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A signal-amplified electrochemical DNA biosensor incorporated with a colorimetric internal control for *Vibrio cholerae* detection using shelf-ready reagents

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ABSTRACT

A novel enzyme/nanoparticle-based DNA biosensing platform with dual colorimetric/electrochemical approach has been developed for the sequence-specific detection of the bacterium *Vibrio cholerae*, the causative agent of acute diarrheal disease in cholera. This assay platform exploits the use of shelf-stable and ready-to-use (shelf-ready) reagents to greatly simplify the bioanalysis procedures, allowing the assay platform to be more amenable to point-of-care applications. To assure maximum diagnosis reliability, an internal control (IC) capable of providing instant validation of results was incorporated into the assay. The microbial target, single-stranded DNA amplified with asymmetric PCR, was quantitatively detected via electrochemical stripping analysis of gold nanoparticle-loaded latex microspheres as a signal-amplified hybridization tag, while the incorporated IC was analyzed using a simplified horseradish peroxidase enzyme-based colorimetric scheme by simple visual observation of enzymatic color development. The platform showed excellent diagnostic sensitivity and specificity (100%) when challenged with 145 clinical isolate-spiked fecal specimens. The limits of detection were 0.5 ng/ml of genomic DNA and 10 colony-forming units (CFU)/ml of bacterial cells with dynamic ranges of 0–100 ng/ml ($R^2=0.992$) and $\log_{10}$ (1–10^4 CFU/ml) ($R^2=0.9918$), respectively. An accelerated stability test revealed that the assay reagents were stable at temperatures of 4–37 °C, with an estimated ambient shelf life of 200 days. The versatility of the biosensing platform makes it easily adaptable for quantitative detection of other microbial pathogens.

1. Introduction

Epidemics of emerging microbial diseases continue to pose a substantial threat not only to global public health, but also socio-economic development and political stability (Ross et al., 2015). According to the World Health Organization (WHO), infectious diseases are a leading cause of morbidity and mortality worldwide, accounting for more than 6.4 million deaths in 2015 (WHO, 2016). In view of the current situation where the general public is at risk of contracting various microbial diseases, it has become imperative to develop sensitive, specific, and rapid diagnostic tools for the timely implementation of control measures to prevent the further spread of disease (Chua and Gubler, 2013). However, diagnostic microbiology laboratories worldwide have traditionally relied on classical isolation methods consisting of culture, biochemical, and immunologic tests for detection of microbial pathogens (Fournier et al., 2014). Although proven to be efficient and have long served as the gold standard for laboratory diagnosis of microbial diseases, these techniques are time-consuming and laborious (Pinto et al., 2015).

An electrochemical DNA biosensor, based on the integration of nucleic acid probes as a biorecognition element and an electrochemical signal transducer presents a promising alternative for detection of microbial pathogens. This type of assay has intrinsic specificity and selectivity provided by probe-mediated sequence-specific hybridization as well as the advantages offered by the electrochemical-based biosensing technology, such as high sensitivity, fast response time, independence from sample turbidity, easy miniaturization via microfabrication, and the capability of quantitative analysis (Rosario and Mutharasan, 2014). Due to these merits, electrochemical DNA biosensing has attracted a tremendous amount of attention in the scientific community in recent years for microbiological diagnostics and has been shown to

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be superior to established classical culture-based methods (Fernandes et al., 2015; Liébana et al., 2016; Low et al., 2011; Vijian et al., 2016; Yean et al., 2008). However, most of these biosensing platforms have not been challenged with actual complex biological samples and, therefore, fail to bridge the gap between prototypic biosensor development and practical applications. In addition, there has also been relatively little empirical work done to resolve the largely unaddressed concern of the shelf life of biosensors, as it is particularly important to develop biosensors that can withstand long-term storage at ambient temperatures in order to further promote their widespread applications in microbiological diagnostics.

We previously described a novel electrochemical DNA biosensor based on magnetic beads (MBs) as the biorecognition surface and gold nanoparticle-loaded latex microspheres (latex-AuNPs) as a signal-amplified hybridization tag for sequence-specific detection of single-stranded DNA (ssDNA) (Low et al., 2015). By integrating latex-AuNP-assisted signal amplification with magnetic separation, the electrochemical DNA biosensor exhibited ultra-high sensitivity towards ssDNA at much low detection limits, thereby facilitating promising diagnostic potential. Notwithstanding, one of the key problems impeding its further application is the absence of an internal control (IC), which is critical for the functional validation of diagnostic assays and to avoid misinterpretation of false negative results. It is important to incorporate an appropriate IC into current molecular diagnostic techniques that strive to obtain maximum reliability, regardless of the number of targets or detection strategies being used (Vinayagamoorthy et al., 2015). In addition, the requirements for multiple preparation steps, costs in terms of cold-chain transportation and storage of assay reagents, and lack of integrated, streamlined, and operator-friendly detection protocols are also factors limiting the extensive use of electrochemical DNA biosensors (Pedrero et al., 2011).

In order to meet the aforementioned challenges, we describe herein a DNA biosensor with a dual colorimetric/electrochemical approach for simultaneous detection of target *Vibrio cholerae*- and IC ssDNA amplified with asymmetric PCR (aPCR). All of the required assay reagents (as shown in Fig. S1 in the Supplementary Data Section) consisting of aPCR mix, hybridization mix, tagging mix, target detection medium, IC detection medium, and assay washing (AW) buffer are shelf-stable and ready-to-use (shelf-ready) to greatly simplify the experimental procedures, shorten the assay times by reducing the number of pipetting steps, and mitigate the possibility of cross-contamination. The detection targets, label-free ssDNA amplicons of the *V. cholerae lol* B gene, were sandwich-hybridized to MB-functionalized target capturing probes (MB/T-CAP) and fluorescein-labeled target detection (T-DET) probes and tagged with latex-AuNPs bioconjugated to anti-fluorescein IgG antibody (latex-AuNP/α-FITC conjugate). In the same reaction tube, the 5′ digoxigenin-labeled ssDNA amplicons of the IC were hybridized to MB-functionalized IC capturing probes (MB/IC-CAP) and tagged with horseradish peroxidase enzyme-linked anti-digoxigenin antibody (α-DIG/HRP). Quantitative electrochemical detection of the target hybridization events was achieved by the differential pulse anodic stripping voltammetry (DPASV) of Au^{3+} ions after dissolution of the AuNPs loaded onto the latex microsphere with target detection medium. The IC hybridization events were analyzed using a simplified HRP enzyme-based colorimetric scheme by simply observing the color development due to the enzymatic reaction with the naked eye following the addition of IC detection medium.

2. Material and methods

The chemicals and apparatus are described in detail in the Supplementary Data Section.

2.1. Preparation of shelf-ready assay reagents

2.1.1. Shelf-ready aPCR mix

Thirty-μl aliquots of the aPCR mix containing 1 × *Taq* buffer with $(NH_4)_2SO_4$, 2.5 mM of $MgCl_2$, 160 μM of dNTP mix, 0.075 U/μl of deglycerolized *Taq* DNA polymerase, 0.67 μM of target forward primer, 0.0067 μM of target reverse primer, 0.53 μM of IC forward primer, 0.053 μM of IC reverse primer, 5 ng/ml of IC recombinant plasmid DNA, and 5% w/v of trehalose were prepared in 0.5 ml tubes using PCR-grade water followed by a vacuum-drying process at 4.8^{-2} mBar pressure for 2 h and stored at ambient temperature until further use.

2.1.2. Shelf-ready hybridization mix

Ten-μl aliquots of the hybridization mix containing 2 μg/μl of MB/T-CAP conjugate, 1.5 μM of T-DET probes, 1 μg/μl of MB/IC-CAP conjugate, 0.075 M NaCl, 0.01% v/v Tween 20, 1% w/v BSA, and 15% w/v of trehalose were prepared in 0.5 ml tubes using phosphate buffer (pH 7.4) followed by a vacuum-drying process and stored at ambient temperature as described above.

2.1.3. Shelf-ready tagging mix

Ten-μl aliquots of the tagging mix containing OD_{310}=6.0 of latex-AuNP/α-FITC conjugate, 0.375 mU/μl of α-DIG/HRP, 0.15 M NaCl, 2% w/v BSA, and 25% w/v of trehalose were prepared in 0.5 ml tubes using phosphate buffer (pH 6.0) and subsequently vacuum-dried and stored as described above.

Additional details for preparation of shelf-ready assay reagents in Sections 2.1.1–2.1.3 are available in the Supplementary Data Section.

2.1.4. Shelf-ready IC detection medium

Twenty-μl aliquot of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate were prepared in 0.5 ml tubes and stored at ambient temperature until further use.

2.1.5. Shelf-ready target detection medium

Three hundred-μl aliquot of 1 M hydrobromic acid containing 0.15 mM bromine were prepared in 1.5 ml tubes and stored at ambient temperature until further use.

2.1.6. Shelf-ready AW buffer

Two-ml aliquot of 0.01 M potassium phosphate buffer, pH 7.4, containing 0.15 M NaCl and 0.01% v/v Tween 20 were prepared in 2.0 ml tubes and stored at ambient temperature until further use.

2.2. Preparation of DNA sample and aPCR amplification

DNA samples used for aPCR amplification were prepared from bacterial cultures and spiked fecal specimens using boiling lysis method as described in the Supplementary Data Section.

To perform aPCR, the shelf-ready aPCR mix was first reconstituted with 30 μl of DNA sample and then amplified using the following parameters: 5 min at 95 °C followed by 35 cycles of 95 °C for 30 s, 63 °C for 30 s and 72 °C for 60 s. The amplification was further incubated for another 30 s at 63 °C and 5 min at 72 °C to extend any incomplete amplicons.

The obtained aPCR amplicons were used directly for DNA biosensing detection without any pretreatment or purification steps.

2.3. DNA biosensing of aPCR amplicons

Ten-μl of aPCR amplicons was added to a tube containing the shelf-ready hybridization mix and incubated for 20 min. The

unbound components were then removed from the MB surface by washing three times with 50 μl of AW buffer via magnetic separation. Meanwhile, the shelf-ready tagging mix was reconstituted in 10 μl of AW buffer and subsequently used to resuspend the washed target- and IC hybrid-bound MB complexes. The tagging procedure proceeded for 5 min and was followed by washing three times with 50 μl of AW buffer via magnetic separation. During the last washing step, 2 μl of the 50 μl of washed suspension (a mixture of tagged target- and IC hybrid-bound MB complexes) was extracted and added to a tube containing a shelf-ready IC detection medium. The reaction mixture was monitored for an instant blue color development due to HRP enzymatic reaction to validate the functionality of the DNA biosensor. Following the extraction of 2 μl for IC colorimetric detection, the remaining washed suspension (48 μl) was subjected to magnetic separation to remove residual supernatant and resuspended in 50 μl of shelf-ready target detection medium for chemical dissolution of AuNPs loaded onto the latex microspheres to Au^{3+} ions. Quantitative electrochemical analyses of the resulting Au^{3+} ions were carried out by transferring whole dissolved Au^{3+} ions onto the sensing area of a simplified two-electrode configuration screen-printed electrode (2-SPE) followed by DPASV detection with the following parameters: electrodeposition at −0.55 V for 390 s followed by potential scanning from 0.0 to +1.0 V (step potential, 6.25 mV; modulation amplitude, 50 mV; modulation time, 50 ms; and scan rate, 50 mV/s). The current value obtained from a voltammogram at +0.65 V was recorded as the analytical signal without searching for a peak height. The 2-SPE was discarded after a single use to avoid sample cross-contamination.

3. Results and discussion

3.1. Design strategy

A schematic diagram of the aPCR-coupled DNA biosensing platform is shown in Fig. 1. This assay demonstrated advantages of ultrasensitivity, reliability, simplicity, robustness, rapidity, and quantitative capacity for the detection of specific DNA sequences.

3.1.1. Ultrasensitivity

To realize an ultrasensitive detection platform, we fabricated a novel nanoparticle tag, which was prepared exactly as reported previously by Low et al. (2015). Briefly, gold nanoparticles (AuNPs) were loaded onto the surface of a polyelectrolyte multilayer film-coated latex microsphere, which was then bioconjugated with α-FITC antibody for specific tagging of hybrid DNA. Fig. S2A in the Supplementary Data Section presents a transmission-electron-microscopy (TEM) image of a latex-AuNP/α-FITC conjugate that allows multiple AuNPs to be tagged in each biorecognition event on the MB surface (Fig. S2B), which consequently led to a significant increase in the sensitivity of quantitative electrochemical detection.

3.1.2. Reliability

The reliability of the developed assay was ascribed to the incorporation of IC detection for validation of assay functionality. The IC plays a pivotal role in the assay platform, as it enables the end-user to simultaneously monitor the integrity of the assay reagents and all procedures while performing aPCR and DNA biosensing. The assay result is considered to be truly negative or positive only if the IC is positive. Hence, the likelihood of a false negative interpretation due to inappropriate specimen processing, the presence of aPCR-inhibitory substances in DNA samples, or technical errors, such as defective equipment, erratic testing procedures, or improper handling of the assay reagents, is considerably minimized by the incorporation of IC detection into the developed assay platform.

3.1.3. Simplicity

The simplicity of the assay was achieved by implementing four performance-enhancing strategies. First, the target *V. cholerae*- and IC DNA sequences were amplified using an alternative aPCR protocol. Unlike conventional PCR, this aPCR protocol predominantly yields ssDNA amplicons, thereby allowing hybridization with the biorecognition elements (DNA probes) without the need for prior

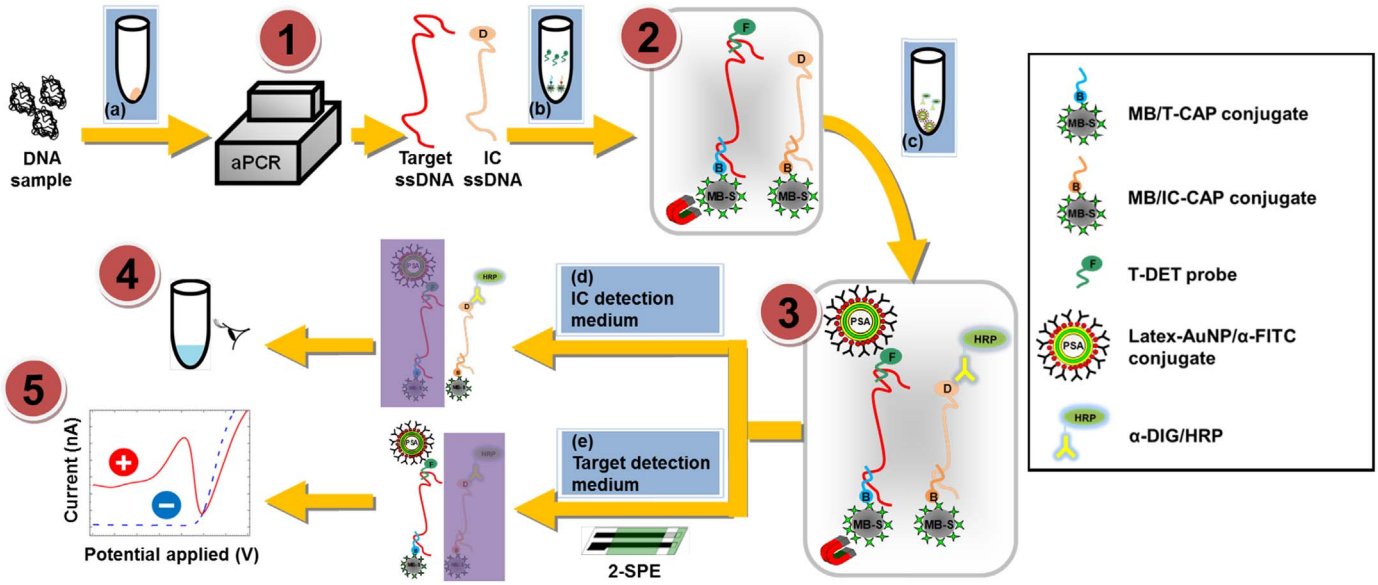

Fig. 1. A schematic of the DNA biosensor for sequence-specific detection and quantification of target ssDNA together with incorporated IC detection for result validation. This assay utilizes shelf-ready reagents consisting of (a) aPCR mix, (b) hybridization mix, (c) tagging mix, (d) IC detection medium, and (e) target detection medium. The experimental procedures used for DNA biosensing identification of the target organism were comprised (1) DNA sample preparation from target organisms followed by aPCR amplification of target ssDNA together with IC amplification to produce IC ssDNA, (2) hybridization with target and IC ssDNA, (3) tagging of hybridization events, (4) colorimetric detection of IC ssDNA by the naked eye following the addition of IC detection medium, and (5) DPASV detection of target ssDNA on a 2-SPE following the addition of target detection medium. The voltammogram depicts typical biosensing responses obtained in the absence (dashed line) and presence (solid line) of target ssDNA.

denaturation (by heating and cooling) and purification of the resulting amplicons (Arora et al., 2007).

The second strategy includes the preparation of shelf-ready assay reagents for both aPCR and DNA biosensing. Assay reagents containing biological components (aPCR mix, hybridization mix, and tagging mix) were subjected to a vacuum-drying process in the presence of an optimal concentration of trehalose as a stabilizer to retain protein structure and biofunctionality during the dehydration process. This vacuum-drying approach allows for the preparation of pre-optimized, pre-mixed, pre-aliquoted, and ready-to-use assay reagents to greatly simplify the aPCR and DNA biosensing experimental procedures.

The third strategy is based on the use of a simplified enzyme-based colorimetric IC detection scheme. The incorporated IC was analyzed by simply observing the color development due to an HRP enzymatic reaction with the naked eye without the need for electrochemical detection. This alternative 'yes or no' response scheme of IC detection provides instant result validation without interfering with the quantitative electrochemical detection of the target ssDNA.

The last assay simplification strategy involves alternating the method of result interpretation in DPASV analysis. In the conventional method, the DPASV results (voltammogram) are interpreted by manually searching the current peak heights using a dedicated algorithm. This troublesome computer-based method of result interpretation renders portable diagnosis in the field difficult. To this end, an alternative method to interpret the DPASV results was proposed in this study, in which only the current value obtained at a fixed potential of +0.65 V is recorded as the analytical signal without searching for a peak height. This simplified DPASV analytical method not only makes the diagnostic assay more user-friendly, but can also facilitates the fabrication of a low-cost portable reader.

3.1.4. Robustness

Robustness is typically defined as the shelf life stability of reagents used in an assay (Epizooties, 2004). A total of six assay reagents, (1) aPCR mix, (2) hybridization mix, (3) tagging mix, (4) target detection medium, (5) IC detection medium, and (6) AW buffer, were used in the proposed aPCR-coupled DNA biosensing platform. Each of these assay reagents were stable over a relatively long period at ambient temperatures and can be transported and stored without refrigeration. The biological assay reagents (1), (2), and (3) were dehydrated in the presence of an optimal concentration of trehalose to achieve ambient temperature stability. The dehydrated biological assay reagents were evaluated using accelerated aging techniques based on the Q_{10} approach (Clark, 1991) and were found to be stable at ambient temperature (28 °C) for at least 200 days when mathematically correlated with its stability at 37 °C for 70 days (detailed discussion is available in the Supplementary Data Section, Fig. S3). The non-biological assay reagents (4), (5), and (6) were not temperature-sensitive and have been empirically shown to be stable for at least 2 years at ambient temperature (28 °C). The target detection medium was used to dissolve the AuNPs loaded onto the latex microspheres during quantitative electrochemical detection of the target ssDNA. The IC detection medium was used for HRP enzyme-based colorimetric detection of the IC ssDNA. This substrate was stable at ambient temperature and supplied as a ready-to-use solution.

3.1.5. Rapidity

We attempted to enhance the speed of the diagnostic assay by introducing the use of MBs as an alternative solid support for DNA biorecognition, which is conventionally carried out directly on the electrode transducer surface. In this study, the T-CAP and IC-CAP probes (Table S1 in the Supplementary Data Section) were immobilized onto the surfaces of MBs for use as biorecognition layers to capture target and IC single-stranded aPCR amplicons in the proposed DNA biosensor. Immobilization of the biorecognition elements on the surface of micron/submicron-sized MBs facilitates rapid kinetics of the DNA hybridization reaction in the solution phase. All hybridization components move freely in solution and randomly collide. These favorable kinetics lead to enhanced hybridization efficiency and, thus, greatly reduce the incubation time required for the hybridization process (Campuzano et al., 2011). Furthermore, the superparamagnetic properties of the MBs allow fast magnetic separation of MB-bound hybrid complexes from solution (in this study, a 30 s exposure to a magnetic field was sufficient to separate the MB-bound complexes) without the need for time-consuming centrifugation steps, and resuspension of the separated MB-bound complexes is easily achieved by simply removing the magnetic field without the need for thorough vortexing or sonication.

3.1.6. Quantitative capacity

The quantitative capacity of the diagnostic assay is attributed to the use of an optimized and sensitive electrochemical platform for DNA biosensing, which produces a measurable current signal that is quantitatively proportional to the amount of target amplicons present in the reaction mixture (Low et al., 2015).

The convergence of the aforementioned strategies will further promote the application of PCR-coupled electrochemical DNA biosensors for clinical diagnosis of microbial pathogens.

3.2. Base mismatch detection

The selectivity of the T-CAP and T-DET probes was evaluated by hybridizing them in a sandwich-type format with different 42-mer synthetic oligomers comprising complementary, single-base-mismatch (1-misA and 1-misB), three-base-mismatch (3-mis), and non-complementary sequences (Table S1 in the Supplementary Data Section). The MB/T-CAP conjugate and T-DET probe were tested against 1 μM of the different 42-mer synthetic oligomers using latex-AuNP/α-FITC conjugate as the hybridization tag. Fig. S4 in the Supplementary Data Section presents the obtained results. The current signals detected for the 3-mis sequences is almost the background signals from the non-complementary sequences, indicating that sequences with as few as three mismatched bases in the probe recognition site can be perfectly discriminated from the complementary target. For both the 1-misA and 1-misB sequences, the obtained current signals are similar to those obtained for the complementary target.

The ability to detect single-base-mismatches was further investigated by adding different concentrations of formamide to the hybridization reaction mixture. This chemical is known to provide a high-stringency condition that makes hybridization difficult (Fuchs et al., 2010). Fig. S5 in the Supplementary Data Section shows the effect of formamide concentration on the current signals obtained in the detection of complementary, 1-misA, and 1-misB sequences. The current signals decreased with increasing concentrations of formamide in the hybridization reaction mixture for all of the tested sequences. For a concentration of 45% v/v of formamide, the current signal obtained for 1-misA and 1-misB is similar to the background signal.

In non-stringent hybridization conditions, the developed DNA biosensor can discriminate between a complementary target and a sequence with a three-base mismatch. For the detection of a single-base mismatch, the use of stringent conditions (45% of formamide in the hybridization reaction mixture) is necessitated, although in this case, the sensitivity of the DNA biosensor is decreased by approximately 50%.

3.3. Incorporation of a colorimetric IC

Two variables affecting the colorimetric IC performance, consisting of enzyme/substrate reaction and MB types, were sequentially evaluated by detection against aPCR amplicons containing IC ssDNA.

Anti-digoxigenin antibodies linked with two different enzymes, horseradish peroxidase (α-DIG/HRP) and alkaline phosphatase (α-DIG/AP) were evaluated to determine their suitability for use as tagging components in the colorimetric detection of IC. The enzymatic activity of HRP was detected using TMB substrate via the formation of a visually observable blue coloration, while the enzymatic activity of AP was analyzed using p-nitrophenyl phosphate (pNPP) substrate via the formation of a yellow coloration. The HRP/TMB reaction was chosen for colorimetric detection of IC because it produced a much more intense color (data not shown) in a much shorter time (~10 s) than the AP/pNPP counterpart (30 min).

MBs with 1.0 and 2.8 μm diameters were evaluated to determine their suitability as biorecognition layers for the colorimetric detection of IC ssDNA. The 2.8 μm MBs were chosen to immobilize IC-CAP probes because they produce a more intense blue color (data not shown) than their smaller 1.0 μm counterpart.

With the incorporation of an optimized colorimetric IC, the developed electrochemical DNA biosensor was subsequently tested against aPCR amplicons yielded by adding different combinations of target and IC DNA templates into shelf-ready aPCR mixes (Fig. 2), which showed that both the target and IC can be independently aPCR-amplified in a single-reaction tube and then DNA biosensing-detected (IC colorimetric validation and target quantitative electrochemical detection) without cross-reacting with each other. The colorimetric IC was subjected to spectrophotometric analysis using the wavelength of maximum absorbance at 650 nm (the absorbance spectrum of the blue-colored product produced in an IC enzymatic reaction is shown in Fig. S6 in the Supplementary Data Section). As seen in Fig. 2A, the absence of an IC DNA template produced no color in IC colorimetric detection with a negligible absorbance value at 650 nm (A_{650}), while the presence of an IC DNA template produced an intense blue color with a significantly higher A_{650} value following a relatively short incubation time of ~10 s. This instant and unambiguous 'yes or no' response greatly facilitates naked eye interpretation of the colorimetric IC.

3.4. Optimization of shelf-ready assay reagents

The influence of different variables on the performance of the shelf-ready hybridization mix, tagging mix, and aPCR mix were sequentially optimized. All optimization studies were performed by positive detection against aPCR amplicons containing target and IC ssDNA. A parallel experiment using aPCR amplicons containing only IC ssDNA was performed as a negative control. The optimal values of the variables were determined based on two criteria: (1) the intensity of blue coloration produced in IC colorimetric detection; and (2) the positive-to-negative (P/N) current ratio calculated in target quantitative electrochemical detection.

Shelf-ready hybridization mix variables consisting of MB/IC-CAP concentration (0–5 μg/μl), MB/T-CAP concentration (0–4 μg/μl), hybridization time (5–40 min), and trehalose concentration (0–30% w/v) were sequentially evaluated and the obtained optimal values were 2 μg/μl, 1 μg/μl, 20 min, and 15% w/v, respectively (Fig. S7 in the Supplementary Data Section).

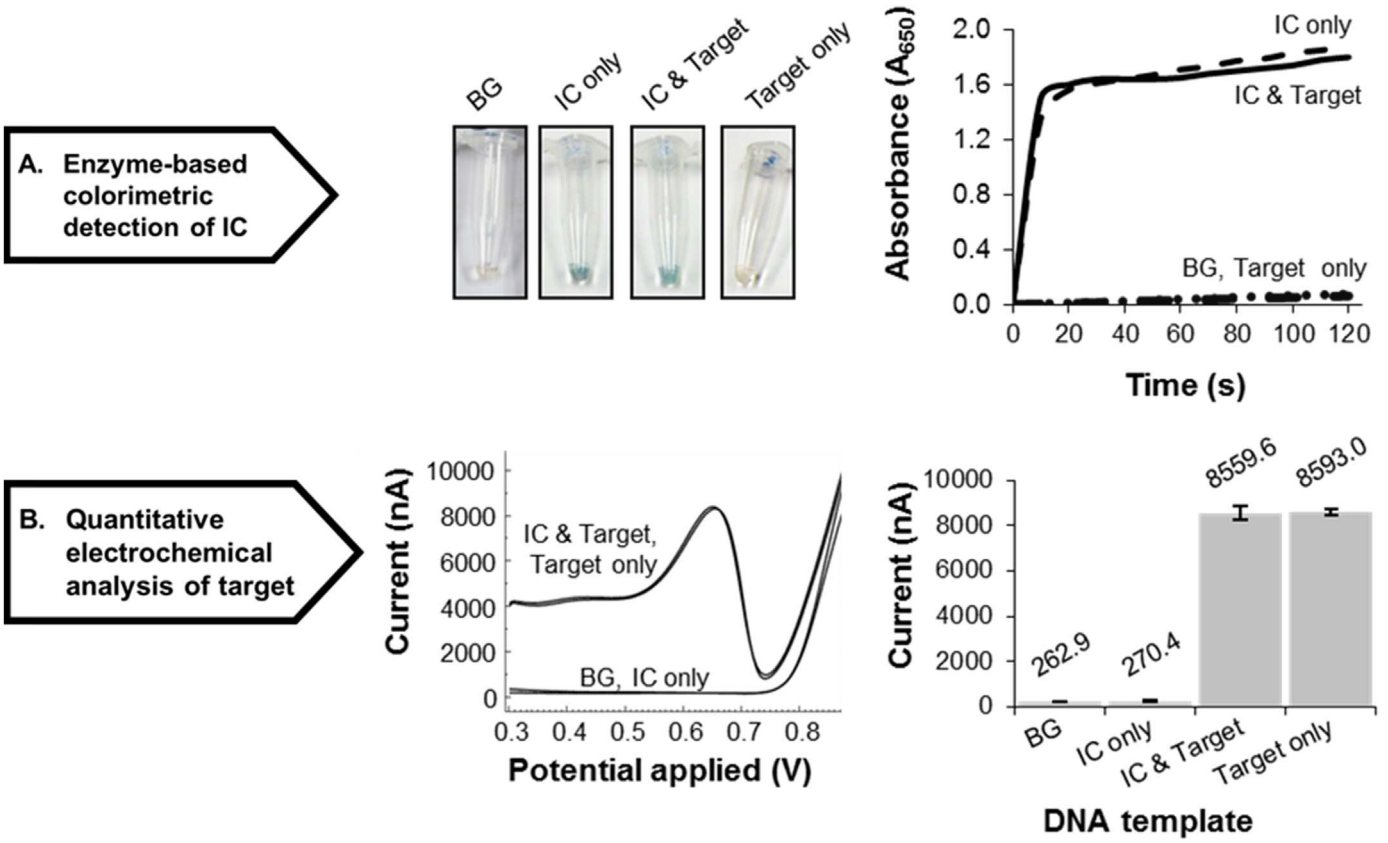

Fig. 2. Evaluation of the DNA biosensor using aPCR amplicons prepared using different combinations of target and IC DNA templates. (A) Visual observation and spectrophotometric analysis of enzymatic color development in IC detection. (B) DPASV quantitative electrochemical analysis of target aPCR-amplified ssDNA. BG, background control without adding DNA template into shelf-ready aPCR mix; IC only, adding IC template only into shelf-ready aPCR mix; IC & Target, adding IC and target templates into shelf-ready aPCR mix; Target only, adding target template only into shelf-ready aPCR mix.

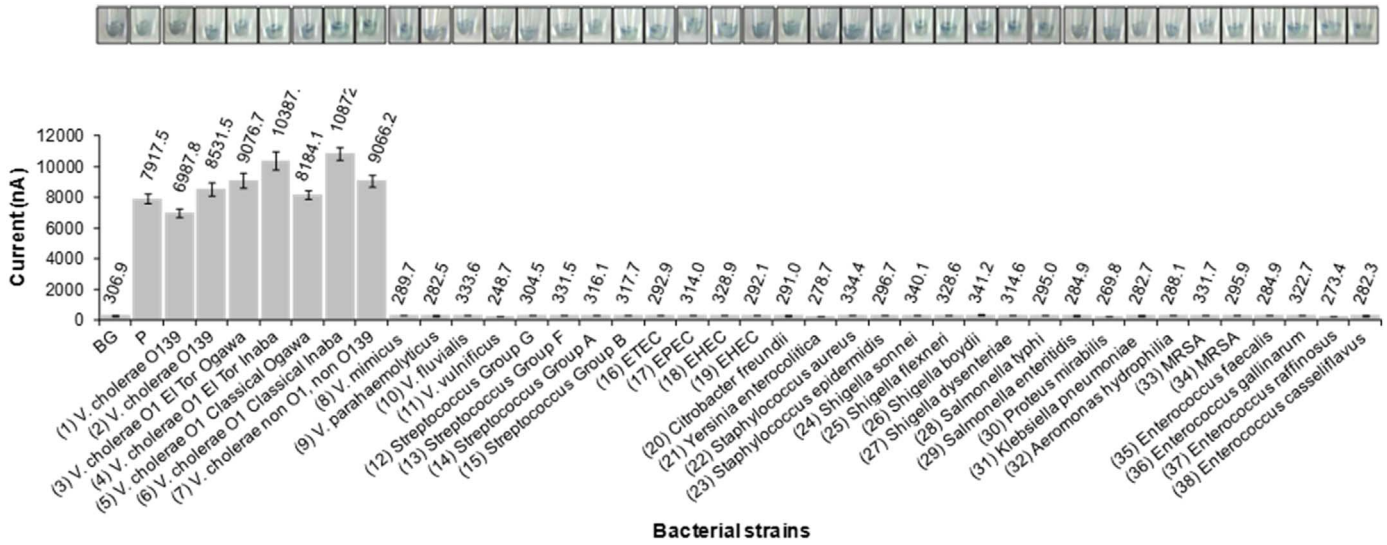

Fig. 3. Analytical specificity evaluation of the DNA biosensor using different bacterial strains. BG, background control without using bacterial strains; P, positive control using target recombinant plasmid as the DNA template. The error bars show the standard deviation for triplicate experiments.

Subsequently, the shelf-ready tagging mix variables consisting of α-DIG/HRP concentration (0–1.5 mU/μl), latex-AuNP/α-FITC concentration (OD_{310}=3–12), tagging time (5–40 min), and trehalose concentration (0–30% w/v) were sequentially evaluated and the obtained optimal values were 0.375 mU/μl, OD_{310}=6, 5 min, and 25% w/v, respectively (Fig. S8 in the Supplementary Data Section).

With the optimized hybridization and tagging conditions, the amplification protocol for aPCR were subsequently evaluated. Several variables affecting the yield of IC and target ssDNA in a shelf-ready aPCR mix, consisting of target forward-to-reverse primer concentration ratio (1:1, 10:1, and 100:1), IC forward-to-reverse primer concentration ratio (1:1, 10:1, and 100:1), IC recombinant plasmid concentration (0–10 ng/ml), annealing temperature (51–70 °C), extension step duration (30–75 s), number of cycles (25–40 ×), trehalose concentration (0–30% w/v), and *Taq* DNA polymerase concentration (0.0375 – 0.1125 U/μl) were sequentially optimized and the obtained optimal values were 100:1, 10:1, 5 ng/ml, 63 °C, 60 s, 35 ×, 5% w/v, and 0.075 U/μl, respectively (Fig. S9 in the Supplementary Data Section).

3.5. Dual colorimetric/electrochemical DNA biosensing

In the present study, the target *V. cholerae lol*B gene together with an incorporated IC TRAP gene of *Plasmodium falciparum* were both aPCR-amplified using a shelf-ready aPCR mix. The resulting aPCR amplicons containing target and IC ssDNA were then captured by a shelf-ready hybridization mix to form target and IC hybrid-bound MB complexes. In the hybridization reaction mixture, the target ssDNA was captured by both the MB/T-CAP conjugate and 3′ fluorescein-modified T-DET probe in a sandwich-type dual hybridization format with the aim to enhance specificity and selectivity of the assay system as well as to confer a free fluorescein hapten label to the target ssDNA. A high concentration of T-DET probes (1.5 μM) was employed in the assay with the aim of hybridizing all of the target ssDNA that was captured by the MB/T-CAP conjugate (Djellouli et al., 2007). The IC ssDNA was labeled with a digoxigenin hapten at the 5′-end during aPCR amplification and captured by an MB/IC-CAP conjugate at the 3′-end during the hybridization step, thereby leaving a free digoxigenin hapten label to the IC ssDNA.

Following hybridization, the resulting target and IC hybrid-bound MB complexes were magnetically separated from the reaction solution and then tagged with a shelf-ready tagging reaction mixture that tagged the target hybrid-bound MB complex with the latex-AuNP/α-FITC conjugate via fluorescein hapten recognition (as shown in the TEM image in Fig. S2B in the Supplementary Data Section), while the IC hybrid-bound MB complex was tagged with an α-DIG/HRP via digoxigenin hapten recognition. The aforementioned DNA biosensing procedures comprising the capture of target and IC ssDNA, followed by the tagging of target and IC hybridization events were all conducted in a single-reaction tube.

Afterwards, the presence of IC ssDNA was singly detected using an enzyme-based colorimetric scheme via the addition of IC detection medium and observed for instant blue color formation. Following IC validation, the presence of target ssDNA was quantitatively detected by electrochemical analysis employing DPASV via dissolution of the AuNPs loaded onto the latex microspheres using a target detection medium.

3.6. Analytical performance of the DNA biosensor

The analytical specificity of the detection platform was evaluated using boiled lysate DNA samples prepared from a panel of 38 reference bacterial strains, which consisted of seven *V. cholerae* strains and 31 non-*V. cholerae* strains. A background experiment was conducted without the use of bacterial cells. The aim of this study was to evaluate the specificity and selectivity of the designed primers and hybridization probes employed in the assay. The results showed that the DNA biosensor was able to clearly discriminate between the target *V. cholerae* and non-target bacterial samples (Fig. 3). IC colorimetric detection in all experiments was positive, as indicated by blue coloration, which validated the functionality of the DNA biosensor. The current signals obtained in this analysis were used to establish a cut-off value for determination of positivity and negativity. The cut-off point for a positive result was defined as a current signal greater than or equal to the mean plus three times the standard deviation of the current signals for the background and non-target samples. A cut-off value of 380 nA was determined from this analysis. Experiments with current signals ≥ 380 nA were interpreted as positive, while those below were defined as negative. Based on the cut-off value, all seven *V. cholerae* strains were positive, while all non-*V. cholerae* strains were negative. Hence, the analytical specificity of the DNA biosensor was 100% and the relative standard deviation (RSD)

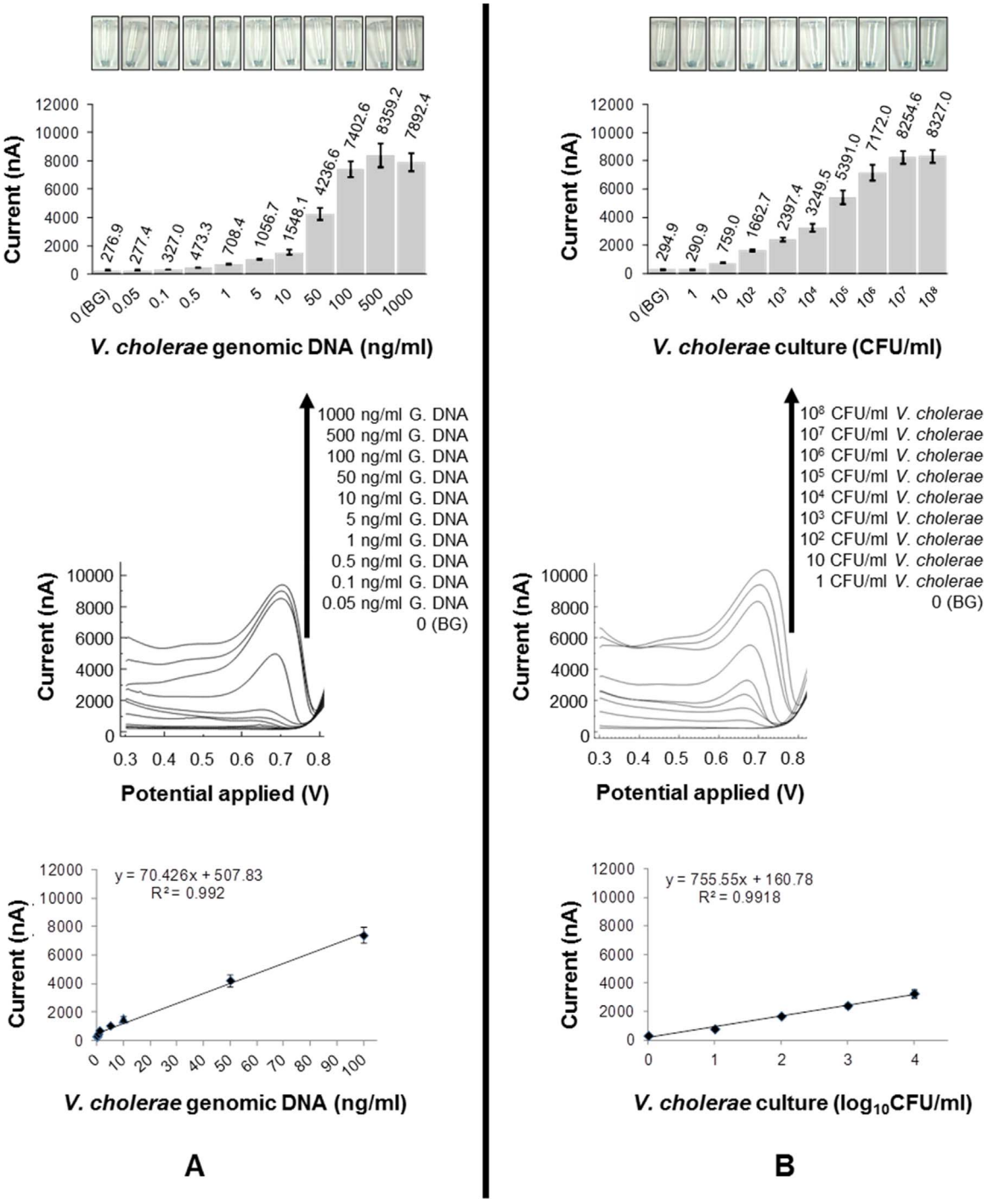

Fig. 4. Analytical sensitivity evaluation of the DNA biosensor using a known amount of serially diluted (A) purified *V. cholerae* genomic DNA and (B) *V. cholerae* culture. The corresponding linear ranges are depicted in respective calibration plots. BG, background experiment. The error bars show the standard deviation for triplicate experiments.

values for triplicate experiments were 0.21–9.95%.

We subsequently performed an analytical sensitivity study by testing against known amounts of DNA and bacterial cells of serially diluted *V. cholerae* purified genomic DNA (0.05–1000 ng/ml) and *V. cholerae* culture (1–10^8 colony-forming units (CFU)/ml), respectively (Fig. 4). With the use of 380 nA as a cut-off point for interpretation of results, the limits of detection for the DNA biosensor at the DNA and bacterial levels were 0.5 ng/ml and 10 CFU/ml, respectively. In addition, the current signal was found to be linearly correlated with the amount of purified genomic DNA and bacterial culture CFU concentration in the range of 0–100 ng/ml (R^2=0.992) and $\log_{10}$ (1–10^4 CFU/ml) (R^2=0.9918), respectively. The RSD values for the DNA and bacterial studies were 5.65–9.95% and 3.20–9.19%, respectively.

3.7. Diagnostic evaluation with spiked fecal specimens

The real applicability of the DNA biosensor was evaluated using fecal specimens spiked with 145 clinical isolates consisting of various types of *V. cholerae* strains and other enteric bacteria. After enrichment in alkaline peptone water for 3 h at 37 °C, boiled lysate DNA samples were prepared from the enriched culture and then subjected to aPCR amplification using shelf-ready aPCR mix and DNA biosensing detection using shelf-ready hybridization and

tagging mixes (Fig. S10 in the Supplementary Data Section). The total assay time from sampling to measurement to produce quantitative results was <6 h. A comparison of the established cut-off value of 380 nA indicated that the current signal of each of the *V. cholerae* clinical isolates was positive with an average current signal-to-cut-off ratio of 22.48. The current signals of all other enteric clinical isolates were correctly interpreted as negative, as each was lower than the cut-off value. The functionality of the aPCR-coupled DNA biosensor was validated by the positive blue coloration produced in IC colorimetric detection. These results showed that the developed assay was able to specifically detect all *V. cholerae* serogroups with 100% diagnostic sensitivity, diagnostic specificity, positive predictive value, and negative predictive value.

4. Conclusions

A variety of signal-amplified, nanoparticle-based electrochemical biosensors have been developed for ultrasensitive detection of microbial DNA as potential alternatives to both classical culture-based methods and modern molecular detection techniques such as real-time PCR, enzyme-linked oligonucleotide assay, and nucleic acid lateral-flow dipstick assays. So far, however, none of these biosensing platforms has been successfully marketed for use in microbial diagnostics, with the typical users expecting the platform to be highly reliable by clearly providing positive, negative, or invalid results; be exceedingly robust by preparing assay reagents capable of remaining stable over time; be greatly simplified by designing integrated, streamlined, and operator-friendly detection protocols. In this study, we successfully developed a novel biosensor for simultaneous detection of target microbial DNA and an IC. The presence of target aPCR-amplified ssDNA was quantitatively detected using the DPASV electrochemical analysis of dissolved AuNPs loaded onto latex microspheres. An alternative method to interpret the DPASV results by recording the current value obtained at a fixed potential without the need for analyzing peak height was proposed in this study. The incorporated IC was not electrochemically analyzed, but instead employed a simplified enzyme-based colorimetric scheme by simply observing with the naked eye the color development due to an enzymatic reaction. An instant blue coloration validated the functionality of the DNA biosensor, thereby verifying the accurate quantitative electrochemical detection of the target analyte. It is pivotal to note that all of the assay reagents used in this aPCR-coupled DNA biosensing platform were shelf-stable with an extended shelf life at ambient temperature and were ready-to-use without the need for any prior pretreatment/incubation steps (shelf-ready). The practicability of using shelf-ready assay reagents and coupling an aPCR assay with an enzyme/nanoparticle-based DNA biosensor with dual colorimetric/electrochemical approach for real diagnostic applications was demonstrated by detecting pathogenic *V. cholerae* spiked in fecal specimens. The aPCR-coupled electrochemical DNA biosensor using shelf-ready reagents together with incorporated colorimetric IC detection to provide instant result validation is exceedingly robust and reliable, and can be applied for the analysis of other microbial pathogens of clinical or food interest simply by designing new specific primers and hybridization probes.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2016.08.064.

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