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An Electrochemical DNA-Hybridization Assay for Acinetobacter baumannii Detection

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The present paper reported the analysis of the *Acinetobacter baumannii* using an electrochemical biosensor fabricated based on a gold electrode (AuE) with the electroactive label of β-cyclodextrin (β-CD). Due to the formation of an Au–S bond, a thiol decorated single-stranded DNA probe was covalently immobilized onto the electrode surface. For the working mechanism of differential pulse voltammetry (DPV) on the DNA hybridization, the electrochemical signals of the β-CD binding reduction to the double-stranded DNA (ds-DNA) were recorded. Moreover, the as-prepared biosensor has the potential of being used for the analysis of *Acinetobacter baumannii* in excrement specimens.

Keywords: DNA; *Acinetobacter baumannii*; Gold electrode; β-cyclodextrin; Differential pulse voltammetry

1. INTRODUCTION

During the past three decades, the *Acinetobacter* genus has experienced remarkable taxonomic modification. *Acinetobacter baumannii*, a main form of this genus, has been listed as one of the top tough pathogens for healthcare institutions in the world [1-3]. Considering its amazing capacity for up-regulating or acquiring resistance determinants, this form of the genus has been paid much attention due to its clinical significance (especially during the past 15 years) [4, 5]. Therefore, it has been listed as an organism that poses risks in the current antibiotic era. *A. baumannii* strains are resistant to all known antibiotics, which makes it necessary for the international healthcare community to take immediate measures [6-8]. The survival ability of *A. baumannii* in a hospital setting for a long period of time is also a threatening factor, which potentiates its capacity for nosocomial spread [9-11]. Usually, the organism takes the most vulnerable hospitalized patients as targets, such as those with

critical illness that have breaches in airway protection or skin integrity. The most common infection induced by the above organism remains hospital-acquired pneumonia, based on reports from the 1970s until now. Unfortunately, more recently, some institutions have been burdened with the troublesome infections associated with the bone, skin, soft tissue, and even central nervous system [12-14]. Recently, public and scientific organizations have been paying increasing attention to *Acinetobacter*. Hence, it is necessary to propose a sensitive method for the analysis of *A. baumannii* [15-17].

Considering the user demands, certain standards must be met for analytical instruments for bacteria, including the high specificity in distinguishing between varying bacteria, desirable adaptability for the analysis of varying analytes, and favorable sensitivity in the analysis of bacteria on-line and in real specimens in the case of no pre-enrichment [18-23]. For the effectiveness of microbiological experiments, the most vital restrictions lie in two factors: sensitivity and time. The analytical apparatus must show high sensitivity, selectivity, and rapidness to effectively screen food specimens [24-26]. Infection might occur even with a single pathogenic organism within food or a body. Moreover, simplicity and low cost are two other important factors for the developed apparatus [27-30]. Biosensor technology has been used to meet the above standards. Generally, biosensors toward the analysis of bacteria contain a biological recognition component, including antibodies, nucleic acids, or receptors, attached to a proper transducer [31-33]. Compared with the conventional analytical methods, electrochemical biosensors are more favorable, due to their operable properties in turbid media, amenability to miniaturization, and high sensitivity [34-37]. The low limit of detection (LOD) could be obtained using modern electroanalytical approaches in the case of a small specimen volume.

Different electrochemical biosensors have been developed in recent years based on the identification of the bacterial nucleic acids. As analytical apparatuses, DNA biosensors involve immobilized DNA probes that are characteristic of specific hybridization to corresponding complementary sequences in a DNA specimen [31, 38-40]. The DNA biosensor works by detecting the DNA probe provided through molecular recognition, along with transforming this recognition into signals via a transducer. The uses of gene probes are usually linked with an ultrasensitive analysis of viruses, microorganisms, and trace levels of special reagents in varying environments. The determination of disease-inducing microorganisms in human tissues, animal, plant, food, and water supplies by gene probes has been reported. In the present report, a state-of-the-art electrochemical biosensor was developed based on its interaction with the gene of *A. baumannii* and the β-CD, and its application to the *A. baumannii* analysis was realized by hybridizing DNA with the self-assembled monolayer (SAM) approach.

2. EXPERIMENTS

2.1. Chemicals

All experimental chemicals (analytical reagent grade) were used with no additional purification. Aldrich was the material source of 6-Mercapto-1-hexanol (MCH). Other reagents were

purchased from Alading. Doubly distilled water was used throughout the preparation of all test solutions. Oligonucleotides (form, lyophilized powder; sequence) were provided by Eurofins MWG Operon. Thiolated DNA probe (*A. baumannii*) was 5′-HS $(CH_2)_6$ AGA CAT GCA AAA AGG TAT-3′. Complementary target DNA (*A. baumannii*) was 5′-AGA CAT GCA AAA AGG TAT-3′. Non-complementary DNA was 5′-GAA TAT GAT TTA CAG TTT ATT TT-3′. Mis-Match DNA (*A. baumannii*) was 5′-AGA CAT GCT AAA AGG TAT-3′. The Tris–HCl buffer that contained 1.0 mM of EDTA was used for the synthesis of the oligonucleotides stock solution (100.0 μM), which was then frozen at −20 °C. On the other hand, the β-CD stock solution (1.0 mM) was prepared by dissolving the β-CD powder into the methanol, in which PBS (0.1 M, pH 7.0) was subsequently added.

2.2. Instrumentations

Electrochemical measurements were carried out using a computer-controlled electrochemical analyzer that contained a three-electrode geometry with a coated AuE as the working electrode, a platinum wire counter electrode, and the reference electrode, which was a saturated calomel electrode (SCE).

2.3. Preparation of DNA sensor

On a smooth polishing fabric, a 1.0 μm and 0.05 μm alumina-water slurry was used to polish the surface of AuE, which was then rinsed with doubly distilled water and left to dry under a nitrogen stream. For the self-assembly of the probe, a 2.5 μL droplet of immobilization buffer solution + *A. baumannii* probe (ss-DNA) (9.0 μM) was deposited onto the surface of the AuE, and then the electrode was incubated at ambient temperature in a high-humidity container for 60 min to yield *A. baumannii* self-assembled AuE (ss-DNA coated AuE), which was followed by the treating of this electrode with the washing solution. After incubation in 1.0 mM of MCH solution for 5 min, the as-prepared electrode was rinsed with ethanol, water (v/v, 80:20) and distilled water. The dsDNA modified AuE was formed after the hybridization of the as-prepared probe with the specimen DNA. For the hybridization, the as-prepared ss-DNA-modified AuE was immersed into hybridization buffer solution (HBS, pH 7.0) + the target oligonucleotide (given the mismatched complementary or non-complementary strand concentrations) for 120 min at ambient temperature.

For the accumulation of β-CD on the as-prepared dsDNA-modified AuE, the as-prepared electrode was immersed in 0.1 M of phosphate buffer solution (PBS) (pH 7.0) + 0.09 mM of β-CD under gentle stirring at 100 rpm for 60 min at zero potential. Afterward, the washing solution was used to rinse this electrode (10 s). The accumulation of β-CD onto the bare AuE was also conducted under comparable conditions.

2.4. Measurements

For electrochemical measurements, the DPV was recorded in PBS (0.1 M, pH 7.0) under the following parameters: step potential, 50 mV; modulation, 0.05 s; and amplitude, 25 mV.

Electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) measurements were carried out in 5.0 mM of $K_3[Fe(CN)_6]$/ $K_4[Fe(CN)_6]$ + 1.0 M KC for the characterization of the as-prepared electrode. For the EIS experiment, the potential amplitude and scan rates were 0.27 V and 5 mV/s, respectively.

3. RESULTS AND DISCUSSION

The solution self-assembly and droplet self-assembly strategies were applied to the immobilization of the ss-DNA. For the former, the bare electrode was immersed into the buffer of immobilization that contained the probe solution (9.0 μM) for 105 min. For the latter method, a 2.5 μL droplet of *A. baumannii* probe solution was introduced onto the bare electrode. This was followed by successive soaking of the two separately coated electrodes into the MCH and β-CD solutions. Based on the successful accumulation of β-CD on the ds-DNA-coated AuE, it can be concluded that the probe self-assembled electrode was successively immersed into the target DNA solution as well as into the β-CD solution. As shown in Figure 1A, the accumulated β-CD on the surface of the ss-DNA-coated AuE before and after hybridization by the complementary DNA using the as-prepared electrodes via these two self-assembly techniques was recorded by DPV.

The current response of the β-CD accumulated on the ss-DNA-coated AuE, prepared via the droplet self-assembly method, was found to have a larger current response compared with the other methods. Moreover, the droplet self-assembly led to a higher current response after the hybridization, compared with the solution self-assembly methods. This indicated that the molecular self-assembly of the probe was more desirable than using the droplet self-assembly method. The accumulated ss-DNA/AuE current response was enhanced by increasing the probe immobilization time up to approximately 105 min, after which the response slightly decreased. This decrease may be caused by complete coverage of the gold electrode surface with the DNA probe after 105 min. Therefore, a time of 105 min was chosen for probe immobilization in subsequent experiments [41].

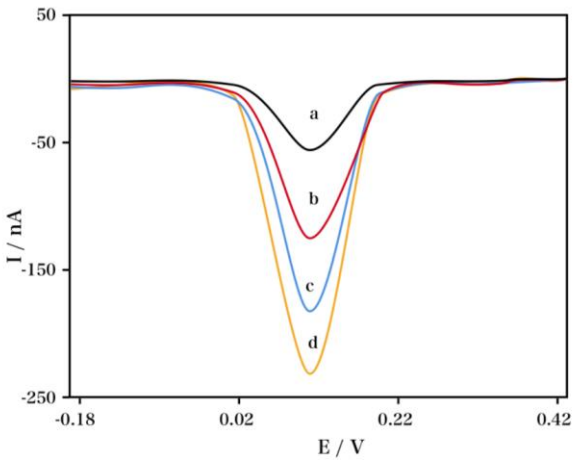

Figure 1. (A) DPV curves for the β-CD accumulated on the ss-DNA-modified AuE surface (a,b) before and (c,d) after hybridization with the complementary DNA using the AuE fabricated via different techniques: droplet self-assembly (b,d) and solution self-assembly (a,c).

For the hybridization of DNA, 3 strategies were used in the present study, and their performances were compared. The droplet self-assembly method was carried out by first depositing a droplet (2.5 μL) of the complementary solution on the AuE coated by ss-DNA for hybridization. In addition, it was incubated in a container (high-humidity) for 120 min at ambient temperature for evaporation prevention. The accumulation of β-CD occurred on the surface of the coated electrode after the coated electrode was completely rinsed in the washing solution under stirring at 100 rpm. The characterization for the accumulated OB on the proposed AuE surface was displayed by DPV in Figure 2. It can be concluded that in the solution protocol, the target DNA has a better chance of attaining the best orientation for hybridization. The preheated solution hybridization method was conducted by soaking the developed ss-DNA-coated electrode in the hybridization solution at 85 °C for 3 min. In addition, the as-prepared solution was stirred mildly. After gradual cooling of this solution at room temperature, the developed electrode was rinsed with the washing solution, onto which the β-CD was accumulated. The accumulated β-CD formed via preheated solution hybridization method was plotted by DPV, as illustrated in Figure 2. After hybridization, the as-prepared AuE was immersed into the β-CD solution. The results suggested that the solution method involving the electrode showed the maximum current response.

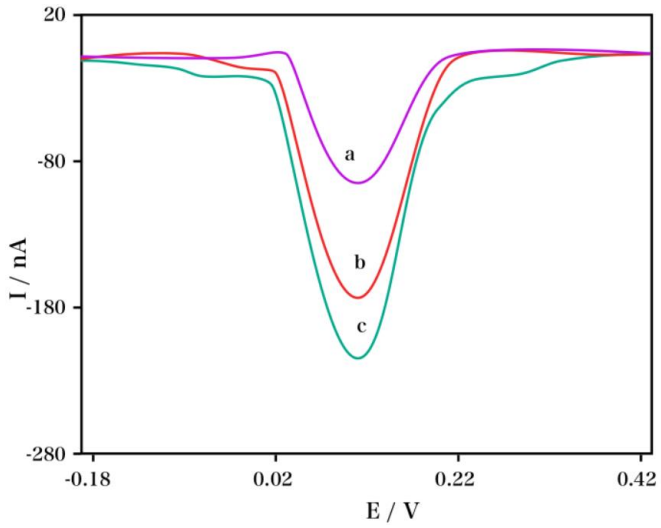

Figure 2. DPV curves for the β-CD accumulated on the ds-DNA/SAM surface synthesized via different hybridization strategies: (a) the drop method, (b) the preheated solution method and (c) the solution hybridization method.

We carried out DPV characterization to study the optimum β-CD (an electroactive label) concentration with ds-DNA using the coated AuE. After hybridization, the coated AuE was immersed into varying concentrations of β-CD for 1.5 h. Figure 3A illustrates that after the immersion of ds-DNA/SAM into the β-CD (varying concentrations), DPV curves were recorded for the accumulated β-CD. With the increasing concentration of the β-CD, a corresponding increase was recorded for the signals of accumulated OB. At the β-CD concentration of 0.9 mM, the highest cathodic current was observed, which then remained stable. Hence, 0.9 mM was determined to be the optimum β-CD

concentration. Moreover, the influence of the β-CD accumulation time was further studied, and 90 min was found to be the optimum accumulation time for β-CD at the ds-DNA/SAM.

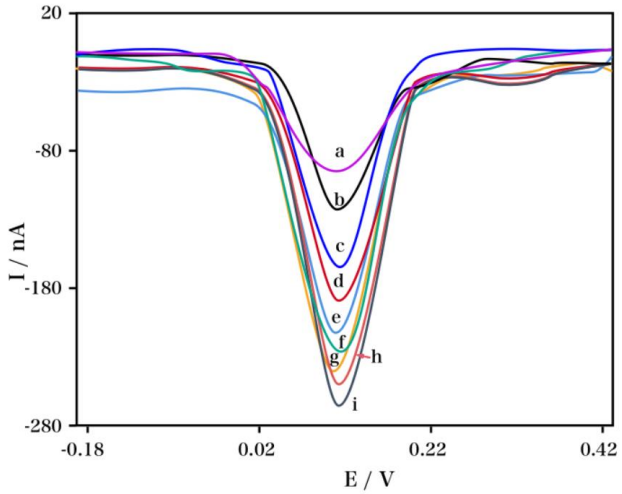

Figure 3. (A) DPV curves for the β-CD accumulated on the ds-DNA/SAM surface synthesized in the presence of β-CD at different concentrations over a range of 0.015-0.135 mM, as shown in (a) to (i).

To investigate the selectivity of the DNA hybridization using the AuE with the probe DNA, we further conducted the hybridization with its complete complementary sequence, a mismatched sequence, and non-complementary sequence. DPV curves in Figure 4 were recorded for the β-CD accumulated on the MCH coated electrode (curve a), ss-DNA-coated AuE before (curve b) and after hybridization with non-complementary (curve c), mismatched (curve d) and complementary (curve e) oligonucleotide solution (2.0 μM).

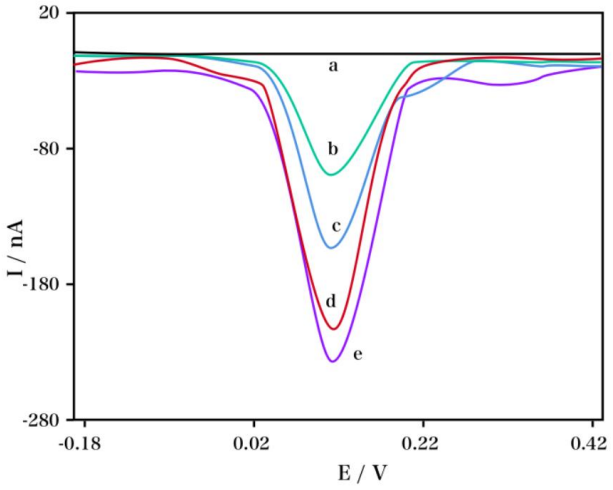

Figure 4. DPV curves of the β-CD accumulated on the (a) MCH coated AuE, ss-DNA-coated AuE before (b) and after hybridization with oligonucleotide solution (2 μM) of the (c) non-complementary, (d) mismatched, and (e) complementary in 0.1 M of phosphate buffer solution (pH 7.0).

The OB displayed an outstanding increase in peak current during its hybridization with its complementary sequence, in comparison with the DPV response of β-CD in the presence of the probe DNA at ss-DNA-coated AuE, as illustrated in Figure 4. A higher current peak was observed during the mismatched DNA-engaged probe hybridization than during the non-complementary sequence-engaged hybridization, which denoted the favorable selectivity of the as-coated electrode toward the hybridization of DNA. The current response of the accumulated ss-DNA increased and then slightly decreased upon increasing the probe concentration. This is in agreement with the findings of Nasirizadeh and co-workers [42] in which massive accumulation of the probe on the electrode resulted in the lower availability of the ss-DNA to DNA.

The characterization of the coated AuE in $K_3[Fe(CN)_6]$ solution (1.0 mM) after each modification step was presented in the CVs of Figure 5A. Compared with the high voltammogram current recorded for the bare electrode, that of the ss-DNA-immobilized electrode was apparently dropped, ascribed to the blocked access of $[Fe(CN)_6]^{3-/4-}$ anions to the surface of AuE (curve (b)). However, it is worth noting that the increased voltammogram current was observed after treating the ss-DNA-coated AuE with MCH, since the orientated ss-DNA was incorrectly removed. A sharp current drop was recorded after hybridizing the ss-DNA with the target DNA, whereas the current was increased during the hybridization with the mismatched target DNA, considering its incomplete hybridization with the ss-DNA present on the electrode.

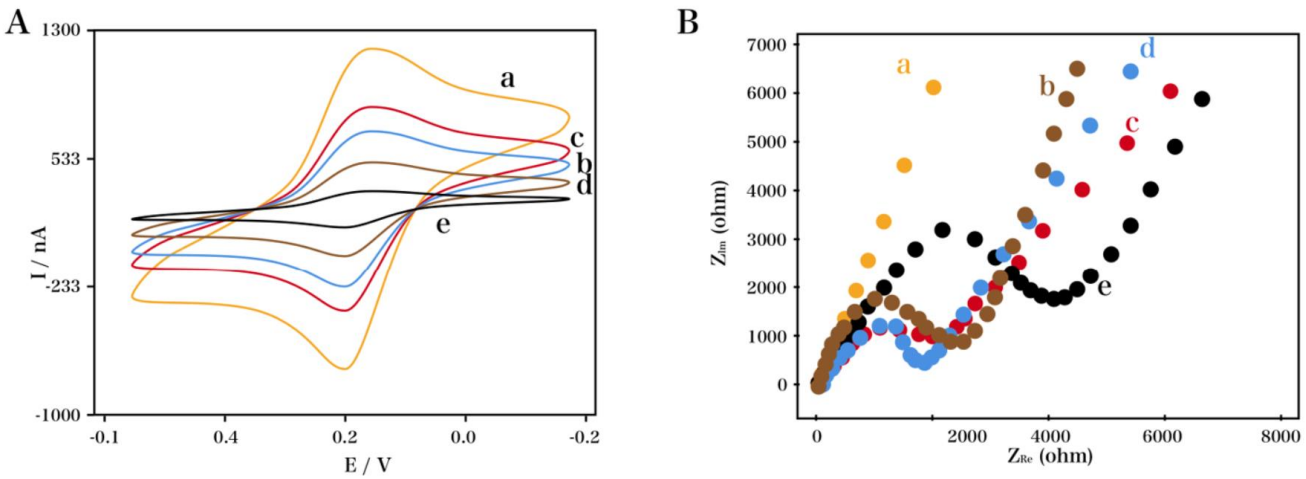

Figure 5. (A) CV recorded the coated electrode in 1.0 mM of $K_3[Fe(CN)_6]$ solution after each electrode modification procedure: (a) bare AuE, (b) ss-DNA-coated AuE, (c) MCH/ss-DNA-coated AuE, (d) mismatched target DNA, and (e) target DNA. (B) The Nyquist plot of these electrodes in 5.0 mM of $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ that contained 1.0 M of KCl: (a) bare AuE, (b) ss-DNA-coated AuE, (c) MCH/ss-DNA-coated AuE, (d) mismatched target DNA, and (e) target DNA.

The different coated AuEs in 5.0 mM of $K_3[Fe(CN)_6]/ K_4[Fe(CN)_6]$ that contained 1.0 M of KCl were characterized by the Nyquist plot displayed in Figure 5B. The bare AuE was characterized in curve (a), with much low-level resistance. A rise was found for the charge transfer resistance (R_{ct}) after immobilizing the ss-DNA onto the AuE, ascribed to the blocked access of $[Fe(CN)_6]^{3-/4-}$ to the

surface of the AuE, as illustrated in curve (b). There was a clear difference in the current for each modification step, which confirmed the successful immobilization and hybridization of each DNA probe. On the other hand, a drop was found for R_{ct} after treating the ss-DNA-coated AuE with MCH, as illustrated in curve (c). The target hybridized/probe-modified AuE showed a significantly higher charge transfer resistance, but the mismatched target had a significantly lower Rct, which could be because of incomplete hybridization of the mismatched DNA compared to the complementary target DNA. The observations from the abovementioned CV and EIS studies are in agreement and confirm the formation of the proposed biosensor through the electrode modification steps and the good selectivity of the biosensor [43].

The plot of DPV hybridization responses *vs.* the increasing complementary sequence target concentrations over a range of 0.3 nM-0.24 μM is illustrated in Figure 6A. The inset in Figure 6B illustrates the peak current increase in the intercalated β-CD with an increasing concentration of the complementary target DNA, with a linear relationship observed. According to $Cm = 3s_{bl}/m$, LOD was calculated as 0.14 nM. To allow for comparison to previous reports, the characteristics of different electrochemical sensors for target DNA concentration are summarized in Table 1.

Table 1. Comparison of the major characteristics of sensors used for the detection of AFP.

Electrode	Linear detection range	Detection limit	Reference
UV dose-response	50 nM - 200 nM	—	[44]
blaOXA-23-like and class 1 integrase gene detection	—	—	[45]
β-CD/ss–DNA coated AuE	0.3 nM - 0.24 μM	0.14 nM	This work

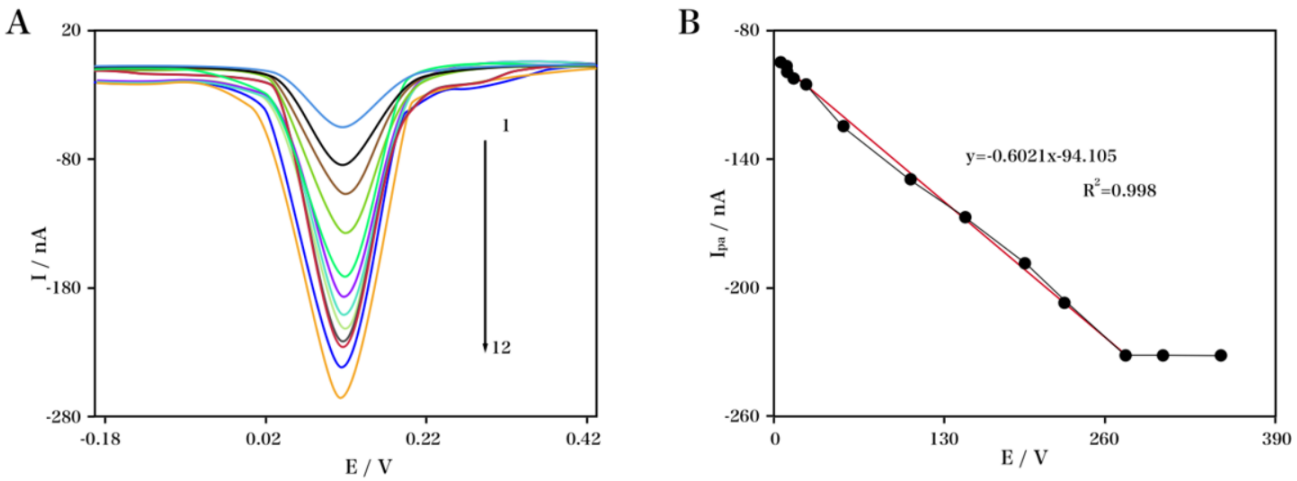

Figure 6. (A) DPV curves for the accumulated β-CD on the hybridized ss-DNA-coated AuE with the complementary (target) DNA at different concentrations in 0.1 M of PBS (pH 7.0). (B) Inset: the variation in the accumulated current response on ds-DNA-coated AuE and ss-DNA/SAM *vs.* the target DNA concentration during hybridization.

The relative standard deviation (RSD) measurement was further carried out for all the tests in the presence of the target DNA (varying concentrations), with a result of 2.8-4.3%. It could be concluded that the repeatability of our proposed biosensor was favorable. Moreover, the preparation process of the biosensor was repeated 5 times, and the biosensor was then measured under comparable circumstances and concentrations. In addition, their reproducibility was studied by comparing the performance results. After the preparation process of the biosensor was carried out 5 times, the RSD was recorded as low as 3.6%, which suggested that the reproducibility of the proposed biosensor was excellent.

Table 2. A. baumannii analysis in real beef samples using the proposed DNA sensor via ELISA technique.

Added (nM)	Immunosensor			ELISA		
	Found (nM)	Recovery (%)	RSD (%)	Found (nM)	Recovery (%)	RSD (%)
5	4.948	98.96	2.3	4.966	99.32	2.2
10	9.776	97.76	2.1	10.051	100.51	3.1
20	20.213	101.07	1.9	19.997	99.99	1.9

We also carried out a recovery test to investigate the accuracy of the as-prepared DNA sensor toward the excrement analysis, where excrement specimens were rinsed by 0.25 μm of ethanol, double-filtered before being stored in the refrigerator at 4 °C when not for immediate use. It can be seen from the results that the consistency between the ELISA method and the electrochemical analysis was excellent. For the investigation of the biosensor accuracy, *A. baumannii* (at varying concentrations) was mixed with the collected specimens, and then the recovery experiment was carried out. It can be seen from Table 2 that the accuracy of the proposed biosensor was remarkable, and it could be successfully used for the analysis of real samples as a substitute method.

4. CONCLUSIONS

In the present report, the interaction of β-CD (electroactive DNA label) was investigated. To fabricate the DNA biosensor used for the analysis, the varying affinities of β-CD to ds-DNA and to ss-DNA were studied. It can be seen from the results that the single-base mismatch in the target DNA can be analyzed by the β-CD–involved DNA biosensor using the solution hybridization as the optimum hybridization method and drop self-assembly as the optimum probe self-assembly approach. Under optimal conditions, it was found that the electrical signal was linearly related to the target DNA concentration (LOD: 0.14 nM).

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