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Enzymatic electrochemical detection of epidemic-causing *Vibrio cholerae* with a disposable oligonucleotide-modified screen-printed biosensor coupled to a dry-reagent-based nucleic acid amplification assay

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ABSTRACT

In this study, we developed a nucleic acid-sensing platform in which a simple, dry-reagent-based nucleic acid amplification assay is combined with a portable multiplex electrochemical genosensor. Preparation of an amplification reaction mix targeting multiple DNA regions of interest is greatly simplified because the lyophilized reagents need only be reconstituted with ultrapure water before the DNA sample is added. The presence of single or multiple target DNAs causes the corresponding single-stranded DNA (ssDNA) amplicons to be generated and tagged with a fluorescein label. The fluorescein-labeled ssDNA amplicons are then analyzed using capture probe-modified screen-printed gold electrode bisensors. Enzymatic amplification of the hybridization event is achieved through the catalytic production of electroactive α-naphthol by anti-fluorescein-conjugated alkaline phosphatase. The applicability of this platform as a diagnostic tool is demonstrated with the detection of toxigenic *Vibrio cholerae* serogroups O1 and O139, which are associated with cholera epidemics and pandemics. The platform showed excellent diagnostic sensitivity and specificity (100%) when challenged with 168 spiked stool samples. The limit of detection was low (10 colony-forming units/ml) for both toxigenic *V. cholerae* serogroups. A heat stability assay revealed that the dry-reagent amplification reaction mix was stable at temperatures of 4–56 °C, with an estimated shelf life of seven months. The findings of this study highlight the potential of combining a dry-reagent-based nucleic acid amplification assay with an electrochemical genosensor in a more convenient, sensitive, and sequence-specific detection strategy for multiple target nucleic acids.

1. Introduction

Infectious diseases impose tremendous health, social, and economic burdens by claiming millions of lives annually and by reducing the quality of life of those with chronic disabilities as a consequence of the disease (Peeling and Mabey, 2010). Therefore, evidence-based diagnoses are crucial for ensuring that afflicted individuals benefit from an appropriate treatment and for preventing the devastating sequelae of diseases. The appropriate use of diagnostic tools will also improve disease detection, reduce disease transmission and avert the emergence of drug-resistant pathogen strains by preventing overtreatment (Mabey et al., 2004). Generally, culture-based methods with lengthy incubation periods are used to isolate etiological agents and life-saving treatments could be even further delayed by the need for additional microscopic, biochemical, and/or serological tests to identify the microorganism (Yager et al., 2008).

The advent of molecular diagnostics has paved the way for more rapid, sensitive, and specific means to detect infectious agents. PCR and its derivatives are the most widely used techniques in nucleic acid-based diagnostics and the application of PCR has already been extended to various fields, ranging from pharmacogenomics and nutrigenomics to biosecurity (Mousumi et al., 2010). PCR is very useful because it allows the simultaneous amplification of several targets in a multiplex format and any potential interference from various nontarget sequences can also be reduced by increasing the signal-to-noise ratio during nucleic acid amplification (Miranda-Castro et al., 2009). However, the post-amplification analysis of PCR amplicons is typically performed with gel electrophoresis, which can generate ambiguous results

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because the separation of DNA fragments is size dependent and not sequence specific.

The limitations of gel electrophoresis have driven the growth of DNA biosensors, with which nucleic acids are rapidly detected via various types of interaction and strategies (Pedrero et al., 2011). A typical biosensor consists of a biorecognition interface and either an optical, electrochemical, or piezoelectrical transducer that converts the recognition event into a useful signal. When oligonucleotide capture probes are immobilized on the biorecognition interface and DNA base-pair recognition are converted into electrical signals, the biosensor is classified as an electrochemical genosensor (Lucarelli et al., 2004). Among the different types of genosensors, the electrochemical genosensor is highly favored because it is compatible with microfabrication technology, which allows a small, simple, compact, low-cost, hand-held device to be produced for point-of-care diagnostic use (Wang, 2006).

In recent years, numerous PCR-coupled electrochemical genosensors have been developed to detect infectious agents, including hepatitis B virus (Erdem et al., 2005), *Salmonella enteric* serovar Typhimurium (Lai et al., 2006), human cytomegalovirus (Djellouli et al., 2007), and *Mycobacterium tuberculosis* (Zhang et al., 2012). The functionality of these electrochemical genosensors is usually established with short synthetic oligonucleotides, and the actual performance of these genosensors with real clinical specimens containing complex biological molecules or repertoires of non-target nucleic acid sequences has not been explored in depth (Pedrero et al., 2011). These PCR-coupled electrochemical genosensors are also susceptible to false negative results because no internal amplification control (IAC) is included in the assay. The IAC plays an important role in all molecular amplification assays because the presence of amplification inhibitors, or technical errors, such as faulty equipment, poor enzyme activity, and inappropriate preparation of the reaction mix, can also generate negative results, similar to the absence of the target DNA (Hoorfar et al., 2004). Therefore, the end-user is unable to discern a true from a false negative result when no IAC is incorporated in the assay.

Previously, we described a novel electrochemical genosensor for the detection of single-stranded amplicons generated with a multiplex linear-after-the-exponential PCR (mLATE-PCR) (Yu et al., 2013). The mLATE-PCR-coupled electrochemical genosensor demonstrated the feasibility of combining two platforms with a multiplexing capacity to sequence-specifically detect multiple target DNAs. However, preparation of the amplification reaction mix involves tedious pipetting steps because multiple pairs of primers must be added to the standard PCR reagents. The risk of contamination and the probability of human error also increase with the increasing number of reagents and pipetting steps involved. All the PCR reagents require cold-chain storage and transportation, entailing a dependence on refrigeration and an electricity supply.

In the present study, the drawbacks of the conventional preparation of an amplification reaction are addressed with the development of a dry-reagent mLATE-PCR mix that is compatible with a downstream detection method using an electrochemical genosensor. The dry-reagent mLATE-PCR mix simplifies the whole workflow of the amplification reaction mix preparation into a two-step assay to which ultrapure water and the DNA template are added. The electrochemical genosensor is further optimized to directly detect the target DNAs with no prior purification of the mLATE-PCR amplicons. The successful combination of these two technologies with multiplexing and miniaturization capacities advances our efforts to increase the accessibility of nucleic acid testing in different types of healthcare settings.

2. Materials and methods

2.1. Reagents and apparatus

The PCR reagents were purchased from Thermo Scientific (Massachusetts, USA) and the oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, USA). The sequences of the oligonucleotides are shown in Table S1. Western blocking reagent was from Roche (Indianapolis, USA). Potassium ferricyanide ($[Fe(CN)_6]^{3-}$), potassium ferrocyanide ($[Fe(CN)_6]^{4-}$), trehalose and raffinose were from Merck (Darmstadt, Germany). Ficoll, bovine serum albumin (BSA), polyethylene glycol (PEG), 6-mercapto-1-hexanol (MCH), diethanolamine (DEA), anti-fluorescein-antibody conjugated to alkaline phosphatase (anti-FITC-ALP), α-naphthyl phosphate (α-NP), and other reagents were from Sigma-Aldrich (Missouri, USA). In all experiments, ultrapure water (>18 MΩ) from a Millipore Milli-Q Water Purification System (Billerica, USA) was used.

The Amicon Ultra-0.5 Centrifugal Unit (30 kDa) was purchased from Millipore, and the NucleoSpin Tissue Kit was from Macherey-Nagel (Düren, Germany). PCR amplification was performed in an Eppendorf Mastercycler (Hamburg, Germany). Samples were lyophilized with a Thermo Scientific Heto Vacuum Concentrator connected to a Heto Lyolab 3000 freeze dryer. Disposable screen-printed gold electrode bisensors (SPGEB) containing two gold working electrodes surrounded by a common gold counter and silver reference electrode were custom-made by DropSens (Asturias, Spain). All electrochemical measurements were made with a portable bipotentiostat, μStat 200, from DropSens and controlled with the DropView 2.2 software.

2.2. Biomodification of SPGEB

The sensing layers on the gold working electrodes were functionalized with mixed self-assembled monolayers (SAM) of capture probes and MCH, as described previously (Yu et al., 2013). Briefly, the SPGEBs were cleaned by cycling the potential between 0 to $+1.25$ V at a 100 mV/s scan rate in 0.1 M sulfuric acid. Each thiol-modified capture probe (1 μM) was then immobilized on a different gold working electrode for 1 h, and then incubated with 10 μM MCH for 1 h. The biomodified working electrodes were assessed with cyclic voltammetry using a 10 mM $[Fe(CN)_6]^{3-/4-}$ redox couple.

2.3. Deglycerolization of Taq DNA polymerase

The glycerol in the storage buffer of *Taq* DNA polymerase was removed by centrifuging 100 U of *Taq* DNA polymerase in a centrifugal filter unit at 14,000*g* for 10 min. The flow-through was discarded and a washing step was performed with 10 mM Tris–HCl (pH 8). The retentate was recovered by placing the filter unit upside down in a new collection tube before centrifugation at 1000*g* for 2 min. The optimal amount of deglycerolized *Taq* DNA polymerase in the dry-reagent mLATE-PCR preparation was determined empirically.

2.4. Preparation of the dry-reagent mLATE-PCR mix

The mLATE-PCR mix containing *Taq* buffer, 4.5 mM $MgCl_2$, 300 nM LaO1-FX, 15 nM LaO1-RL, 300 nM LaCT-FX, 60 nM LaCT-RL, 300 nM LaO139-FX, 15 nM LaO139-RL, 500 nM IAC-FX, 25 nM IAC-RL, 320 μM dNTP mix, 1.5 U of deglycerolized *Taq* DNA polymerase, 50 pg of IAC template, 5% (w/v) raffinose, 0.25 mg/ml BSA, and 0.1 mg/ml Orange G dye in a total volume of 20 μl per tube was lyophilized at $5–8\times10^{-2}$ mBar and -50 °C for 2 h. All amplification tubes containing the dry-reagent mLATE-PCR mix were

stored in a desiccator or sealed in aluminum pouches with silica gel for long-term storage.

2.5. Dry-reagent mLATE-PCR assay

The dry-reagent mLATE-PCR amplification reaction mix was reconstituted by rehydrating the lyophilized pellet with 18 μl of ultrapure water before the addition of 2 μl of the DNA template. The thermocycling conditions for the amplification reaction were: initial denaturation at 95 °C for 5 min, 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 10 min.

2.6. Electrochemical detection of mLATE-PCR amplicons

The unpurified mLATE-PCR amplicons were analyzed by adding an equal volume of 12 × saline sodium citrate (SSC) buffer (pH 7) to the amplification product before the mixture was loaded onto the capture probe-modified SPGEBs. After incubation at room temperature for 30 min, the capture probe–amplicon hybrids were enzymatically labeled for 15 min with 2.5 U/ml anti-FITC-ALP in DEA buffer (0.1 M, pH 9.6) containing 0.5% (v/v) western blocking reagent. After incubation for 30 s with 10 mM α-NP substrate, the presence of electroactive α-naphthol was detected amperometrically by applying a constant potential at 0.25 V for 120 s.

2.7. Diagnostic evaluation with spiked stool samples

Spiked stool samples were prepared as described by McOrist et al. (2002), with slight modification. Briefly, 10 g of stool was suspended in 90 ml of peptone water and vortexed vigorously. Any remaining solid matter was removed by centrifugation at 65*g* for 5 min, and the supernatant was subjected to another centrifugation step at 12,000*g* for 5 min. The pellet was washed twice with 25 ml of TE buffer (10 mM Tris, pH 8, containing 1 mM EDTA) and finally resuspended in 100 ml of TE buffer. The suspension was divided into 200 μl aliquots and each aliquot was spiked with a single colony of the bacterial strain to be tested. Alkaline peptone water (10 ml) was then inoculated with the spiked stool samples and incubated at 37 °C for 6 h. The boiling-lysis method was used to prepare the cell lysates for use as DNA templates in the dry-reagent mLATE-PCR assay. The bacterial strains used for diagnostic evaluation are listed in Table S2.

3. Results and discussion

3.1. Design strategy

A schematic illustration of the genosensing platform is shown in Fig. 1. A simple nucleic acid amplification reaction was performed by rehydrating the dry-reagent mLATE-PCR mix before adding the DNA template. LATE-PCR was selected as the nucleic acid amplification scheme because multiple DNAs of interest can be targeted for amplification in a single reaction by using specific primer pairs, and the single-stranded amplicons can be simultaneously labeled using hapten-modified primers. In this study, all forward excess primers were modified with fluorescein, so that the resulting single-stranded amplicons were labeled at the 5′ end with fluorescein during the amplification process. The sequence-specific detection of the amplicons was based on the hybridization reaction between the complementary target DNAs and the capture probes immobilized on the working electrodes of the SPGEB. The capture probe–amplicon hybrids were enzymatically labeled by the addition of the anti-FITC-ALP conjugate, which binds to the fluorescein tag on the single-stranded DNA (ssDNA) amplicons and amplifies the hybridization signals by converting electroinactive α-NP into electroactive α-naphthol. The production of α-naphthol is then measured amperometrically with a portable bipotentiostat. The use of disposable SPGEBs prevents carry-over contamination and circumvents the need for a bulky electrochemical cell and electrode surface regeneration with tedious mechanical or electrochemical methods.

3.2. Biomodification of SPGEB

The spontaneous end-point attachment of the capture probes to the gold working electrodes was achieved through a self-

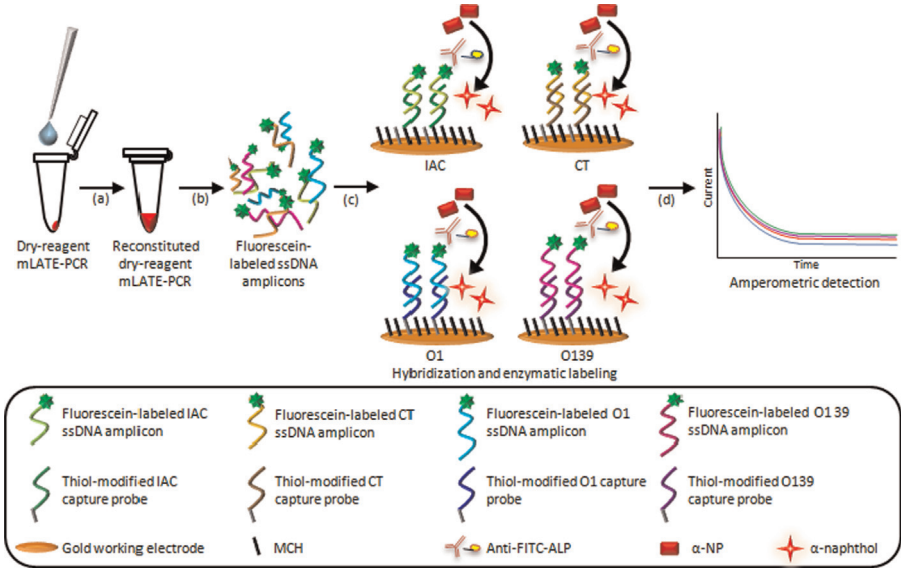

Fig. 1. Schematic illustration of the procedures involved in nucleic acid detection with a dry-reagent mLATE-PCR-coupled multiplex electrochemical genosensor; (a) two-step protocol for setting up the mLATE-PCR mix by adding ultrapure water and the DNA template; (b) generation of fluorescein-labeled ssDNA amplicons during amplification; (c) hybridization between ssDNA amplicons and complementary capture probes that have been self-assembled on the gold working electrodes followed by binding of anti-FITC-ALP to fluorescein-labeled ssDNA amplicons and conversion of electroinactive α-NP into electroactive α-naphthol by ALP; and (d) amperometric detection of α-naphthol at a working potential of 0.25 V.

assembly process known as SAM. SAM exploits the strong affinity of thiols for gold surfaces, which has been equated to the strength of covalent bonding (Lucarelli et al., 2004). The subsequent introduction of MCH to form a mixed SAM further increases the hybridization efficiency by removing weakly adsorbed DNA bases from the gold surface and by improving the accessibility of the capture probe through mutual electrostatic repulsion (Peterson et al., 2001). The optimal composition of the mixed SAM to enhance the efficiency of the hybridization reaction was investigated by varying the immobilization times of the capture probes (5 min to 2 h) and MCH (30 min to 2 h). The optimal immobilization time for both the capture probes and MCH was 1 h based on the best signal-to-background (S/B) ratios (Fig. S1). The successful formation of a sensing layer with mixed SAM was demonstrated by the cyclic voltammograms that were generated in the presence of the $[Fe(CN)_6]^{3-/4-}$ redox couple (Fig. S2). A progressive reduction in reversibility was seen in the cyclic voltammograms generated after the immobilization of the capture probes and MCH compared with that of the bare gold electrodes as the peak currents decreased and the peak potential separation increased. The changes in the cyclic voltammogram were attributable to the phosphate backbones of the DNA capture probes and the hydroxyl group of MCH, which caused the electrode surface to become negatively charged, resulting in the repulsion of the $[Fe(CN)_6]^{3-/4-}$ redox couple. Therefore, the rapid formation of a sensing layer with mixed SAM on SPGEB was achieved without the need for complicated procedures.

3.3. mLATE-PCR for the detection of toxigenic V. cholerae O1 and O139

Vibrio cholerae is the etiological agent of cholera, a disease characterized by the massive secretion of “rice watery” stools, and when left untreated, severe cases are associated with dehydration, hypovolemic shock, and death (Sack et al., 2004). Cholera is endemic in several regions of the world, especially Africa, and cholera outbreaks are increasing in both their frequency and severity (Ryan, 2011). Therefore, important determinants of *V. cholerae* isolates, especially their epidemic potential, were considered when the present genosensing platform was developed, using *V. cholerae* as the model organism. The two serogroups of *V. cholerae* that are exclusively associated with pandemic cholera can be distinguished by inherent differences in their genes involved in O-antigen biosynthesis (Bik et al., 1995). Therefore, primers targeting the *wbeM* and *wbfR* genes were designed to identify *V. cholerae* O1 and O139, respectively. Primers targeting the conserved *ctxA* gene, which encodes the cholera toxin (CT) A subunit, were included to detect the principal virulence factor in toxigenic *V. cholerae* strains. All LATE-PCR primers were designed according to the criteria described by Pierce et al. (2005). A noncompetitive IAC primer pair flanking a 112 bp region of the thrombospondin-related adhesive protein gene of *Plasmodium falciparum* (Ang et al., 2012) was incorporated into the mLATE-PCR assay to rule out false negative results.

3.4. Preparation of dry-reagent mLATE-PCR mix

The two integral factors in developing a dry-reagent mLATE-PCR mix were investigated, the amount of deglycerolized *Taq* DNA polymerase included and the stabilizer formulation. Hygroscopic glycerol was first removed from the enzyme by centrifugal filtration and varying amounts of deglycerolized *Taq* DNA polymerase (0.75–2 U) were added to the mLATE-PCR mix to determine the optimal amount required to maintain the amplification efficiency after lyophilization. A percentage recovery of more than 90% was only achieved with a lyophilized mLATE-PCR mix containing 1.5 U or 2 U of deglycerolized *Taq* DNA polymerase when the signals were compared with those of a conventionally prepared mLATE-PCR mix containing 0.75 U of *Taq* DNA polymerase (Fig. 2A). The incorporation of 0.75 U or 1 U of deglycerolized *Taq* DNA polymerase in the lyophilized mLATE-PCR mix resulted in a dismal percentage recovery of less than 70%. This loss of *Taq* DNA polymerase functionality may be attributable to the lyophilization-induced denaturation of the protein. Therefore, 1.5 U of deglycerolized *Taq* DNA polymerase was selected to ensure that sufficient *Taq* DNA polymerase activity was retained after the lyophilization process.

The thermostabilizing effects of trehalose and raffinose, which undergo vitrification during lyophilization, were tested by incorporating 1–10% (w/v) of each stabilizing sugar into the mLATE-PCR mix before lyophilization. Different amounts of stabilizing sugar were tested because an insufficient amount of stabilizer would not confer the necessary protection against lyophilization and storage-related stresses, such as freezing, desiccation, dehydration, and heat, whereas excessive stabilizer would reduce the storage stability of the lyophilizate by increasing the risk of sugar crystallization (Carpenter et al., 2002). The lyophilized mLATE-PCR mix was subjected to thermal stress by storing it at an elevated temperature of 56 °C to allow the rapid assessment of the protective effect of each stabilizer. After storage at 56 °C for one day, only the lyophilized mLATE-PCR mix containing 3% (w/v) trehalose and 1–5% (w/v) raffinose retained more than 90% of the signal (Fig. 2B). However, a dramatic drop in the percentage signal recovered, to less than 4%, was observed for all the stabilizers tested after storage at 56 °C for seven days, with the exception of 5% (w/v) raffinose, where 13–18% of the signal was recovered (Fig. 2C).

These findings show that the use of carbohydrate alone is insufficient, so bulking agents, such as BSA, Ficoll, and PEG, were tested alone or in combination. Bulking agents can be essential in a lyophilization formulation containing a very low concentration of protein because they prevent protein loss during dehydration. Bulking agents have also been reported to assist in the formation of a strong and porous cake structure (Shamblin, 2004). The presence of BSA, with or without Ficoll, significantly enhanced the thermal stability of the lyophilized mLATE-PCR mix, and more than 90% of the signal was recovered after storage at 56 °C for seven days (Fig. 2D and E). The effectiveness of combining raffinose with BSA was clear because it was the only dry-reagent formulation tested that retained more than 90% signal recovery after storage at 56 °C for 30 days (Fig. 2F).

3.5. Electrochemical detection of mLATE-PCR amplicons

The working potential for amperometric detection of α-naphthol was investigated by testing potentials ranging from 0.10 V to 0.40 V. The range of potential was selected based on the formal peak potential of α-naphthol (0.25 V) which was used as a reference point. Non-specific current responses were observed when the potential applied was within 0.10–0.15 V as well as within 0.30–0.40 V as the background currents generated were greater than the amperometric signals of α-naphthol (Fig. S3). The optimal potential for amperometric detection of α-naphthol was found to be 0.25 V based on the best S/B ratio.

The parameters affecting the hybridization reaction between the capture probe and the target DNA were also optimized. Various types of hybridization buffers, including 6 × SSC, 6 × saline sodium phosphate-EDTA (SSPE), 1 M NaCl, and 1 M $MgCl_2$, were tested and the best S/B ratio was obtained when 6 × SSC was used (Fig. S4A). The duration of the hybridization reaction was then varied between 10–120 min (Fig. S4B). The amperometric signal increased with increasing hybridization time, before reaching a plateau at 30 min. No further increase in the amperometric signal

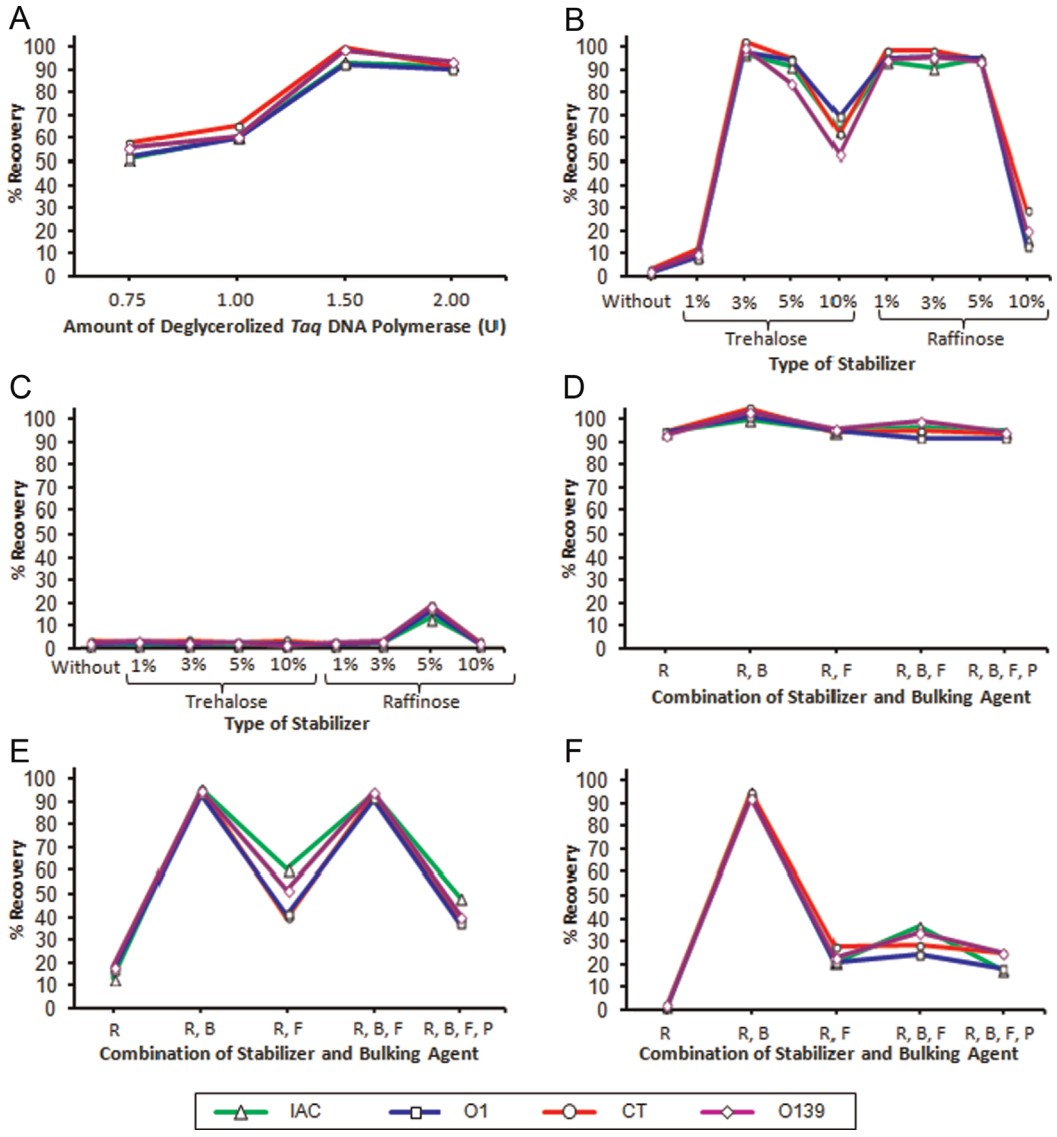

Fig. 2. Percentage signal recovered with (A) different amounts of deglycerolized *Taq* DNA polymerase; different types of stabilizers after storage at 56 °C for (B) one day and (C) seven days; and with different combinations of raffinose and bulking agents after storage at 56 °C for (D) one day, (E) seven days, and (F) 30 days. Each data point expresses the average of three replicate values. R, raffinose; B, bovine serum albumin; F, Ficoll; P, PEG 10,000.

was observed with longer hybridization times because the capture probes were saturated. Therefore, 30 min was selected as the optimal hybridization time.

The hybrids formed on the SPGEBs were enzymatically labeled by binding anti-FITC-ALP conjugates to the fluorescein label on the captured amplicons. To prevent the nonspecific adsorption of anti-FITC-ALP onto the SPGEB, different blocking reagents, including BSA, skim milk, western blocking reagent, and PVP, were tested (Fig. S5A). BSA and western blocking reagent were found to be more effective than other blocking reagents tested and western blocking reagent was selected because it produced a higher S/B ratio. Different proportions of the selected blocking reagent (0.1–2% [v/v]) were tested and 0.5% (v/v) western blocking reagent gave the most satisfactory results (Fig. S5B).

3.6. Analytical performance of the genosensing platform

The detection limit of the genosensing platform was assessed with purified genomic DNAs and bacterial cell suspensions of *V. cholerae* O1 and O139. Overall, the amperometric responses displayed an upward trend as the amount of genomic DNA increased before a signal plateau was reached at 100 pg and 250 pg for the O1 and CT targets, respectively, for *V. cholerae* O1, whereas for *V. cholerae* O139, the signals plateaued at 250 pg for both the O139 and CT targets (Fig. S6). Linear ranges between 0.1 and 10 pg were recorded for the O1 and CT targets of *V. cholerae* O1 and for the O139 and CT targets of *V. cholerae* O139. The limits of detection based on the mean of the blank measurements plus three standard deviations of the blank measurements were 0.4 pg and 0.5 pg of genomic DNA for *V. cholerae* O1 and O139, respectively (Fig. 3A and B).

Fig. S7 shows the increase in the amperometric response with increasing bacterial cells. A signal plateau was observed at 10^5 colony-forming unit (CFU)/ml for both serogroups of *V. cholerae*. The linear ranges of the O1 and CT targets for *V. cholerae* O1 and the O139 and CT targets for *V. cholerae* O139 were 1–10^4 CFU/ml (Fig. 3C and D). The limit of detection was 10 CFU/ml for both *V. cholerae* O1 and O139. Detection limit of the present genosensing platform was found to be lower than that reported by Low et al. (2013) and Rao et al. (2006) which were 10^3 and 10^5 CFU/ml of *V. cholerae*, respectively.

Dry-reagent mLATE-PCR mixes stored at various temperatures

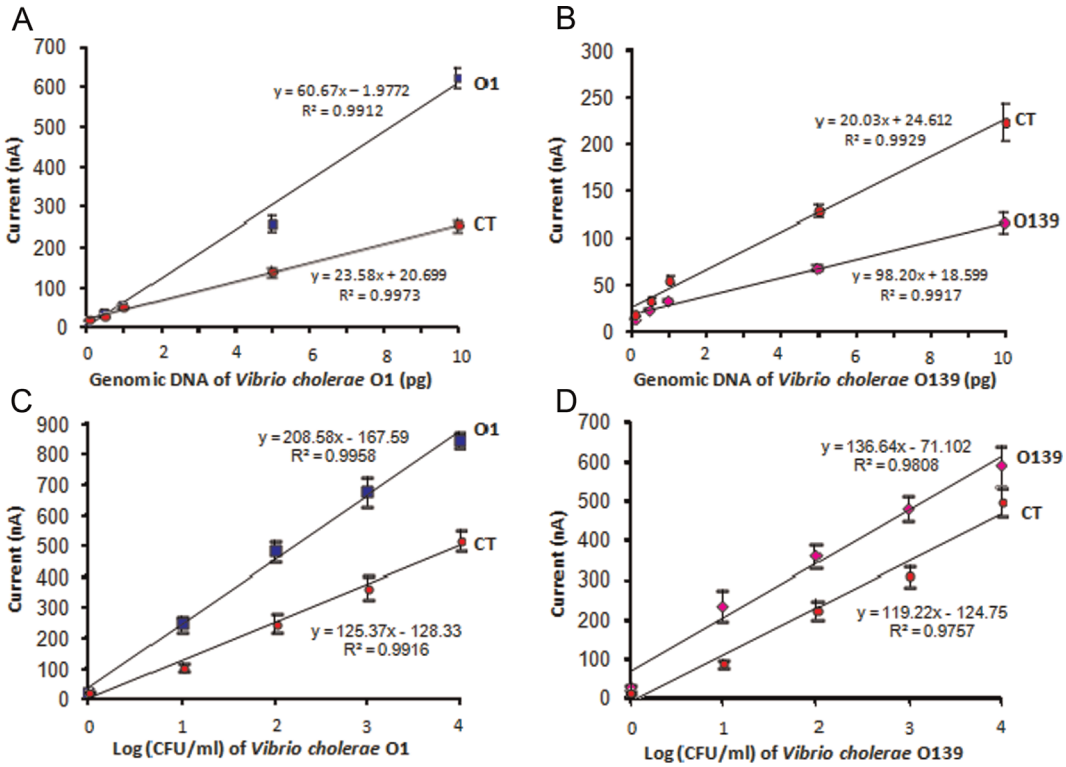

Fig. 3. Calibration plots constructed with (A, B) genomic DNAs and (C, D) bacterial cells of *V. cholerae* serogroups O1 (left) and O139 (right). Each data point represents the average of three replicates and the error bars indicate ± SD.

(4 °C, 25 °C, 37 °C and 56 °C) for one month were stable because the percentage signal recovery exceeded 90% when tested under optimized conditions (Fig. S8). An estimated shelf-life of 216 days or approximately seven months was calculated based on the accelerated aging test (Clark, 1991).

3.7. Diagnostic evaluation with spiked stool samples

The genosensing platform was evaluated with a panel of bacterial strains consisting of 95 positive samples and 73 negative samples. The threshold values used to distinguish between a positive and a negative result for O1, O139, CT, and IAC were derived in an earlier study (Yu et al., 2013), and the average background current from the negative samples plus three standard deviations of the negative sample measurements were used as the cut-off points. The genosensing platform identified all toxigenic *V. cholerae* serogroup O1 and O139 samples with no cross-reactivity with other nontarget pathogens. All negative samples were validated with a positive amperometric response for IAC, which ruled out false negative results. Diagnostic sensitivity and specificity of 100% were recorded with the platform developed in this study. Representative results of the diagnostic evaluation are presented in Fig. 4.

4. Conclusions

We have successfully developed a nucleic acid-sensing platform by combining two nucleic acid-based technologies that are not only highly versatile, sensitive, and selective but also amendable to multiplexing, miniaturization, and mass fabrication. The use of a dry-reagent-based nucleic acid amplification mix significantly reduces the assay complexity and the hands-on time, and minimizes the dependence on skilled persons, a cold chain, and utilities such as electricity. In this article, we have showcased the simplicity of the ready-to-use dry-reagent mLATE-PCR mix by demonstrating that the lyophilized pellet only requires rehydration before the DNA sample is added. The postamplification analysis was achieved with a multiplex electrochemical genosensor, which provides a digital output and therefore eliminates

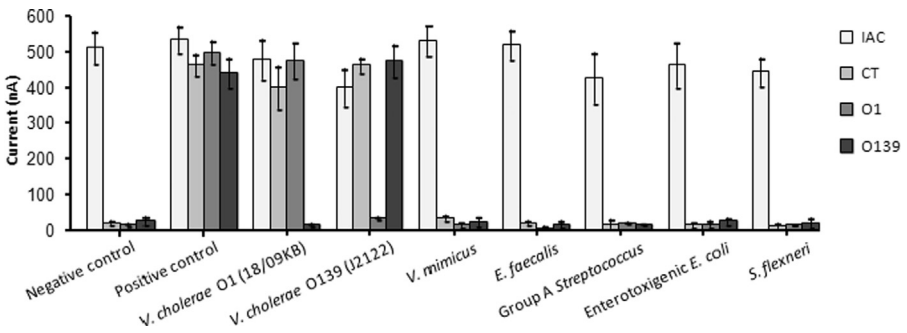

Fig. 4. Representative amperometric responses of the genosensing platform to different bacterial cells in a diagnostic evaluation. IAC, internal amplification control; CT, cholera toxin.

ambiguity and operator bias in the interpretation of the results. The practicability of combining a dry-reagent mLATE-PCR assay with a multiplex electrochemical genosensor for diagnostic applications was demonstrated with the simultaneous detection of several epidemic determinants of *V. cholerae*. The capacity to extract multiple data from a single reaction is a much-sought-after feature in clinical diagnostics because various types of epidemiological information, including strain typing, pathogenicity, and antibiotic susceptibility, can be collected to improve the intervention and the execution of preventive measures, as well as supporting evidence-based treatments. The use of a commercially available, hand-held, battery-operated PCR thermocycler with the dry-reagent mLATE-PCR mix and multiplex electrochemical genosensor described here extends the possibility of nucleic acid testing in various settings, including resource-limited regions.

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Appendix A. Supplementary Information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2015.03.048.

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