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A new methodology for electrostatic immobilization of a non-labeled single strand DNA onto a self-assembled diazonium modified gold electrode and detection of its hybridization by differential pulse voltammetry

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ABSTRACT

In this work, a self-assembled diazonium modified gold electrode (D-MGE) was used for the fabrication of an electrochemical DNA biosensor. For preparation of D-MGE, initially 2-amino-5-mercapto-1, 3, 4-thiadiazole (AMT) self-assembled on gold electrode and a simple diazonation reaction was used to prepare D-MGE. Then, non-labeled single strand DNA (NL-ssDNA) directly was immobilized on D-MGE through electrostatic interaction. Cyclic voltammetry and electrochemical impedance spectroscopy (EIS) characterized the DNA biosensor fabrication process with the use of ferro-ferric cyanide as an electrochemical redox indicator. The hybridization capacity of the developed biosensor was monitored with differential pulse voltammetry using $[Fe(CN)_6]^{4-}$ as an indicating probe. A wide dynamic detection range (7.9×10^{-11}–1.2×10^{-7} mol L^{-1}) and a low detection limit (1.4×10^{-11} mol L^{-1}) were achieved for the complementary sequence. In addition, the hybridization specificity experiments showed that the sensing system could accurately discriminate complementary sequence from non-complementary sequences.

1. Introduction

In recent years, the interest for DNA-based diagnostic tests has been growing. The development of systems allowing DNA detection is motivated by applications in many fields: DNA diagnostics, gene analysis, fast detection of biological warfare agents, and forensic applications. Detection of genetic mutations at the molecular level opens up the possibility of performing reliable diagnostics even before any symptom of a disease appears. Optical [1], electrochemical [2,3], and micro-gravimetrical [4] DNA transductions have been widely studied. The electrochemical DNA biosensor is, among all, able to offer a simple, rapid yet accurate, low-cost point of care detection of selected DNA sequences, and this has been the topic of considerable interest to many researchers in recent times [5,6]. During the past few decades, many efforts have been devoted to explore new supporting materials and immobilization methods for the fabrication of DNA biosensors.

Several methods for surface-capturing DNA probes onto transducer surfaces are known, such as chemical adsorption, covalent-binding, electrostatic attraction, co-polymerization, and via the avidin–biotin affinity system. Diazonium salts are known to react easily with nucleophilic groups like thiols, amines or even aromatic compounds [7,8]. Biological molecules as nucleic acids and proteins are natural polymers of nucleotides or amino acids, respectively, which present different chemical groups as aromatics, thiols, phenol rings, phosphates, primary amines, imidazole rings, and guanidine functions [9,10].

Several metal complexes such as cobalt phenanthroline [11,12], Hexamine–ruthenium (III) chloride (RuHex) [13], and organic dyes such as methylene blue [14] were used as labels for the detection of hybridization. In previous, researchers used $[Fe(CN)_6]^{4-}$ for characterization of electrode process [15] or determination of sensitivity of biosensor by electrochemical impedance spectroscopy (EIS) [16,17]. But, to our knowledge, there is no report on the use of $[Fe(CN)_6]^{4-}$ for quantification of DNA concentration. In this work, we used $[Fe(CN)_6]^{4-}$ for the determination of DNA by differential pulse voltammetric (DPV) method for the first time. We introduce diazonium salt to gold electrode by self-assembly technique for electrostatic immobilization of non-labeled DNA. The oxidation peak of the $[Fe(CN)_6]^{4-}$ in DPV is used for the detection of DNA. The anodic peak currents (i_{pa}) are related to the concentration of DNA sequence between 7.9×10^{-11} and 1.2×10^{-7} mol L^{-1} with a detection limit of 1.4×10^{-11} mol L^{-1} ($S/N=3$). The fabricated biosensor showed promising performance, e.g., high sensitivity, good selectivity, and regeneration ability. The probe showed high sensitivity and selectivity.

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2. Material and methods

2.1. Reagents

All synthetic oligonucleotides were purchased from Aminsan Company (Tehran, Iran). Their base sequences are as follows:

Probe DNA sequence: 5′-PO_4-AGCATGGACCTCACCAACACTG-3′;

Complementary target DNA sequence: 5′-CAGTGTTGGTGAGGT CCATGCT-3′;

Non-complementary target DNA: 5′-GCACTGTGCGCTTAAAG AAGCC-3′.

Potassium nitrate, 2-amino-5-mercapto-1,3,4-thiadiazole (AMT), $K_3Fe(CN)_6$, and $K_4Fe(CN)_6$ were purchased from Merck (Germany).

2.2. Instrumentation

Voltammetric experiments were performed with an EN50081-2 electrochemical workstation (Declaration of company, Netherlands) and electrochemical impedance spectroscopy (EIS) measurements were carried out on an Autolab 302 electrochemical workstation. A conventional three-electrode system was used with a working electrode (un-modified or modified), a 3.5-M KCl Ag/AgCl reference electrode, and a platinum wire counter electrode (Azar Electrode, Iran). A Metrohm-827 pH/mV meter (Switzerland) was used for pH adjustments. The measurement was carried out at room temperature.

2.3. Fabrication of diazonium modified gold electrode (D-MGE)

Diazonium modified gold electrode (D-MGE) was formed according to the literature [11] by a little modification. In brief, AMT-MGE was prepared by immersing the clean gold electrode in an ethanolic solution of 5.0×10^{-4} mol L^{-1} AMT for 12 h at room temperature. In this process the AMT self-assembled to the surface of the gold electrode. The electrode was then rinsed with ethanol and distilled water in order to remove the unbonded thiol. Then AMT-MGE immersed in 30 mL of 0.1 M HCl solution at 2–4 °C, afterward 100 mg of sodium nitrite was added gently and shakes for 30 min to reaction take place in surface of the electrode. Finally, the electrode removed and immediately rinsed with cool water. The obtained electrode denoted as D-MGE.

2.4. DNA immobilization and hybridization

A 1.0×10^{-7} mol L^{-1} stock solution of Non-labeled ssDNA (NL-ssDNA) in phosphate buffer solution of pH 7.0 (physiological pH) was prepared, and stored at 4 °C. A 10 μL portion of DNA solution was immobilized onto D-MGE by drop-cast method and dried for 1 h at room temperature. For the execution of DNA hybridization, NL-ssDNA immobilized electrode was immersed into stirred phosphate buffer solution (pH 7.0) containing different concentration of target DNA for 30 min at 37 °C. Then, the electrodes were taken out and rinsed with double distilled water. The whole DNA biosensor fabrication process was schematically demonstrated in Fig. 1.

2.5. Electrochemical measurements

The biosensor fabricated was transferred to an electrochemical cell including 10 mL of 0.10 M PBS (pH 7.0) containing 5.0 mmol L^{-1} $[Fe(CN)_6]^{4-}$, 0.1 mol L^{-1} KNO_3, and different concentrations of DNA. The DPV measurements were performed in the potential range from 0.0 to 0.5 V. The DPV parameters were 50 mV pulse amplitude, pulse width 50 ms, and a scan rate of 20 mV/S. The EIS measurements were performed in 1.0 mmol L^{-1} $[Fe(CN)_6]^{3-/4-}$ containing 0.10 mol L^{-1} KNO_3 with the frequency range from 10^4 to 0.1 Hz.

3. Result and discussion

3.1. Electrochemical behaviors of $[Fe(CN)_6]^{4-}$ at D-MGE

Fig. 2 shows the cyclic voltammograms of 5 mM $[Fe(CN)_6]^{4-}$ using different electrodes. There was a pair of well-defined redox peaks observed on the bare gold electrode with the anodic (E_{pa}) and cathodic (E_{pc}) peak potential of 0.244 and 0.169 V, respectively, and a peak to peak potential separation of about 75 mV (a). These peaks could be definitely attributed to the redox behaviors of $[Fe(CN)_6]^{4-}$. The self-assembly of AMT on electrode surface inhibited the peak currents of $[Fe(CN)_6]^{4-}$ (b). This could be easily

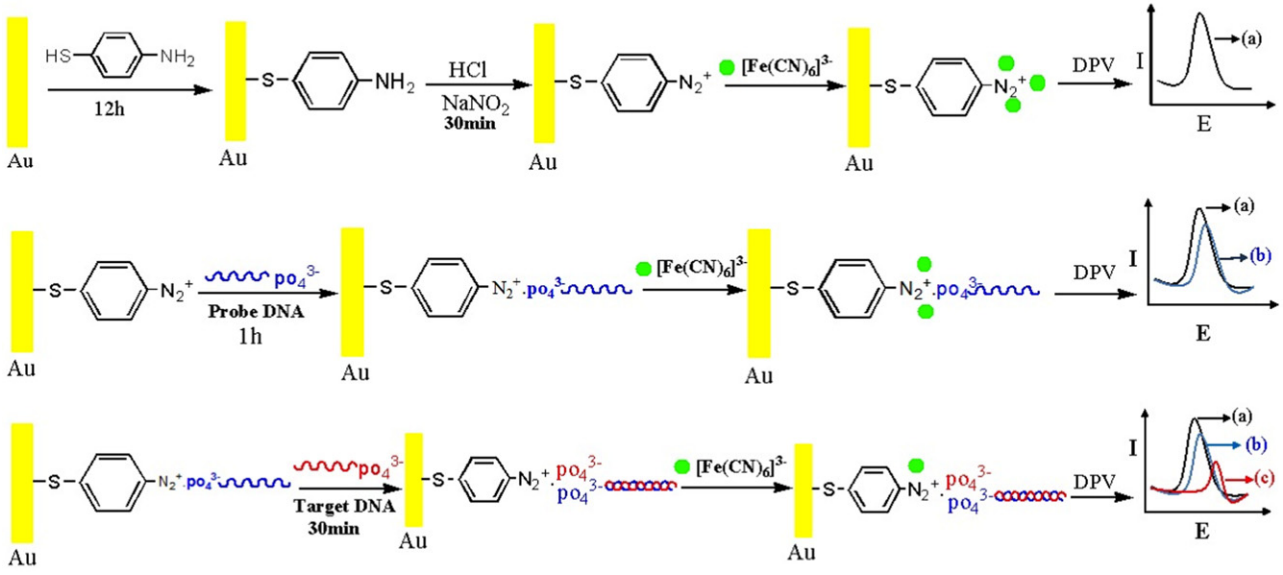

Fig. 1. Schematic illustration of the fabrication steps of the electrochemical DNA biosensor. The DPVs are for $[Fe(CN)6]^{4-}$ at (a) diazonium modified; (b) NL-ssDNA immobilized; and (c) hybridized with complementary target DNA electrodes.

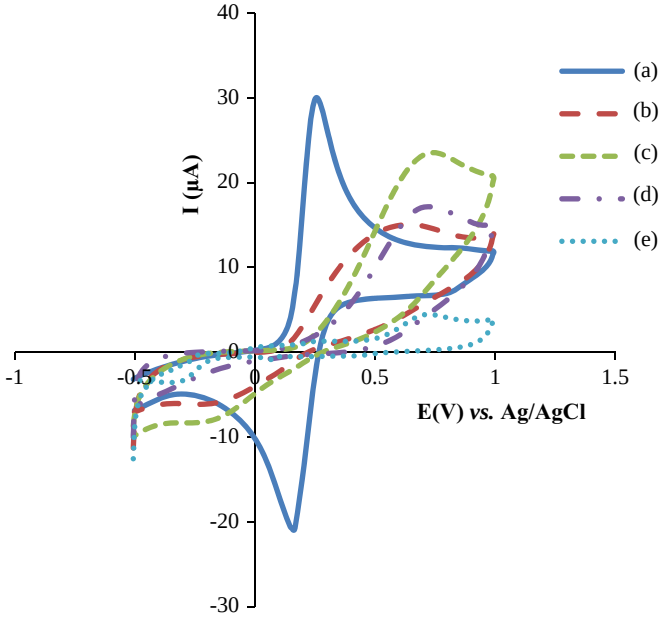

Fig. 2. Cyclic voltammograms obtained for bare (a), AMT modified (b), diazonium modified (c), NL-ssDNA immobilized (d), and hybridized with complementary target DNA (e) at 50 mV s^{-1} in 5.0 mM $[Fe(CN)_6]^{4-}$ containing 0.1 mol L^{-1} KNO_3.

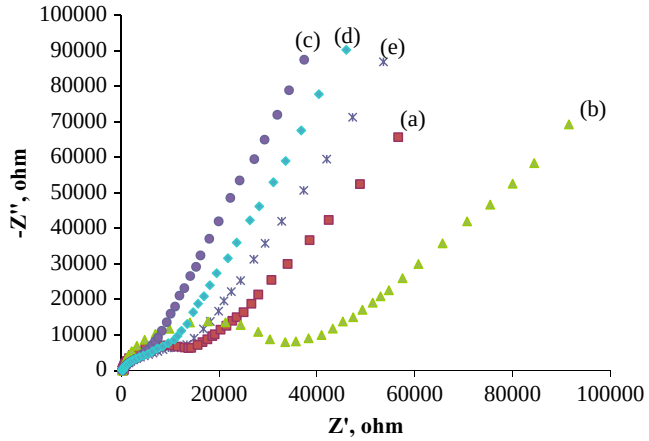

Fig. 3. Electrochemical impedance spectroscopy of 5.0 mM $[Fe(CN)_6]^{3-/4-}$ recorded on bare gold electrode (a), AMT-MGE (b), D-MGE (c), NL-ssDNA-(D-MGE) (d), and target-NL-ssDNA-(D-MGE) (e) in 0.1 mol L^{-1} KNO_3.

understood that a relatively compact monolayer of AMT formed on the electrode surface that prevents from diffusion of $[Fe(CN)_6]^{4-}$ toward the electrode surface. The next modification of diazonium on the electrode surface induced an increase of peak current (c). Such significant increase might be ascribed to the excellent conductivity of the diazonium that accelerate redox reaction of $[Fe(CN)_6]^{4-}$ [18] and facilitation of diffusion of $[Fe(CN)_6]^{4-}$ onto electrode surface through electrostatic interaction between positive charge of diazonium salt and negative charge of $[Fe(CN)_6]^{4-}$. It could be observed that the redox peaks of $[Fe(CN)_6]^{4-}$ significantly decreased in the studied potential range after probe DNA immobilization (d), indicating that the probe DNA has been successfully immobilized on the electrode surface and the diffusion of $[Fe(CN)_6]^{4-}$ toward electrode surface was largely repelled by the negative charged phosphate backbone of probe DNA. The $[Fe(CN)_6]^{4-}$ current in the studied potential range was further decreased after hybridization of probe DNA modified electrode with targets DNA (e). This could be well assigned that the introduction of complementary DNA increases the negative charge responsible for the increased repellence of redox species.

Electrochemical impedance spectroscopy (EIS) analysis was performed to provide further evidence for interface assembly on electrode and to study the interface properties of the developed biosensor. Fig. 3 showed that the Nyquist plots obtained on bare Au electrode (a), AMT-MGE (b), D-MGE (c), NL-ssDNA immobilized (d), and after hybridization with complementary target DNA (e). As seen, significant differences in the electron transfer resistances (R_{Ct}) were observed upon the stepwise formation of the modified electrode. Such differences in peak shape and position can be justified as same as voltammogram peak one, because in both method, prevention from diffusion of $[Fe(CN)_6]4^-$ salts to electrode surface, play important role. As seen in Fig. 3, diameter of the semicircle, indicating the corresponding R_{ct}, increased for the case of NL-ssDNA and target DNA immobilization on the D-MGE. This is attributed to increase in the repulsive interaction (electrostatic and steric) between the negative charge of redox probe and the phosphate group of NL-ssDNA and target DNA at electrode surface; the repulsion impedes the charge-transfer through the interface. These results are in good agreement with the results of cyclic voltammetry represented in Fig. 2. Compare this EIS analysis with that reported by Enayati and Mehrgardi [11], show that, more shift happen in EIS graph when we introduce NL-ssDNA and target DNA onto modified electrode, that proves stronger interaction of NL-ssDNA rather NH_2-labaled DNA with D-MGE. A large number of electrostatic interaction between numerous phosphate groups of probe DNA and diazonium film on the electrode surface is superior to one covalent bond between NH_2 group of probe DNA and electrode surface diazonium salt described in Ref. [11]. Hence, this factor affect on the sensitivity, detection limit, and concentration range of the developed biosensor.

3.2. Optimization of the probe DNA

The sensitivity of the developed biosensor was investigated by varying the concentration of NL-ssDNA sequences with differential pulse voltammetric (DPV) technique. As shown in Fig. 4, with increase of NL-ssDNA concentration from 10^{-10} to 10^{-7} mol L^{-1}, the peak current of modified electrode decreases because phosphate groups of incorporated NL-ssDNA prevent from diffusion of $[Fe(CN)_6]^{4-}$ onto surfaces modified electrode. Further increase in NL-ssDNA concentration, however, resulted in no decrease in the peak current of modified electrode, most probably due to saturation of the electrode surface. Thus, the optimum immobilization concentration for probe was chosen as 10^{-7} mol L^{-1} in order to obtain the full surface coverage of D-MGE.

3.3. Analytical performance

The calibration curve of the biosensor was explored using different concentrations of the complementary target, according to the described procedure. With increasing concentration of the target DNA in the solution, the oxidation peak current of $[Fe(CN)_6]^{4-}$ was decreased gradually in DPV as shown in Fig. 5. This indicating more hybridization reaction occurred on the electrode surface and hence increases phosphate groups of target DNA in electrode surface prevent from diffusion of $[Fe(CN)_6]^{4-}$ to electrode surface.

DNA concentration ranging from 1.0×10^{-12} to 1.0×10^{-6} mol L^{-1} was used for the target DNA calibration curve. The peak current in the DPV response probe decreases with the increase in the complementary target DNA concentration.

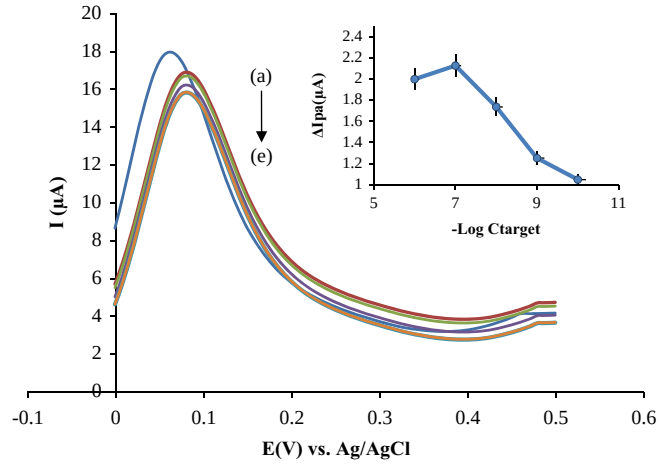

Fig. 4. The DPV response of the phosphate buffer solution (pH 7.0) containing 5.0 mM $[Fe(CN)_6]^{4-}$ and 0.1 mol L^{-1} KNO_3 recorded for the (D-MGE) that incorporated with various concentrations of NL-ss DNA: (a) 1.0×10^{-10}; (b) 1.0×10^{-9}; (c) 1.0×10^{-8}; (d) 1.0×10^{-7}; (e) 1.0×10^{-6} (mol L^{-1}). Inset: decrease of peak current (ΔI_p) vs. logarithm of concentration of NL-ss DNA. DPV parameters were 25 mV pulse amplitude, pulse width 50 ms and a scan rate of 20 mV/S.

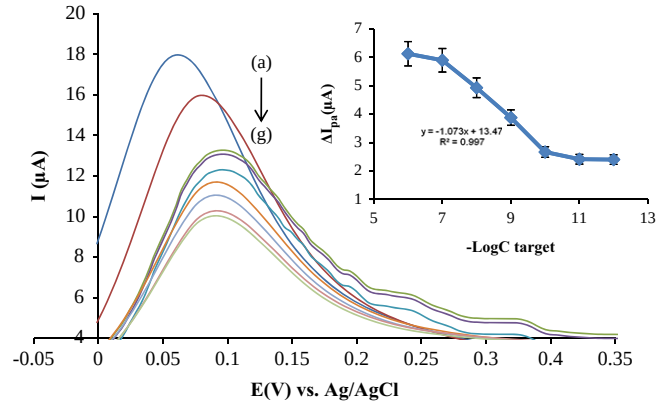

Fig. 5. The DPV response of phosphate buffer solution (pH 7.0) containing 5.0 mM $[Fe(CN)_6]^{4-}$ and 0.1 mol L^{-1} KNO_3 recorded for the (D-MGE) incorporated with 10^{-7} mol L^{-1} NL-ss DNA and hybridized with different concentrations of target DNA, pulse amplitude was 50 mV. The target concentrations are (a) 1.0×10^{-12}; (b) 1.0×10^{-11}; (c) 1.0×10^{-10}; (d) 1.0×10^{-9}; (e) 1.0×10^{-8}; (f) 1.0×10^{-7}; and (g) 1.0×10^{-6} (mol L^{-1}). Inset: decrease of peak current (ΔI_p) vs. logarithm of concentration of target DNA sequence. DPV parameters were 25 mV pulse amplitude, pulse width 50 ms and a scan rate of 20 mV/S.

The relationship between the peak current variation of target DNA and the natural logarithm of the target DNA concentration is illustrated in Fig. 5. As seen, the decrease in anodic peak currents was in linear relation with the concentration of target DNA in a range of 7.9×10^{-11}–13×10^{-7} mol L^{-1}. The detection limit from the calibration curve with $r^2=0.997$ was obtained 1.4×10^{-11} mol L^{-1}.

3.4. Selectivity and regeneration of DNA biosensor

The selectivity of DNA biosensor was tested by using DNA probes to hybrid with different target DNA, such as complementary sequence and non-complementary sequence [15]. The response of non-complementary sequence was almost the same with DNA probes, and complementary sequence decreases the oxidation peak current of $[Fe(CN)_6]^{4-}$ significantly (Fig. 6).

The regeneration of DNA biosensor is extremely important to practical applications such as clinical diagnoses and biological monitoring. It was found that the fabricated DNA biosensor could

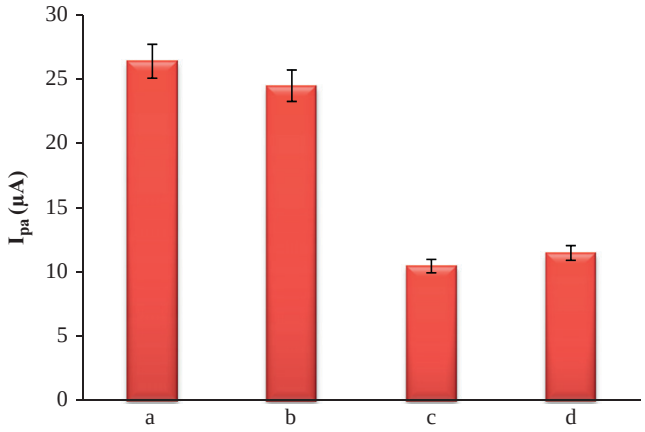

Fig. 6. Histograms of the DPV peak current of $[Fe(CN)_6]^{4-}$ recorded for the probe ssDNA (a); hybridized with 1.0×10^{-8} mol L^{-1} noncomplementary (b); 1.0×10^{-8} mol L^{-1} complementary oligonucleotide sequence before (c); and after regeneration (d). All measurements carried out in phosphate buffer solution (pH 7.0) containing 5.0 mM $[Fe(CN)_6]^{4-}$ and 0.1 mol L^{-1} KNO_3. DPV parameters were 25 mV pulse amplitude, pulse width 50 ms and a scan rate of 20 mV/S.

be regenerated five times with about 12% loss of the original signal by dipping the electrode in hot water (80 °C) for 10 min, followed by a rapid cooling in an ice bath for 10 min [18].

3.5. Stability, repeatability, and reproducibility of the DNA biosensor

The stability of the DNA biosensor was investigated by hybridizing with the target sequence and the results indicated that the ssDNA sequence could be tightly immobilized on the electrode surface due to the covalently reaction. After 12 days storage of the modified electrode in the refrigerator, 98.6% of the initial sensitivity remained, indicating this modified electrode was a stable platform as an electrochemical DNA biosensor.

The repeatability was estimated through the relative standard deviation of seven replicate measurements of a 0.1 mol L^{-1} PBS (pH 7) containing 1.0×10^{-8} mol L^{-1} target DNA. The relative standard deviation (RSD) of 2.11% ($n=7$) revealed a good repeatability.

The reproducibility of the biosensor was studied by five parallel-made modified electrodes in detection of 1.0×10^{-8} mol L^{-1} target DNA, and the results showed that a relative standard deviation (R.S.D.) of 3.52% ($n=5$), showing a high reproducibility of the constructed DNA biosensor.

4. Conclusion

By utilizing a simple and convenient self-assembling technique mercapto-diazonium salt on gold electrode surface prepared and a novel and effective route for fabrication of DNA electrochemical biosensor based on electrostatic immobilization of probe NL-ssDNA was demonstrated. The developed DNA electrochemical biosensor possessed high sensitivity and regeneration ability. The promising performance of the developed DNA electrochemical biosensor makes this methodological study and application attractive in DNA analysis.

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