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Bismuth Oxide Nanoparticles/Chitosan/Modified Electrode as Biosensor for DNA Hybridization

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An electrochemical DNA biosensor based on nanoparticles bismuth oxide Bi_2O_3 for DNA immobilization and hybridization detection is presented. The nano Bi_2O_3/chitosan-modified gold electrode (AuE) was fabricated and oligonucleotides were immobilized onto the AuE surface with the use of activating reagents – water soluble 1-ethyl-3(3'-dimethylaminopropyl)-carbodiimide (EDC) and N-hydroxy sulfosuccinimide (NHS). Hybridization was induced by exposure of the ssDNA-containing AuE to complementary target DNA in solution. The electrochemical behavior of methylene blue (MB) on the ssDNA and dsDNA modified electrode were carefully monitored by cyclic voltammetry (CV) analysis. The increases in the anodic peak current of MB were observed upon hybridization of probe with the target complementary DNA. Selectivity and sensitivity of this assay were also investigated in order to determine the specificity of the hybridization detection. This is the first application of chitosan combined with nano Bi_2O_3 for the fabrication of an electrochemical DNA biosensor with a favorable performance for the detection of specific hybridization.

Keywords: Nanoparticles bismuth oxide, methylene blue, dna hybridization, electrochemical DNA biosensor

1. INTRODUCTION

The detection of DNA hybridization is of central importance to the diagnostic and treatment of genetic diseases, for the detection of infectious agents, and for reliable forensic analysis [1]. DNA biosensors consist of a biological recognition layer, usually single stranded DNA and a transducer converting the recognition event into a measurable signal [2]. The technique for the detection of DNA hybridization (transducer) has attracted much attention. Many methods for detecting hybridization have been developed, including electrochemical, optical, microgravimetric methods and surface plasma resonance. As compared with other detection techniques, the main advantages of electrochemical techniques are their low cost, small size, and convenience for integration and

microminiaturization. Hence, DNA electrochemical biosensors have been rapidly developed recently [3-8].

Electrochemical detection of DNA hybridization usually involves monitoring of a current response, resulting from the Watson–Crick base-pair recognition event, under controlled potential conditions [9-11]. The probe-coated electrode is commonly immersed into a solution of a target DNA whose nucleotide sequence is to be tested. When the target DNA contains a sequence which matches that of the immobilized oligonucleotide probe DNA, a hybrid duplex DNA is formed at the electrode surface. Such hybridization event is commonly detected via the increased current signal of an electroactive indicator (that preferentially binds to the DNA duplex), in connection to the use of enzyme labels or redox labels, or from other hybridization-induced changes in electrochemical parameters (e.g. capacitance or conductivity) [12].

Methyelene blue (MB), an aromatic heterocycle molecule, is often employed as an electrochemical indicator toward selective discrimination of single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) [13]. The binding of ssDNA with MB is mainly through electrostatic interaction [14], whereas MB binds with dsDNA via intercalation and groove-binding interaction [15]. In particular, the cationic charge of MB (Figure 1) would improve the DNA binding affinity by electrostatic interaction with phosphate backbone [16]. Possibility of using MB as an electrochemical indicator was proven by some reports in the previous work. Ozsoz et al. [16] reported a difference between the voltammetric signals of MB at ssDNA and dsDNA modified carbon paste electrodes and thought that MB was a promising novel hybridization indicator of DNA electrochemical biosensors. Barton et al. [17] used a 15-base pair oligonucleotides modified electrode to study the intercalation of MB to dsDNA on the electrode surface. Sadik and co-workers [18] reported an electrochemical DNA biosensor prepared on a gold electrode through specific adsorption of 17-mer DNA probe and observed the increase in peak current of MB upon the adsorption of a probe and the hybridization with complementary DNA. Ju et al. [19] used MB as an electrochemical indicator to monitor the recognition of immobilized yeast DNA to yeast DNA sequence.

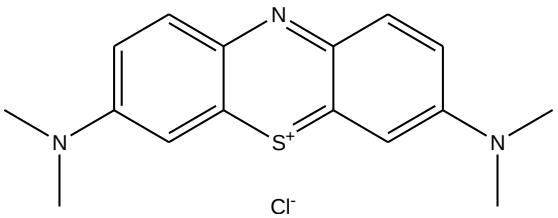

Figure 1. Molecular structure of methylene blue

A wide variety of nanomaterials, especially nanoparticles possess some unique chemical, physical and electronic properties caused by nanoscale effect (owing to their small size; normally in the range of 1 – 100 nm) [20-22]. This is different from those of bulk material, and can be used to construct novel and improved sensing devices; in particular, electrochemical sensors and biosensors [22]. Different kind of nanoparticles can play different roles in different electrochemical sensing

systems, such as enzyme sensors, immunosensors and DNA sensors [23,24]. One of the philosophies of biosensor is biomimicry. Thus, biosensor development necessitates working within the biomolecular 'convenience' of the bioreceptors. This means keeping the bioreceptors (DNA) in its natural (physiological) structural conformation and environment as much as possible. Thus the key step in the design of electrochemical DNA biosensor is the immobilisation of the DNA probe [25]. Ying et al. reported that the amount of immobilized DNA probe will influence the accuracy, sensitivity, selectivity, and life of a DNA biosensor directly. The success of immobilisation lies on the immobilisation layer or platform and the chemistries of immobilization [25]. Because of the high surface-to-volume ratio and excellent biological compatibility, nano-materials (especially oxide nanoparticles) can enlarge the sensing surface area to increase the amount of immobilized DNA greatly, and the DNA mixed with nano-materials can keep its biological activity well [26].

DNA hybridization biosensors based on nanomaterials are widely reported in previous literatures. As a versatile semiconductor, a ZnO nanomaterial has been investigated for signal enhancement in DNA hybridization detection. Zhi et al. developed a DNA biosensor based on micropatterned amine-functionalized ZnO/SiO2 core/shell nanorod arrays on nanocrystalline diamond substrates [27]. Lee and co-workers observed a signal enhancement during the immobilization and hybridization of oligonucleotides on ZnO nanorods, indicating the potential application of ZnO nanorods for signal enhancement in DNA biosensors [28]. Zhu et al. [29] presented a simple and practical DNA hybridization detection based on MB and zirconia (ZrO_2) thin film modified gold electrode. Yang et al. [30] reported an efficient DNA immobilization matrix based on a MWNTs/nano-ZrO_2/chitosan composite film. This assay based on carbon nanotubes and nano- ZrO_2, could enhance the ssDNA loading quantity and improve the detection sensitivity for DNA hybridization. Table 1 shows previous work related to electrochemical DNA biosensor using different ways to immobilize DNA, and their detection range of target DNA.

Table 1. Literature on electrochemical DNA based biosensor

Films for DNA immobilization	Detection method	Detection range of target DNA (mol l^{-1})	Reference
ZrO2/Au	DPV with MB as indicator	2.25×10^{-10} to 2.25×10^{-8}	[29]
ZrO2/NG/GCE	DPV with MB as indicator	1.0×10^{-10} to 1.0×10^{-6}	[36]
MAA/Au	CV with MB as indicator	5.0×10^{-8} to 1.0×10^{-4}	[32]

In our work, we report the electrochemical detection of DNA hybridization using a gold electrode modified with Bi_2O_3 nanoparticle mixed with chitosan. DNA sequence from the *Escherichia*

coli (E. coli) pathogen was used for probe immobilization at modified gold electrodes. The hybridization was carried out by exposure of the ssDNA-modified electrode to target DNA in solution. Here, we use MB as an electrochemical indicator to monitor the peak current changes between ssDNA and dsDNA. The immobilization procedure, optimization of hybridization condition, and the analytical performances were also studied.

2. EXPERIMENTAL

2.1. Reagents and materials

The 25-mer oligonucleotides were purchased from 1st BASE Laboratories Sdn Bhd, Selangor, Malaysia. Their base sequences were as follows:

- Probe DNA: 5'-CAG GAT ATG TGG CGG ATG AGC GGC A-3'
- Complementary target DNA: 5'-TGC CGC TCA TCC GCC ACA TAT CCT G-3'
- Single-base-mismathed DNA: 5'-TGC CGC TCA TCA GCC ACA TAT CCT G-3'
- Non-complementary DNA: 5'-GGC CAT CGT TGA AGA TGC CTC TGC C-3'

All oligonucleotides stock solutions of these oligomers (100 mg/l) were prepared using TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and kept frozen. More diluted solutions were obtained by dilution the stock solution with the same buffer solution. Bismuth oxide nanoparticles were obtained from catalysis laboratory, Department of Chemistry, UPM, Selangor, Malaysia.

Activating reagents, 5 mM N-hydroxysulfosuccinimide sodium salt (NHSS, Sigma Aldrich, Switzerland) and 2 mM 1-ethyl-3(3'-dimethylaminopropyl)-carbodiimide (EDC, Fluka) solution were prepared in 0.05 M phosphate buffer (pH 7.0).

Methylene blue (MB) was purchased from R&M Chemicals (Essex,UK). Stock solution of MB (1 mM) was prepared in 0.1 M KCl (pH 7.2). More dilute solutions were prepared by using the same buffer solution.

0.1 M KCl solution (R&M Chemicals, Essex, UK) (pH 7.2) was used as the supporting electrolytes, 10 mM Tris-HCl (Fisher, New Jersey) buffer solution containing 1 mM EDTA (pH 8.0) and a 30 mM saline-sodium citrate (R&M Chemicals, UK) buffer 2×SSC containing 300 mM NaCl (PROLABO, Paris) (pH 7.0) were used as hybridization buffer.

All chemicals were of analytical grade and used without further purification. All solutions were prepared with deionized water.

2.2. Apparatus and electrodes

Electrochemical investigations were carried out using ECO Chemie Autolab PGSTAT 10 potentiostat (Metrohm) with a conventional three-electrode system.

A Metrohm gold electrode was used as working, Ag|AgCl (3 M KCl) as reference and platinum as counter electrode.

2.3. Preparation of Bi_2O_3/CHIT solution

2% CHIT solution was prepared by dissolving CHIT flakes into 1% acetic acid and stirred for 4 h at room temperature until it was completely dissolved. An appropriate amount of Bi_2O_3 was dissolved in chitosan solution under 30 min sonication. The mass ratio of chitosan: Bi_2O_3 was 1:5.

2.4. Electrode modification

Gold electrode was polished with a 3-μm alumina/water slurry on a polishing cloth for 1 min. Then it was rinsed with deionized water and dried thoroughly under a N_2 flow. Firstly, 10 μl of Bi_2O_3/CHIT solution was dropped coated on the gold electrode surface and dried at room temperature. NHSS and EDC were used to activate 5'-phosphate group of probe ssDNA suspended in TE buffer and the activation was done for at least 18 hrs at 4 ^oC. The immobilization of DNA probe was carried out by immersing the Bi_2O_3/CHIT/AuE modified electrode in the solution-containing ssDNA for about 24 hours at room temperature. Then, a ssDNA-modified electrode was obtained and denoted as ssDNA/Bi_2O_3/CHIT/Au.

2.5. Hybridization on the electrode surface

The ssDNA/Bi_2O_3/CHIT/Au was immersed in 300 mM NaCl+30 mM sodium citrate solution (2×SSC buffer, pH 7.0) containing target ssDNA for 60 min at 30 ^oC and then cooling to room temperature. After hybridization, the electrode was rinsed using deionized water. Thus, the dsDNA modified electrode was obtained and denoted as dsDNA/Bi_2O_3/CHIT/Au.

The control experiments were performed in the same way using two different kinds of DNA; non-complementary target DNA and single base-mismatch DNA at the hybridization stage.

2.6. MB accumulation and electrochemical measurement

The results of modified gold electrode and DNA hybridization was investigated by measurement of electrochemical signal of MB accumulated on electrode surface. The electrode was immersed into 0.1 M KCl (pH7.2) containing 5.0×10^{-5} M MB for 2 min. After accumulation of MB, the electrode was rinsed with 0.1 M KCl buffer. The electrochemical response of MB was measured using the DNA-modified gold electrode in supporting electrolyte (0.1 M KCl). Cyclic voltammetry (CV) was carried out using the potential range of -0.5 to 1.8 V with the scan rate 100 mV/s unless specified. All experiments were conducted at room temperature (unless stated).

3. RESULTS AND DISCUSSION

3.1. Electrochemical characterization of different modified electrodes

Cyclic voltammograms of MB oxidative current at (a) bare AuE, (b) Bi_2O_3/CHIT/AuE and (c) ssDNA/Bi_2O_3/CHIT/AuE were shown on Figure 2. The peak current of MB increased in the order of bare AuE, Bi_2O_3/CHIT/AuE and ssDNA/Bi_2O_3/CHIT/AuE. Increasing on MB peak current of Bi_2O_3/CHIT/AuE compared to bare AuE indicate that Bi_2O_3/CHIT modified electrode has a larger electroactive surface and high surface free energy [22]. Immobilization of the ssDNA onto the Bi_2O_3/CHIT layer modified electrode resulted in a significant increase in anodic peak current of MB, which was attributed to the affinity of MB for guanine bases on DNA molecules [31]. The adsorption of biomolecules onto the surfaces of nanoparticles also can retain their bioactivity because of the biocompatibility of nanoparticles [22]. In conclusion, Bi_2O_3/CHIT films appears to be a good matrix for DNA molecules immobilization.

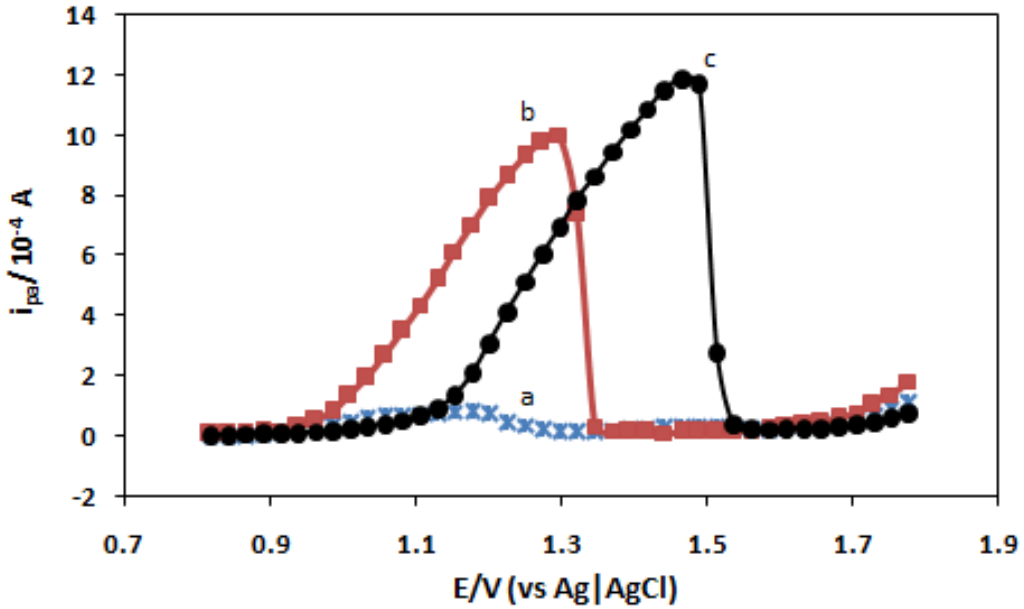

Figure 2. Cyclic voltammograms of (a) bare AuE, (b) Bi_2O_3/CHIT/AuE, (c) ssDNA/Bi_2O_3/CHIT/AuE, all in 0.1 M KCl buffer solution (pH 7.2) containing 5.0×10^{-5} M MB at scan rate of 100 mV/s.

3.2 Effect of scan rate on peak current of MB

The effect of varying scan rates on the CV response of MB using a hybridized DNA/Bi_2O_3/CHIT-modified gold electrode is shown in Figure 3. With the increase of scan rate, both reductive and oxidative peak currents increased.

The electron-transfer properties of MB using hybridized DNA modified electrode was studied by plotting a graph of log oxidation current (log i_{pa}) versus log scan rate (log v) as shown in Figure 4.

A linear relationship was observed with the slope of 0.422, near to 0.5 which is the ideal value of diffusion-controlled process [19,32].

The electrochemical process of MB on this modified electrode is prove to be a diffusion-controlled process.

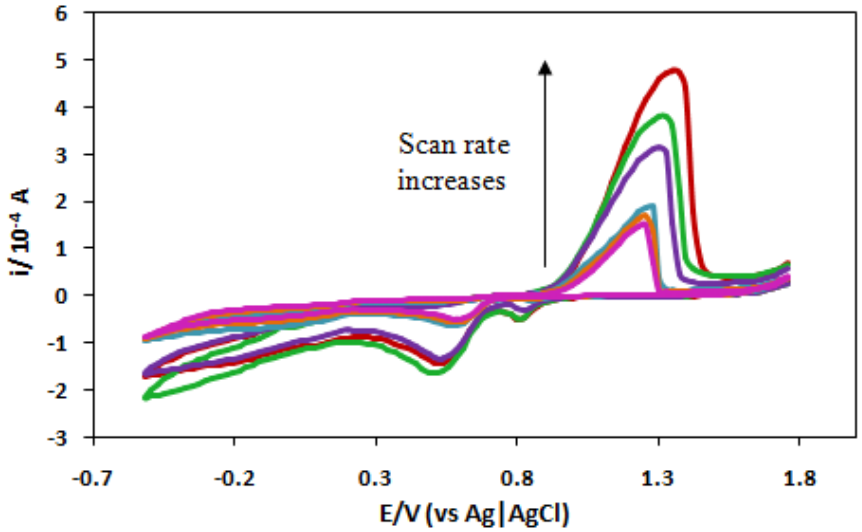

Figure 3. Effect of varying scan rates on the cyclic voltammograms of 5.0×10^{-5} M MB in 0.1 M KCl (pH 7.2) at a hybridized DNA/Bi_2O_3/CHIT-modified gold electrode. Scan rates used are 50, 70, 100, 300, 500 and 700 mV/s.

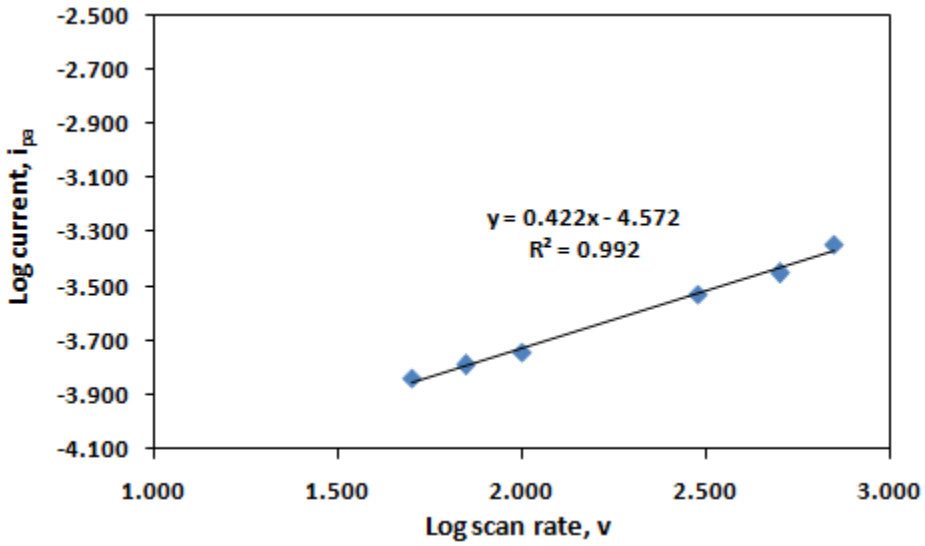

Figure 4. Plot of log oxidation current (log i_{pa}) versus log scan rate (log v).

3.4. Optimization of hybridization conditions

Additional factors that affected the detection are hybridization temperature and hybridization time.

3.4.1. Effect of hybridization temperature

The effect of hybridization temperature on hybridization signal is shown in Figure 5. The temperature of hybridization studies were in the range of 20 ^{o}C to 60 ^{o}C. Highest peak current was obtained when the hybridization was carried out at 30 ^{o}C. This indicated that 30 ^{o}C was the optimum temperature for hybridization process. As the melting point, T_m of this DNA is at 64.9 ^{o}C, it is possible that at temperature 60 ^{o}C and higher, the dsDNA tend to dissociate [33,34].

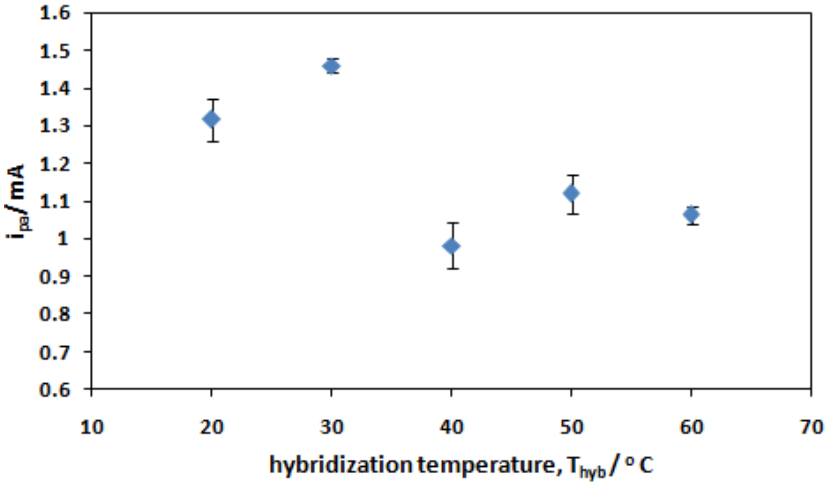

Figure 5. Effect of hybridization temperature on hybridization signal. [MB]: 5.0 × 10^{-5} M. MB accumulation time: 2 min; Supporting electrolyte: 0.1 M KCl (pH 7.2); scan rate: 100 mV/s.

3.4.2. Effect of hybridization time

The time taken for target DNA in hybridization buffer to contact with the probe DNA at the surface of gold electrode (hybridization time) also affected the CV signals.

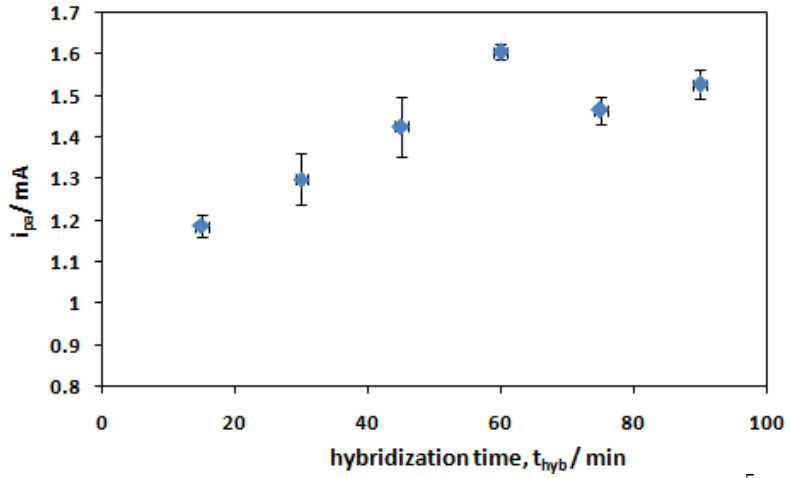

Figure 6. Effect of hybridization time on hybridization signal. [MB]: 5.0 × 10^{-5} M. MB accumulation time: 2 min; Supporting electrolyte: 0.1 M KCl (pH 7.2); scan rate: 100 mV/s.

The effect of hybridization time on oxidation current peaks was investigated and the result is shown in Figure 6. The results demonstrates with the prolong in hybridization time from 10 to 90 minutes, the current response increase dramatically and then tends to change only slightly. Highest peak current was observed when hybridization was carried out for 60 min. Therefore, the optimum hybridization time was 60 min, which indicate at this time, the ssDNA was completely hybridized with target DNA.

3.5. Electrochemical detection of DNA hybridization

3.5.1. Selectivity of the developed DNA biosensor

The selectivity of this study was examined by using the probe ssDNA-modified electrode to hybridize with different kinds of DNA sequences. Figure 7 showed the CV of ssDNA-modified AuE (a) after hybridization with the non-complementary DNA sequences (b) the single-base mismatched DNA sequence (c) and complementary DNA (d). As can be seen, almost no increment of current was observed when non-complementary DNA sequence was used for hybridization, indicating that hybridization reaction did not happen [29,35,36]. After hybridization with single-base mismatched sequence, the anodic peak current of MB slightly increased than the one observed for the ssDNA-modified electrode before hybridization. This small response can be attributed to a little intercalation of MB, and a low hybridization efficiency of a single-base mismatched DNA compared with complementary DNA sequence [34].

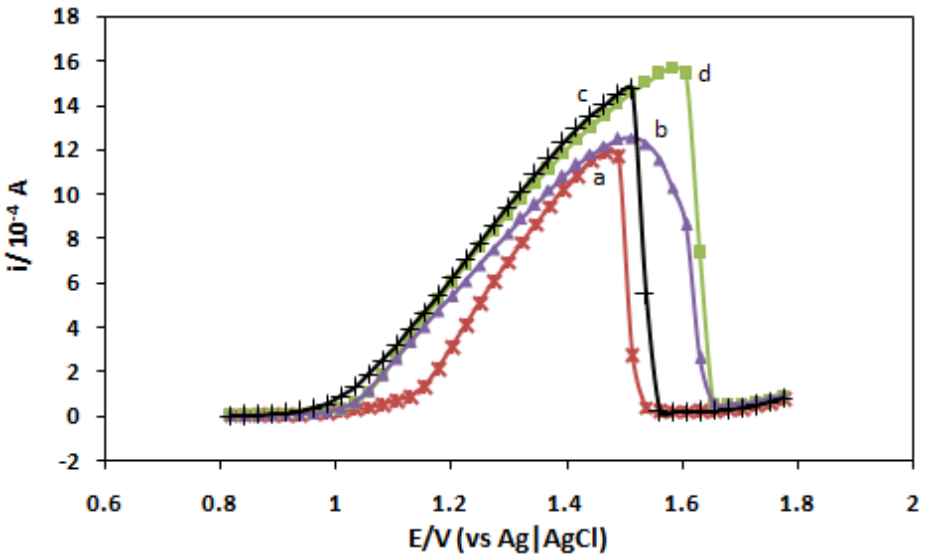

Figure 7. Cyclic voltammograms of 5.0 × 10^{-5} M MB as the redox intercalator at the (a) ssDNA/Bi_2O_3/CHIT/AuE, ssDNA/Bi_2O_3/CHIT/AuE hybridized with; (b) non-complementary DNA sequence, (c) single-base mismatched DNA sequence, and (d) complementary DNA sequence. MB accumulation time: 2 min; Hybridization time: 60 min; Hybridization temperature: 30 ^{o}C; Supporting electrolyte: 0.1 M KCl (pH 7.2); scan rate = 100 mV/s.

The peak current increased greatly after hybridization with complementary DNA, which is due to intercalation of MB with dsDNA. The MB molecules had intercalated into the double helix structure of immobilized DNA [32]. The results implied that MB can be considered as an efficient intercalator to distinguish between hybrids, non-complementary and mismatch DNA sequences. It can be observed that this electrochemical DNA biosensor displayed a high selectivity for the hybridization detection.

3.5.2. Sensitivity of the developed DNA biosensor

The sensitivity of the hybridization detection was examined by varying the concentration of target DNA. Differential pulse voltammetry (DPV) was used to measure the peak current values of MB under the optimum conditions according to the procedure described. As shown in Figure 8, the oxidation current of MB gives a linear relationship with respect to the logarithm value of the concentration of target DNA over the range from 1.33×10^{-10} to 1.33×10^{-8} mol l^{-1}. The sensitivity, expressed as the slope of the linear regression of the calibration curve is 5.23 μA/mol l^{-1} with the regression correlation coefficient (R^2) of 0.993.

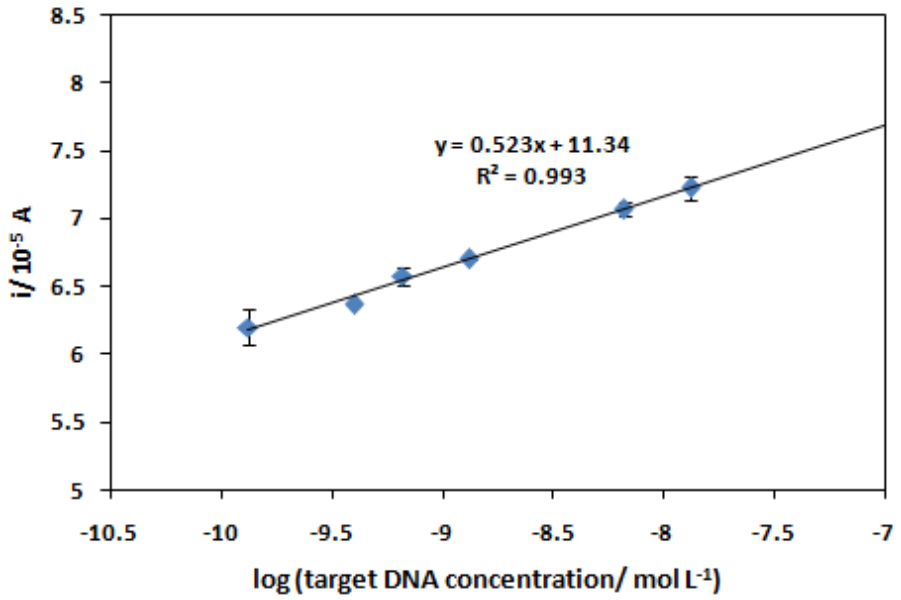

Figure 8. Relationship between log (concentration of target DNA) and peak current.

3.6. Reproducibility of the DNA biosensor

The possibility to use the developed DNA sensor in several successive hybridization detection was also investigated. The surface of the electrode could be renewed by polishing on a polishing cloth, followed by the usual method to prepare the hybridized DNA-modified electrode. The CV response was examined under the same condition described in section 2.5. Table 2 shows the oxidative current values of MB at hybridized DNA-modified electrode, for five replicates. The response current values

showed an acceptable R.S.D of 1.608 % (n=5). It indicated that a satisfactory reproducibility could be obtained by this system.

Table 2. Reproducibility study of the DNA biosensor.

Number of replicates	Current, i_{pa} (mA)
1	1.447
2	1.472
3	1.412
4	1.441
5	1.423

4. CONCLUSION

Characterization of DNA biosensors using gold electrodes modified with Bi_2O_3 nanoparticles/CHIT, and methylene blue as electrochemical label has been carried out. The ssDNA is succesfully immobilized on the Bi_2O_3/CHIT-modified gold electrode using EDC and NHSS as activating agent. Under the optimized experimental conditions, hybridization event can be detected based on the current signals obtained after accumulation of MB on the DNA sensors. The results obtained, confirm that this developed DNA biosensor can discriminate complementary target DNA sequence, non-complementary sequence and single-base mismatched sequence. To our knowledge, this is the first application of Bi_2O_3 nanoparticles combined with chitosan to fabricate an electrochemical DNA biosensor for the detection of DNA hybridization.

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