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An electrochemical DNA biosensor fabricated from graphene decorated with graphitic nanospheres

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Abstract

Graphene decorated with graphitic nanospheres functionalized with pyrene butyric acid (PBA) is used for the first time to fabricate a DNA biosensor. The electrode was formed by attaching a DNA probe onto PBA, which had been stacked onto a graphene material decorated with graphene nanospheres (GNSs). The nanomaterial was drop-coated onto a carbon screen-printed electrode (SPE) to create the GNS-PBA modified electrode (GNS-PBA/SPE). A simple method was used to produce GNS by annealing graphene oxide (GO) solution at high temperature. Field emission scanning electron micrographs confirmed the presence of a spherical shape of GNS with a diameter range of 40–80 nm. A stable and uniform PBA-modified GNS (GNS-PBA) was obtained with a facile ultrasonication step. Thus allowing aminated DNA probes of genetically modified (GM) soybean to be attached to the nanomaterials to form the DNA biosensor. The GNS-PBA/SPE exhibited excellent electrical conductivity via cyclic voltammetry (CV) and differential pulse voltammetry (DPV) tests using potassium ferricyanide ($K_3[Fe(CN)_6]$) as the electroactive probe. By employing an anthraquinone monosulfonic acid (AQMS) redox intercalator as the DNA hybridization indicator, the biosensor response was evaluated using the DPV electrochemical method. A good linear relationship between AQMS oxidation peak current and target DNA concentrations from 1.0×10^{-16} to 1.0×10^{-8} M with a limit of detection (LOD) of less than 1.0×10^{-16} M was obtained. Selectivity experiments revealed that the voltammetric GM DNA biosensor could discriminate complementary sequences of GM soybean from non-complementary sequences and hence good recoveries were obtained for real GM soybean sample analysis. The main advantage of using GNS is an improvement of the DNA biosensor analytical performance.

Keywords: DNA biosensor, graphene nanosphere, differential pulse voltammetry, electrochemical biosensor, DNA hybridization

(Some figures may appear in colour only in the online journal)

1. Introduction

Graphene is a promising material for bio-immobilization purposes due to its inert nature and a surface that can be modified easily with functional groups via a chemical reaction. The immobilization of biological materials onto graphene will neither alter their biological properties nor interfere with the surface biochemical reactions [1]. The excellent mechanical properties possessed by graphene allows it to be manufactured into many shapes and composites have been exploited and introduced in the construction of sensors and biosensors [2].

A wide variety of biosensors have been reported utilizing nano-interfaces in a spherical shape. Some examples are gold nanoparticles (AuNPs) [3], acrylic nanospheres [4], silica nanopheres [5–7], carbon nanospheres [8] and fullerenes [9]. These spherical shaped nanoparticles have been proven to improve the performance of many biosensors, especially on the lower detection limit of the sensor. Most recently, graphene nanospheres (GNS) were also synthesized using various methods [10–13]. These spherical shaped graphene nanoparticles may have good potential for biosensor applications, especially for DNA biosensing.

Immobilization of biological molecules is crucial in the development of a DNA biosensor as this can influence the response and operational stability of the resulting nucleic acid biosensor. Close contact between the bio-receptors and the transducer can be achieved through chemical and physical immobilization methods. The usage of nano-supporting materials such as nanospheres with a large surface area would allow a higher number of DNA probes to be immobilized on the matrix to produce a highly sensitive DNA biosensor. The three-dimensional spherical structure possesses high surface area to volume ratios, which permit the diverse functional surfaces to react with the bio-analyte more efficiently. Other than having large immobilization surface areas, graphene-based nanomaterials also have good electrical conductivity to promote the charge transfer rate on the nanoparticle surface. The biological layer that is immobilized close to the conductive electrode surface could also enhance the sensitivity in the biorecognition event, and lead to a high-performance sensing platform.

Graphitic nanomaterials do not normally have appropriate surface functional groups that could chemically bind with biomolecules via covalent bonding. Consequently, surface modifications with aromatic molecules through non-covalent bonding such as π-π stacking interactions have been widely used. Among them, pyrene and its derivatives have been reported to interact with carbon-based materials [14, 15] and graphene [16]. Such interactions are mostly via stacking with the basal planes of carbon materials and retain the conductivity and mechanical properties of the carbon materials.

In this study, we explored the possibility of using graphene decorated with nanospheres modified with pyrene butyric acid (GNS-PBA) as a nanomaterial for DNA immobilization to prepare a biosensor of improved performance. Aminated DNA probes for genetically modified (GM) soybean were immobilized on the GNS-PBA nano-material and coated onto a screen-printed carbon paste electrode (SPE). The immobilization was performed via a peptide covalent link with carboxylic acid functional groups of the PBA molecules using the carbodiimide coupling reaction. The hybridization of DNA probe with target DNA was then indicated by the anodic peak current (i_{pa}) of the intercalator anthraquinone-2-sulfonic acid. The schematic preparation of the electrochemical DNA biosensor based on GNS-PBA modified SPE and DNA hybridization process is shown in figure 1.

2. Methods

2.1. Apparatus and electrodes

Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) experiments were performed with the Autolab PGSTAT 12 potentiostat/galvanostat (Autolab, Metrohm). SPE modified with GNS-PBA nanospheres (GNS-PBA/SPE) was used as a working electrode. The SPEs used were designed by Universiti Kebangsaan Malaysia and manufactured by Scrint Technology Pvt. Ltd., Malaysia with an electrode diameter of 4 mm and an active surface area estimated at 12.57 mm^2. Carbon pencil and Ag/AgCl electrodes were used as auxiliary and reference electrodes, respectively. All potentials measured in this study refer to the Ag/AgCl electrode. A Galanz microwave oven was employed in the heating treatment during the synthesis of GNS. Homogeneous mixtures were prepared using an Elma S30H sonicator bath and IKA C-MAG HS7 hotplate stirrer. UV–Vis spectrophotometer (Varian-Cary Win UV 50) was used to determine the maximum absorption band of graphene oxide (GO) and GNSs. The size and morphology of the as-prepared GNS were examined by field emission scanning electron microscopy [FESEM, JEOL JSM-7800 F (JEOL, Peabody, MA, USA)].

2.2. Chemicals

Aldrich supplied the GO, 1,4-dioxane and PBA. 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydroxyl chloride (EDC) and N-hydroxysuccinimide (NHS) were provided by Systerm and Across, respectively. The potassium ferricyanide (K3[Fe(CN)6]) came from Merck and the anthraquinone monosulfonic acid (AQMS) was from Acro organics. All aqueous solutions were prepared using deionized water. GM soybean standards were obtained from Fluka Chemical Co. (Switzerland). Table1 shows the list of 20-base synthetic oligonucleotides purchased from Sigma-Aldrich. The DNA probe was designed with an additional amine functional group at base C7 of the gene. The stock solution of the DNA probe was diluted with 0.05 M K-phosphate buffer (pH 7.0), and the complementary DNA (cDNA) solution was diluted with 0.05 M Na-phosphate buffer at pH 6.5.

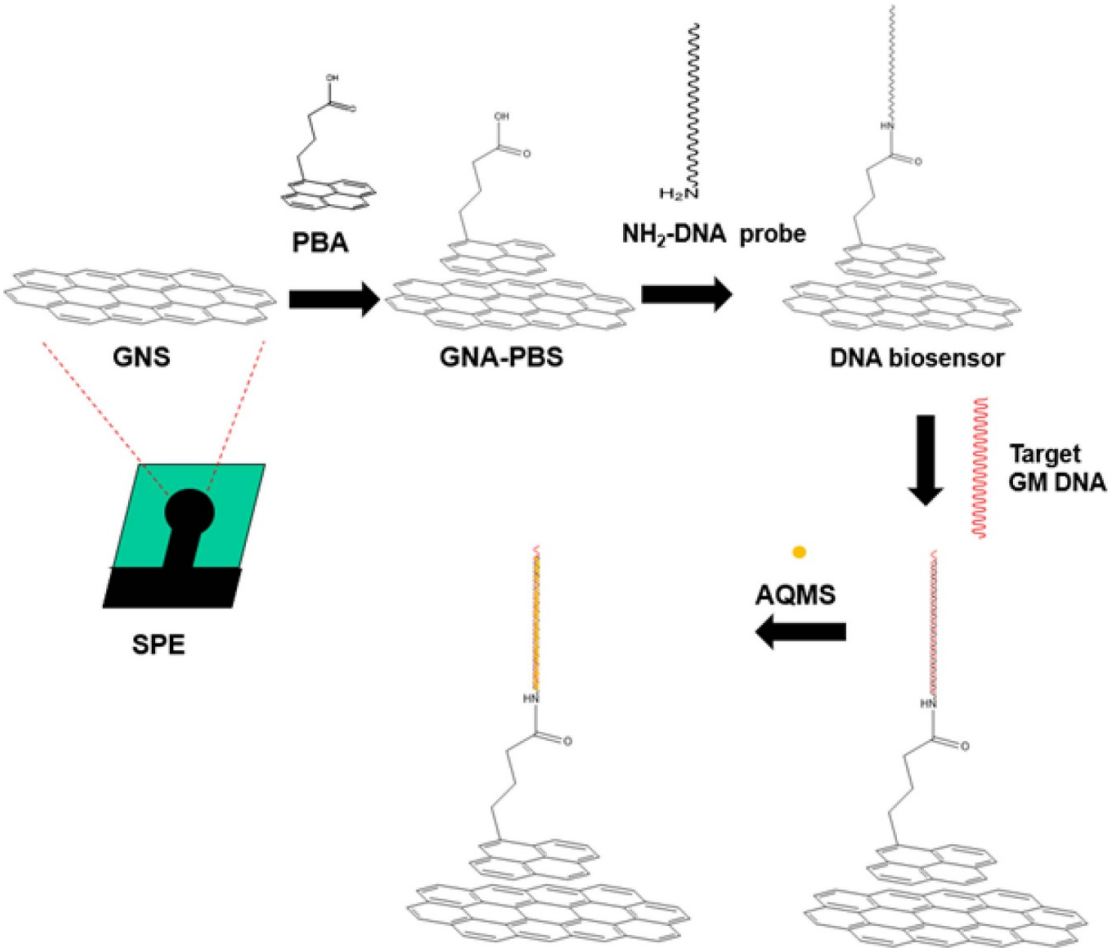

Figure 1. The design of electrochemical DNA biosensor based on GNS-PBA mixture coated on an SPE with AQMS as DNA hybridization label.

Table 1. Sequences of oligonucleotides used in the present research.

DNA	Base sequences
DNA probe	5′TATCCGGAAACCTCCTCGGA(AmC_7)
cDNA	5′TCCGAGGAGGTTTCCGGATA
Non-complementary DNA (ncDNA)	5′GTAGCATGAACTGTCATCGA
2-base mismatched DNA (NC 2)	5′ GTCGAGGAGGTTTCCGGATA
7-base mismatched DNA (NC 7)	5′ GTAG CATGGGTTTCCGGATA
12-base mismatched DNA (NC 12)	5′ GTAG CATGAACT GTC AGATA
16-base mismatched DNA (NC 16)	5′ GTAG CATGAACT GTC ATCGA

2.3. Synthesis of GNSs

GNS were synthesized according to previously reported methods [10–12] with slight modifications. The synthesis of GNS generally involved two main steps. First, the production of GNS through the assistance of microwaves and then the dispersion of GNS in an organic solution through sonication and centrifugation. In brief, about 20 mg of GO was dissolved in 100 ml of 1,4-dioxane in a scintillation vial and stirred for 2 d. The suspension was then centrifuged at 2000 rpm for 15 min. Next, some 20 ml of GO suspension from the supernatant was added with 5 g of natural graphite powder in another vial and stirred for another 2 d. The mixture was then heated in a microwave oven (2450 MHz, 700 W) at 101 °C for 60 s followed by sonication for 30 min until a homogenous suspension was obtained. The GNS were isolated by centrifugation at 2000 rpm for 15 min and the suspension obtained was subjected to UV–Vis spectrophotometer and FESEM characterizations.

2.4. Preparation of GNS-PBA for SPE and DNA immobilization

Prior to SPE electrode modification with graphene material, the SPE was pre-treated by using CV in 0.1 M KCl in the potential range of − 1.5 V to 0 V with a scan rate of

100 mV s^{-1} for four cycles. The GNS-PBA mixture was prepared by dissolving 1.0 mg of GNS in 200 μl of 1,4-dioxane containing 0.01 M PBA and sonicated for 4 h. A 5 μl of GNS-PBA suspension was then drop-coated on the SPE and left to dry at room temperature at 25 °C. For the immobilization of DNA probes on the GNA-PBA modified electrode, the carboxyl-terminal from the PBA was activated by immersing the GNS-PBA coated electrode in 300 μl of 5.0×10^{-6} M DNA probe in 0.05 M Na-phosphate buffer (pH 7.0) containing 0.005 M EDC and 0.008 M NHS for 24 h. Hybridization with the target DNA was carried out by exposing the DNA SPE (DNA probe/GNS-PBA/SPE) in 5.0 μM cDNA solution in 0.05 M Na-phosphate buffer (pH 6.5) followed by immersion in 1.0 mM AQMS at room temperature. Measurement of electrochemical DNA biosensor reponse via CV or DPV was carried out in 0.05 M Na-phosphate buffer (pH 7.0).

2.5. Optimization of GM DNA biosensor for genetically modified soybean determination

The effect of PBA concentration on the electrochemical GM DNA biosensor response was conducted by varying the volume ratio of GNS suspension to PBA at 1:1, 1:5, 1:10, 1:15 and 1:20 (v/v) in a fixed amount of GNS to form GNS-PBA nanomaterial suspension. All the GNS-PBA suspensions were then vortexed quickly before being drop-coated on the SPE. The electrode was then left to dry at ambient conditions to form the GNS-PBA electrode. The modified SPE was later covalent grafted with 5.0×10^{-6} M DNA probe followed by hybridization with 5.0 μM cDNA solution and AQMS intercalation at 1.0 mM. DPV measurement of the GM DNA biosensor response was performed in 0.05 M Na-phosphate buffer at pH 7.0.

The effect of DNA probe loading was carried out by changing the DNA probe concentration from 1.0 to 5.0 μM in 0.05 M Na-phosphate buffer (pH 7.0) containing 0.005 M EDC and 0.008 M NHS before binding to the GNS-PBA electrode. The pH effect and buffer capacity studies were performed by preparing cDNA solution at different pHs (between pH 6.0−8.0) and buffer concentrations from 0.01 to 0.50 M, respectively, using a Na-phosphate buffer. The effect of ionic strength on the DNA hybridization response was tested by adding sodium chloride salt in the concentration range of 0.1–2.0 M into 0.05 M Na-phosphate buffer solution at pH 6.5. The optimum DNA probe immobilization duration on the GNS-PBA electrode was determined by varying the DNA probe immobilization time between 1 h and 24 h; whilst DNA hybridization duration was examined by increasing the DNA hybridization time from 0.5 to 3.0 h. The long term stability of the DNA biosensor was investigated by using replicates of DNA biosensors that were stored at 4 °C during the testing period. Three modified SPEs were tested for their electrochemical response with 5.0×10^{-6} M cDNA at pH 6.5 on each experimental day for 100 d.

The linear response range of the DNA biosensor was determined by immersing different individual DNA electrodes into a series of cDNA solutions from 1.0×10^{-10} to 1.0×10^{-2} μM in 0.05 M Na-phosphate buffer (pH 6.5) containing 0.5 M NaCl for 1 h. The DPV response was recorded after immersion in 1.0 mM AQMS for another 1 h. The reproducibility of the DNA biosensor was assessed with two different cDNA concentrations, i.e. 1.0×10^{-5} μM and 1.0×10^{-8} μM by using five different individual DNA electrodes reproduced by a similar fabrication method. The DPV response was taken once for each individual electrode under the same experimental conditions. The percentage of relative standard deviation (%RSD) was calculated based on DPV response produced by the hybridized DNA biosensor. The selectivity of the electrochemical GM DNA Biosensor was evaluated with a 2-base mismatched DNA (NC 2), 7-base mismatched DNA (NC 7), 12-base mismatched DNA (NC 12) and 16-base mismatched DNA (NC 16) at 5.0 μM, and compared with the response obtained by a blank sample without target DNA as well as cDNA.

2.6. Validation of DNA biosensor based on GNS-PBA

The DNA biosensor response was validated by using several genomic soybean samples containing different amounts of genomic GM DNA. For that purpose, standard certified genomic GM samples for soybean of 0% and 100% GM contents (Roundup Ready GM-soybean, Monsanto, USA) were used [4]. These certified soybean samples were extracted using a GeneEx Plant Kit where the total genomic DNA was extracted. The extraction procedure followed that recommended by the Kit supplier. An EzSep filter column was applied during the extraction to further isolate the genomic DNA. The extracted genomic DNA from non-GM soybean samples (0% GM) were mixed accordingly with certain amounts of genomic 100% GM soybean samples to yield GM concentrations ranging from 2.00×10^{-8} to 5.00×10^{-4} μg ml^{-1}.

The electrochemical DNA biosensor was then applied under optimized conditions to determine the GM DNA content of soybean genomic DNA extracts. The electrochemical voltammetric response obtained was compared with the original certified GM DNA concentrations. The percentage of GM DNA recovery of the DNA biosensor was also calculated.

3. Results and discussion

3.1. Physical characterization of GNS

This study exploited an easy synthesis method to produce GNS through the annealing of GO with natural graphite in 1,4-dioxane at high temperature, using a microwave spark assistance process. When natural graphite flakes were dispersed in a GO organic solution and then irradiated by microwaves, sparks would be induced by the graphite flakes. The sparks induced by the absorption of microwaves by the graphite flakes could reach >1000 °C. As a result, the temperature of the mixture solution reached its boiling point. Thus, regions of GO close to the hot graphite were annealed and transformed into reduced graphene oxide (rGO). A small area of hot rGO was usually surrounded by the much colder GO, causing a large difference in the temperature between the two materials. Such difference

in temperature caused surface strain and the rGO sheets rolled up to form spheres. The residual GO continued to roll up layer by layer on the hot sphere until complete GNS were formed by the final annealing [10–12].

The products of the GNS formation processes can be monitored via UV–Vis spectroscopy, which is based on the electronic conjugation of graphene. UV–Vis absorption spectrum of GO shows a maximum peak (λ_{max}) at 230 nm, which is related to π-π^* transitions of the aromatic C–C bond. A shoulder at about 300 nm corresponds to n-π^* of the C = O bond transition. As the GO is undergoing the annealing process at high temperature from microwave absorption, the resulting spherical graphene particles formed, giving a maximum absorption at 260 nm. The initial UV absorption band of GO at 230 nm has shifted to 260 nm after the reduction process. This confirmed a complete reduction of GO to form reduced GO, i.e. rGO [17] (figure 2(a)).

The GNS production approach may produce non-uniformity for the structure, which is the mixture of nanosphere and nanosheets, which formed the nanomaterial for the biosensor coating. As can be seen in figures 2(b)–(c), the annealing of GO produces spheres in the diameter range of 30–80 nm. The nano-sized GNS are perfectly round. This three-dimensional shape is envisaged to enhance the DNA probe loading capacity on the electrode to provide ultra-sensitive detection of target DNA by accelerating the electron transfer rate at the electrode-solution interface.

The reduction of GO into reduced GO was tested by both UV–Vis spectroscopy and FTIR. The π network restoration in reduced GO can be observed from the redshift in the UV–Vis spectrum and oxygen functionalities removal can be confirmed by reduced oxygen-related peaks in FTIR. These are the most direct approaches to confirm the reduction of GO into rGO. A similar testing approach was used in another report by Fernandez-Merino *et al* [18]. The GNS was modified with PBA via the −COOH bond of the acid. Using EDC, the aminated DNA probes could be attached to the PBA of the GNS with an amide −CONH bond. The success of the DNA probe immobilization on the GNS-PBA was confirmed by FITR studies. The FTIR spectra (figure 3) showed that for GNS alone, the FTIR peak was not obvious (figure 3(a)). After reacting with PBA, peaks at 3400 cm^{-1} and 1650 cm^{-1} appeared, and this indicated the presence of -OH dan C=O (figure 3 (b)). These peaks became less prominent after the aminated DNA was attached to the GNS-PBA (figure 3 (c)).

The Raman spectrum of the GNS (figure 4) shows two characteristic G and D bands at 1589 cm^{-1} and 1347 cm^{-1}, respectively. The G band is related to the presence of graphitic domains while the D band is associated to the structural defects in the graphene. The ratio of ID/IG for GNS is computed to be 1.09, which indicates the increase of defect sites in graphitic planes. This is consistent with previous study [19].

3.2. Electrochemical characterization of GNS-PBA/SPE and DNA hybridization with cDNA

The results of electrochemical characterizations of GNS modified SPE (GNS/SPE) and GNS-PBA modified SPE (GNS-PBA/SPE) are shown in figure 5(a). The cyclic voltammograms were obtained in 1.0 mM $K_3[Fe(CN)_6]$. A pair of well-defined redox peaks were observed for ferri/ferrocyanide ($[Fe(CN)_6]^{3-/4-}$) redox-couple with SPE modified with GNS when compared with bare SPE. This demonstrated that the redox peaks become more enhanced in the presence of the GNS. The observation may be attributed to the fast electron transfer rate which occurred at the GNS-SPE owing to the excellent conductivity properties of the GNS. Also, the round shape of GNS has maximized the electrochemically active surface area of SPE. However, with further modification of the GNS/SPE with non-conductive PBA, the redox signal of GNS-PBA/SPE declined. This is because the redox activity of $Fe(CN)_6{}^{3-/4-}$ was retarded by electrostatic repulsion between negatively charged PBA and $Fe(CN)_6{}^{3-/4-}$ ions. This has also proven that the GNS-PBA nano-material has been successfully immobilized onto the carbon paste SPE surface and was demonstrated to enhance the heterogeneous electron transfer rate by the GNS materials, which indicates the suitability of GNS electrode as biosensor transducer.

Before the DNA probe was immobilized on the GNS-PBA/SPE, it was exposed to AQMS. The CV scan between the potential range of − 0.8 V and 0 V indicates no redox signal of the quinone derivative (figure 5(b)). This showed that there was no specific adsorption of anionic AQMS on the GNS-PBA nano-material at the electrode surface. The absence of absorption was due to the repulsion between the same charge sulfonate functional group of AQMS and the carboxyl functional group of PBA. Similarly, that the electrode GNS-PBA-DNA/SPE did not show any redox response in the presence of AQMS was also due to the negatively charged repulsion between DNA probe (i.e. the negatively charge phosphate backbone) and the anionic AQMS (figure 4(a)).

Hybridization of the DNA probe with cDNA on the GNS-PBA/SPE electrode exhibited an enhanced redox signal (figure 4(b)). This suggests that intercalation of AQMS into the hybridized DNA duplexes have been taken place to generate enhanced electron transfer on the GNS-PBA/SPE [20]. The AQMS redox intercalator acted as the DNA hybridization indicator operated by long-range transfer mechanisms through the immobilized DNA duplexes. Potential mechanisms for the long-range electron transfer include guanine relays for hole jumps and superexchange [21]. However, the response of the GNS-PBA/SPE electrode towards ncDNA showed no such electrochemical response. This indicates high selectivity of the DNA biosensor for discriminating complementary sequences from non-complementary sequences.

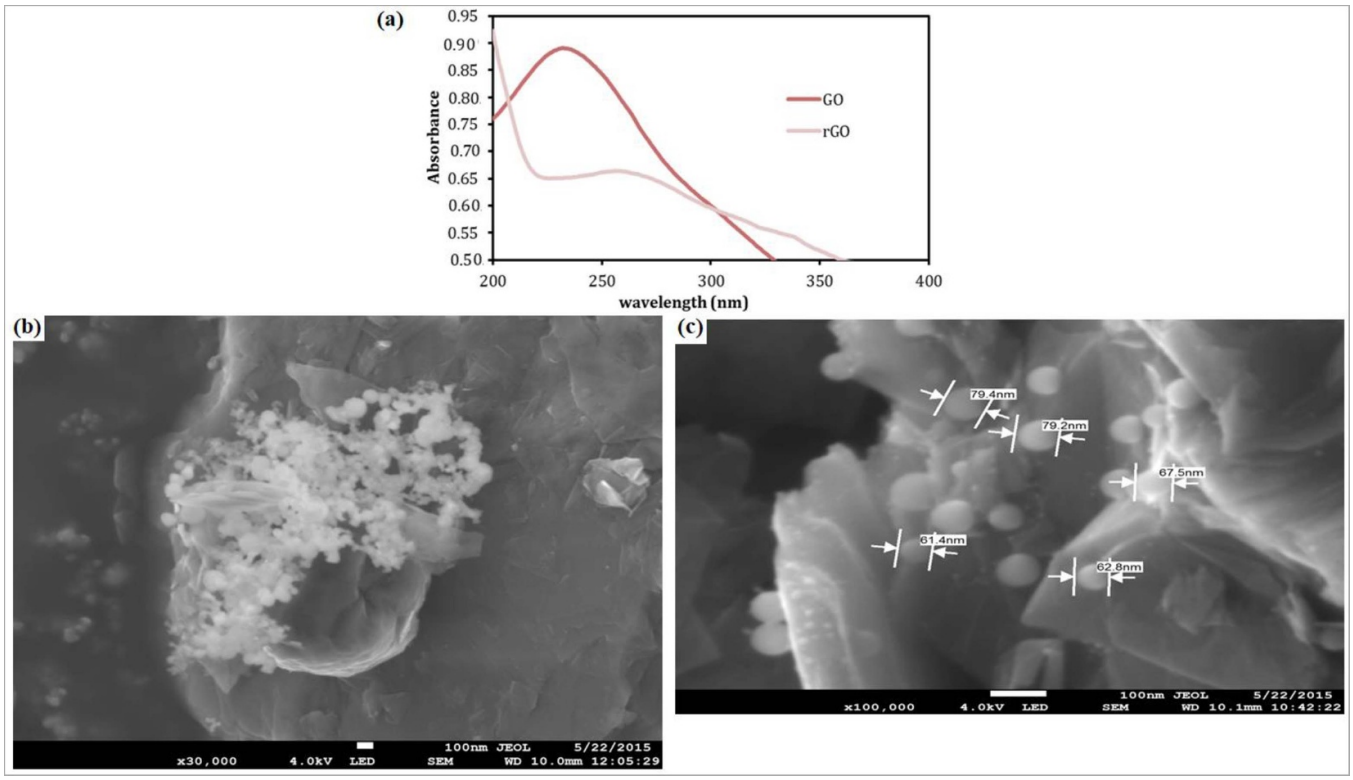

Figure 2. (a) UV–Vis absorption spectra of GO, $\lambda_{max} = 230$ nm and GNS (rGO), $\lambda_{max} = 260$ nm after undergoing a microwave spark assistance process. (b) & (c) The FESEM images of the synthesized GNS at 30 K and 100 K magnifications, respectively.

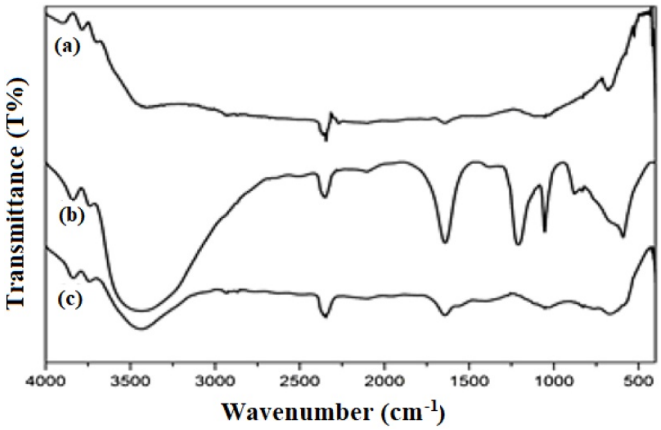

Figure 3. The FTIR spectrum of (a) GNS, (b) GNS-PBA reacted with EDC and (c) aminated DNA attachment on GNS.

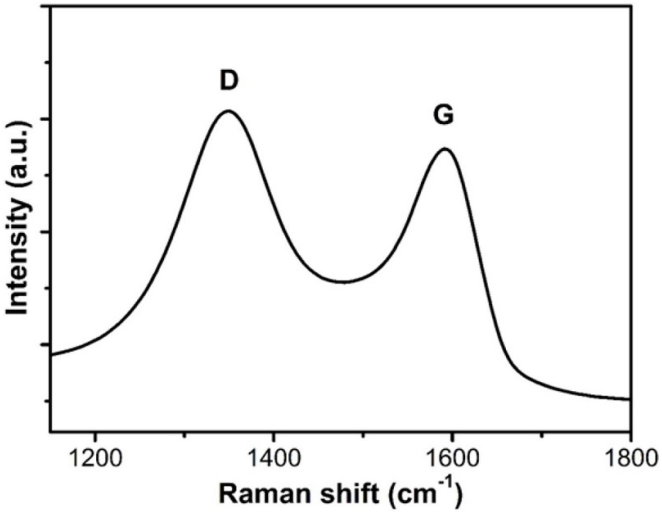

Figure 4. The Raman spectrum of the as-obtained GNS by microwave spark assistance process.

3.3. The effect of PBA concentration and DNA probe loading towards GM DNA determination

In this study, PBA is bound to the GNS through π-π stacking interactions without interfering with the physical or chemical structure of GNS to safeguard the electronic properties of GNS. The DPV anodic peak current response in figure 6(a) is noted to decrease when increasing the volume ratio of GNS suspension to PBA (GNS:PBA) from 1:1 to 1:20 (v/v) on the modified SPE. At low GNS:PBA volume ratio i.e. 1:1 and 1:5, the DNA biosensor exhibited sufficiently high DPV peak current response in the determination of 5.0 μM cDNA (in 1.0 mM AQMS). This is attributed to the high electrically conductive nature of GNS, which was capable of accelerating the electron transfer at the DNA electrode surface [17]. In contrast, an obvious decline in the DPV response of the DNA biosensor occurred when GNS:PBA volume ratio was increased from 1:10 and onwards. The high loading of non-conductive

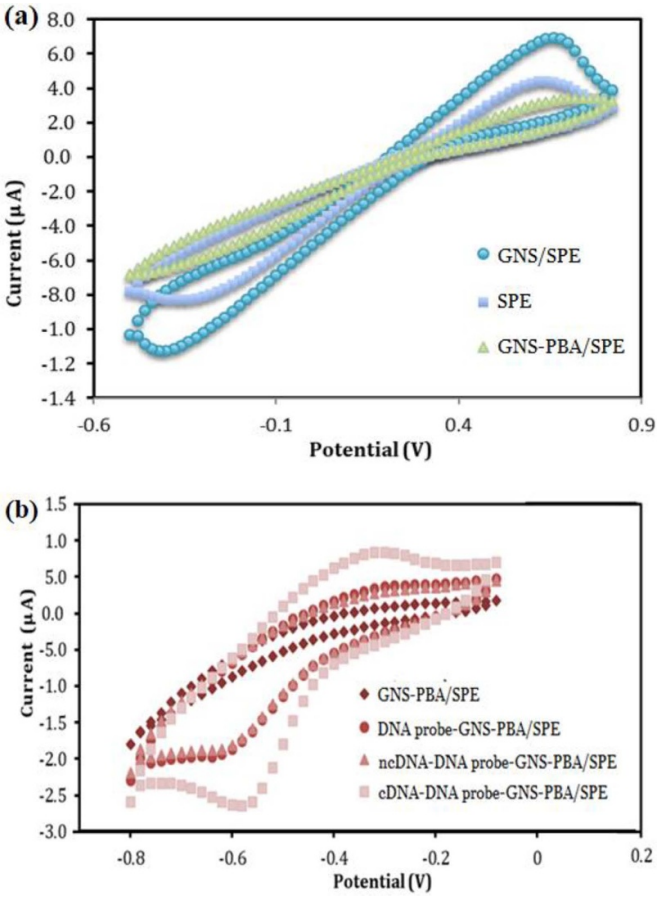

Figure 5. (a) Cyclic voltammograms of bare SPE, GNS/SPE and GNS-PBA/SPE in 1.0 mM $K_3[Fe(CN)_6]$ containing 0.1 M KCl and (b) Cyclic voltammograms of GNS-PBA/SPE, GNS-PBA-DNA/SPE and hybridization with ncDNA (ncDNA-DNA probe-GNS-PBA/SPE) and cDNA (cDNA-DNA probe-GNS-PBA/SPE) in the presence of 1.0 mM AQMS redox label.

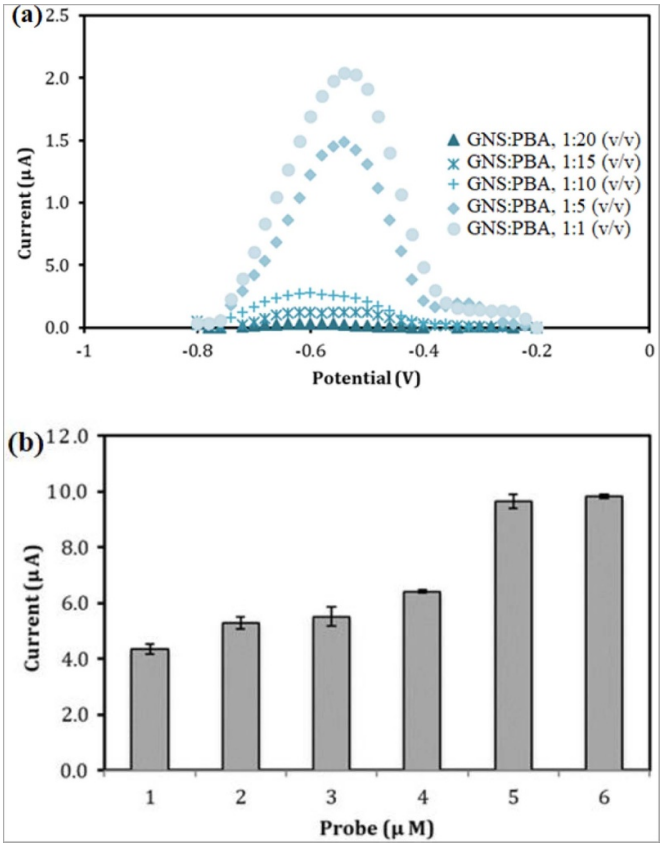

Figure 6. (a) The effect of PBA concentrations on the DNA biosensor for GM soybean in 5.0 μM cDNA and 1.0 mM AQMS in 0.05 M Na-phosphate buffer (pH 7.0). (b) The AQMS oxidation peak current response of the DNA biosensor by increasing the amount of DNA probe immobilized on the GNS-PBA/SPE towards the detection of 5.0×10^{-6} M target DNA in 0.05 M Na-phosphate buffer at pH 6.5.

PBA linker at this stage might have hindered the smooth electron transfer of AQMS intercalated into the double stranded DNA (dsDNA) [22].

The carboxyl terminal functional group of PBA acts as the specific immobilization site for coupling to the aminated DNA probe through amide covalent bonds. Hence the DNA probe may be able to orientate perpendicularly to the electrode surface. Figure 6(b) shows the DPV response of AQMS labels with different DNA probe concentrations used towards hybridization with 5.0×10^{-6} M target DNA. The increasing DPV response is anticipated with increasing amount of DNA probe (1.0×10^{-6} M – 5.0×10^{-6} M) loaded on the GNS-PBA/SPE electrode. This is because it allows proportional increment of AQMS from intercalation with DNA hybridization [23, 24]. A saturation state is attained when high DNA probe concentrations, i.e. between 5.0×10^{-6} M and 6.0×10^{-6} M was used in DNA probe immobilization. The operation of the DNA biosensor for GM soybean based on GNS-PBA-DNA/SPE was then optimized further at 5.0×10^{-6} M DNA probe loading.

3.4. Effect of pH, buffer concentration and ionic strength on the DNA biosensor response

pH is one of the most important factors in DNA hybridization reactions. By increasing the pH from pH 6.0 to pH 6.5 in the DNA hybridization medium, the electrochemical DNA biosensor response increased favorably (figure 7(a)) as it reduced protonation of hydroxyl groups on the DNA probe molecules, thus enabling more DNA hybridization reactions to take place. However, when further increasing the buffer pH beyond pH 6.5, denaturation of DNA occurred when hydrogen bonding between nucleotides was disrupted.

Buffer concentration plays a significant role in stabilizing the pH of the DNA hybridization buffer by binding or releasing hydrogen ions in response to pH changes. Figure 7(b) shows the effect of different buffer concentrations on the DPV response of the GM DNA biosensor. Maximum AQMS oxidation peak current was attained with 0.05 M Na-phosphate buffer at pH 6.5. This denotes an optimum buffer capacity of the DNA hybridization medium that could facilitate stabilization of the DNA double helix configuration, which consists of negatively charged phosphodiester bonds connecting between nucleotide units.

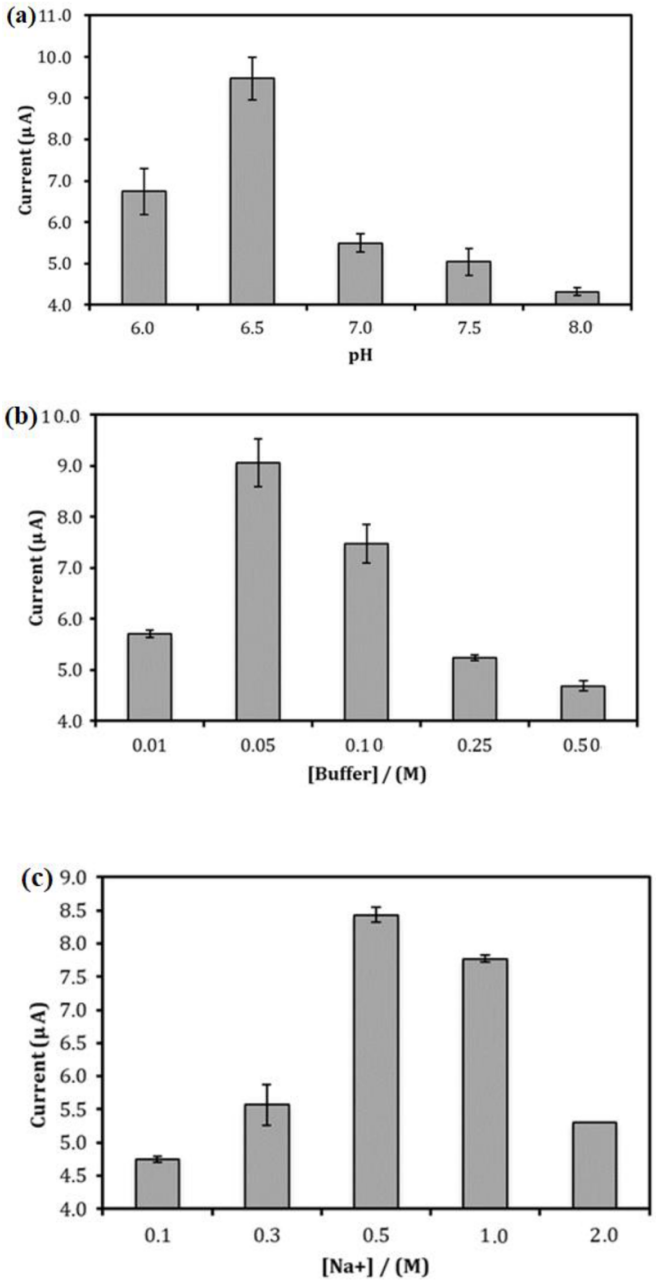

Figure 7. The effect of (a) pH (pH 6.0–8.0) (b) buffer capacity (0.01–0.50 M) and (c) ionic strength using NaCl (0.1–2.0 M) in 0.05 M Na-phosphate buffer (pH 6.5) containing 1.0 mM AQMS for DPV detection 5.0×10^{-6} M target DNA with DNA biosensor based on GNS-PBA/SPE.

The salt content in the DNA hybridization buffer must be optimized to reduce steric hindrance and electrostatic repulsion between the negatively charged phosphate group of DNA probe and cDNA. By adding NaCl between 0.1 M and 2.0 M into 0.05 M Na-phosphate buffer at pH 6.5, it neutralized the negatively charged DNA sugar-phosphate backbone. This eased DNA hybridization reactions, therefore higher DPV signal was acquired with the GM DNA biosensor (figure 7(c)). However, when further promoting the ionic strength of the DNA hybridization buffer above 0.5 M NaCl, electrostatic attraction of positively charged Na^+ ions over the negatively charged phosphate group of the DNA probe and cDNA prevents re-establishment of the repulsive force between DNA probe and target DNA. Therefore, lower DPV response was observed with high ionic strength DNA hybridization buffer.

3.5. Effect of DNA probe immobilization and hybridization duration on the GM DNA biosensor response and lifetime of the DNA electrode

Figure 8(a) represents the GM DNA biosensor response fabricated of the GNS-PBA SPEs that were modified with DNA probes with different immobilization duration. The DPV response of the DNA electrode with 6 h DNA probe immobilization time appeared to give the optimum electrochemical signal for target DNA quantification. After which point a plateau response was achieved with DNA probe immobilization time longer than 6 h. The optimized DNA probe immobilization time was then applied to prepare the rest of the DNA electrodes for subsequent DNA biosensor optimization studies.

The time taken for optimum DNA hybridization on GNS-PBA-DNA electrode was investigated from 30 min to 3 h. Based on the results depicted in figure 8(b), approximately 70% of DNA hybridization can be achieved in less than 1 h. The same response trend of increasing DNA hybridization time with increasing electrochemical signal has been reported by Meric *et al* [25] and Du *et al* [26].

A lifetime study of the DNA biosensor was conducted to determine the stability of the DNA electrode. They were stored in the refrigerator for a certain period throughout the studies. Figure 8(c) reveals that the GM DNA biosensor could retain 70% of its initial DPV response after 19 d. Even after 33 d of storage, 51% of its initial response could still be obtained.

3.6. Linear response range, reproducibility and selectivity of the GM DNA biosensor

The sensitivity of this GM DNA biosensor based on GNS-PBA electrode was investigated by measuring its electrochemical response with a series of target DNA solutions at different concentrations. Figure 9(a) shows the DNA biosensor's electrochemical response increased steadily with increasing cDNA from 1.0×10^{-10} to 1.0×10^{-2} μM. This implies more DNA duplex was formed on the electrode through DNA hybridization reactions followed by AQMS intercalation. The linear response range of the DNA biosensor from 1.0×10^{-10} to 1.0×10^{-2} μM target DNA gave a satisfactory correlation coefficient value of $R^2 = 0.9934$ with a limit of detection (LOD) calculated based on the sample blank value plus three standard deviation was obtained at 4.59×10^{-10} μM.

A reproducibility study was conducted to determine the closeness of the agreement between the results of measurements produced by the electrochemical GM DNA biosensor at the AQMS oxidation peak response of −0.55 V after 50 min of depositing the DNA SPE into cDNA solution. Based on

Table 2. The recovery percentages of GM DNA in soybean samples by using GNS-PBA based DNA electrode.

Sample	GM DNA concentration in certified GM soybean plant materials (μg ml^{-1})	GM DNA concentration determined by GNS-PBA DNA electrode (μg ml^{-1})	Recovery (%)
1	2.00×10^{-8}	1.94×10^{-8}	97.0
2	4.00×10^{-7}	3.85×10^{-7}	96.2
3	5.00×10^{-6}	4.94×10^{-6}	98.8
4	6.20×10^{-5}	6.03×10^{-5}	97.3
5	5.00×10^{-4}	5.09×10^{-4}	101.8

Table 3. The comparison of the analytical performance of the DNA biosensor based on graphene nanospheres with other previously reported GM DNA biosensors.

GM DNA biosensors	Linear range (M)	Detection limit (M)	Reproducibility (%)	Hybridization time (min)	References
GNS-PBA nanospheres/SPE	1.0×10^{-16}–1.0×10^{-8}	1.0×10^{-16}	3.1–3.6 ($n = 5$)	30	This study
Acrylic microspheres/rGO/SPE	1.0×10^{-15}–1.0×10^{-8}	1.0×10^{-15}	3.7–4.6	30	Jamaluddin *et al* [33]
Glassy carbon electrode (GCE) modified with EDC and NHS	5.0×10^{-9}–1.2×10^{-7}	5.0×10^{-9}	-	90	Xu *et al* [32]
GCE/platinum nanoparticles	2.1×10^{-9}–2.1×10^{-7}	2.1×10^{-9}	5.9% ($n = 5$)	60	Wang *et al* [29]
Gold electrode/mecaptoacetic acid	1.2×10^{-12}–4.8×10^{-8}	1.2×10^{-12}	-	-	Sun *et al* [30]
Carbon pencil electrode/single walled carbon nanotubes/poly-L-lysine	1.0×10^{-12}–1.0×10^{-7}	3.1×10^{-13}	3.2% ($n = 7$)	10	Jiang *et al* [31]

the bar chart illustrated in figure 9(b), no significant discrepancy in DPV response was obtained by five individual DNA electrodes for both DNA testing sets with 1×10^{-14} M and 1×10^{-11} M cDNA. The reproducibility of this DNA biosensor was found to be good with a %RSD yield in the range of 3.1–3.6%. This suggests a relatively high reproducibility of the DNA electrode being batch-produced with a manual preparation process, as the individual DNA electrode gave a reproducible electrochemical response with RSD value below 5%, which is normally considered to be good reproducibility.

Figure 9(c) depicts the selectivity of the proposed DNA biosensor towards different DNA sequences. Hybridization of the DNA biosensor with cDNA gave the highest DPV peak current response. The DPV response obtained from DNA hybridization reactions using 2-base mismatched DNA (NC 2) showed an obvious decrease in the DPV response compared with the target DNA as non-matching sequences have less hybridization during recognition events [3]. The 2-base pairs mismatch tested in the selectivity of GNS-PBA/SPE-based DNA biosensor is the minimum of 10% mismatch on 20-base oligonucleotides used frequently in this type of studies [4, 27, 28]. As the number of mismatched nucleotide base pairs increased in the target sequence, the greater the reduction in the DNA biosensor electrochemical response can be perceived. It is interesting to note that 12-base mismatched DNA (NC 12) and 16-base mismatched DNA (NC 16) exhibited the level of DPV response that was close to the response of the blank sample without cDNA. This proves the capability of the GM DNA biosensor for efficient discrimination of complementary sequences from non-complementary sequences of DNA.

3.7. Application of DNA biosensor for DNA determination in GM soybean

The electrochemical DNA biosensor based on GNS-PBA electrode was validated by determining the DNA of genetically modified soybean samples. From the results in table 2, the concentration of GM DNA in soybean samples agreed with that prepared from standard GM soy bean materials commercially available [4]. The recovery values obtained

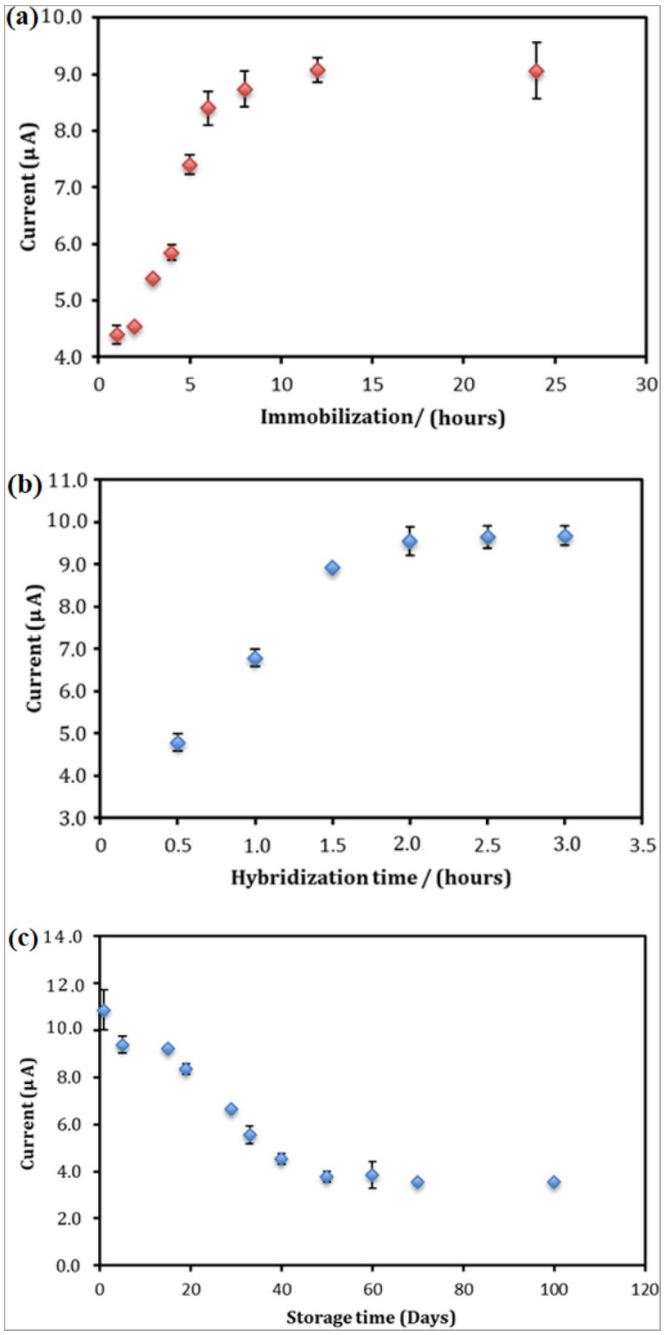

Figure 8. DPV responses of GM DNA biosensor based on (**a**) Different DNA probe immobilization duration (1–24 h) (**b**) Different DNA hybridization times (0.5–3.0 h) and (**c**) Different DNA electrode storage times (1–100 d) by using 1.0 mM AQMS towards determination 5.0×10^{-6} M target DNA in 0.05 M Na-phosphate buffer at pH 6.5 containing 0.5 M NaCl.

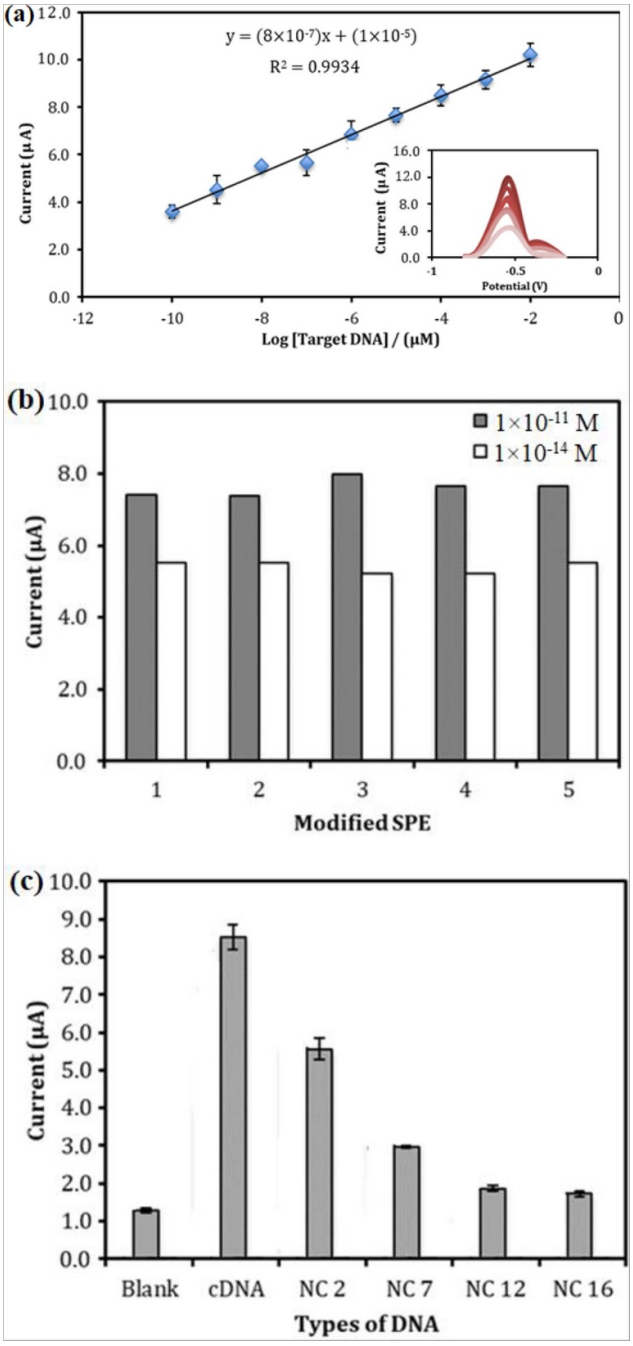

Figure 9. (a)The linear response range for hybridization reaction between GNS-PBA-DNA/SPE with various target DNA concentrations from 1.0×10^{-10} to 1.0×10^{-2} µM in 0.05 M Na-phosphate buffer (pH 6.5) containing 0.5 M NaCl. Inset shows the DPV anodic peak current of the DNA biosensor. (b) The reproducibility of the DNA biosensor towards two different target DNA concentrations at 1×10^{-14} M and 1×10^{-11} M ($n = 5$). (c) The selectivity of the GM DNA biosensor for discriminating of complementary sequences from non-complementary sequences.

from GM DNA analysis using the proposed DNA biosensor is between 96.2% and 101.8%. This confirms the satisfactory selectivity of the DNA biosensor towards the DNA of GM soybeans. Therefore, the DNA biosensor can be used for the determination of GM DNA in GM food samples.

3.8. Comparison of the DNA biosensor performance with previously reported DNA biosensors for GM food detection

A brief comparison of the analytical performance of the proposed DNA biosensor with previously reported

electrochemical DNA biosensors for GM DNA detection is outlined in table 3. The proposed GNS-PBA nanospheres based DNA electrode demonstrated better sensing performance in terms of wide linear response range and lower detection limit compared to other electrodes based on carbon materials and gold [29–32]. Comparing with a similar DNA biosensor for soybean GM DNA analysis using a composite of acrylic microspheres/rGO electrode coating [33], there is a 10 fold improvement in the LOD of the biosensor when GNS is used as an electrode coating material. This may be attributed to the three-dimensional spherical geometry of the GNS-PBA nanomaterials that allowed higher loading of DNA probe molecules immobilized on the electrode. The excellent electrical conductivity property of the GNS is assumed to enable amplification of the voltammetric response, and hence yielded a better lower detection limit for the DNA biosensor.

4. Conclusions

Graphene decorated with nanosphere-PBA is used for the first time to fabricate a DNA biosensor. The DNA biosensor was based on stacking PBA on GNSs material for the attachment of the DNA probe through covalent bonding. Applying the biosensor for the analysis of GM DNA of soybeans showed a large DPV response range and a very low detection limit towards GM DNA. Additionally, the DNA biosensor showed high selectivity towards target DNA determination, discriminating DNA mismatches in the oligonucleotide sequences. This biosensor was successfully utilized in identifying GM DNA in soybean samples with good recovery percentages of GM DNA concentrations in GM food samples. Thus, graphene decorated with nanospheres can be a potential matrix material for the fabrication of highly sensitive and improved performance DNA biosensors.

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