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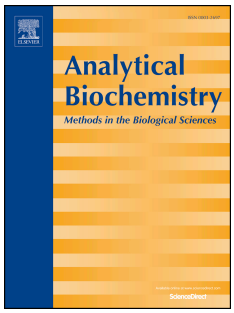

A novel electrochemical DNA biosensor for Ebola virus detection

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A novel electrochemical DNA biosensor for Ebola virus detection

Hoda Ilkhani, Siamak Farhad
Advanced Energy & Sensor Lab., Department of Mechanical Engineering, The University of Akron, Akron, Ohio 44325, United States

Corresponding Authors: hilkhani@uakron.edu and sfarhad@uakron.edu

Abstract

The aim of this study was to fabricate a novel electrochemical-based DNA-sensing device for Ebola virus DNA diagnostic by an enzyme-amplified detection, which improves the sensitivity and selectivity of the sensor. A thiolated DNA capture probe sequence was immobilized on the screen printed electrode surface and hybridized with biotinylated target strand DNA for the fabrication of Ebola DNA-sensing devices. Prior to the electrochemical detection of the enzymatic product by differential pulse voltammetry (DPV) method, the biotinylated hybrid was labelled with a streptavidin-alkaline phosphate conjugate on the surface of the working electrode. All the experiment steps were optimized using electrochemical impedance Spectroscopy (EIS) and the optimum condition for biosensor fabrication was achieved. The last step, the selectivity, reproducibility and sensitivity of fabricated electrochemical DNA biosensor was obtained.

Keywords: Electrochemical DNA biosensor, Ebola virus, Electrochemical impedance spectroscopy, Hybridization

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1 Introduction

The sequence-specific DNA detections, also called DNA biosensors, has attracted an extensive attention in many clinical and environmental studies, including cancer diagnostic [1], bacteria and virus detection [2, 3], gene expression analysis [4, 5], drug delivery and discovery [6-8] and environmental pollution detection [9-11]. Various DNA biosensors have been used in different approaches such as electrochemical [12, 13], SPR [14, 15] and spectroscopic techniques [16]. Electrochemgical DNA biosensors stand out among them due to their unique advantages including low-cost, rapid response, simplicity, proper selectivity and high sensitivity. Thus, the electrochemical biosensors have been widely used for specific gene sequences detection [17-20].

One of the well-established applications of electrochemical DNA biosensors is the DNA/RNA sequencing. The Ebola virus that causes the deadly Ebola disease is a negative strand-RNA virus from the Filoviridae family [21]. The Ebola virus causes high mortality hemorrhagic fevers, for which no vaccine or treatment currently exists [22]. There are five Ebola virus species, including Zaire Ebola virus, Sudan Ebola virus, Bundibugyo Ebola virus, Tai Forest Ebola virus and Reston Ebola virus, with the first (1976) and major (2014) outbreaks caused by the type species Zaire Ebola virus [23]. Frequent outbreaks of Ebola are worrying and continue escalating, with over 25,000 cases and 10,000 deaths from the virus mainly in Guinea, Liberia and Sierra Leone, according to the World Health Organization database [24]. Promising vaccine candidate tests are being rushed to face the epidemics and could be available soon [25]. Various experimental diagnostics and therapies including recombinant viral vectors [26], or antibodies that target the viral glycoprotein are continuing [22, 27]. However, innovative approaches are still required to develop the diagnosis tools and identification of virus. The design and development of new diagnostic methods for Ebola virus can help to better understand

the virus outbreak that enables the researchers to develop effective treatment methods.

In this study, a novel electrochemical DNA biosensor was fabricated for the detection of Ebola virus. The electrochemical biosensor was fabricated by modifying the gold surface of screen-printed electrode using single strand DNA as capture probe via S-Au bounds. The single strand DNA (target) is commercially labelled with SH group. In the next step, the free and active space of electrode surface was covered and deactivated using bovine serum albumin (BSA) solution. Then, the hybridization was occurred between capture probe strand DNA and biotinylated target strand DNA. For electrochemical detection step, the biotinylated target strand DNA was interacted with streptavidin alkaline phosphatase enzyme via biotin-streptavidin conjugation bond. To the best of our knowledge, so far there is no study reporting Ebola DNA detection using electrochemical biosensors. The electrochemical impedance spectroscopy (EIS) and differential pulse voltammetry (DPV) methods were used to detect the hybridization process. The conductivity and negative-charge changes upon DNA immobilization and hybridization on this sensitive modified screen-printed electrode could be easily monitored by EIS method and adopted as the signal for label-free DNA hybridization detection. The immobilization of DNA on the electrode surface resulted in the increase of electrochemical impedance value due to the blocking of $[Fe(CN)_6]^{3-/4-}$ redox indicator by single strand capture probes. Additionally, the enzymatic substrates to detect the hybridization process were studied. The enzyme catalyzed the 4-Aminophenyl phosphate to 4-Aminophenol that is an electroactive product and was detected by DPV method.

2 Experimental

2.1 Reagents and Materials

The chemicals were reagent-grade and were used as received without additional purification. Diethanolamine was purchased from Sigma-Aldrich and was used for preparing DEA buffer (pH 9.6). Phosphate buffer (pH 7.4), 6-mercapto-1-hexanol (MCH), Sodium Ferro cyanide, Potassium ferricyanide (III), streptavidin-alkaline phosphatase, bovine serum albumin (BSA) and 4-Aminophenyl phosphate monosodium salt hydrate were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Potassium chloride was obtained from Merck (Darmstadt, Germany). The compositions of the DEA buffers was included 0.1 mol L^{-1}diethanolamine, 1 mmol L^{-1} $MgCl_2$ and 0.1 mol L^{-1} KCl, pH 9.6 and DEA/BSA buffer was included 10 mg mL^{-1} of BSA in mentioned DEA buffer. Other reagents were commercially available and analytical reagent-grade. All solutions were prepared with ultrapure water from a Millipore Milli-Q system.

The oligonucleotides sequences were purchased from Eurofins Genomics. Table 1 lists the oligonucleotides sequences used in this study. The single strand capture probe sequence is labeled with SH group and complementary strand DNA is labeled with Biotin group.

Table 1. Oligonucleotides sequences used in the present study.

DNA primer	Sequence
Capture probe	5′- TTG TAC GAA GCT GTA CAT AAA TT-(CH2)-SH
Complementary target	5′-AAT TTA TGT ACA GCT TCG TAC AA-3′-Biotin
Non-Complementary target	5′-CGA AAG GAC GTC TTG AAC ATG CC-3′-Biotin

2.2 Procedures

2.2.1 Immobilization of a thiolated single strand capture probe

Figure 1 demonstrates a schematic of different steps to fabricate the electrochemical DNA biosensor. For single strand capture probe immobilization, the gold SPEs were modified by transferring 10 µL of 1 µmol L^{-1} capture probe (in PBS buffer) on its surface. Chemisorption was allowed to proceed for 45 min. During this period, the electrodes were kept in Petri dishes to protect the solutions from evaporation and environmental pollutions. The modified electrodes were then washed three times by 0.5 mol L^{-1} PBS buffer with the pH of 7.4 to remove weakly adsorbed capture probes.

To improve the quality and stability of the capture probe monolayer and block all free active sites on surface of the working electrode, a second MCH monolayer was deposited onto the capture probe strand modified working electrode. A 10 µL of the 1.0 mM aqueous solution of MCH was placed on the capture probe strand modified working electrode surface for 60 min. The resulting electrode was then washed with 0.5 M PBS buffer with the pH of 7.4 three times for removing any unbounded chemicals (See Figure 1).

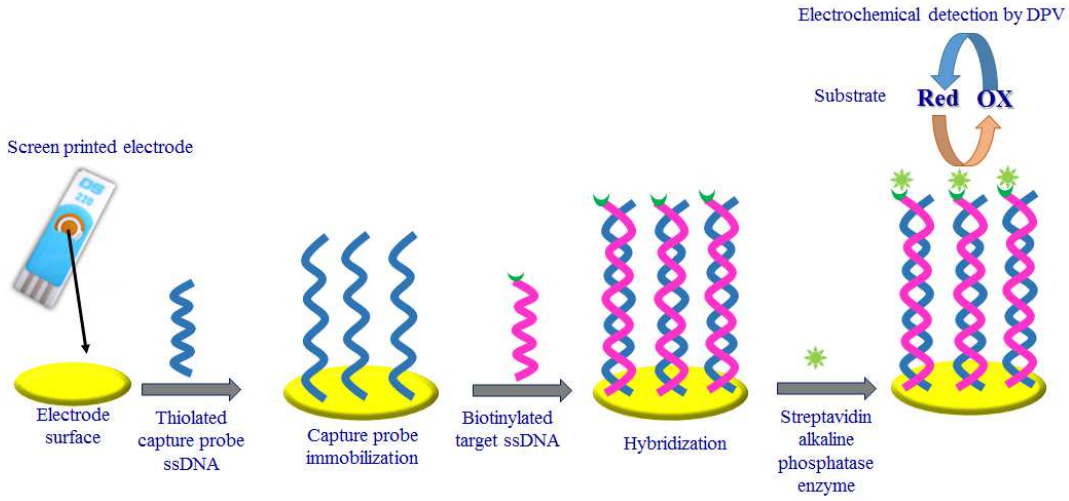

Figure 1. Schematic representation of different steps for the fabrication of electrochemical DNA biosensor.

2.2.1 Hybridization Process

The thiolated single strand capture probes modified on working electrode surface were exposed to 10 µL of the biotinylated target strand DNA solution in 0.5 mol L^{-1} PBS buffer with the pH of 7.0. The assembly was kept at room temperature and in a petri dish for 120 min to proceed the hybridization reaction. The electrodes were then washed with DEA buffer with the pH of 9.6 to remove the non-specifically bound oligonucleotides.

2.2.2 Enzymatic Process

The biotinylated hybrid obtained on the electrode surface was reacted with 8 µL of a solution containing 0.36 U/ml of the streptavidin–alkaline phosphatase conjugate and 0.01 g mL^{-1} of BSA in DEA buffer with the pH of 9.6. After 20 min, the enzyme conjugated biosensor was washed with DEA buffer with the pH of 9.6.

For DPV electrochemical detection, the electrode surface was covered with 50 µL of 4-Aminophenyl phosphate solution (1 mg/mL in DEA buffer). After 15 min of incubation, the 4-Aminophenyl phosphate was reduced to 4-Aminophenol via enzymatic reaction. Finally, the oxidation signal of the enzymatically-produced 4-Aminophenol was measured by DPV method and its peak height was taken as the analytical signal. The DPV measurements (modulation time 0.05 s; interval time 0.1 s; scan rate 0.07 V s^{-1}; modulation amplitude 70 mV; potential scan: from -0.05 to 0.6 V) were performed with a BioLogic (SP-150 potentiostat) electrochemical setup. The EIS measurements were performed using the same BioLogic electrochemical setup. The electrode surface was covered with 10 mM K3Fe(CN)6/ 10 mM K4Fe(CN)6 containing 0.01M KCl as electrolyte. A sinusoidal potential modulation of ±10 mV amplitude in the 0.1–100 kHz frequency range, spaced logarithmically, and a potential of 0.1 V was superimposed

onto the formal potential of the redox couple [Fe(CN)6]3−/4−. The experiments carried out at room temperature.

3 Results and Discussion

3.1 EIS detection

In recent years, EIS has been used for the DNA hybridization sensing to realize a sensitive label-free detection of the gene sequence [28-30]. The immobilization of DNA on the electrode surface changes the R_{et} value. It is totally clear that there is an electrostatic repellent interaction of $[Fe(CN)_6]^{3-/4-}$with the negatively charged phosphate backbone of DNA molecule. If $[Fe(CN)_6]^{3-/4-}$solution is used as the supporting electrolyte solution for the impedance measurement, the R_{et} value would increase after the immobilization of DNA on the electrode surface due to mentioned electrostatic repellent interaction. Therefore, the immobilization of DNA can be monitored via the impedance measurements. The EIS was used as monitoring method for DNA biosensor optimization steps.

3.2 Optimization of Capture Probe Immobilization

The immobilization time and capture probe concentration were optimized to achieve the high performance of the biosensor. Figure 2 exhibits the optimization of capture probe strand DNA (1 µM) immobilization time. The immobilization times were set from 30 min to 120 min. As it can be seen from Figure 2, the immobilization times of 45 min and 60 min had almost the same R_{et} amount that means the hybridization process was completed during those immobilization times. The immobilization time of 45 min was selected as optimum since it was shorter and effective enough for immobilization.

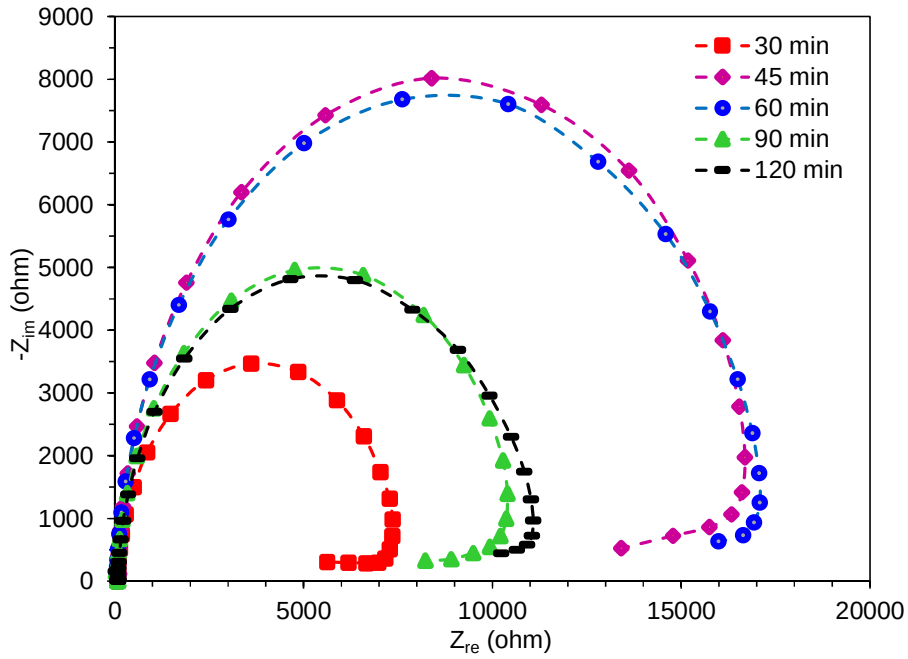

Figure 2. The Nyquist plots after immobilization of capture probe strand DNA (1 µM) on gold SPE from 30 min to 120 min. All *measurements were performed in 10* mM, $K_3Fe(CN)_6/K_4Fe(CN)_6$ dissolved in 10 mM KCl solution over frequency range of 0.1–100 kHz and potential of 0.1 V.

As mentioned earlier, MCH was used for covering and deactivation of surface electrode's free sites. Figure 3 displays the optimization of MCH incubation time. To achieve this goal, 1 mM MCH was added onto the capture probe modified electrode surface and incubated for different times from 30 min to 90 min. As it can be seen from Figure 3, the 60 min incubation time was the optimum MCH incubation time because it had the largest R_{et} value.

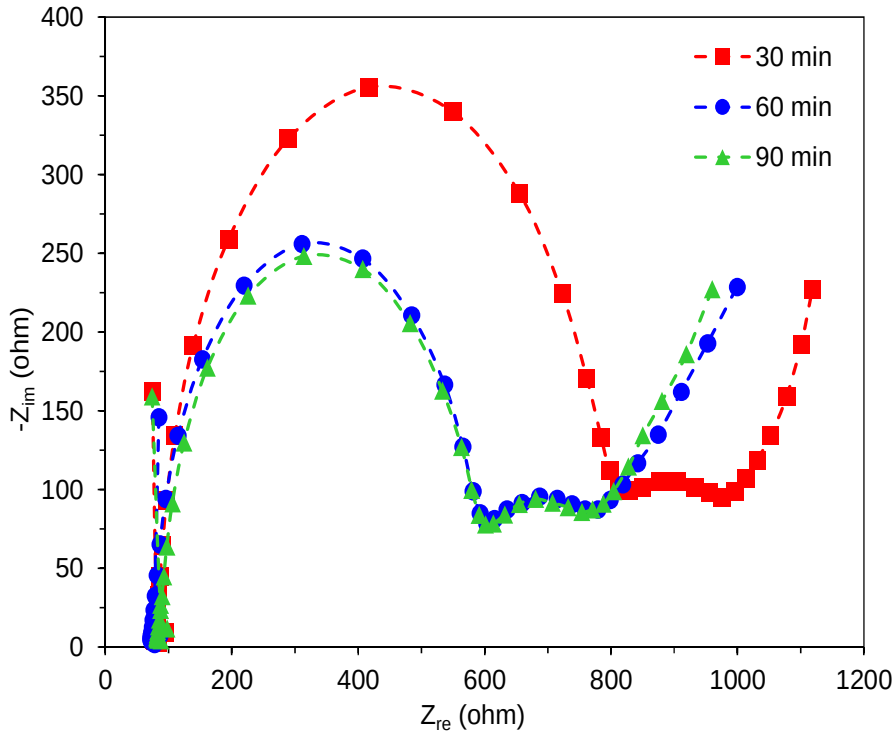

Fig. 3. The Nyquist plots for MCH (1mM) incubation time during 30 min, 60 min and 90 min on capture probe modified electrode surface. All *measurements were performed in 10* mM, $K_3Fe(CN)_6/K_4Fe(CN)_6$ solved in 10 mM KCl solution over frequency range of 0.1–100 kHz and potential of 0.1 V.

3.3 Detection of Target Strand DNA

The capture probe modified working electrode was used to carry out the DNA hybridization analysis. Effect of the hybridization time on differential pulse signal was investigated with the concentration of the complementary target strand DNA equal to 20 nM. It can be seen from Figure 4 that the electrochemical signal increased gradually as the hybridization time prolonged, and reached a constant value after 60 min. Thus, 60 min was selected as hybridization time for the following experiments.

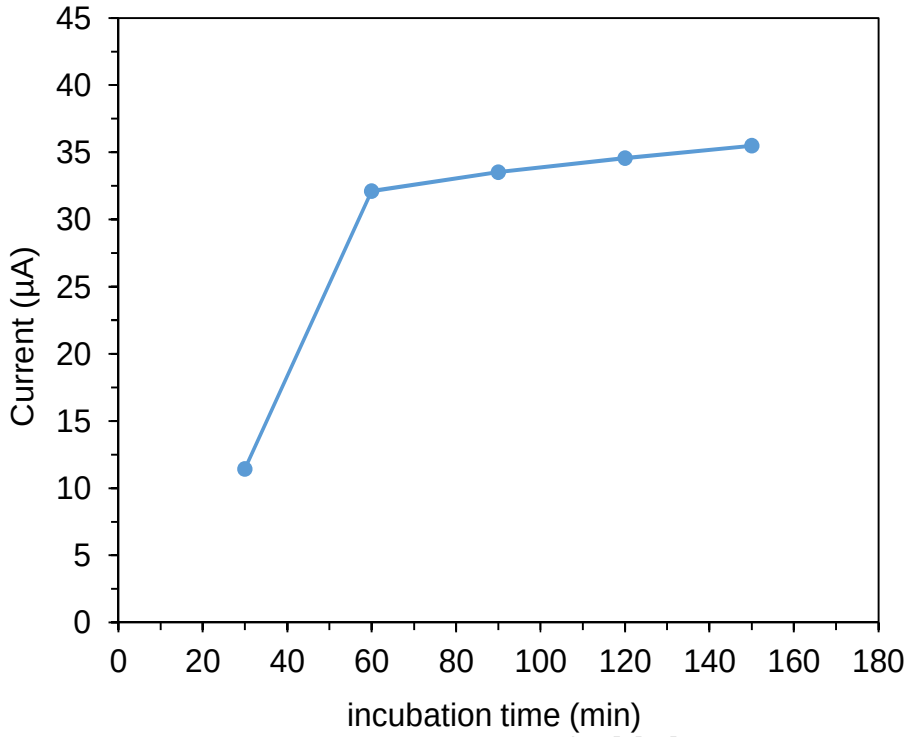

Fig. 4. The optimization of target strand DNA hybridization time from 30 min to 150 min. The concentration of target strand DNA was 20 nM.

3.4 Calibration Curve

The selectivity, sensitivity and reproducibility of any biosensing platform is important in DNA hybridization detection. In the present study, the reproducibility of this label-free impedance DNA hybridization sensing was examined by DPV method. A new electrode for each reproducibility test was used and each experiment was repeated at least five times. When DPV was used as detection method, after hybridization and labelling steps the modified electrode was exposed to 4- Aminophenyl phosphate and the oxidation signal obtained of the enzymatic product was recorded using DPV. If the hybridization process was occured, the enzyme could be attached to the biotinylated target strand DNA via biotin-streptavidin conjugation and the enzymatic reduction of 4- Aminophenyl phosphate was happened. Figure 5 shows the

differential pulse voltammograms after hybridization with complementary and non-complementary sequences. It can be seen from Figure 5 that the DPV current height was plotted versus complementary and non-complementary target strand DNA concentrations. The regression equation was obtained as y=1.45x+4.31 (x is the complementary target strand DNA concentration (nM) and y is ΔI_p, (μA, n=5)) for complementary target strand. The regression coefficient of the linear curve is 0.9905. A detection limit of 4.7 nM complementary oligonucleotides could be estimated using 3σ, where σ is the standard deviation of the blank solution, n=5.

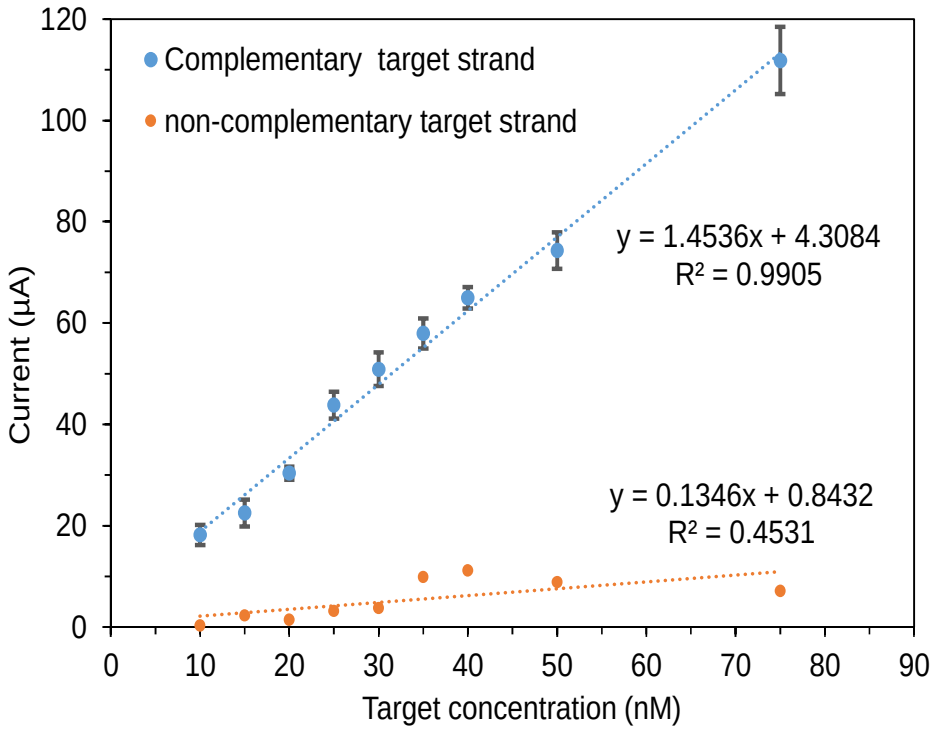

Fig. 5. DPV current height *vs.* the concentration of complementary and non-complementary target strand DNA. The concentration range is from 10 to 75 nM for both experiments.

The experiments were repeated using non-complementary target strand DNA solutions with the same concentrations as complementary solutions for finding the biosensor selectivity. As it can be seen from Figure 5, the non-complementary calibration curve has a regression equation of $y = 0.13x + 0.84$ and regression coefficient of 0.45. The hybridization of single strand capture probe with the non-complementary sequence led to an enhancement in the DPV response. However this enhancement is 10-fold lower in the case of the non-complementary sequence. The evidences indicate that the designed biosensor possesses high selectivity and sensitivity.

4 Conclusions

The DNA hybridization is a basic method in molecular biology for providing new possibilities in various biomedical biochemical areas. In this study, an electrochemical DNA biosensor, which offers fast and sensitive results for the Ebola DNA detection was introduced using hybridization process. The immobilization time for each step of biosensor fabrication was optimized due to achieve the best performance of DNA detection. The optimization steps included capture probe immobilization time, MCH incubation time, and hybridization incubation time. The obtained results indicated that the Ebola virus DNA can be detected using electrochemical biosensor with high reproducibility, sensitivity and selectivity. This method can be employed for the detection of Ebola virus DNA in real samples as medical diagnostic method. Additionally, this method can be employed to assess environmental bacterial pollution such as water or food resources.

Acknowledgments

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- A novel biosensor was fabricated for the enzyme-amplified detection of Ebola virus DNA.
- The biosensor fabrication conditions were optimized by electrochemical methods.
- The optimization was performed for all fabrication steps of the electrochemical DNA biosensor.
- The fabricated electrochemical DNA biosensor exhibited high selectivity and sensitivity.

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