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A thermostabilized magnetogenosensing assay for DNA sequence-specific detection and quantification of *Vibrio cholerae*

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ABSTRACT

Vibrio cholerae is a human pathogen that causes mild to severe diarrheal illnesses and has major public health significance. Herein, we present a thermostabilized electrochemical genosensing assay combining the use of magnetic beads as a biorecognition platform and gold nanoparticles as a hybridization tag for the detection and quantification of *V. cholerae lol*B gene single-stranded asymmetric PCR amplicons as an alternative to the time-consuming classical isolation method. This thermostabilized, pre-mixed, pre-aliquoted and ready-to-use magnetogenosensing assay simplified the procedures and permitted the reaction to be conducted at room temperature. The asymmetric PCR amplicons were hybridized to a magnetic bead-functionalized capture probe and a fluorescein-labeled detection probe followed by tagging with gold nanoparticles. Electrochemical detection of the chemically dissolved gold nanoparticles was performed using the differential pulse anodic stripping voltammetry method. The real-time stability evaluation of thermostabilized assay was found to be stable for at least 180 days at room temperature (25–30 °C). The analytical specificity of the assay was 100%, while its analytical sensitivity was linearly related to different concentrations of 200-mer synthetic target, purified genomic DNA, and bacterial culture with a limit of detection (LoD) of 3.9 nM, 5 pg/μl, and 10^3 CFU/ml, respectively. The clinical applicability of the assay was successfully validated using spiked stool samples with an average current signal-to-cut-off ratio of 10.8. Overall, the precision of the assay via relative standard deviation was $< 10\%$, demonstrating its reliability and accuracy.

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1. Introduction

Vibrio cholerae is classified into three broad serogroups: O1, O139, and non-O1/non-O139 (Baron et al., 2007). Both *V. cholerae* O1 and O139 are the etiological agents of epidemic and pandemic cholera owing to their toxigenicities. Cholera is an acute infectious disease characterized by rapid onset of severe secretory diarrhea with the production of "rice water" stools (Ghose, 2011). Without immediate rehydration treatment, death by fluid loss can occur within hours or days (Weil et al., 2012). *V. cholerae* non-O1/non-O139 typically do not produce cholera toxin and is nonepidemic, but can be pathogenic marked by incidents of extraintestinal infections, bacteremia (Petsaris et al., 2010), and sporadic outbreaks of acute gastroenteritis (Khuntia et al., 2008; Punpanich et al., 2011).

The gold standard method for *V. cholerae* identification in clinical and environmental samples is by classical isolation procedures (Bopp et al., 1999; Choopun et al., 2002) consisting of cultural, biochemical, and immunological assays. However, this classical methodology is time-consuming (requiring several days to complete), difficult, and labor-intensive. Due to the virulence of *V. cholerae*, the development of a low-cost, rapid, sensitive, and specific analytical method is extremely important and highly desirable. To this end, an electrochemical genosensing (DNA-based electrochemical biosensing) assay would be an ideal approach to identify pathogenic *V. cholerae*. It is because this assay has inherent specificity and selectivity provided by nucleic acid sequence-specific hybridization as well as the advantages offered by electrochemical-based biosensing technology such as cost effectiveness, rapid and accurate analysis, simplicity, possibility of miniaturization, and the capability of point-of-care diagnosis. The use of electrochemical genosensing for sequence-specific detection of pathogenic microorganisms using different detection strategies has drastically increased in recent years (Campuzano et al., 2011; Geng et al., 2011; Siddiquee et al., 2010).

The use of nanoparticles as labels in electrochemical genosensing systems is well established (Castañeda et al., 2007; Merkoçi, 2007). Among them, the application of gold nanoparticles (AuNPs) in biosensing assays has been of particular interest owing to its favorable characteristics such as facile chemical synthesis with size-controllability, good stability with a relatively defined nano-scaled

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size distribution, large specific area for surface modification, and excellent biocompatibility with a wide variety of biomolecules. In addition, the electrochemical properties of AuNPs allow detection using simple, portable, and inexpensive electrochemical devices.

Conventional electrochemical genosensing assays are based on a biorecognition layer formed on various kinds of electrode surfaces. However, an inherent problem of this approach is the difficulty of optimizing DNA biorecognition events and electrochemical detection on the same electrode surface (Paleček and Fojta, 2007). In this context, the emergence of magnetic bead (MB)-based detection offers an irresistibly attractive option for overcoming this problem. MBs can serve as an alternative solid support for bioreactions in which probe immobilization and DNA hybridization events can be conducted apart from the electrotransducive surface. The MB-bound complex can be easily separated from the reaction solution using an external magnetic field without the need for centrifugation. In comparison with electrode surface, MBs provide a larger surface area for bioreactions, thereby dramatically improving assay sensitivity (Loaiza et al., 2011).

Since the field of electrochemical genosensing has advanced sufficiently for detection of microorganism (Pedrero et al., 2011), the efforts to design ready-to-use components/reagents that are stable at room temperature are urgently needed to advance the development of automated, integrated, sensing systems. The utility of room temperature-stable reagents has been widely reported in conventional and real-time PCR assays (Qu et al., 2010; Siegmund et al., 2005). In this approach, the PCR reaction mixture is subjected to a drying process in the presence of a stabilizer to retain protein structure during dehydration. The dehydrated components in the mixture are stable at room temperature and, therefore, the requirement of cold transportation and storage can be eliminated. In addition, this stabilization approach reduces pipetting steps and limits the risk of carry-over contamination (Ramanujam et al., 1993).

In this study, a thermostabilized electrochemical genosensing assay combining the use of MBs as a biorecognition platform and AuNPs as a hybridization tag is described for sequence-specific detection and quantification of pathogenic *V. cholerae*. All assay reagents, including the asymmetric PCR reaction components, were dehydrated using carbohydrate polymers (a mixture of trehalose and sucrose) to achieve room temperature stability (thermostabilized). Further, the performance of the magnetogenosensing assay was evaluated using thermostabilized reagents. In order to enhance the specificity and selectivity of the detection platform, a sandwich-type dual-hybridization strategy was employed in which a pair of DNA probes was used to flank the target single-stranded DNA (ssDNA) amplified from the *V. cholerae lol*B gene using asymmetric PCR (aPCR). The *lol*B gene is highly specific to all serogroups of *V. cholerae* (Lalitha et al., 2008). Electrochemical detection of the chemically dissolved AuNPs was performed on a simplified screen-printed electrode using the differential pulse anodic stripping voltammetry (DPASV) method.

2. Material and methods

2.1. Chemicals

Streptavidin-coupled magnetic beads (MB-S) with 1 μm diameters (Dynabeads® MyOne™ Streptavidin T1, 10 mg/ml) were purchased from Invitrogen Dynal (Oslo, Norway).

Hydrobromic acid (HBr) 48%, potassium carbonate (K_2CO_3), ethylenediaminetetraacetic acid (EDTA), Tween-20 (T20), trehalose, and sucrose were obtained from Sigma (Missouri, USA), while ImmunoPure® mouse monoclonal anti-fluorescein IgG (α-FITC), gold(III) chloride trihydrate ($HAuCl_4 \cdot 3H_2O$), sodium citrate tribasic dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$), bovine serum albumin (BSA), bromine (Br_2), and sodium chloride (NaCl) were supplied by Pierce Biotechnology (Rockford, USA), Aldrich (Missouri, USA), Fluka (Buchs, Germany), Amresco (Ohio, USA), R&M Chemical (Essex, UK), and Merck (Darmstadt, Germany), respectively.

PCR components (*Taq* DNA polymerase, *Taq* buffer with $(NH_4)_2SO_4$, $MgCl_2$, and dNTP mix) and PCR-grade water were obtained from Fermentas (Vilnius, Lithuania) and Gibco (NY, USA), respectively. All DNA sequences listed in supplementary data section, Table S1 were synthesized by First BASE Laboratories Sdn. Bhd. (Selangor, Malaysia) and provided in lyophilized form. Reconstitutions and stock aliquots (20 μM) were made in PCR-grade water.

The buffer solutions used in this study included phosphate buffer (PB; 0.01 M potassium phosphate buffer, pH 8.0), PB/BSA (PB with 1% w/v BSA), phosphate-buffered saline/T20 (PBS/T20; 0.01 M potassium phosphate buffer, pH 7.4, containing 0.15 M NaCl and 0.01% v/v T20), and TEN/T20 (5 mM Tris-HCl, pH 7.5, containing 0.5 mM EDTA, 1 M NaCl, and 0.01% v/v T20). All aqueous solutions were made in type 1 ultrapure water (UPW) obtained from a PURELAB Option Q-7BP MK1 purification system (ELGA, Lane End, UK).

2.2. Apparatus

The electrochemical transducer was a simplified two-electrode configuration screen-printed electrode (2-SPE) fabricated by Quasense (Bangkok, Thailand) consisting of a carbon working electrode and an Ag/AgCl combined reference and counter electrode. The electroanalytical performance of 2-SPE was evaluated in a previous study (Low et al., 2012a). All electroanalytical measurements were performed using a μAUTOLAB (III) electrochemical potentiostat/galvanostat (Eco Chemie, Utrecht, The Netherlands) with controlling software NOVA 1.6. The separation of MB from the supernatant was carried out using a DynaMag-Spin magnetic separator (Invitrogen Dynal, Oslo, Norway). The PCR amplification was performed in a Bio-Rad DNA Engine thermal cycler (CA, USA).

2.3. Preparation of thermostabilized AuNP/α-FITC conjugate

AuNPs with an average diameter of 15 nm were chemically synthesized via the reduction of $HAuCl_4$ by sodium citrate (Pinijsuwan et al., 2008; Reddy et al., 2007). The bioconjugation of the AuNPs was performed by mixing 40 ml of AuNPs (adjusted to pH 8.0 using 0.2 M K_2CO_3) with 4 ml of 0.1 mg/ml α-FITC in PB buffer and incubated with tilting mixing for 45 min. The AuNP/α-FITC conjugate was then blocked for 30 min by adding 4.89 ml of 10% w/v BSA in PB to yield a final 1% w/v BSA concentration. The unconjugated α-FITC and free BSA were removed by centrifugation at 16,000g 4 °C for 15 min followed by washing 1 × with an equal volume of PB/BSA buffer. The soft sediment obtained was resuspended in the same buffer and kept at 4 °C as concentrated stock. The optical density at a wavelength of 520 nm (OD_{520}) of the concentrated AuNP/α-FITC conjugate was determined using the PB/BSA buffer as a blank.

For thermostabilization, 10-μl aliquots of PB/BSA buffer comprising AuNP/α-FITC conjugate and carbohydrate polymers with a final concentration of OD_{520}=2% and 25% w/v, respectively, were prepared in 0.5 ml tubes. The aliquots were then subjected to vacuum-drying for 2 h at a pressure of 4.8^{-2} mBar. The dehydrated conjugate was used as a tagging reagent for the assay.

2.4. Preparation of thermostabilized MB-S/capture probe conjugate

A biotinylated capture probe was conjugated onto the MB-S surface to form a biorecognition layer to capture the target ssDNA.

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