Regular Article

ANALYTICAL AND BIOANALYTICAL CHEMISTRY RESEARCH

Published by the Iranian Chemical Society

Anal. Bioanal. Chem. Res., Vol. 9, No. 2, 163-171, April 2022.

A Simple and Sensitive Electrochemical DNA Biosensor of the Bacteria *Chlamydia Trachomatis*

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(Received 27 March 2021 Accepted 25 October 2021)

Bacterial infection is a global problem, and detection of bacteria is the first step for solving such a problem. Herein, we developed an electrochemical biosensor for the detection of the bacteria *Chlamydia Trachomatis*. The hybridization-based biosensor was made by modifying the screen-printed gold electrode (SPGE) with the thiolated specific detection probes, which were complementary sequences to the target DNA molecule of the bacteria. The Oracet blue was used as an electrochemical label which was intercalated between two DNA sequences, and its reduction peak current was recorded by DPV method as an output signal of the biosensor. Conventional electrochemical characterization techniques, including cyclic voltammetry (CV) and Electrochemical impedance spectroscopy (EIS), were used to confirm the fabrication of the modified electrode. In addition, the Atomic Force Microscopy (AFM) imaging was performed to assess the electrode surface. The dynamic range of the biosensor was from 4 to 3000 pM with a detection limit of 1.3 pM. The simplicity and performance mentioned above of the biosensor, alongside the low cost and repeatability of the production, make it a great candidate for clinical applications for *Chlamydia Trachomatis* detection. Plus, it can be used for another species of bacteria with just a change of the thiolated probe.

Keywords: Electrochemical sensor, Bacteria, Detection, DNA, *Chlamydia Trachomatis*

INTRODUCTION

Worldwide, bacterial infections have affected health, safety and even could cause death, especially in developing countries, and it is estimated that infectious diseases account for nearly 40% of the total 50 million annual estimated deaths [1-3]. In addition, major infection diseases caused by pathogenic bacteria could even lead to severe diseases like cancer [4]. Different types of methods have been developed for bacterial detection due to their importance in choosing a correct therapy method or selecting suitable medication [5]. These major methods are ranging from a simple culture and colony counting method [6], immunology-based/ ELISA methods [7,8] and genomics and proteomics-based studies [9-11]. Among them, the genomic-based methods have been attended mostly, especially those like biosensors which could detect the bacteria fast, inexpensive, specific and reliable [12].

Chlamydia trachomatis (*C. trachomatis*) is known as an important cause of sexually transmitted disease and also one of the major causes of infertility [13,14]. Their infections often are asymptomatic or non-specific in their clinical

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course and also are capable of transmitting to a sexual partner(s) [15]. Therefore, their detection in clinical samples such as urine is very important for clinicians [14-16]. Detection of the bacteria based on molecular biology methods such as PCR has been performed. But, using biosensors, the process of detection would be simpler and less time-consuming [17,18].

Biosensors are chemical sensors in which the recognition system utilizes a biochemical mechanism. They consist of a biological recognition part, transducer and analyzer [19,20]. Based on their transducer type, they are categorized into main types of biosensors, including electrochemical, optical, mechanical, electrical, *etc.* [21-23]. These different types of biosensors have been used vastly in bacterial detection so far, for example electrochemical methods [24-26], colorimetric [27,28], fluorescent methods [29,30] , Surface Plasmon Resonance [31,32], Raman-based techniques [33,34], paper-based lateral flow [35,36] and even microfluidics and chip-integrated biosensors [37,38]. Among the biosensors for bacteria detection, electrochemical biosensors have been attended mostly, especially in medicine and bacteria detection, due to their simplicity, in-expensive, fast and precise working mechanism [39,40]. Many innovations have been made so far in the application of different types of electroactive labels to enhance the sensitivity of these biosensors [41-45]. Oracet blue, as a natural anthraquinone, has been studied before for oligonucleotide electrochemical biosensors, and its functionality as an intercalating electroactive label has been approved [40,41,46].

There are many previous publications about developing novel electrochemical DNA biosensors for bacteria detection purposes. Through this, scientists have invented more sensitive, faster and low-cost biosensors by taking advantage of electrochemical methods and many different assembling and working methods and mechanisms. Specifically speaking about the *Chlamydia Trachomatis,* few biosensors have been developed so far using different methods such as Quartz Crystal Microbalance [18], optical nanosensor [47], microchip-Based method [48], and most importantly, electrochemical methods [49-52].

Hereby, we have tried to detect the bacteria *Chlamydia Trachomatis* by developing a simple, cost- and time-effective DNA electrochemical biosensor based on a self-assembled thiolated probe on the gold electrode and use of Oracet blue as an electroactive label.

MATERIALS AND METHODS

Materials, Oligonucleotides and Solutions

While the 6-Mercapto-1-hexanol (MCH) was provided from Aldrich Company, the Oracet blue (Disperse blue24), alongside the other materials used here, were purchased from Merck Company. In addition, oligonucleotides were supplied (as lyophilized powder) from Eurofins MWG Operon, with the following sequences: Complementary DNA target (*Chlamydia Trachomatis*): 5'-AAAAAGGAGAAAAAGTATGAA-3'. Thiolated DNA probe (Probe): 5'- HS (CH2)6 TTCATACTTTTTCTCCTTTT-3'. Non-supplementary DNA: 5'- GAA TAT GAT TTA CAG TTT AT -3'. Single base mismatch target DNA: 5'- TTT GTT ACT GGG GTA GAT AC-3' (This oligonucleotide was similar to supplementary DNA with a surrogate (T/G) at site 11).

All the solutions were made based on the previous publication by Nasirizadeh and colleagues in 2011 [42], except for the Oracet blue solution that its concentration and incubation time have been optimized here and concluded to be 0.1 mM and 75 min, respectively.

Instruments

The Autolab potentiostat/galvanostat model PGSTAT 30 (EcoChemic Utrecht, Netherlands) and a GPES 4.9 software at laboratory temperature (25 ± 1 °C), were used for electrochemical measurements with a connection to a screen-printed gold electrode (SPGE) (Dropsens DRP-220AT, Spain) which was composed of a high temperature curing ink gold working electrode with a 4 mm diameter, where the counter electrode was also gold, and the reference electrode was silver.

Preparation of Probe-modified Electrode and DNA Hybridization

Firstly, the surface of SPGE was washed thoroughly with 80:20 (v/v) ethanol: water and then distilled water and then dried by N_2 stream. Then, 2.5 µl immobilization buffer

solution containing 8.0 µM probe (ss-DNA) was dropped on the AuE electrode surface for the probe self-assembly. Self-assembly of the probe was performed by incubation of the electrode at room temperature (25 ± 1 °C) for 2.0 h in the high humidity container to prevent evaporation. Finally, the modified electrode (ss-DNA/AuE) was rinsed with the washing solution and was incubated in 1.0 mM MCH for 5 min. Afterward, the electrode was washed out with 80:20 (v/v) ethanol:water and distilled water, respectively. The hybridization was performed by immersing the ss-DNA/AuE into the hybridization buffer solution (pH 7.0) containing a distinct concentration of the target oligonucleotide (complementary, mismatch or non-complementary) at room temperature (25 ± 1 °C) for 2.0 h.

Oracet Blue Accumulation on the Modified Electrode

Oracet blue was accumulated on the ss-DNA/AuE by dropping 2.0 µl of 0.1 mM phosphate buffer solution (pH 7.0) containing 0.1 mM Oracet blue and methanol on the working electrode area for 90 min. Then, this electrode was rinsed with a washing solution for 10 s. A similar procedure was applied for the accumulation of Oracet blue on a cleaned AuE electrode and probe-modified electrodes following hybridization with DNA samples.

Measurement Procedures

The electrochemical experiments were done by differential pulse voltammetry (DPV), with an amplitude of 25 mV, a modulation time of 0.05 s, and a step potential of 50 mV in 0.1 M phosphate buffer solution (pH 7.0). The experimental data were discussed applying the Savitzky and Golay filter (level 2) of the GPES software, followed by the GPES software moving average baseline modification applying a 'peak width' of 0.01.

In addition, for evaluating the steps of the biosensor preparation, cyclic voltammetry (CV) was performed in 1.0 mM $K_3[Fe(CN)_6]$ in PBS buffer at the potential range of 0.025 to 0.33 V and a sweep rate of 0.02 V s^{-1}. To reconfirm the CV results, electrochemical impedance spectroscopy (EIS) was performed on the biosensor in a solution of 5.0 mM $K_3[Fe(CN)_6]$ containing 1.0 M KCl, from 100 kHz to 0.01 Hz with an amplitude of 5 mV and potential of 0.27 V.

The Atomic Force Microscopy (AFM) imaging was performed to analyze the roughness of the electrode surface before and after modification steps. For this purpose, the non-contact mode AFM imaging was carried out by Nanosurf easyScan 2 AFM instrument (Nanosurf AG, Switzerland).

Real Sample Analysis of the Biosensor

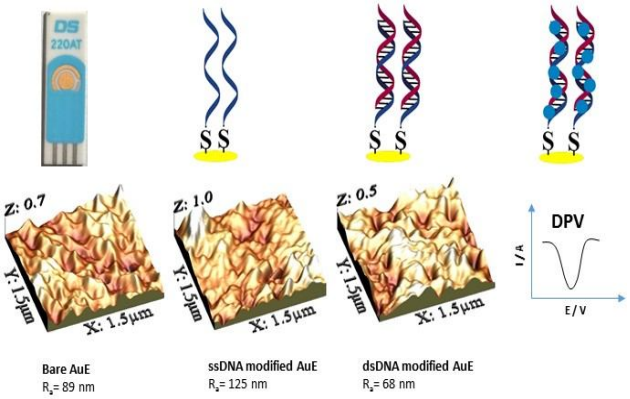
In order to evaluate the functionality of the proposed biosensor in the real genomic DNA of the bacteria for future clinical applications, the real sample studies were done. The DNA was extracted from the cultured bacteria *Chlamydia Trachomatis* using the ExiPrep Plus Bacteria Genomic DNA Kit from Bioneer (South Korea). The extracted DNA was assumed as a real sample environment for testing the biosensor functionality to detect the specific sequence among other oligonucleotide sequences and other potential contaminant compounds and interference moieties.

RESULTS AND DISCUSSION

The fabricated biosensor was simply made of an ssDNA probe which is complementary to the target DNA sequence and an electrochemical indicator named Oracet blue. The sensing strategy is basically based on the selective hybridization process of the complementary DNA sequences in which the Oracet blue molecule is intercalating between two DNA. The reduction signals of the Oracet blue are recorded by the DPV method, and the calibration curve is shown in Fig. 6, in which the measured reduction peak can be associated with a distinct amount of target DNA.

AFM Imaging of the Work Electrode Surface

The biosensor has been made of an SPGE electrode modified with the specific DNA probe designed for the target DNA segment of the bacteria. The electrochemical label in this work was Oracet blue which its reduction signals were measured through DPV. Scheme 1 is representing different steps of the working electrode (Au) modification and their regarding AFM images for better understanding the roughness of the surface after each step. As it can be seen, the bare electrode had an average roughness (R_a) of 89 nm, where the ssDNA modified gold electrode represented the R_a = 125. Due to the hybridization



Scheme 1. A schematics of the electrochemical biosensor developed for the detection of bacterial DNA and AFM imaging of the working electrode in each modification steps

of the target DNA with immobilized probe oligoes on the electrode surface, the roughness was decreased significantly (R_a = 68 nm). The reasonable trend of the surface modification, could represent the rightness of the modification steps of the electrode to build the biosensor. The average roughness change upon DNA modification on the electrode, has been proved in previous studies too [53-55].

CV and EIS Study of Biosensor Fabrication

Using two common electrochemical methods, including CV and EIS, the biosensor fabrication procedure was evaluated step by step in $[Fe(CN)_6]^{3-/4-}$ electrolyte. The cyclic voltammograms shown in Fig. 1A indicate that the obtained current of bare SPGE (a) decreased after its modification with thiolated probes (b). This can be because of filling the area of the electrode surface by ssDNAs and also repellent negative charges of the DNAs, which can be a barrier for $[Fe(CN)_6]^{3-/4-}$ anions. Adding MCH resulted in a slight reduction of peak current (c), probably because of filling the remaining area between ssDNAs on the electrode surface. However, a significant decrease has happened after hybridization of the target DNA with immobilized probe (d), which can be explained due by the resistance effect of the formed layer against electron transfer on the SPGE surface.

The results of the EIS study are shown in Fig. 1B. It can be observed that the charge transfer resistance (R_{ct}) of bare SPGE (a) increases after assembling the probes (b) due to the barrier function of the negative charges of the ssDNA molecules and their space-filling assembly on the electrode surface. The Rct value is reduced very slightly after MCH treatment mainly because the MCH molecules correct the ssDNA probes orientations on the electrode. Therefore, the $[Fe(CN)_6]^{3-/4-}$ anions can reach the electrode surface and transfer the electron easier (c). But, the hybridization of probe/DNA target causes a considerable elevation in recorded R_{ct} (d). There are various points of view for explaining these data; nevertheless, it is believed that the trends of CV and EIS results approve each other and show that the biosensor fabrication procedure has been desirably accomplished.

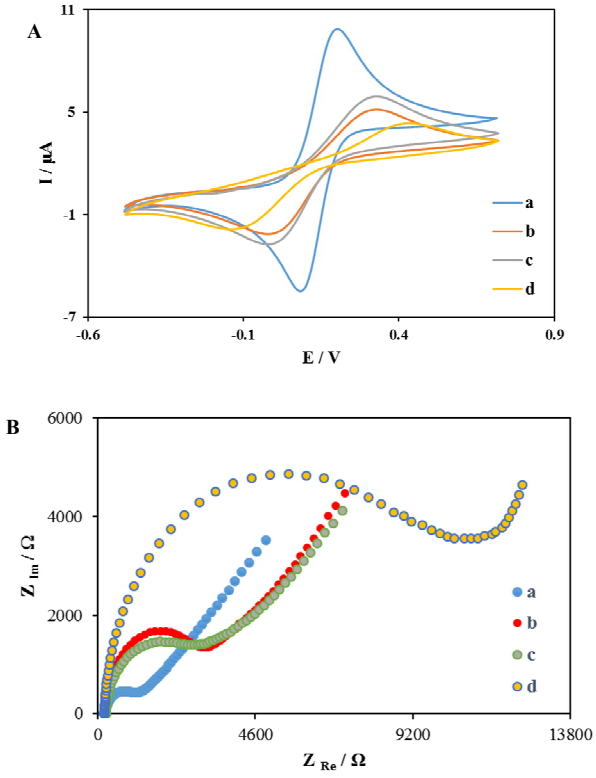


Fig 1. Biosensor fabrication assessment, using (A) Cyclic Voltammetry (CV) in 1.0 mM $[Fe(CN)_6]^{3-/4-}$ and (B) electrochemical impedance spectroscopy (EIS) in 5.0 mM $[Fe(CN)_6]^{3-/4-}$. In both CV and EIS studies, curve (a) is for bare gold electrode, (b) is ssDNA modified, (c) MCH-ssDNA modified and (d) dsDNA modified gold electrode.

Optimization of the Parameters

In order to reach the best biosensing performance, it is essential to optimize crucial steps and parameters such as probe immobilization process, concentration and incubation time of the probe on the Au electrode surface, hybridization condition and incubation time of interacting target DNA with the immobilized probe on the electrode surface, and also, the Oracet blue concentration and time span of treatment.

SH-Probe immobilization method, concentration and time span. In this study, two different common techniques were used for stabilizing the thiolated capture probes (SH-probe) on the surface of SPGE: droplet self-assembly and solution self-assembly. Figure 2A indicates the comparison results of the droplet self-assembly and solution self-assembly method, including obtained curves before (a, c) and after (b, d) the probe/target hybridization, respectively. As it can be seen, using the droplet self-assembly method resulted in broader current ranges before and also after the hybridization of target DNA with the capture probe.

In order to determine the optimized concentration of capture probe, six concentration ranging from 1 to 11 µM was compared based on their DPV results and the current of DPV reduction peaks (Fig. 2B and 2C). As Figs. 2B and 2C show, the probe concentration of 7.0 µM was chosen and used in the later experiments.

Furthermore, the probe-immobilization step was carried out at different incubation times (20 to 140 min), and 100 min, which had a higher final current than others, was selected as the most appropriate incubation time for immobilizing probe on the electrode (Figs. 2D and 2E).

Hybridization method and time span of the target DNA with the immobilized probe. Since the hybridization interaction between probe and complementary DNA target directly affects the biosensor performance, selecting the efficient hybridization method and optimizing the hybridization time span are the key parameters to achieve better electrochemical signal transduction. Figure 3A indicates the DPV results obtained from using three hybridization methods, including drop hybridization method (a), preheated solution hybridization method (b), and solution hybridization method (c). In a comparison view, it is obvious that the current related to the solution

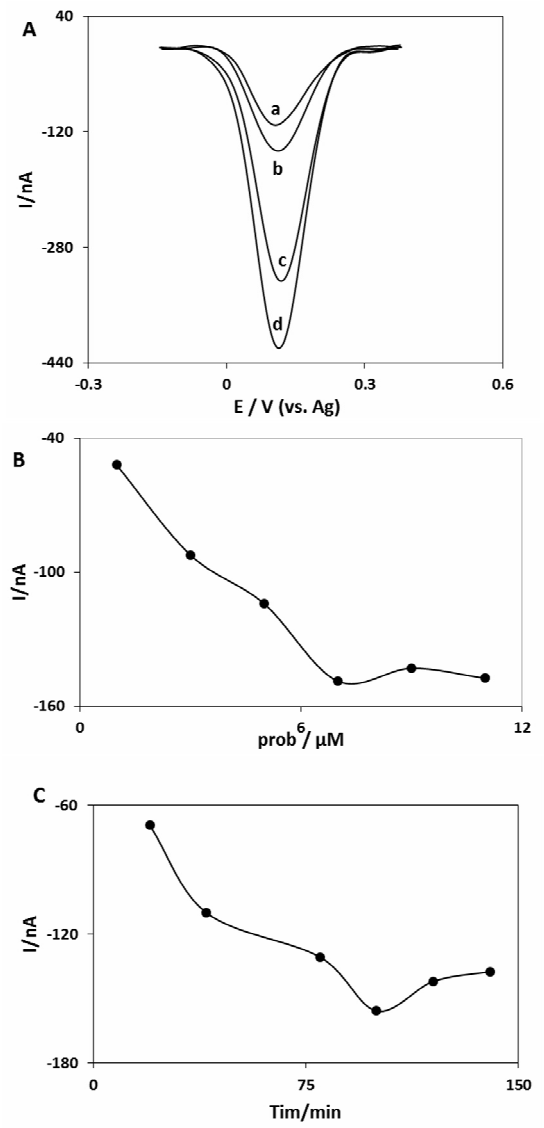


Fig. 2. SH-Probe (ssDNA) parameters: (A) Probe immobilization method, (B) optimization of concentration, and (D) incubation time of the SH-Probe.

hybridization method is higher, and therefore, this method was selected as a hybridization method for all the experiments.

Finally, by testing different hybridization time periods (45 to 180 min), the 120 min time span was chosen as the optimum time period for probe/target hybridization in biosensor fabrication (Fig. 3B).

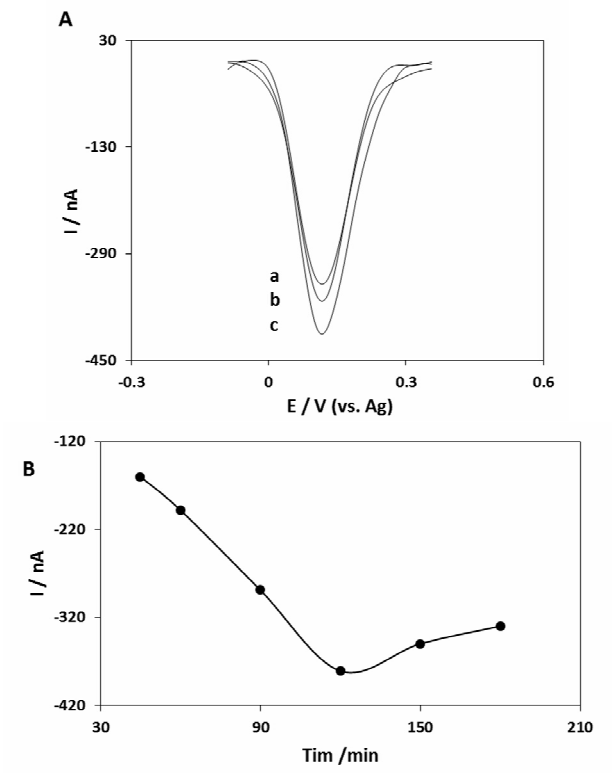


Fig. 3. Probe-Target DNA hybridization parameters: (A) comparison of the three hybridization methods. (B) Plot of time span of the hybridization *versus* reduction peak currents of the DPVs.

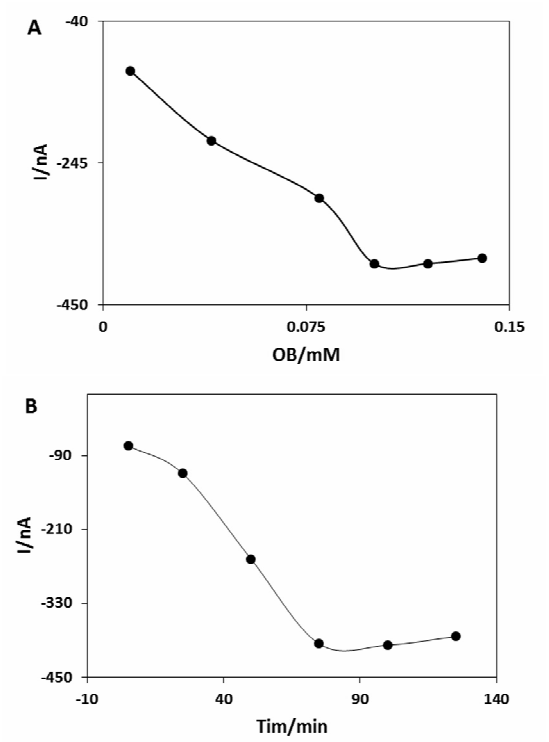


Fig. 4. Optimization of the (A) Oracet blue concentration and (B) time span.

Concentration and time span of Oracet blue. The final signal of biosensor analysis was reached due to the electrochemical behavior of Oracet blue. Therefore, its concentration and incubation time on the electrode significantly influence the whole obtained results. Based on the DPV measurements of nine different concentrations of the Oracet blue (0.01 to 1.4 mM), the optimum concentration of Oracet blue is determined to be 0.1 mM (Figs. 4A and 4B).

The biosensing analysis was also done in different time periods of Oracet blue accumulation, and the results proposed a time span of 75 min for Oracet blue exposure to get the best electrochemical reduction signals (Fig. 4C).

Selectivity of the Biosensor

In order to evaluate the practical selectivity of our proposed biosensor, the DPV measurements were carried out using non-complementary and mismatched sequences. As Fig. 5 shows, the differential pulse voltammograms were

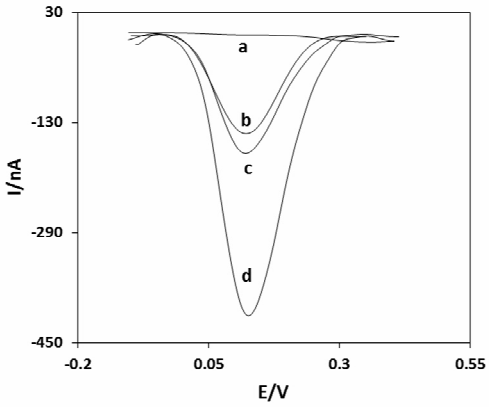


Fig. 5. DPV voltammograms of the Oracet blue accumulated on the hybridized oligonucleotides with the immobilized thiolated probes on the electrode, representing the selectivity of the biosensor.

recorded for MCH treated SPGE without probe (a), single-base mismatched sequence (b), SH-probe modified SPGE (c), non-complementary sequence (d), and target DNA sequence (e). It is pretty obvious that the DPV current related to target DNA is much more strong compared to other non-specific conditions. This phenomenon resulted from good selectivity of biosensor performance which is considered a valuable feature in detecting specific DNA targets, especially in real sample analysis.

Analytical Performance and Repeatability of the Biosensor

The proposed electrochemical biosensor was used to measure a serial dilution of target DNA, and its responses were recorded as the differential pulse voltammograms of Oracet blue accumulation (Fig. 6). As mentioned before, the DPV studies were done in phosphate buffer solution (0.1 M, pH 7.0). Afterward, the mean current value of resulted reduction peaks (the assays were done in triplicates) was used for plotting the standard curve, which showed a linear correlation between the DPVs and the target DNA concentration in the range of 4 to 3000 pM (inset of Fig. 6). Consequently, the detection limit of the proposed biosensor was calculated to be 1.3 pM using the well-known function $Cm = 3s_{bl}/m$, where s_{bl} is the standard deviation of 14 repeated DPV of the blank sample (the signal of the Oracet blue on the non-hybridized ss-probe), and m is the slope of the calibration plot.

The results of analytical performance indicate that our Oracet blue-based biosensor provides such a vast linear range (4-3000 pM) and considerable low detection limit (1.3 pM), which presents its superiority in comparison with most previously reported electrochemical DNA biosensors. Moreover, considering its selective and fast responses, easy handling, and simple fabrication, the presented electrochemical biosensor can meet the criteria of promising DNA biosensors for practical applications. It seems that the use of Oracet blue (as the signal generator) accompanied by SPGE (as the biosensing platform) is responsible for such high performance of this biosensor.

Real Sample Tests

The analytical performance of a biosensor in real or simulated samples (making a sample by adding some potential interference molecules to the buffer to be like a real sample environment) is very important for its future applications. The presence of some elements or compounds could badly affect major steps of assay or interfere with the signal generation and thus cause false results. Therefore, the assessment of each developed biosensing assay in a real sample environment could help the scientists to evaluate the functionality of the assay for potential clinical applications. In our study, the real extracted genomic DNA sample from the bacteria was chosen to assess the functionality of our developed biosensor. The initial concentration of the DNA was evaluated with the biosensor through the regular working procedure of the biosensor, then the exact concentrations of the synthetic DNA were added to the extracted DNA solution, and the measurement was done again. The results are shown in Table 1, which contains the recovery percentage and relative standard deviations. It can be seen that our device has high recovery and low RSD for both concentrations, which is really valuable in biosensor performance. Thus, the proposed biosensor does have the potential to be used in real extracted DNA samples for future applications.

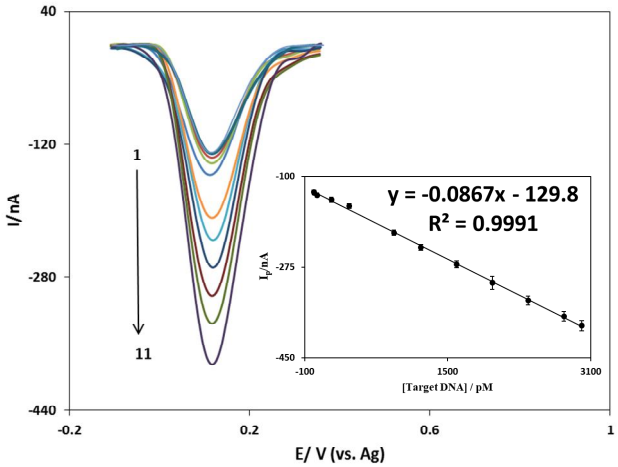


Fig. 6. DPV voltammograms of the Oracet blue accumulated on the different concentrations of the target DNA hybridizedwith immobilized probe on the electrode surface. Inset shows the plot of reduction peak currents of the Oracet blue *versus* the concentration of the target DNA as the calibration curve.

Table 1. The Real Sample Assay of the Biosensor in the Extracted DNA Sample of Bacteria Instead of Synthetic Environment (Three Replications)

Initial detected DNA concentration	Spiked DNA	Detected DNA	Recovery percentage	Relative standard deviation percent
11.8 pM	10.0 pM	21.46 pM	98.44%	6.32%
11.7 pM	20.0 pM	32.31 pM	101.88%	5.88%

CONCLUSIONS

The proposed electrochemical biosensor for the detection of the bacteria *Chlamydia Trachomatis* has shown a great performance and functionality due to the detection limit of 1.3 pM, great selectivity and also good real sample responses. The basic structure of the biosensor was made of a screen-printed gold electrode (SPGE) modified with the thiolated detection probes and Oracet blue as an electrochemical label. The simplicity and aforementioned performance of the biosensor, alongside the low cost and repeatability of the production, make it a great candidate for clinical applications for the *Chlamydia Trachomatis* detection, and comparison of the results to the previously published papers for bacteria biosensors has proved its superiority over majority of electrochemical biosensors for *Chlamydia Trachomatis* [49-51]. Plus, it can be used for another species of bacteria with just a change in a thiolated probe.

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