Biosensors and Bioelectronics 38 (2012) 61–66

Contents lists available at SciVerse ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

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

Gustavo A. Nascimento [a,*], Elaine V.M. Souza [a], Danielly S. Campos-Ferreira [a], Mariana S. Arruda [a], Carlos H.M. Castelletti [a,b], Marcela S.O. Wanderley [a], Marek H.F. Ekert [a], Danyelly Bruneska [a,c], José L. Lima-Filho [a,c]

[a] Laboratório de Imunopatologia Keizo Asami (LIKA), Universidade Federal de Pernambuco-UFPE, Av. Prof. Moraes Rego, s/n, Campus Universitário, 50670-901 Recife, PE, Brazil
[b] Instituto Agronômico de Pernambuco (IPA), Av. General San Martin, 1371, Bongi, 50761-000, Recife, PE, Brazil
[c] Departamento de Bioquímica, Universidade Federal de Pernambuco-UFPE, Av. Professor Moraes Rego, s/n, Campus Universitário, 50670-901 Recife, PE, Brazil

ARTICLE INFO

Article history:
Received 29 January 2012
Received in revised form
28 April 2012
Accepted 30 April 2012
Available online 31 May 2012

Keywords:
Bovine papillomavirus
Electrochemical DNA biosensor
Screen-printed electrode

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.

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

* Corresponding author. Tel.: +55 81 2126 8484; fax: +55 81 2126 8485.
E-mail address: galvesn23@gmail.com (G.A. Nascimento).

0956-5663/$ - see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bios.2012.04.052

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