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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

3.2. Preparation of multi-layer latex particles with polyelectrolytes

PSA particles (5 mg mL^{-1}) were sequentially incubated in 1 mL of PAA, PSS and PAA (1 mg mL^{-1} in 0.5 M NaCl each). Each step was performed for 30 min followed by three washing steps with sterile deionized water before proceeding to the next incubation step. The multi-layer latex particles with polyelectrolytes were stored in deionized water.

3.3. Preparation of AuNP–latex particles

Colloidal AuNPs were prepared by the sodium citrate reduction method [15]. The AuNPs were adsorbed onto the multi-layer latex particles by adding an aliquot of latex particles into colloidal gold solution in the volume ratio of 1:20 and incubated for 30 min. The AuNP–latex particles were recovered by filtration with 0.2 μm cellulose acetate filter to remove unbound AuNPs. The AuNP–latex particles were kept in 0.5 mL sterile distilled water at 4 °C until use.

3.4. Conjugation of DNA reporter probes to AuNP–latex particles

DNA reporter probes were conjugated to the AuNP–latex via avidin–biotin binding. Firstly, colloids of AuNP–latex were conjugated to avidin as described by Mao et al. [16]. Avidin solution (3 mg mL^{-1}) was added to AuNP–latex suspension in 1:5 ratio; the solution was adjusted to pH 10 and incubated at room temperature for 15 min under stirring. After that, the solution was kept at 4 °C for 5 min and then centrifuged at 10,000 rpm for 15 min, followed by washing with sterile 0.1 M phosphate buffer (PB) solution. The pellet was resuspended in 0.1 M PB. 100 μL of biotinylated DNA reporter probe and blocking probe mixture (at 1:500 ratio) were added into 0.5 mL of the avidin conjugated AuNP–latex particles and incubated for 2 h. BSA (0.2% w/v) was added as a blocking agent and incubated for 30 min. Excess BSA was removed by centrifugation at 10,000 rpm for 15 min, followed by washing step with 0.1 M PB, pH 7.0. The DNA-AuNP–latex complex were then re-dispersed in 5 × SSPE buffer (0.75 M NaCl, 50 mM NaH_2PO_4 and 5 mM EDTA) and kept at 4 °C until use.

3.5. Preparation of electrode surface

SPE was cleaned by washing in 0.1 M PB, pH 7 and dried with N_2 gas. 20 μL of DNA capture probe (1 μM) was applied onto the electrode surface to cover both carbon working and Ag/AgCl reference electrodes. DNA capture probe was immobilized on the electrode surface by electrostatic adsorption. A potential of +100 mV was applied for 30 s, followed by 3 washing steps using sterile distilled water. Carbon working electrode was blocked by incubation in 0.2% BSA at room temperature for 20 min. The SPE was washed again with sterile distilled water and dried with N_2 gas.

3.6. DNA hybridization with AuNP–latex particles

Two hybridization procedures were tested: step-by-step (sequential) and premix sandwich hybridization. An overview of the DNA hybridization procedures is shown in Fig. 1. In the step-by-step hybridization procedure, 10 μL of target DNA was applied onto the carbon working electrode and allowed to hybridize with the immobilized capture probe for 20 min at room temperature, followed by washing 3 times with sterile 0.1 M PB, pH 7.0. After that, AuNP–latex particles were added onto the carbon working electrode and incubated again for 20 min. The SPE was washed 3 times with 0.1 M PB and dried with N_2 gas.

The premix sandwich hydridization procedure is a slight modification of the step-by-step procedure, whereby pre-hybridization of target DNA to AuNP–latex particles was performed before application onto SPE. Target DNA was pre-hybridized with 10 μL AuNP–latex particles for 20 min in a microcentrifuge tube before applying onto the carbon working electrode. Hybridization of capture probe to the target DNA-AuNP–latex was performed for 20 min, followed by washing 3 times with 0.1 M PB and then drying with N_2 gas. For testing the specificity of the target response, non-complementary

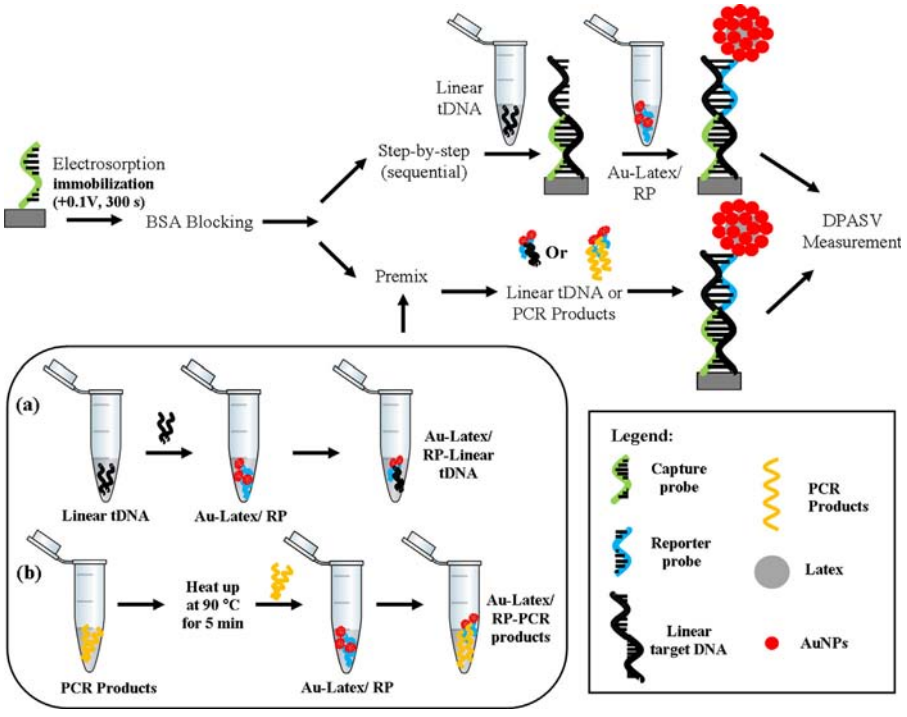

Fig. 1. Schematic diagram of the genosensor assay based on multi-layer latex–gold nanoparticles. Bottom left inset shows the hybridization strategy for linear target DNA (a) and PCR product (b).

and 3 bp mismatched DNA were used instead of complementary target DNA.

3.7. Electrochemical detection of DNA hybridization

The carbon working electrode was cut from the SPE and immersed in acid detection medium (1 M HBr/0.1 mM Br_2) to perform DPASV [16]. All measurements were performed in five replicates.

3.8. Detection of PCR product from A. invadans

A. invadans genomic DNA was kindly provided by Aquatic Animal Health Research Institute (AAHRI), Department of Fisheries, Thailand. PCR was performed with the primers ITS11 and ITS23 to generate a 555 bp PCR product. The 555 bp PCR product was used as template for a second round of nested PCR with internal primers ITS-F and ITS-R to produce a smaller PCR product of 208 bp. PCR product of 237 bp amplified from *V. cholerae* was used as non-complementary target. PCR products were purified using a PCR purification kit. Before hybridization, the PCR product was heated at 90 °C for 5 min to denature the double-stranded PCR product to single-stranded DNA. Hybridization was performed using the premix sandwich hybridization procedure as described in the previous section.

4. Results and discussion

4.1. Characterization of synthesized AuNP–latex particles

The maximum absorbance wavelength of colloidal AuNPs solution was measured at 520 nm, which indicated the diameter of the synthesized AuNPs was 13 nm as shown in Fig. 2 [15]. The amount of synthesized AuNPs in a 0.25 mM solution was calculated as 2.22×10^{12} particles mL^{-1} from the density of Au at 19.3 g cm^{-3} (assuming that 100% of Au ions were converted to AuNPs) [17]. Hence, it was estimated that ~1460 AuNPs were adsorbed onto the polyelectrolyte-coated latex via electrostatic interaction.

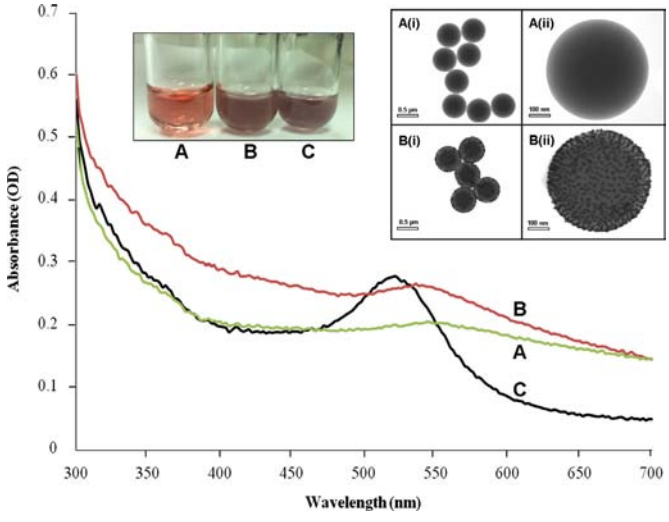

Fig. 2. Absorbance reading of colloidal AuNP solution (A), AuNPs–latex–avidin complex (B) and AuNPs–latex–avidin complex conjugated with DNA reporter probes (C). Top left inset shows the color change of the colloidal AuNPs solution before (A) and after modification (B and C). Top right inset shows the TEM images of polyelectrolyte-coated latex (at 2 different magnifications) before [A(i) and (ii)] and after [B(i) and (ii)] conjugation with AuNPs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Avidin–biotin interaction was used in this study to functionalize the surface of the AuNP–latex with biotin-labeled reporter probe. The strong and stable non-covalent binding between avidin and biotin prevent loss of the AuNP–latex particles during multiple washing steps. After adsorption of avidin onto AuNPs, the color of the solution changed from wine red to maroon (top left inset, Fig. 2). The maximum absorbance wavelength of the solution shifted right to 545 nm due to a change in the surface plasmon resonance of the colloidal AuNPs.

Latex particles were modified with multiple layers of polyelectrolytes to increase and provide positively charged surface for the adsorption of AuNPs onto the latex particles. The AuNP–latex particles were visualized using TEM, as shown in Fig. 2 (top right inset). After AuNPs conjugation, the smooth surface of the polyelectrolyte-coated latex spheres (Fig. 2A) was uniformly covered by the AuNPs [Fig. 2B(i) and B(ii)].

Based on the TEM images, the diameter of the AuNP–latex particles was estimated as ~564 nm with a mass of 9.865×10^{-14} g per particle (density of PSA: 1.05 g cm^{-3}) [10]. The size of the polyelectrolyte-coated latex was within the 500 nm size range, as previously described [10].

4.2. DNA hybridization and electrochemical detection

In the sandwich hybridization assay, target DNA binds to capture probes that were immobilized onto the SPE surface, followed by hybridization with reporter probes labeled with AuNP–latex. The sandwich hybridization method is more suitable for real sample detection than direct hybridization, whereby target DNA is directly applied onto the SPE surface, followed by hybridization with labeled reporter probes. The sandwich hybridization method allows prior immobilization of the SPE with capture probes before the experiment is started. Therefore, multiple samples can be processed and undergo hybridization at the same time. This strategy also improves the selectively of the signal and reduces the hybridization of mismatched targets.

The sandwich hybridization assay was performed using two procedures, step-by-step (sequential) or premix hybridization. In the latter, target DNA was pre-hybridized first with reporter probe, i.e. the premix step, before applying onto the electrode for hybridization with capture probe.

The specificity of the assay was tested with 1 pM of complementary, non-complementary and 3 bp mismatched target DNA of *A. invadans*. Complementary target DNA produced a higher current response (> 60%) compared to the 3 bp mismatched and non-complementary DNA. A blank sample that contained only DNA-free buffer gave a low background signal. Hence, the capture and reporter probes were highly specific towards the *A. invadans* complementary DNA and were able to distinguish a 3 bp mismatch in the middle of the sequence.

Next, the signal responses for various concentrations of *A. invadans* DNA were tested. The concentration of both capture and reporter probes was kept constant at 1 μM, while the concentration of target DNA varied from 5 fM to 5 pM. The cut-off value was set as 3 standard deviation (S.D.) above the signal of non-complementary DNA. For the step-by-step hybridization assay, the lowest limit of detection was 5 fM, defined as an average current peak response that was three times higher than the response produced by 50 fM non-complementary DNA (dashed line in Fig. 3). This corresponds to approximately 0.912 fg (0.1 amole) of target DNA (~60,000 DNA molecules). When the premix hybridization assay was performed with DNA concentration ranging from 0.05 fM to 1 pM, the lowest limit of detection was 0.5 fM, which is equivalent to approximately 45.5 ag (4.99 zmol) of target DNA (~3,000 DNA molecules) (solid line in Fig. 3). The premix hybridization method produced better sensitivity, i.e. a ten-fold lower

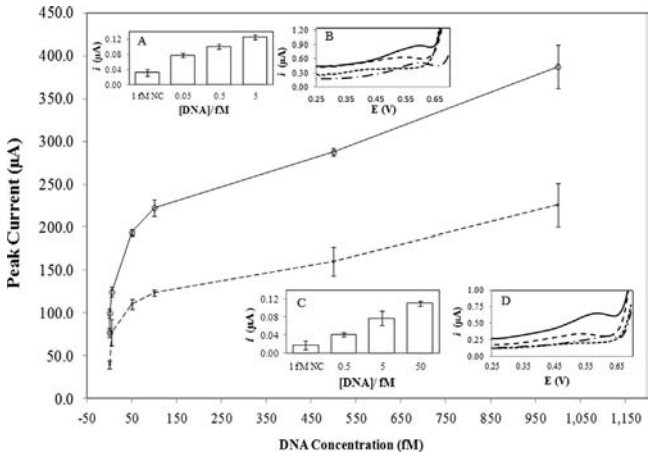

Fig. 3. Detection of target DNA using the premix (solid line) and step-by-step (dash line) sandwich hybridization assays. Inset A and C show the average peak current response of the 3 lowest concentrations of target DNA compared to non-complementary DNA for premix (A) and step-by-step (C). Inset B and D show the DPASV responses of the 3 lowest concentrations (dash–dot line, dash line and solid line, respectively) of target DNA compared to non-complementary DNA (square dot line) for premix (B) and step-by-step (D).

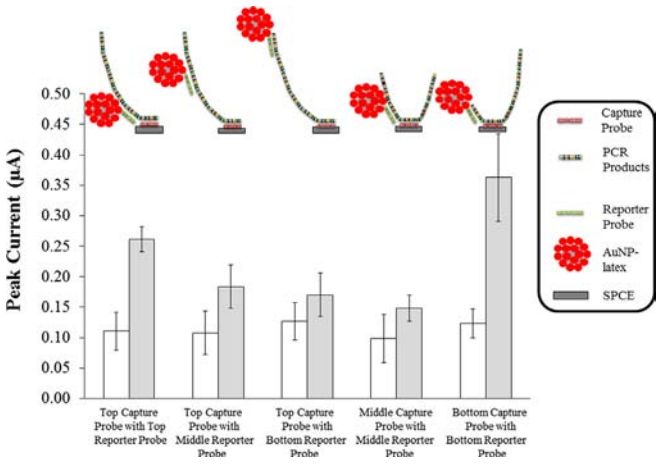

Fig. 4. Average peak current response from the hybridization of 5 fM (unfilled bar) and 100 pM (gray bar) of *A. invadans* PCR product. An illustration of the different combinations of capture and reporter probes with their binding positions is shown on top.

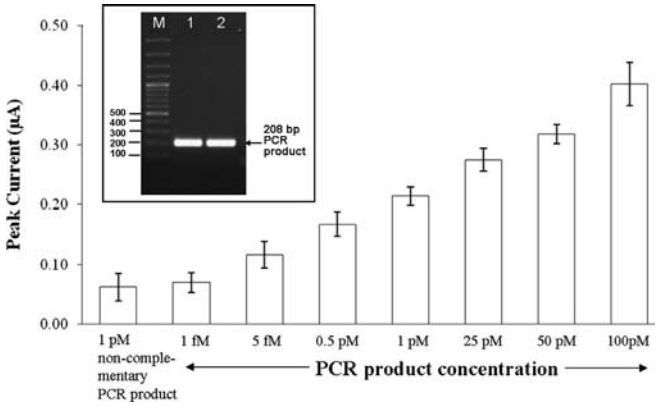

Fig. 5. Histogram showing the average peak current response from the hybridization of 1 fM–100 pM of *A. invadans* PCR product, and 1 pM of non-complementary PCR product. Inset shows the 208-bp PCR product of *A. invadans* detected by gel electrophoresis (M: 100 bp DNA ladders; lanes 1 and 2: *A. invadans* PCR product).

limit of detection, compared to the step-by-step method. In addition, the premix hybridization method is easier and more practical since it reduces the hybridization time by half as this step can be performed in parallel to the capture probe immobilization step. When the immobilization step is completed, hybridization of the target DNA-AuNP–latex complex to the capture probe can proceed immediately without waiting for the sequential hybridization of target DNA to capture probe followed by reporter probe-AuNP–latex. Multiple samples can be processed simultaneously with this method.

4.3. Evaluation of different probe binding regions on hybridization efficiency

Hybridization with long target DNA such as PCR products presents additional challenges as the PCR products are double-stranded and prone to secondary structures. Stable secondary structures at the probe binding regions of the PCR product can hamper probe binding to their complementary sequences. Steric hindrance and electrostatic repulsion of long PCR products or capture probes may also affect hybridization efficiency, thus lowering the sensitivity of the assay. Thus, probe binding regions should be carefully selected during probe design.

Capture and reporter probes that bind to 5 different regions within the 208 bp PCR product of *A. invadans* were designed to determine the best region that gave the best signal response. Capture and reporter probes were designed to bind to the "top" (within 50 bp from the 5′ end of the sense strand), "middle" (within 80–130 bp from the 5′ end of the sense strand) and "bottom" (within 50 bp from the 3′ end of the sense strand) regions of the PCR product (Fig. 4).

Using 100 pM of PCR product, the combination of "bottom" capture and "bottom" reporter probes produced the best result. This probe binding position provided the best accessibility to both capture and reporter probes for binding to the PCR product. A similar study by Zhang et al. [23] also observed that the signal was highest when both probes were located at the distal end of the target. The second highest signal was obtained with "top" capture and "top" reporter probes. The combination of "bottom" and "top" probes, which binds to both ends of the PCR product gave a lower signal. This lower signal could be attributed to the loss of the reporter probe-AuNP–latex complex as the single stranded capture probe could not support the large size of the complex, leading to its collapse.

4.4. Detection of PCR product from A. invadans

Real sample detection of *A. invadans* was performed using 7 concentrations of PCR products ranging from 1 fM to 100 pM when 1 pM of non-complementary PCR product (*V. cholerae*) was tested. A steady increase in peak current response with concentration was observed (Fig. 5). This sensor shows that detection was possible to as low as 1 fM of amplified PCR products, which corresponds to 1.35 fg (10 amol) of target DNA in 10 μL immobilization solution, i.e., $\sim 5.92 \times 10^3$ DNA molecules. The sensitivity of the assay is better than the previously published limit of detection of ~500 fg of PCR product of *A. invadans* [5,6]. We also found that the sensitivity of our AuNPs–latex-based hybridization method is comparable to other previously reported gold nanoparticle-based sensors (Table 2). Zhang et al. [23] reported a similar sensitivity level of 60 fg of *E. coli* PCR product using a sandwich-type electrochemical biosensor with enzyme labeling.

The high sensitivity of the assay makes it a suitable screening test for EUS in the absence of observable gross or histopathological symptoms in the fish tissues.

Table 2
Comparison of the sensitivity of DNA sensors that are based on the detection of gold nanoparticle label.

Label	Target gene/organism	Detection method[a]	Detection limit	Ref.
DNA-AuNP	Human cytomegalovirus	ASV/DPV	5 pM	[12]
DNA-AuNP/Au enh	Human cytomegalovirus	ASV/CV	0.6 fM	[19]
MNP-DNA-AuNP	BRCA breast cancer	DPV	0.198 μg mL^{-1}	[20]
DNA-AuNP/latex	*E. coli*	ASV/DPV	0.5 fM	[10]
DNA-MNP/AuNP	*Mycobacterium sp.*	DPV	0.01 ng μL^{-1}	[21]
MNP-DNA-AuNP/α-FITC	*Vibrio cholerae*	ASV/DPV	3.9 nM	[22]
DNA-AuNP/latex	*A. invadans*	ASV/DPV	1 fM	*This work*

[a] ASV, anodic stripping voltammetry; DPV, differential pulse voltammetry; CV, cyclic voltammetry; enh, enhancer; MNP, magnetic nanoparticle; FITC, fluorescein isothiocyanate.

5. Conclusions

In summary, we are the first to describe an electrochemical detection assay for the EUS fish pathogen, *A. invadans*. The use of AuNPs–latex particles coupled with the rapid and convenient premix sandwich hybridization assay provides a practical and ideal biosensor platform for detection of PCR products from real sample of *A. invadans*. Lower limit of detection was obtained compared to previously reported PCR assays for the fungus. The sensor has shown good selectivity with negligible response to non-complementary DNA sequences. In addition, the cost-effectiveness and portability of the biosensor make it highly suitable for use in the field, especially for sampling suspected fish cases in the wild or in farms.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.09.016.

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