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DNA-based Biosensor for Detection of *Ganoderma boninense*, an Oil Palm Pathogen Utilizing Newly Synthesized Ruthenium Complex $[Ru(phen)_2(qtpy)]^{2+}$ Based on a PEDOT-PSS/Ag Nanoparticles Modified Electrode

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An electrochemical DNA biosensor has been developed for detection of *ganoderma boninense*, an oil palm pathogen utilizing newly synthesized ruthenium $[Ru(phen)_2(qtpy)]^{2+}$ complex as hybridization indicator. The sensor incorporated the use of a gold electrode (AuE), modified with a conducting nanocomposite of poly(3,4-ethylene- dioxythiophen) - poly(styrenesulfonate) (PEDOT-PSS) and silver nanoparticles (AgNPs). A specific sequence of a *ganoderma boninense* DNA probe has been immobilized on the modified electrode and the hybridization event was monitored via intercalation of the ruthenium complex to the hybridized DNA. Effect of hybridization temperature and time was evaluated and found to be optimal at 45 ^{o}C in 25 minutes for the hybridization. Detection of target DNA ranged from 1.0 x 10^{-15} M to 1.0 x 10^{-9} M was performed, and a correlation relationship of 0.9756 and detection limit of 5 x 10^{-16} M were obtained. The newly synthesized ruthenium complex was able to be used is a novel redox marker and can be adopted for routine detection of DNA.

Keywords: Biosensor, *Ganoderma boninense*, DNA, PEDOT-PSS, Ruthenium complex

1. INTRODUCTION

Recently, electrical conduction through DNA molecules has received much attention, particularly as DNA emerged one of the best candidate materials for device applications such as

biosensors and molecular electronics [1]. Different techniques have been developed for DNA detection and much effort has been put in order to upgrade the detection sensitivity and selectivity of electrochemical DNA biosensor [2, 3]. The primary target of an electrochemical DNA biosensor is to achieve a precise molecular orientation of probe DNA for hybridization of target DNA fragment [4]. The level of skill required to achieve this includes knowledge of the parameters that enhance molecular orientation of successful DNA immobilization and hybridization. Characterization of a new device for detection ability is of paramount importance, especially when dealing with sensitive organism detection such as specific base sequence of bacteria, fungi or viral specie in molecular diagnostics and genomics analysis.

The ability to detect specific DNA sequences is important because nucleic acid sequences are unique to every living organism, and so far there exist 4000 known inherited diseases [5] of which their underlying mutations need to be identified. Oil palm E*laeis guineensis* is an important economic driver across Asia, specifically Malaysia and Indonesia, where it is found and widely planted for economic purposes. In recent years, oil palm trees have been suffered from fungal attack by *ganoderma boninense*, causing basal stem rot (BSR) disease, resulting in severe losses of palm oil production [6]. It is therefore, necessary for its spread to be controlled. The identification of *ganoderma boninense* colonies has been based on traditional culturing methods that require lengthy investigation periods. The lack of specificity and many sources of interference occur in the conventional chemical methods, making the process often susceptible to errors, as there are many ganoderma species that appear to be very similar in their culturing conditions.

The trend of DNA studies involved the use of polymerase chain reaction (PCR) as nucleic acid diagnostic tool that emerged in 1983 from the work of Kary Mullis [7]. In PCR, pre-amplification of genomic DNA is required prior to analysis to enhance the selectivity. However, many uncertainties were observed from PCR results [8-10]. Research activities then focused on molecular interactions between surface-linked DNA and target pollutants in order to develop devices that are fast, sensitive, selective and reliable platform for screening of compounds [5, 11]. Thus, the development of electrochemical biosensors has emerged and has grown rapidly because of its ability to detect biological binding events [12, 13], and for miniaturization and provision of portable instrumentation at a point of care.

Currently, the emergence of nanomaterials has enabled electrode modification system using nanocomposite materials to begin to replace the traditional electrode system in electrochemical biosensing techniques [14]. There are a great number of benefits offered by nanoparticles in sensor research, and these are explained extensively in a review by Upadhyayula [15]. Nanoparticles such as gold and silver nanoparticles have been used together with poly (3, 4ethylene dioxythiophen) PEDOT and poly (3,4ethylenedioxythiophen) – poly (styrenesulfonate) PEDOT-PSS for electrode modification [16, 17, 18, 19]. These techniques are used in an effort to achieve correct molecular orientation of the probe DNA on the modified surface for hybridization of the target DNA.

The blend of metal nanoparticles with conjugate polymers to form nanocomposite is intended to increase electrical conductivity of the electrode [20, 21]. When a polyelectrolyte $(PSS)^{n-}$ is incorporated into PEDOT, the polyanion of PSS would compensate for the positive charge of PEDOT [22], which results in high ionic conductivities. The use of PEDOT has attracted the interest of many

researchers because of its very good film forming properties, high stability and high charge mobility. We considered PEDOT-PSS could be excellent for electrode coating in our device because of its good electrical characteristics, and work function (Φ) ~ 5 eV [23]. In a related study, Lin and co-workers [24] have used PEDOT-PSS by simple drop-coating technique in modification of electrode for detection of NO_2 gas. The use of PEDOT with PSS enables PEDOT dispersion in water and thus, thin film can be obtained on the substrate surface [21]. The low cost, and the special chemical and physical properties of silver nanoparticles compared to other nanoparticles such as gold, make it attractive for electrochemical biosensor applications [25-28].

The DNA identification is basically performed with the application of a hybridization indicator. In recent development, studies of the interactions of ruthenium complexes are gaining popularity as alternative redox indicators in DNA biosensor applications [29]. Redox active cations and DNA bind strongly to modified surfaces and produce the expected electrochemical signals. Metal complexes can, if desired, be used as DNA-intercalating and electrochemical label, of which the properties is related to facile interchange of its ligand [30]. Complexes with phen ligand, $[Ru(phen)_3]^{2+}$ (phen=1,10-phenanthroline) [31] are reported to have low affinity for binding to DNA. In another study, $[Ru(phen)_3]^{2+}$ has a binding mode of intercalation at Δ-enantiomer and minor groove at complementary Λ-enantiomer [30]. In another previous research [32, 33], complexes having both phen and dppz ligands $[Ru(phen)_2(dppz)]^{2+}$ has been reported to have a good intercalation properties [32, 33], leading to their description as "molecular light switches" for DNA [34]. The unique combination of chemical stability, redox properties, excited state reactivity, luminescence emission and excited state lifetime [35] of this metal complex has attracted the interest of researchers. In this work, we considered the use of PEDOT-PSS blend with silver nanoparticle to form a nanocomposite film membrane for gold electrode surface modification using a simple drop coating method. A newly synthesized ruthenium complex $[Ru(phen)_2(qtpy)]^{2+}$ [phen = 1, 10- phenanthroline; qtpy = 2, 2': 4, 4′′ : 4′, 4′′′- quaterpyridyl] was used as hybridization indicator, for the detection of *ganoderma boninense*, pathogen of the oil palm.

2. MATERIALS AND METHODS

2.1. Reagents and solutions

The ruthenium complex was synthesized according to literature [36]. Stock solution of the ruthenium complex $[Ru(phen)_2(qtpy)]^{2+}$ was prepared in deionized water. Dilute solutions of 25 μM, 20 μM and 10 μM was then prepared from stock. Supporting electrolyte solution of Tris-EDTA [10 mM Tris-HCl and 1 mM EDTA] (pH 7.15) was prepared in deionized water (Di-water) obtained from a Millipore Milli-*Q* purifier. An activation solution of 5 mM *N* -hydroxysulfosuccinimide (NHSS) + 2 mM 1-ethyl–3-[3 – dimethylamino- propyl] carbo- diimide hydrochloride (EDC) + 50 mM Sodium-phosphate was prepared in deionized water. All chemicals used were of analytical grade. Oligomers (20-mer probe ssDNA 5′-NH_2-CCT GCT GCG TTC TTC TTC AT-3, ' 35-mers target DNA 5′-TTG GCT CTC GCA TCG ATG AAG AAG AAC GCA GCA GG-3′ and 21-mers mismatch 5′-AGA TGC GTT ACA TCG CAA TAC-3′) were synthesized by First BASED Laboratories Sdn Bhd, Selangor,

Malaysia. DNA oligonucleotide (100 μM) stock solutions and other dilute concentrations 1 x 10^{-9} M to 1 x 10^{-15} M of the DNA was prepared in TE buffer solution (pH 8.0) and kept frozen. The DNA solution was defrosted when needed and returned immediately to frozen store when not in use.

2.2. Preparation of coating solution

Silver nanoparticles were blended according to the literature [19] into a solution of PEDOT-PSS in the ratio of 10 μl AgNPs:10 ml PEDOT-PSS, and the blended solution was kept at -10 ^{o}C when not in use. An aqueous suspension of the PEDOT-PSS and silver nanoparticles in ethylene glycol were purchased from Sigma-Aldrich and all used as received.

2.3. Apparatus and electrode

Voltammetry measurements were obtained using μAUTOLAB (Ecochemie, The Netherlands) potentiostat incorporated with General-Purpose Electrochemical System (GPES 4.9, Eco Chemie) software. The electrochemical cell used was a three-electrode system of Metrohm gold electrode (AuE) as the working electrode, platinum (Pt) wire as the counter electrode and Ag/AgCl/KCl 3M as the reference electrode.

2.4. Electrochemical characterization of ruthenium complex $[Ru(phen)_2(qtpy)]^{2+}$

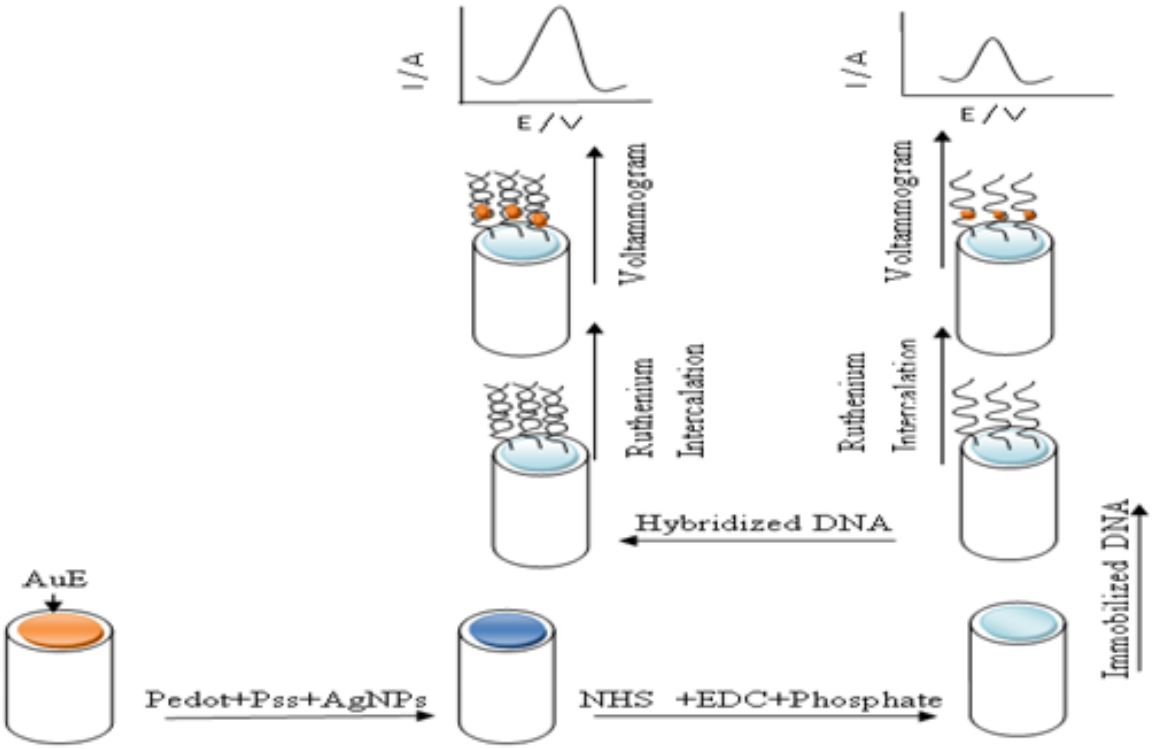
The prepared Tris-EDTA (TE) buffer was used as supporting electrolyte for the voltammetry experiments. The cyclic voltammogram was performed with a bare gold electrode (bare AuE) at potential from 2000 mV to 50 mV, at a scan rate of 100 mV/s. Differential pulse voltammetry (DPV) was also performed with and without ruthenium complex solution at potential from 200 mV to 1200 mV and step potential of 5.1 mV.

2.5. Modification of the electrode

The bare gold electrode was pre-treated for modification by cleaning it with alumina slurry, sonicated and rinsed in deionize water, immersed in concentrated sulphuric acid for 5 min**,** rinsed and sonicated in TE washing buffer. It was dried under nitrogen gas for 30 seconds and dried at room temperature for 45 min. The AuE was drop-coated with a mixture of PEDOT-PSS/AgNPs and was oven-dried at 70 ^{o}C for 15 h. The nanocomposite membrane (PEDOT-PSS/AgNPs/AuE) was then cleaned with TE buffer to remove unbound remnants. The modified gold electrode (PEDOT-PSS/AgNPs/AuE) was then dried at room temperature for 45 min. Electrochemical investigation of the modified electrode was carried out using cyclic voltammetry (CV) and DPV with TE buffer as the supporting electrolyte.

2.6. Immobilization of DNA probe

The modified gold electrode PEDOT-PSS/AgNPs/AuE was rinsed with TE buffer and dried at room temperature for 45 minutes. It was then incubated in 5 mM NHSS and 2 mM EDC containing 50 mM phosphate buffer (pH 5.2) solution for 1 h at room temperature. After the reaction, the modified electrode was rinsed with TE buffer and thoroughly dried under a stream of nitrogen gas flow. The modified electrode was then incubated in DNA probe solution for 12 h. The attachment of DNA probe (represented in scheme 1) is adapted from the traditional method of combining EDC and *N*-hydroxysuccinimide (NHS) to form covalent amide bonds for immobilization of 5'-NH_2-ends of DNA on to carboxyl-containing substrates, whereby the carboxyl is being replaced with a sulfonate group from PSS. The probe-modified electrode was labeled ssDNA/PEDOT-PSS/AgNPs/AuE**.**



Scheme 1. Schematic representation of electrochemical detection of DNA immobilization and hybridization

2.7. Hybridization of DNA

The probe-modified electrode (ssDNA/PEDOT-PSS/AgNPs/AuE) was hybridized with target DNA at a concentration of 100 µM. Different hybridization time and temperature was evaluated in order to optimize the experimental parameter. On each occasion, 25 µl of 25µM ruthenium [Ru $(phen)_2(qtpy)]^{2+}$ complex was used to confirm the hybridization. The hybridized system was denoted as dsDNA/PEDOT-PSS/AgNPs/AuE. The same procedure was applied for mismatched DNA

sequences at optimized condition. The sensitivity of the detection system was evaluated using different concentration of target DNA ranging from 1 x 10^{-9} M to 1 x 10^{-15} M prepared in TE buffer (pH 7.15).

3. RESULTS AND DISCUSSIONS

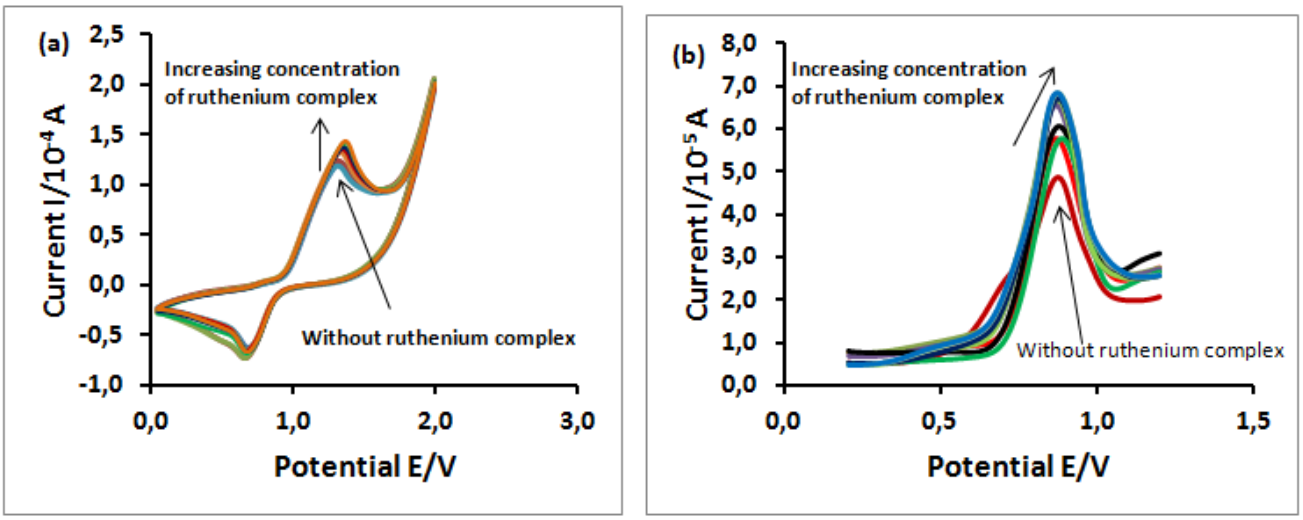


Figure 1. (a) CV and (b) DPV of ruthenium complex [Ru $(phen)_2(qtpy)]^{2+}$ using AuE in TE buffer

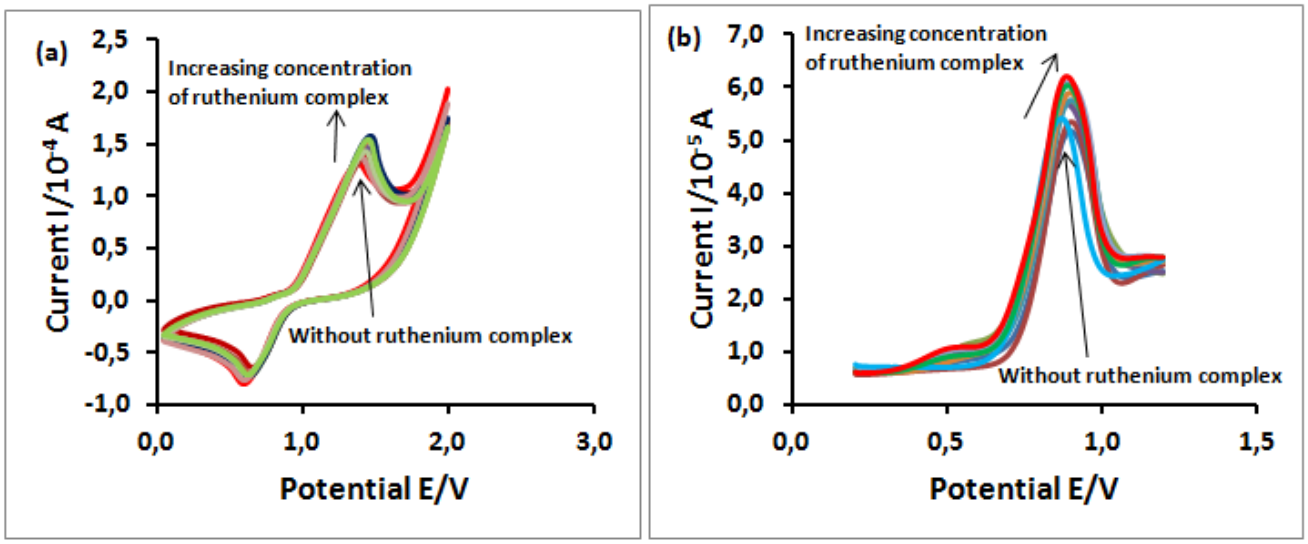


Figure 2. (a) CV and (b) DPV of Modified (PEDOT-PSS/AgNPs/AuE) electrode in ruthenium complex [Ru $(phen)_2(qtpy)]^{2+}$ solution (in TE buffer).

A variety of methods are used to assess electrochemical transduction of redox indicators. Each has its advantages and drawbacks. Initially, the current signal peaks of the ruthenium complex indicator obtained using a bare AuE through CV and DPV voltammogram presented in Fig.1 (a and b) revealed an increased in current with increasing concentration of ruthenium complex. It is observed

that by using DPV, the response increments are more distinct than by using CV, signifying higher sensitivity response in DPV compared to CV.

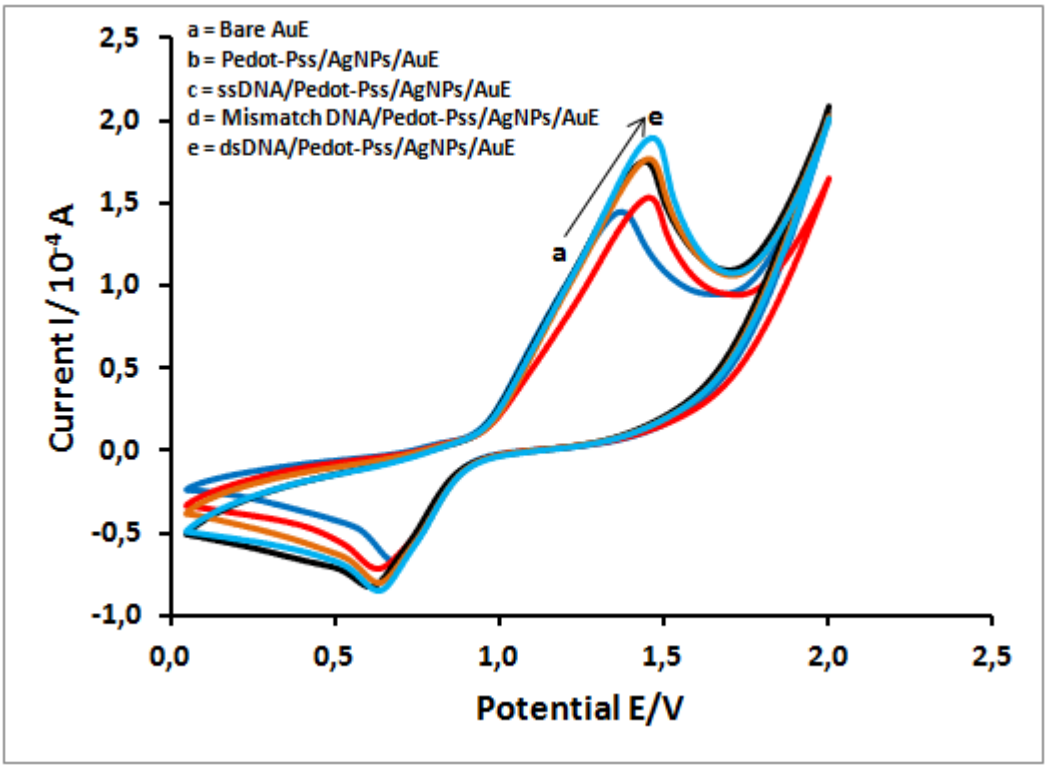
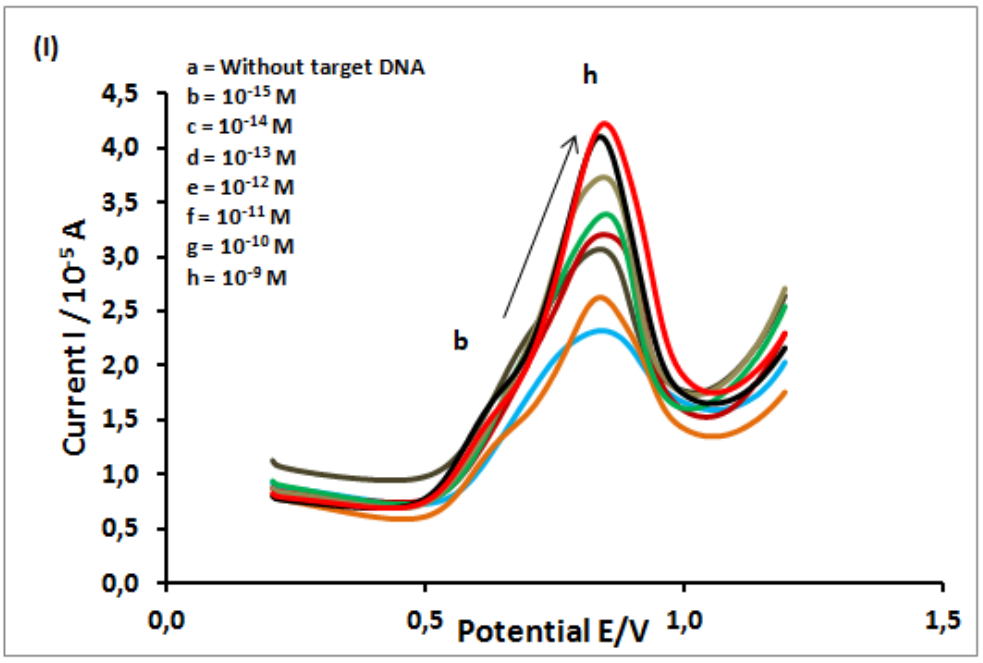


Figure 3. CV of (a) Bare AuE, (b) PEDOT-PSS/AgNPs/AuE, (c) ssDNA/PEDOT-PSS/ AgNPs /AuE, (d) mismatchDNA/PEDOT-PSS/AgNPs/AuE, (e) dsDNA/PEDOT-PSS/AgNPs/AuE



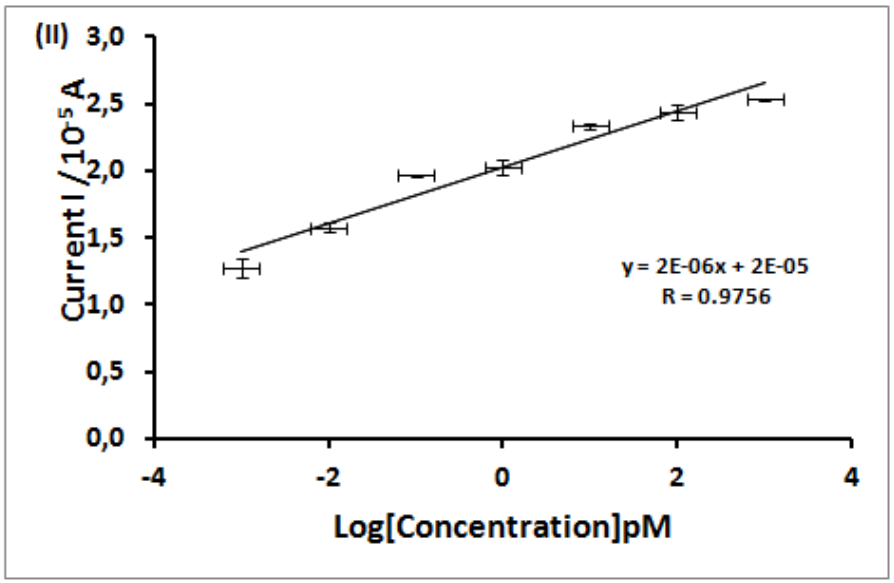


Figure 4. (I) Differential pulse voltammograms (DPV) on effect of target DNA concentration of (a) without target DNA, (b) 1 x 10^{-15} M, (c) 1 x 10^{-14} M, (d) 1 x 10^{-13} M, (e) 1 x 10^{-12} M, (f) 1 x 10^{-11} M, (g) 1 x 10^{-10} M, (h) 1 x 10^{-9} M (II) log(concentration) of target DNA against peak current

However, the difference in peak current obtained after and prior modification in Fig. 1a and 2a is insignificant. The highest current was obtained for hybridization with target DNA rather than with mismatched DNA and probe DNA, as shown in Fig. 3. This indicates that there is a higher ruthenium intercalation when hybridized with complementary target DNA compared to with mismatch DNA and DNA probe.

The increased in current during hybridization is due to the amount of ruthenium [Ru $(phen)_2(qtpy)]^{2+}$ intercalated into the DNA duplex on the surface of the modified electrode.

The developed system could detect target DNA with concentration ranging from 1.00 x 10^{-9} M to 1.00 x 10^{-15} M, as shown in Fig. 4, revealing that it is highly sensitive. The limit of detection (LOD) is calculated as 5.00 x 10^{-16} M. Table 1 summarized the comparison of the performance of other DNA electrochemical biosensors found in the literature based on metal complexes and nanoparticles. The present biosensor has lower detection limit and acceptable linear range as compared to previous research [3, 37, 38].

Table 1. Comparison of detection limit between different metal complexes with various modifiers

Modifying films	Methods	Linear range (µM)	LOD (µM)	Reference
PEDOT-PSS/AgNP/AuE	DPV with $[Ru(dppz)_2(qtpy)]\ Cl_2$	1 x 10^{-9} – 1 x 10^{-3}	6.20 x 10^{-10}	[37]
$NiOx_{np}$/GCE	DPV with $[Ru(NH_3)Cl]PF_6$	4 x 10^{-4} – 1 x 10^{-2}	6.80 x 10^{-5}	[38]
AuNP/AuE	DPV with $[Ru(NH_3)_6]^{3+}$	1 x 10^{-6} – 1 x 10^{-1}	1.00 x 10^{-6}	[3]
PEDOT-PSS/AgNP/AuE	DPV with $[Ru(phen)_2(qtpy)]^{2+}$	1 x 10^{-9} – 1 x 10^{-3}	5.00 x 10^{-10}	This work

GCE glassy carbon electrode, $NiOx_{np}$ nickel oxide nanoparticles

4. CONCLUSION

The DNA biosensor was developed based on a modified gold electrode with a nanocomposite membrane; where by an immobilized DNA probe was immobilized on the thin-film nanocomposite membrane of a blended PEDOT-PSS polymer and silver nanoparticles. The modification of the electrode maintain the conductivity of the system, of which the interaction between the new ruthenium complex $[Ru(phen)_2(qtpy)]^{2+}$ and DNA molecule was investigated. The use of the new ruthenium complex is effective as a hybridization indicator. The developed sensor was found to be selective, sensitive, and able to detect as low a concentration as 1×10^{-15} $molL^{-1}$ of the target DNA. The results indicated that the developed biosensor by using the newly synthesized ruthenium complex provides a promising platform for DNA hybridization based detection. The result of R = 0.9756 obtained confirmed a strong coefficient of relationship of the DNA concentration detected and the 5×10^{-16} M limit of detection, demonstrating the sensitivity level of the developed biosensor.

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