FUNCTIONALIZED GOLD NANO-COMPOSITE ELECTROCHEMICAL SENSOR FOR THE RECOGNITION OF GENOMIC DNA

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Abstract- Electrochemical sensors are designed to observe current or potential changes as a result of interaction between the sample matrix surface and the sensor. The major challenges with the application of sensors is the recognition of small sequences in large amounts of double stranded DNA. Gold nanoparticles solution was synthesized and characterized using Uv-vis spectroscopy and XRD for the formation of nanocomposite with a conductive Pedot-Pss film on gold electrode which was also characterized using FE-SEM. Bare and the modified gold electrode surfaces were characterized using cyclic voltammetry (CV) technique for the active surface area. A designed Poly(3,4-ethylenedioxythiophen)–poly (styrenesulfonate) (Pedot-Pss) with gold nanocomposite modified gold electrode was functionalized with 3,3'-dithiodipropionic acid that enhanced the sensitivity of the sensor for easy recognition of DNA. Immobilization of a 20-mer single stranded peptide nucleic acid (ssPNA) probe as the bioreceptor of the sensor was achieved by covalent attachment of the amine group of the capture probe to a carboxylic group of an activated 3,3'-dithiodipropionic acid layer using EDC/NHSS.The sensitivity of the sensor was optimized using differential pulse voltammetry (DPV) and the sensor demonstrated specific detection for the target concentration ranged between 1.0×10^{-15}M to 1.0×10^{-9}M with a detection limit of 1.55×10^{-18} M. Hybridization of bioreceptor with perfectly matched target DNA related to Ganoderma boninense (G. boninense) fungal disease was successful in TE supporting electrolyte and monitored with a new ruthenium complex [Ru $(dppz)_2(qtpy)Cl_2$]; dppz=dipyrido [3,2–a:2',3'-c] phenazine; qtpy=2,2',-4,4".4'4"'-quarterpyridyl redox indicator using cyclic votammetry (CV). The sensor was also able to detect genomic DNA of (G. boninense) extracted via DNeasy plant mini kit procedure.

Keywords- Sensor; peptide nucleic acid; Pedot-Pss nanocomposite; G. boninense

I. INTRODUCTION

Electrochemical DNA biosensors exploit the affinity of single-stranded DNA (ssDNA) probe for complementary strands of DNA (Zhang et al, 2009) and are used in the detection of specific sequences of DNA with a view towards developing portable analytical devices. The design of the sensor described in this paper could be a general concept applicable to any genomic DNA but a white rot fungus, G. boninense was chosen in this study. G. boninense is a pathogen that causes a devastating bio threat of basal and stem rot to palm oil tree. However, the sensitivity, selectivity and rapid hybridization of DNA is still a crucial phenomenon that require improvement. Among the new strategies for the enhancement is the use of peptide nucleic acid (PNA) described by Nielsen's group in 1991 (Nielsen et al, 1991) as a mimic of DNA. The entire negatively-charge sugar-phosphate backbone of DNA is substituted with a neutral « peptide like » backbone with repeated N-(2-aminoethyl) glycine units linked by amide bonds (Singh et al 2010), however, PNA contains the same number of backbone bonds between bases and the same number of bonds between the backbone and the bases, as DNA. The use of PNA has brought a promising path to achieve the required improvement with regard to sensitivity and selectivity (Eva and Claire-Marie, 2010). Therefore, PNA is a new powerful biomolecular tool that has polyamide backbone with nucleobases capable of binding complementary nucleic acid strands. Due to the neutrality of the PNA backbone, there is no electrostatic reaction (repulsion) that may probably occur between PNA and DNA strands (as experienced between the negatively charged sugar-phosphate backbone of ssDNA probe and the hybridized target DNA), which further enhances the PNA – DNA hybridization properties (Arlinghaus et al., 2004). The unique properties of single-stranded PNA (ssPNA) and its physic-chemical differences from DNA, gave it more advantage for use in surface characterization techniques, for label-free nucleic acid target detection in biological samples (Eva and Claire-Marie, 2010) Over the past decades, efforts are made in quest of simple portable devices of sequence-specific DNA detection and this led for the first introduced concept of 'the electrochemical DNA hybridization biosensor' in 1993 by Millan and Mikkelsen (1993), and since then this exciting research area has been explored by many research groups. Consequently, in the 17th century, colloidal nanosized particles of silver (Ag), gold (Au) and copper (Cu) were used in coating glasses (Jain, et al. 2007) and however, experimental evidence thereafter has shown that nanoparticles of Au and Ag can be embedded into a polymeric layer which largely increase the modification surface area. The use of Ag,

Au , platinum (Pt) and palladium (Pd) are reported to have strong binding affinity towards amines, thiols, and disulfides (Nasir and Nur, 2008 ; El-Sayed, et al. 2005), therefore, this idea has greatly contributed in the recent shift of electrode modification protocols utilizing conducting polymer with metal nanoparticles (nanocomposite) film for electrode modification. The use of grapheme (GN) nanomaterials in life sciences and clinical diagnosis are also found extremely promising for improvement of GN-based biosensors, therapy monitoring and its related biomedical applications (Numan et al., 2016).

Cyclic voltammetry (CV) has been the most popular electrochemical technique (Gulaboski and Fereira, 2008) used for initial voltammetry studies to acquire qualitative and quantitative data from electrochemical reactions through the working electrode. In typical CV, the solution components are electrolyzed as they come into contact with the electrode, thereby making the surface sufficiently positive or negative in voltage to induce electron transfer. Interestingly, the use of intercalating dyes such as ruthenium and ethidium bromide make it possible to detect with fluorescence microscopy a single stained DNA molecules in solution. Interaction between analyte and receptor on the solid biosensor surface is detected by a change in (i.e current) signal. The signal can be basically monitored in the following different pathways for electrochemical DNA hybridization detection;

(1) A decrease/increase in the oxidation/reduction peak current of the label, which selectively binds with dsDNA/ssDNA.

(2) A decrease/increase in the oxidation/reduction peak current of electroactive DNA bases such as guanine or adenine.

(3) The electrochemical signal of the substrate after hybridization with an enzyme-tagged probe.

(4) The electrochemical signal of a metal nanoparticle probe attached after hybridization with the target.

However, from the above mentioned pathways, signals of current peaks could be at sequential increase/decrease depending on the sensor orientation (i.e, electrode modification system) for transduction. A number of electrons move from the electrode to the analyte solution during reduction and return during oxidation. The peak current for a reversible system is described by the widely known Randles-Servick equation (Gau et al., 2005) as follows

$$i_p = (2.69 \times 10^5) n^{3/2} A D^{1/2} C v^{1/2} \text{ ---------- (1)}$$

Where i_p is peak current (A), n is the number of electrons, A is electrode area (cm^2), D is the diffusion coefficient (cm^2/s), C is concentration (mol/cm^3), and v is scan rate (V/s). Accordingly i_p increases with $v^{1/2}$ and is directly proportional to concentration, C.

II. MATERIALS AND METHODS

2.1 Reagents and Solutions

The ruthenium complex [Ru $(dppz)_2(qtpy)Cl_2$]; = [Ru(dppz)] was synthesized according to a previously method described and documented by Haslina Ahmad in literature (Ahmad et al 2011). All the reagents used are of analytical grades and are purchased from a standard company in Malaysia used without further purification. Deionized water used throughout the experiment was prepared from a Milli-Q purifier.

2.2 Preparation of Solutions

A washing buffer and supporting electrolyte of 10 mM trisaminomethane-HCl (Tris-HCl) containing 1 mM EDTA (TE) (pH 7.15) are prepared with deionized water. Another supporting electrolyte of 1 mM Potassium Hexacyano ferrate III ($K_3Fe(CN)_6$) was prepared for the characterization of electrode active surface area. More solution of 10 mM trisaminomethane-HCl (Tris-HCl) containing 1 mM EDTA (TE) (pH 8.0) was prepared for further dilution of DNA. Further, an ethanolic solution of 3 mM 3,3'-dithiopropionic acid (DPA) was also prepared as a linker. A stock solution of 25 µM [Ru(dppz)] was prepared in 50 mM Tris-HCl, 20 mM NaCl (volume 90%) and methanol (volume 10%). An activation solution of 5 mM N-hydroxysulfosuccinimide (NHSS) sodium salt in a mixture of 2 mM 1-ethyl-3-[3-dimethylaminopropyl]carbo-diimidehydro chlioride (EDC) containing 50 mM sodium-phosphate was prepared in deionized water. The 5' end of 20-mers probe PNA was modified with an amino (NH_2) group (5`-/5AmMC6/CCT GCT GCG TTC TTC TTC AT-3`) and 20-mers complementary target (5`-ATG AAG AAG AAC GCA GCA GG-3`) were synthesized by First Based Laboratories Sdn Bhd, Selagor, Malaysia. The stock solutions 100 µM of the DNA and PNA molecule were prepared upon arrival and other dilute concentrations of 1.0×10^{-15}M to 1.0×10^{-9}M of the complementary target were prepared.

2.3 Apparatus and electrode

Voltammetric measurements were performed using AUTOLAB (Eco Chemie, the Netherland potentiostat linked to General-Purpose Electrochemical System [GPES 4.9] Echo Chemie software. The electrochemical cell was a three electrode system of Metrohm gold electrode as working electrode, platinum wire as counter electrode and silver-silverchloride KCl 3M as reference electrode.

2.4 Synthesis of gold nanoparticles

Synthesis of gold nanoparticles (AuNPs) was based on earlier reported procedures (Nasir and Nur, 2008) in which AuNPs was prepared by the sodium citrate reduction of $HAuCl_4.3H_2O$ solution. A yellow solution was obtained by dissolving 79 mg $HAuCl_4.3H_2O$ in 200 mL deionized water (1 mM), under vigorous stirring and refluxing the solution was brought to boiling. Afterwards, an addition of 20 mL (40 mM) sodium citrate dehydrate solution was followed in a drop wise. While making the addition at continuous stirring for about 1 min the solution turned to colorless then to deep-red, refluxing continued for further 15 min. The heater was turned off and the solution was finally allowed to cool under

stirring at room temperature. A UV-Vis spectrophotometry (UV-Vis-1650 PC SHIMADZU B) was used to determine the absorbance in the synthesized sample solution and X-ray diffraction (XRD) was also performed to determine the crystal structure and chemical composition of the sample. Furthermore, the size of the particles was observed under TEM and so the morphology by FESEM.

2.5 Pre-treatment of gold electrode

The bare gold electrode was polished with an alumina slurry followed by sonication for 5 min in deionized water. The electrode was then dried under nitrogen flow and thereafter immersed in a concentrated sulphuric acid and rinsed with deionized water. It was again sonicated in absolute ethanol for 5 min before rinsed with deionized water and subsequently in TE washing buffer. It was finally dried under gentle flow of nitrogen gas for 1 min and kept for further drying at room temperature for 45 min. A cyclic voltammetry (CV) at scan rates 50, 100, 150, 200, 250, 300, 350 mV/s was performed in a 1.0 mM $K_3Fe(CN)_6$.

2.6 Modification of electrode

A pre-treated bare gold electrode (AuE) was drop coated with prescribed volume of Pedot-Pss and exposed to ovum dry at 70 ^{0}C for 15 h. The film surface of the cured Pedot-Pss on the electrode was rinsed with deionized water and TE washing buffer to remove the unbound Pedot-Pss on the gold electrode surface. It was dried gently under nitrogen gas flow and further dried at room temperature and Pedot-Pss/AuE modified electrode was obtained. The modified electrode was then immersed in a colloidal gold nanoparticles solution for 24 h. The washing and drying protocols explained above was followed and AuNps/Pedot-Pss/AuE modified electrode was obtained. A cyclic voltammetry (CV) at scan rates 50, 100, 150, 200, 250, 300, 350 mV/s was performed in a 1.0 mM $K_3Fe(CN)_6$ and also in TE supporting electrolyte at scan rate of 100 mV/s

2.7 Immobilization of probes

Layer of DPA was formed on the AuNps/Pedot-Pss/AuE modified electrode by submerging the modified electrode in an ethanolic solution of 3 mM DPA for 45 min. It was rinsed with absolute ethanol, Millipore water and TE washing buffer respectively then dried. The DPA layered AuNps/Pedot-Pss/AuE electrode was incubated in 5 mM NHSS and 2mM EDC solution for 1 h at 25 ^{o}C to activate the carboxylic group of the DPA for easy bonding with amine group of the probe PNA (ssPNA) sequence related to G. boninense. It was rinsed with TE washing buffer and then dried. A 30 µL of 100 µM PNA was aseptically accumulated on the EDC activated modified electrode for 12 h at room temperature to cultivate the probe modified electrode labeled ssPNA/AuNPs/Pedot-Pss/AuE. Fig.1 illustrates the mechanism involved in the attachment of the PNA probe on the modified electrodes. The CV measurement of each probe in TE supporting electrolyte at scan rate of 100 mV/s using ruthenium [Ru(dppz)] redox maker was performed.

2.8 Extraction of Genomic DNA

The mycelia of G. boninense fungus cultivated in potato dextrose broth were used for the DNA extraction using DNeasyPlant Mini Kit according to the manufacturer's instruction with slight modifications (Hushiarian et al., 2015).

2.9 Hybridization of DNA

The immobilized probe of ssPNA/AuNPs/Pedot-Pss/AuE was hybridized with complementary target DNA at different concentrations prepared in the range 1.00 x 10^{-9} M to 1.00 x10^{-15} M. The target DNA was denatured at 45 ^{o}C for 35 min for hybridization and allowed to anneal at 25 ^{o}C for 90 min. The hybridization event was monitored using [Ru(dppz)] redox maker and a DPV measurement was used to study the response in TE supporting electrolyte. The probe was used to study the hybridization of 100 µM complementary target DNA (CDNA) sequence related to G. boninense and the extracted genomic DNA (GDNA) of the G. boninense. The hybridization was monitored using [Ru(dppz)] redox indicator and a CV voltammogram was used to study the response in TE supporting electrolyte.

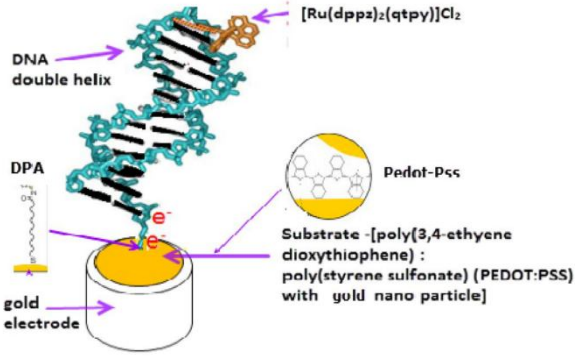


Fig.1. Schematic representation of the designed electro-chemical biosensor revealing its major components after hybridization

III. RESULTS AND DISCUSSION

3.1 Characterization of synthesized gold nanoparticles

The Uv-vis spectra and the X-ray diffraction (XRD) pattern of synthesized gold nanoparticles shown in Fig.2 are based on aqueous solution of 1 mM $HAuCl_4$. $3H_2O$ in 40 mM sodium citrate. The absorbance in the Uv-vis appears at wavelength (λ) 545 nm. The absorbance of gold nanoparticle appears at ranged from about 500 nm to 600nm, depending on their particle size (Apte et al., 2013).The absorption peak located at 545 nm in Fig.2(I) indicate the formation of gold nanoparticles.

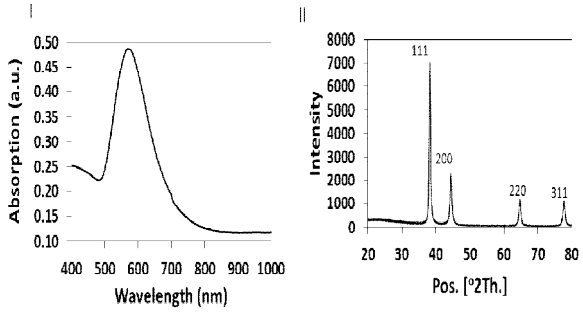


Fig.2 (I) **UV**-Vis spectra (II) **XRD** pattern of synthesized gold nanoparticles

In the XRD Fig.2(II), the strong diffraction peaks with 2θ values located at 38.2˚, 44.3˚, 64.5˚, and 77.5˚ correspond to Bragg's reflection from (111), (200), (220) and (311) planes of the face-centered cubic (fcc) metal gold nano structures. The Bragg reflection obtained correspond with that obtained on gold nanomaterials (Yan et al., 2008).

Transmission electron microscopy (TEM) Fig.3, was employed to study the size of the synthesized gold nanoparticles while field emission scanning electron (FESEM) microscopy was performed to study the particles surface morphology. The TEM micrograph reveals nano particles with average size of 22 nm, whereas the FESEM Fig.4(I) shows a mono and poly dispersion of the particles that are mostly spherical with few triangular in shapes. The EDX Fig.4(II) performed from the FESEM has revealed the elemental composition of pure gold nanoparticles.

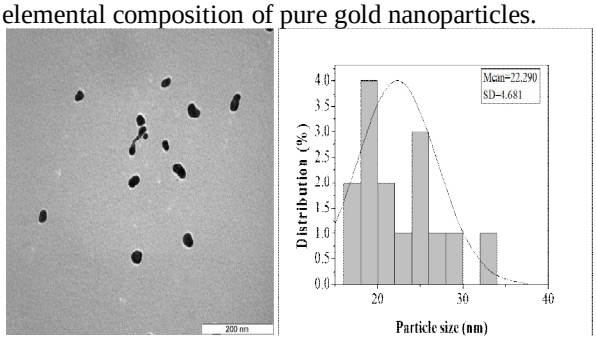


Fig.3 **TEM** micrographs of AuNPs distribution

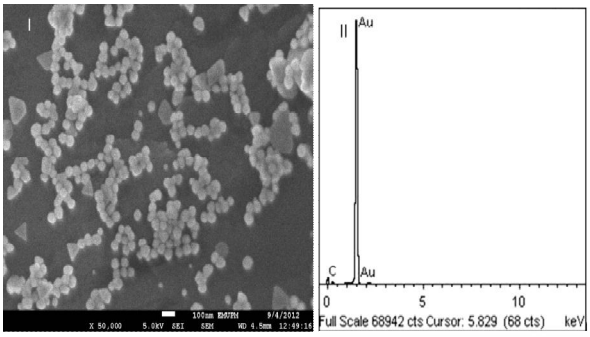


Fig.4 (I) **FE-SEM** micrographs and (II) EDX of AuNPs

3.2 Characterization of modified gold electrode

An FESEM of Fig.5 (I) Pedot-Pss (Pedot-Pss/AuE) and Fig.5(II) Pedot-Pss nano composite (AuNPs/Pedot-Pss/ AuE) modified gold electrodes shows the successful modification pattern prior to immobilization of the probe for DNA hybridization. The interaction of Pedot-Pss with gold nanoparticles exceed the level of physical adsorption, a chemical bond occur between sulfur of the Pedot and gold nanoparticles due to the affinity of sulfur for gold. A peak current versus square root of scan rates obtained from cyclic voltammograms of Fig.6(I) Pedot-Pss/AuE and Fig.6(II) AuNPs/Pedot-Pss/AuE modified gold electrode in 1.0 mM $K_3Fe(CN)_6$ are presented in Fig.5, the active surface area of Pedot-Pss/AuE modified electrode was found using Randles-Sevcik formula as 0.047 cm^2. In further modification with gold nanoparticles AuNPs/Pedot-Pss/AuE, the effective surface area increases to 0.104 cm^2 which is 2.2 folds greater than Pedot-Pss/AuE modification.

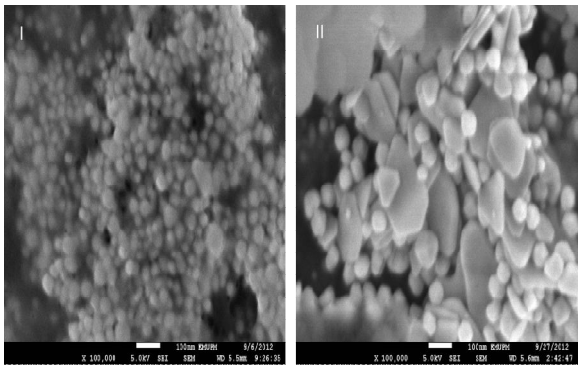


Fig.5 **FE-SEM** micrograph (I) PEDOT-PSS (II) PEDOT-PSS with AuNPs

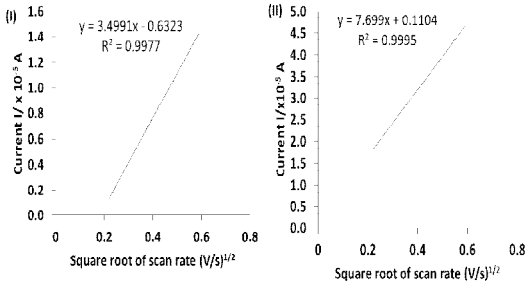


Fig.6 **CV** current versus square root of scan rates(50, 100, 150, 200, 250, 300, 350 mV/s) of **(I)** PEDOT-PSS/AuE **(II)** AuNPs/PEDOT-PSS/AuE modifications in a 1.0 mM $K_3Fe(CN)_6$

3.3 Hybridization of different concentration of target DNA

Differential pulse voltammograms (DPV) was used to study hybridization effect of different concentrations of target DNA to examine the sensitivity of the developed biosensor. It is observed that the DPV peak current increases with the increase in target DNA concentration as shown in Fig.7(I) and the covalent attachment of the target DNA to the probe enable more interaction via intercalation of the ruthenium complex with the DNA helix. The correlation between the DPV peak current and the target DNA concentrations are clearly explained from the plot of peak current versus log[concentration] of the target DNA shown in Fig.7(II). The detection limit of the sensor calculated from the known equation 3.3σ/S as 1.55×10^{-18} M.

3.4 Selectivity of the developed sensor

The selectivity of the developed biosensor shown in Fig.8 was examined via CV based on the electrochemical transduction of the probe ssPNA/AuNPs/Pedot-Pss/AuE. It was hybridized with complementary target DNA (CDNA) and the extracted genomic DNA (GDNA) of G. boninense. The hybridization was monitored using [Ru(dppz)] redox indicator. The genomic DNA (GDNA) has anodic peak current (i_{pa} = 2.030 x 10^{-4} A) lower than that of target DNA (CDNA) sequence (i_{ap} = 2.160 x 10^{-4} A). This may attribute to possible trace impurities (such as protein) in the extracted genomic DNA that lead to less ruthenium complex intercalation to the hybridized helix for current enhancement.

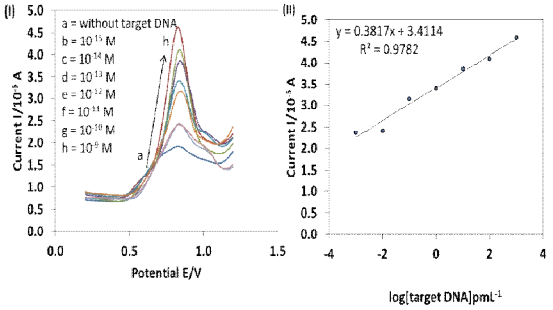


Fig.7 (I) DPV of dsDNA/AuNPs/PEDOT-PSS/AuE and (II) Linear plot of current against log[target DNA]pmL^{-1} in TE with [Ru(dppz)]$^{2+}$ of different target DNA concentrations (ranged 1.0 x 10^{-15} M to 1.0 x 10^{-9} M) at scan rate 100 mV/s.

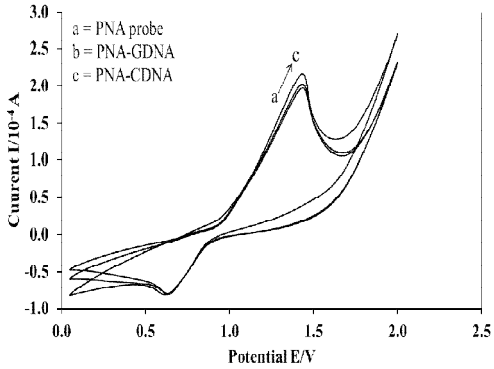


Fig.8 CV of genomic DNA (GDNA) and complementary target DNA (CDNA) hybridization using PNA probe in TE supporting electrolyte

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