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Electrochemical DNA biosensor for the detection of specific gene related to *Trichoderma harzianum* species

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ABSTRACT

A new electrochemical biosensor is described for voltammetric detection of gene sequence related to *Trichoderma harzianum*. The sensor involves immobilization of a 20 base single-stranded probe (ssDNA), which is complementary to a specific gene sequence related to *T. harzianum* on a gold electrode through specific adsorption. The DNA probe was used to determine the amount of target gene in solution using methylene blue (MB) as the electrochemical indicator. The covalently immobilized probe could selectively hybridize with the target DNA to form a hybrid on the surface despite the bases being attached to the electrode. The changes in the peak currents of methylene blue (MB), an electroactive label, were observed upon hybridization of probe with the target. Peak currents were found to increase in the following order: hybrid-modified AuE and the probe-modified AuE which localized to the affinity of MB. Control experiments with the non-complementary oligonucleotides were performed to assess whether the DNA biosensor responds selectively, *via* hybridization, to the target. DNA biosensor also able to detect microorganism at the species levels without nucleic acid amplification. The redox current was linearly related to the concentration of target oligonucleotide DNA, ranged from 1–20 ppm. Numerous factors, affecting the probe immobilization, target hybridization and indicator binding reactions are optimized to maximize the sensitivity and reduce the assay time.

1. Introduction

Current developments in nucleic acid-based technologies are continuing to improve the specificity, sensitivity, rapidity, user-friendliness and cost effectiveness of molecular diagnostics. Nucleic-acid based assays are a direct means of pathogen detection and characterization, and particularly valuable where conventional means of isolation, culture or characterization have proven to be tedious, hazardous, timely and uninformative or even impossible [1]. DNA is also more resistant to denaturation than proteins and can survive for long periods under appropriate conditions. Basic principles of cloning, the use of restriction enzymes for plasmid fingerprinting, as well as the use of hybridization probes for the use in dot blots as well as Southern and Northern blotting all played important contributory roles in the initial detection and/or characterization of nucleic acids of various pathogens. Initially hybridization reactions required the separation of hybrid molecules from unhybridized probes using ultracentrifugation or chromatography and filtration.

For DNA analysis, the polymerase chain reaction (PCR) is one of the most widely used analytical methods [2,3]. The PCR method requires preamplification of genomic DNA prior to analysis in order to selectively increase the concentration of the target DNA sequence relative to unrelated sequences. However, the amplification process makes it difficult to quantitatively analyses target genes because of contaminations and non-linearities in amplification number with cycle number [4,5].

Recently, the microarray-based DNA chip has been developed to achieve high-throughput DNA analysis [6–8]. Conventional methods for the analysis of specific gene sequences are based on direct sequencing or DNA hybridization methods. Because of its simplicity, the second option is more commonly used in diagnostic laboratories. There is no doubt that this technology has revolutionized genetic research for high throughput DNA hybridization analyses. However, the non-negligible cost of a spotting instrument, analysis time is still a concern. Because array-based hybridization assays still generally depend on the diffusion of target DNA to the surface-bound probes, leading to hybridization times of several hours or more [9,10].

DNA biosensor technologies are rapidly developing as an alternative to the classical gene assays, due to several advantages such as low cost, rapid analysis, simplicity and possibility of miniaturization [11]. Genosensors (or DNA biosensors) are devices that combine, as a biological recognition agent, a single-stranded DNA (ssDNA) called DNA probe, with a transducer. The selectivity of this device is due to the former, while its sensitivity is provided by the latter [12].

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Genosensors make use of the hybridization event to detect a target DNA sequence [13].

The analysis of specific gene sequences in the diagnostic laboratory is usually based on DNA hybridization. Here, the target gene sequence is identified by a DNA probe able to form a double stranded (ds) hybrid with its complementary nucleic acid with high efficiency and specificity [14]. In DNA hybridization, the target gene sequence is identified by a DNA probe that can form a double stranded hybrid (dsDNA) with its complementary nucleic acid (target sequence) with high efficiency and extremely high specificity in the presence of a mixture of much different, non-complementary nucleic acid. Information concerning the immobilization of single-stranded DNA (ssDNA) and duplex DNA (dsDNA) on a surface is paramount to the development of DNA-based electronic biosensors. Electrochemical detection of DNA immobilization and hybridization usually involves monitoring a current response under controlled potential conditions. Self-assembly of DNA monolayer based on the formation of a gold-thiolate bond is an important method for preparing stable, closely packed monolayers with well-defined structures [15,16].

Recently, a lot of progress has been made in the development of electrochemical DNA hybridization biosensors. These biosensors rely on the conversion of the base-pair recognition event into a useful electrical signal. The sensing events on the electronic transducers are designed to alter the interfacial properties at the transducer/solution interface.

The DNA immobilization procedure on the electrode surface is a very important aspect since it influences the characterization of the DNA probe, the sensor response, and its performance [17]. Thus, a key issue with a DNA biosensor is the accessibility and molecular orientation of the probe DNA, which requires a high degree of control over the immobilization of the probe oligonucleotides. Self assembled monolayers (SAMs) of alkanethiols, which have shown to provide molecular level control over the immobilization of several types of biomolecules, have been used as active films on which DNA segments can be attached using covalent linkers [18]. Electrochemical biosensors use DNA probe molecules attached on electrically active surface and measure current or resistance changes caused by hybridization of target DNA [19]. By coupling hybridization to a redox-active dsDNA intercalator with a catalytic mediator [20] the sensitivity is increased to make detection possible without amplification.

Electrochemical DNA sensors based on the voltammetric transduction of the formation of double stranded (ds) oligonucleotide–DNA complexes have been reported by following the direct electrical response of the ds-assembly [21], and the electrical response of the ds-assembly of the transition metal complexes [22] or dyes [23] that are intercalated or electrostatically attracted to the double stranded assembly. Many electroactive indicators such as cationic metal complexes [24], anticancer drugs [25] or organic dyes [26] have, thus, been employed in DNA hybridization biosensors.

The electrochemical response of these labels or indicators changes upon DNA hybridization, usually increasing when the hybridization process occurs due to an increase of the indicator concentration at the electrode surface. Methylene blue (MB), an organic dye that belongs to the phenothiazine family, is a redox indicator, which, due to its interaction with the guanine bases in DNA, displays significantly different voltammetric signals in the presence of ssDNA or dsDNA modified electrodes [27]. The interaction between MB and DNA has been studied by means of spectrophotometric and electrochemical methods [18,28,29]. So, Kelley et al. [30,31] investigated the charge transfer mechanism of Au electrodes covered with dsDNA in the presence of MB concluding that MB show high affinity for immobilized DNA ($K \approx 4 \times 10^6 M^{-1}$) and that MB binding sites are localized mainly to the solution accessible periphery of the monolayer. It has also been reported that the peak potential of the MB square wave voltammetric signals at thiol terminated probe-modified AuEs was 10–15 mV more positive than the one at thiol terminated hybrid-modified AuEs [32].

The electrochemical transduction of DNA hybridization is achieved using the voltammetric signals of methylene blue (MB) at AuE surface. Erdem et al. [27] reported that MB could be used as a redox-active indicator for the electrochemical detection of mismatched bases in DNA. The detection of hybridization was accomplished using the specific interaction of MB with guanine. The evidence of the direct interaction of MB with guanine based on the DNA coated carbon paste electrodes (CPEs) was also investigated by Yang et al. [33]. Rohs et al. [34] reported a modelling study for MB binding to DNA with alternating guanine–cytosine base sequence. Enescu et al. [35] investigated the conformation of MB–guanine complexes by molecular dynamics simulation. The position and orientation of MB–guanine complexes were found to be in three modes: T-shaped, non-stacked and face-to-face. This manuscript describes the use of methylene blue (MB) as electrochemical hybridization indicators for the detection of *Trichoderma harzianum* gene sequences.

Thus, the main objective of this article is to design a new electrochemical, nucleic acid biosensor for the detection of *T. harzianum* species which can achieve high specificity and sensitivity, and allows quantization of microorganisms without prior any nucleic acid amplification. The development of an electrochemical biosensor for detecting the hybridization occurring at the surface of modified gold electrode with a SAM of 3-mercaptopropionic acid (MPA) derivatised with thiolated DNA.

2. Experimental

2.1. Apparatus and electrodes

Voltammetric measurements were carried out with an μAUTOLAB (Ecochemie) potentiostat using the software package General Purpose Electrochemical System (GPES 4.9, Eco Chemie, The Netherlands). A Metrohm gold disk electrode (3 mm) was used as electrode to be coated with the respective SAMs: MPA and further covalent immobilization of the probe oligonucleotide. 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.

2.2. Reagents and solutions

Methylene blue (MB) was purchased from Sigma. 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.

The PCR amplified real samples were collected from 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:

- 20-mer probe: 5′ HS-$(CH_2)_6$-ACT CCC AAA CCC AAT GTG AA′3;
- 20-mer target DNA: 5′ CAG CCG TTA AAC ACC CAA CT′3;
- 20-mer non-complementary: 5′ ATC GAT GCC AGA ACC AAG AG′3.
- 20-mer target DNA of non-harzianum (*T. longibrachiatum*): 5′ CCA CCC TCG AGT GAA CGT AT′3.

DNA oligonuleotide stock solutions (nominally 1000 ppm conc. 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).

Probe immobilization is obtained *via* self assembly of the 5 or 3 thiolated probe purchased from First BASE Laboratories Sdn Bhd. 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. Other solutions employed, prepared in deionized water were 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 of cyclic voltammetric measurements and also used for washing buffer. In hybridized 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.

2.3. Procedures

2.3.1. Pretreatment of the gold electrode (AuE)

Before carrying out the deposition of the SAMs, the gold disk electrode (AuE) was pretreated as described previously by Katz and Solovev [36]. The AuE was polished with 3-μm diamond powder (BAS MF-2059) for 2 min. Then, it was sonicated in deionized water for 1 min, and immersed for 1 h in a hot 2 M KOH solution. Next, the electrode was rinsed with water, immersed in concentrated H_2SO_4 for 10 min, rinsed with water, immersed in concentrated HNO_3 for 10 min and rinsed again with deionized water. Finally the electrode was dried thoroughly under a N_2 flow.

2.3.2. Preparation of the MPA-modified AuE

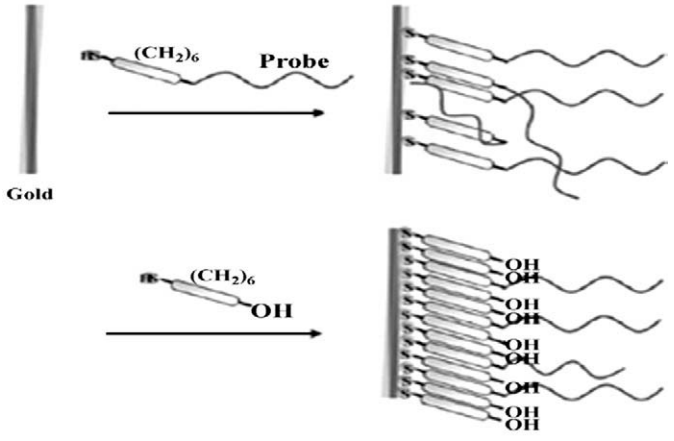
MPA-SAMs were formed by immersion of the pretreated AuE in a 40 mM MPA solution in EtOH/H_2O (75/25, v/v) for at least 15 h. Then, the modified electrode was rinsed with deionized water.

2.3.3. Probe immobilization

The SAM/MPA/Au electrode was modified with a thiol derivatised probe (Oligo-C_6-SH, TE buffer, pH 8.0 solution) for at least 24 h at room temperature (Scheme 1) [37]. After that it was washed with washing solution in (50 mM Tris–HCl + 20 mM NaCl, pH 7.2) for 2 min to remove unbound oligonucleotides.

2.3.4. Hybridization

The probe-modified electrode (ssDNA/SAM/MPA/AuE) was then hybridized with different concentration target DNA in 2×SSC buffer stirred for different times and temperatures, and then cooled gradually at room temperature. The electrode surface was then washed with the washing buffer for 1 min to remove the unbound oligonucleotides. These treatments allowed the specific sequence target DNA to hybridize with immobilized ssDNA to form dsDNA on the surface of gold electrode (dsDNA/SAM/MPA/AuE). The same protocol was applied to the probe-modified electrode in order to test the hybridization reactions of the probe with non-complementary oligonucleotide sequences.



Scheme 1. The schematic illustration of the processes of the immobilization of thiolated DNA onto the gold electrode [37].

2.3.5. Label binding to the 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 5 min without applying any potential. After accumulation of MB, the electrode was rinsed with 50 mM Tris–HCl buffer (pH 7.2) for 10 s to remove the non-specifically bound MB and transferred into the blank buffer solution (50 mM Tris–HCl + 20 mM NaCl, pH 7.2) for the voltammetric measurements.

2.3.6. Voltammetric transduction

The reduction signal of the accumulated MB was measured using cyclic voltammetric (CV) by scanning the potential from −1.50 to +1.50 V with amplitude of 100 mV/s performed in 50 mM Tris–HCl buffer (pH 7.2). A constant potential of +0.459 V vs Ag|AgCl was applied to the electrode for 75 s. The formation of MB polymer film and of gold oxides occurred at +1.0 V. The raw data were also treated using the Savitzky and Golay filter (level 1) of the GPES software, followed by the moving average baseline correction with a "peak width" of 0.1. Repetitive measurements were carried out by renewing the surface and repeating the above assay format. All experiments were conducted at room temperature.

2.3.7. Crude DNA extraction from isolates

The isolates were cultured in liquid media to obtain mycelia mass for DNA extraction. Agar discs were cut out from an actively growing *Trichoderma* mycelia with a 5 mm-diameter cork borer and placed into 100 ml potato dextrose broth (PDB) (Difco; USA) as starter cultures. The flasks were maintained as still cultures for 7–10 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 slurry obtained was stored at −20 °C or used immediately for crude DNA extraction.

Total fungal DNA was extracted by the Phenol-Chloroform Method [38]. About 50 mg of the ground mycelium was added 500 μl 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. Then after added with 350 μl buffered phenol and 150 μl chloroform, manually mixed in an up-down motion for 10 min and then centrifuged at 13,000 *g* at 4 °C for 10 min. The upper aqueous layer was collected into sterile microcentrifuge tubes to which was added 3 μl RNAse solution. The mixture was incubated at 38 °C in a water bath for 15 min. An equal volume of chloroform was added to the sample, gently mixed manually for 10 min and then centrifuged a second time (at 13,000 *g*/4 °C/10 min). The upper aqueous phase was again collected into a new microcentrifuge tube and the DNA was precipitated with 250 μl iso-propan-2-ol; the samples were kept overnight at −20 °C. The tubes were centrifuged the next day (at 13,000 *g*/4 °C/10 min), and the pellet thoroughly washed twice with 500 μl of 70% ethanol, vacuum-dried and diluted in ddH_2O and kept at −20 °C or directly proceed to the next step, which was to check the quality of the DNA samples by performing electrophoresis on 1.5% agarose gel using lambda DNA as marker. Electrophoresis was performed in a 1× TBE (0.045 M Tris-borate and 1 mM EDTA [pH8.2]) running buffer at 70 V for 1 to 2 h. The appearance of bands on the gel after by agarose gel electrophoresis indicated in the presence of DNA and thus allowed to further use DNA sensors.

3. Results and discussion

3.1. MB accumulation onto the modified electrode and probe-DNA

Modification of gold surfaces with 5′-thiol-terminated DNA was established by cyclic voltammetric (CV) reduction signals of MB labeled oligonucleotides on AuE. The electrochemical cell of 50 mM Tris–HCl buffer (pH 7.2) at gold extends from −1.50 and +1.50 V.

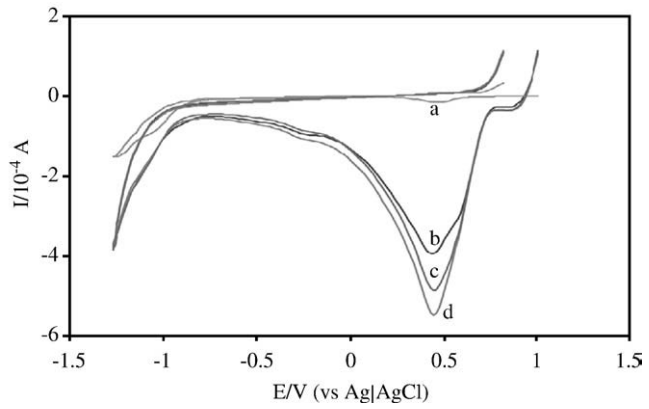
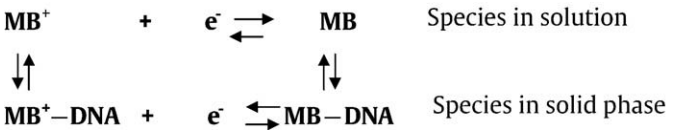


Fig. 1. Cyclic voltammetric obtained for; a) a bare gold electrode without MB, b) a bare gold electrode with MB, c) probe-modified electrode without MB, and (d) probe-modified electrode with MB at a scan rate of 100 mV/s vs Ag|AgCl. (I — current, E — potential).

The cyclic voltammetric measured at a bare gold electrode (AuE) with MB, a bare AuE without MB, probe-modified electrode (AuE/MPA/SAM) with MB and probe-modified electrode (AuE/MPA/SAM) without MB are shown in Fig. 1. The voltammetric signals of MB at these electrodes were then compared with the signals obtained at a bare AuE. Immobilization of the probe onto the SAM-modified electrode resulted in a larger increase in MB peak currents (AuE/MPA/SAM/probe in Fig. 1) due to the affinity of MB. The accumulation of MB, the peak current rapidly increased at all. This resulted in much larger voltammetric-peak-currents than was observed for the immobilized ssDNA (thiol-derivatised probe). Generally, MB-DNA interaction process can be described by the following scheme, which is similar to the one proposed by Yan et al. [26] in which both the oxidized and reduced forms of MB were bound to the DNA molecule in solid substrate (Scheme 2).



The electron-transfer rate between the electrode and the DNA monolayer was attenuated by the intercalation of MB onto the DNA monolayer and the rate was significantly faster at a bare gold than the derivatised surface. Loaiza et al. [39] concluded that MB was optimized by checking the sensor response between 2 and 20 μM. The peak current responded was observed up to 10 μM MB with a level off for higher concentrations, while an accumulation time of 1 min at open circuit was used. The overall performance of the gold electrode surface reaction was strongly dependent upon experimental variables that might influence the hybridization efficiency.

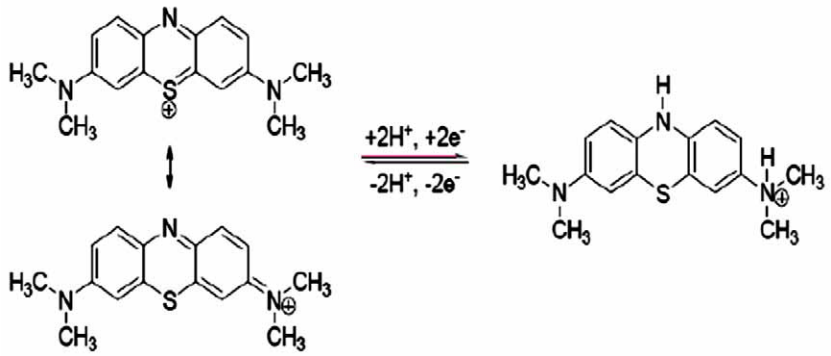
3.2. Hybridization specificity of T. harzianum

The DNA biosensor relies on the electrochemical transduction of the hybridization between the probe and target sequences. In all cases, MB was used as hybridization indicator. This molecule produces different signals in the presence of ssDNA or dsDNA or non-harzianum ssDNA at 1000 ppm conc. DNA. Taking into account, the significant difference between the voltammetric signals of MB obtained the hybridization of *T. harzianum* and non-harzianum with target DNA, the capture probe was investigated for the response of non-complementary DNA. The amounts of accumulated MB were in the order of complementary hybrid dsDNA > ssDNA > non-complementary DNA > non-harzianum ssDNA (*T. longibrachiatum*) (Fig. 2).

For response of non harzianum target DNA, a small peak current was obtained, can be attributed to very little intercalation of MB, and showed a lower hybridization efficacy compared with target DNA of *T. harzianum*. These probe molecules were the complementary target sequences of the specific part of the amplicon related to a specific gene sequence of *T. harzianum*. The electrochemical biosensors also showed an effective tool to differentiate the hybridization of *T. harzianum* versus non-harzianum with a good selectivity and sensitivity. In our knowledge, this is the first time described a sequence-specific electrochemical biosensor based on the immobilization of a 20 base single-stranded probe, specific to the *T. harzianum* gene, onto a modified gold disk electrode *via* covalent binding of MB.

The increase in the level of the voltammetric reduction signals of MB thus reflected the extent of the hybrid formation. These results showed a reasonable agreement with common concept for an intercalator [31,39]. In other words, more MB can be incorporated into dsDNA than ssDNA under similar experimental conditions. Kelley et al. [30,31] also reported the redox chemistry of MB (Scheme 3) on monolayer of DNA on gold electrodes. They also concluded that MB shows high affinity for immobilized dsDNA and that MB binding sites are localized mainly to the solution-accessible periphery of the monolayer. Therefore, the difference between the obtained MB voltammetric signals in the presence of the single-stranded DNA probe, and after the hybridization process, was employed as indicator of the extent of this process. As can be seen, the signals obtained after the hybridization process were always higher than those obtained in the presence of the single-stranded DNA probe. This behaviour can be attributed to the interaction of MB with two guanine bases in the ss-DNA [26,40], as well as to the intercalation of MB in the DNA double helix, if there are at least two G–C base pairs [41].

Control experiments were performed to assess whether the biosensor responded selectively, *via* hybridization, to the target. The



Scheme 2. Methylene blue reduction at the electrode surface [26].

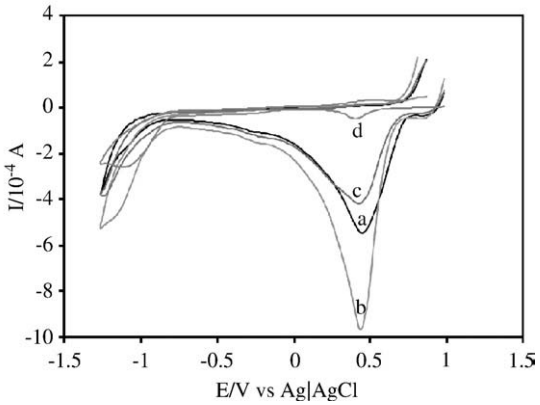


Fig. 2. Cyclic voltammetric using MB as the redox indicator for: (a) a probe-modified gold electrode, (b) after hybridization with the probe-ssDNA, (c) non-complementary DNA with the probe-ssDNA and (d) non-harzianum target DNA (*T. longibrachiatum*) with the probe-ssDNA at a scan rate of 100 mV/s vs Ag|AgCl. (*I* — current, *E* — potential).

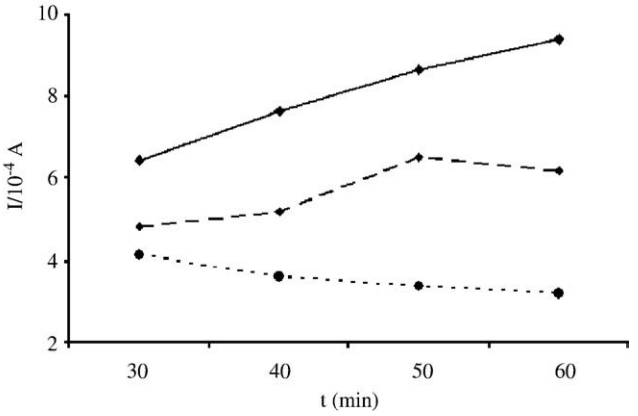


Fig. 3. Cyclic voltammetric measured using MB as the redox indicator for different temperatures (a) 30 °C, (b) 40 °C and (c) 50 °C) and times (30, 40, 50 and 60 min) of target DNA at modified-AuE at a scan rate of 100 mV/s vs Ag|AgCl. (*I* — current, *t* — time).

peak current of MB always obtained less signal comparing between a probe DNA and the non-complementary DNA.

3.3. Effect of hybridization times and temperatures

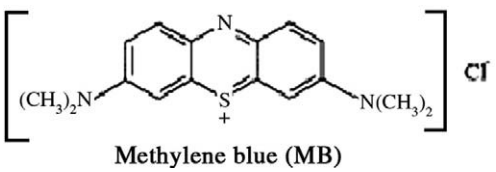
The efficiency of hybridization of the target DNA (1000 ppm conc. DNA) is dependent onto the hybridization times and temperatures, optimized *via* CV method. The modified gold electrode was hybridized with target nucleic acid [Tm = (60.03 ± 0.026) °C] and probe DNA [Tm = (60.21 ± 0.04) °C] at different temperatures, such as 30, 40 and 50 °C, and for different times such as 30, 40, 50 and 60 min. The hybridization efficiency and stability can be evaluated by interaction of MB. The larger the value was the higher hybridization percentage. The effects of hybridization temperatures and the hybridization times on the CV peak current of MB are shown in Fig. 3.

At 30 °C, the hybridization was reached the maximum hybridization efficiency and specificity with time for 60 min. Times can influence the labeling reaction of hybridization. As hybridization proceeded, the peak current increased because the increased local concentration of dsDNA on the electrode surface allowed greater quantities of MB to be intercalated into dsDNA.

At 50 °C, the hybridization rapidly decreased with times between 30 and 60 min. At 40 and 50 °C, hybridization of the peak current of MB was decreased with time for 60 min. This indicates that hybridized DNA was stable at 30 °C for 60 min. Thus, the optimal hybridization temperature was selected as 30 °C and the optimal hybridization time as 60 min with ensures high efficiency.

3.4. Electrochemical response of a different concentration of target DNA

The electrochemical signal is directly proportional to the amount of analyte (target ssDNA). The sensitivity of the AuE modification based electrochemical hybridization assay was investigated by varying the oligonucleotide concentrations in the range of 1–20 ppm. The hybridization reaction completed after 1 h at 30 °C, when a plateau or maximum value of the peak current was obtained. The linear regression equation for target DNA was: $y = 1.0031x + 32.7$ ($R^2 = 0.9664$) (x is the concentration of target DNA, y is the reduction current). The response for the reduction of accumulated MB peak current increased with increasing amounts of the target DNA.



Methylene blue (MB)

Scheme 3. Structure of common indicator used in DNA biosensor.

3.5. Crude DNA assessment between T. harzianum and non-harzianum isolates

The DNA biosensor confides on the electrochemical transduction of the crude DNA among *T. harzianum* (isolate: T32), *T. inhamatum* (isolate: T127), *T. koningii* (isolate: S10), *T. longibrachiatum* (isolate: T28), *T. virens* (isolate: T128) and *T. aureoviride* (isolate: T45). In all cases, MB was used as a redox indicator, which displayed significantly different voltammetric signals in the presence of *T. harzianum* and/or non-harzianum (*T. inhamatum*, *T. koningii*, *T. longibrachiatum*, *T. virens* and *T. aureoviride*) with modified AuE at 30 °C for 60 min. Peak currents were found to increase in the following order: *T. harzianum* > *T. inhamatum* > *T. aureoviride* > *T. virens* > *T. longibrachiatum* > *T. koningii* (Fig. 4). For results into a explanation, a diminutive voltametric signals difference between *T. harzianum* and *T. inhamatum.* Accordingly, Bissett [42] reported that *T. inhamatum* was a synonym of *T. harzianum* based on the morphological analysis. Using molecular tools, there was found a close relationship between *T. harzianum* and *T. inhamatum,* and it was not able to differentiate *T. inhamatum* from *T. harzianum.* So, they declared *T. inhamatum* a synonym of *T. harzianum*/*H. lixii* clade [43,44].

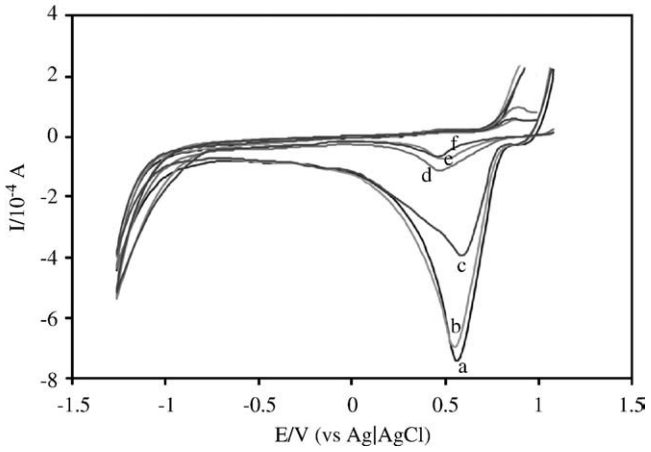


Fig. 4. Cyclic voltammetric obtained using a crude DNA with accumulation of MB peak currents recorded at each stage in the fabrication of modified-AuE by; a) *T. harzianum*, b) *T. inhamatum*, c) *T. aureoviride*, (d) *T. virens* e) *T. longibrachiatum* and (f) *T. koningii* at scan rate of 100 mV/s vs Ag|AgCl. (I — current, E — potential).

Therefore, DNA Biosensor results also supported that *T. inhamatum* belongs to *T. harzianum*.

In response to other non harzianum crude DNA, a less peak current was obtained comparing to crude DNA of *T. harzianum*. The electrochemical biosensors showed an effective tool to differentiate of *T. harzianum* versus non-harzianum species, without nucleic acid amplification. In our knowledge, this is the first time that described a crude DNA used electrochemical biosensor based on the immobilization of a 20 base single-stranded probe, specific to the *T. harzianum* gene, onto a modified gold disk electrode *via* covalent binding of MB.

4. Conclusions

The construction of *Trichoderma harzianum* specific DNA biosensor using a SAM/MPA/AuE-modified gold electrode, as described in this paper, allows the enhancement of the efficiency of the DNA hybridization process with the complementary thiolated DNA on the gold surface. The potentialities of a simple and specific DNA hybridization detection method based on the cyclic voltammetric response of MB on modified AuE with a thiol derivatised probe–DNA duplex after hybridization has been evaluated by resorting to the analysis of the ITS 1 region genes of *T. harzianum*. A practically new technique of electrochemical DNA biosensor could be able to detect microorganism at the species levels without the use of PCR amplification. The sensor has shown to be an effective tool to differentiate the hybridization versus non-hybridization with a good selectivity and sensitivity. These results confirmed that this detection approach provides a quick, sensitive, reusable and convenient methodology for the detection of *T. harzianum*.

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