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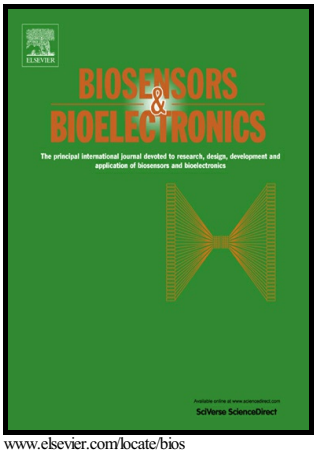

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Fabrication of an electrochemical DNA-based biosensor for *Bacillus cereus* detection in milk and infant formula

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Abstract

This paper describes fabrication of a DNA-based Au-nanoparticle modified pencil graphite electrode (PGE) biosensor for detection of *Bacillus cereus*, causative agent of two types of food-borne disease, i.e., emetic and diarrheal syndrome. The sensing element of the biosensor was comprised of gold nanoparticles (GNPs) self-assembled with single-stranded DNA (ssDNA) of *nheA* gene immobilized with thiol linker on the GNPs modified PGE. The size, shape and dispersion of the GNPs were characterized by field emission scanning electron microscope (FESEM). Detection of *B. cereus* was carried out based on an increase in the charge transfer resistance (R_{ct}) of the biosensor due to hybridization of the ss-DNA with target DNA. An Atomic force microscope (AFM) was used to confirm the hybridization. The biosensor sensitivity in pure cultures of *B. cereus* was found to be 10^0 colony forming units per milliliter (CFU/mL) with a detection limit of 9.4×10^{-12} mol L^{-1}. The biosensor could distinguish complementary from mismatch DNA sequence. The proposed biosensor exhibited a rapid detection, low cost, high sensitivity to bacterial contamination and could exclusively and specifically detect the target DNA sequence of *B. cereus* from other bacteria that can be found in dairy products. Moreover, the DNA biosensor exhibited high reproducibility and stability, thus it may be used as a suitable biosensor to detect *B. cereus* and to become a portable system for food quality control.

***Keywords*:** DNA biosensor, *Bacillus cereus*, self-assembly, electrochemical, food quality control, Gold nanoparticle.

1. Introduction

Bacillus cereus is a spore-forming, gram-positive bacilli that can be found in diverse environmental conditions, including soil and food such as dairy products, rice and vegetables. It is causative agent for two type of food-borne disease, i.e., emetic and diarrheal syndrome (Mols *et al*., 2011).

The emetic syndrome is caused by consumption of dodecadepsipeptide, cereulide that produced in food before ingestion (Jeßberger *et al*., 2014). This toxin is resistant to acidic pH, proteolytic enzymes and food processing including autoclaving (Jääskeläinen *et al*., 2004). It could produce three types of enterotoxins able to cause the diarrheal syndrome including a single protein toxin, cytotoxin K1 (CytK1) and two enterotoxin compelexes i.e., haemolysin BL (Hbl) and nonhaemolytic enterotoxin (nhe) (Wehrle *et al*., 2010). Symptoms could appear in consumer if either viable cells or spores are ingested (Jeßberger *et al*., 2014). Amongst genes involved in biosynthesis of above mentioned enterotoxins, *nheA* group are the most prevalent in all subspecies of *B. cereus.*

The spores formed by this microorganism may resist pasteurization of milk. Spores present in some food products may germinate and the vegetative cells can grow if conditions are suitable; leading to spoilage of the food product and production of toxins in the food or in the host (Biesta-Peters *et al*., 2011). In addition to safety concerns, growth of *B. cereus* causes some defects such as off-flavours, sweet curdling and bitty in dairy products (Molva *et al*., 2009). According to Codx standard for infant formula maximum acceptable number of *B. cereus* is 10^2 colony forming units per milliliter (CFU/mL) (Codex, 1994).

Conventional culture based detection methods for *B. cereus* are time consuming (from 2 to 7 days for confirmation) and labor and cost-intensive. Also, injured and non-culturable cells not detected in culture media. Methods based on molecular biology and immunoassay are usually costly, requires professional experts and expensive instrument (Kang *et al*., 2013). There are antigen-antibody and cell based methods to quantify *B.cereus* but to the best of our knowledge as yet, no electrochemical DNA biosensors has been reported for diagnosis of *B.cereus*. In general, a DNA based biosensor for onsite, sensitive, specific, time and cost-efficient diagnosis of *B. cereus* could be very useful.

Electrochemical DNA biosensors convert base-pair recognition event into an electrical signal and could be very useful in basic and applied research by detection of pathogenic bacteria because of its ease of use, speed, low cost and high sensitivity. Luo and co-workers (2013) was reported an electrochemical DNA biosensor for detection of *Enterobacteriaceae* bacteria with a limit of detection down to 40 CFU/mL in milk samples, in 3.5 h. Electrochemical DNA biosensor based on multiwall carbon nanotubes (MWCNTs)-chitosan−bismuth with PCR amplification of target DNA and purification of PCR products was reported for detection of *Staphylococcus aureus* with a detection limit of 1.23 ng mL^{-1} in fresh beef samples (Abdalhai *et al*., 2014).

Pencil graphite electrode (PGE), as a transducer, was selected because of its low price, availability, renewable and suitable mechanical surface, which does not need to additional regeneration. PGE was used for supporting oligonucleotide probes and transducer for the DNA hybridization (Pournaghi-Azar *et al.*, 2006). PGE demonstrate an arresting stripping performance for trace nucleic acids, compare conventional carbon paste electrodes.

For the first time in this study a quantitative DNA based (*nheA*) electrochemical biosensor, without PCR amplification of sample, was designed and developed to detect *B. cereus* in dairy products as a fast efficient and non-expensive method. Hybridization of the ss-DNA (self-assembled at the surface of gold nanoparticle-modified PGE, GNPs-PGE) with target DNA was used to detect *B. cereus* using electrochemical impedance spectroscopy (EIS). Another advantage of the DNA biosensor is the ability to separate virulent bacteria from avirulent members of the same species by targeting virulence associated gene (*nhe*A) as a DNA-probe. In addition to identifying vegetative cells, spores are detected too.

2. Experimental

2.1. Reagents

Gold chloride ($HAuCl_4.3H_2O$) was purchased from Sigma-Aldrich and $K_3Fe(CN)6$, $K_3Fe(CN)_6$ and methylene blue (MB) were purchased from Merck. All solutions were prepared in deionized water (DI water).

A 25 mer ssDNA (as a probe) with HS-$(CH_2)_6$ modification at the 5'end and all oligonucleotides with BIO-RP purification were purchased from Bioneer Corporation (South Korea). Stock solutions (100 pmole μL^{-1}) of the DNA sequences were prepared with sterile distilled water (SD water) and stored at a refrigerator at –20°C.

Sequence of *nhe*A gene was obtained from the public data base GenBank (accession No. NC_006274.1). Specificity of the oligonucleotide sequences were investigated with Basic Local Alignment Search Tool (BLAST), the oligonucleotide sequences designed were as follows:

25 mer thiolated sequence of ssDNA (probe): 5' –SH-CAG CCA GAC ATT AAG GTA AAC GCG A-3'.

25 mer complementary sequence (target DNA): 5'-TCG CGT TTA CCT TAA TGT CTG GCT G-3'.

40 mer complementary sequence: 5'- TCG TCA AAC TAC TCA TCG CGT TTA CCT TAA TGT CTG GCT G.

25 mer single mismatch sequence: 5'-TCG CGT TTA TCT TAA TGT CTG GCT G-3'.

2.2. Apparatus

Electrochemical measurements were performed in an analytical system (Ivium potentiostat/galvanostat, pocketSTAT, Netherlands) linked to a computer using the Ivium 2.231 software that connected to a three-electrode cell (Metrohm) including a modified GNPs-PGE (Pentel, 0.7 mm diameter) as a working electrode, Ag/AgCl (3.0 mol L^{-1} KCl) as a reference electrode and platinum wire as an auxiliary electrode, which were inserted in a solution containing 5.0 mmol L^{-1} of $Fe(CN)_6^{4-/3-}$ in 0.1 molL^{-1} KCl. EIS was performed between 100 kHz to 10 mHz at a potential of 150 mV and cyclic voltammetry (CV) was swept between potential range of - 0.20 to 0.60 V at a scan rate of 50 mV s^{-1}. Field Emission Scanning Electron Microscope (FESEM) (Hitachi S-4160, Japan) was used to characterize the Au nanoparticle that was electrodeposited on the PGE surface. Atomic force microscope images were obtained using AFM from Bruker Nanos, Germany)

2.3. Fabrication of the DNA biosensor

First of all, PGE was activated using voltammetric method. For this purpose, a PGE was treated at a constant potential (+1.40 V) in acetate buffer solution (0.1 mol L^{-1}, pH 4.8) for 300 s. For electrodeposition of gold nanoparticles on the surface of the treated PGE, the treated PGE was immersed into a 3.0 mmol L^{-1} ($HAuCl_4.3H_2O$) solution at a potential of –0.20 V for 120 s. The electrode was then dipped into a 0.1 mol L^{-1} KNO_3 and cyclic voltammogram was run in a potential range of –0.30 to +1.50 V (10 cycles).

To immobilize the single-stranded DNA (ssDNA) at the surface of GNPs/PGE, the electrode (4 mm) was immersed into the ssDNA solution (25 μmol L^{-1}) for 60 min at room temperature. The electrode was then gently rinsed with DI water to remove any unbounded ssDNA at the electrode surface and dried under nitrogen atmosphere. The fabricated electrode was stored at 4 °C. The schematic representation of the DNA biosensor is shown in Scheme 1. **"Here Scheme 1"**

2.4. Hybridization and regeneration of the biosensor

The charge transfers resistance (R_{ct}) of ssDNA/GNPs/PGE before its interaction with the target DNA was measured by EIS in $Fe(CN)_6^{4-/3-}$ solution containing 0.1 mol L^{-1} KCl. The hybridization temperatures were controlled between 45°C (with interval of 5 °C) with incubation times of 5 to 35 min, to define the effective hybridization condition. The binding of ssDNA (on the electrode surface) and complementary DNA was performed by immersion of ssDNA/GNPs/PGE (4 mm) in 8 μL of the target DNA solution. This electrode was then washed with DI water to remove nonspecifically adsorbed DNA. Finally, impedimetric measurement was carried out using 5.0 mmol L^{-1} $Fe(CN)_6^{4-/3-}$solution containing 0.1 mol L^{-1} KCl. The charge transfers resistance

of the fabricated DNA biosensor before and after immersing in the target DNA solution was used to monitor the hybridization efficiency.

In order to investigate the hybridization efficiency of the fabricated DNA biosensor with the complementary and mismatch DNAs, as well as EIS measurements, differential pulse voltammetry (DPV) was also carried out using MB (20.0 μmol L^{-1} solution) as an electroactive probe (Ensafi *et al*., 2013). In order to load MB into the electrode, the fabricated electrodes at each stages were immersed into a 20.0 μmol L^{-1} MB (pH 7.0) with stirring for at least 2 h until the loading equilibrium was reached. Then, the electrodes were rinsed with DI water and the DPV signals were recorded in the potential range of –0.70 to +0.30 V, in phosphate buffer solution (0.1 mol L^{-1}, pH 7.0).

In order to regenerate the biosensor, the hybridized electrode was immersed into a 0.1 mol L^{-1} NaOH solution for 7 min and then it was washed with DI water. The complementary sequence was removed from the surface of the electrode to obtain ssDNA/GNPs/PGE. Zhang *et al*., (2008) were reported that regeneration of DNA sensor occurs in 0.5 M NaOH solution for 2 min.

2.5. Bacterial culture development

B. cereus PTCC1015 was used as a target bacterium to determine the efficacy and sensitivity of the biosensor. As in dairy products *B. cereus* is present in combination with other bacteria, thus target DNA with non-target DNA to be found in them. Therefore, *Escherichia coli* O157:H7, *Bacillus subtilis*, and *Staphylococcus. aureus* PTCC 1337 were used to examine the specificity of the biosensor.

Before DNA extraction, a single colony of each bacterial culture (from a Nutrient agar plate grown at 37 °C and kept at 4 °C for maximum one week) was used to prepare an overnight culture of each bacterium in Nutrient broth and then they were centrifuged at 14000 × g for 10 min at 4 °C to obtain pellet of the bacteria.

Total genomic DNA from *B. cereus* and other bacteria were extracted using GeneAll®Exgene™Cell SV mini (107-101/107-152). The extracted DNA of *B. cereus* was serially diluted, using 9.0 mL of normal saline and 1.0 mL of DNA (10-fold serial dilutions) to prepare 10^0 to 10^2 CFU mL^{-1}. The fabricated DNA biosensor was dipped into a 0.9% NaCl solution and used as a blank solution. EIS was used to prepare the calibration curve, by plotting of the resulted R_{ct} *vs.* the different cell concentrations. Each experiment was repeated at least three times.

2.6. Preparation of real samples

To extract total DNA from dairy products, dairy products including raw milk, pasteurized milk and infant formula were centrifuged at 14000 × g for 10 min at 4 °C. The obtained pellet was after kept at –80 °C for 30 min; a fat layer was formed on the inter wall of Falcon tube was removed and then the pellet was thawed. The precipitated cells were used for DNA extraction (Cocolin *et al*., 2002) using GeneAll®Exgene™ Cell SV mini kit for DNA extraction from gram positive bacteria. Finally, SD water added as required and the extracted DNA was stored at –20 °C. The quality and quantity of the extracted DNA were evaluated by gel electrophoresis using 1% agarose and Picodrop (Pico200, England), respectively. The final concentration of the extracted DNA was 0.805 μg/μL. The extracted

DNA was kept at water bath at temperature of 95 °C for 5 min to obtain ssDNA and immediately kept on an ice bath for another 5 min to stabilized ssDNA.

3. Results and discussion

3.1. Characterization of the fabricated biosensor

Different techniques including FESEM, cyclic voltammetry, EIS and AFM were used to characterize the electrodes.

The morphological properties of the electrodes were investigated using FESEM. Fig. 1A shows the surface morphology with different deposition time of Au at PGE. As increasing the deposition time of Au on the electrodes surface the uniformity of GNPs increases both in size and distribution. Further increase in the deposition time is normally led to aggregation of GNPs. As shown in Fig. 1A, as increasing in the deposition time from 90 s (a) to 120 s (b), the minimum particle size of GNPs decreased from 120 nm to about 50 nm and the particles are more uniform in size and distribution (i.e the density has increased). Further, increasing in the deposition time to 180 s (c) led to more aggregation of GNPs, as expected. Therefore, 120 s was used to deposit GNPs at the surface of PGE.

Electrochemical characterization of the electrode surface was carried out to investigate the immobilization of the ssDNA on GNPs/PGE and its hybridization with the complementary DNA. Here, cyclic voltammetry was used in 5.0 mol L^{-1} $Fe(CN)_6^{4-/3-}$ solution containing 0.1 mol L^{-1} KCl. As shown in Fig. S1A (b), coating of the PGE surface with GNPs caused increasing kinetic of the electron transfer, with reversible behavior of $Fe(CN)_6^{4-/3-}$. To immobilize the ssDNA at the surface of GNPs/PGE, monolayer of thiolated ssDNA was self-assembled at the surface of GNPs/PGE. The cyclic

behavior of the biosensor in $Fe(CN)_6^{4-/3-}$ solution is shown in Fig. S1-A (c). The overpotential of the redox reaction was increased (0.21 to 0.26 V), whereas the peaks current decreased. This is due to the electrostatic repulsion between negative charge of the DNA surfaces and $Fe(CN)_6^{4-/3-}$ (the obtained peak current for GNPs/PGE, ssDNA/GNPs/PGE and dsDNA/ssDNA/PGE were 72, 32 and 20 µA, respectively).

In addition to cyclic voltammetry, EIS was used to investigate the immobilization efficiency of the ssDNA at the surface of GNPs/PGE and its hybridization with the complementary sequence (Fig. S1-B). Immobilization of ssDNA at the electrode surface formed a resistant layer at the electrode surface and therefore, increased the charge transfer, R_{ct} of the electrode (in a solution containing 5.0 mol L^{-1} $Fe(CN)_6^{4-/3-}$in 0.1 mol L^{-1} KCl). It was clear that hybridization of the ssDNA with the complementary DNA was further increased the R_{ct} of the biosensor, as shown in Fig. S1-B, curve d (Kafka *et al*., 2008; Wang *et al*., 2011). Immobilization of the capture probe in the immobilization layer resulted increasing in the capacitance. As the ssDNA has very hydrophilic property, it attracts the electrical double layer closer to the electrode surface, resulting in an increasing in the capacitance. The capacitance depends on the thickness and dielectric behavior of the dielectric layers. DNA acts as dielectric and the capacitance changes over time. As showed in Fig. S-2 (supplementary material) after 40 min, the percentage change in the Cd vs. R_{ct} is ignorable, hence the hybridization efficiency can be determined by R_{ct} measurement.

AFM images of ssDNA and dsDNA is presented in Fig. 1B. As expected, the height of the dsDNA layer was increased compare to that of ssDNA layer. Increase in the height of dsDNA (curve b) is usually attributed to increase in the strength *vs.* ssDNA and

concomitantly decrease in the flexibility (elastic deformation). This is due to the hybridization with the complementary DNA, which forms dsDNA (Mirmomtaz *et al*., 2008). **"Here Fig. 1"**

3.2. Optimization of immobilization of ssDNA and its hybridization

In order to obtain the best performance of the biosensor, immobilization time and temperature, concentration of ssDNA (as a probe) and hybridization conditions were investigated. All experiments were performed in triplicate. In order to optimize the time of immobilization of the ssDNA on GNPs/PGE, 50 μmol L^{-1} DNA with different time (20, 40, 60, 80, 100 and 120 min) were tested. The results indicated that by increasing the immobilization time up to 60 min, the charge transfer resistance was increased and then leveled off (Fig. 2A).

To optimize the ssDNA concentration, GNPs/PGE was immersed in different concentration (10, 12.5, 25, 50, 75, 100 μmol L^{-1}) of ssDNA for 60 min to choose the optimum concentration of ssDNA. The results showed that 25 μmol L^{-1} ssDNA was the optimized concentration of the DNA (Fig. 2B).

To optimize the hybridization temperature, the fabricated biosensor was immersed in a solution of target DNA (2.0×10^{-6} mol L^{-1}) at different temperatures (30, 35, 40, 45, 50, 55 °C) for 40 min. Then, the electrode was immersed into a solution containing 5.0 mol L^{-1} $Fe(CN)_6^{4-/3-}$ in 0.1 mol L^{-1} KCl. The results indicated that by increasing temperature to 45 °C, R_{ct} increased and the hybridization was maximum at 45 °C (Fig. S3-A). Higher temperatures caused separation of the immobilized ssDNA from the electrode surface and disabled any hybridization. Similar result has been reported before (Wang *et al*., 2011).

Also, Herdt and co-workers (2006) was reported that DNA dissociation and degradation from gold nanoparticle surfaces occur faster at higher temperature. Some recent studies showed that degradation and oxidation of Au-thiol bond occur easily and rapidly in ambient conditions (e.g., exposure to air, water and/or light) (Willey *et al*., 2005; Liu *et al*., 2006; Bhatt *et al*., 2011).

The influence of the hybridization time on the response of the biosensor was also investigated. The fabricated DNA biosensor was immersed into a solution of target DNA (2.0×10^{-6} mol L^{-1}) at 45 °C for different times of incubation (5 min up to 35 min). Then, the impedance spectra of the electrode were recorded in a solution containing 5.0 mol L^{-1} $Fe(CN)_6^{4-/3-}$ in 0.1 mol L^{-1} KCl. The results indicated that 25 min was optimized for the hybridization time (Fig. S3-B). Therefore, 45 °C and 25 min were selected as the optimum temperature and incubation time of the hybridization.

To check the hybridization of the ssDNA with the complementary DNA and mismatch DNA, EIS method (in 5.0 mol L^{-1} $Fe(CN)_6^{4-/3-}$ in 0.1 mol L^{-1} KCl) and DPV method (in 20.0 µmol L^{-1} MB in 0.10 mol L^{-1} phosphate buffer, pH 7.0) were used. Fig. 2C shows that the EIS of the fabricated DNA biosensor interacted with the 25-mer mismatch and with the target DNA (25 and 40-mer). The results indicated that the mismatch sequence could not have stable binding with the ssDNA at the GNPs/PGE surface. Therefore, the R_{ct} of the biosensor was not change significantly (Fig. 2C, curve b). When the biosensor was hybridized with 40-mer target DNA (Fig. 2C, curve d) the R_{ct} increased in comparison with 25-mer complementary sequence (Fig. 2C, curve c) due to the fact that non-hybridized part of the target DNA sequence was oriented on the electrode surface (overhang of 15 nucleotides), which resulted increasing of the R_{ct}.

Moreover, to detect the DNA hybridization efficiency, MB was used as an electroactive probe, because it can intercalate with ds-DNA. Fig. 2D shows the DPV responses of loaded MB on the electrode surface (in the potential range of –0.70 to 0.30 V) before and after interaction of the biosensor with the complementary DNA (25 mer) and with one base mismatches (25 and 40 mer), from 20 μmol L^{-1}MB solution (in phosphate buffer, pH 7.0. With immobilization of ssDNA at the surface of GNPs/PGE, a peak current of 18.9 ± 0.02 μA was obtained. After hybridization of the ssDNA with the target DNA, MB was intercalated between the produced dsDNA and the oxidation/reduction reaction signal was appeared and increased. Therefore, the efficiency of DNA hybridization was estimated by increasing in MB signal (Fig 2D curve of (c), (d)). After hybridization of the DNA probe with both 25 and 40 mer target DNA, signals with a current of 29.2 ± 0.03 and 27.4 ± 0.05 μA were observed, respectively. The DPV signal of the biosensor after interacted with DNA mismatch solution did not increase significantly and the height and position of the peak was nearly equal, compared with the signal of MB in ssDNA (Fig. 2D, curves (a) and (b)). This is due to the fact that MB could not intercalate with the ssDNA at the surface of the biosensor, also the mismatch DNA could not interact with the ssDNA at the electrode surface.

"Here Fig. 2"

3.3. Figures of merit

EIS was used to prepare the calibration curve by plotting the R_{ct} of the biosensor (in a solution containing 5.0 mol L^{-1} $Fe(CN)_6^{4-/3-}$ in 0.1 mol L^{-1} KCl) *vs.* the target DNA concentrations. For each concentration, R_{ct} was measured three times. The calibration curve of the fabricated DNA biosensor showed two linear dynamic ranges, 0.02–10 nmol

$L^{-1}$and 0.01–10 μmol L^{-1} (Fig. 3A and 3B) with correlation equations of y = 181.37x + 942.33 (R^2 = 0.997) and y = 157.55x + 2964.6 (R^2 = 0.966), respectively where x is concentration of the complementary DNA and y is R_{ct}.

The limit of detection (LOD) of the biosensor was calculated using the DNA extracted from a real bacterial sample (section of 2.5). The biosensor as used to measure R_{ct} in DNA solution extracted from bacterial solution (Fig. S4). The calculated LOD was (based on cell and target DNA concentration) 10^0 CFU/mL and 9.4×10^{-12} mol L^{-1} (estimated based on $3S_{bk}/m$), respectively. Moreover, to confirm the LOD of the biosensor, determination of LOD was done by real time PCR (RTi-PCR) of serial dilution of pure culture of *B. cereus* samples, with LOD of 10^0 CFU/mL as shown in Fig. S5.

The reproducibility of the DNA biosensor was checked using EIS method (in a solution containing 5.0 mol L^{-1} $Fe(CN)_6^{4-/3-}$ in 0.1 mol L^{-1} KCl) with five separate DNA biosensors, to detect the target DNA (2.0×10^{-9} mol L^{-1}). The relative standard deviation (RSD%) of 3.6% was calculated, demonstrating an acceptable reproducibility of the DNA biosensor. To investigate the stability of the DNA biosensor, different sensors were made separately in the same conditions and they were kept at 4 °C for 30 days. The R_{ct} of each of them was measured in two-day interval (in a solution containing 5.0 mol L^{-1} $Fe(CN)_6^{4-/3-}$ in 0.1 mol L^{-1} KCl). During 20 days, no difference observed in their R_{ct}, and after that, the R_{ct} started to gradually decrease and after 30 days there was only 4.1% decrease in the R_{ct}, which suggest desirable stability of the DNA biosensor. Based on the obtained results, 4 °C was suitable condition for long storage of the biosensor. This result is consistent with finding of (Bhatt *et al*., 2011) reported that at 4 °C release kinetics is notable reduced.

Regeneration of biosensor was carried out according to section 2.4. Our studied showed that the fabricated DNA biosensor could be repeatedly used for hybridization–denaturation circles for 7 times. Only 4.8% decrease in R_{ct} compare to that of first test was obtained. Therefore, 0.1 mol L^{-1} NaOH solution is a suitable reagent to regenerate the biosensor. The calculated RSD% for five replicate measurements of the target DNA (2.0×10^{-9} mol L^{-1}) with a single biosensor was 3.8%.

In order to investigate the biosensor selectivity, the fabricated *B. cereus* DNA biosensor was subjected to bacteria those almost found in dairy products (mentioned above). *Escherichia coli* O157:H7, *Bacillus subtilis*, and *Staphylococcus aureus* PTCC 1337 were used to test the selectivity of the fabricated DNA biosensor, as these are usually found in contaminated dairy products. The results showed that these bacteria can not affect the detection of *B. cereus*. Also, no hybridization with the immobilized ssDNA was observed and thus no signal was detected (R_{ct} changes <5%). The results indicated that the DNA biosensor of *B. cereus* can be used for quantitatively detection of *B. cereus* in food samples (Fig. S6).

3.4. Real sample analysis

In order to detect *B. cereus* in dairy products such as raw milk, pasteurized and infant formula, the fabricated biosensor was used. For this purpose, DNA extraction was carried out from different samples (section of 2.7.1). The DNA biosensor was immersed into the extracted DNA (45 °C for 25 min) those obtained from different dairy products. To detect *B. cereus* based on culture method, 0.1 mL of the dairy products were cultured in mannitol egg yolk polymyxin (MYP) media that no colony was detected in MYP plate

culture (reported as less than 10 CFU/mL), whereas the fabricated DNA biosensor could detect *B. cereus*. The results are given in Table 1. The results confirmed that the DNA biosensor can be used for detection of *B. cereus* in food samples. The important advantage of using fabricated DNA biosensor compare to that of culture based method is detection of non-culturable and injured cells.

Compared with the other electrochemical biosensors those were reported for detection of *B. cereus*, the fabricated biosensor in this study provides efficient detection of *B. cereus* due to lower detection limit and wide linear range. Other methods such as RT-PCR, are not only costly, requiring expensive instrumentation and consumables, but also they do not provide an instant (at least 3hours is needed for diagnosis), onsite means of detection, and thus are not suitable for food safety and quality control applications.

4. Conclusions

The results demonstrated that the fabricated biosensor could be effectively detect *B.cereus*. The DNA biosensor is easy to operate and is rapid, has low cost and could efficiently detect *B. cereus* specifically in dairy products. It is label free and does not need indicators. The proposed DNA biosensor could be well applied for *B. cereus* detection for infant formula duo to its LOD of 10^0 CFU/mL particularly in catering due to rapid detection. The results indicated that the proposed DNA biosensor has high accuracy in the measuring *nhe*A gene in real samples (Table 1). Also, hybridization efficiency was evaluated in $Fe(CN)_6^{4-/3-}$ and MB probe." The fabricated DNA biosensor compared with previously reported biosensors for detection of *B. cereus*, offered lower LOD and wider linear dynamic range. The other advantages of the biosensor are desirable stability and

high reproducibility. The biosensor is highly sensitive and selective to detect 10^0 CFU/mL *B. cereus*. The LOD of the proposed biosensor is better than that reported for antigen-antibody and cell based sensor (Table 2).

Future research will focus on design of DNA biosensor devices based on lab on chip for food safety and quality control.

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Table 1: Detection of *B. cereus* in dairy products (n= 3).

Sample	Added (nmol L^{-1})	Found (nmol L^{-1}) #	Recovery (%)	RSD (%)
Infant formula	---	Less than detection limit	---	
Infant formula	1.50	1.48 ± 0.1 1.47 ± 0.05 1.43 ± 0.06	97.3%	4.8
Pasteurized milk	---	0.02 ± 0.001	---	
Pasteurized milk	2.00	1.94 ± 0.20 1.99 ± 0.11 1.97 ± 0.03	98 %	5.6
Raw milk	---	0.20 ± 0.05	---	
Raw milk	5.00	5.01 ± 0.10 5.11 ± 0.18 5.08 ± 0.16	101.2%	2.8

Average of three determinations plus the standard deviation.

Table 2: Comparison of the proposed method with several electrochemical approaches for the detection of *B. cereus*.

Method	Electrode	LOD (CFU/mL)	Linear dynamic range (CFU/mL)	Reference
Antigen–antibody (polyclonal antibodie)	Silver electrode	10^1 to 10^{2} *	1.2×10^1, 3.4×10^1 and 4.4×10^{2}**	Pal *et al*., 2007
Antigen–antibody (monoclonal antibodies)	Glassy carbon electrode	10.0	5.0×10^1-5.0×10^4	Kang *et al*., 2013
Analyte interaction with mammalian cells	B lymphocyte Ped-2E9 cellline, encapsulated in collagen matrix in 3D scaffold	10^3	10^2–10^4	Banerjee and Bhunia, 2010
Immunomagnetic separation	Screen-printed carbon electrode	40	4.0×10^0-3.9×10^2	Setterington and Alocilja, 2012
DNA biosensor for detection of *Enterobacteriaceae* bacteria by ExonucleaseIII-assisted signal amplification	gold electrode	40	0.01 pM to 1 nM	Luo *et al*., 2013
DNA biosensor based on HRP-mimicking hemin/G-quadruplex for detection of *Escherichia coli* O157:H7 (*E.coli* O157:H7)	Graphene oxide (GOx)–Thi–Au@SiO2	0.01 nM	0.02 to 50.0 nM	Li *et al*., 2015
RT-PCR teqnique (Magneto-DNA system)	-	10^0	dynamic range of detection could be controlled by changing the PCR cycle number	Chung *et al.*, 2013
RT-PCR teqnique based on SYBR green I	-	10^1	NA	Wehrle *et al*., 2010
DNA hybridization	GNPs/PGE	10^0	10^0 to 10^7 or 0.02–10 nmol L^{-1} and 0.01–10 µmol L^{-1}	This work

*10^1 CFU/mL for polyaniline protonated with hydrochloric acid and emeraldine salt and 10^2 CFU/mL for polyaniline protonated with perchloric acid.
**polyaniline protonated with hydrochloric acid, emeraldine salt and polyaniline protonated with perchloric acid at a concentration of 0.25 g/mL were determined to be 1.2×10^1, 3.4×10^1 and 4.4×10^2 CFU/mL, respectively.
***Not Available.

Figures and scheme caption

Fig. 1: A): FE-SEM images of the bare PGE (a), and different electrodeposition time of Au (b, c and d for 90, 120 and 180 s, respectively) in 3.0 mmol L^{-1} $HAuCl_4$ at a potential of −200 mV (*vs.* Ag/AgCl). **B):** AFM images of the ssDNA (a) and the ssDNA after hybridization with the complementary DNA (b).

Fig. 2: A): Effect of immobilization time for immobilization of the ssDNA. **B**): Influence of concentration of the ssDNA on its immobilization at the electrode surface. **C):** Nyquist plots of ssDNA/GNPs/PGE (a); After interacted with 25-mer one-base mismatch (b); After interacted with 25-mer of the complementary DNA (c); and after interacted with 40-mer of the complementary DNA (d). Conditions: In 5.0 mmol L^{-1} $Fe(CN)_6^{4-/3-}$ containing 0.1 mol L^{-1} KCl and scanning potential between –0.2 and +0.6 V in phosphate buffer solution (pH 7.0). **D):** DPV of the loaded MB at (a): ssDNA/GNPs/PGE; (b): ssDNA/GNPs/PGE immersing in 25-mer one-base mismatch DNA solution; (c): ssDNA/GNPs/PGE immersing in 40-mer complementary DNA solution; (d) ssDNA/GNPs/PGE immersing in 25-mer complementary DNA solution; Conditions: Scanning potential between -0.70 and +0.30 V in phosphate buffer solution (pH 7.0). All the concentrations of the hybridized DNA were 5.0×10^{-10}.

Fig. 3. Calibration curve for dsDNA in the presence of the complementary DNA as: **A):** (a) 2.0×10^{-11}; (b) 2.0×10^{-10}; (c) 2.0×10^{-9}; (d) 5.0×10^{-9}; (e) 8.0×10^{-9} mol L^{-1}; 1.0×10^{-8} (f) **B):** 1.0×10^{-8} (g); 8.0×10^{-7} (h); 2.0×10^{-6} (i); 5.0×10^{-6} (j); 8.0×10^{-6} (k); 1.0×10^{-5} mol L^{-1}. 25 min was used for the hybridization at 45 °C. (Error bar is the standard deviation of three determinations).

Scheme 1. Schematic diagram for the immobilization of the ssDNA on GNPs/PGE and its hybridization.

HIGHLIGHTS

- An electrochemical DNA-based biosensor was introduced for *Bacillus cereus.*
- Gold nanoparticles were used to prepare modified pencil graphite electrode.
- The biosensor could detect *Bacillus cereus* as low as 10^0 CFU/mL.
- The efficiency of the biosensor was determined in real food sample.

Figure 1

Fig 1

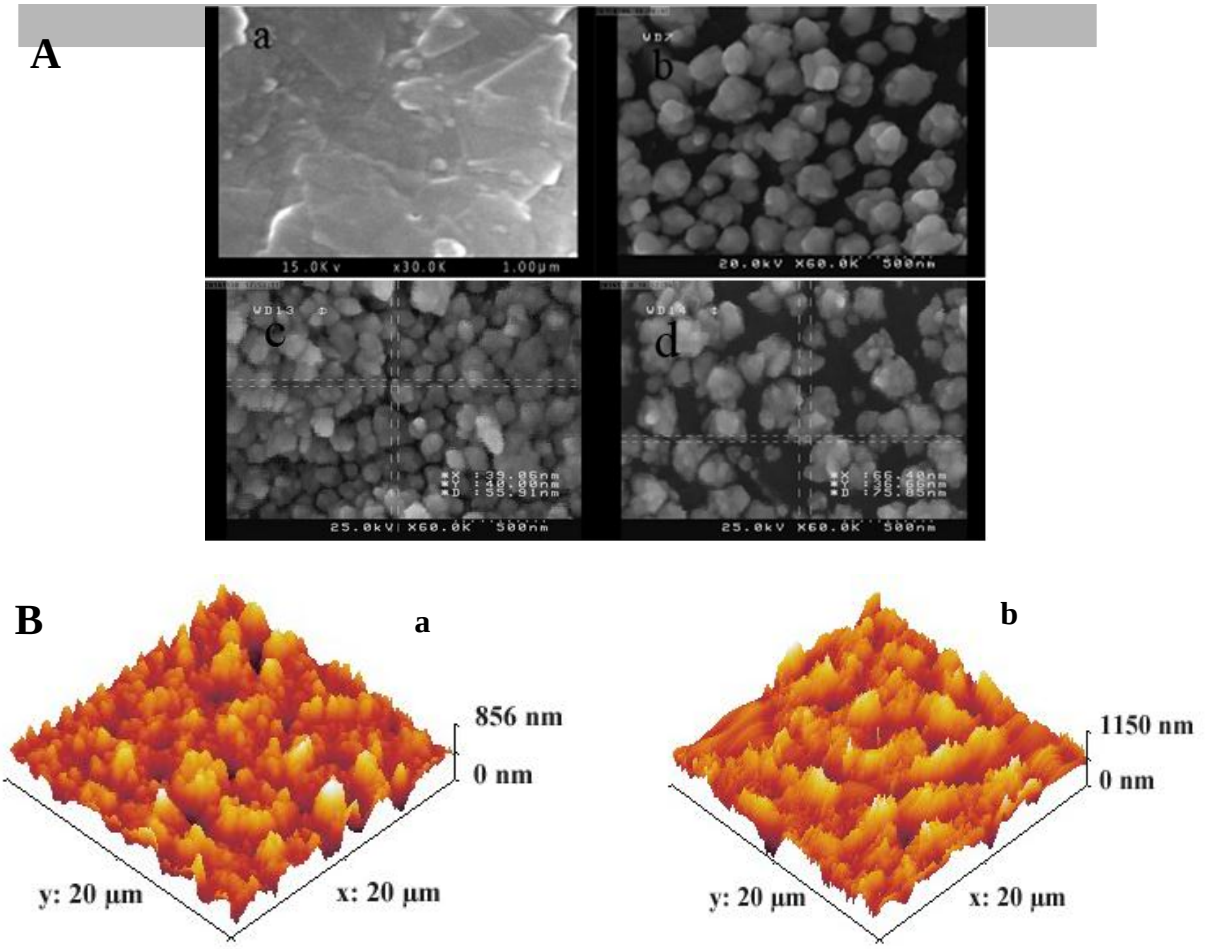


Figure 2

Fig 2

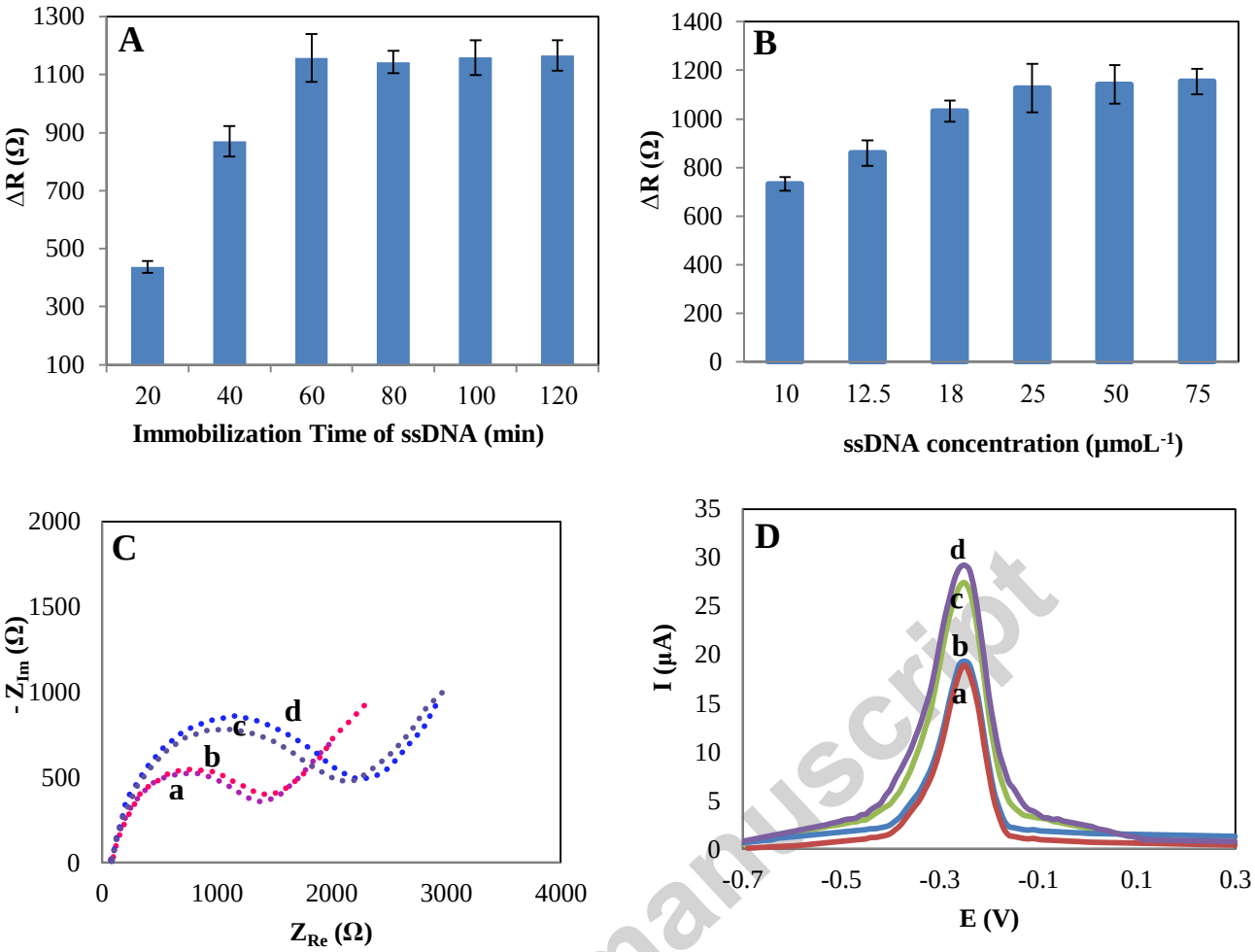
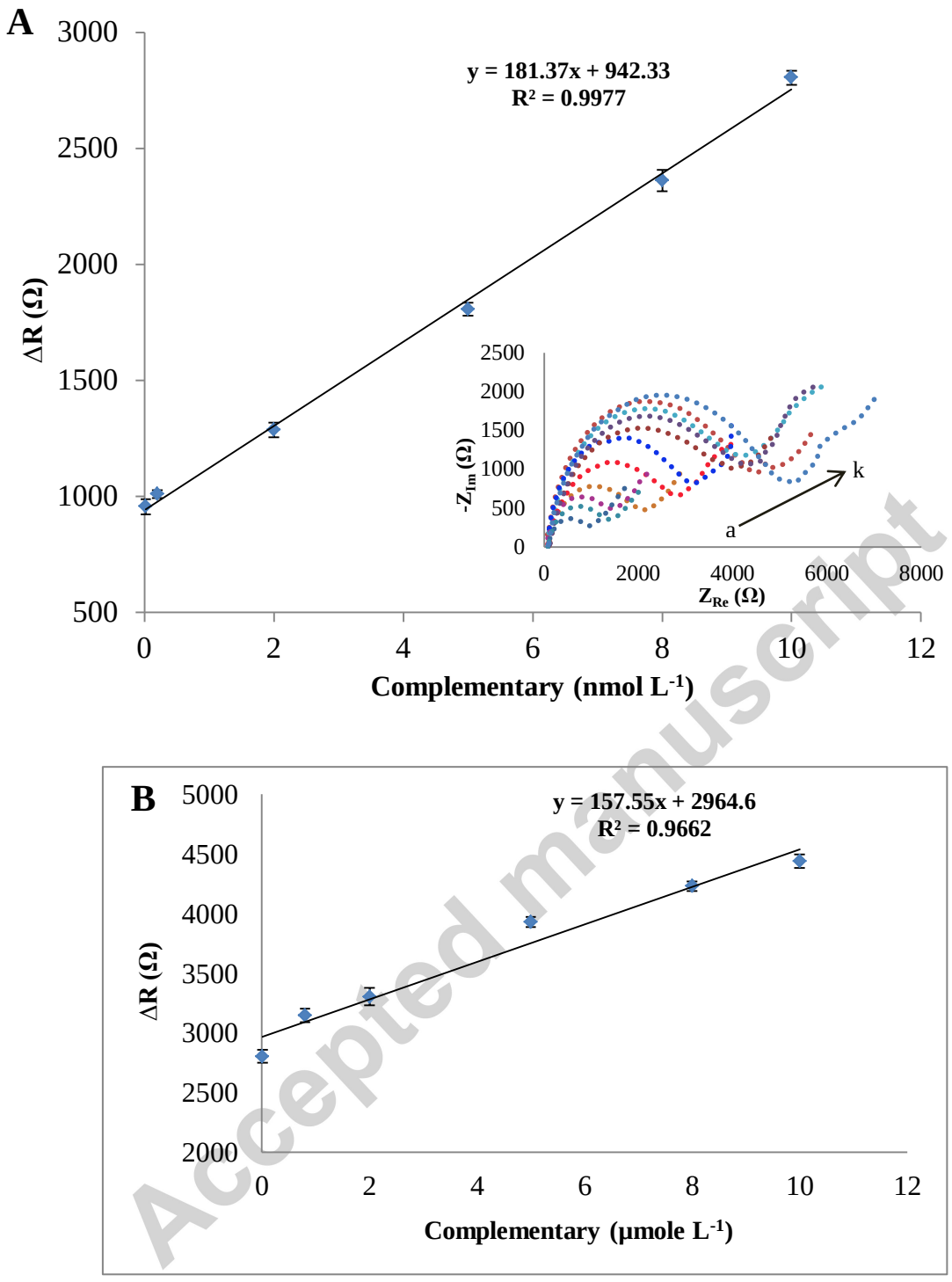


Figure 3

Fig 3



Scheme 1

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