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Electrochemical DNA biosensor for bovine papillomavirus detection using polymeric film on screen-printed electrode

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ABSTRACT

A new electrochemical DNA biosensor for bovine papillomavirus (BPV) detection that was based on screen-printed electrodes was comprehensively studied by electrochemical methods of cyclic voltammetry (CV) and differential pulse voltammetry (DPV). A BPV probe was immobilised on a working electrode (gold) modified with a polymeric film of poly-L-lysine (PLL) and chitosan. The experimental design was carried out to evaluate the influence of polymers, probe concentration (BPV probe) and immobilisation time on the electrochemical reduction of methylene blue (MB). The polymer poly-L-lysine (PLL), a probe concentration of 1 μM and an immobilisation time of 60 min showed the best result for the BPV probe immobilisation. With the hybridisation of a complementary target sequence (BPV target), the electrochemical signal decreased compared to a BPV probe immobilised on the modified PLL-gold electrode. Viral DNA that was extracted from cattle with papillomatosis also showed a decrease in the MB electrochemical reduction, which suggested that the decreased electrochemical signal corresponded to a bovine papillomavirus infection. The hybridisation specificity experiments further indicated that the biosensor could discriminate the complementary sequence from the non-complementary sequence. Thus, the results showed that the development of analytical devices, such as a biosensor, could assist in the rapid and efficient detection of bovine papillomavirus DNA and help in the prevention and treatment of papillomatosis in cattle.

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1. Introduction

Bovine papillomatosis is an infectious disease caused by a double-stranded DNA virus, bovine papillomavirus (BPV) (Campo, 2006; Claus et al., 2008). This virus can induce lesions in the stratified squamous epithelium of the skin and mucous membranes and fibroepithelial tumours (Antonsson and Hansson, 2002; Campo, 2002; Yuan et al., 2010). The BPV belong to the *Papillomaviridae* family, which contains 16 genera (Antonsson and Hansson, 2002; Ogawa et al., 2004). Ten types (BPV1–10) have been well characterised by studies on their biology and genome homology (Claus et al., 2009; Nasir and Campo, 2008). In general, three primers, MY09/MY11, GP5+/GP6+ and FAP59/FAP64, are widely used for papillomavirus identification in humans (first two pairs) and in bovine and other animals (last pair) (Lee et al., 2010; Rai et al., 2011). Research into BPV diagnostics has attracted considerable interest due to problems in the infected animals, such as dermatitis and cancer in different body sites (Borzacchiello and Roperto, 2008; Brandt et al., 2011; Campo, 2002). Consequently, these problems can contribute to economic losses in the agricultural industry. Currently, BPV diagnosis has been made through histological observations of skin and tissues. These models often do not lead to conclusive results and consequently produce a slow diagnosis.

Electrochemical DNA biosensor technologies have been developed due to their several advantages, such as specificity, selectivity, low cost, rapid diagnosis and the possibility of miniaturisation (Siddiquee et al., 2010). A DNA biosensor or genosensor recognises a complementary sequence (target) to a single-stranded DNA (probe) (Labuda et al., 2010; Souza et al., 2011). For a DNA hybridisation sensor, a probe with a defined nucleotide sequence is immobilised on a conductive surface by using immobilisation methods, such as adsorption, cross-linking, encapsulation, an avidin–biotin complex or a covalent attachment (Wang et al., 2008). The key for manufacturing a DNA biosensor is the oligonucleotide immobilisation on different types of transducer surfaces (Wang et al., 2010). Many polymers have been used in

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the development of DNA biosensors (Ghanbari et al., 2008; Jiang et al., 2008; Li et al., 2005) and have the ability to form thin layers on electrodes surfaces. They also have functional groups that can bind with DNA molecules, which allow for better performance in the immobilisation. For instance, poly-L-lysine (PLL) and chitosan are polymers widely used in the construction of DNA biosensors. The PLL can be electrodeposited or adsorbed on several types of electrodes, and it has a functional group that can link with DNA (Stobiecka and Hepel, 2011; Wang et al., 2010). Chitosan is a polysaccharide biopolymer, which is widely used in several industries, biomedicine and biosensors (Wang et al., 2011), that also binds with DNA molecules (Mandong et al., 2007). Thus, the purpose of this present study was to develop a new electrochemical DNA biosensor for bovine papillomavirus detection using polymer films on screen-printed electrodes.

2. Materials and methods

2.1. Reagents and materials

All reagents used were of a high purity. Methylene blue (MB), L-lysine and chitosan were purchased from Sigma-Aldrich (USA). UltraPure™ DNase/RNase-Free distilled water was purchased from Invitrogen (USA). All ssDNA oligonucleotides were synthesised by Integrated DNA Technologies (USA). Screen-printed electrodes were purchased from The Gwent Group (UK) for electrochemical detection.

2.2. Apparatus

A conventional three-electrode system was employed in this work. Gold was used as a working electrode (surface area of approximately 3 mm^2), silver/silver chloride (Ag/AgCl) was used as a reference electrode and carbon was used as a counter electrode. The electrochemical analysis was performed with a potentiostat (Autolab PGSTAT) that was equipped with GPES (4.0.007) software. All hybridisation experiments were carried out in a Hybridisation Oven/Shaker (Amersham Pharmacia Biotech).

2.3. Bovine papillomavirus oligonucleotides

All oligonucleotides were purchased as a lyophilised powder, diluted with ultrapure water and stored as a stock solution in a freezer. The oligonucleotides were diluted from the stock solution in 0.5 M acetate buffer pH 5 for the experiments. The following three oligonucleotides sequences, which were designed by bioinformatics tools, were used in this study:

BPV probe : 5′-TGG AAA TCT TTT TTT GAA AGG CTT TGG-3′
BPV target : 5′-CCA AAG CCT TTC AAA AAA AGA TTT CCA-3′
Non−complementaryDNA : 5′-CCC CGG ATC ACT GAG ACG-3′

2.4. Papilloma samples

15 blood samples were collected from cattle with cutaneous papillomatosis in the Itambé experimental station of Instituto Agronômico de Pernambuco (IPA), which is located in Itambé, Pernambuco, Brazil. Blood samples were kept in EDTA vacutainer tubes and refrigerated at 4 °C until viral DNA extraction.

2.5. Viral DNA extraction and PCR assay

Viral DNA was extracted from the blood samples by using an automated Maxwell® 16 Clinical Instrument System. The Maxwell® 16 Blood DNA Purification Kit was used for DNA purification according to the protocol specifications. After purification, the extracted DNA was amplified with the primer pair MY09 (forward: 5′-CGT CCM ARR GGA ACT GAT C-3′) and MY11 (reverse: 5′-GCM CAG GGC ATA AYA ATG G-3′) to amplify a fragment of 450 bp from the L1 gene. PCR was performed using the GoTaq® qPCR Master Mix kit and the following conditions: 1 μL of the extracted DNA, 6.25 μL of GoTaq® qPCR Master Mix, 1 μL of each primer and 3.25 μL of ultrapure water. The amplification was performed in the following conditions: (i) 94 °C for 3 min, (ii) 34 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min and (iii) a final extension at 72 °C for 10 min. The PCR product was visualised by electrophoresis in a 2% agarose gel containing ethidium bromide (1 mg/mL) and TBE buffer pH 8.4 (89 mM Tris, 89 mM boric acid and 2 mM EDTA) under UV light.

2.6. Preparation of polymer-modified screen-printed electrodes

The polymers chitosan and poly-L-lysine (PLL) were used to allow the immobilisation of the DNA probe onto the working electrode. The chitosan solution (1%) was prepared in an acetic acid solution (1%). The L-lysine solution (0.001 M) was prepared in 0.1 M phosphate-buffered saline (PBS) pH 7.4. For preparation of the chitosan film, 3 μL of the chitosan solution was added onto the working electrode and then incubated until dry at 30 °C for 20 min. For preparation of the PLL film, the L-lysine solution was electropolymerised on the working electrode by cyclic potential scanning from −1.8 to 2.0 V for 10 cycles with a scan rate of 100 mV s^{-1}.

2.7. Experimental design

The influence of the independent variables of the polymer (PLL and chitosan), probe concentration (1 μM and 8 μM of the BPV probe) and immobilisation time (30 min and 60 min) on the dependent variable of the MB electrochemical reduction was evaluated from the results obtained by the 2^3 full factorial design. In this design, a set of 24 experiments was performed in triplicate to allow for the estimation of pure experimental error. The results were analysed by an analysis of variance (ANOVA) at a significance level of $p < 0.05$. All statistical and graphical analyses were carried out with the Statistica 8.0 programme (StatSoft Inc.).

2.8. Probe DNA immobilisation and target DNA hybridisation

The BPV probe in an acetate buffer solution (0.5 M, pH 5) was immobilised by adsorption on a gold electrode surface that was modified with a polymer layer, and the immobilisation time was obtained in the factorial design. Then, the unbound oligonucleotides were removed from the working electrode by washing with PBS.

In the hybridisation process, a solution containing the BPV target in acetate buffer (0.5 M, pH 5) was added onto the working electrode with the immobilised BPV probe and incubated at 55 °C for 10 min with a stirred speed of 300 rpm to link the complementary sequences. This temperature was the best for annealing sequences based on the company's descriptions. A Tris–HCl buffer (20 mM, pH 7.0) was used to wash the electrode and remove the non-hybridised sequences. The same procedure was applied for the interaction of the BPV probe with a non-complementary sequence. For the hybridisation of the papilloma samples with the BPV probe on the modified working electrode, the extracted viral DNA was first heated to 94 °C for 4 min to allow the denaturation of the double helix and then added to the working electrode surface with the immobilised BPV probe. The formation

of a hybrid between the extracted viral DNA and BPV probe was also performed at 55 °C for 10 min with a stirred speed of 300 rpm, and the electrode was then washed with a Tris–HCl buffer (20 mM, pH 7.0) to remove the non-hybridised sequences.

2.9. Electrochemical analysis

For the electrochemical signal analysis, the differential pulse voltammetry (DPV) method was used for the measurement system of the current signals. After the immobilisation and hybridisation process, a 500 μM MB solution in Tris–HCl buffer (20 mM, pH 7.0) was accumulated on the modified working electrode for 5 min, which was then washed with the Tris–HCl buffer. The DPV measurement was performed in the Tris–HCl buffer for the MB electrochemical reduction under the following conditions: a potential sweep between −0.8 and 0 V, modulation amplitude of 50 mV and scan rate of 20 mV s^{-1}.

3. Results and discussion

3.1. Preliminary investigations

In this work, polymers were used to allow for the immobilisation of the probe on the gold working electrode surface. Polymers PLL and chitosan were chosen to be the electropositive compounds, which thus allow for the link with the DNA molecules that are electronegative. With these polymers, it is possible to produce thin films on different supports, such as gold electrodes.

The binding of DNA on PLL or chitosan occurred in a similar way. The immobilisation of the DNA on a PLL-gold surface was primarily due to the electrostatic attraction of the negatively charged phosphate groups from the DNA to the positively charged PLL. It is likely that hydrogen bonding between the NH^{+3} groups of PLL and the oxygens from the phosphate groups in the DNA may contribute to strengthen the attachment of the DNA to PLL (Stobiecka and Hepel, 2011). Chitosan also can form a stable complex with the polyanionic phosphodiester backbones of DNA (Singh et al., 2010). It is susceptible to chemical modifications due to the presence of reactive amino and hydroxyl functional groups and provides a hydrophilic environment for the immobilisation of desired biomolecules (Mandong et al., 2007). From the similarity of the interactions between the DNA and the polymers investigated, an experimental design was carried out to evaluate the efficiency of the polymer layer on screen-printed electrodes during the immobilisation process.

The performance of the biosensor was investigated by differential pulse voltammetry with the redox indicator methylene blue (MB). It is widely known that MB has an affinity to DNA, and several authors have demonstrated two possible mechanisms of interaction. These links can occur by an electrostatic interaction with the negatively charged phosphate groups or by an interaction between the guanine bases, which are mainly in ssDNA (Ortiz et al., 2011; Tran et al., 2011). In this work, the binding of MB occurred by an interaction between the guanine bases in ssDNA (probe) because the phosphate groups were linked to the polymer layer on the gold surface.

3.2. Factorial design analysis in the probe immobilisation

The probe immobilisation efficiency was evaluated from the 2^3 full factorial design, whose independent variables of the polymer, probe concentration (BPV probe) and immobilisation time were investigated. Fig. 1A illustrates the average of the current peak results for the low and high levels of polymer (PLL and chitosan), probe concentration (1 μM and 8 μM) and immobilisation time

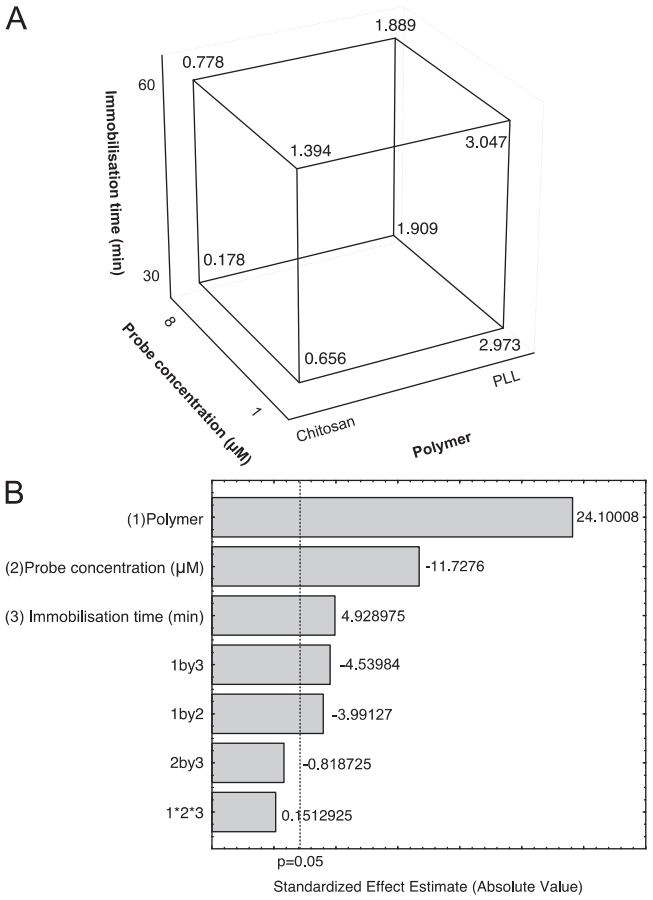

Fig. 1. (A) Graphical representation of the relationships between independent variables polymer, probe concentration and immobilisation time that were analysed by a 2^3 full factorial design. Each independent variable corresponds to an axis, with its respective low and high levels. All results were plotted with the averages of the current peaks (μA) performed in triplicate under the conditions investigated. (B) Pareto bar chart of the standardised effect estimate (absolute value) of the independent variables polymer (1), probe concentration (2) and immobilisation time (3) on MB electrochemical reduction for the 2^3 full factorial design.

(30 min and 60 min). From the 24 experiments that were performed under the conditions investigated, it was observed that the best result for the BPV probe immobilisation process on the MB electrochemical reduction was obtained with the PLL polymer, a probe concentration of 1 μM and an immobilisation time of 60 min (Fig. 1A). The chitosan most likely had no significant results on the MB electrochemical reduction compared to PLL because of its poor electrical conductivity that often results in a low sensitivity for the determination of the analytes (Wang et al. 2011).

The Pareto bar chart (Fig. 1B) represents the estimated effects of the variables and their interactions on the MB electrochemical reduction in decreasing order of magnitude. The length of the bars is proportional to the standardised effect. The vertical line is used to judge which effects are statistically significant. Bars extending beyond this line correspond to the statistically significant effects at a confidence level of 95% (Lima et al., 2009). Statistical analysis showed that the independent variables polymer and immobilisation time had a significant positive effect on the MB electrochemical reduction. Additionally, the independent variable probe concentration and the polymer-immobilisation time and polymer–probe concentration interactions had significant negative effects on the MB current peak. However, the independent variable polymer had the most significant effect, suggesting that PLL can improve the immobilisation process and, consequently, the electrochemical signal. The probe concentration-immobilisation time and polymer–probe

concentration-immobilisation time interactions had no significant effect (Fig. 1B).

The Pareto bar chart (Fig. 1B) also showed that a decrease in the probe concentration could improve the current signal. Therefore, a BPV probe concentration curve was performed using the best conditions observed in the 2^3 full factorial design, which were the modified PPL-gold electrode and an immobilisation time of 60 min. The goal of this experiment was to observe the BPV probe concentration effect on the immobilisation through the MB electrochemical reduction. In Fig. 2, it was observed that the concentrations of 1 μM and 1.5 μM had the highest current peaks; however, the concentration of 1 μM had a better reproducibility compared to 1.5 μM. The other concentrations (0.01 μM, 0.1 μM, 0.25 μM and 0.5 μM) showed lower current signals due to a low amount of immobilised probe on the working electrode surface. Thus, the probe concentration of 1 μM was chosen for the interaction with complementary sequences.

3.3. Optimisation of the hybridisation process

The biosensor recognition array was applied in experiments on the hybridisation reaction between the BPV probe and the BPV target DNA fragment. In this study, the hybridisation was performed with differential potential voltammetry on the MB electrochemical reduction. The goal of this experiment was to determine the optimal hybridisation conditions for the probe and target on the modified PLL-gold electrode while minimising the non-specific interaction. In this regard, the hybridisation was performed with different concentrations of the complementary target sequence. In Fig. 3, it was observed that the current signal increased with the increasing target concentration of up to 100 nM, and then it stabilised at 250 nM. At these concentrations, it was observed that the highest current signal was 0.9 μA (Fig. 3). However, this current signal in the hybridisation decreased when compared to the current signal with the BPV probe (3.4 μA) immobilised on the modified PLL-gold electrode. MB had a strong affinity for the free guanine in the ssDNA. Thus, this difference occurred due to a weak interaction between the dsDNA and MB compared with the ssDNA and MB, which resulted in the decreased intercalation of MB within the dsDNA (Lin et al., 2007; Souza et al., 2009).

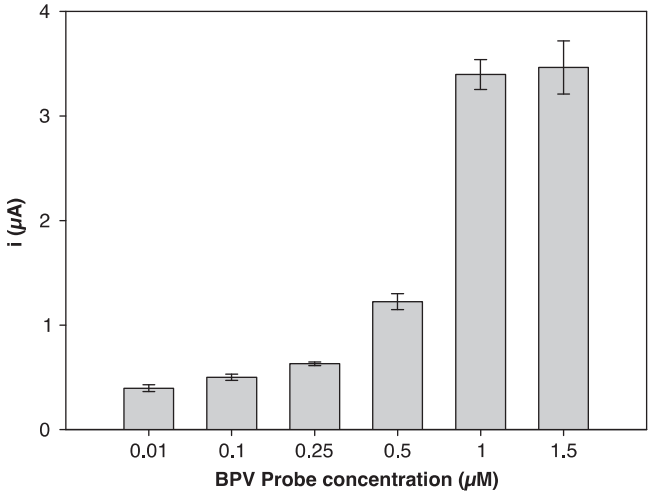

Fig. 2. Histogram of the BPV probe concentration effect on the MB electrochemical reduction during the immobilisation process. The differential pulse voltammetry method was used to analyse the current signals in the following conditions: initial potential −0.8 V, end potential 0 V, modulation amplitude 50 mV and scan rate 20 mV s^{-1}. All results plotted were the averages of triplicates that were performed at different BPV probe concentrations.

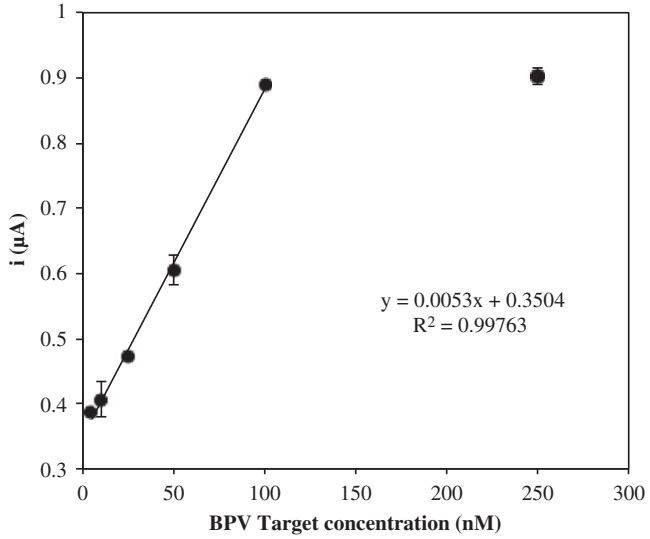

Fig. 3. The effect of the BPV target concentration on the MB electrochemical reduction during hybridisation. The differential pulse voltammetry method was used to analyse the current signals in the following conditions: initial potential −0.8 V, end potential 0 V, modulation amplitude 50 mV and scan rate 20 mV s^{-1}. All results plotted were the averages of experiments that were performed at different concentrations of the BPV target. The black line represents the linear regression at a concentration range of 5–100 nM.

The linear regression obtained from the electrochemical signal regarding the different concentrations of the BPV target is shown in Fig. 3. The calibration curve ($y=0.0053x+0.3504$) is linear between 5 nM and 100 nM with a correlation coefficient of 0.99763 ($p<0.00048$, $n=5$). A detection limit of 4.35 nM could be estimated by equation $3\sigma/a$, where σ is the standard deviation of the intercept and a is the slope of the linear regression (Gumustas and Ozkan, 2011). The relative standard deviation (RSD) was 0.056% over three independent probe-modified electrodes that were measured at 100 nM of the BPV target, which indicated a remarkable reproducibility of the detection method.

3.4. Papilloma sample analysis

In the present paper, the viral DNA extraction from the blood samples was proposed to reduce the steps in the processing of biological samples compared to skin samples (Munday and Knight, 2010). Consequently, the elimination of these steps allowed for the rapid detection of BPV. Two of the fifteen samples were amplified by the primer pair MY09/MY11. This ratio of amplification number to sample number was due to a low concentration of viral DNA in the blood. According to the literature, the identification of BPV was possible in blood samples collected from cows with papillomatosis (Wosiacki et al., 2005). In Fig. 4, it is possible to observe two amplification products of 450 bp in the electrophoresis gel that correspond to the papillomavirus L1 gene. The primer MY is widely used for the amplification and identification of the human papillomavirus L1 gene (Rai et al., 2011). However, many authors have demonstrated the amplification of the BPV L1 gene with primer MY (Ogawa et al., 2004; Silva et al., 2010). Positive and negative controls for the papillomavirus were compared with the PCR products of the bovine samples. After identification of the papillomavirus in the blood samples by PCR, we evaluated the performance of the biosensor with these positive samples. In this assay, we used the extracted viral DNA unlike other studies that used PCR products (Gao et al., 2011; Wu et al., 2011).

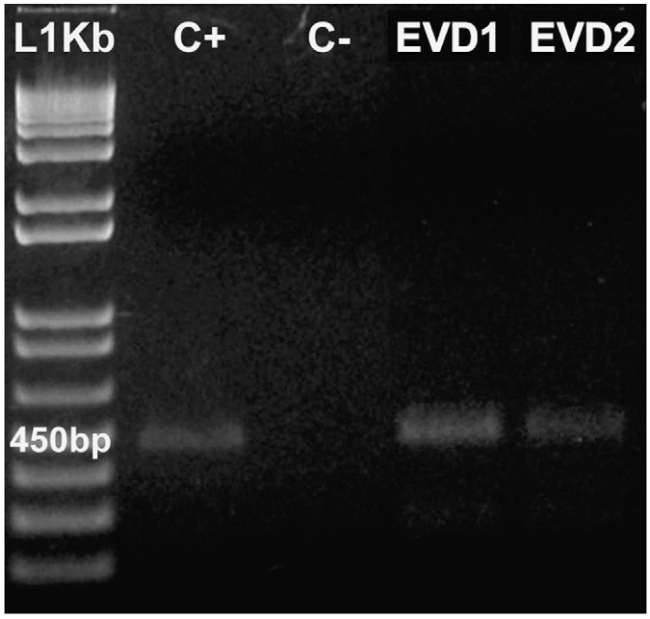

Fig. 4. The amplification of the L1 gene (papillomavirus) with the primer pair MY09/MY11 from viral DNA that was extracted from bovine blood samples. Lane L1(Kb): 1 Kb Plus DNA ladder (Invitrogen); lane C+: positive control for the papillomavirus L1 gene in pBR322.HPV16 plasmid; lane C−: negative control (ultrapure water); lane EVD1: extracted viral DNA 1 and lane EVD2: extracted viral DNA 2.

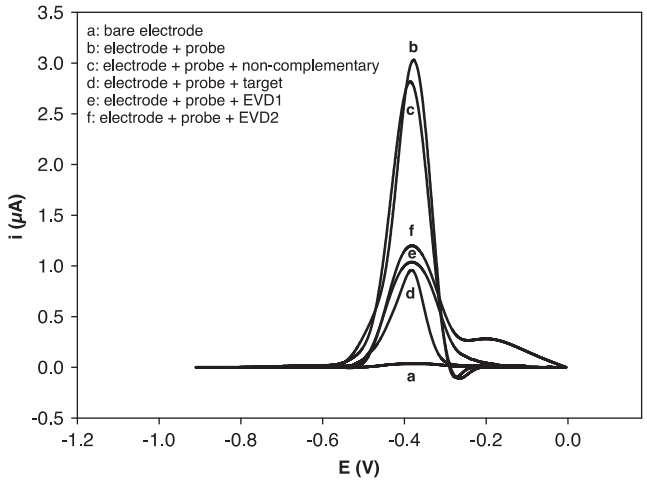

Fig. 5. The differential pulse voltammograms for the MB electrochemical reduction of (a) bare modified PLL-gold electrode; (b) 1 μM BPV probe immobilised on modified PLL-gold electrode before hybridisation; (c) 1 μM BPV probe immobilised on modified PLL-gold electrode after hybridisation with a non-complementary sequence; (d) 1 μM BPV probe immobilised on modified PLL-gold electrode after hybridisation with 100 nM BPV target sequence; (e) 1 μM BPV probe immobilised on modified PLL-gold electrode after hybridisation with 46.90 μg/mL EVD1 and (f) 1 μM BPV probe immobilised on modified PLL-gold electrode after hybridisation with 62.15 μg/mL EVD2.

3.5. Detection of bovine papillomavirus

The performance of the electrochemical DNA biosensor was investigated by checking the hybridisation of the BPV probe, which was immobilised on the modified PLL-gold electrode, with the BPV target sequence, a non-complementary sequence and the viral DNA that was extracted from the papillomatosis samples. Fig. 5 shows a voltammogram with the profiles of current peaks that were generated in different experiments. It is possible to observe that the BPV probe showed the highest current peak when compared to the other curves. This increase occurred due to a strong affinity between MB and the 7 free guanines that are present in the BPV probe. After hybridisation of the BPV probe with the complementary BPV target, the extracted viral DNA 1 (EVD1) and extracted viral DNA 2 (EVD2) current peaks were decreased, which confirmed the occurrence of hybridisation in the proposed detection system. The current peak change could be explained by the steric inhibition of MB packing between the duplex (Tran et al., 2011). The specificity of the biosensor was studied by incubating the non-complementary sequence on the probe electrode. Fig. 5 showed that the electrochemical signal after hybridisation of the BPV probe with non-complementary sequence increased when compared to the hybrid formed. The significant increase in the current signal between the BPV probe and a non-complementary sequence showed that the biosensor was highly selective. However, the experiments with a non-complementary sequence showed a current peak slightly lower than the immobilised BPV probe on the modified PLL-gold electrode, which was most likely due to the non-specific links between non-complementary sequences (Pournaghi-Azar et al., 2007). The bare modified PLL-gold electrode (curve a) did not present an electrochemical signal, which indicated the absence of guanine on the working electrode (Fig. 5). According to the analysed data, the decrease of the current peak represented a positive diagnosis for a bovine papillomavirus infection.

4. Conclusions

In this present work, it was demonstrated a new electrochemical DNA biosensor for detection of the bovine papillomavirus using synthesised oligonucleotides and extracted viral DNA with a high specificity and sensitivity. The modified gold electrode surface can be characterised by a differential pulse voltammetry. PLL presented a better performance than the chitosan during the immobilisation process. In the detection model proposed, the DNA biosensor can detect hybridisation. Therefore, it was possible to identify samples positive for the bovine papillomavirus. The BPV probe after hybridisation resulted in a decrease of the MB electrochemical reduction compared to the BPV probe before hybridisation. This decrease in the MB electrochemical reduction was related to the presence of a BPV infection in the cattle. The data obtained with the biosensor showed viability for the bovine papillomavirus diagnostic, thereby allowing for the development of a new portable detection system for viruses.

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