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Gold-nanoparticle based electrochemical DNA sensor for the detection of fish pathogen *Aphanomyces invadans*

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ABSTRACT

Epizootic ulcerative syndrome (EUS) is a devastating fish disease caused by the fungus, *Aphanomyces invadans*. Rapid diagnosis of EUS is needed to control and treat this highly invasive disease. The current diagnostic methods for EUS are labor intensive. We have developed a highly sensitive and specific electrochemical genosensor towards the 18S rRNA and internal transcribed spacer regions of *A. invadans*. Multiple layers of latex were synthesized with the help of polyelectrolytes, and labeled with gold nanoparticles to enhance sensitivity. The gold–latex spheres were functionalized with specific DNA probes. We describe here the novel application of this improved platform for detection of PCR product from real sample of *A. invadans* using a premix sandwich hybridization assay. The premix assay was easier, more specific and gave higher sensitivity of one log unit when compared to the conventional method of step-by-step hybridization. The limit of detection was 0.5 fM (4.99 zmol) of linear target DNA and 1 fM (10 amol) of PCR product. The binding positions of the probes to the PCR amplicons were optimized for efficient hybridization. Probes that hybridized close to the 5′ or 3′ terminus of the PCR amplicons gave the highest signal due to minimal steric hindrance for hybridization. The genosensor is highly suitable as a surveillance and diagnostic tool for EUS in the aquaculture industry.

1. Introduction

The emergence and spread of fish diseases are a major concern to the aquaculture industry. Epizootic ulcerative syndrome (EUS), which is also known as mycotic granulomatosis, red spot disease or ulcerative mycosis, is one of the most devastating diseases affecting several commercially important wild and cultured freshwater and estuarine finfish species [1–3]. EUS is caused by an invasive oomycete fungus, *Aphanomyces invadans* (*A. invadans*). EUS is diagnosed by culturing, gross observation and histopathological examination of mycotic granulomas in infected fish tissues [4]. Molecular techniques such as polymerase chain reaction (PCR) [5,6] and fluorescent in-situ hybridization (FISH) [6] have also been used to facilitate diagnosis of EUS.

Electrochemical DNA biosensors have been used to detect diseases as they are of low cost, offer higher sensitivity, portability, greater analyte discrimination, are less time consuming and easy to use. Several electrochemical approaches for DNA biosensors have been studied, such as direct detection of DNA capture probe and target hybridization using electrochemical labeling with enzymes, redox active species or nanoparticles to enhance the signal responses. Nanoparticles such as carbon-nanotubes [7], quantum dots [8], silver [9] and gold nanoparticles [10] have been used as labels in biological assays. Nanoparticles are capable of

Abbreviations: Ag/AgCl, silver–silver chloride; AuNPs, gold nanoparticles; BSA, bovine serum albumin; CP, capture probe; DPASV, differential pulse anodic stripping voltammetry; EUS, epizootic ulcerative syndrome; ITS, internal transcribed spacer regions; 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; TEM, transmission electron microscope

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improving signal response; thus, improving the sensitivity of DNA biosensors [11–14]. Among the various nanomaterials, gold nanoparticles are the most frequently used in biological assays since they have several attractive features, such as high surface-to-volume ratios and excellent surface immobilization properties. Hence, gold nanoparticle-based DNA biosensors are suitable platforms for development of a portable, highly sensitive and specific on-site diagnostic tool for EUS in the aquaculture industry.

In this study, we describe the novel application of electrochemical genosensor using gold nanoparticles (AuNPs) coated on multiple layers of latex for the detection of the EUS pathogen, *A. invadans*. We demonstrate the use of this genosensor with a 208 bp PCR product of the 18S rRNA-internal transcribed spacer regions (ITS) from real sample of the fungus. Premix sandwich hybridization assay was employed, whereby capture probes were immobilized on screen printed carbon paste electrode surface to capture the PCR product. Subsequently, reporter probes conjugated to the AuNP–latex spheres were hybridized with the PCR product. After hybridization, the gold nanoparticles were detected by differential pulse anodic stripping voltammetry (DPASV).

2. Materials and methods

2.1. 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) and bovine serum albumin (BSA) were purchased from Sigma Aldrich, USA. Styrene and acrylic acid were purchased from Fluka, USA. Ammonium persulfate (APS) was from Riedel-de Haën. Hydrobromic acid (HBr) and bromine water (Br_2) was purchased from R&M Chemicals, UK. PCR reagents, DNA ladders and the genomic DNA purification kit were purchased from Fermentas, Lithuania. PCR primers and probes were purchased from 1st BASE, Malaysia. Biotin-modified probes were purchased from Integrated DNA Technologies, USA. PCR purification kit was purchased from Promega, USA.

Primers and probes sequences were designed based on the 18S rRNA and ITS regions of *A. invadans* (GenBank accession number AY283642). Sequence alignment with closely-related species was performed using ClustalW2 software (http://www.ebi.ac.uk/Tools/msa/clustalw2). For optimization of the assay, one set of capture (EUS-CP1) and reporter (EUS-RP1) probes were used. For studying the effect of different probe binding positions on hybridization efficiency, three sets of capture and reporter probes targeting the 5′ region ("top"), middle and 3′ region ("bottom") of the sense strand of the PCR product were designed. All reporter probes sequences were followed by ten adenine bases and modified with a biotin moiety at the 3′ end. Blocking probes consisted of ten adenine bases and a biotin at the 3′ end. The primers and probes sequences are listed in Table 1.

2.2. Instruments

Electrochemical experiments were performed using an Autolab PGSTAT 10 computer-controlled potentiostat with GPES version 4.9 software (Eco Chemie, Netherlands). Transmission electron microscopy (TEM) was carried out with a JEOL model JM-2100 (JEOL Ltd., Japan). Nucleic acid quantitation was performed using a UV–visible spectrophotometer (DU8000 Beckman Coulter, USA). Disposable electrochemical screen-printed carbon electrodes (SPE) were purchased from Quasense Co. Ltd., Thailand. The SPE consisted of a carbon track as working electrode and Ag/AgCl track as reference electrode. Platinum (Pt) wire was used as counter electrode in DPASV measurement. PCR was performed using 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 Gel Doc™ XR+ system (Bio-Rad, USA).

3. Methods

3.1. Synthesis of latex particles

Polystyrene-co-acrylic acid (PSA) or latex particles with 500 nm diameter were synthesized as described by Pinijsuwan et al. [10]. Briefly, 19 g of deionized water was purged with nitrogen gas for 1 h under stirring at 350 rpm in a three-necked flask submerged in a water bath. 20 g of styrene and 0.5 g of acrylic acid were added under stirring at 350 rpm, 70 °C while continuously purging with nitrogen gas. Polymerization was started by adding 0.2 g of APS into 10 mL of deionized water and allowed to proceed for 7 h. The resulting PSA latex particles were recovered by centrifuging with distilled water twice at 13,000 rpm for 20 min.

Table 1
Sequences of primers and probes used in this study.

Name	Sequence (5′→3′)	Length	References
PCR Primers			
ITS11	GCCGAAGTTTCGCAAGAAAC	555 bp	Phadee et al. [5]
ITS23	CGTATAGACACAAGCACACCA		This study
ITS-F	TACTGAAACCTTAGCCATCAG	208 bp	
ITS-R	GTATTAACGGACACTGATACA		
Capture probes (CP)			
EUS-CP1	ACGAAATGTCAGTAC	15 Bases	This study
EUS-CP-top	GATAGCTTGTAATCA	15 Bases	This study
EUS-CP-middle	GCGAACTGCGATACG	15 Bases	This study
EUS-CP-bottom	GCACTTTCGGGTTAG	15 Bases	This study
Reporter probes (RP)			
EUS-RP1	TATGGATGTTTGGGCAAAAAAAAAA-Biotin	25 Bases	This study
EUS-RP-top	TACAACTTTCAACAGAAAAAAAAAA-Biotin	25 Bases	This study
EUS-RP-middle	TAATGCGAATTGCAGAAAAAAAAAA-Biotin	25 Bases	This study
EUS-RP-bottom	TCCTGGAAGTATGTCAAAAAAAAAA-Biotin	25 Bases	This study
Blocking probe (BP)			
EUS-BP	AAAAAAAAAA-Biotin	10 Bases	This study