefore 20 min. suggested as a suitable time for probe immobilization at electrode surface.

Probe immobilization solution pH is another factor affecting probe adsorption on GO modified electrode and studied. Results (fig. 2C, curve a) showed that, maximum adsorption of probe DNA on the surface of GO/PGE achieved at pH 5 and then decreased by raising pH. This result may be is due to increasing the electrostatic repulsion between DNA and GO at higher pH values.

Researches showed that there are two opposite interactions between DNA and GO. One of these interactions is electrostatic repulsion between defective amorphous regions (oxidized) of GO that contain the anionic functionalization and negatively charged backbone of DNA. Second one is π-π stacking hydrophobic physical adsorption and van der Waals attraction between the aromatic and hydrophobic rings in DNA structure and the hexagonal cells of GO [44- 48]. To overcome repulsion forces and bring DNA close to the GO surface for binding and facilitate these short-range interactions, electrolytes are needed. For this purpose, the effect of ionic strength on the probe adsorption was studied by using probe immobilization buffers containing various NaCl concentrations. The obtained results (fig. 2C, curve b) showed that by increasing the NaCl concentration up to 100 mM, probe oxidation signal increased and then remained unchanged for higher values. Thus 100 mM selected as optimum value for NaCl concentration at probe immobilization.

3.3. Monitoring of Hybridization

Variation in the guanine oxidation signal of PHCV1a modified GO/PGE upon duplex formation between the probe and complementary target DNA was used for DNA hybridization detection. Fig. 3A shows a typical DPV response of bare PGE, activated PGE and GO/PGE before and after interaction with target complementary DNA. As seen in Fig. 3A, probe DNA don't immobilize on the surface of bare PGE. However, guanine oxidation signal of probe immobilized activated PGE and GO/PGE after interaction with complementary sequence HCV1a decreases, but current decrease in the GO/PGE is several times greater than activated PGE. This is firstly due to that the peak height for probe immobilized GO/PGE is about 4 times higher than that of activated PGE probably because of the high surface area and rapid electron transport and more ss-DNA adsorption capability of GO which leads to the higher current value in comparison with unmodified activated PGE.

Secondly, signal change on the surface of activated PGE is due to the partially availability of redox active groups of guanine bases after hybridization, but in the case of GO/PGE, preferential adsorption of single-stranded DNA (ssDNA) to GO over ds-DNA, result in desorption of formed ds-DNA from electrode surface and greater signal decrease. To establish that this signal decrease is due to the release of ds-DNA from electrode surface and not due to the partly availability of guanine bases in the hybrid, we used $[Fe\ (CN)_6]^{3-/4-}$ as electrochemical active species. Immobilization of negatively charged probe DNA on the electrode surface will inhibit the electron transfer between the electro-active species $[Fe(CN)_6]^{3-/4-}$ and the electrode because of the electrostatic repulsion. After hybridization if the formed ds-DNA releases from electrode surface this result in the electrostatic repulsion decrease, and if not, electrostatic repulsion increases. The obtained results (fig. 3B) showed that hybridization of probe with target leads to desorption of duplex from electrode surface and decreasing charge transfer resistance.

3.4. Optimization of the hybridization conditions

In order to obtain the optimal biosensor operating conditions, effect of some experimental variables such as pH value and reaction time affecting the sensing performance were studied (fig. 4).

One of the important factors which can affect the hybrid formation is pH. Subsequently we studied the effect of hybridization solution pH on the hybridization efficiency and on the basis of obtained results (fig. 4A) pH 7 selected as optimum pH for duplex formation. This result may be due to the fact that the best interaction between two complementary oligonucleotides occurs under the neutral pH condition.

The effect of hybridization time on the guanine oxidation signal of immobilized probe after hybridization with complementary target DNA (a) as well as after interaction with non-complementary DNA (b) at different hybridization times was investigated. As shown in fig.

4B, by increasing hybridization time up to 20 minute difference between complementary and non-complementary DNA signal increases. Consequently, for increasing selectivity and sensitivity of the biosensor 20 minute selected as optimum time for hybridization.

Washing time has effects on removing undesired oligomers (any unbound or weakly bound substances) from the electrode surface. Subsequently we studied the effect of this parameter on the signal of probe modified electrode after interaction with complementary and non-complementary sequences. The obtained results (fig. 4C) showed that by increasing the washing time up to 5 min, signal of complementary oligonucleotides decrease, but signal of non-complementary DNA remain unchanged. Therefore we selected 5 min as washing time before each measurement.

Results obtained here are similar to results of previous paper with low differences in hybridization time [40]. The resulted more interaction time in the case of DNA biosensor compared to aptasensor, may be related to the repulsive force and lower affinity between two complementary ss-DNA compared to Aptamer-analyte.

3.5. Selectivity of the Method in Optimized Conditions

To investigate the selectively of the proposed biosensor to the target DNA, the sensing interface was incubated with target DNA and three non-complementary and three-base mismatched DNA at the same concentrations, respectively. As shown in Fig. 4, the interaction between non-complementary or three base mismatched oligonucleotides with probe at the surface of GO/PGE does not led to a significant decrease in the guanine oxidation signal and maximum ΔI value obtained from complementary DNA hybridization due to the higher hybridization efficiency. Moreover, guanine oxidation signals for the hybridized HCV1a alone (curve b) and in the presence of a mixture of non-complementary and complementary DNAs are almost the same. These results indicate that the suggested

biosensor can discriminate effectively between complementary and non-complementary targets.

3.6. Diagnostic performance

Under the optimized test conditions, the difference between the guanine oxidation signal of the probe modified GO/PGE in the presence and absence of target DNA (ΔI) was increased with increasing the complementary target concentration and leveled off at ca. 0.5 µM (Fig. 5). Thus, at this concentration of target, the maximum capacity of the probe available on the electrode surface is involved in hybridization event. As shown in inset B of Fig. 5, the increased DPV signal was directly related to the logarithmic concentration of HCV1a in the range of 0.1 nM and 0.5 µM with correlation coefficient of 0.995. The detection limit calculated by means of equation: $y_{LOD}\ (\Delta I) = y_B + 3S_{y/x}$ and regression equation: $y\ (nA) = 637.63\ log\ (c/10^{-10}\ M) + 0.92$ was about 4.3×10^{-11}M under optimal condition (signal/noise = 3). The comparison of performance of developed system with various types of carbon electrode based biosensors has been made and presented at Table 1. Considering other label free biosensors based on guanine oxidation signal in the Table, proposed biosensor has acceptible sensitivity (lower LOD) with wide linear range.

3.7. Reproducibility and stability of the biosensor

The reproducibility of the biosensor was evaluated and results showed that the relative standard deviation over five independently probe modified electrodes measured at 5 nM of HCV1a was 4.9 % indicating a remarkable reproducibility of the detection method. The stability of the biosensor was also investigated. Results showed that when the probe-modified electrode was stored at 4˚C, it retained 92% of its initial current after one week storage which displays good stability of the biosensor.

4. Conclusion

A simple and sensitive label-free electrochemical DNA biosensor for the determination of HCV1a gene was developed by employing graphene oxide modified pencil graphite electrode. Under optimized conditions, the decrease of guanine oxidation signal is linearly related to the target oligonucleotide concentration with a detection limit of 0.5 nM. Compared with the existing methods for DNA detection, the strategy eliminated the requirement for DNA labeling, representing a comparatively simple method. This method may also hold promise for potential applications in DNA biosensing and DNA nanostructure framework construction. This method may also be considered as a fundamental platform for the development of further electrochemical DNA detection techniques and more studies will be conducted following this work.

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Table 1. The comparison of performance of developed system with other label free electrochemical biosensors

electrode	method	Linear range	LOD	References
PGE	DPV	0.05 to 0.75 μM	6.5 nM	12
PGE	DPV	10 – 250 nM	5.78 nM	49
PGE	DPV	10 to 100 nM	3.09 nM	50
MnO-Nms/GCPE	DPV	3.75×10^{-8} to 1.5×10^{-6} M	1.31 nM	15
Graphene/PGE	DPV	——	2.09 μg/mL	32
GO/PGE	DPV	0.1 nM and 0.5 μM	4.3×10^{-11}	This work

Legend of figures

Scheme1. Schematic illustration of the label-free electrochemical biosensor for HCV detection.

Fig. 1. The SEM image of (A) bare PGE; (B) GO modified PGE; (C) DPV response of bare GO/PGE (curve a), PHCV1a immobilized GO/PGE (curve b), HCV1a immobilized GO/PGE (curve c), ds-HCV1a immobilized GO/PGE (curve d). Electrode conditions: immersion in 6 μM DNA solution for 20 minute; other experimental and voltammetric conditions were as described in section 2.

Fig. 2. Optimization of probe immobilization conditions; (A) DPV response of PHCV1a immobilized GO/PGE using various probe concentrations in 30 min. immobilization time, Inset: Variation of DPV response versus probe concentrations; (B) DPV response of PHCV1a immobilized GO/PGE using various immobilization times with 6 μM probe concentration, Inset: Variation of DPV response versus probe immobilization times; (C) Effect of probe immobilization solution pH on the obtained guanine oxidation signal (a) and Effect of NaCl concentration of probe immobilization buffer solution on the obtained guanine oxidation signal (b) Experimental and voltammetric conditions were as described in section 2.

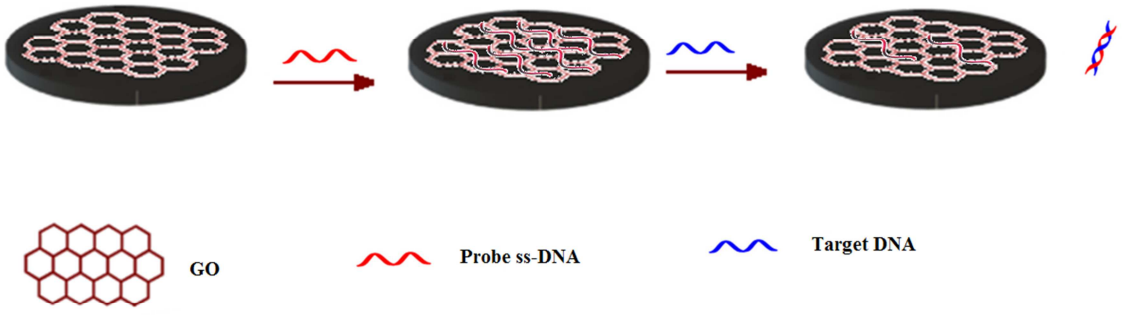
Fig. 3. (A) DPV response of PHCV1a immobilized PGE (a), activated PGE (b, d), GO/PGE (c, e) before (a, b, c) and after (d, e) hybridization with 0.5 μM complementary HCV1a in 10 mM tris-HCl buffer solution; (B) DPV response of bare GO/PGE (curve a), PHCV1a immobilized GO/PGE before (curve b) and after hybridization with complementary HCV1a (curve c) in 10 mM tris-HCl buffer solution containing 5 mM $[Fe(CN)_6]^{3-/4-}$ with pH 6. Experimental and voltammetric conditions were as described in Fig. 1.

Fig. 4. Optimization of PHCV1a immobilization and hybridization condition (A) The effect of pH of hybridization solution on the obtained guanine oxidation signal; (B) Histograms related to the effect of hybridization time and (C) Washing time on the obtained guanine

oxidation signal after interaction with 0.5 µM complementary (a) and non-complementary (b) DNAs. Experimental and voltammetric conditions were as described in Fig. 1.

Fig. 5. Selectivity of the biosensor to HCV1a compared to HCV2, R2X8B, F4X7B, three base miss matched HCV1a, and a mixture of HCV1a and non-complementary oligonucleotides. Experimental and voltammetric conditions were as described in Fig. 4. Electrode conditions: immersion in 6 µM PHCV1a for 20 minute; incubation time: 20 min; washing time: 300 s.

Fig. 6. DPV responses of the PHCV1a/GO/PGE after interaction with different concentration of target HCV1a; Inset A: Variation of the difference between the oxidation signal of the probe modified GO/PGE in the presence and absence of the target sample (ΔI) versus target concentration. Inset B: related calibration graph at concentration range 0.1 nM – 0.5 µM.



Scheme 1

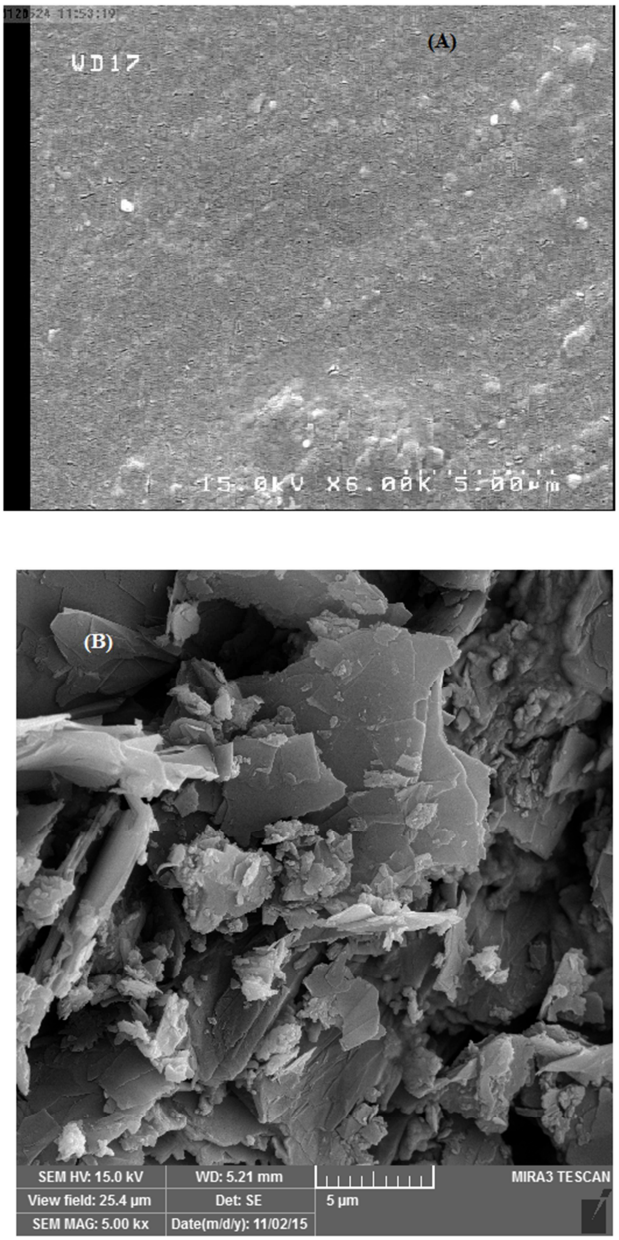

(A)
WD17
15.0kV X6.00K 5.00µm
(B)
SEM HV: 15.0 kV
WD: 5.21 mm
MIRA3 TESCAN
View field: 25.4 µm
Det: SE
5 µm
SEM MAG: 5.00 kx
Date(m/d/y): 11/02/15
(C)
I/ nA
E/ V vs SCE
a
b
c
d
2500
2000
1500
1000
500
0
-500
0.7
0.8
0.9
1
1.1
1.2

Fig. 1

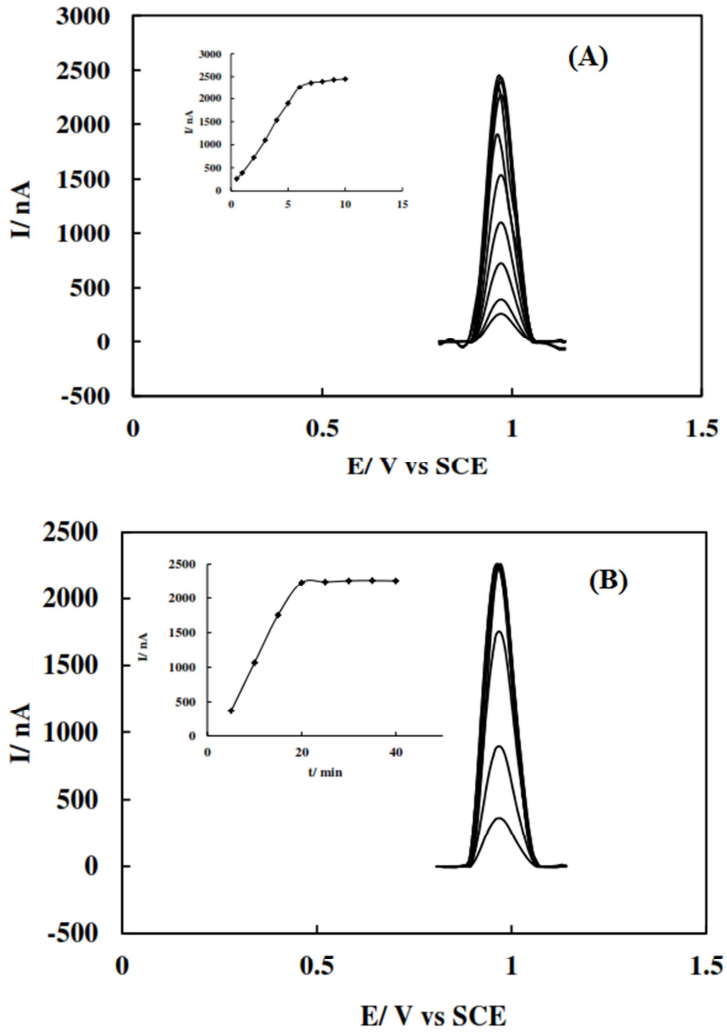


Fig. 2

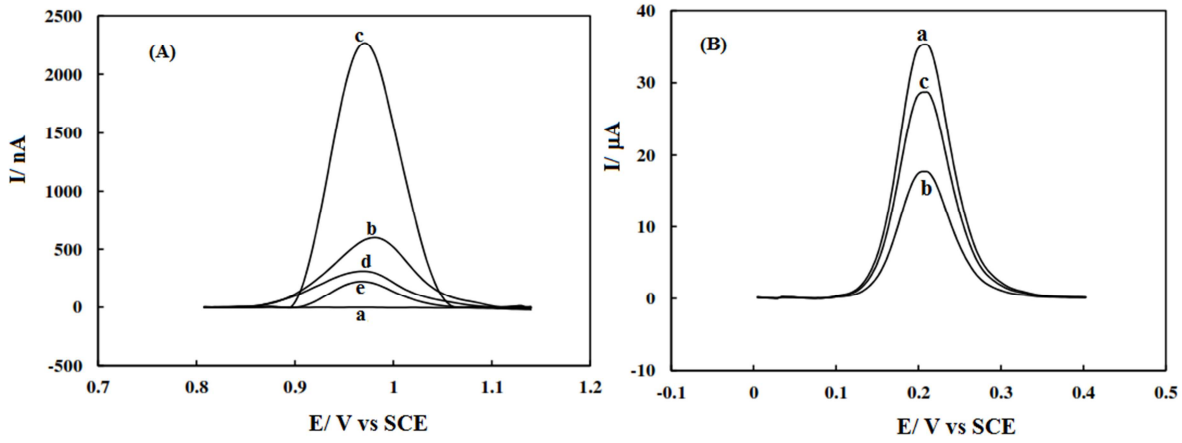


Fig. 3

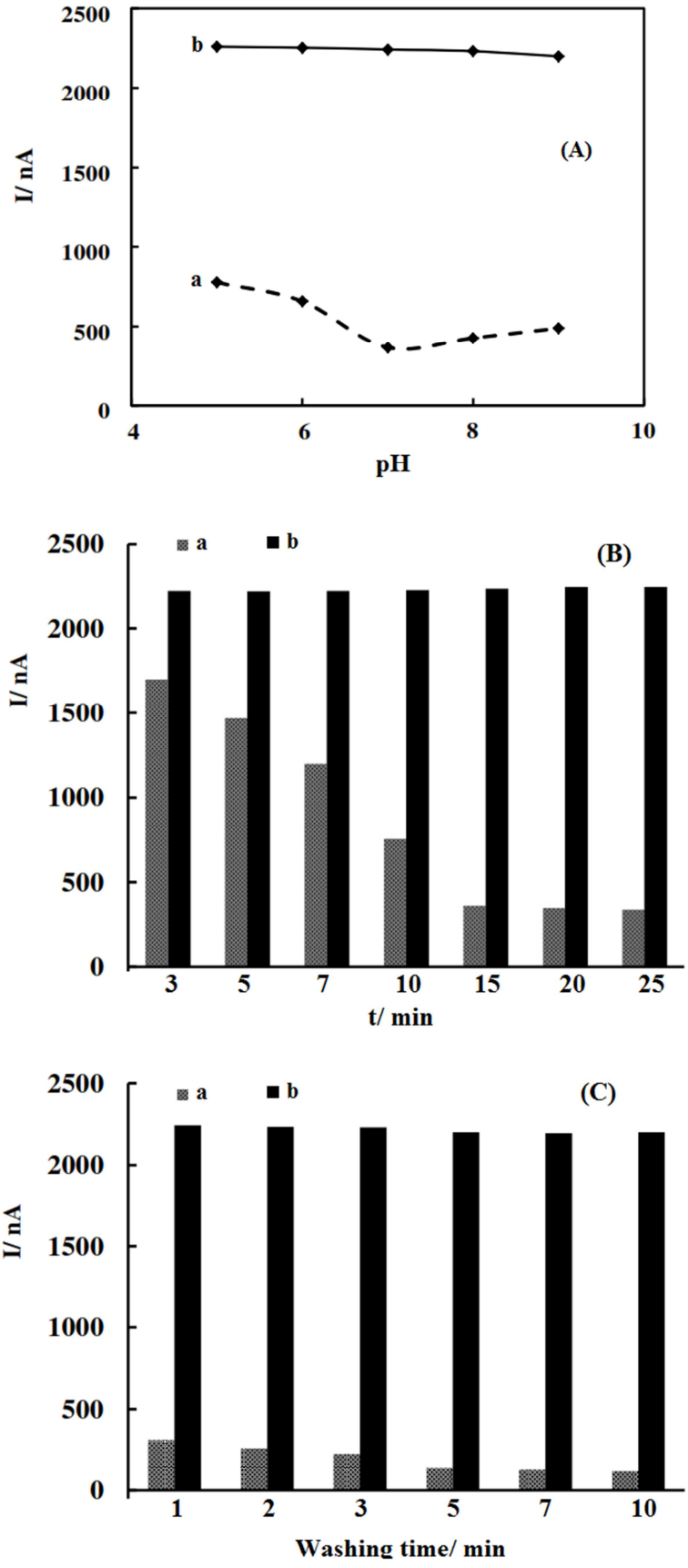

2500
2000
1500
1000
500
0
I/ nA
b
a
(A)
4
6
8
10
pH
a
b
(B)
3
5
7
10
15
20
25
t/ min
(C)
1
2
3
5
7
10
Washing time/ min

Fig. 4

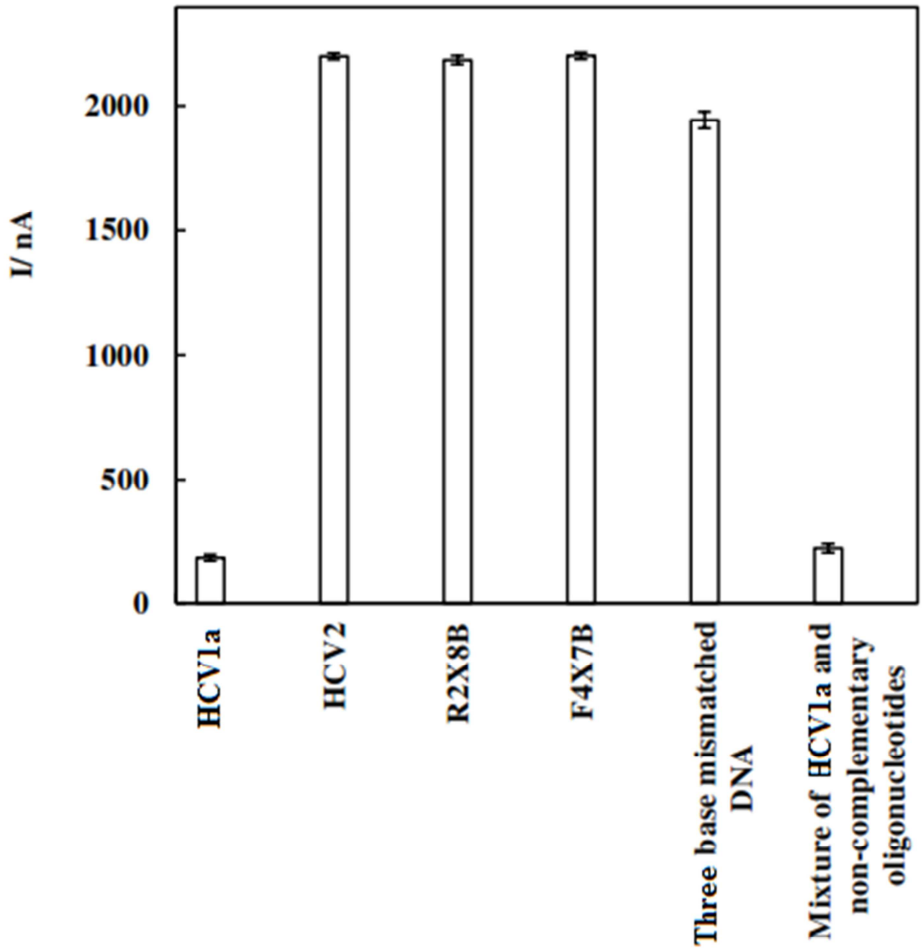


Fig. 5

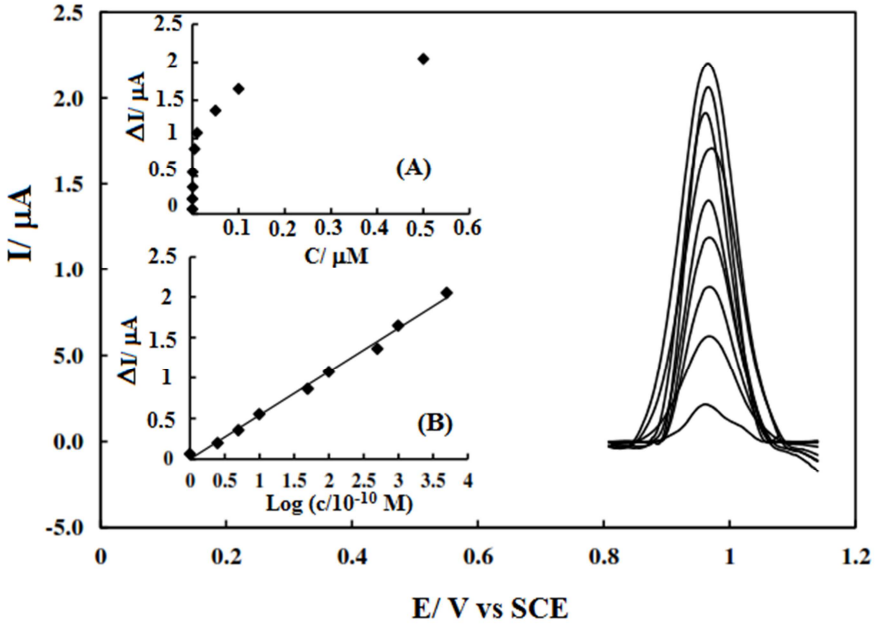


Fig. 6