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Nanoparticle-enhanced electrochemical biosensor with DNA immobilization and hybridization of *Trichoderma harzianum* gene

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ABSTRACT

The genus *Trichoderma* is a soil-borne fungi which in numerous reports has been successfully used as a biological control agent against various plant pathogens. The identification of *Trichoderma* species worldwide is currently deduced from micro-morphological descriptions which are tedious and prone to error. Electrochemical approaches are currently being developed for the detection and analysis of DNA. In the present study, an electrochemical DNA biosensor was successfully developed based on ionic liquid (e.g., 1-ethyl-3-methylimidazolium trifluoromethanesulfonate ([EMIM][Otf])), ZnO nanoparticles and a chitosan (CHIT) nanocomposite membrane on a modified gold electrode (AuE). A single-stranded DNA probe was immobilized on this electrode. Methylene blue (MB) was used as the hybridization indicator to monitor the hybridization reaction of the target DNA. Under optimal conditions using differential pulse voltammetry (DPV), the target DNA sequences were detectable at concentration ranges of 1.0×10^{-18}–1.82×10^{-4} mol L^{-1}, and the detectable limit was 1.0×10^{-19} mol L^{-1}. The developed DNA biosensor enables the study of hybridization with crude DNA fragments and the results of this study confirm that this DNA biosensor provides a fast, sensitive and convenient way for the species level identification of *Trichoderma harzianum*.

1. Introduction

The genus *Trichoderma* is a saprophytic fungi which can be found in all climatic zones of the world. The genus *Trichoderma* was first described in 1794 by Persoon [1] and there are reportedly almost 130 species [2]. The conventional technique used to identify and classify *Trichoderma* species is based on phenotypic traits which include morphological and biochemical characteristics; however, it is quite difficult to differentiate between very closely related species. DNA sequences can be used for the identification of fungi at the species level, but approximately 40% of the GenBank database sequences of *Trichoderma* species have been erroneously identified or remain unidentified at the species level [3,4]. There are a large number of sequences deposited in the GenBank that are incorrectly labeled and unless remedied they will continue to be assigned to the wrong taxa [5,6]. Under these conditions, the best conceivable conception of the molecular data and morphological characteristics of isolates is achieved using detailed photographs or drawings of the specimens to prevent any controversial identification at the species level [7]. To easily recognize and ensure the quality of results it is also possible to go back to the main source of the information. Otherwise, species level identification is difficult to do correctly, especially when it is necessary to rely on a source that has made a misidentification.

Currently, several conventional identification techniques have been established such as polymerase chain reaction (PCR) [8], culture and colony counting [9], immunological techniques [10] and fluorescence-based assays using organic dye molecules [11]. The majority of these approaches is laborious, complex, time consuming, and lack the necessary levels of detectability and specificity towards the target [12]. Therefore, new, rapid, selective and sensitive detection techniques are required in clinical diagnosis, disease control, environmental monitoring and food safety. DNA biosensor technologies are rapidly developing as an alternative to the classical gene assays, due to the advantages of low cost, rapid analysis time, simplicity of operation, and possibility of

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miniaturization [13]. Moreover, it is a device that combines a DNA probe consisting of a biological recognition agent and a single-stranded DNA with a transducer. The selectivity of this device is due to the former, while its sensitivity is provided by the latter [14]. Biosensors take advantage of hybridization events to detect target DNA sequences [15].

Nucleic acid based techniques are widely used for analytical applications due to their powerful recognition properties [16,17]. Selection of the nucleic acid for a DNA-based biosensor mainly depends on the event to be sensed. The main purpose of the biosensor is to detect a DNA sequence using a single-stranded DNA with a short oligonucleotide as the biosensing element. Several aspects that are crucial in the development of hybridization biosensors are sensitivity, detection of low concentrations of DNA and ability to detect a point mutation. Traditional methods of detecting the hybridization event are too slow and need special preparation. Thus, there is great interest in developing biosensors based on electrochemical hybridization.

The use of nanoparticles (NPs) in biosensors has gained importance as an emerging area of research. The integration of NPs into biodevices has been reported by several researchers [18–21]. Nanobiosensors have been invented for the specific detection of biological molecules, e.g., nucleic acids [22], proteins [23] and enzymes [24] and as well as infectious agents [25]. The nanostructures have great advantages including high surface area, nontoxicity, good environmental acceptability, inexpensive, electrochemical activity and high electron communication features. Zinc oxide (ZnO) nanoparticles are one of the most important nanomaterials due to their unique electronic, metallic and structural characteristics [26]. Most nanoparticle-sensing research has focused on the ability of surface-confined ZnO to promote electron-transfer reactions with electroactive species. Electrochemical biosensors have taken great advantage of NPs to increase the surface area of the electrode, and to enhance electronic properties and electrocatalytic activity in order to improve their speed, detection ability and selectivity [18].

Ionic liquids (ILs) consist of large organic cations and various kinds of anions that exist in the liquid state at high temperatures of more than 100 °C [27,28]. ILs have been receiving increased attention due to their unique chemical and physical properties, such as high chemical and thermal stability, negligible vapor pressure, high ionic conductivity, low toxicity, and ability to dissolve a wide range of organic and inorganic compounds [29,30]. ILs are extensively used as modifiers on electrode surfaces in the fabrication of gas sensors [31] and biosensors [32] due to their unique electrochemical properties, such as high ionic conductivity and relatively wide electrochemical window. Moreover, ILs also hold great promise for green chemistry applications in general and for electrochemical applications in particular. Chitosan (CHIT) as a biocompatible polymer was selected for the application in this study due to its low-cost, hydrophilicity, nontoxicity, and excellent film-forming ability. The combination of CHIT–ILs as a composite material has great potential in the application of electrochemical biosensors.

The main objective of this research was to develop a simple and fast method to create a well-defined recognition surface for the immobilization and hybridization of the *Trichoderma harzianum* gene. The incorporation of ILs, ZnO nanoparticles and CHIT nanocomposite membrane were explored to increase the electrochemical signals of the redox indicator and to enhance the sensitivity for DNA detection. The analytical performance of the designed electrochemical biosensor was evaluated for the detection of a specific sequence related to a *T. harzianum* gene based on the internal transcribed spacer 1 and 2 regions of the rDNA. The developed DNA biosensor was also applied in the analysis of crude DNA fragments.

2. Materials and methods

2.1. Apparatus and electrodes

The voltammetry measurements were carried out with a μAUTOLAB (Ecochemie, Netherlands) potentiostat using the software package General Purpose Electrochemical System (GPES 4.9, Ecochemie). A Metrohm gold disk electrode (3 mm) was used as the electrode to be coated for the covalent immobilization of the oligonucleotide probe. An Ag|AgCl|KCl 3M reference electrode and a platinum (Pt) wire counter electrode were also employed. The detection was carried out in a 10 ml standard electrochemical cell comprised of AuE as working electrode, an Ag|AgCl|KCl 3M electrode as reference electrode, and a platinum wire as counter electrode. The AuE surface was cleaned by Ultrasonic cleaner model clean-02 (above the 18/20 kHz range), after that polished on a weighing paper to a smoothed finish before use. The solution pH was measured with a model pH-2700 (Eutech Instruments). The convective transport was provided by a magnetic stirrer. All experiments were carried out under room temperature condition of 25 ± 2 °C.

2.2. Reagents and solutions

Methylene blue (MB) was purchased from Sigma (USA). Stock solutions of MB (1 mM) were prepared in a 50 mM Tris–HCl, 20 mM NaCl buffer solution (pH 7.2). Diluted solutions were prepared by appropriate dilution with the same buffer solution.

The PCR amplified real samples were collected from the Mycology and Plant Pathology Laboratories, Faculty of Science, Universiti Putra Malaysia. The tested oligonucleotides were synthesized by First BASE Laboratories Sdn Bhd, Selangor, Malaysia. The sequences are listed in Table 1.

DNA oligonucleotide stock solutions (nominally 1.82×10^{-4} mol L^{-1} concentration DNA) were prepared in a TE buffer solution containing 10 mM Tris–HCl and 1 mM EDTA (pH 8.0) and kept frozen. Additional dilute solutions of the oligomers were prepared in a 50 mM Tris–HCl and 20 mM NaCl buffer solution (pH 7.2). A 40 mM mercaptopropionic acid (MPA, Research Chemicals Ltd.) solution, prepared in a 75/25% (v/v) ethanol/water mixture, was employed for the formation of the monolayer. A 5 mM *N*-hydroxysulfosuccinimide (NHSS) sodium salt (Fluka) and 2 mM 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) (Sigma) solutions were prepared in a 0.05 M phosphate buffer (pH 5.5).

A 50 mM Tris-(hydroxymethyl) aminomethane–HCl (Tris–HCl) (Sigma, USA) buffer solution containing 20 mM NaCl (Sigma, USA) (pH 7.2) was used as a supporting electrolyte buffer for the differential pulse voltammetry (DPV) measurements and as a washing buffer. This was prepared in deionized water and then used. The hybridization buffer was prepared in a 0.3 M NaCl and 30 mM sodium citrate buffer solution, pH 7.0 (2× SSC buffer). All chemicals used in the experiments were of analytical-reagent grade. Deionized water was obtained from a Millipore Milli-Q purification system.

2.3. Self assembly monolayer (SAM)

The pretreated gold electrode (AuE) was immersed in a 40 mM mercaptopropionic acid (MPA) solution in EtOH/H_2O (75/25, v/v) for at least 15 h. Then, the electrode was rinsed with deionized water to remove unbound MPA. The MPA modified electrode was immersed in a thiol derivatized probe solution (Oligo-C_6-SH, Tris–HCl buffer, pH 8.0) for at least 24 h at room temperature. Then it was washed with washing solution (50 mM Tris–HCl + 20 mM NaCl, pH 7.2) for 30 s to remove unbound oligonucleotides.