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Electrochemical genosensor assay using lyophilized gold nanoparticles/latex microsphere label for detection of *Vibrio cholerae*

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ABSTRACT

Vibrio cholerae is a Gram-negative bacterium that causes cholera, a diarrheal disease. Cholera is widespread in poor, under-developed or disaster-hit countries that have poor water sanitation. Hence, a rapid detection method for *V. cholerae* in the field under these resource-limited settings is required. In this paper, we describe the development of an electrochemical genosensor assay using lyophilized gold nanoparticles/latex microsphere (AuNPs–PSA) reporter label. The reporter label mixture was prepared by lyophilization of AuNPs–PSA–avidin conjugate with different types of stabilizers. The best stabilizer was 5% sorbitol, which was able to preserve the dried conjugate for up to 30 days. Three methods of DNA hybridization were compared and the one-step sandwich hybridization method was chosen as it was fastest and highly specific. The performance of the assay using the lyophilized reagents was comparable to the wet form for detection of 1 aM to 1 fM of linear target DNA. The assay was highly specific for *V. cholerae*, with a detection limit of 1 fM of PCR products. The ability of the sensor is to detect LAMP products as low as 50 ng μl^{-1}. The novel lyophilized AuNPs–PSA–avidin reporter label with electrochemical genosensor detection could facilitate the rapid on-site detection of *V. cholerae*.

1. Introduction

Vibrio cholerae is a highly motile Gram-negative bacterium that possess a single polar flagellum attached to its curved rod body [1]. This micro-size bacteria is the causative agent of cholera, which threatens millions of human lives every year [2]. It transmitted to human via contaminated drinking water, food or feces of infected persons. However, only *V. cholerae* serogroups O1 and O139 are identified as toxigenic species that cause epidemic outbreaks [1]. Affected persons may start to experience watery diarrheal at first. Sickness may become serious once severe dehydration and acidosis occur and may cause death if no immediate treatment is given [2]. Other *Vibrio* species may cause mild diarrheal sickness. In order to effectively reduce morbidity and mortality arising from cholera outbreaks, early detection of this bacterium from contaminated sources is urgently required.

Traditionally, laboratory diagnosis of *V. cholerae* is done based on culturing, raising of specific antibodies, enzyme-linked immunosorbent assay (ELISA), passive hemagglutination test or biochemical tests [1]. Molecular-based detection was discovered to be an improvement to traditional laboratory methods. Examples of molecular-based tests for *V. cholerae* include polymerase chain reaction (PCR) [3,4], loop mediated isothermal amplification (LAMP) [5,6], nucleic acid sequence-based amplification (NASBA) [7,8] and DNA microarray [9,10]. However, molecular-based tests are usually conducted in a laboratory setting and are often time-consuming. Trained personnel are required to perform and interpret the test results. In order to overcome these present difficulties, electrochemical-based method is found to be highly suitable due to its fast signal transduction, simplicity, portability and high sensitivity and selectivity [11–16].

Abbreviations: Ag/AgCl, silver–silver chloride; AuNP, gold nanoparticles; PSA, polystyrene co-acrylic acid; BSA, bovine serum albumin; CP, capture probe; DPASV, differential pulse anodic stripping voltammetry; PAA, poly(allylamine) hydrochloride; PB, phosphate buffer; PCR, polymerase chain reaction; PSA, polystyrene-co-acrylic acid; PSS, poly(sodium 4-styrene) sulfonate; Pt, platinum; RP, reporter probe; SPE, screen printed electrode; LAMP, loop mediated isothermal amplification

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In this study, we describe the development of an electrochemical genosensor test using lyophilized gold nanoparticles/latex microsphere (AuNPs–PSA) as reporter label for detection of pathogenic *V. cholerae*. This electrochemical genosensor platform, which uses AuNPs adsorbed on layer-by-layer modified latex, was previously shown to be highly sensitive [17,18]. However, the genosensor reagents, such as AuNPs–PSA–avidin-reporter probe conjugates require cold storage, which makes transportation of the test reagents problematic and unsuitable for prolonged use in the field. With this in mind, we have modified the existing genosensor platform to a novel lyophilized reagent-based test and evaluated its stability at room temperature. The performance of the assay using lyophilized reagent was tested with PCR products, which were amplified from genomic DNA of *V. cholerae*. Electrochemical measurement was performed using differential pulse anodic stripping voltammetry (DPASV). We also performed preliminary detection of loop mediated isothermal amplification (LAMP) products, with the aim of simplifying the amplification process using LAMP instead of PCR.

2. Materials and methods

2.1. Instruments

Electrochemical measurements were carried out using an Autolab PGSTAT 10 computer-controlled potentiostat with GPES version 4.9 software (Eco Chemie, Netherlands). Transmission electron microscopy (TEM) was performed with a JEOL model JM-2100 (JEOL Ltd., Japan). Nucleic acid concentration was measured using a UV–visible spectrophotometer (DU8000 Beckman Coulter, USA). Disposable electrochemical screen-printed carbon electrodes (SPE) were obtained from Quasense Co. Ltd., Thailand, which consisted of two carbon tracks as working electrode, reference electrode and counter electrode in DPASV measurement. PCR was performed using a MyCycler thermal cycler (Bio-Rad, USA). Gel electrophoresis was performed in a Mini-Sub Cell GT System (Bio-Rad, USA) and the gel was viewed by a Gel Doc™ XR+ system (Bio-Rad, USA). Vacuum drying of genosensor reagents was done using CentriVap micro IR Vacuum Concentrators (LabConco, USA).

2.2. Materials

Hydrogen tetrachloroaurate ($HAuCl_4 \cdot 3H_2O$), avidin from egg white, poly(allylamine) hydrochloride (PAA, MW ~56,000), poly (sodium 4-styrene) sulfonate (PSS, MW ~70,000), D(+)-Trehalose, bovine serum albumin (BSA), $MgSO_4$, and betaine were purchased from Sigma-Aldrich, USA. Styrene and acrylic acid were purchased from Fluka, USA. Ammonium persulfate (APS) was purchased from Riedel-de Haën. D-(Sorbitol), hydrobromic acid (HBr) and bromine water (Br_2) were purchased from R&M Chemicals, UK. D(+)-Sucrose was purchased from QRëC, New Zealand. PCR reagents, DNA ladders, dNTPs and the genomic DNA purification kit were purchased from Fermentas, Lithuania. PCR purification kit was purchased from Promega, USA. *Bst* DNA polymerase was purchased from New England Biolabs, USA. Oligonucleotides and biotin-modified probes were synthesized by 1st BASE, Malaysia. The primer and probe sequences were designed based on the *lol*B gene of *V. cholerae* (GenBank accession number AF227752.1). LAMP primers were designed using primer Explorer version 4 software (http://primerexplorer.jp/elamp4.0.0/index.html). The primers and probes sequences are listed in Table 1.

Table 1
Sequences of primers and probes used in this study. The primer and probe sequences were designed based on the *lol*B gene of *V. cholerae* (GenBank accession number AF227752.1). Reporter probe sequence was modified with 10 adenine bases and a biotin moiety at the 3′ end. Blocking probe consisted of 10 adenine bases and a biotin moiety at the 3′ end.

Name	Sequence (5′→3′)	Length (-mer)
Capture probe		
VCP_1	TCATCGACCTGTAAG	15
Reporter probe		
VCRP_1-Biotin	TTCAGCACGGTTTGAAAAAAAAAAA-Biotin	25
Complementary linear target		
VCLT	TCAAACCGTGCTGAACTTACAGGTCGATGA	30
Non-complementary linear target		
NCLT	GCCCAAACATCCATAGTACTGACATTTCGT	30
Blocking probe		
VCBP	AAAAAAAAAA-Biotin	10
PCR forward primer		
VHMF	TGGGAGCAGCGTCCATTGTG	20
PCR reverse primer		
VHA-AS5	CTCACTGAACCACACTAACGG	21
LAMP forward inner primer		
VC-FIP	TGCGCGGGTCGAAACTTATGATAATTGCGGATCAGGCTTTGT	42
LAMP backward inner primer		
VC-BIP	TTGCTTAAACGCAGTGAGAGTCGTTCAACTTTCAATGGC	39
LAMP forward primer		
VC-F3	TCAAGCTGTTCAACGGGAAT	20
LAMP backward primer		
VC-B3	TTGCTTAAACGCAGTGAGAG	20

3. Methods

3.1. Preparation of SPE surface

Preparation of the carbon SPE surface was done according to Guan et al. [18]. 1 μM of VCP_1 capture probe (Table 1) was immobilized onto a clean SPE by applying 0.1 V for 300 s. After 3 steps of washing with 0.1 M PB, blocking was performed with 0.2% w/v BSA for 20 min. The SPE was washed again with 0.1 M PB for 3 times and kept at 4 °C until use.

3.2. Preparation of AuNP–PSA–avidin conjugate

Colloidal AuNPs were prepared by a Turkevich sodium citrate reduction method [19]. Layer-by-layer modification of latex or polystyrene-co-acrylic acid (PSA) with 500 nm diameter was performed as described by Pinijsuwan et al. [17]. The modified PSA solution was added into colloidal AuNPs at a ratio of 1:20 and allowed to incubate for 30 min. The AuNPs–PSA solution was filtered through 0.2 μm cellulose acetate membrane to remove the excess AuNPs. Next, 3 mg mL^{-1} of avidin (1:5 ratio) was added into the AuNPs–PSA solution and incubated at room temperature for 15 min under stirring condition followed by incubation at 4 °C for 5 min. The mixture was centrifuged at 6000 × *g* for 15 min and washed with sterile 0.1 M phosphate buffer (pH 7.0) twice. The AuNPs–PSA–avidin conjugate was resuspended in 0.5 mL of 0.1 M phosphate buffer (pH 7.0).

3.3. Preparation of AuNPs–PSA–avidin conjugate in lyophilized form

Four types of stabilizers were evaluated for preservation of the AuNPs–PSA–avidin conjugate in lyophilized form. The stabilizers and concentrations that were evaluated are sorbitol (1%, 5%, 10% w/v), sucrose (1%, 5%, 10% w/v), trehalose (1%, 5%, 10% w/v) and BSA (0.25%, 1%, 2% w/v). The AuNPs–PSA–avidin conjugates were mixed with the different concentrations of stabilizers in 0.5 ml tubes and vacuum dried for 4 h. AuNPs–PSA–avidin conjugates without added stabilizer