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Point-of-care rapid detection of *Vibrio parahaemolyticus* in seafood using loop-mediated isothermal amplification and graphene-based screen-printed electrochemical sensor

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ABSTRACT

Vibrio parahaemolyticus is one of the most important foodborne pathogens that cause various life-threatening diseases in human and animals. Here, we present a rapid detection platform for *V. parahaemolyticus* by combining loop-mediated isothermal amplification (LAMP) and disposable electrochemical sensors based on screen-printed graphene electrodes (SPGEs). The LAMP reactions using primers targeting *V. parahaemolyticus toxR* gene were optimized at an isothermal temperature of 65 °C, providing specific detection of *V. parahaemolyticus* within 45 min at the detection limit of 0.3 CFU per 25 g of raw seafood. The LAMP amplicons can be effectively detected using unmodified SPGEs, redox active molecules namely Hoechst-33258 and a portable potentiostat. Therefore, the proposed system is particularly suitable as a point-of-care device for on-site detection of foodborne pathogens.

1. Introduction

Gram-negative halophilic bacterium, *Vibrio parahaemolyticus,* commonly found in estuarine and marine environment, is an important foodborne pathogen that can cause devastating outbreaks throughout the world. Consumption of *V. parahaemolyticus*-contaminated seafood, especially raw oyster and shellfish, can result in acute gastroenteritis, a severe diarrheal disease (Butt et al., 2004; Su and Liu, 2007). The traditional cell-culture-based method for detection of *V. parahaemolyticus* described in the US Food and Drug Administration Bacterial Analytical Manual is laborious and time consuming, taking 5–7 days for sample processing (Kaysner and DePaola, 2004). In addition, the method is unable to differentiate *V. parahaemolyticus* from other related *Vibrio* species such as *V. vulnificus* and *V. mimicus* (Su and Liu, 2007). Various molecular-based approaches, including immunological detection and polymerase chain reaction (PCR)-based methods, have been developed for rapid and specific detection of *V. parahaemolyticus* in foods, water and environment. Unfortunately, the operational procedure for most of the developed molecular-based techniques are highly complicated and still require sophisticated infrastructures, expensive reagents, specialized equipment and highly trained personnel, limiting the implementation in field areas (Croci et al., 2007; Dileep et al., 2003; Kim et al., 1999; Rosec et al., 2009).

Loop-mediated isothermal amplification (LAMP), a kind of isothermal deoxyribonucleic acid (DNA) amplification method, is one of the promising approaches for on-site nucleic acid detection due to its high sensitivity, high specificity and simplicity (Notomi et al., 2000). Additionally, the LAMP reagents can be preserved in a lyophilized form

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that does not need to be stored at a very low temperature like polymerase chain reaction (PCR) reagents, making it even more practical for field applications (Klatser et al., 1998). In the past decade, LAMP assays have been optimized to detect various foodborne pathogens such as *Salmonella enterica* (Ohtsuka et al., 2005), *V. parahaemolyticus* (Chen and Ge, 2010; Yamazaki et al., 2008), *V. vulnificus* (Han and Ge, 2008), *V. cholerae* (Srisuk et al., 2008), *Escherichia coli* (Wang et al., 2012). The LAMP products can be detected by many approaches including gel electrophoresis, optical devices or visual inspection (Arunrut et al., 2016; Kiatpathomchai et al., 2008; Sappat et al., 2011; Suebsing et al., 2013a, 2013b). Although these techniques are simple and low cost, their reliability is rather limited due to the subjective interpretation of changes in optical characteristics including the turbidity and color of LAMP solution. Electrochemical biosensor (EC) has recently gained momentum as an alternative potential technology for cell and DNA detections (Shi et al., 2018; Ahmed et al., 2009; Hernández-Santos et al., 2004; Lucarelli et al., 2004).

In particular, the detections of *V. parahaemolyticus* have recently been reported based on some electrochemical methods and electrode materials, mediators. Firstly, *V. parahaemolyticus* cells were amperometrically detected over the concentrations ranging from 7×10^3 to 3×10^4 cells/ml using hydroquinone/benzoquinone mediators and gold screen-printed electrode with immobilized antibody via avidin/biotin linkers, achieving a detection limit of 4×10^2 cells/ml (Laczka et al., 2014). In addition, electro-reduced graphene oxide/TiO_2 nanowires/chitosan/carbon ionic liquid electrodes with immobilized single-stranded DNA operated in differential pulse voltammetry (DPV) using methylene blue (MB) as a mediator could detect *V. parahaemolyticus* DNA in the concentration range of 1.0×10^{-12} to 1.0×10^{-6} M with the detection limit of 3.17×10^{-13} M (Sun et al., 2015). Moreover, electrochemical immunosensors for *V. parahaemolyticus* detection were developed based on faradaycage-type anodic stripping voltammetry combined with antibody (cAb)-immobilized Fe_3O_4 particles/GO-supported silver nanoparticles modified on magnetic glassy carbon electrodes, offering the dynamic range of 10^2 to 10^8 CFU ml^{-1} and the detection limit of 33 CFU ml^{-1} (Song et al., 2017). Additionally, screen-printed carbon electrodes modified with polylactidestabilized gold nanoparticles operated in DPV mode using MB could detect *V. parahaemolyticus* over the concentrations ranging from 2×10^{-8} to 2×10^{-11} M with a low detection limit of 2.16 pM (Nordin et al., 2017). Furthermore, DNA of pork, chicken and bovine meats were identified using a LAMP method and a linear sweep voltammetry (LSV) with a disposable electrochemical printed (DEP) chips and Hoechst 33258 mediator, achieving the detection limits of 20.33 ng/μL (3×10^4 copies/reaction), 78.68 pg/μL (300 copies/reaction), and 23.63 pg/μL (30 copies/reaction) for pork, chicken and bovine species, respectively (Ahmed et al., 2010). It can be seen that the detection performances of these electrochemical biosensors depend significantly on their redox mediators and electrode materials. Redox probes are often required in electrochemical sensing systems to enhance electrochemical signals for detections of biological species, which are usually not highly electroactive. Among them, Hoechst 33258, a popular fluorescent staining dye, is a particularly effective redox probe for DNA detection (Ahmed et al., 2007). The electro-oxidation of Hoechst 33258 will be rapidly quenched causing a reduction of electrochemical current when a double-stranded DNA preferentially binds on Hoechst 33258 (Safavieh et al., 2012).

Concerning electrode materials, graphene, a unique two-dimensional nanocarbon structure, has emerged as a very attractive choice for electrochemical biosensing owing to its high effective surface area, high electron transfer capability, high biocompatibility, good stability and low cost (Pumera, 2011; Sezgintürk, 2016; Robinson et al., 2012; Forsyth et al., 2017; Zhu, 2017). Several works have demonstrated effective biological functionalization on graphene and highly sensitive graphene-based electrochemical biosensors (Wisitsoraat et al., 2017; Chauhan et al., 2017; Ambrosi et al., 2014; Shi et al., 2017a; Cinti and Arduini, 2017; Lawal, 2015; Nurzulaikha et al., 2015). Specifically, many researches have applied graphene-based electrochemical sensing platforms for monitoring various pathogen DNA such as Escherichia coli (Giuliodori et al., 2017; Akbari et al., 2015; Huang et al., 2011), *M. Tuberculosis* (Nguyen et al., 2014; Liu et al., 2014; Zaid et al., 2017) and *Human immunodeficiency virus* − 1 (Safavieh et al., 2017; Y. Wang et al., 2015). Graphene can generally be produced by various approaches including chemical oxidation/reduction (Liu et al., 2014), electrochemical oxidation/reduction (Shi et al., 2017b; Sriprachuabwong et al., 2012), mechanical exfoliation (Hernandez et al., 2008), vapor-phase syntheses (Bhaviripudi et al., 2010), organic syntheses (Cai et al., 2010) and self-assembly (Shi et al., 2014). Among these, electrolytic exfoliation, an electrochemical oxidation process, is particularly effective since it can synthesize high-quality graphene nanosheets with a low oxygen content in a few steps at low cost (Sriprachuabwong et al., 2012).

Hence, it is persuasive to employ graphene-based electrode for electrochemical detection of *V. parahaemolyticus* DNA amplified by LAMP. Graphene-based electrodes can be constructed by various fabrication techniques such as drop coating, inkjet printing, screen-printing and spray coating. Among these, screen-printing technology is highly popular for the production of disposable biosensor electrodes due to the ease of design, high production rate, good reproducibility and low cost (Metters et al., 2011). Presently, carbon screen-printed electrodes (SPCEs) are the most widely used electrochemical electrodes in various applications. However, the performances of SPCEs are quite limited and insufficient for advanced electrochemical applications. Thus, SPCEs have been modified by different materials including graphene by various modification approaches including surface and bulk modifications to improve their electrochemical properties (Ostoji´ et al., 2017). Surface modification methods are simple and more widely used in literature but they are not reliable and suitable for practical use. Recently, SPCEs have been directly modified into screen-printed graphene electrodes (SPGEs) by mixing graphene into carbon paste or replacing carbon particles with graphene. SPGEs have been reported to exhibit superior electrochemical properties including higher electron transfer rate and larger effective electrochemical surface area for various analytes than SPCEs (Brownson and Banks, 2010; Karuwan et al., 2013; Randviir et al., 2014). However, there is no report of the electrochemical detection of *V. parahaemolyticus* DNA using SPGEs without the surface modification. In this work, an integrated point-of-care platform is developed by combining the field-ready lyophilized LAMP method with the electrochemical detection (LAMP-EC) using SPGEs, Hoechst 33258 (H33258) redox probe and a portable potentiostat for rapid detection of *V. parahaemolyticus* in seafood.

2. Material and methods

2.1. Optimization of LAMP reaction

LAMP primers as listed in Table 1 were designed based on the published sequence of the *toxR* gene (GenBank accession no. JF930553) using Primer Explorer version 4 software (Eiken Chemical, Japan). LAMP assay comprised 25 ml of a reaction mixture containing 2 μM FIP, 2 μM BIP, 0.2 μM F3, 0.2 μM B3, 2 μM LF primer, 2 μM LB primer, 1x thermopol-supplied reaction buffer, 0.6 M betaine (USB Corporation, OH, USA), 6 mM $MgSO_4$ (New England Biolabs, MA, USA), 1.4 mM dNTPs (Thermo Fisher Scientific, MA, USA), 8 U *Bst* DNA polymerase (New England Biolabs) and 2 μL of DNA template. Gel electrophoresis (GEP) was used to evaluate the optimal reaction temperature and time.

2.2. Determination of sensitivity and specificity of the LAMP assay

Twenty-six known foodborne pathogens from culture collections as listed in Table 2 were used in this experiment to determine the specificity of the LAMP reactions for the detection of *V. parahaemolyticus*.

Table 1
LAMP primers designed for rapid detection of *V. parahaemolyticus*.

Primers	Sequences (5–3′)	Position
Vp-F3	AGATTGGTGAGTATCAGAACGTA	639–661
Vp-B3	ATACGAGTGGTTGCTGTCATG	841–821
Vp-FIP	GCACTGCTCAATAGAAGGCATTTTCAGTGATGACACCTGTAAATCA	705–724/663–684
Vp-BIP	CACCATGCAGAAGACTCGTTTTTGCTGGTTATTTTGTCCGCC	743–761/803–785
Vp-LF	CCAGTTGTTGATTTGCGG	703–668
Vp-LB	CAGTGGAAGTGATTGCCAC	765–783

Table 2
Panels of bacterial pathogens used for specificity evaluation and detection results.

Number	Bacterial strains	Detection result
1	*Vibrio parahaemolyticus* ATCC 17802 (Target)	+
2	*Vibrio anguillarum* DMST 22082	–
3	*Vibrio cholerae* O1, Inaba DMST 22115	–
4	*Vibrio cholerae* O1, Ogawa DMST 22125	–
5	*Vibrio cholerae* o139, DMST 22135	–
6	*Vibrio cholerae* non-O1, non-O139 DMST 22140	–
7	*Vibrio mimicus* DMST 22089	–
8	*Vibrio vulnificus* DMST 5258	–
9	*Vibrio vulnificus* DABU	–
10	*Vibrio fluvialis* DMST 24049	–
11	*Vibrio fluvialis* DMST 22085	–
12	*Bacillus* sp. ATCC 49342	–
13	*Bacillus cereu* BCC 6386	–
14	*Bacillus subtilis* BCC 6327	–
15	*Clostridium perfringens* ATCC 13124	–
16	*Enterobacter cloacae* Laboratory strain	–
17	*Escherichia coli* O157:H7 ATCC 35150	–
18	*Enterobacter aerogenes* DMST 1333	–
19	*Listeria monocytogenes* ATCC 19115	–
20	*Listeria ivanovii* ATCC 700402	–
21	*Listeria innocua* DMST 9011	–
22	*Listeria welshimeri* DMST 20559	–
23	*Staphylococcus epidermidis* TISTR 518	–
24	*Salmonella typhi* Laboratory strain	–
25	*Salmonella typhimurium* ATCC 13311	–
26	*Salmonella choleraesuis* DMST 5580	–
27	*Salmonella dublin* DMST 30404	–
28	*Salmonella enteritidis* ATCC 13076	–
29	*Salmonella hadar* DMST 10634	–
30	*Salmonella infantis* DMST 26426	–
31	*Salmonella mbandaka* DMST 17377	–
32	*Salmonella senftenberg* DMST 17013	–
33	*Salmonella Virchow* DMST 32758	–
34	*ETEC* Laboratory strain	–
35	*Pseudomonas Aeroginosa* Laboratory strain	–
36	*Shigella sonnei* Laboratory strain	–

The stock suspensions of all bacterial isolates were stored in media containing 20% glycerol at − 80 °C before use. DNA samples were either extracted from cultured bacterial cells, which were grown at 37 °C for 24 h in tryptic soy broth (TSB; Difco, NJ, USA), or from homogenized specimen, which were pre-enriched in alkaline peptone water (APW; HiMedia Laboratories, LLC., Mumbai, India) at 37 °C for 4 h. The pellets from 1 ml of pre-enriched or cultured bacteria were resuspended in 500 L of 25 mM NaOH and boiled at 95 °C for 5 min. Subsequently, the suspensions were neutralized and centrifuged at 15,000 rpm for 5 min. Supernatant was then used as the template for LAMP amplification. The specificity and sensitivity of the LAMP assay were assessed with a 10-fold serial dilution of stock samples. Standard culture methods in various selective media, including thiosulfate-citrate-bile-salt-sucrose (TCBS) agar (HiMedia Laboratories, LLC.) and CHROMagar Vibrio agar (CV; CHROMagar, Paris, France), were also performed for the identification of *V. parahaemolyticus* in different contaminated seafood specimens (Matsumota et al., 2000; Ono et al., 2006).

2.3. Validation of the LAMP assay with seafood samples

For detection of foodborne pathogen in food specimen, selected fresh squids with no foodborne pathogen contamination using the standard culture method in TCBS for 18 h at 37 °C were spiked with a log-phase culture (OD_{600} = 0.4–0.5) of *V. parahaemolyticus*. Spiked seafood was pre-enriched in alkaline peptone water for 2, 4, 6, 8 and 24 h. To evaluate the sensitivity of the technique, the uncontaminated squids were spiked with 2, 5, 10, and 100 CFU *V. parahaemolyticus* in 25 g of seafood. The detection results particularly sensitivity of LAMP-EC were compared with that of the LAMP-GEP and PCR-GEP.

2.4. PCR assay

The DNA extracted from bacterial samples were used as the template for PCR amplification. The sequences of the forward and reverse primers were 5′-GTCTTCTGACGCAATCGTTG-3′ and 5′-ATACGAGTGG TTGCTGTCA-3′, respectively (Kim et al., 1999). The PCR amplification was carried out in a 20 µL reaction mixture containing 10x PCR buffer, 50 mM $MgCl_2$, 10 µM PCR primers, 200 µM dNTPs, 2 U of *Taq* DNA polymerase (Invitrrogen, USA) and different amounts of DNA templates. The following PCR conditions were used: 30 cycles at 94 °C for 30 s, 65 °C 30 s, and 72 °C for 30 s, followed by a final extension at 72 °C for 7 min. The PCR products were analyzed by 2% agarose gel electrophoresis.

2.5. Preparation for dry reagents based (DRB) LAMP

The LAMP reaction mixture contains all reagents similar to that of the conventional LAMP reaction but the LAMP reagents were preserved in a lyophilized form using a standard freeze dryer (Thermo electron corporation). 10% trehalose was added to preserve the enzyme. To determine the shelf life of the DRB-LAMP reagents, all DRB reaction mixtures were checked for activities and reliability after storage at room temperature and − 20 °C up to 8 weeks.

2.6. Fabrication of graphene modified carbon paste electrodes

Graphene nanoparticles (GPNPs) were synthesized using electrolytic exfoliation method as described elsewhere (Karuwan et al., 2012, 2013; Sriprachuabwong et al., 2012). Briefly, two carbon rods (Electron Microscopy Science, PA, USA) were immersed in a polystyrene-sulfonate (PSS) electrolyte solution (Sigma-Aldrich, MO, USA). A constant potential of 8 V was then applied between rods for 15–18 h at room temperature (25–27 °C) to generate low-oxygen GPNPs dispersed in PSS solution. The solution was washed several times in deionized water and ethanol before drying at 100 °C for 12 h. Dried GPNPs were then added to carbon paste at 10% (w/w) before screen-printing onto a clean polyvinylchloride (PVC) substrate (Navanakornplastic Co. Ltd., Pathum Thani, Thailand) using a MK-MINI screen printer (Minami, Japan). The reference electrodes were then formed by screen-printing of Ag/AgCl ink (Gwent Electronic Materials Ltd., Pontypool, UK). Finally, an insulating ink was screen-printed over some part of electrodes in order to define the detection area as shown in Fig. 1. The structural characteristics of GPNPs and SPGEs characterized by transmission electron

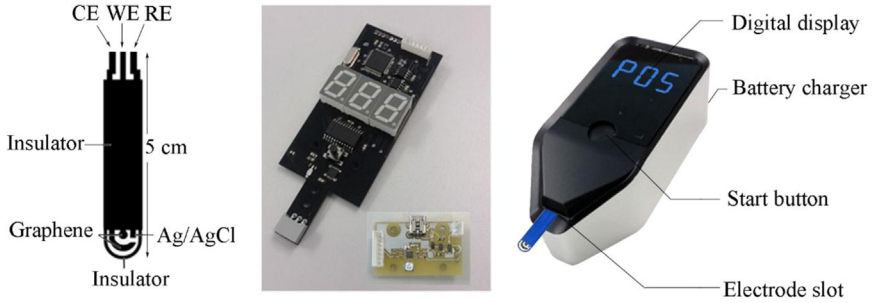

Fig. 1. Design of screen-printed graphene electrode (SGPE) comprising counter electrode (CE), working electrode (WE) and reference electrode (RE) and photographs of printed circuit board (PCB) and housing of portable mini-potentiostat along with a SGPE.

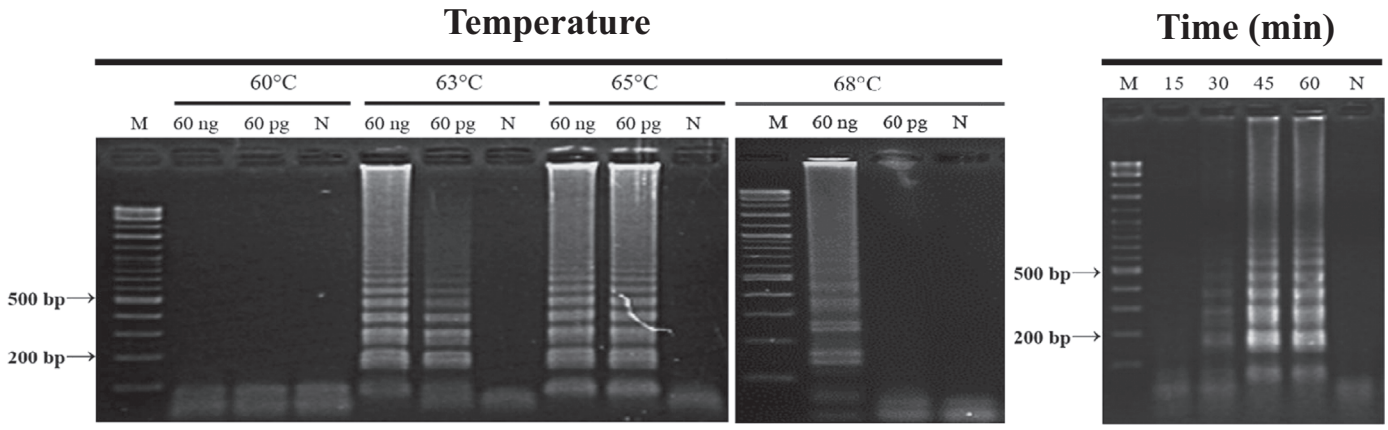

Fig. 2. Gel electrophoresis images of LAMP-amplified DNA samples with four different LAMP temperatures (60, 63, 65 and 68 °C) at 45 min and four different amplification times (15, 30, 45 and 60 min) at 65 °C. Samples: 60 ng and 60 pg of isolated *V. parahaemolyticus* DNA (before amplification), Marker (M: 2-log DNA Ladder Reference) and negative (N: sterile distilled water).

microscopy, field emission scanning electron microscopy, Raman spectroscopy and Fourier-transform infrared spectroscopy were extensively studied previously (Karuwan et al., 2013; Sriprachuabwong et al., 2012).

2.7. Construction of portable mini-potentiostat

A simple and easy-to-use mini-potentiostat was developed for on-site detection of LAMP amplicons. The circuit was designed to be compact with low power consumption and operable with a small battery as shown in Fig. 1. The case of portable mini-potentiostat was prototyped using a 3D printer and then manufactured using a computational numerical control (CNC) machine. The software of mini-potentiostat was developed and written using C programming. Presently, the mini-potentiostat could perform two electrochemical methods including cyclic voltammetry (CV) and chronoamperometry. The parameter settings were the same as those of a standard potentiostat but the potential range and resolution were limited to 0–1 V and 0.05 V, respectively. Additionally, cut-off values for positive and negative decisions could be set from the scanned current responses of known samples. The measurement accuracy and repeatability of the mini-potentiostat were verified by recording the CV of 1 mM NADH for 10 times with the same SPGE electrode using the standard potentiostat and mini-potentiostat as illustrated in the supplementary information. The results confirmed that the mini-potentistat provided repeatable measurements with the relative standard deviation (RSD, n = 10) of 5.3% comparable with 4.6% of the standard potentiostat (AUTOLAB, PGSTAT204). In addition, the mini-potentiostat provided accurate data with the accuracy of more than 92.5% relative to the standard potentiostat. Hence, the mini-potentiostat could directly be used for electrochemical measurements of LAMP amplicons.

2.8. Electrochemical measurement of LAMP amplicons

To detect LAMP amplicons using the developed electrochemical method, the LAMP products were mixed with the redox probe containing 500 μM Hoechst-33258 (2′-(4-hydroxyphenyl) − 5-(4-methyl-1-piperazinyl) − 2,5′-bi(1H-benzimidazole): Sigma-Aldrich, MO, USA) at a ratio of 1:9. The oxidation signals of H33258 before and after interaction with LAMP amplicons were measured using cyclic voltammetry (CV) in the potential range between 0.0 and 800 mV with a step potential and a scan rate of 50 mV and 50 mV/s, respectively. Electrochemical experiments were carried out using the developed mini-potentiostat. The data were analyzed using NOVA 1.10 software package (Metrohm Autolab B.V., Netherland).

3. Results and discussion

3.1. Optimization of LAMP reaction for V. parahaemolyticus

The LAMP reactions were carried out for 60 min at four different temperatures (60, 63, 65 and 68 °C) using 60 pg and 60 ng of DNA extracted from *V. parahaemolyticus* as a template in order to determine the optimum temperature. The amplification results were evaluated using the standard gel electrophoresis as demonstrated in Fig. 2. It is apparent that the samples after LAMP processing at 60 °C display no DNA bands like the negative samples, indicating that LAMP primers are still not active at this temperature. As the temperature increases from 60° to 65 °C, the samples with 60 pg of DNA show increasingly strong DNA bands, demonstrating increasingly effective LAMP reactions. For the samples with 60 ng of DNA, their DNA bands are similarly strong due to the saturation of amplicon density after completed LAMP amplifications with the high starting DNA concentration at both

temperature. However, the DNA bands are fading at the highest LAMP temperature of 68 °C, because LAMP primers become less effective. Thus, the optimal temperature of the LAMP process is 65 °C. To determine the optimum time for the LAMP process, DNA samples were amplified at 65 °C with four different reaction times of 15, 30, 45 and 60 min, respectively. The resulting gel electrophoresis images are also presented in Fig. 2. At the shortest time of 15 min, no DNA band can be observed, indicating that the DNA concentration remains very small. As the time increases from 15 to 45 min, the intensities of DNA bands increase rapidly, confirming the increase of DNA concentration with increasing reaction time. However, the intensities of DNA bands remain approximately the same as the time increases further to 60 min, suggesting that the reaction time of 45 min is sufficient to complete the LAMP reaction.

3.2. Comparison between the sensitivity of LAMP-GEP, PCR-GEP and LAMP-EC

The sensitivity of the LAMP- EC was evaluated in comparison with PCR-GEP and LAMP-GEP using ten-fold serial dilutions of DNA extracted from *V. parahaemolyticus* with varying concentrations from 8 × 10^6 to 8 CFU/ml. The gel electrophoresis images as displayed in Fig. 3 (upper) show that the LAMP samples with the initial DNA contents of more than 8 CFU/ml exhibits strong DNA bands while PCR samples display considerably weaker bands, which can only be observed at the initial DNA concentration of more than 800 CFU/ml. Thus, the LAMP-GEP method offers a low minimum detected concentration of 80 CFU/ml, which is around 100-fold lower than that of PCR-GEP. Therefore, the developed LAMP method is much more effective for amplification of *V. parahaemolyticus* than the standard PCR. The LAMP samples were then detected by the EC methods using the portable potentiostat as demonstrated in Fig. 3 (lower). The inset plot demonstrates the calibration curve displaying the oxidation peak current as a function of *V. parahaemolyticus* concentration. It is seen that the negative sample (0 CFU/ml) exhibits the highest current response with an oxidation peak at ~0.55 V, which corresponds to the oxidation of H33258 (Safavieh et al., 2012). With the addition of DNA at the lowest concentration (8 CFU/ml), the oxidation peak current is moderately lower because some H33258 molecules are bound by *V. parahaemolyticus* DNA, reducing the rate of H33258 oxidation (Ahmed et al., 2007). As the DNA content increases to 80 CFU/ml, the oxidation peak current reduces greatly due to the large enhancement of the DNA density and DNA-bound H33258 complexes. In addition, the peak potential tends to shift towards a lower voltage value with increasing DNA concentration due to the reducing concentration of free H33258 molecules as a lower potential will be needed to drive the mass transport at a lower H33258 concentration (X.

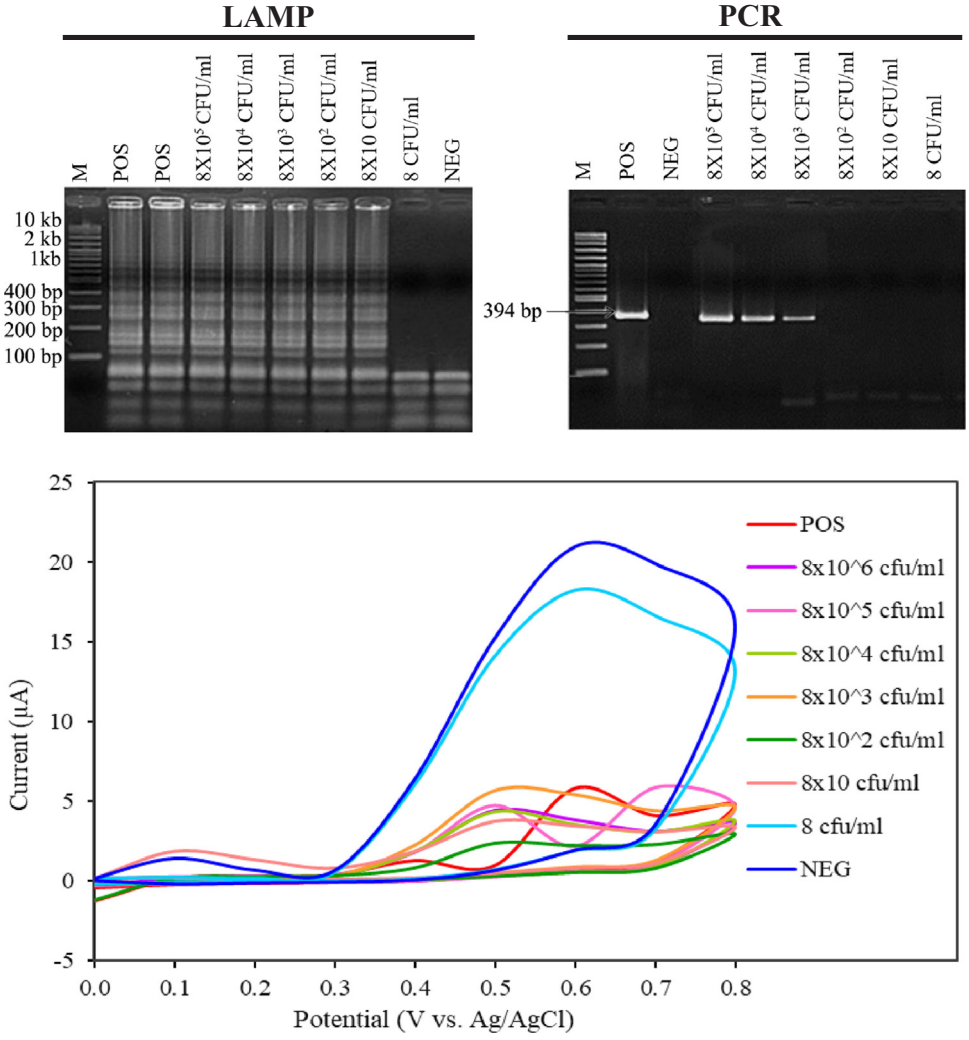

Fig. 3. Gel electrophoresis images (upper) of DNA samples amplified by LAMP at 65 °C for 45 min compared with the standard PCR process and corresponding cyclic voltammograms (lower) measured using SPGEs and the portable mini-potentiostat (Inset: calibration curve with error bars representing variations of 3 SPGEs fabricated in the same batch). Samples: 8, 8 × 10, 8 × 10^2, 8 × 10^3, 8 × 10^4, 8 × 10^5 and 8 × 10^6 CFU/min of *V. parahaemolyticus* DNA (before amplification), Marker (M: 2-log DNA Ladder Reference), positive (POS: 200 ng of DNA extracted from *V. parahaemolyricus* ATCC 17802) and negative (NEG: sterile distilled water).

Wang et al., 2015). However, the oxidation current and peak potential only slightly change as the DNA content increases additionally from 80 to 8 × 10^6 CFU/ml, indicating the saturation of H33258 quenching because most H33258 molecules already bind with the LAMP-amplified DNA at the lower initial DNA concentration of ~80 CFU/ml. Hence, the LAMP-EC method can sensitively measure very low concentrations of *V. parahaemolyticus* DNA in the range of 8–80 CFU/ml. The magnitude of anodic current reduction can be related to the amount of pathogen DNA in the range of 8–80 CFU/ml. In addition, this method provides a lower minimum detected concentration than that of the LAMP-GEP, which is already 100 times better than the PCR-GEP method. The theoretical detection limit (3 S/N) is estimated to be as low as 0.15 CFU/ml. The attained detection limit is much lower than those of other reported biosensors, whose detection limits are higher than 10 CFU/ml (Laczka et al., 2014; Sun et al., 2015; Song et al., 2017; Nordin et al., 2017; Ahmed et al., 2010). Furthermore, the method provides good reproducibility with 5.1% RSD (n = 10 sensors fabricated in the same batch). Additionally, the integration of LAMP with the electrochemical method substantially reduces the measurement time down to a few minutes compared with a few hours of detection using gel electrophoresis or days of conventional culture methods. Besides, the detection results can be reported in a clear and simple format, reducing the need of trained personnel to perform experiment and interpret the data. Moreover, the portability of miniature potentiostat designed in this work makes the method highly useful for field applications including routine interrogation of foodborne pathogen contamination in seafood and on-site monitoring of the pathogen epidemic in a high-risk area.

3.3. The specificity of LAMP reaction

In order to evaluate the specificity of LAMP assay, the panel of 36 bacterial strains as listed in Table 2 were assayed and detected by EC and GEP. The detection results by LAMP-EC and LAMP-GEP in Table 2 show only the positive identification for *V. parahaemolyticus* and negative outcomes for all other bacterial strains. The results confirm that the LAMP assay performed in this study is highly specific to *V. parahaemolyticus*.

3.4. Detection in spiked seafood samples

According to the 2011 Food and Agriculture Organization (FAO)/World Health Organization (WHO) recommendation, the content of the *V. parahaemolyticus* should be less than 10^3 CFU/g in seafood specimen in order to reduce the risk of infectious diseases caused by *V. parahaemolyticus*. To validate the efficacy of the method with real samples, uncontaminated raw squids were used as the blank samples. The specified amounts of *V. parahaemolyticus* ranging from 2 to 100 CFU/25 g were spiked into the raw seafood prior to the test by LAMP-GEP, PCR-GEP and LAMP-EC. The gel-electrophoresis images in Fig. 4 (upper) reveal that the smallest DNA content of 2 CFU/25 g in raw squids can be detected by the LAMP and PCR methods but the LAMP method provides higher sensitivity. The results also indicate that both assays can specifically amplify the LAMP amplicons of *V. parahaemolyticus* since the detection results are not affected by other unidentified substances or chemical contaminants in the seafood. Similarly, the LAMP-EC method using Hoechst 33258 redox probe for EC detection with the portable mini-potentiostat can feasibly detect *V. parahaemolyticus* at the lowest DNA content of 2 CFU/25 g as demonstrated in Fig. 4 (lower). The corresponding calibration curve (inset) shows similar dependence of oxidation peak current on the concentration of *V. parahaemolyticus* in standard solution (Fig. 3) with the rapid decrease of oxidation peak current at low concentrations (0–5 CFU/25 g) and almost steady current values at higher concentrations. In addition, the peak potential also shifts toward lower voltage with increasing concentration due to increasing diffusion mass transport of diminishing free H33258 molecules. The theoretical detection limit (3 S/N) is determined to be as low as 0.3 CFU/25 g, which is much lower than the required detection limit for food safety. The reproducibility of measurement in seafood samples (5.7% RSD, n = 10) is similarly as good as that in the standard solution. The results confirm that the LAMP-EC method is effective for the detection of *V. parahaemolyticus* with negligible interference effects of matrices in raw and processed seafood tested in this work. However, more experiments will be needed to verify the interference effects of matrices in other types of food.

3.5. Storage and the sensitivity of DRB-LAMP

To extend the use of the LAMP as a point-of-care approach, the LAMP reagents were lyophilized using a standard freeze dryer. The addition of 10% trehalose into the freezing solution did help preserve the functionality of the *Bst* DNA polymerase enzymes and all other necessary reagents. To test the storage conditions, the lyophilized reaction mixture was kept for 2 days at room temperature after the freeze-drying process and then stored at − 20 °C or room temperature. It was found that the reagents stored at room temperature led to a decrease in sensitivity at the storage time of more than two weeks, indicating that some of the LAMP reagents even in the lyophilized form were not stable at room temperature. In contrast, the reaction assay kept at − 20 °C offered good detection sensitivity without significant deterioration over 8 weeks. The ability to keep the LAMP assay in a lyophilized form would be very useful for point-of-care applications since it would be easier to be transported to the sites needed for the diagnosis. Therefore, the system not only highlighted the integration of SPGE with LAMP-EC for detection of foodborne pathogen in seafood but also provided a promising platform for cheap, fast and reliable diagnosis of foodborne pathogens.

4. Conclusion

In conclusion, a point-of-care device for rapid detection of *V. parahaemolyticus* was developed based on the integration of DRB-LAMP, SPGE and portable mini-potentiostat. For rapid detection of the bacteria, the LAMP reactions were conducted at an isothermic temperature of 65 °C within 45 min using a simple heating block. Additionally, the LAMP reagents including the enzymes could be freeze-dried, allowing them to be carried to a point-of-care location. Upon dropping the LAMP reaction product to the SPGE, a change in cathodic current caused by intercalating property of redox probe to double-stranded DNA were readily determined by CV, allowing a rapid detection of the LAMP product. The screen-printing of conductive graphene ink could manufacture cheap, simple, sensitive and disposable SPGEs at a high production rate suitable for field applications. A simple portable mini-potentiostat was designed to fit with SPGEs to support on-site LAMP-EC measurement. The LAMP-EC method offered highly sensitive, highly specific and rapid detection of *V. parahaemolyticus* in the raw seafood samples without modification of SPGE surface. In particular, the method could recognize 2 CFU of *V. parahaemolyticus* per 25 g of raw or process seafood satisfying the food safety requirement imposed by FAO/WHO. Therefore, the developed easy-to-use point-of-care diagnostic system would be particularly useful for the determination of pathogen contamination in food and the monitoring of *V. parahaemolyticus* pandemics. Furthermore, the system might be extended using a more complex integration of purification, amplification and detection modules in order to minimize human intervention and reduce potential carry-over contaminations.

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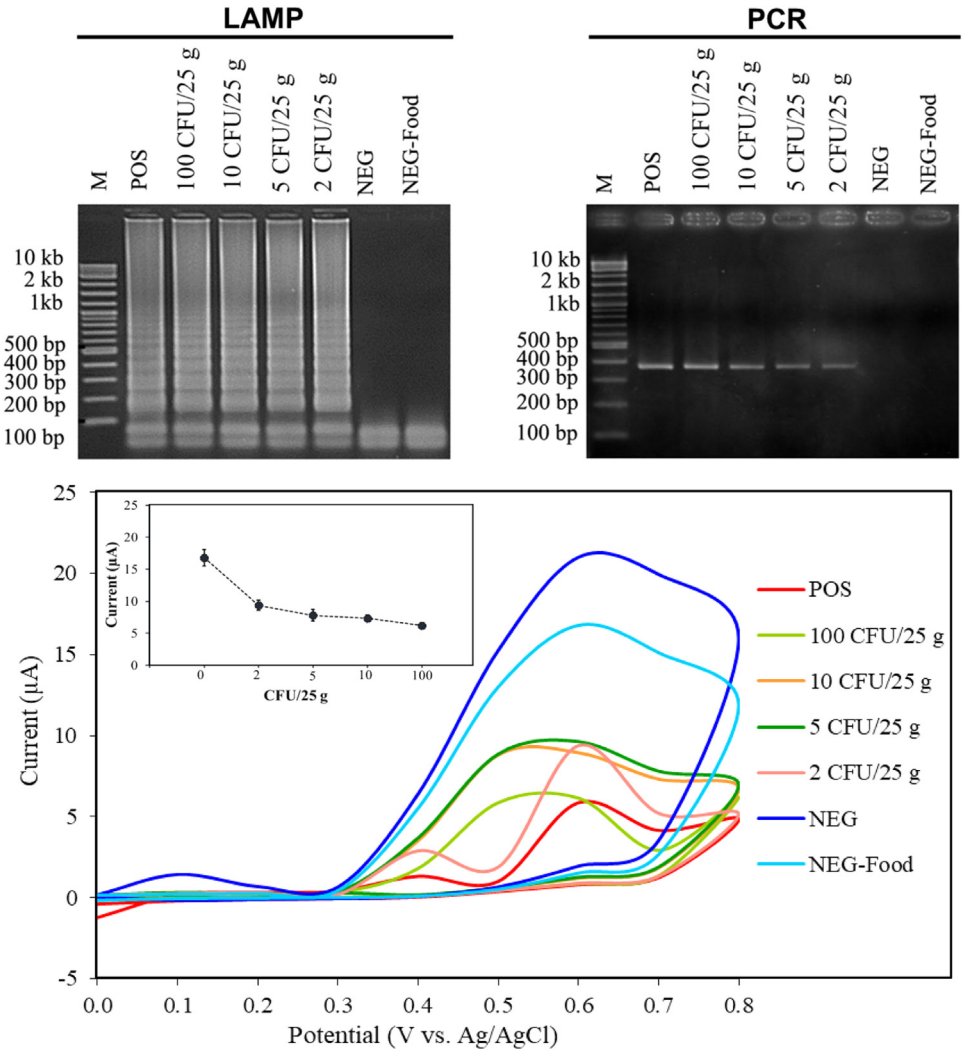

Fig. 4. Gel electrophoresis images (upper) of samples amplified by LAMP at 65 °C for 45 min compared with the standard PCR process and corresponding cyclic voltammograms (lower) measured using SPGEs and the portable mini-potentiostat (Inset: calibration curve with error bars representing variations of 3 SPGEs fabricated in the same batch). Samples: seafood samples spiked with *V. parahaemolyticus* DNA at 2, 5, 10 and 100 CFU/25 g (before amplification), Marker (M: 2-log DNA Ladder Reference), positive (POS: 200 ng of DNA extracted from *V. parahaemolyticus* ATCC 17802), negative (NEG: sterile distilled water) and food negative (NEG-food: seafood with no *V. parahaemolyticus* DNA).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bios.2019.02.060.

References

Ahmed, M.U., Hasan, Q., Hossain, M.M., Saito, M., Tamiya, E., 2010. Food Control 21, 599–605.
Ahmed, M.U., Idegami, K., Chikae, M., Kerman, K., Chaumpluk, P., Yamamura, S., Tamiya, E., 2007. Analyst 132, 431–438.
Ahmed, M.U., Saito, M., Hossain, M.M., Rao, S.R., Furui, S., Hino, A., Takamura, Y., Takagi, M., Tamiya, E., 2009. Analyst 134, 966–972.
Akbari, E., Buntat, Z., Afroozeh, A., Zeinalinezhad, A., Nikoukar, A., 2015. IET Nanobiotechnol. 9, 273–279.
Ambrosi, A., Chua, C.K., Bonanni, A., Pumera, M., 2014. Chem. Rev. 114, 7150–7188.
Arunrut, N., Kampeera, J., Sirithammajak, S., Sanguanrut, P., Proespraiwong, P., Suebsing, R., Kiatpathomchai, W., 2016. PLoS One 11 (3), e0151769.
Bhaviripudi, S., Jia, X., Dresselhaus, M.S., Kong, J., 2010. Nano Lett. 10 (10), 4128–4133.
Butt, A.A., Aldridge, K.E., Sanders, C.V., 2004. Lancet Infect. Dis. 4, 201–212.
Brownson, D.A.C., Banks, C.E., 2010. Analyst 135, 2768–2778.
Cai, J., Ruffieux, P., Jaafar, R., Bieri, M., Braun, T., Blankenburg, S., Muoth, M., Seitsonen, A.P., Saleh, M., Feng, X., Mllen, K., Fasel, R., 2010. Nature 466 (7305), 470–473.
Chauhan, N., Maekawa, T., Nair, D., Kumar, S., 2017. J. Mater. Res. 32, 2860–2882.
Chen, S., Ge, B., 2010. BMC Microbiol. 10, 41.
Cinti, S., Arduini, F., 2017. Biosens. Bioelectron. 89, 107–122.
Croci, L., Suffredini, E., Cozzi, L., Toti, L., Ottaviani, D., Pruzzo, C., Serratore, P., Fishetti, R., Goffredo, E., Loffredo, G., Mioni, R., the Vibrio parahaemolyticus working, 2007. J. Appl. Microbiol. 102, 229–237.
Dileep, V., Kumar, H.S., Kumar, Y., Nishibuchi, M., Karunasagar, I., Karunasagar, I., 2003. Lancet Infect. Dis. 36, 423–427.
Forsyth, R., Devadoss, A., Guy, O.J., 2017. Diagnostics 7, E45.
Giuliodori, A.M., Brandi, A., Perrozzi, F., Ottaviano, L., Spurio, R., Fabbretti, A., 2017. PLoS One 12, e0183952.
Han, F., Ge, B., 2008. Foodborne Pathog. Dis. 5, 311–320.
Hernández-Santos, D., Diaz-González, M., González-Garcia, M.B., Costa-Garcia, A., 2004. Anal. Chem. 76, 6887–6893.
Hernandez, Y., Nicolosi, V., Lotya, M., Blighe, F.M., Sun, Z., De, S., McGovern, I.T., Holland, B., Byrne, M., Gun'ko, Y.K., Boland, J.J., Niraj, P., Duesberg, G., Krishnamurthy, S., Goodhue, R., Hutchison, J., Scardaci, V., Ferrari, A.C., Coleman, J.N., 2008. Nat. Nanotechnol. 3 (9), 563–568.
Huang, Y., Dong, X., Liu, Y., Li, L.-J., Chen, P., 2011. J. Mater. Chem. 21, 12358–12362.
Karuwan, C., Sriprachuabwong, C., Wisitsoraat, A., Phokharatkul, D., Sritongkham, P., Tuantranont, A., 2012. Sens. Actuators B Chem. 161, 549–555.
Karuwan, C., Wisitsoraat, A., Phokharatkul, D., Sriprachuabwong, C., Lomas, T., Nacapricha, D., Tuantranon, A., 2013. RSC Adv. 3, 25792–25799.
Kaysner, C.A., DePaola, A., 2004. Chapter 9: Vibrio in Bacteriological Analytical Manual, 8th ed. Revision A, U.S. Food and Drug Administration, Gaithersburg, MD, USA.
Kiatpathomchai, W., Jaroenram, W., Arunrut, N., Jitrapakdee, S., Flegel, T.W., 2008. J. Appl. Microbiol. 153, 214–217.
Kim, Y.B., Okuda, J., Matsumoto, C., Takahashi, N., Hashimoto, S., Nishibuchi, M., 1999. J. Clin. Microbiol. 37, 1173–1177.
Klatser, P.R., Kuijper, S., Van, I., Kolk, A.H.J., 1998. J. Clin. Microbiol. 36, 1798–1800.
Laczka, O., Labbate, M., Doblin, M., 2014. Anal. Methods 6, 2020–2023.
Lawal, A.T., 2015. Talanta 131, 424–443.
Liu, C., Jiang, D., Xiang, G., Liu, L., Liu, F., Pu, X., 2014. Analyst 139, 5460–5465.
Lucarelli, F., Marrazza, G., Turner, A.P.F., Mascini, M., 2004. Biosens. Bioelectron. 19, 515–530.
Matsumota, C., Okuda, J., Ishibashi, M., Iwanaga, M., Garg, P., Rammamurthy, T., Wong, H.C., Depaola, A., Kim, Y.B., Albert, M.J., Nishibuchi, M., 2000. J. Appl. Microbiol.

38, 578–585.
Metters, J.P., Kadara, R.O., Banks, C.E., 2011. Analyst 136, 1067–1076.
Nguyen, L.H., Nguyen, T.D., Tran, V.H., Dang, T.T.H., Tran, D.L., 2014. Adv. Nat. Sci.: Nanosci. Nanotechnol. 5, 035005.
Nordin, N., Yusof, N.A., Abdullah, J., Radu, S., Hushiarian, R., 2017. AMB Expr. 7, 41.
Nurzulaikha, R., Lim, H.N., Harrison, I., Lim, S.S., Pandikumar, A., Huang, N.M., Lim, S.P., Thien, G.S.H., Yusoff, N., Ibrahim, I., 2015. Sens. Bio-Sens. Res. 5, 42–49.
Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., Hase, T., 2000. Nucleic Acids Res. 28, E63.
Ohtsuka, K., Yanagawa, K., Takatori, K., Hara-Kudo, Y., 2005. Appl. Environ. Microbiol. 71, 6730–6735.
Ono, T., Park, K.S., Ueta, M., Iida, T., Honda, T., 2006. Infect. Immun. 74, 1032–1042.
Ostoji´, J., Herenda, S., Beši´, Z., Miloš, M., Gali´, B., 2017. Molecules 22, 1120.
Pumera, M., 2011. Mater. Today 14, 308–315.
Randviir, E.P., Brownson, D.A.C., Metters, J.P., Kadara, R.O., Banks, C.E., 2014. Phys. Chem. Chem. Phys. 16, 4598–4611.
Robinson, N.D., Edman, L., Chhowalla, M., 2012. Phys. Scr. T146, 014023.
Rosec, J.P., Simon, M., Causse, V., Boudjemaa, M., 2009. Int. J. Food Microbiol. 129, 136–145.
Safavieh, M., Ahmed, M.U., Tolba, M., Zourob, M., 2012. Biosens. Bioelectron. 31, 523–528.
Safavieh, M., Kaul, V., Khetani, S., Singh, A., Dhingra, K., Kanakasabapathy, M.K., Draz, M.S., Memic, A., Kuritzkes, D.R., Shafiee, H., 2017. Nanoscale 9, 1852–1861.
Sappat, A., Jaroenram, W., Puthawibool, T., Lomas, T., Tuantranont, A., Kiatpathomchai, W., 2011. J. Virol. Methods 175, 141–148.
Sezgintürk, M.K., 2016. TrAC Trends Anal. Chem. 76, 1–14.
Shi, L., Rong, X., Wang, Y., Ding, S., Tang, W., 2018. Biosens. Bioelectron. 102, 41–48.
Shi, L., Wang, Y., Ding, S., Chu, Z., Yin, Y., Jiang, D., Luo, J., 2017a. Biosens. Bioelectron. 89, 871–879.
Shi, L., Li, Y., Rong, X., Wang, Y., Ding, S., 2017b. Anal. Chim. Acta 968, 21–29.
Shi, L., Chu, Z., Liu, Y., Jin, W., Xu, N., 2014. Adv. Funct. Mater. 24, 7032–7041.
Song, X., Xv, L., Wu, L., Hu, Y., Wang, S., Guo, Z., Su, X., 2017. J. Electrochem. Soc. 164, B704–B709.
Sriprachuabwong, C., Karuwan, C., Wisitsorrat, A., Phokharatkul, D., Lomas, T., Sritongkham, P., Tuantranont, A., 2012. J. Mater. Chem. 22, 5478.
Srisuk, C., Chaivisuthangkura, P., Rukpratanporn, S., Longyant, S., Sridulyakul, P., Sithigorngul, P., 2008. Lett. Appl. Microbiol 50, 36–42.
Su, Y.C., Liu, C., 2007. Food Microbiol. 24, 549–558.
Suebsing, R., Prombun, P., Kiatpathomchai, W., 2013a. Lett. Appl. Microbiol. 56, 428–435.
Suebsing, R., Kampeera, J., Tookdee, B., Withyachumnarnkul, B., Turner, W., Kiatpathomchai, W., 2013b. Lett. Appl. Microbiol. 57, 317–324.
Wang, F., Jiang, L., Ge, B., 2012. J. Clin. Microbiol. 50, 91–97.
Wang, Y., Bai, X., Wen, W., Zhang, X., Wang, S., 2015a. ACS Appl. Mater. Interfaces 7, 18872–18879.
Wang, X., Li, G., Liu, L., Cheng, Y., Zheng, W., Wu, S., Wu, F., Sun, W., 2015b. Anal. Methods 7, 2623–2629.
Wisitsoraat, A., Mensing, J.P., Karuwan, C., Sriprachuabwong, C., Jaruwongrungsee, K., Phokharatkul, D., Daniels, T.M., Liewhiran, C., Tuantranont, A., 2017. Biosens. Bioelectron. 87, 7–17.
Yamazaki, W., Seto, K., Taguchi, M., Ishibashi, M., Inoue, K., 2008. BMC Microbiol. 8, 94.
Zaid, M.H.M., Abdullah, J., Yusof, N.A., Sulaiman, Y., Wasoh, H., Noh, M.F.M., Issa, R., 2017. Sens. Actuators B 241, 1024–1034.
Zhu, Z., 2017. Nano-Micro Lett. 9, 1–25.