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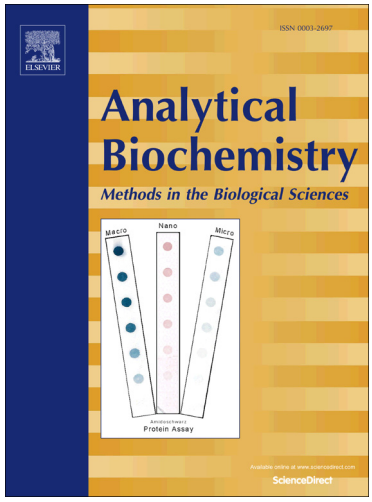

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An ultra-sensitive and selective DNA biosensor based on AuNPs/RGO/GCE for detection of breast cancer

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Abstract

We have designed a simple and novel electrochemical biosensor based on glassy carbon electrode (GCE) for DNA detection. GCE was modified with reduced graphene oxide (RGO) and gold nanoparticles (AuNPs) by electrochemical method, which is helpful for immobilization of thiolated bioreceptors. The electrode modification processes were characterized by SEM and electrochemical methods. Then a single stranded DNA (ssDNA) probe for *BRCA1* 5382 insC mutation detection was immobilized on the modified electrode for a specific time. The experimental conditions such as probe immobilization time and target DNA (Complementary DNA) hybridization time and temperature with probe DNA were optimized using electrochemical methods. The electrochemical response for DNA hybridization and synthesis was measured using electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) methods. The calibration graph contains two linear ranges, the first part is in the range of 3.0×10^{-20} to 1.0×10^{-12} M and the second segment part is in the range of 1.0×10^{-12} to 1.0×10^{-7} M. The biosensor showed excellent selectivity for the detection of the complementary sequences from non-complementary sequences so it can be used for detection of breast cancer.

Keywords: DNA biosensor, Gold nanoparticles, EIS, hybridization, Breast cancer detection, graphene

Introduction

DNA biosensors have an important role in disease diagnosis, cancer detection, gene mutation and legal applications [1]. High specificity and sensitivity are two main characteristics of the DNA biosensors. Therefore many attempts [2-7] have been made to generate inexpensive, sensitive and rapid-response devices for detection of DNA hybridization. The DNA determination by electrochemical biosensors [8-12] comparing with other methods offer major advantages such as simplicity, high speed, cheap and high sensitivity. Increasing the immobilization amount of probe DNA and improving its DNA hybridization efficiency has a key issue in the performance properties of DNA biosensor. In order to achieve this aim, nanomaterials could be used as an interesting material [13-19]. So, we used the combination of reduced graphene oxide (RGO) and gold nanoparticles (AuNPs) for increasing of electrode surface and enhancement of electronic and catalytic properties. The high surface area is a powerful tool for more immobilization of the probe DNA. Graphene is a single layer of carbon atoms in a closely honeycomb two-dimensional lattice and has modern properties such as good mechanical capability, large specific surface area and elevated conductivity. Because of being a strong affinity between sulfur and gold atoms, gold nanoparticles (AuNPs) are usually applied to react with organo sulfur compounds that are responsible to covalently attach biomolecules to the surface of AuNPs [7]. Excellent catalytic activity, great surface area, effective mass transport and compatible environment are main advantages of gold nanoparticles. Recently, graphene–metal nanoparticles composite has attracted enormous attention in many

fields [20, 21]. Graphene–metal nanoparticles composite exhibits extra structural, electronic and chemical properties, and makes large surface area.

Cancer starts due to out of control growth of abnormal cells and causes damage DNA. Damaged cells are not repaired in cancer cells but the cells do not die. There are many types of cancer such as breast cancer that is a malignant tumor. Breast cancer is determined by mammogram, magnetic resonance imaging (MRI) and breast biopsy test. These detection techniques are time-consuming, invasive and expensive, which usually need surgical techniques and sometimes without any positive results. Therefore, development of a cost effective and highly sensitive method for detection of the breast cancer at initial stage is essential.

Electrochemical impedance spectroscopy (EIS) is very sensitive to changes on the electrode surface and can be used as a ‘‘label-free’’ detection of a wide range of molecular distinction. Therefore, this method is a powerful tool for DNA detection. This technique offers major advantages such as high sensitivity, easiness of signal quantification and ability to separate the surface binding events from the solution resistance.

Cyclic voltammetry (CV) is a noticeable and suitable method for confirming the features of modified electrode surfaces. The changes in peak current and the separation of peak potentials in CVs at different electrodes are dependent to the electron transfer rate constant.

In this work, for the first time we reported a new electrochemical biosensor for *BRCA 1* mutation detection based on a glassy carbon electrode modified by reduced graphene oxide (RGO) and AuNPs by CV and EIS methods. The advantages of this electrochemical biosensor are detecting DNA without the use of additional labels, ease of preparation, low detection limit, high sensitivity and stability. In our previous

work we reported an electrochemical biosensor based on gold nanoparticle [5] and in another work we described a kind of biosensor based on RGO modified glassy carbon electrode (GCE) for sex determination [6]. The aim of this fabricated biosensor is to detect breast cancer disease in the localized stage, because of early detection of cancer offer the best chance for cure. Extremely low detection limit of this method confirming that proposed biosensor can be used in the detection of *BRCA 1* mutation. Also, DNA synthesis was done at the surface of electrode to enhance its selectivity and sensitivity. The advantages of the present electrochemical biosensor compared with our pervious works [5, 6] are qualitative and quantitative investigation of *BRCA 1* mutation detection and its wider linear range which were obtained using composition of electrochemically produced RGO and AuNPs.

Experimental

Chemical and reagents

Probes and specific primers were designed based on breast cancer informations obtained from the Gen Bank database. Primers were dissolved in sterile distilled water to form 100 µM stock solution and were kept frozen at -20 °C. Tris-base buffer (20 mmol L^{-1} + 0.1 mol L^{-1}NaCl, pH 7.4) was used as the buffer solution. $HAuCl_4$ and all other chemicals were analytical reagent grade and obtained from Merck. The double distilled water and all the buffers were sterilized using autoclave. All solutions were prepared with deionized water (DI: 18 MΩ cm^{-1} resistivity). Graphene oxide (GO) nanosheets were prepared according to the literatures [7, 22]. 6-Mercapto-1-hexanol (MCH) was purchased from Aldrich. The sequences of the probe and complementary were as follows:

Probe sequence (P1):

5'-SH-AAGCGAGCAAGAGAATTCCAG-3'

Complementary (P1C):

5'-GTGAAAGTATCTAGCACTGCTGGAATTCTCTTGCTCGCTT-3'

Noncomplementary sequence (P1nC):

5'-TGTGAAAGTATCTAGCACTGTGGGAATTCTCTTGCTCGCT-3'

Apparatus and electrochemical measurements

Electrochemical measurements and EIS were applied by Autolab potentiostat/galvanostat model PGSTAT 30 (Eco Chemic, Utrecht, Netherlands) and NOVA 1.7 software at laboratory temperature (25±1°C). The applied three-electrode system was composed of a glassy carbon working electrode (surface area of 7.07 mm^2), an Ag/AgCl (1.0 M KCl) reference electrode, and a platinum auxiliary electrode. The pH measurements were performed using a Metrohm model 691 pH/mV meter. CV and EIS measurements were performed in 0.5 mM $[Fe(CN)_6]^{3-/4-}$ in the supporting electrolyte of 0.1 M KCl. The scan rate in CV plots was 100 mV/s. EIS measurements were recorded at constant DC potential of 0.20 V and the AC voltage was changed between 100 kHz and 0.01 Hz with 5 mV amplitude. Scanning electron microscopy (SEM) was performed with TESCAN instrument model VEGA3.

Modification of GCE and preparation of dsDNA/AuNP/RGO/GCE

Before fabrication, non-modified GCE was polished by 0.05 µm alumina slurry to mirror like, and then the mirror GCE was washed successively with anhydrous alcohol and water. Then steps was followed by ultrasonication for 5 min. 30 µL homogeneously dispersed solution GO (0.050 g mL^{-1}) was pipetted onto the working electrode surface and dried under ambient condition. The GO on GCE was

reduced at a constant potential of -0.9 V for 40 s in PBS (0.1 M, pH=4.1) solution. Then AuNPs were deposited on the modified electrode by reduction of 0.3 mM $HAuCl_4$ solution at a constant potential of -0.2 V for 40 s [23]. This electrode named AuNP/RGO/GCE. The AuNP/RGO/GCE was washed with double-distilled water. Then 50 µL of the DNA probe solution (1 µM) was dropped on the electrode surface for 12 h in a wet chamber. This electrode was named as ssDNA/AuNP/RGO/GCE. To prevent of nonspecific adsorption of DNA at the AuNP/RGO/GCE surface that have not been covered by DNA molecules, the electrode was kept in the MCH solution (1.0 mM) at room temperature for 30 min. This electrode named as MCH/ssDNA/AuNP/RGO/GCE.

For hybridization of MCH/ssDNA/AuNP/RGO/GCE, fabricated electrode was immersed in 100 µL Tris-HCl buffer solution containing a specific concentration of complementary DNA for 50 min at room temperature. This electrode was named dsDNA/AuNP/RGO/GCE.

Results and discussion

Characterizing of RGO and AuNPs by SEM

Fig. 1A shows scanning electron microscopy (SEM) of bare GCE. This figure shows unmodified GCE has a homogeneous surface. Fig. 1B shows SEM image of the RGO/GCE. The differences between two images demonstrate that deposition of RGO on the GCE. The image reveals a large surface area made of petal-like graphene nanoflakes, random directions and sharp edges. Fig. 1C shows SEM image of RGO/GCE modified by AuNPs with electrochemical deposition. As shown in Fig. 1C, many spherical AuNPs are electrodeposited on the surface of RGO/GCE and assembly of AuNPs on RGO/GCE is uniform.

HERE Fig. 1.

Electrochemical characterization of the fabricated electrode

In this experiment, both of EIS and CV were used to monitor the fabrication process of the biosensor. As shown in Fig. 2, compared with the CV response of unmodified GCE, an obvious increase in the peak current (i_p) of GCE/RGO electrode are observed. The enhanced peak current intensity may be due to the high electric transfer kinetics, the huge surface and excellent conductivity of RGO [24]. When AuNPs were electrodeposited on the GCE/RGO, the cathodic and anodic peak currents increased compared to the GCE/RGO, implying that the introduction of AuNPs at the electrode surface increase the electrochemical activity the obtained electrode. By immobilizing probe on the surface of the electrode the peak current decreased and the peak potential separations (ΔE_p) increased significantly due to repellence of $[Fe(CN)_6^{3-/4-}]$ by the negative charged phosphate backbone of probe DNA and blocking the electrode surface by probe DNA. The introduction of complementary DNA increases the negative charge makes increasing repulsion of redox species. On the other side, it has been revealed that the DNA immobilization and hybridization could be affected on current of fabricated electrode surface [25]. The impedance experiments for different modification steps were in good agreement with the results obtained by CV experiments. According to Fig. 3, by modification of the bare electrode with RGO, the surface of the electrode is improved and because of high surface area and excellent conductivity of RGO, R_{ct} decreases compared to bare GCE. By coating AuNPs at RGO/GCE an almost straight line can be seen as the Nyquist plot which implied the characteristic of a diffusion limiting step of the electrochemical process. The respective semicircle diameter at the high frequencies

range, corresponding to the R_{ct} of the electrode surface, increases by immobilization DNA probe on the AuNPs/RGO/GCE surface. After blocking with MCH the value of the R_{ct} is increased, indicating displacement of nonspecifically bound oligonucleotides. In the last step, hybridization of the DNA probe with the complementary DNA the diameter of the semicircle of Nyquist plot significantly enlarged implying a high electron transfer resistance because of hybridization and more repulsion and preventing [$Fe(CN)_6^{3-/4-}$] ions from reaching to the electrode surface. The change in specific electron charge transfer resistance ($\Delta R_{ct} = (R_{ct})_{final} - (R_{ct})_{initial}$) was adopted as the measurement signal.

HERE Fig. 2.

HERE Fig. 3.

Optimization of experimental conditions

Optimization of immobilization time of probe DNA

To progress the detection ability of DNA biosensor, increasing the immobilization amount of probe is important. We investigated different times of probe (1×10^{-6} M) immobilization on the electrode surface from 1 hour to 24 hour. It was observed that with increasing time, ΔR_{ct} increases significantly and the detection sensitivity can be improved by the extension of time. However, when the time is more than about 14 hour, ΔR_{ct} starts to level off, considering the largest ΔR_{ct}, an immobilization time of 14 hour was selected for further studies.

Optimization of MCH incubation time

The effect of incubation in the MCH solution on the fabricated biosensor was studied. Removing the nonspecifically adsorbed probe molecules from the electrode surface; ssDNA/AuNPs/RGO/GCE was incubated in the solution of 1 M MCH for a specific time. Because of nonvertical attachment of DNA molecules on the electrode during self-assembled monolayers (SAM) formation, some DNA molecules might not orient in the right direction at electrode surface during SAM formation. MCH attaches to the nonspecific sites on ssDNA/AuNPs/RGO/GCE, also it makes DNA molecules orient in the right direction and removes nonspecifically adsorbed DNA molecules from the electrode surface. ΔR_{ct} enhanced with the increasing time between 5 and 30 min, and then response signal has been stable. Therefore, the optimum time of 30 min was selected for the incubation of MCH.

Optimization of time and temperature of hybridization

The effect of hybridization time and temperature were optimized. As shown in Fig. 4, the impedance values of target were a function of hybridization time. Hybridization time was changed from 5 min to 1 h and hybridization temperature and complementary target DNA concentration were kept constant at 25 ^{o}C and 1 μM, respectively. As shown in Fig. 4, time 50 min was the optimum hybridization time and was employed for all of experiments.

HERE Fig. 4.

The temperature of hybridization has a key role for hybridization reaction. Results revealed that the ΔR_{ct} value is constant to about 35°C and rapidly decreased with the increase of the temperature. Higher temperature leads to decrease of ΔR_{ct} because of removing some complementary DNA molecules from the surface

electrode. Thus the temperature of 25 °C was chosen as optimum hybridization temperature (Fig. 5).

HERE Fig. 5.

Sensitivity of target sequence recognition

The proposed biosensor was used to assay of different concentration of BRCA 1 and this has been shown in Fig. 6. Inset A of Fig. 6 shows the Randles equivalent circuit, was used to analyze the Nyquist plots of calibration curve. In this equivalent circuit, R_s is the electrolyte (0.5 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl) resistance and was between 73.9 and 85.0 Ω; W_{01}, is Warburg impedance resulting from the diffusion of ions and ranged from 111.0–143.0 $\mu\Omega^{-1}$; CPE is constant phase element and was in the range of 3.91–4.8 μF cm^{-2} ; and R_{ct} is the electron transfer resistance. Among these electrical parameters, we focused on the R_{ct} value recorded after each step, since electron transfer process between $[Fe(CN)_6]^{3-/4-}$ and electrode surface was strongly influenced by electrode modification and hybridization process.

By increasing the concentrations of complementary target DNA, the R_{ct} increased and this can be explained that more sites of surface are occupied by DNA molecules by increasing concentration of target DNA. Under the optimized conditions, as shown in inset B of Fig. 6, ΔR_{ct} versus target concentration is constituted from two linear segments, the first segment of linear correlation is in the range of 3.0×10^{-20} to 1.0×10^{-12} M of target DNA with a regression equation of ΔR_{ct} =1.172 Log C (M) +26.94 and the second segment is from 1.0×10^{-12} to 1.0×10^{-7} M with a regression equation of ΔR_{ct} =0.8269 Log C (M) +22.58. The detection limit based on 3_{sb} and using calibration equation was obtained to be 1.0×10^{-20} M. This results show that combination of RGO and AuNPs due to their unique properties

exhibited high sensitivity. Some characteristics of the proposed electrode were compared with similar reported electrodes [6, 26-30] in Table 1. According to this table detection limit and linear range obtained in this work are extremely better than other works.

HERE Fig. 6.

Syntheses of BRCA 1 by DNA polymerase enzyme

The applied method for DNA synthesis by DNA polymerase enzyme in this research is shown in Scheme 1. Briefly for the *BRCA1* DNA synthesis in the solution the probes initially were immobilized on the surface of modified electrode and then hybridization was occurred between probe and complementary sequences. After hybridization, synthesis procedure was performed by adding enzyme. A solution containing 2 µL of buffer, 2 µL of Tag enzyme (5 U/µL), 2 µL of dNTP (5 mM), and 14 µL of water was pipetted on the dsDNA/AuNPs/RGO/GCE for 1 h until strain ssDNA was constructed according to the model as shown in scheme 1. At last, for denaturation process, electrode was immersed in the hot water (90 °C) for 10 min. Curve a in Fig. 7 shows the EIS response of MCH/ssDNA/AuNPs/RGO/GCE in ferro/ferric cyanide solution. Then complementary (P1C) DNA which is longer than probe DNA and contains a part of complementary sequence for hybridization with probe DNA was introduced in order to hybridize onto its corresponding probe. Curve b of Fig. 7 shows the Nyquist plot of the electrode after hybridization of ssDNA at the electrode surface with complementary sequence. As curve b shows R_{ct} increased compared with MCH/ssDNA/AuNPs/RGO/GCE that indicates hybridization of P1C strains was done with ssDNA probes. After synthesis, R_{ct} (curve c of Fig. 7) increased

compared with dsDNA/AuNPs/RGO/GCE that indicates some segments were added to it due to synthesis by enzyme. The electrode was immersed in water at 93 °C for 10 min to denature the dsDNA. As shown in curve d, R_{ct} was higher than AuNPs/RGO/GCE modified only by original probe. Hence, this indicated that extended probe after synthesis was longer than the initial probe therefore, this confirmed synthesis of *BRCA1* DNA by enzyme along the probe. Also synthesis process was investigated for 10^{-16} M P1nC sequence. Curve e of Fig. 7 shows EIS response for 10^{-16} M P1nC before syntheses and curve f shows EIS response for 10^{-16} M P1nC after syntheses. The results showed that obvious change was not observed in R_{ct} indicating that DNA synthesis was not occurred while in the same condition ssDNA probe hybridized with P1C took part in enlarging probe well. Therefore, it revealed excellent selectivity of the modified electrode.

HERE Fig.7.

The selectivity of the developed biosensor for target sequence recognition

One of the main purposes of fabricating of new biosensors is to recognize differences between the targets DNA from others. To achieve this goal the selectivity of the developed DNA electrochemical biosensor was investigated by using the proposed electrode to hybridize with different kinds of DNA sequences including target P1C and P1nC. The results show that proposed electrode after hybridization with P1C and P1nC have different EIS responses. High change in charge transfer resistance (ΔR_{ct}=14.5 kΩ) was obtained with the 10^{-10} M P1C sequence and there was very low change (ΔR_{ct}=3.5 kΩ) in R_{ct} of P1nC. The phenomenon confirmed the ability of the designed biosensor to differentiate targets DNA from others.

Reproducibility and stability of DNA biosensor

The reproducibility is one of the important characters of a DNA biosensor. To investigate the reproducibility, three independent DNA sensors were prepared in the same condition and the same day. Three parallel immobilizations of RGO and AuNPs on the GCE, modification of the electrode surface by 1.0×10^{-6} M ssDNA and then hybridization with 1.0×10^{-14} M target DNA were examined. An acceptable relative standard deviation of 4.1% (n=3) for ΔR_{ct} ($\Delta R_{ct}=(R_{ct})_{\text{after hybridization}}-(R_{ct})_{\text{before hybridization}}$) was obtained. It indicated that a satisfactory reproducibility could be obtained by this biosensor. The stability of the modified electrode was also tested when a MCH/ssDNA/AuNPs/RGO/GCE was used for determination of 1.0×10^{-8} M target DNA and after measurement it was stored in the 0.1 M PBS buffer solution (pH 7.4) in the refrigerator at 4 °C for at least 15 days. No obvious change ($\Delta R_{ct} < 5\%$) in R_{ct} was observed up to 8 days compared to the fabricated electrode in the first day, but after 10 days the ΔR_{ct} value 6.2% decreased compared to the initial designed electrode. It demonstrates the suitable stability of the proposed DNA sensor.

Conclusion

In this research, we described the setup of a novel DNA label-free electrochemical biosensor for *BRCA1* mutation detection, based on AuNPs/RGO/GCE. Combination of AuNPs and RGO could greatly increase the active sites and enhance the sensitivity of the proposed biosensor. In optimum conditions, this label-free electrochemical biosensor showed a wide dynamic range with a quite low detection limit of 1.0×10^{-20} M for target DNA. This designed impedimetric biosensor with high selectivity and sensitivity can recognize complementary and non-complementary sequences. In addition some benefits of this

electrochemical biosensor are wide linear range, low detection limit, good stability, suitable reproducibility and no needs to indicators. In addition, in order to enhance selectivity and sensitivity of the proposed electrode DNA synthesis was done at the surface of electrode.

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Caption of figures:

Fig. 1. SEM image of bare GCE (A), SEM image of the RGO/GCE (B), SEM image of AuNPs/RGO/GCE

Fig. 2. Cyclic voltammograms obtained for 0.5 mM $[Fe(CN)_6]^{3-/4-}$ in 0.1 M PBS at the surface of different electrodes (scan rate 100 mV s^{-1})

Fig. 3. Electrochemical Impedence Spectroscopy (EIS) obtained for 0.5 mM $[Fe(CN)_6]^{3-/4-}$ in 0.1 M PBS at the surface of different mentioned electrodes. EIS conditions: constant DC potential of 0.200 V, ac potential amplitude 5 mV, frequency range 10 kHz to 0.1 Hz

Fig. 4. Effect of time of hybridization for 1.0×10^{-16} M target DNA at 25 °C, Inset: Comparison of ΔR_{ct} ($\Delta R_{ct}=(R_{ct})_{\text{after hybridization}}-(R_{ct})_{\text{before hybridization}}$) versus the consumed time of hybridization

Fig. 5. Effect of hybridization temperature for 1.0×10^{-16} M target DNA and hybridization time of 60 min. Inset: Comparison of ΔR_{ct} versus the temperature of hybridization

Fig. 6. Nyquist diagrams recorded at ssDNA immobilized electrode after hybridization with different concentration of complementary DNA. Inset A: equivalent circuit used to model impedance data in the presence of redox couples and Inset B: plot of the dependence of ΔR_{ct} versus the concentration of target

Fig. 7. EIS obtained in 0.5 mM $[Fe(CN)_6]^{3-/4-}$ at (a) MCH/ssDNA/AuNPs/RGO/GCE, (b) MCH/ssDNA/AuNPs/RGO/GCE after hybridization with 1×10^{-16} M of complementary DNA, (c) dsDNA/AuNPs/RGO/GCE after synthesis, (d) dsDNA/AuNPs/RGO/GCE after denaturation, (e) MCH/ssDNA/AuNPs/RGO/GCE immersed in 1×10^{-16} M of noncomplementary

DNA, and (f) as (e) after synthesis. EIS parameters: ac potential 5 mV, frequency range 10 kHz to 0.1 Hz

Table 1 Comparison of analytical performances of several electrochemical DNA sensors

DNA sensor	Technique	Linear range (M)	Detection Limit (M)	Hybridization time (min)	Reference
RGO/GCE	EIS	1.0×10^{-20}- 1.0×10^{-14}	3.2×10^{-21}	60	[6]
DNA/Aunano/PLL/GC	CC	1.0×10^{-13}-1.0×10^{-11}	3.5×10^{-14}	40	[25]
DNA/polyaniline/GCE	DPV	22.5×10^{-9}-2.25×10^{-12}	1.0×10^{-12}	30	[26]
ssDNA/Au/PABA/MWCNTs	CC	5.0×10^{-9}-1.0×10^{-12}	3.5×10^{-12}	40	[27]
DNA/AuNP/cys/PGA	DPV	0.09×10^{-9}-4.8×10^{-9}	0.042×10^{-9}	20	[28]
Ag nano/PPAA/MWCNTs	DPV	0.009×10^{-9}-9.0×10^{-9}	3.2×10^{-12}	60	[29]
RGO/AuNPs/GCE	EIS	3.0×10^{-20}-1.0×10^{-12} 1.0×10^{-12}-1.0×10^{-7}	1.0×10^{-20}	50	This work

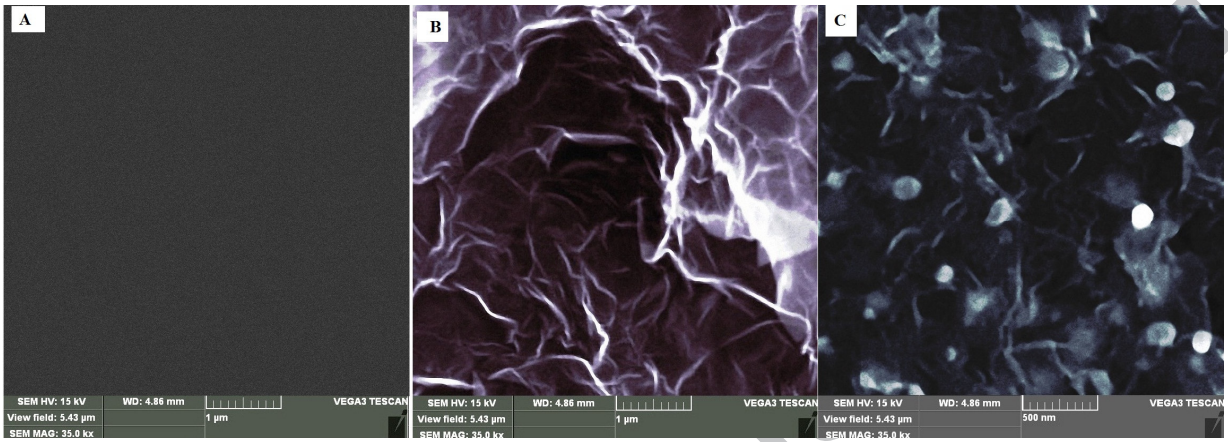


Fig. 1

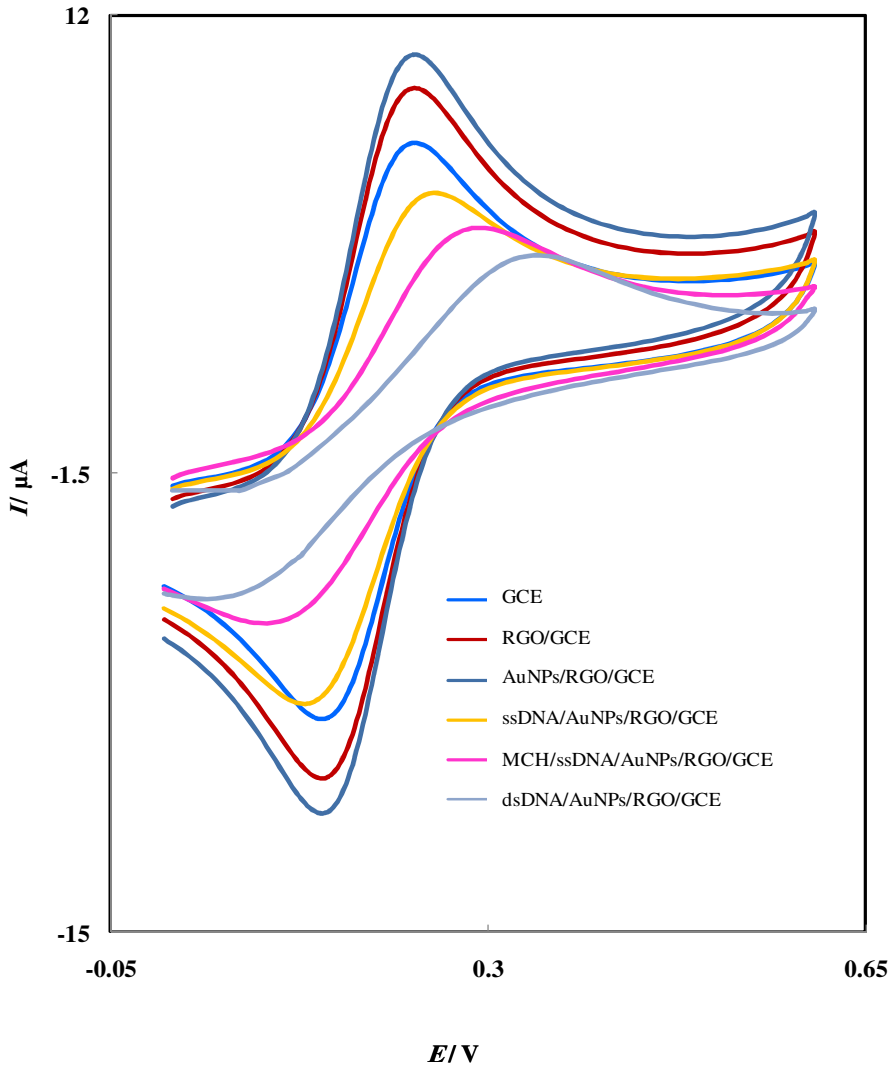
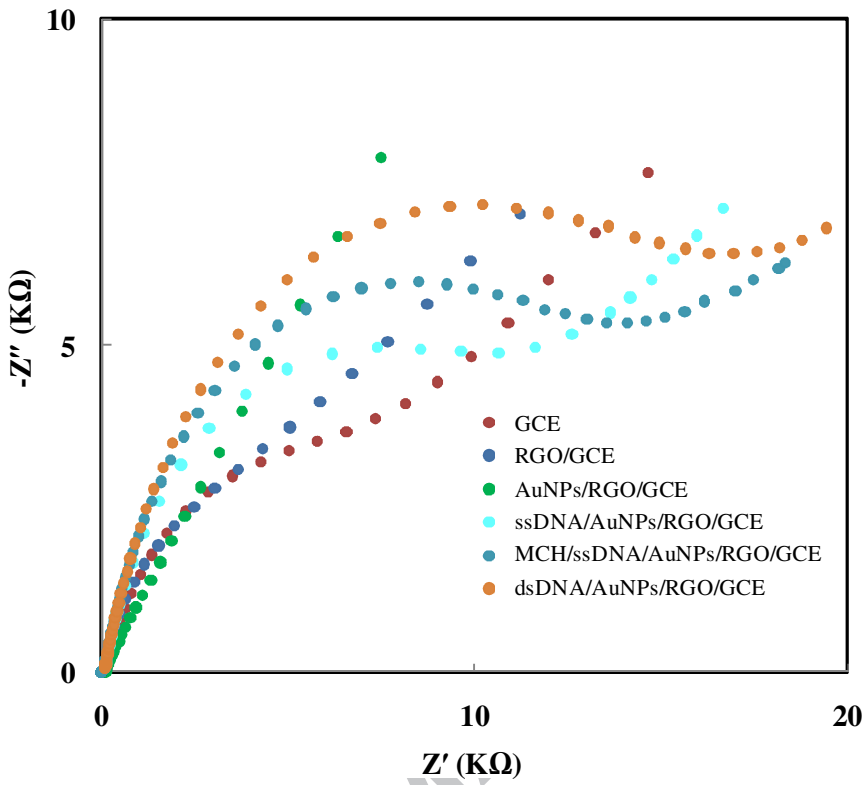


Fig. 2



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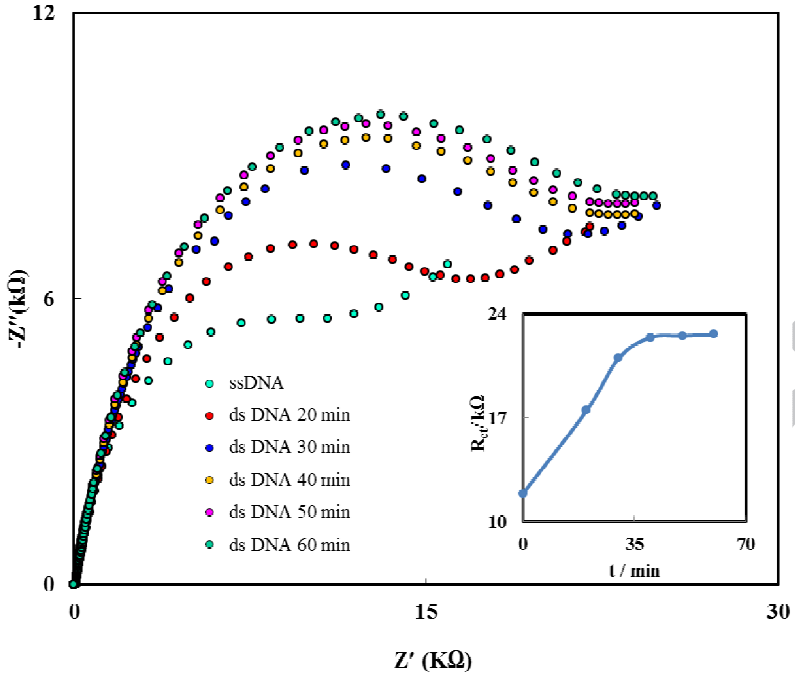


Fig. 4.

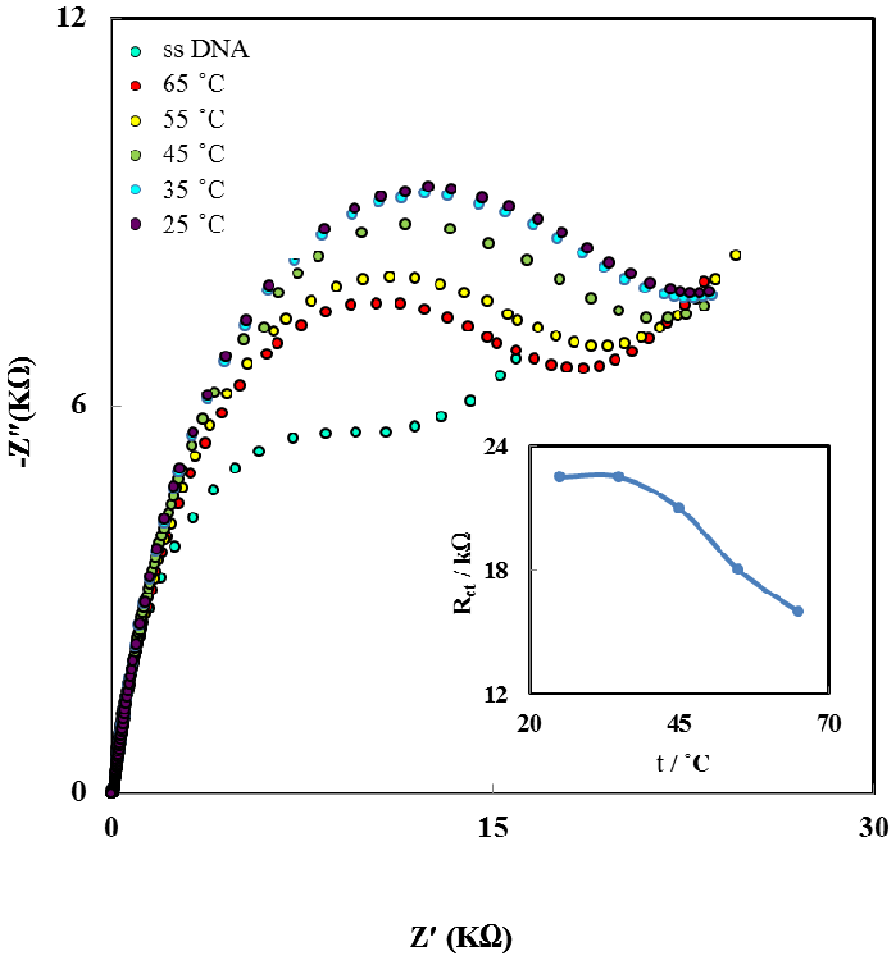
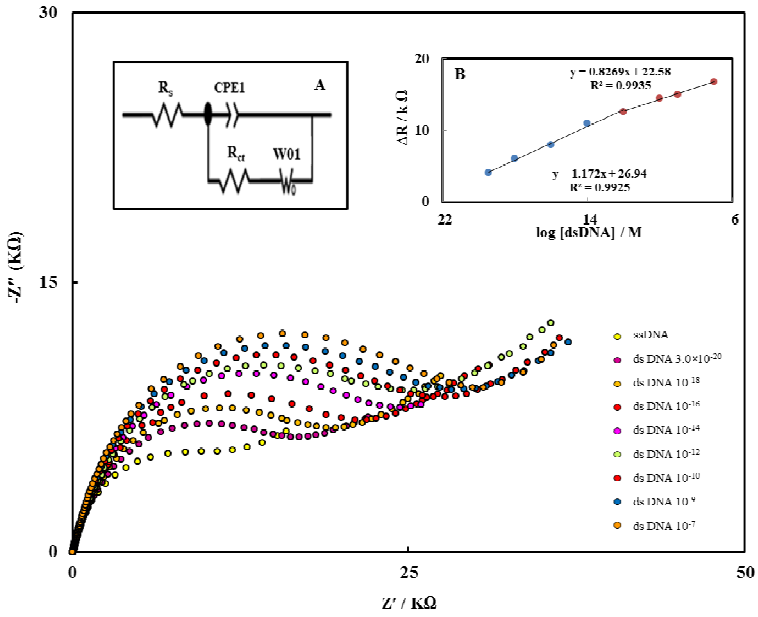


Fig. 5.



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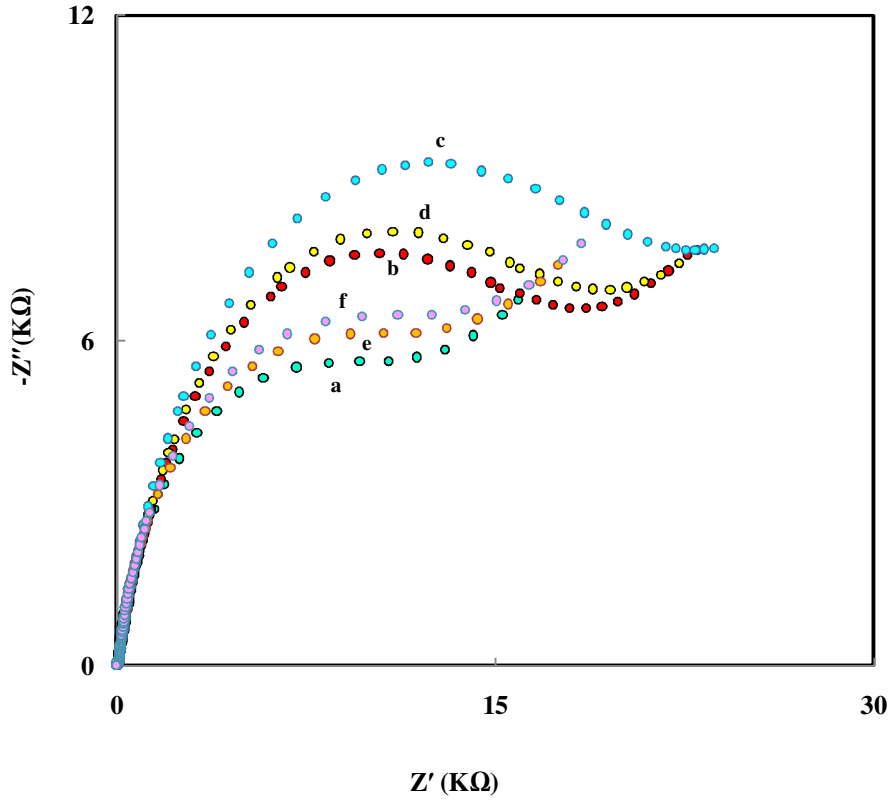
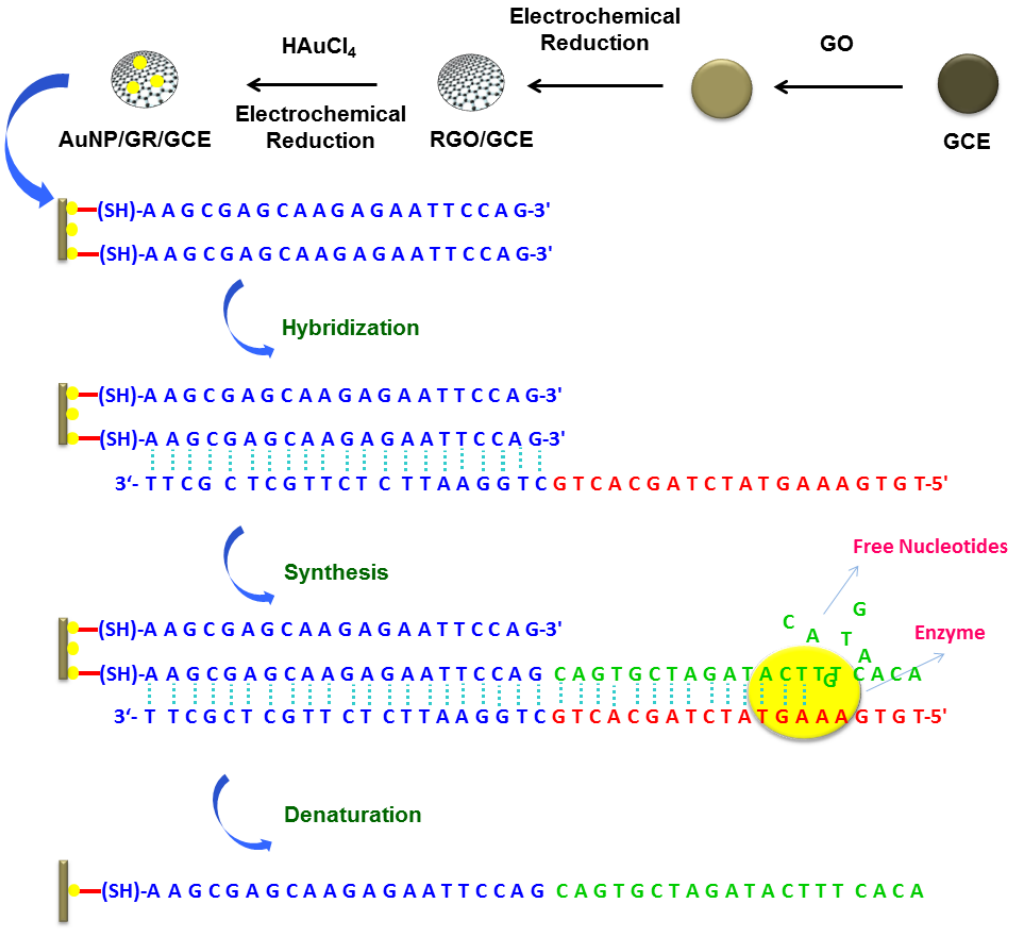


Fig. 7.



Scheme 1. Electrode modification processes and schematic diagram of the DNA synthesis in the presence of DNA polymerase at the surface of modified electrode