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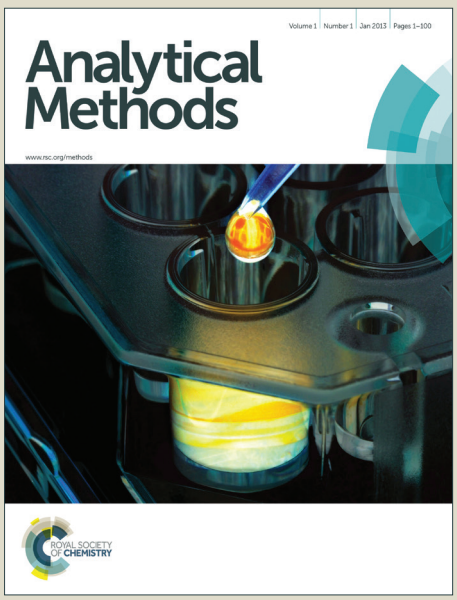

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ARTICLE

Single-step and reagentless analysis of genetically modified soybean DNA with an electrochemical DNA biosensor

Cite this: DOI: 10.1039/x0xx00000x

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

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Abstract: In many electrochemical deoxyribonucleic acid (DNA) based biosensors, a redox indicator is required to be introduced separately to indicate the DNA hybridization event. In this work, we developed a simple procedure for voltammetric determination of cauliflower mosaic virus CaMV 35S gene modified DNA without the need of introduction of a redox indicator. The DNA biosensor contains immobilized DNA probe and also a methylene blue (MB) redox indicator that is able to be slowly released during the hybridization event. The biosensor was constructed from a screen printed carbon paste electrode (SPE) coated with acrylic microspheres (AMs)-gold nanoparticles (AuNPs) composite immobilized with single-stranded DNA probes, whilst methylene blue (MB) was immobilized in the hydrogel poly(2-hydroxyethyl methacrylate) membrane located next to the electrode. Genetically modified (GM) DNA was examined based on the reduction in MB cathodic peak current (i_{pc}) signal, which was ascribed to the DNA hybridization event, via differential pulse voltammetry (DPV) method. The i_{pc} current signal of MB after DNA hybridization with target CaMV 35S gene DNA was linearly related to the logarithmic target DNA concentration ranging from 2.0×10^{-12} to 2.0×10^{-7} M ($R^2 = 0.989$) with a limit of detection (LOD) at 1.40×10^{-13} M. The proposed AMs-AuNPs composite DNA electrode gave satisfactory reproducibility performance with <10% (n = 5) relative standard deviation (RSD). The recoveries between 94.1±2.2% and 103.7±8.2% (n = 5) were obtained when the DNA biosensor was used for GM DNA determination in GM soybean DNA sample. The DNA biosensor based on AMs-AuNPs composite deposited SPE and immobilized MB exhibited higher sensitivity by single-step analysis compared with conventional electrochemical sensor methods.

Introduction

Technological advances have produced genetically modified (GM) food that is resistant to insects and viruses.[1,2,3,4] Besides, the food can be produced in large quantities in a short time frame. Several types of crops such as soybeans, corns, potatoes, etc. are commonly employed as GM food sources. However, the impact of GM foods on the environment and health is still not known.[5,6] In recent years, several countries including China, Japan and member states in the European Union have set up food labelling laws to incorporate threshold limits for biosafety reason concerning transgenic plants.[7] Polymerization chain reaction (PCR) is an established standard method for GM DNA determination, however DNA biosensors based on electrochemical transducer become more popular today for detecting genetically modified organisms (GMOs).[5,6,7,8,9,10,11] The stability, reproducibility and sensitivity of an electrochemical DNA biosensor is largely dependent on the DNA immobilization technique applied on different kinds of transducer surfaces, and biocompatibility of the DNA probe immobilization matrix.[7] Additionally, the use of small-scale materials, such as three-dimensional structured nanoparticles or microspheres having larger surface area compared[12,13] to one-dimensional structured membrane, allow greater number of DNA probes to be immobilized on the miniature matrix to promote better biosensor sensitivity.

In most reported electrochemical DNA biosensors, DNA hybridization and indicator intercalation were performed separately.[5,6,14,15,16] In this study, we have designed a DNA biosensor where both DNA hybridization and indicator intercalation detections can be performed in a single-step. Aminated DNA recognition probes for GM soybean were covalently attached to the succinimide functionalized AMs-AuNPs composite on a screen printed carbon paste electrode (SPE). Methylene blue (MB) redox active indicator was immobilized in a low molecular weight poly(2-hydroxyethyl methacrylate) or poly(HEMA) membrane next to the composite material on the SPE. The redox indicator was immobilized in such a manner in order to allow slow leaching out of the membrane and

diffuse into the DNA composite area to react with the immobilized DNA hybrid, and it is disposable after one measurement. This could eliminate the additional redox indicator introduction step and allow DNA hybridization and indicator intercalation to be carried out at once. The proposed DNA biosensor design and the schematic representation of DNA immobilization and hybridization are illustrated in Fig. 1.

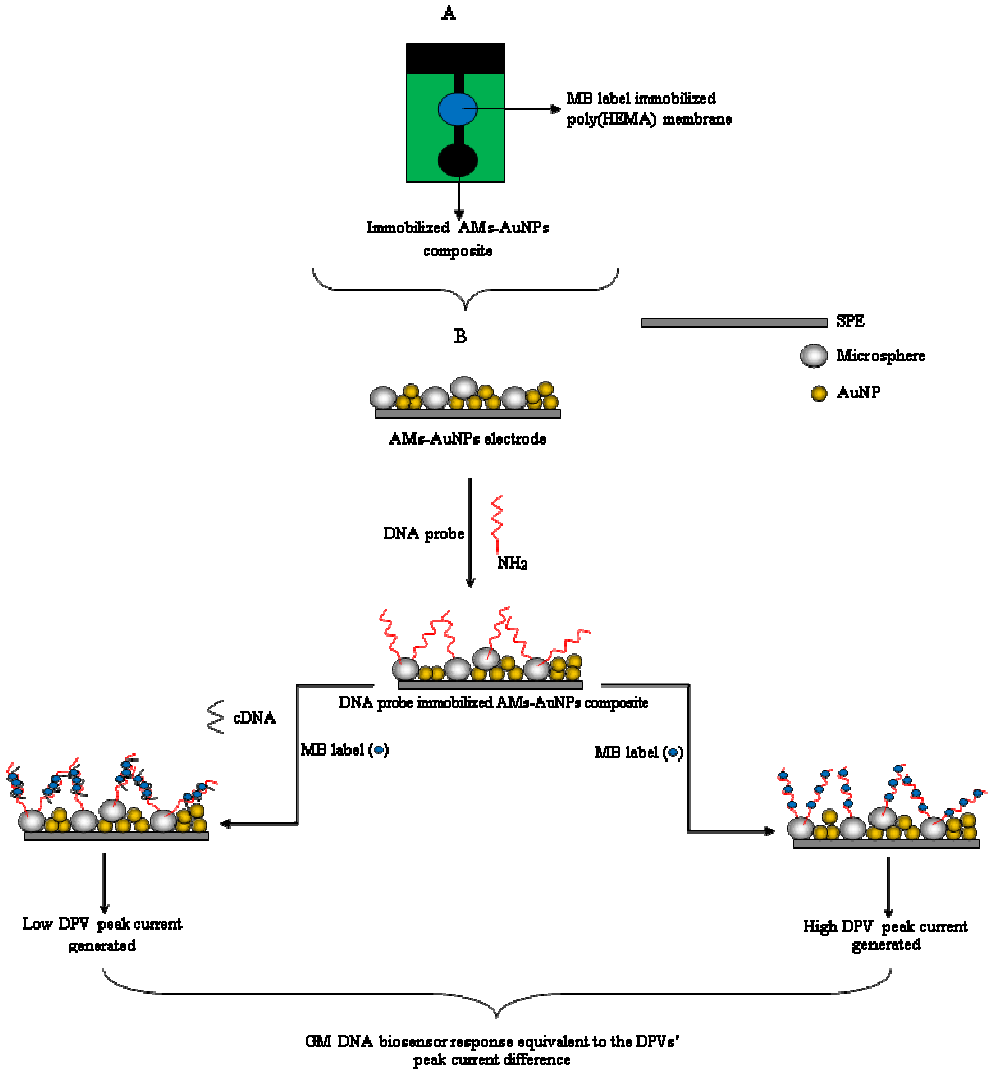

Fig.1 DNA biosensor design (A) and the schematic representation of DNA immobilization and hybridization (B).

N-acryloxysuccinimide (NAS) modified poly(n-butyl acrylate) [poly(nBA)] microspheres (AMs) synthesized from nBA monomer under UV lithography are hydrophobic in character, low polarity, insensitive to foreign ions, low glass transition temperature (T_g) and having large reaction surface area.[17,18,19] The immobilized succinimide functional group can spontaneously react with amine functional group at DNA probe to form amide covalent bond.[20,21] The large surface area of the micro-sized AMs would further allow higher loading of DNA probes at the microspheres surfaces and reduces the barrier to diffusion for reactants and products, and that the biosensor performance can be improved in terms of response time and linear response range.[19] Besides, the low T_g value of the acrylic micro matrix may facilitate the deposition of the self-adhesive microspheres on the SPE substrate.

Experimental

Chemicals

Aldrich supplied n-butyl acrylate (nBA), 2-2-dimethoxy-2-phenylacetophenone (DMPP), 1,6-hexanadiol diacrylate (HDDA), methylene blue (MB) and AuNPs (<100 nm particle size). 1,4-dioxane and N-acryloxysuccinimide (NAS) were purchased from Across. Sodium dodecyl sulfate (SDS), and NaCl were supplied by Systerm and Univar, respectively. 20 μg/mL Roundup Ready GM-soybeans (Monsanto, U.S.A) with 0%, 0.1%, 0.5%, 1.0%, 2.0 % and 5.0% GMO contents were obtained from Fluka Chemical Co. (Switzerland). Poly(2-hydroxylethyl methacrylate) [poly(HEMA)] and 20-mer synthetic oligonucleotides (Table 1) were obtained from Sigma-Aldrich. All oligonucleotides' stock solutions at 100 μM were diluted with Tris-EDTA (TE) buffer solution (10 mM Tris-HCl, 1.0 mM EDTA) at pH 7.8 and kept frozen when not in use.

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Table 1 The DNA sequences of oligonucleotides used in the present research

DNA	Base Sequences
DNA probe	5' TATCCGGAAACCTCCTCGGA(AmC_7)
Complementary DNA (cDNA)	5'TCCGAGGAGGTTTCCGGATA
Non-complementary DNA (ncDNA)	5'GTAGCATGAACTGTCATCGA

Emulsion photopolymerization of Poly(nBA) microspheres (AMs)

Poly(nBA) microspheres (<5 μm) were synthesized based on previous reported method.[18,19] In brief, a mixture of 7 mL nBA monomer, 0.01 g SDS, 450 μL HDDA, 0.1 g DMPP, 6 mg NAS and 15 mL deionized water was sonicated for 10 min. The resulting emulsion solution was then photocured for 600 s with UV light under continuous nitrogen gas flow. AMs were then collected by centrifugation at 4000 rpm for 30 min, and washed three times with 0.05 M K-phosphate buffer (pH 7.0) via centrifugation followed by air dried at room temperature.

Immobilization of MB in Poly(HEMA) membrane and leaching test

Poly(HEMA) solution was prepared by dissolving 1.0 g of the polymer in 100 mL of 20% 1,4-dioxan solution. 0.712 mg MB was then dissolved with 1000 μL of poly(HEMA) solution. Then, 10 μL of the MB-containing poly(HEMA) solution was casted on the SPE next to the electroactive area and dried overnight at room temperature. The extent of immobilized MB leaching out of the poly(HEMA) membrane was conducted by soaking the MB-immobilized SPE into 4.5 mL 0.05 M Na-phosphate buffer at pH 6.0, and DPV peak current signal at -0.2 V was taken every 10 min for 60 min.

Preparation of AMs-AuNPs composite GM DNA biosensor

About 10.0 μL of AuNPs suspension (1.0 mg / 300.0 μL) in mixed ethanol/H_2O solution was casted on the electroactive area of a SPE and air dried for 20 min before casted with another layer of 10.0 μL NAS-modified AMs suspension (1.0 mg / 100.0 μL) in ethanol. After 20 min of drying period, the AMs-AuNPs modified SPE was soaked in 4.0 μM DNA probe solution at pH 7.0 (K-phosphate buffer), and stored at 4 °C overnight to allow spontaneous covalent binding of the DNA probes. Further DNA hybridization and labelling was done by soaking the GM DNA biosensor into 4.0 μM complementary DNA (cDNA) solution in 0.05 M Na-phosphate buffer (pH 7.0) for 30 min at 25 °C. All the DPV investigations in this study were performed on a Autolab PGSTAT 12 potentiostat (Metrohm). The DNA probes immobilized AMs-AuNPs composite SPE with MB immobilized in poly(HEMA) an adjacent area served as a working electrode. Carbon pencil and Ag/AgCl electrodes were used as auxiliary and reference electrodes, respectively. The effects of DNA probe concentration, pH, buffer capacity, ionic strength and reproducibility of the GM DNA biosensor were also examined.

Recovery of GM DNA biosensor using GM soybean DNA extract

Roundup Ready GM-soybeans with 0%, 0.1%, 0.5%, 1.0%, 2.0% and 5.0% GMO contents at 20 μg/mL DNA extracts were diluted with 0.05 M Na-phosphate buffer (pH 6.0) containing 1.5 M NaCl to obtain a series of GM DNA concentrations from 2.0×10^{-8} to 1.0×10^{-4} μg/μL. The developed GM DNA biosensor was then used to determine the concentration of transgenic soybean DNA extracts for recovery study using electrochemical DPV method.

Results and discussion

Electrochemical characteristics of surface modified SPE

The cyclic voltammograms of MB immobilized on differrent working electrodes are shown in Fig. 2. The electrodynamic results are summarized in Table 2. The peak potential difference for oxidation and reduction of MB (ΔE_p) increased in the electrode order of AuNPs SPE<AMs-AuNPs SPE<bare SPE. The larger the ΔE_p value of an electrode indicates a slower electron transfer rate of the system.[22,23] The SPE modified with electroconductive AuNPs showed higher electron transfer rate via MB redox reaction at electrode-solution interface. Upon deposition of AMs, it slightly reduced the electron transfer rate effect. Reversible cyclic voltammetric response of MB was observed with all three working electrode designs. However, the oxidation rates at AuNPs SPE and bare SPE were substantially greater than the reduction rate. This is also implied by the anodic peak current to cathodic peak current ratio (i_{pa}/i_{pc}), whereby $i_{pa}/i_{pcc}>1$ when AuNPs SPE and bare SPE were used. Equilibrium MB redox reaction can be obtained from AMs-AuNPs SPE with $i_{pa}/i_{pc}\approx1$, as a result of the non-conducting AMs which have reduced the electron transfer rate from MB to the electrode surface. The AMs-AuNPs SPE was then immobilized with DNA probe to construct an electrochemical GM DNA biosensor using MB label.

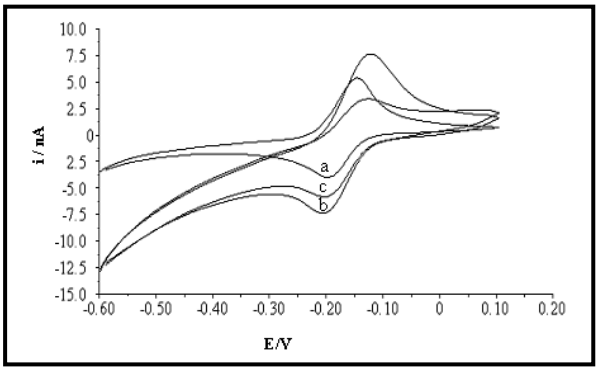

Fig. 2 Cyclic voltammograms of MB redox indicator immobilized on bare SPE (a) AuNPs SPE (b) and AMs-AuNPs SPE (c).

Table 2 The potential difference for oxidation and reduction of MB (ΔEp) and anodic peak current to cathodic peak current ratio (i_{pa}/i_{pc}) for different working electrode designs

Electrode	ΔE_p (V)	i_{pa}/i_{pc}
Bare SPE	0.07	1.73
AuNPs SPE	0.05	1.55
AMs-AuNPs SPE	0.06	0.95

Leaching test for immobilized MB and DNA hybridization response

The study found no significant difference in DPV peak current signal for MB redox label immersed in 0.05 M Na-phosphate buffer (pH 6.0) for an immersion time of more than 10 min. This indicates that all the immobilized MB in the poly(HEMA) membrane has diffused into the electrolyte for subsequent functionalization as a marker for DNA hybridization.

Fig. 3 shows DPV peak current signals for MB at AMs-AuNPs SPEs. The highest DPV peak current for AMs-AuNPs SPE immobilized with DNA probe was due to the accumulation of MB at exposed guanine bases of immobilized ssDNA probe. The DPV peak current signal declined significantly after the immobilized DNA probe hybridized with cDNA to form DNA duplex. This finding is consistent with those previous reported studies,[24,25,26,27] whereby the MB intercalated between double stranded DNA (dsDNA) was hampered from reduction at the electrode surface.[28] DNA probe immobilized AMs-AuNPs SPE peak current declined slightly after hybridization with non-complementary DNA (ncDNA) as 20% bases in the ncDNA sequence were complementary with the immobilized ssDNA probe, in which the extent of exposed guanine bases at DNA for direct interaction with MB redox indicator was higher. No DPV peak current signal for MB was perceived for AMs-AuNPs SPE indicated no adsorption of MB on the SPE, where all immobilized MB has leached out into the carrier solution.

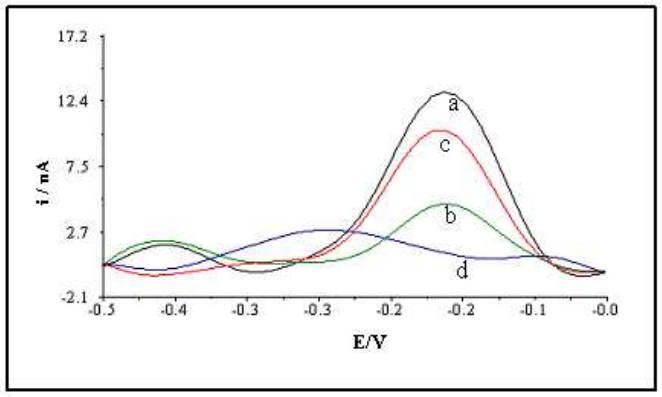

Fig.3 Differential pulse voltammograms of DNA probe immobilized AMs-AuNPs SPE electrode (a) upon hybridization with cDNA (b), ncDNA (c) and AMs-AuNPs SPE (d). The experiment was conducted in 0.05 M K-phosphate buffer (pH 7.0) with a scan rate of 0.5 V/s versus Ag/AgCl electrode.

Effect of ssDNA probe loading on the GM DNA biosensor response

The amount of ssDNA probe immobilized on the AMs-AuNPs SPE was varied between 1.25 nmol/cm^2 and 4.96 nmol/cm^2 in the absence of cDNA. The DPV peak current signal of MB increased with increasing DNA probe loading from 1.25 nmol/cm^2 to 2.46 nmol/cm^2 as the increasing immobilized ssDNA probe promoted the increasing MB accumulation at guanine bases of the immobilized ssDNA probes (Fig. 4). The peak current signal remained unchanged until 4.96 nmol/cm^2 DNA probe when the NAS-modified AMs has been saturated with immobilized ssDNA probe at 2.46 nmol/cm^2. The same DNA probe concentration effect on the DNA biosensor response has also been reported previously by Kerman et al.[29] and Loaiza et al.,[30] whereby increasing the DNA probe loading would certainly increase the DNA biosensor response.

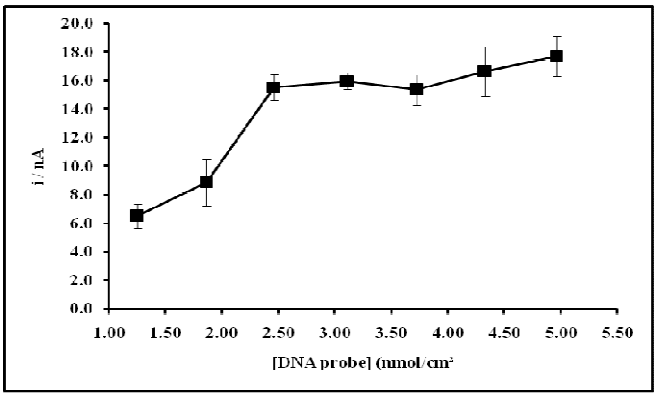

Fig. 4 Effect of ssDNA probe loading on the GM DNA biosensor response in 0.05 M Na-phosphate buffer at pH 7.0.

Effects of pH and ionic strength on DNA hybridization

The pH effect on DNA hybridization response of the GM DNA biosensor was studied between pH 5.5 and pH 8.0 using 0.05 M Na-

phosphate buffer. As shown in Fig. 5, the lowest DPV peak current signal of MB was obtained at pH 6.0 as maximum DNA hybridization occurred through hydrogen bonding between ssDNA bases (i.e. adenine, guanine, thymine and cytosine) to form immobilized dsDNA at this pH. Low DNA hybridization events were found at pH 5.5, pH 7.0−8.0. The formation of hydrogen bonds between the bases of ssDNA can be influenced by pH.[31] In acidic condition, DNA molecule has minimum solubility,[14] and the increase number of protons in solution can affect the hydrogen bonding through interactions with O and N atoms in DNA bases. Hence, when DNA molecule solubility was lower, the interaction between proton and O or N atoms in DNA bases reduced the DNA hybridization reaction rate. In a more basic medium, reduction in the quantity of protons increased the electrostatic repulsion between negatively charged phophodiester-bearing DNA molecules. This has also resulted a decline in DNA hybridization reaction rate.[32,33,34] Therefore, Na-phosphate buffer solution at pH 6 was selected as optimum pH for DNA hybridization in subsequent biosensor studies.

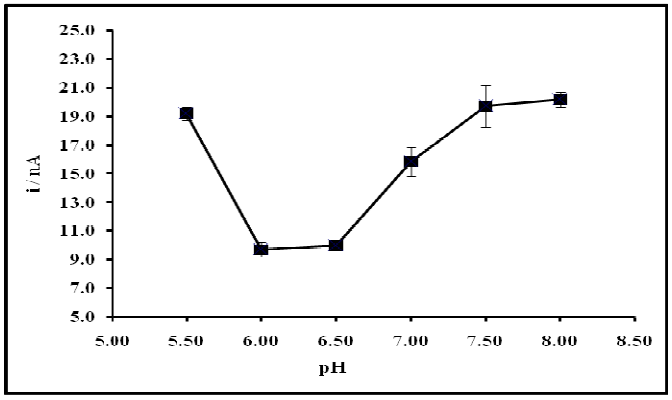

Fig. 5 The effect of pH on DNA hybridization in 0.025 M Na-phosphate buffer solution.

Parameters such as buffer capacity and ionic strength must be taken into account for optimum DNA hybridization. GM DNA biosensor gave significant characteristic response via proportional reduction in DPV peak current signal of MB when 0.01 M to 0.05 M Na-phosphate buffer and 0.11 M to 1.51 M Na^+ ion were introduced (Fig. 6). The electrostatic repulsion between DNA phosphodiester chains would reduce the DNA hybridization attraction rate.[31] By adding positively charged Na^+ cation, it can reduce the electrostatic repulsion between ssDNA as the electrostatic reaction between Na^+ ion and phosphodiester chain of DNA neutralized the DNA molecule, and increased the DNA hybridization reaction rate.[31,32,33] The higher the buffer capacity and ionic strength of the DNA hybridization medium the higher the DNA hybridization reaction rate. Hence, 1.51 M Na^+ ion in 0.05 M Na-phosphate buffer at pH 6.0 were used as optimum ionic strength for DNA hybridization. However, extremely high ionic strength would reduce DNA solubility and DNA hybridization reaction rate.[31]

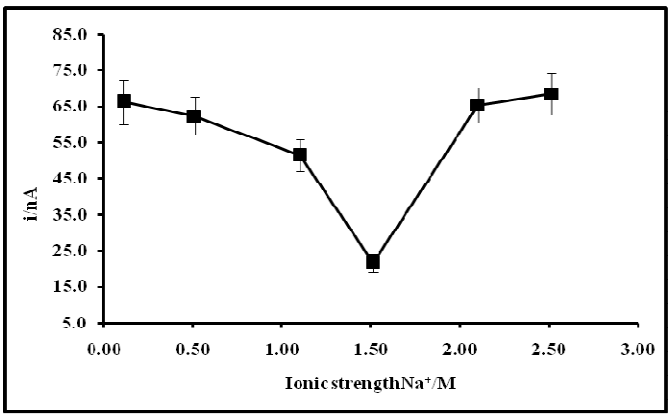

Fig. 6 The ionic strength of Na^+ ion on DNA Hybridization in 0.05 M Na-phosphate buffer (pH 6.0).

Dynamic range and recovery of GM DNA biosensor

The linear response range of the electrochemical GM DNA biosensor was assessed by exposing the DNA biosensor with 2.0×10^{-12} to 2.0×10^{-7} M cDNA with 30 min DNA hybridization at 25 °C. Fig. 7 shows the dynamic range of the GM DNA biosensor (R^2 = 0.989). The detection limit of the GM DNA biosensor is estimated to be 1.40×10^{-13} M.

The GM DNA biosensor was then used to determine the DNA concentration of transgenic soybean extract from Roundup Ready GM-soybean for recovery study. The recoveries between 94.12±2.19% and 103.70±8.24% (n = 5) imply that the GM DNA biosensor is applicable for accurate DNA assay in GM food with reproducible results between 3.1% and 8.2% RSD (n = 5).

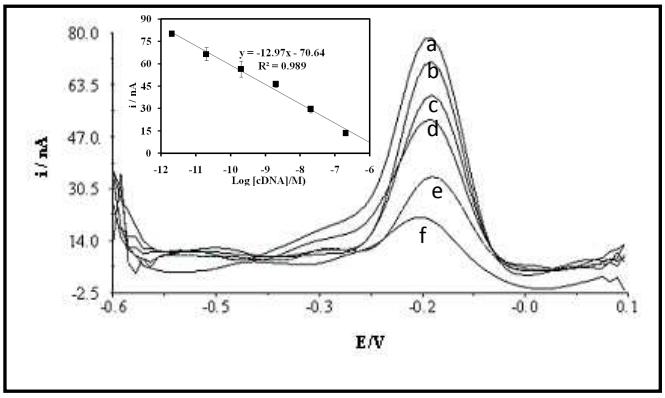

Fig. 7 Differential pulse voltammograms of GM DNA biosensor response to 2.0×10^{-12} (a), 2.0×10^{-11} (b), 2.0×10^{-10} (c), 2.0×10^{-9} (d), 2.0×10^{-8} (e) and 2.0×10^{-7} M cDNA (f). Inset shows the proportionality between i_{pc} of MB and cDNA concentration.

Comparison with other DNA biosensors

Table 3 presents the comparative performance of developed GM DNA biosensor with other reported electrochemical DNA biosensors with respect to linear response range, LOD and DNA hybridization duration. The proposed DNA biosensor using poly(nBA) microspheres as

DNA immobilization matrix has shown much improved biosensing performance as indicated by a wider dynamic linear range and lower LOD compared to other DNA biosensors using a variety of matrices for DNA immobilization. The micro sized AMs, functionalized with succinimide, are beneficial for higher loading of DNA probe and enhancing the biosensor sensitivity. The good adhesion property of the AMs material allows for bonding on the electrode.

Table 3 Comparison between the proposed electrochemical GM DNA biosensor based on immobilized MB label and other previously reported electrochemical DNA biosensors based on free MB redox label

DNA Biosensor Method	Linear Range (M)	Detection Limit (M)	Reproducibility (%)	Hybridization Time (min)	References
AMs-AuNPs SPE	2.0×10^{-12} - 2.0×10^{-7}	1.40×10^{-13}	3.1 - 8.2	30	Present work
CeO_2/chitosan film modified glassy carbon electrode	1.59×10^{-11} - 1.16×10^{-7}	1.0×10^{-12}	4.0	60	14
N-hydroxysulfosuccinimide (NHS) modified glassy carbon electrode	1.25×10^{-7} - 6.75×10^{-7}	5.9×10^{-8}	4.8	30	15
Zirconia (ZrO_2) modified Au electrode	2.25×10^{-10} - 2.25×10^{-8}	1.0×10^{-10}	-	30	17
Thiolated DNA immobilized Au electrode	2.0×10^{-8} - 2.0×10^{-6}	1.0×10^{-8}	-	60	35
Avidin modified Au electrode	-	3.9×10^{-12}	19 - 28	15 - 20	36

Conclusion

A reagentless electrochemical GM DNA biosensor based on AMs-AuNPs composite and MB immobilized on single SPE electrode was constructed. DNA hybridization and accumulation of redox indicator were performed in a single-step for detection of CaMV 35S gene-modified DNA. This makes electrochemical DNA assay become more convenient merely requiring adding of DNA sample into the detection cell. The micro structured acrylic immobilization matrix had enhanced the biosensing performance with regard to dynamic range and LOD. Evaluation and optimization of the GM DNA biosensor via CV and DPV confirmed the reproducibility of the DNA biosensor with promising recovery performance.

Acknowledgements

We would like to thank the National Biotechnology Directorate of the Ministry of Science, Techonology and Innovation of Malaysia for a top-down research grant and Universiti Kebangsaan Malaysia for financial support via research operational grants DIP-2012-11.

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Notes and references

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Single-step and reagentless analysis of genetically modified soybean DNA with an electrochemical DNA biosensor

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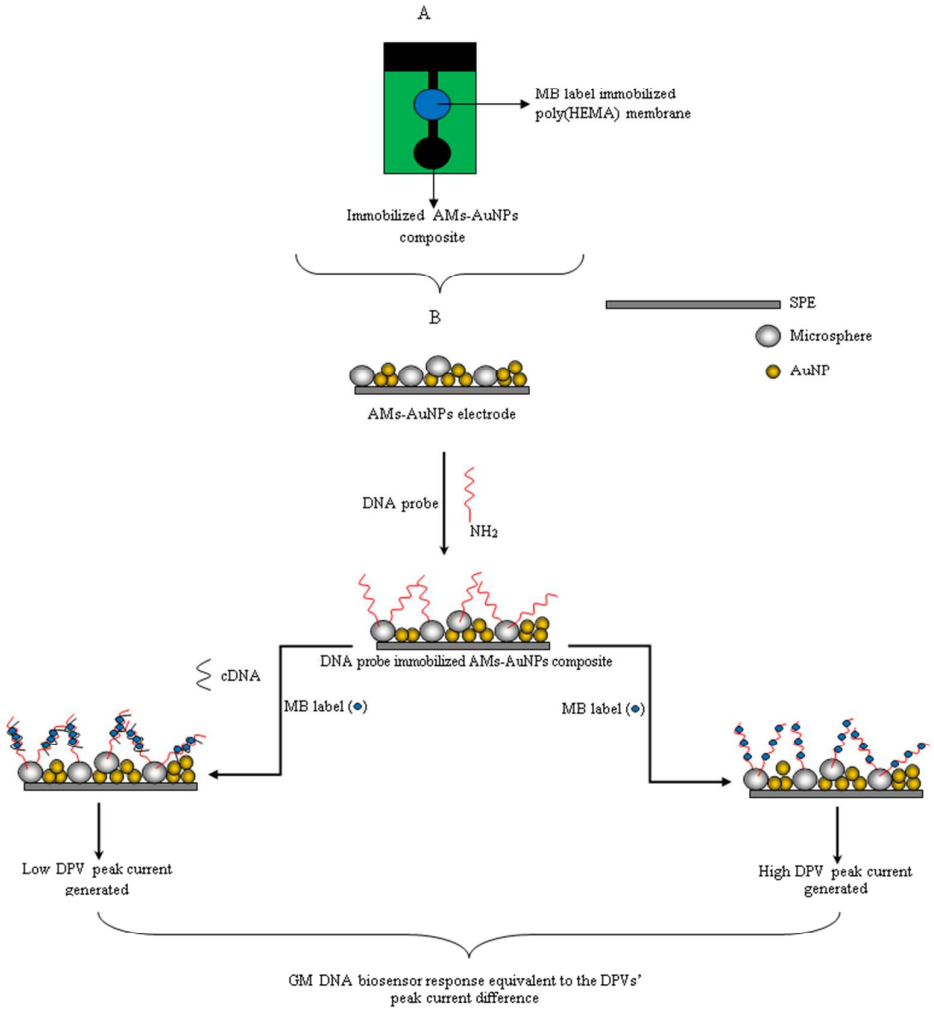

Abstract: In many electrochemical DNA based biosensors, a redox indicator is required to be introduced separately to indicate the DNA hybridization event. In this work, we developed a simple procedure for voltammetric determination of CaMV 35S gene modified DNA without the need of introduction of a redox indicator. The DNA biosensor contains immobilized DNA probe and also a methylene blue redox indicator that is able to slow release during the hybridization event. The biosensor was constructed from a screen printed carbon paste electrode (SPE) coated with acrylic microspheres (AMs)-gold nanoparticles (AuNPs) composite immobilized with single-stranded DNA probes, whilst methylene blue (MB) was immobilized in the hydrogel poly(2-hydroxyethyl methacrylate) membrane located next to the electrode. Genetically modified (GM) DNA was examined based on the reduction in MB cathodic peak current (i_{pc}) signal, which was ascribed to the DNA hybridization event, via differential pulse voltammetry (DPV) method. The i_{pc} current signal of MB after DNA

hybridization with target CaMV 35S gene DNA was linearly related to the logarithmic target DNA concentration ranging from 2.0×10^{-12} to 2.0×10^{-7} M (R^2 = 0.989) with a limit of detection (LOD) at 1.40×10^{-13} M. The proposed AMs-AuNPs composite DNA electrode gave satisfactory reproducibility performance with <10% (n = 5) relative standard deviation (RSD). The recoveries between 94.1±2.2% and 103.7±8.2% (n=5) were obtained when the DNA biosensor was used for GM DNA determination in GM soybean DNA sample. The DNA biosensor based on AMs-AuNPs composite deposited SPE and immobilized MB exhibited higher sensitivity by single-step analysis compared with conventional electrochemical sensor methods.