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# An Electrochemical DNA Biosensor for *Ganoderma Boninense* Pathogen of the Oil Palm Utilizing a New Ruthenium Complex, $[Ru(dppz)_2(qtpy)]Cl_2$

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A new DNA biosensor for *Ganoderma boninense*, pathogen of the oil palm has been developed. The system was developed based on a gold electrode (AuE) modified with a conducting polymer film of poly(3,4-ethylenedioxythiophen)-poly(styrenesulfonate) (PEDOT-PSS) containing silver nanoparticles (AgNPs). Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) techniques were employed to characterize and optimized the detection system. The modified electrode exhibited higher electrical conductivity compared to the bare electrode. A DNA probe for *Ganoderma boninense* was immobilized on the modified electrode and a new ruthenium complex was employed as a marker for monitoring hybridization of target DNA. The effect of hybridization temperature and time was studied and was found to be optimal at 45 $^{o}$C with hybridization time of 35 minutes. Effect of different concentration of target DNA ranged from 1.00 x $10^{-15}$ M to 1.00 x $10^{-9}$ M was studied and calibration plot with correlation of 0.969 and a detection limit of 6.20 x $10^{-16}$ M was obtained. The newly synthesized ruthenium complex has shown a good affinity towards hybridized DNA and proven to be a good hybridization indicator. This work is the first ever reported biosensor based detection method for *Ganoderma boninense*.

**Keywords:** Electrochemical Sensor, Modified Electrode, *Ganoderma boninense*, Ruthenium, DNA.

## 1. INTRODUCTION

Knowledge of the parameters that promote the monitoring of DNA hybridization, immobilization and intercalation of redox with DNA provide a convenient means of the applications of electrochemical biosensors in the area of DNA detection. In particular, the most crucial aspect of electrochemical DNA biosensor performance is the immobilization of probe on the electrode surface [1] and the goal is to achieve a precise molecular orientation, of the single-stranded DNA (ssDNA) probe [2], for hybridization of the target DNA fragment. However, Immobilization procedure of probe (ssDNA) used onto modified electrode surface is critical as it influence the accuracy, sensitivity, selectivity and lifetime of the DNA biosensor [3]. Electrochemical characterization experiments have been performed to determine the correct protocol for detecting specific DNA sequences. Characterization of detection ability of a particular technique is paramount when detecting a specific base sequence of bacteria, fungi, or viral species in molecular diagnostics and genomics analysis.

The oil palm E*laeis guineensis* is widely planted across Asia—specifically Malaysia—and in recent years the oil palm trees have been prone to fungal attack by G*anoderma boninense*. The *Ganoderma boninense* pathogen has caused severe losses of palm oil production [4], therefore the control of its spread is critical. The identification of *Ganoderma boninense* colonies has been based on conventional culturing methods that require lengthy periods (9–10 days) of investigation. Consequently, the conventional chemical techniques suffer from a lack of specificity, a number of interfering factors, and are often susceptible to errors as there are many *Ganoderma* species that appear very similar in their culture conditions, as well as being slow, expensive, and not suitable for monitoring in the field. In recent studies, the trends of electrode and or surface modification [5] using nanocomposite materials such as silver nanoparticles have been the common practice. This is due to their electrical conductivity, unique structural and catalytic properties, high loading of biocatalysts, good stability, and excellent penetrability [6]. In particular gold and silver nanoparticles are used with polymers for surface modification in order to achieve proper molecular orientation of probe DNA for high accessibility of target DNA fragment. Different applications for the use of polymer containing silver and gold nanoparticles are summarized in Table 1.

**Table 1.** Polymer containing silver and or gold nanoparticles for surface modification

| Polymer | Nanoparticles | Purpose | Reference |
|---|---|---|---|
| PEDOT | AuNPs | Gold electrode coating | [7] |
| PEDOT | AgNPs | Glassy carbon electrode coating | [8] |
| PEDOT-PSS | AgNPs | Source-drain electrode ink printing | [9] |
| PEDOT-PSS | ___ | ITO covered glass coating | [10] |
| Polyaniline | AuNPs | Nanotube membrane | [11] |
| Polyaniline | AgNPs | Electrochemical oxidation of hydrazine | [12] |

AuNPs=Gold Nanoparticles, AgNPs= Silver Nanoparticles, ITO= Indium Tin Oxide

To increase the electrical conductivity of the conducting surface, metal nanoparticles are added to conjugate polymers [13-15]. We envisaged that PEDOT-PSS may have good potential for electrical and optical characteristics because of its work function (Φ) ~5 eV [16]. A novel approach, therefore, was sought for the complimentary and effective molecular orientation of probe DNA with silver nanoparticles and PEDOT-PSS in DNA detection, utilizing a new ruthenium compound { [ Ru $(dppz)_2$ (qtpy) ] $Cl_2$ = $(dppz)^{+2}$ }; dppz = dipyrido [3, 2 – a:2′ , 3′- c] phenazine; qtpy = 2, 2', - 4, 4′′ . 4′, 4′′′- quaterpyridyl as a hybridization indicator. The complex of PEDOT with PSS allows PEDOT dispersion in water to obtain thin films on surfaces because PEDOT alone cannot be easily processed [15].

The polymer on its own behaves as an insulator. The term "conducting polymer" should not be misunderstood, though it is often used with two different meanings in scientific literature [17], i.e., the blends of electrically conductive additives with thermoplastic polymers, and duromers are also occasionally referred to as conductive polymers [18]. The unique properties of conducting polymers have made them better candidates for conducting capabilities between biorecognition elements and electrode surfaces [19] in bioelectrochemical sensors.

The special properties exhibited by silver which gave it greater advantage over gold and its analogues includes; high extinction coefficient, sharper extinction bands, high ratio of scattering to extinction, and extremely high field enhancements [20]. The aforementioned properties have made it a good candidate, for bioelectrochemical sensor [21-23], catalysis [6, 24-25], antimicrobial and therapeutic [26] applications.

Ruthenium complexes are gaining popularity as alternative redox indicators in biosensor applications [27-28]. Redox active cations and DNA bind strongly to the modified surfaces and produce the expected electrochemical signals. Metallo-intercalators, such as ruthenium containing dppz are noted for their intercalation capability via photoelectrochemistry [29-30] and were found to have a high affinity (K= $10^6$ - $10^7$ $M^{-1}$**)** with dsDNA [31]. Complexes with a dppz ligand show strong intercalation with DNA [32] due to the extended aromatic heterocyclic surface that extrudes from the central core of the complex and in contrast, $[Ru(phen)_3]^{2+}$ (phen=1,10-phenanthroline) [33] has low affinity for binding to DNA whereas $[Ru(phen)_2(dppz)]^{2+}$ complexes have been reported to intercalate [29-30] leading to their description as "molecular light switches" for DNA [34].The structures of metal polypyridyl complexes of Ru with ligand can be transformed to suit metal-to-ligand charge transfer and/or the ligands can be oriented with electron donors and acceptors to probe electron transition [35]. The unique combination of chemical stability, redox properties, excited state reactivity, luminescence emission, and excited state lifetime [36] has attracted interest and there are currently many hundreds of derivatives with structural variations that are limited only by the researcher's imagination from original molecules. It is interestingly noted, that ligands or complexing agents containing sulphur (S-H) bearing group, amino compound (-NH2-), or carboxyl compound (-COOH-) can strongly form complexes species with a number of metal ions through coordinate covalent bond [37].

In this work we consider blends of PEDOT-PSS with silver nanoparticle for the electrode modification, and a new ruthenium complex $[Ru(dppz)_2(qtpy)]Cl_2$ as the intercalating material for the interaction and detection of the G*anoderma boninense*.

## 2. MATERIALS AND METHODS

### *2.1. Reagents and solutions*

The ruthenium complex was synthesized according to the literature [38]. Stock solution of the ruthenium complex was prepared in 50 mM Tris-HCl, 20 mM NaCl (volume 90%) and methanol (volume 10%). Dilute solutions (25 μM, 20 μM, and 10 μM) were then prepared from the stock. Buffer solutions of Tris-EDTA (TE) [10 mM Tris-HCl and 1 mM EDTA] (pH 7.15), 50 mM phosphate (pH 7.15), deionized water, and Tris-NaCl [50 mM Tris-HCl containing 20 mM NaCl] (pH 7.15) were prepared in deionized water (Di-water) obtained from a Millipore Milli-*Q* purifier respectively. An activation solution of 5 mM *N*-hydroxysulfosuccinimide (NHSS) + 2 mM 1-ethyl–3-[3-dimethylaminopropyl] carbo-diimide hydrochloride (EDC) + 50 mM sodium phosphate was prepared in deionized water. All chemicals used were of analytical grade. Oligomers (20-mer probe 5′-/5AmMC6/CCT GCT GCG TTC TTC TTC AT-3′, 35-mer target DNA 5′-TTG GCT CTC GCA TCG ATG AAG AAG AAC GCA GCA GG-3′ and 21-mer 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 1.00 x $10^{-9}$ M to 1.00 x $10^{-15}$ M of the DNA were prepared in TE buffer (pH 8.0) and kept frozen when not in use.

### *2.2. Preparation of coating solution*

Silver nanoparticle solution was blended according to the literature [9] 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.

### *2.3. Apparatus and electrode*

Voltammetry measurements were obtained using a 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 with a Metrohm gold electrode (AuE) as the working electrode, a platinum (Pt) wire as the counter electrode, and Ag/AgCl/KCl 3M as the reference electrode.

### *2.4. Electrochemical characterization of the novel ruthenium complex*

The prepared buffers were used as the analyte for cyclic voltammetry (CV) experiments with and without the prepared concentrations of ruthenium complex using 25 μl, 15 μl, and 10 μl volumes respectively. The CV was carried out with bare gold electrode under a set electrode potential of best fit, at 1800 mV to -5 mV**, s**can rate 100 mV/s for phosphate buffer; 1500 mV to -5 mV**,** scan rate 100 mV/s for Di-water; 2000 mV to -1000 mV, scan rate 100 mV/s for Tris-NaCl buffer and 2000 mV to

50 mV, scan rate 100 mV/s for TE buffer. DPV was performed without and with 25 μM, 25 μl ruthenium complex in Tris-EDTA at set potential 205mV to 1200mV and 5.1mV step potential.

### *2.5. Modification of Electrode*

The bare gold electrode (bare AuE) was pre-treated for modification by cleaning with alumina slurry, deionized water, and concentrated sulfuric acid**,** then sonicated and rinsed in TE washing buffer. It was dried under nitrogen gas for 30 seconds and kept at room temperature for 45 minutes for further drying. The AuE surface was drop-coated after drying with the prepared blended PEDOT-PSS containing AgNPs and was oven-dried at 70 $^{o}$C for 15 h. It was then cleaned with TE washing buffer to remove unbound remnants on the film surface. The modified gold electrode (PEDOT-PSS/AgNPs/AuE) was dried at room temperature for 45 minutes. Electrochemical investigations using DPV were performed with analyte TE buffer containing 25 μl of 25 μM ruthenium complex solution.

### *2.6. Immobilization of probe DNA*

The modified gold electrode PEDOT–PSS/AgNPs/AuE was rinsed with TE washing 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 washing buffer and dried under a stream of nitrogen. The probe DNA was then accumulated on the EDC-activated modified electrode surface for 12 h. The attachment of DNA probe was 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 onto carboxyl-containing substrates whereby the carboxyl is replaced with the sulfonate group from Pss. The probe-modified electrode was labeled ssDNA/PEDOT-PSS/AgNPs/AuE**.** The CV of the modified electrode was carried out in TE buffer at potential of +200 mV to +50 mV at scan rate 100 mV/s.

### *2.7. Hybridization of DNA*

Hybridization was carried out in TE buffer (pH 7.15) at 35 $^{o}$C**,** 45 $^{o}$C**,** and 55 $^{o}$C for 25, 35, 45, and 55 minutes. On each occasion 25 μl of 25μM ruthenium marker was used to detect the hybridization. The hybridized electrode was then denoted as dsDNA/PEDOT-PSS/AgNPs/AuE and the CV of each was obtained. The same protocol was applied to the probe-modified electrode for testing the hybridization reaction to mismatched sequences of target DNA. The effect of different concentrations of target DNA ($1.00 \times 10^{-9}$ M to $1.00 \times 10^{-15}$ M) in TE buffer (pH 7.15)) was also investigated. The DPV electrochemical measurements were obtained at set potential +205 mV to +1200 mV.

## 3. RESULTS AND DISCUSSIONS

Initial characterizations of the biosensor were aimed at optimizing buffers so that sensitive, distinct, and clear current peaks could be obtained for oxidation and reduction reactions. Based on Fig. 1, a distinct redox peak and the highest current was obtained when TE buffer was used as the supporting electrolyte, therefore this system was applied for further analysis.

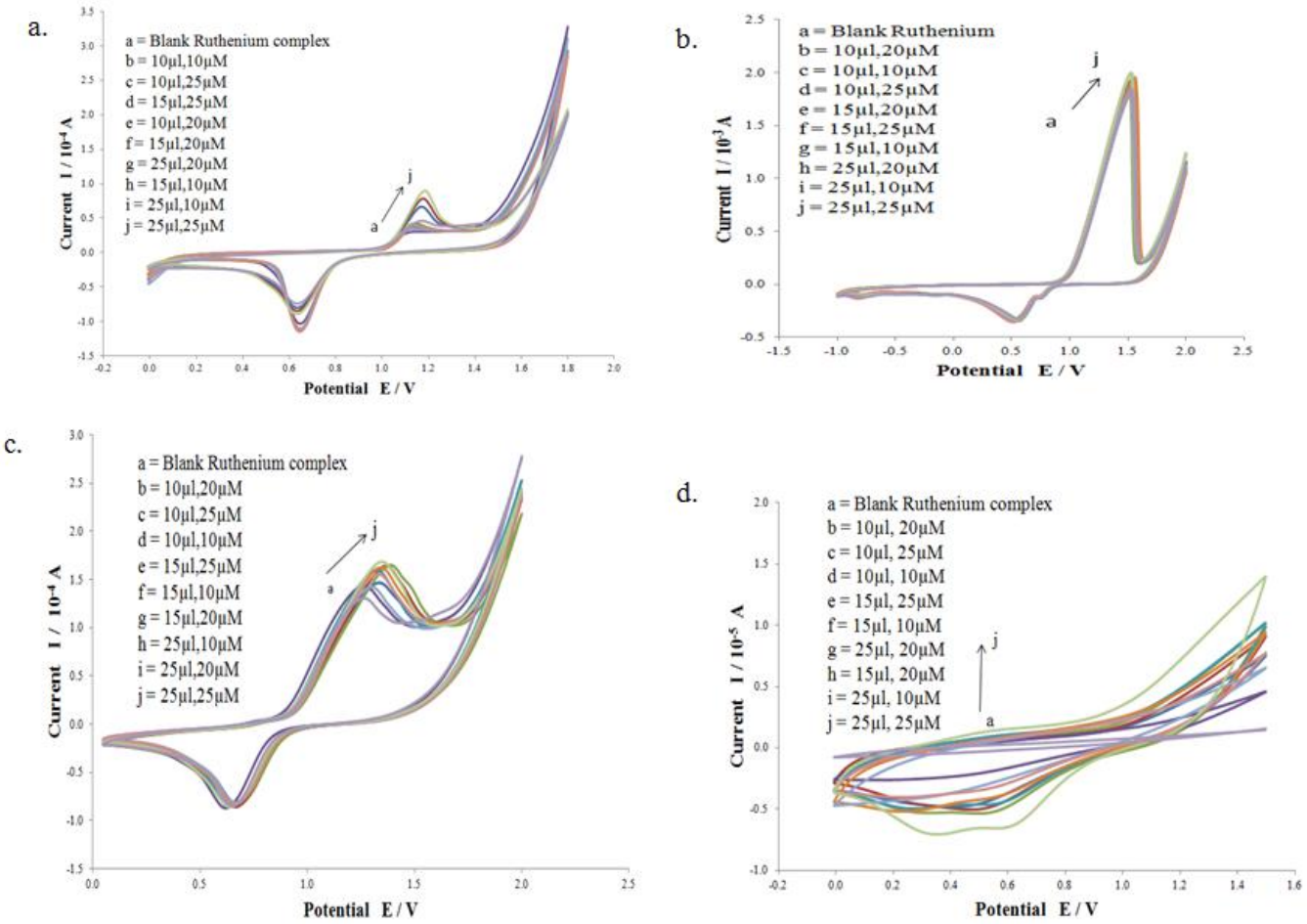


**Figure 1.** CV of ruthenium complex using (a) phosphate buffer (b) Tris-NaCl buffer (c) TE buffer (d) di-water as supporting electrolyte

The peaks obtained from the modified electrode PEDOT-PSS/AgNPs/AuE in the DPV presented in Fig. 2 indicates good conductivity improvement compared with that of bare AuE, both with and without the ruthenium complex. The modified film surface PEDOT-PSS/AgNPs/AuE of the electrode was formed from the blended aqueous solution in the ratio of 2 µl AgNPs to 2 ml PEDOT-PSS. The resistance of PEDOT-PSS doped with AgNPs in a solvent ratio of 20 : 1 (v/v) was less compared to 10 : 1 (v/v) found in the literature**,** where it was reported that conductivity improved with PEDOT-PSS than when AgNPs was added to the solution [9]. The highest current was obtained for hybridization with target DNA indicating a perfect match (Fig. 3) whereas mismatch DNA produced a lower current due to less ruthenium intercalation.

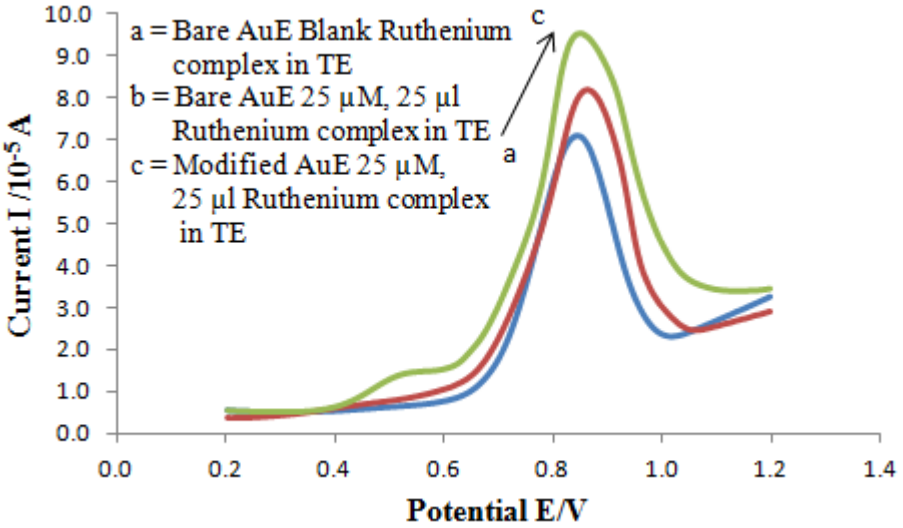


**Figure 2.** DPV carried out in TE buffer of (a) bare AuE without, (b) bare AuE with, (c) modified AuE with Ruthenium complex

Hybridization time and temperature was optimized and the result is presented in Fig. 3. The sensor showed sensitive detection of the target DNA at temperatures of 35 $^{o}$C, 45 $^{o}$C, and 55 $^{o}$C. At 45 $^{o}$C, highest current was obtained indicating an increase in hybridization process. Theoretical studies on DNA hybridization and melting in solution can be monitored using the parameter, melting temperature ($T_m$). Melting temperature is the temperature at which 50 percent of the duplexes initially present are denatured or unfolded into single strands. Many biological applications, such as PCR, northern and southern blots, and sequencing use $T_m$ value to determine the conditions for optimum performance [39]. The calculation of $T_m$ of a duplex can be perform using the base stacking standard thermodynamical parameters of a particular sequence for its coil-to-helix transition using;

$$T_m(°C) = \frac{\Delta H° * 1000}{\Delta S° + R * \left(C_T/_x\right)} - 273.15 + 16.6log[Na^+]$$

Where $C_T$ is the total strand concentration (mol/l), R is the gas constant and x equals 4 for non-self-complementary and equals 1 for self-complementary duplexes, [Na+] is the salt concentration in solution, $\Delta H^o$ is the amount of heat produced or taken up at constant pressure and $\Delta S^o$ is an entropy. Hybridization temperature ($T_{hyb}$), theoretically is 5 – 10 $^{o}$C lower than $T_m$. Optimized hybridization obtained in this study is at hybridization temperature 45 $^{o}$C which is near to the $T_m$ of the target DNA.

Current signal for hybridization with different concentration of target DNA ranging from 1.00 x $10^{-9}$ M to 1.00 x $10^{-15}$ M is presented in Fig. 4.

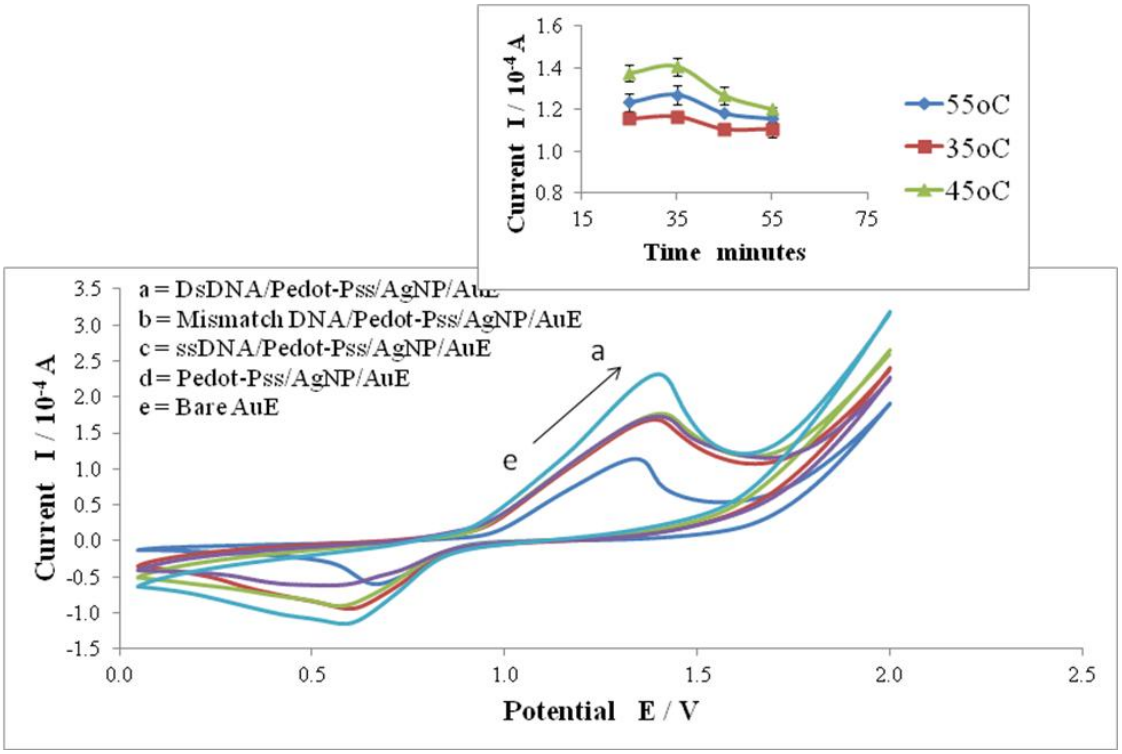


**Figure 3.** Redox signals for (a) dsDNA/PEDOT-PSS/AgNPs/AuE, (b) mismatch DNA/PEDOT-PSS/AgNPs/AuE, (c) ssDNA/PEDOT-PSS/AgNPs/AuE, (d) PEDOT-PSS/AgNPs/AuE, (e) bare AuE at scan rate 100 mV/s

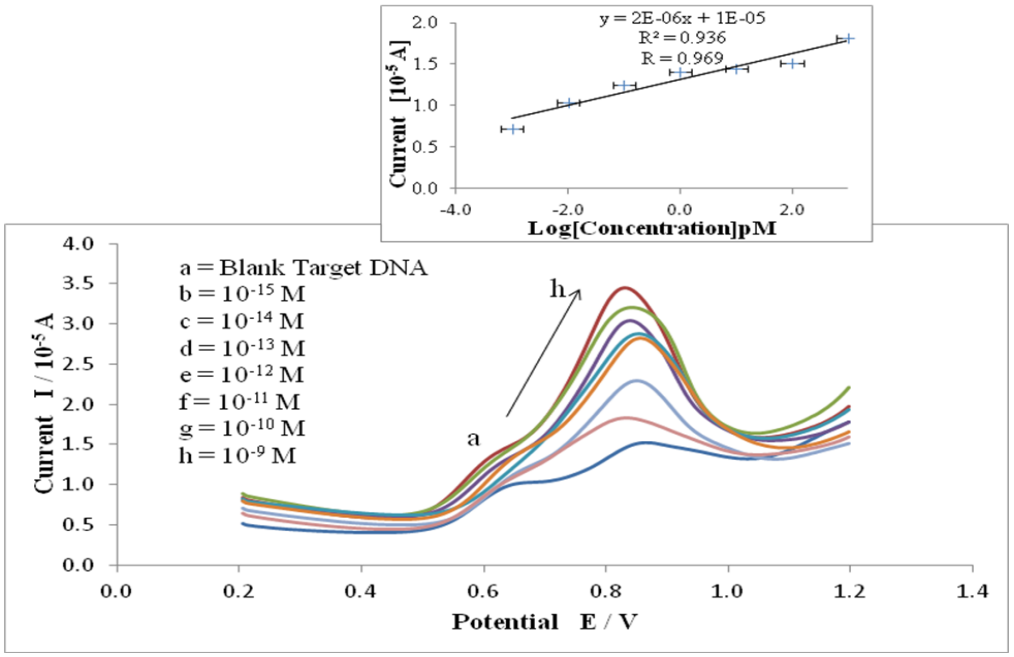


**Figure 4.** Differential pulse voltammograms (DPV) of the electrochemical analysis of the probe DNA on a nanocomposite platform showing current drop to target DNA concentration of (a) blank, (b) 1.00 x $10^{-15}$ M, (c) 1.00 x $10^{-14}$ M, (d) 1.00 x $10^{-13}$ M, (e) 1.00 x $10^{-12}$ M, (f) 1.00 x $10^{-11}$ M, (g) 1.00 x $10^{-10}$ M, (h) 1.00 x $10^{-9}$ M at scan rate 100mV/s.

According to David and Terry [40] the maximum apparent analyte concentration expected as replicates of blank sample having no analyte tested is considered the limit of blank (LoB). The limit of detection that is feasible, which is the lowest analyte concentration identified from the LoB of the system, was calculated to be 6.20 x $10^{-16}$ M.

A comparison between previous researches on the detection limits obtained using different metal complexes and modification process with that obtained in this work is summarized in Table 2. We have reported a wider linear range than the others and obtained the lowest LOD value which indicates that our developed sensor has better sensitivity compared to previous work on DNA biosensor.

**Table 2.** Comparison of detection limit between different metal complexes with various modifiers

| Modifying films | Methods | Linear range (µM) | LOD (µM) | Reference |
|---|---|---|---|---|
| Pt-nano/GCE | SWV with $[Co(phen)_3]^{3+}$ | 2.14 x $10^{-1}$ – 2.14 x $10^{-3}$ | 1.00 x $10^{-3}$ | [41] |
| PEDOT/AuNP/AuE | SWV with $[Fe(CN)6]^{-3/-4}$ | 0.10 x $10^{-3}$ – 100 x $10^{-3}$ | 0.02 x $10^{-3}$ | [7] |
| $NiOx_{np}$/GCE | DPV with $[Ru(NH_3)Cl]PF_6$ | 4.00 x $10^{-4}$ – 1.00 x $10^{-2}$ | 6.80 x $10^{-5}$ | [42] |
| AuNP/AuE | DPV with $[Ru(NH_3)_6]^{3+}$ | 1.00 x $10^{-6}$ – 1.00 x $10^{-1}$ | 1.00 x $10^{-6}$ | [43] |
| PEDOT-PSS/ AgNP/AuE | DPV with $[Ru(dppz)_2(qtpy)]Cl_2$ | 1.00 x $10^{-9}$ – 1.00 x $10^{-3}$ | 6.20 x $10^{-10}$ | This work |

GCE = glassy carbon electrode, SWV = square wave voltammogram, $NiOx_{np}$ = nickel oxide nanoparticles

## 4. CONCLUSIONS

In this study, a DNA electrochemical biosensor based on modified gold electrode with a nanocomposite membrane on which a DNA probe was immobilized has been developed for the interaction between the new ruthenium complex $[Ru(dppz)_2(qtpy)]Cl_2$ and DNA. The binding effects of the new ruthenium complex indicate that it exhibited good intercalation into the DNA helix. There were current signals obtained in the reactions between the ruthenium and the buffer of phosphate, Tris-NaCl, Tris-EDTA, and di-water used in this study and thus can be used for similar studies. The developed sensor was found to be sensitive at low concentrations up to 1.00 x $10^{-15}$ $molL^{-1}$ of the target DNA during hybridization; this was due to the unique properties of the nanocomposite. High sensitivity and selectivity were achieved by DPV measurements with the new ruthenium complex as a novel intercalator for electrochemical detection of DNA hybridization. The apparent stronger intercalative capability of the new ruthenium complex with DNA may be explained by the denaturation studies that revealed optimum hybridization at 45 $^{o}$C for 35 minutes. Consequently, the results indicate that using PEDOT-PSS/AgNPs nanocomposite membrane on a gold electrode surface

can provide a promising platform for electrochemical biosensor development for ruthenium complex interaction with DNA molecule detection, as it has remarkably enhanced the detection sensitivity of DNA hybridization. The R = 0.969 obtained in the calibration graph, Figure 4, indicates a strong coefficient of relationship of the DNA concentration detected and the 6.20 x $10^{-16}$ M limit of detection shows the sensitivity level of the developed biosensor.

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