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

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**A B S T R A C T**

The genus *Trichoderma* is a soil-borne fungus 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 liquid-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 \(1.0 \times 10^{-15} - 1.82 \times 10^{-19}\) mol L\(^{-1}\), and the detectable limit was \(1.0 \times 10^{-19}\) 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*.

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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 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
miniatrization [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 biosensing devices 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 methods

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/H₂O (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-C₃-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.
2.4. ZnO nanoparticles

ZnO nanoparticles were obtained from Biosensors and Bioelectronics Lab, Department of Chemistry, Faculty of Science, Universiti Putra Malaysia.

2.5. Preparation of the [EMIM][Otf]/ZnO/CHIT modified gold electrode

A 2% CHIT (Sigma, USA) solution was prepared by dissolving an appropriate amount of CHIT flakes into 1% acetic acid and stirred for at least 4 h at room temperature until complete dissolution. An appropriate amount of the ZnO nanoparticles was dispersed in the 2% CHIT solution. It was then sonicated for 20 min after stirring for 8 h. The mass ratio of ZnO: CHIT was 1:5. After that, ionic liquid (1-ethyl-3-methylimidazolium trifluoromethanesulfonate, [EMIM][Otf]) was dispersed in the ZnO/CHIT composite, and then sonicated for 3 h to produce a homogeneous suspension. The ratio of [EMIM][Otf] was fixed at 3.0% (v/v) in the experiments.

Before modification, the AuE was polished with 3-mum diamond powder (BAS MF-2059) for 2 min. Then, the electrode was sonicated in deionized water for 2 min. The electrode was then rinsed with deionized water and immersed in concentrated H2SO4 for 10 min and again rinsed with deionized water. Finally, the electrode was dried thoroughly under a N2 flow. After that, 30 μL of the [EMIM][Otf]/ZnO/CHIT suspension was casted onto the AuE and then dried at room temperature for at least 4 h to obtain a uniform coated membrane of [EMIM][Otf]/ZnO/CHIT on the electrode.

2.6. Immobilization and hybridization of DNA

The [EMIM][Otf]/ZnO/CHIT/AuE was incubated in a 50 mM phosphate buffer (pH 5.5) containing 2 mM EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride) and 5 mM NHS (N-hydroxysulfosuccinimide). EDC and NHS were used to activate the 5’ phosphate group of the ssDNA probe and the activation was done for about 12 h at room temperature (in dark conditions). It was then dried at room temperature for at least 4 h and washed with the washing buffer solution for 30 s to remove any unbound ssDNA from the probe. Finally, this probe-captured electrode was then denoted as ssDNA/[EMIM][Otf]/ZnO/CHIT/AuE.

The probe-modified electrode (ssDNA/[EMIM][Otf]/ZnO/CHIT/AuE) was then hybridized with a different concentration of the target DNA in a 2× SSC buffer stirred for 60 min at 30 °C. The electrode surface was then washed with the washing buffer for 30 s to remove any unbound-hybridized DNA. Finally, this hybridized electrode was denoted as dsDNA/[EMIM][Otf]/ZnO/CHIT/AuE. The same protocol was applied to the probe-modified electrode in order to test the hybridization reactions of the probe with non-complementary and non-harzianum oligonucleotide sequences.

2.7. MB accumulation onto the DNA-modified electrodes

MB was accumulated onto the hybrid surface 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 the accumulation of MB, the electrode was rinsed with 50 mM Tris–HCl buffer (pH 7.2) for 30 s to remove the non-specifically bound MB. It was then transferred into a blank buffer solution (50 mM Tris–HCl + 20 mM NaCl, pH 7.2) for voltammetric measurements.

2.8. Voltammetric transduction

The oxidation signal of the accumulated MB was measured using differential pulse voltammetry (DPV). The scanning potential for measurement was from +0.40 to +1.40 V vs Ag/AgCl with a scan rate of 100 mV/s in the analytical buffer (50 mM Tris–HCl buffer, pH 7.2). The raw data were treated using the Savitzky and Golay filter (level 2) of the GPES software, followed by moving the average baseline correction with a “peak width” of 0.2. Repetitive measurements, as mentioned above, were carried out by renewing the surface and repeating the above assay conditions. All experimental works were conducted at room temperature unless otherwise stated.

2.9. Extraction of DNA from Trichoderma isolates

DNA extraction was done by the Phenol–Chloroform method previously described by Siddiquee et al. [33]. Agar discs were cut out from actively growing Trichoderma mycelia with a 5 mm diameter cork borer. The discs were then placed into 100 ml potato dextrose broth (PDB) (Difco, USA) as starter cultures. The flasks were maintained as starter cultures, under ambient laboratory conditions for 10 days. The mycelial mats were then filtered through a double layered muslin cloth and harvested. They were washed several times with sterile water and then ground with a mortar and pestle and swabbed with ethanol prior to their use or stored at −20 °C if not used immediately.

After that, 50 mg of ground mycelial mats were 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 sulfate, SDS). The reaction tubes were then 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 homogenously mixed for 10 min. Subsequently, the resulting suspension was 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 3 μl of RNAase solution was added. This was then incubated at 38 °C in a water bath for 15 min. After incubation, an equal volume of chloroform was gently mixed and added to the sample for 10 min. The mixture was then centrifuged once more (13,000×g/10 min/4 °C). The upper aqueous phase was again collected and transferred into a new tube. The DNA was precipitated with 250 μl of iso-propanol 2-ol and kept overnight at −20 °C. The tube was centrifuged the next day (13,000×g/10 min/4 °C). The resultant pellets were thoroughly washed twice with 500 μl of 70% ethanol, and then vacuum-dried, and diluted in ddH2O. Finally, the DNA pellets were suspended in 50 μl of ddH2O. The concentration of template DNA was 1500 ng/μl and kept at −20 °C if not used immediately.

Table 1
Set of oligonucleotides used in this work.

| Name                  | Base sequence 5’-3’ | Reference          |
|-----------------------|---------------------|--------------------|
| Probe DNA of T. harzianum | ACT CCC AAA CCC AAT GTG AA | Siddiquee et al. [12] |
| Target DNA of T. harzianum | CAG CGG TTA AAC ACC CAA CT | –                  |
| Non-complementary of T. harzianum | AAG TTC AGC GGG TAT TCC TC | –                  |
| Target DNA of T. longibrachiatum | CCA CCC TCG AGT GAA CGT AT | –                  |
| Target DNA of T. virens | TTA TTT TAT ACC CCC TGC CG | –                  |
| Target DNA of T. aureoviride | CCG AGG AAG AAA AAA CCA AA | –                  |
| Target DNA of T. koningii | CTC CCA AAC CCA ATG TGA AC | –                  |
the quality of the DNA samples was checked by performing gel electrophoresis on 1.5% agarose horizontal minigels using a 50bp (Promega®, USA) ladder as a marker. The electrophoresis was carried out 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 15–30 min the gels were visualized under UV-light. The appearance of bands indicated the presence of the DNA template and which thereby confirmed the usability of this electrochemical DNA biosensor.

3. Results and discussion
3.1. Enhancement of voltammetry signals using nanoparticles

The electrochemical DNA biosensor was designed to explore the hybridization of immobilized probe ssDNA into its target DNA. In this experiment, probe DNA was immobilized onto two types of modified electrodes using by SAM/MPA/AuE and ZnO/CHIT/AuE. The differential pulse voltammetry (DPV) signals were measured in the following order: dsDNA/ZnO/CHIT/AuE (Fig. 1, curve a), dsDNA/SAM/MPA/AuE (Fig. 1, curve b), ssDNA/ZnO/CHIT/AuE (Fig. 1, curve c), ssDNA/SAM/MPA/AuE (Fig. 1, curve d), dsDNA/ZnO/CHIT/AuE (Fig. 1, curve e) a non-complementary, and dsDNA/SAM/MPA/AuE (Fig. 1, curve f) a non-complementary and bare gold electrode with MB (AuE/MB) (curve g). The curves are respectively shown in Fig. 1. When the ssDNA molecule was used as the capture probe, the hybridization reaction was recorded through the increases in current signals after the duplex formation on the electrode surface. For the ZnO nanoparticles, the current response always increased indicating a higher affinity for MB on the electrode surface by the DPV signal with accumulation of MB on the surface of the CHIT film through electrostatic attraction. The addition of the ZnO nanoparticles to the CHIT coating increased the interface area and this increased the loading amount of DNA and improved the sensitivity of the DNA biosensor. Thus, it was confirmed that the ZnO nanoparticles in the nanocomposite membrane improved the amount of dsDNA immobilized on the electrode surface by the DPV signal with accumulation of MB on a modified AuE.

Subsequently, the ssDNA/ZnO/CHIT/AuE probe was hybridized with a single-base non-complementary sequence (Fig. 1, curve e). A low hybridization efficacy was obtained in comparison to the target DNA of Trichoderma harzianum. The decrease in the peak current of MB when incubated with non-complementary DNA suggests that the hybridization was weak.

The reproducibility of this developed DNA biosensor was tested by measuring a 1.82 × 10⁻⁴ mol L⁻¹ concentration of the target DNA. The response currents of the five electrodes were prepared independently under the same conditions for 2.40, 2.39, 2.38, 2.37 and 2.36 (× 10⁻⁴ A), respectively. A series of five replicate measurements of the oxidation current signals produced reproducible results with a relative standard deviation (RSD) of 0.66%.

3.2. Electrochemical analysis of the ionic liquid modified-AuE

DNA immobilization on the surface of the modified-electrodes is used to detect DNA hybridization by electrochemistry. The electrochemical analysis of the ILs modified electrode was investigated using the ssDNA/([EMIM][Otf])/ZnO/CHIT/AuE probe to hybridize with target and non-complementary DNA sequences of the ITS 1 and 2 regions of the rDNA from fungi related to the genus Trichoderma. Five similarly constructed DNA biosensors using ILs modified electrodes were used to detect the target DNA, T. harzia-

![Fig. 1. DPV obtained for (a) dsDNA/ZnO/CHIT/AuE, (b) dsDNA/SAM/MPA/AuE, (c) ssDNA/ZnO/CHIT/AuE, (d) ssDNA/SAM/MPA/AuE, (e) dsDNA/ZnO/CHIT/AuE of non-complementary, (f) dsDNA/SAM/MPA/AuE of non-complementary DNA and AuE/MB at a scan rate of 100 mV/s vs Ag/AgCl.](image)

![Scheme 1. Methylene blue reduction at the electrode surface](image)
which confirms that this electrochemical DNA biosensor is highly reproducible.

After the hybridization of the target DNA, the dsDNA/[EMIM][Otf]/ZnO/CHIT/AuE showed the highest oxidation signals with peak currents as shown in Fig. 2, curve a. For the dsDNA/[EMIM][Otf]/ZnO/CHIT/AuE, the oxidation signal was about 1.80 times stronger than that of the ssDNA/[EMIM][Otf]/ZnO/CHIT/AuE probes (Fig. 2, curve b). Curve c in Fig. 2 represents the hybridization with the non-complementary sequence. The results show that the peak current was much lower than that obtained from the hybridization of the target DNA (Fig. 2, curve a). The results indicate that the presence of ILs ([EMIM][Otf]), ZnO nanoparticles and CHIT film greatly improved the surface area between the intercalated MB and the AuE.

The DPV signals provided useful information about the ssDNA probes immobilized on the modified electrode ([EMIM][Otf]/ZnO/CHIT/AuE). Higher peak current signals were obtained for the ssDNA/[EMIM][Otf]/ZnO/CHIT/AuE (Fig. 2, curve b) than the ssDNA/ZnO/CHIT/AuE probe (Fig. 1, curve c). The results found that the ssDNA/[EMIM][Otf]/ZnO/CHIT/AuE probes oxidation currents were about 1.11 times higher than that of the ssDNA/ZnO/CHIT/AuE probes. Curve c in Fig. 2 represents the hybridization with a non-complementary sequence. The results show a low hybridization efficacy was obtained in comparison to the target DNA.

Recently, a key issue in the development of new materials is the solvent in which the materials are synthesized. ILs received intensive interest for use in electrochemistry due to their relatively large potential windows, outstanding electrochemical stability and extremely high ionic conductivity [36,37]. In addition, ILs also have a special group of electrolytes consisting of ions and are free of molecular solvent. Wei and Ivaska [38] mentioned that ILs are mainly used as the supporting electrolyte and act as the modifier in chemically modified electrodes. ILs are also used as supporting liquid and integrated into the fabrication of biosensors and bioelectronics [36,39].

ILs/nanoparticles/CHIT nano-composite materials have been applied in some developed DNA biosensors. For examples, Wang et al. [40] mentioned that CHIT integrated with BMIMBF4 and multi-walled carbon nanotubes (MWNTs) (BMIMBF4/MWNTs/CHIT) and manually cast on glassy carbon electrodes (GCE) was able to achieve a lower detection limit and distinguish β-nicotinamide adenine dinucleotide (NADH) for its anti fouling properties. Safavi et al. [41] have reported the utilization of octylpyridinium hexafluorophosphate (OPPP) as a binder to make a carbon ionic liquid electrode (CLE), which showed some specific characteristics including better reversibility, higher sensitivity and a lower potential for electroactive compounds.

3.3. Selectivity of the developed DNA based biosensor

The selectivity of the ILs modified DNA biosensor was explored by measuring its responses towards different gene sequences related to the Trichoderma genus. After the hybridization of the probe DNA with target DNA, T. harzianum, the peak current increased greatly, which suggests that dsDNA was formed at the modified electrode surface (Fig. 3, curve a). No significant increment in peak current was observed after the probe DNA was hybridized with non-harzianum target DNA, indicating that it was poorly hybridized. The results suggest that the developed DNA biosensor based on ionic liquid modified electrodes is highly selective towards T. harzianum compared to non-harzianum target DNA.

3.4. Electrochemical response of different concentrations of target DNA

The developed electrochemical DNA biosensor was studied using the ssDNA/[EMIM][Otf]/ZnO/CHIT/AuE probe to hybridize with various concentrations of the target DNA of T. harzianum gene as shown in Fig. 4. The developed DNA biosensor was used to detect target DNA in concentrations ranging from $1 \times 10^{-18}$ to $1.82 \times 10^{-14}$ mol L$^{-1}$. The detection limit was calculated to be $1 \times 10^{-19}$ mol L$^{-1}$ ($n=5$). This DNA biosensor has a lower detection limit and a wider linear range as indicated in Fig. 5 for the target DNA sequence being analyzed.

The developed electrochemical DNA biosensor was able to reach a lower detection limit (LOD) of 2.5 fM for DPV. Wei et al. [42] have integrated the concepts of μTAS and microarrays, which are able to diminish the sample and reagent volumes to 1 μL, and the hybridization time to less than 10 min, reaching a detection limit of 19 aM for the CV method. Oszoz et al. [43] reported the Au-tagged 23-mer capture probes attached with the synthetic 23-mer target were able to obtain LODs as low as 0.78 fM via the CV method. Takenaka et al. [44] also reported the electrochemical measurement of ferrocenylnaphtalene-diimide (Nc-Bis[4-[3-aminopropyl]-piperazinyl]propyl]-napthalene-1,4,5,8-tetracarb oxylic acid diimide), which has displayed higher affinity for a double-chain of target DNA, with sensitivities in the 10 zM range using DPV methods.

![Fig. 2. DPV obtained for (a) target DNA with [EMIM][Otf]/ZnO/CHIT/AuE, (b) probe with [EMIM][Otf]/ZnO/CHIT/AuE, (c) non-complementary DNA with [EMIM][Otf]/ZnO/CHIT/AuE at a scan rate of 100 mV/s vs Ag|AgCl.](image)

![Fig. 3. DPV obtained using the target DNA for (a) T. harzianum, (b) T. longibrachi-atum, (c) T. aureoviridea, (d) T. koningii and (e) T. virens on the ssDNA/[EMIM][Otf]/ZnO/CHIT/AuE probe at a scan rate of 100 mV/s vs Ag|AgCl.](image)
3.5. Application of the developed DNA biosensor on crude DNA

The probe DNA sequence immobilized on the [EMIM][Otf]/ZnO/CHIT/AuE was immersed into a hybridization buffer (2/C2 SSC) solution containing crude DNA fragments from different species among isolates of the genus Trichoderma. The DNA biosensor was applied for the analysis of five T. harzianum isolates (isolates: T32, FA26, FA29, FA44 and FA30) and four non-harzianum isolates (Trichoderma koningii (isolate: S10), Trichoderma longibrachiatum (isolate: T28), Trichoderma virens (isolate: T128) and Trichoderma aureoviride (isolate: T45)). The hybridization reactions of crude DNA were 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 is highly specific because non-harzianum isolates of crude DNA fragments did not show a significant enhancement in peak currents compared to T. harzianum isolates. These results show that the intensity of the oxidation current increased in the order of T. harzianum > T. aureoviride > T. longibrachiatum > T. virens > T. koningii.

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. These results indicate that peak currents were less varied among isolates of crude DNA from the same species.

4. Conclusion

An electrochemical DNA biosensor is successfully developed based on ILs/ZnO nanoparticles/CHIT/AuE then applied for the detection of DNA immobilization and hybridization from T. harzianum gene and crude DNA taken from real samples. ZnO nanoparticles homogeneously dispersed in CHIT and ILs film enhanced the consignment interface for the immobilization of probe DNA and increased the detection sensitivity level for DNA hybridization. This developed DNA biosensor was applied in the detection of T. harzianum gene sequences in a wide concentration range of 1.0 × 10^{-18}–1.82 × 10^{-4} mol L^{-1} (n = 5) using DPV method and methylene blue used as an electrochemical indicator. The designed biosensor has several advantages such as a simple preparation procedure, high selectivity, low cost, fast response, and a wide linear range. This developed biosensor also offers a convenient research
tool for the identification of *T. harzianum* in contrast to non-
*harzianum*.

Conflict of interest

The authors confirm that there is no conflict of interest to declare.

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