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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.

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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

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