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ORIGINAL PAPER

Development of electrochemical DNA biosensor for *Trichoderma harzianum* based on ionic liquid/ZnO nanoparticles/chitosan/gold electrode

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Abstract Electrochemical DNA biosensor was successfully developed by depositing the ionic liquid (e.g., 1-ethyl-3-methylimidazolium trifluoromethanesulfonate ([EMIM][Otf])), ZnO nanoparticles, and chitosan (CHIT) nanocomposite membrane on a modified gold electrode (AuE). The electrochemical properties of the [EMIM][Otf]/ZnO/CHIT/AuE for detection of DNA hybridization were studied. Under optimal conditions using cyclic voltammetry, the target DNA sequences could be detected in the concentration range of 1.0×10^{-18} to 1.82×10^{-4} mol L^{-1}, and with the detection limit of 1.0×10^{-19} mol L^{-1}. This DNA biosensor detection approaches provide a quick, sensitive, and convenient method to be used in the identification of *Trichoderma harzianum*.

Keywords DNA electrochemical biosensor · DNA hybridization · ZnO nanopaticles · Ionic liquid · Chitosan

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Introduction

Trichoderma harzianum is a ubiquitous soil species and is used as a biological control to protect plants against root, seed, and foliar diseases, and storage rots [1, 2]. Results from field trials showed that the isolates are well adapted to different environmental conditions, protecting several crops, as well as controlling various plant pathogens [3]. Therefore, numerous number of *T. harzianum* strains could be selected for their activity against the casual pathogens on different crops and specific environmental factors [4]. Thus, rapid and sensitive detection methods are required to meet the challenge for the detection of *T. harzianum* strains. Practical challenges for timely and effective viability detection include speed and portability.

DNA is a fundamental biomolecule which stores genetic information as it plays a vital role in determination of hereditary characteristics. From this approach, DNA is considered as the major target interacting with various molecules [5]. The inherent stability of a biomolecule is an important issue in the development of the DNA-based biosensor that influenced directly on the sensor response [6]. Many types of nanoparticles (NPs) of different sizes and compositions are available to support the electrochemical-related applications

in enzyme-based sensors, immunosensors, and DNA sensors [7, 8]. Accordingly, this article is devoted to the use of ionic liquid and ZnO nanoparticles on a modified gold electrode for the construction of electrochemical biosensors with enhanced analytical performance.

Room temperature ionic liquids (ILs) are attracting intensive interest in the area of electrochemistry for their relatively large potential windows, outstanding electrochemical stability and extremely high ionic conductivity [9]. ILs are composed of organic cations and inorganic/or organic anions, which remained liquid at temperature below 100°C. It has been widely used in the fields of electrochemistry and electroanalysis due to the advantages such as high chemical and thermal stability, negligible vapor pressure, high ionic conductivity, wide electrochemical windows, low toxicity, and the ability to dissolve a wide range of organic and inorganic compounds [10, 11].

Ionic liquids (ILs) can be used as not only the supporting electrolyte but also as the modifier in chemically modified electrode. For examples, Safavi et al. [12] reported the utilization of octylpyridinium hexafluorophosphate as a binder to make a carbon ionic liquid electrode (CILE), which showed some specific characteristics including better reversibility, higher sensitivity, and a lower potential of electroactive compounds. Xiang et al. [13] studied the direct electron transfer of cytochrome c immobilized on the gold nanoparticles/ionic liquid/carbon nanotubes nanohybrid film prepared by layer-by-layer self assemble technique. The detection of DNA hybridization by electrochemistry, DNA immobilization on the surfaces of modified electrodes, and electronic materials are important criteria in developing different forms of biosensors. To date, ILs open up new prospects for the development of biosensors, biomacromolecules, and so on.

Zinc oxide (ZnO) is an inorganic semiconductor oxide with a high thermal stability, chemical inertness, lack of toxicity, and a high isoelectric point (~9.5) [14], which is positively charged on the surface under acidic conditions. It has been widely used for the immobilization of biomolecules [15]. In ZnO, Zn is acting as a deep acceptor and oxygen is acting as a deep donor. So, ZnO films are composed of nano-sized metal-oxide particles which have been intensively explored for the use in self-assembly dye/ZnO thin films [16].

As a biocompatible polymer, chitosan (CHIT) is a focus of study due to its cheapness, hydrophilicity, nontoxicity, excellent film-forming ability, and remarkable biocompatibility. CHIT–IL composite materials have a potential application in electrochemical biosensor. Wang et al. [17] reported that the integrated CHIT with BMIMBF4 and multi-walled carbon nanotubes (MWNTs) (BMIMBF4/MWNTs/CHIT) which manually cast on a glassy carbon electrode (GCE) showed a lower detection limit and able to distinguish β-nicotinamide adenine dinucleotide for the antifouling properties.

This study aims at developing a simple and effective constructed method to provide a well-defined recognition surface for immobilization and hybridization. The integration of ionic liquid, ZnO nanoparticles and chitosan nanocomposite membrane was explored to increase the electrochemical signals of the redox indicator and to enhance the sensitivity of DNA detection. The analytical performances of the newly designed electrochemical biosensor were evaluated for the detection of a specific sequence related to a *T. harzianum* gene based on the internal transcribed spacers 1 and 2 regions of the rDNA. The developed DNA biosensor was also applied on analysis of crude DNA fragments.

Materials and methods

Apparatus and electrodes

Voltammetry measurements were carried out with an μAUTOLAB (Ecochemie, The Netherlands) potentiostat using the software package General Purpose Electrochemical System (GPES 4.9, Eco Chemie). A Metrohm gold electrode (3 mm) was used as the working electrode. An Ag|AgCl|KCl 3 M reference electrode and a platinum (Pt) wire counter electrode were also employed. A 10-mL glass electrochemical cell was used in the experiments.

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 suitable dilution with the same buffer solution. An ionic liquid of 1-ethyl-3-methylimidazolium trifluoromethanesulfonate ([EMIM][Otf]) was purchased from Mark Chemical Company Ltd (Germany).

The PCR-amplified real samples were collected from the Mycology and Plant Pathology Laboratories in the Faculty of Science, Universiti Putra Malaysia. The tested oligomers were synthesized by FIRST base Laboratories Sdn Bhd, Selangor, Malaysia. Their base sequences were as follows:

Oligonucleotides of *T. harzianum*

- 20-mer probe: 5′ GAA CGT TAC CAA ACT GTT GC ′3
- 20-mer target DNA: 5′ CAG CCG TTA AAC ACC CAA CT ′3
- 20-mer non-complementary: 5′ AAG TTC AGC GGG TAT TCC TC ′3

Oligonucleotides of non-*T. harzianum* origin

- 20-mer target DNA of *Trichoderma longibrachiatum*: 5′ CCA CCC TCG AGT GAA CGT AT ′3
- 20-mer target DNA of *Trichoderma virens*: 5′ TTA TTG TAT ACC CCC TCG CG ′3
- 20-mer target DNA of *Trichoderma aureoviride*: 5′ CGG AGG AAG AAA CAA CCA AA ′3
- 20-mer target DNA of *Trichoderma koningii*: 5′ CTC CCA AAC CCA ATG TGA AC ′3

DNA oligonucleotide stock solutions (nominally 1.82×10^{-4} mol L^{-1} concentrated DNA) were prepared in a TE buffer solution containing 10 mM Tris–HCl and 1 mM EDTA (pH 8.0) and kept frozen. More dilute solutions of the oligomers were prepared in a 50 mM Tris–HCl and 20 mM NaCl buffer solution (pH 7.2). The other solutions employed which was prepared in deionized water, was a 50 mM Tris–(hydroxymethyl) aminomethane–HCl (Tris–HCl) (Sigma, USA) buffer solution containing 20 mM NaCl (Sigma, USA) (pH 7.2) as a supporting electrolyte buffer for voltammetrics measurements and as a washing buffer. The hybridization buffer was prepared in a 0.3 M NaCl, 30 mM sodium citrate buffer solution, pH 7.0 (2× SSC buffer). All chemicals used were of analytical reagent grade, and deionized water was obtained from a Millipore Milli-Q purification system.

Preparation of [EMIM][Otf]/ZnO/CHIT/AuE

A 2% chitosan (CHIT) solution was prepared by dissolving an appropriate amount of CHIT flakes into a 1% acetic acid and stirred for at least 4 h at room temperature until completely dissolved. An appropriate amount of ZnO nanoparticles were dispersed in a 2% CHIT solution and then were sonicated for 20 min after stirring for 8 h. The mass ratio of ZnO:CHIT was 1:5. After that, an ionic liquid ([EMIM][Otf]) was dispersed in the ZnO/CHIT composite sonicated and stirred for 3 h to give a homogeneous suspension. Finally, a uniform [EMIM][Otf]/ZnO/CHIT nanocomposite suspension was obtained. The [EMIM][Otf] was fixed at 3.0% (*v*/*v*) for all experimental procedures.

Before modification, the AuE was freshly polished prior to each experiment with 3-μm diamond powder (BAS MF-2059) for 2 min. The electrode was then sonicated in deionized water for 2 min. Later, the electrode was rinsed with deionized water, immersed in concentrated H_2SO_4 for 10 min, rinsed with deionized water, immersed in concentrated HNO_3 for 10 min with deionized water. Finally, the electrode was dried thoroughly under a N_2 flow. After that, 30 μL of the [EMIM][Otf]/ZnO/CHIT suspension was dropped onto a modified gold electrode (AuE) and dried at room temperature for at least 4 h to obtain a uniform coated membrane of [EMIM][Otf]/ZnO/CHIT on the electrode.

DNA probe immobilization and hybridization

The obtained [EMIM][Otf]/ZnO/CHIT/AuE was incubated in a 50 mM phosphate buffer (pH 5.5) solution containing 2 mM EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride) and 5 mM NHSS (*N*-hydroxysulfosuccinimide. EDC and NHSS were used to activate 5′ phosphate group of the ssDNA probe (1.82×10^{-4} mol L^{-1} concentration oligomer from ITS1 and 2 regions), and the activation was done for about 12 h at room temperature (in dark condition) (Scheme 1). It was then dried at room temperature for at least 4 h followed by washing with the washing buffer solution (50 mM Tris–HCl+20 mM NaCl, pH 7.2) for 30 s to remove any unbound ssDNA probe. Finally, this probe-captured electrode was then denoted as ssDNA/[EMIM][Otf]/ZnO/CHIT/AuE.

Hybridization reaction was conducted by immersing the ssDNA/[EMIM][Otf]/ZnO/CHIT/AuE into hybridization solution (2× SSC buffer) containing 1.82×10^{-4} mol L^{-1} concentration of target DNA for 60 min at 30°C. Then, the electrode surface was washed with the washing buffer for 30 s to remove any unhybridized DNA. This hybridized electrode was denoted as dsDNA/[EMIM][Otf]/ZnO/CHIT/AuE. The same protocol was applied to the ssDNA probe electrode in order to test the hybridization reactions of the probe with non-complementary and non-*T. harzianum* oligonucleotide sequences. The modification of electrode and the electrochemical characterization of the modified electrode are summarized in Scheme 2.

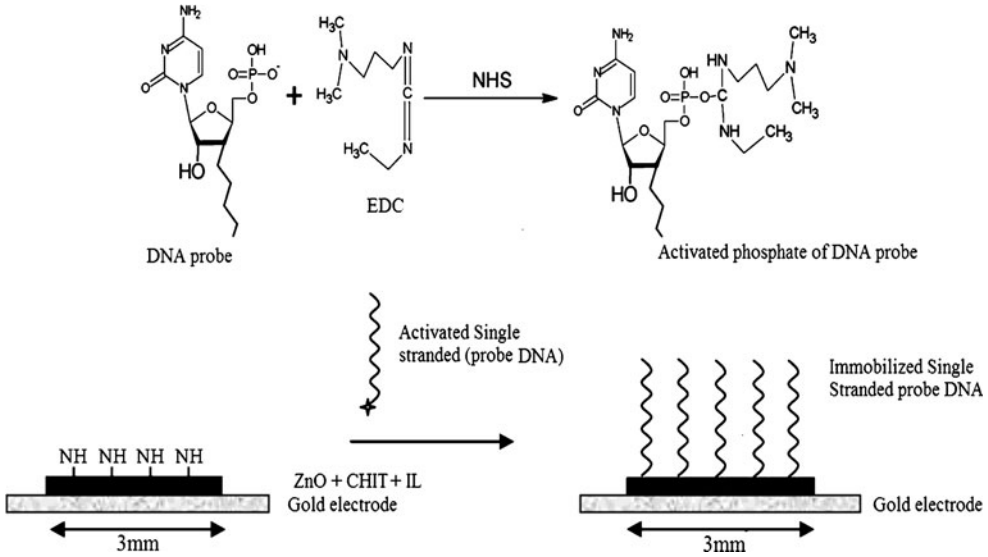
Label binding of DNA-modified electrodes

MB was accumulated onto the surface hybrid by immersing the electrode into stirred 50 mM Tris–HCl buffer (pH 7.2) containing 10 μM MB with 20 mM NaCl for 2 min without applying any potential. After accumulation of MB, the electrode was rinsed with a 50 mM Tris–HCl buffer (pH 7.2) for 30 s to remove any non-specifically bounds and then transferred into the blank buffer solution (50 mM Tris–HCl+20 mM NaCl, pH 7.2) for voltammetric measurements.

Voltammetric transduction

The reduction signal of the accumulated MB was measured using cyclic voltammetry (CV). These were done by scanning the potential from −1.50 to +1.50 V vs Ag|AgCl in an analytic buffer (50 mM Tris–HCl, pH 7.2) solution at scan rate of 100 mV/s. The raw data were treated using the Savitzky and Golay filter (level 2) of the GPES software,

Scheme 1 A schematic representation of the probe DNA-IL/ZnO/CHIT/Au electrode

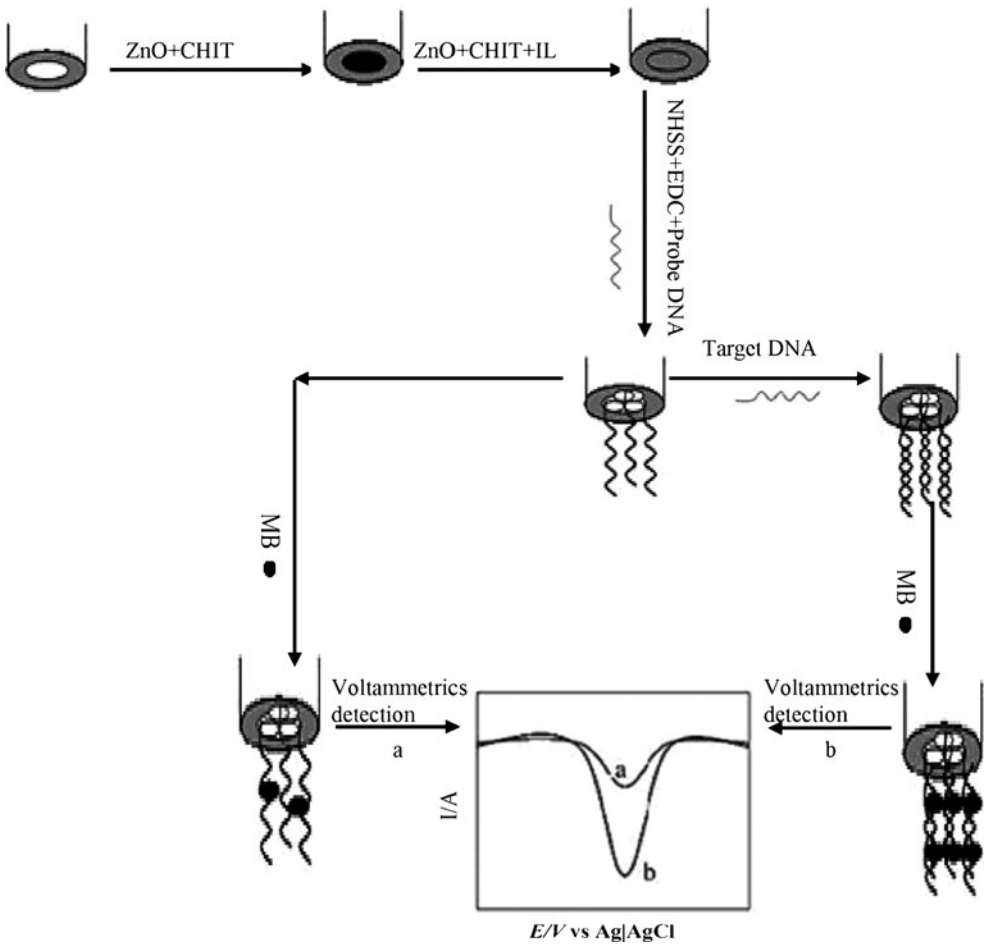


followed by moving average baseline correction with a "peak width" of 0.1. Repeat measurements were carried out by renewing the surface and then repeating the above assay conditions. All experiments were conducted at room temperature unless otherwise stated.

Extraction of crude DNA fragments

Isolates were cultured in liquid media to obtain mycelia mass for DNA extraction. Agar discs were cut out from actively growing *Trichoderma* mycelia with a 5-mm

Scheme 2 Schematic representation of electrochemical detection of DNA immobilization and hybridization



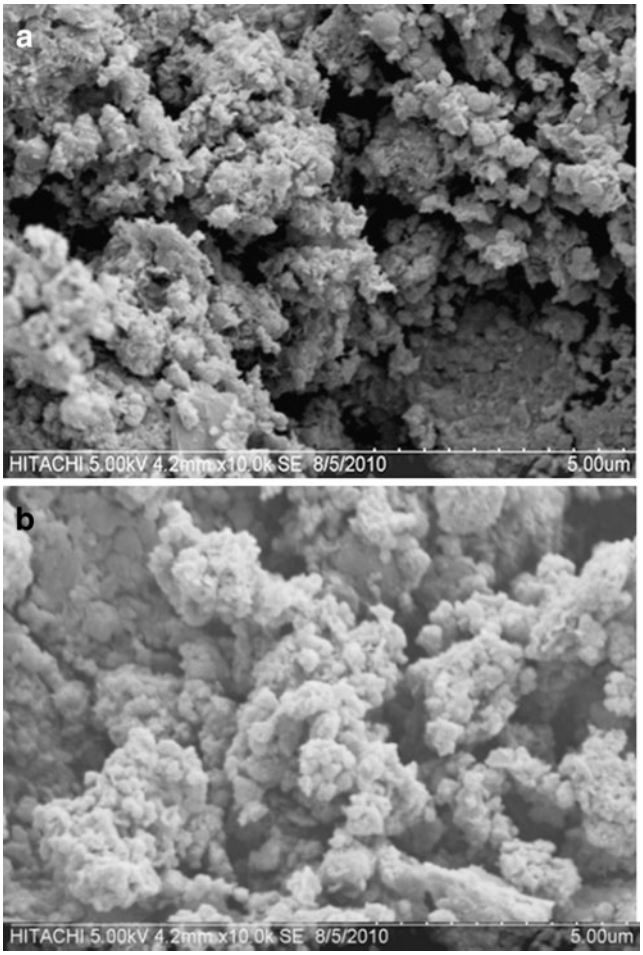


Fig. 1 SEM images of ZnO nanoparticles/chitosan (**a**) and ionic liquid/ZnO nanoparticles/chitosan (**b**) composite film on the electrode

diameter cork borer and placed into 100 ml potato dextrose broth (PDB) (Difco, USA) as starter cultures. The flasks were maintained as starter cultures for 7 to 10 days under ambient laboratory conditions. The mycelia mats were harvested by filtration through a double layered muslin cloth, washed several times with sterile water, and then ground with a mortar and pestle swabbed with ethanol prior to its use. The obtained slurry was stored at −20°C if not used immediately for crude DNA extraction.

Total fungal DNA was extracted by the phenol-chloroform method previously described by Reader and Broda [18]. A 50 mg of ground mycelium was added to 500 μl of extraction buffer (1 M Tris–HCl [pH 8.5], 1 M NaCl [pH 8.5], 1 M EDTA [pH 8.0], and 10% sodium dodecyl sulphate, SDS), and the reaction tubes were placed in a water bath for 8 h at 38°C. After incubation, 350 μl of buffered phenol and 150 μl of chloroform were added and then homogenously mixed for 10 min. The resulting suspension was subsequently centrifuged at 13,000×*g* and 4°C for 10 min. The upper aqueous layer was collected and transferred to a sterile centrifuge tube, to which was added 3 μl of RNAse solution and then incubated at 38°C in a water bath for 15 min. After incubation, an equal volume of chloroform was added to the sample with gently mixing for 10 min. The mixture was then centrifuged for second time (13,000×*g*/10 min/4°C), and the upper aqueous phase was again collected and transferred into a new tube. The DNA was precipitated with 250 μl of iso-propan-2-*ol* and kept overnight at −20°C. The tube was centrifuged the next day (13,000×*g*/10 min/4°C), and the pellet was thoroughly washed twice with 500 μl of 70% ethanol, vacuum-dried, and diluted in ddH_2O. Finally, the DNA pallets were then suspended in 50 μl of ddH_2O and kept at −20°C. The next step was to check the quality of the DNA samples by performing gel electrophoresis on 1.5% agarose horizontal minigels with a 50 bp (Promega®, USA) ladder as a marker. The electrophoresis was performed in a 1× TBE (0.045 M Tris–borate and 1 mM EDTA [pH 8.2]) running buffer at 70 V for 1 to 2 h. The gels were stained with ethidium bromide (0.5 μl/ml) and after 15 to 30 min; the

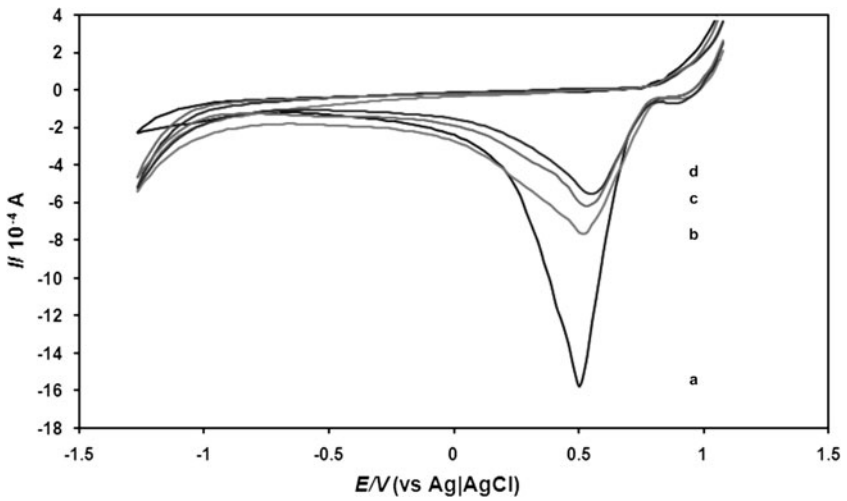


Fig. 2 CV obtained for **a** target DNA with [EMIM][Otf]/ZnO/CHIT/AuE, **b** probe with [EMIM][Otf]/ZnO/CHIT/AuE, **c** probe with ZnO/CHIT/AuE, and **d** non-complementary DNA with [EMIM][Otf]/ZnO/CHIT/AuE at a scan rate of 100 mV/s vs. Ag|AgCl

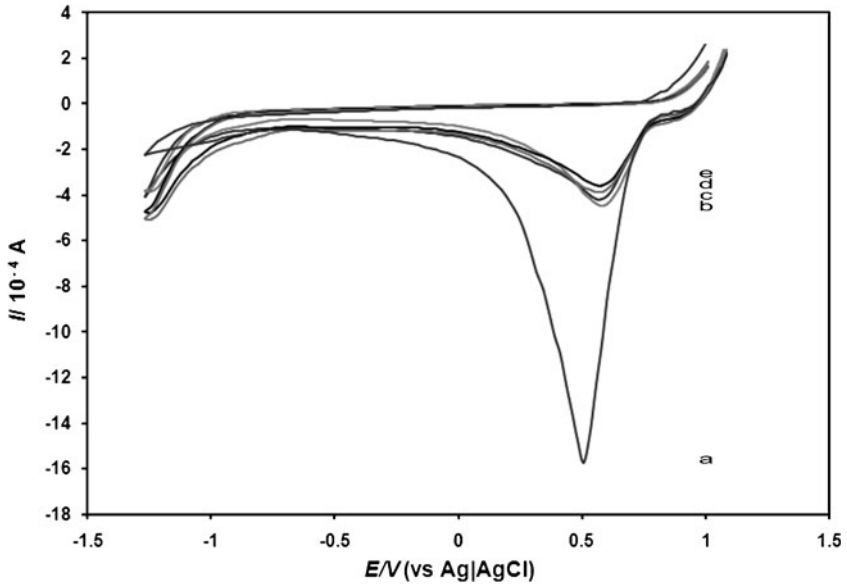


Fig. 3 CV obtained using target DNA at the ssDNA/[EMIM][Otf]/ZnO/CHIT/AuE probe for **a** *T. harzianum*, **b** *T. virens*, **c** *T. aureoviride*, **d** *T. longibrachiatum*, and **e** *T. koningii* at a scan rate of 100 mV/s vs. Ag|AgCl

gels were visualized under UV-light. The appearance of bands indicated the presence of a DNA template and thus allowed their use as DNA biosensors.

Results and discussion

Morphology of ionic liquid/ZnO nanoparticles/chitosan film

The morphologies of the as-synthesized ZnO nanoparticles/chitosan and liquid/ZnO nanoparticles/chitosan nanocomposite could be formed as films on the surface of used electrode for the ssDNA immobilization, which were studied using a scanning electron microscopy (SEM). It can be seen from Fig. 1a, b that ZnO nanoparticles were uniformly dispersed in chitosan and ionic liquid solution respectively, exhibiting uniform porous structure.

Electrochemical studies of the modified electrode on DNA hybridization detection

The selectivity of DNA biosensor was investigated by using the ssDNA/[EMIM][Otf]/ZnO/CHIT/AuE probe to hybridize with different target and non-complementary DNA sequences on the ITS 1 and 2 regions of the rDNA from fungi related to the genus *Trichoderma*. The dsDNA/[EMIM][Otf]/ZnO/CHIT/AuE (Fig. 2, curve a) showed the highest reduction current compared to the ssDNA/[EMIM][Otf]/ZnO/CHIT/AuE probe (Fig. 2, curve b).

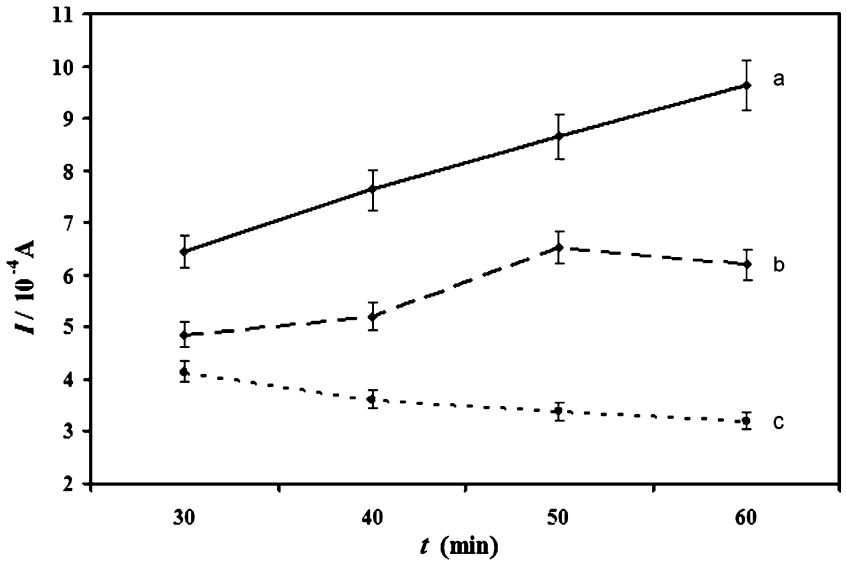


Fig. 4 CV measured using MB as a redox indicator for different temperatures [**a** 30°C, **b** 40°C, and **c** 50°C] and times (30, 40, 50, and 60 min) of target DNA

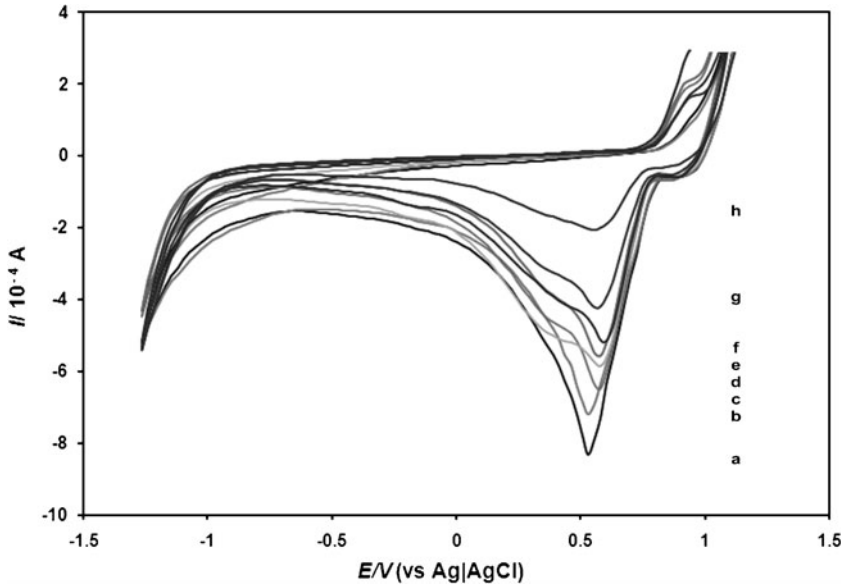


Fig. 5 CV measured for **a** 1.82×10^{-4}, **b** 3.32×10^{-6}, **c** 8.31×10^{-7}, **d** 1.66×10^{-7}, **e** 1×10^{-12}, **f** 1×10^{-14}, **g** 1×10^{-16}, and **h** 1×10^{-18} mol L^{-1} at the ssDNA/[EMIM][Otf]/ZnO/CHIT/AuE probe at a scan rate of 100 mV/s vs. Ag|AgCl

Curve d in Fig. 2 represents hybridization with the non-complementary sequence. The result showed that the peak current (Fig. 2, curve d) is much lower than that obtained from the hybridization of the target DNA (Fig. 2, curve a).

The results indicated that the presence of ionic liquid ([EMIM][Otf]), ZnO nanoparticles, and CHIT film greatly improved the surface area and accelerate the electron transfer between the redox couple in a blank solution and the electrode. The modified electrode surface attached with the mono-bases through their 5′-phosphate group via the formation of a phosphoramidate bond with free amino groups of chitosan. CHIT is a polycationic polymer and effectively employed for the immobilization of DNA which easily interacted with DNA and other polyanions materials [19]. With the addition of nanomaterials in the CHIT film, an increase of surface area with more coarseness appeared, which helped to increase the loading of DNA amount and improved the sensitivity of the biosensor.

ZnO nanoparticles also provide good electronic property and widely used in the construction of electrochemical DNA biosensors [16, 20]. Room temperature ionic liquids (ILs) have a special group of electrolytes consisting of ions and free of molecular solvent. Wei and Ivaska [21] mentioned that ILs not are only used as the supporting electrolyte but can also be used as the modifier in chemically modified electrode. Applications of ILs are mostly focused on two aspects: (1) ILs are used as the supporting liquid and (2) ILs are integrated into the fabrication of biosensors and bioelectronics [9, 10].

Selectivity of the developed DNA biosensor

The selectivity of DNA biosensor was explored by measuring the responses towards different sequences gene related to the genus of *Trichoderma*. After hybridization of the probe DNA with target DNA of *T. harzianum*, the peak

Table 1 Comparison of the analytical parameters with other detection limits

	Zhang et al. 2008 [10]	Xiao et al. 2008 [23]	Zhang et al. 2009 [24]	Yang et al. 2009 [25]	Sun et al. 2010 [26]	This work
Modifying films	ZrO_2/nano gold	OMIMPF6-SWNT/GCE	CeO_2–SWNTs-BMIMPF6/GCE	PANI/MWNT/CHIT/CPE	CTS-V2O5-MWCNTs/CILE	ZnO/CHIT/[EMIM][Otf]/AuE
Detection method	DPV with MB as indicator	CV	CV with $[Fe(CN)6]^{3-}/^{4-}$ as indicator	CV with MB	DPV with MB as indicator	CV with MB as indicator
Linear range (mol L^{-1})	1.0×10^{-10}–1.0×10^{-6}	2.0×10^{-9}–4.0×10^{-6}	1.0×10^{-12}–1.0×10^{-7}	1.0×10^{-13}–1.0×10^{-7}	1.0×10^{-11}–1.0×10^{-6}	1.0×10^{-18}–1.82×10^{-4}
Detection limit (mol L^{-1})	7.5×10^{-11}	1.0×10^{-9}	2.3×10^{-13}	2.7×10^{-14}	1.76×10^{-12}	1.0×10^{-19}

SWNT Single-walled carbon nanotube, *GCE* glassy carbon electrode, *OMIMPF6* 1-octyl-3-methylimidazolium hexafluorophosphate, *CILE* carbon ionic liquid electrode, *MWCNTs* multi-walled carbon nanotubes, *BMIMPF6* 1-butyl-3-methylimidazolium hexafluorophosphate, *PANI* polyaniline nanofibers, *CPE* carbon paste electrode

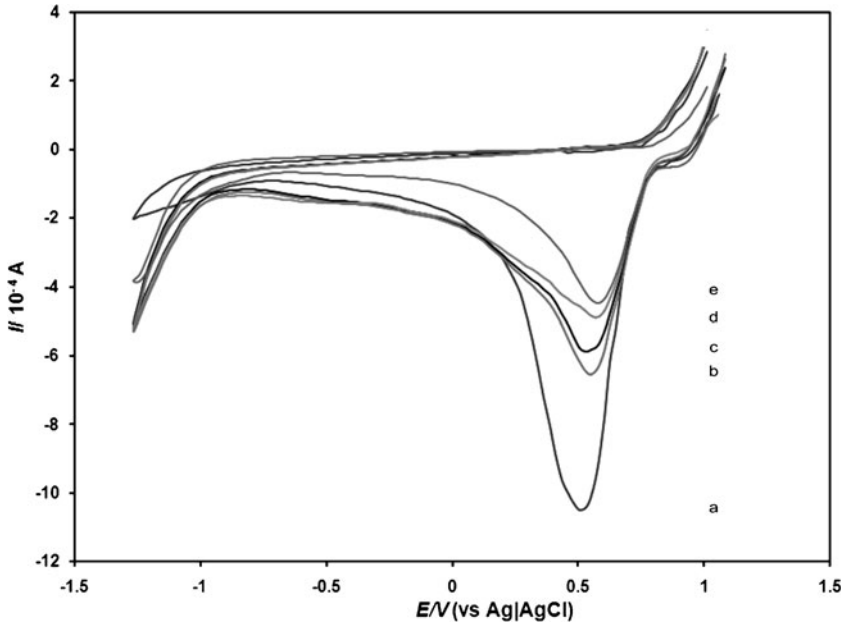


Fig. 6 CV responses obtained using crude DNA for **a** *T. harzianum*, **b** *T. koningii*, **c** *T. virens*, **d** *T. aureoviride*, and **e** *T. longibrachiatum* at the ssDNA/[EMIM][Otf]/ZnO/CHIT/AuE probe at a scan rate of 100 mV/s vs. Ag|AgCl

current highly increased obviously, suggesting that the hybrid (dsDNA) was formed at the electrode surface (Fig. 3, curve a). No significant increment of peak current was observed after the probe DNA was hybridized with non-*T. harzianum* target DNA, indicating that it was poorly hybridized (Fig. 3). The results suggested that the developed DNA biosensor showed a very high selectivity towards *T. harzianum* when compared to non-*T. harzianum* target DNA.

Effect of hybridization time and temperature

The efficiency of hybridization of the target DNA was dependent on the hybridization time and temperature, which optimized via CV method, previously described by Siddiquee et al. [22]. The modified gold electrodes were hybridized together with the target DNA [Tm=(60.03±0.026)°C] and probe DNA [Tm=(60.21±0.04)°C] at different temperature such as 30°C, 40°C, and 50°C, and at different time such as 30, 40, 50 and 60 min. The hybridization efficiency and stability could be evaluated by the interaction of MB with the probe DNA. The results of hybridization temperature and the hybridization time on the reduction peak current of MB are shown in Fig. 4.

At 30°C, the hybridization process obtained at the maximum hybridization was obtained at 60 min. As hybridization proceeded, the peak currents increased due to the increased local concentration of dsDNA on the modified electrode surface which allowed greater quantities of MB to be intercalated into the dsDNA. At 50°C, the hybridization rapidly decreased when time was increased from 30 to 60 min, because the melting temperature of the probe DNA and the target DNA are around 60.21±0.04°C. Increased temperature, for example 50°C, which is closer to the melting point, results in denaturing of DNA. Based on this, the hybridized reactions were more suitable to be carried out at 30°C. MB response currents obtained after hybridization increased with increasing hybridization time

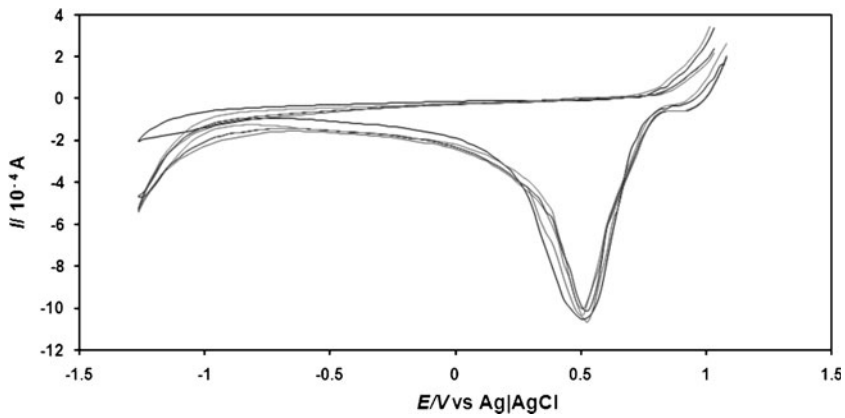


Fig. 7 CV obtained using crude DNA of five isolates of *T. harzianum* on the ssDNA/[EMIM][Otf]/ZnO/CHIT/AuE probe at a scan rate of 100 mV/s vs. Ag|AgCl

from 30 to 60 min. Therefore, the optimal hybridization temperature was selected at 30°C, and the optimal hybridization time as 60 min.

Responses towards different concentrations of target DNA

The sensitivity of this electrochemical DNA biosensor was studied by using the immobilized ssDNA/[EMIM][Otf]/ZnO/CHIT/AuE probe to hybridize with different concentrations of target DNA of a *T. harzianum* gene as shown in Fig. 5. The hybridization reactions were completed after 60 min at 30°C. The sensor was used for the detection range of 1×10^{-18} to 1.82×10^{-4} mol L^{-1}, and the detection limit was calculated to be 1.0×10^{-19} mol L^{-1} (n=5). The performances of the constructed biosensor and the other DNA electrochemical biosensors based on the nanoparticles and ionic liquid were compared [10, 23–26], and the results are shown in Table 1. It can be seen that this DNA biosensor has a lower detection limit and a wider linear range for the target DNA sequence being analyzed. The linear regression equation for the calibration plot was calculated to be y=0.407x+9.4437 (R^2=0.9719), where x is the concentration of target DNA, and y is the reduction current.

Detection of *T. harzianum* from crude DNA fragments

The probe DNA sequence immobilized on the [EMIM][Otf]/ZnO/CHIT/AuE was immersed into a hybridization buffer (2× SSC) solution containing crude DNA fragments taken from different species among isolates of the genus *Trichoderma*. The DNA biosensor was applied to the analysis of five isolates of *T. harzianum* (isolates: T32, FA26, FA29, FA44, and FA30) and four isolates of non-*T. harzianum* (*T. koningii* (isolate: S10), *T. longibrachiatum* (isolate: T28), *T. virens* (isolate: T128), and *Trichoderma aureoviride* (isolate: T45)). The hybridization reactions of crude DNA was done under the same conditions as for the DNA oligonucleotides.

The developed DNA biosensor was able to hybridize with the crude DNA fragments taken from real samples as shown in Fig. 6. This developed DNA biosensor was highly specific because non-*T. harzianum* isolates of crude DNA fragments did not show a significantly enhanced peak current compared to *T. harzianum* isolates. These results indicated that the ssDNA/[EMIM][Otf]/ZnO/CHIT/AuE probe surface had a higher affinity for crude DNA of *T. harzianum* compared to non-*T. harzianum* crude DNA. These results prove that the developed DNA biosensor has a very high potential for rapid, sensitive, and selective usage for crude DNA analysis.

When five isolates of *T. harzianum* crude DNA were hybridized at the ssDNA/[EMIM][Otf]/ZnO/CHIT/AuE probe, no significant variation was observed among the isolates as shown in Fig. 7. The developed DNA biosensor has shown a reproducible signal even when applied with crude DNA fragments.

Conclusions

DNA electrochemical biosensor has developed based on the ionic liquid/ZnO nanoparticles/chitosan membrane modified gold electrode. DNA is able to incorporate via the biocompatibility of nano-ZnO, the good film-forming ability of CHIT and room temperature ionic liquid. The modified gold electrode using [EMIM][Otf]/ZnO/CHIT had increased the surface area of the electrode and further increase the efficiency of the immobilization of probe DNA. It has remarkably enhanced the detection sensitivity levels of DNA hybridization. This electrochemical DNA biosensor can be applied to the detection of *T. harzianum* gene sequence with a dynamic concentration in the range of 1.0×10^{-18} to 1.82×10^{-4} mol L^{-1}. These results suggested that this developed electrochemical biosensor offers a simple, fast response, good selectivity, high sensitivity, wide detection range, and convenient method for use in microorganism research laboratories.

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References

1. Harman GE, Latorre B, Agosin E, Martin RS, Riegel DG, Nielsen PA, Tronsmo A, Pearson RC (1996) Biol Control 7:259–266
2. Adams PB (1990) Annu Rev Phytopathol 28:59–72
3. Chet I (1987) In: Chet I (ed) Innovative approaches to plant disease control. Wiley, New York, pp 137–160
4. Papavizas GC (1985) Annu Rev Phytopathol 23:23–54
5. Diculescu VC, Piedade JAP, Oliveira-Brett AM (2007) Bioelectrochem 70:141–146
6. Chiorcea AM, Oliverira Brett AM (2004) Bioelectrochem 63:229–232
7. Merkoci A, Aldavert M, Marin S, Alegret S (2005) Trends Anal Chem 24:341–349
8. Katz E, Willner I, Wang J (2004) Electroanalysis 16:19–44
9. Xiong HY, Chen T, Zhang XH, Wang SF (2007) Electrochem Commun 9:1648–1654
10. Zhang X, Jiao K, Wang X (2008) Electroanal 20:1361–1366
11. Pandey S (2006) Anal Chimi Acta 556(1):38–45
12. Safavi A, Maleki N, Moradlou O, Sorouri M (2008) Electrochem Commun 10:420–423
13. Xiang CL, Zou YJ, Sun LX, Xu F (2008) Electrochem Commun 10:38–41
14. Zhang YJ, Wen Y, Liu Y, Li D, Li JH (2004) Electrochem Commun 6(11):1180–1184

15. Zhu XL, Yuri I, Gan X, Suzuki I, Li GX (2007) Biosens Bioelectron 22:1600–1604
16. Yoshida Y, Muroi K, Otsuka A, Saito G, Takahashi M, Yoko T (2004) Inorg Chem 43(4):1458–1462
17. Wang Q, Tang H, Xie Q, Tan L, Zhang Y, Li B, Yao S (2007) Electrochim Acta 52:6630–6637
18. Reader U, Broda P (1985) Lett Appl Microbiol 1:17–20
19. Xu C, Cai H, Xu Q, He P, Fang Y (2001) Fresenius' J Anal Chem 369:428–432
20. Feng X, Liu Y, Kong Q, Ye J, Chen X, Hu J, Chen Z (2010) J Solid State Electrochem 14(6):923–930
21. Wei D, Ivaska A (2008) Anal Chim Acta 607(2):126–135
22. Siddiquee S, Nor Azah Y, Salleh AB, Fatimah AB, Lee YH (2010) Bioelectrochem 79:31–36
23. Zhang W, Yang T, Zhuang X, Guo Z, Jiao K (2009) Biosens Bioelectron 24:2417–2422
24. Xiao F, Ruan C, Liu L, Yan R, Zhao F, Zeng B (2008) Sens Actuator B:Chem 134:895–901
25. Yang T, Zhou N, Zhang Y, Zhang W, Jiao K, Li G (2009) Biosens Bioelectron 24:2165–2170
26. Sun W, Qin P, Gao H, Li G, Jiao K (2010) Biosens Bioelectron 25:1264–2170