Analytica Chimica Acta 804 (2013) 258–263

Contents lists available at ScienceDirect

Analytica Chimica Acta

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

Electrochemical DNA biosensor for human papillomavirus 16 detection in real samples

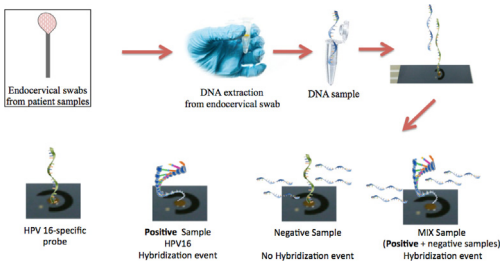
Danielly S. Campos-Ferreira[a,*], Gustavo A. Nascimento[a], Elaine V.M. Souza[a,c], Maria A. Souto-Maior[a], Mariana S. Arruda[a], Deborah M.L. Zanforlin[a], Marek H.F. Ekert[a], Danyelly Bruneska[a,b], José L. Lima-Filho[a,b]

[a] Laboratório de Imunopatologia Keizo Asami (LIKA), Universidade Federal de Pernambuco – UFPE, Av. Prof. Moraes s/n, 50670-901 Recife, PE, Brazil
[b] Departamento de Bioquímica, Universidade Federal de Pernambuco – UFPE, Av. Professor Moraes Rego s/n, 50670-901 Recife, PE, Brazil
[c] Departamento de–Enfermagem, Universidade Federal de Alagoas–UFAL, Campus Arapiraca, Av. Manoel Severino Barbosa, s/n, 57309-005, Bom Sucesso, Arapiraca, AL, Brazil

HIGHLIGHTS

- Electrochemical biosensor was used to detecting human papillomavirus (HPV) in real samples from endocervical swabs.
- The detection limit was of 18.13 nM.
- No hybridization with non-complementary sequence showed that the method is selective.
- It can be an excellent approach to detect human papillomavirus (HPV) in real samples.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 21 March 2013
Received in revised form 8 October 2013
Accepted 16 October 2013
Available online 25 October 2013

Keywords:
Electrochemical DNA biosensor
Human papillomavirus 16
Methylene blue
Screen-printed electrode

ABSTRACT

An electrochemical DNA biosensor for human papillomavirus (HPV) 16 detection has been developed. For this proposed biosensor, L-cysteine was first electrodeposited on the gold electrode surface to form L-cysteine film (CYSFILM). Subsequently, HPV16-specific probe was immobilized on the electrode surface with CYSFILM. Electrochemistry measurement was studied by differential pulse voltammetry method (DPV). The measurement was based on the reduction signals of methylene blue (MB) before and after hybridization either between probe and synthetic target or extracted DNA from clinical samples. The effect of probe concentration was analyzed and the best results were seen at 1000 nM. The hybridization detection presented high sensitivity and broad linear response to the synthetic-target concentration comprised between 18.75 nM and 250 nM as well as to a detection limit of 18.13 nM. The performance of this biosensor was also investigated by checking probe-modified electrode hybridization with extracted DNA from samples. The results showed that the biosensor was successfully developed and exhibited high sensitivity and satisfactory selectivity to HPV16. These results allow for the possibility of developing a new portable detection system for HPVs and for providing help in making an effective diagnosis in the early stages of infection.

1. Introduction

Epidemiological studies showed that infection with high-risk types (HR) of human papillomavirus (HPV), with large predominance of the genotype 16, is associated with nearly 100% of cases of cervical cancer and with a significant number of vaginal, vulvar, penile, and anal cancers [1,2].

* Corresponding author. Tel.: +55 81 2126 8484; fax: +55 81 2126 8000.
E-mail addresses: daniellylsantos@hotmail.com, daniellylsantos@gmail.com (D.S. Campos-Ferreira).

0003-2670/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.aca.2013.10.038

The oncogenic properties of HR-HPVs reside in the E6 and E7 genes [3]. Expression of these genes can lead to immortalization of keratinocytes, the natural host cells of HPV, suggesting that they significantly contribute to the oncogenic potential of HR-HPVs [4,5].

Currently, HPV diagnosis has been made through cytologic evaluation or visual inspection of the skin and tissues. These techniques have their own limitations, mainly because of poor specificity [6]. However, recent advances in molecular technologies have emerged for HPV detection, as well as the viral types identification. For instance, digene HC2 High-Risk HPV DNA, PapilloCheck®, COBAS® 4800 HPV, The Linear array®, CLART® human papillomavirus 2, INNO-LiPA, Clinical arrays® HPV and others are tests that have shown an excellent sensitivity for the detection of precancerous lesions of the cervix [7,8]. On the other hand, many of these tests do not distinguish individual HPV types, require PCR products, the cost is still relatively high, and require specific apparatus [7,8]. Thus, the development of a miniaturized, rapid, free-PCR and affordable test for HPV DNA makes this a viable alternative compared to current diagnostics for HPV [8–10].

DNA biosensors are analytical devices that are designed to detect a specific sequence of DNA (target) by hybridization with complementary probes immobilized on a solid substrate [11]. Among the different methods that have been used to detect hybridization, the electrochemical methods are more advantageous due to their portability, cost effectiveness, small size, and ease of use [12–14].

Many amino acids polymers have unique properties, which make them excellent platforms for the immobilization of biomolecules on electrodes. They have functional groups that can bind with DNA molecules, which allow for a better performance in the immobilization procedure [15]. The amino acid L-cysteine is a very promising surface modifier. It possesses a thiol group, which can bond strongly to silver or gold electrodes, carboxylic and amino groups, which can interact with several biological molecules, like DNA [16]. Thus, it is ideal to investigate biological systems in an electrochemical environment.

Due to the high rates of prevalence and the difficulties involved in making an effective diagnosis in the early stages of infection, the aim of this study was to develop a platform for the differential diagnosis of HPV16.

2. Materials and methods

2.1. Reagents and materials

All reagents were of analytical purity grade and all solutions were prepared using ultra pure water purchased from Invitrogen (USA). Methylene blue (MB) and L-cysteine were purchased from Sigma–Aldrich (USA). All single-stranded DNA (ssDNA) oligonucleotides were synthesized by Intregrated DNA Technologies (USA). Screen-printed electrodes were purchased from The Gwent Group (UK) for electrochemical detection.

2.2. Aparatus

The electrochemical analysis was carried out with a potentiostat (Autolab PGSTAT) equipped with GPES (4.0.007) software. The voltammetric experiments were performed on a three-electrodes system, consisting of a working, gold electrode (surface area of approximately 3 mm^2), a carbon auxiliary electrode and an Ag/AgCl reference electrode. All electrodes in the above-mentioned system were screen-printed. All hybridization experiments were carried out in hybridization Oven/Shaker (Amersham Pharmacia Biotech).

2.3. Human papillomavirus oligonucleotides

All oligonucleotides (as lyophilized powder) were diluted with ultrapure water and stored as a stock solution in a freezer. The stock solutions of oligonucleotides were diluted in 0.1 M phosphate-buffered saline (PBS) pH 7.4. The following three oligonucleotides sequences were used in this study:

HPV16 probe: 5′-ATG CAC CAA AAG AGA ACT GCA AT-3′
HPV16 target: 5′-AT TGC AGT TCT CTT TTG GTG CAT-3′
Non-complementary DNA: 5′-GTT CCG CCA CGG CAT CAC C-3′.

2.4. DNA Extraction from endocervical swab patient samples, PCR assay and HPV genotyping

Endocervical swabs from 10 patients were collected from Unidade de saúde da família de Jardim Fragoso at Olinda, Brazil. The genomic DNA was extracted from swabs by Wizard Genomic DNA Purification kit (Promega – USA) following the manufacturer's instructions. The genomic DNA extraction was amplified with the primer pair GP5+/GP6+ (forward 5′-TTTGTTACTGTGGTAGATACTAC-3′ and reverse5′-GAAAAATAAACTGTAAATCATATTC-3′). The GP5+/GP6+ primer pair amplifies a fragment of 150 bp from L1 gene.

The 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 ultra pure 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 visualized by electrophoresis in a 2% agarose gel containing ethidium bromide (1 mg mL^{-1}) and TBE buffer pH 8.4 (89 mM Tris, 89 mM boric acid and 2 mM EDTA) under UV light.

The extracted DNA from endocervical swabs was also used to identify HPV types. HPV genotyping was carried out by PapilloCheck® assay (Greiner Bio-one, Germany) according to manufacturer's recommendations.

2.5. Preparation of the modified electrodes

The screen-printed electrode was washed thoroughly with ultra-pure water. Before modification, the bare electrode was scanned in 0.5 M H_2SO_4 between 0.5 and 1.5 V at 50 mV s^{-1} until a reproducible cyclic voltammogram (CV) was obtained.

The cysteine film was used to allow the immobilization of the DNA probe onto the working electrode. The L-cysteine solution (10 mM) was prepared in 0.1 M PBS pH 7.4. For preparation of the cysteine film, the L-cysteine solution was electrodeposited on the working electrode by cyclic potential scanning from −0.2 to 1.5 V for 15 cycles with a scan rate of 50 mV s^{-1}.

2.6. Probe DNA immobilization

The HPV16 probe was immobilized by adsorption for 30 min at 30 °C on the gold electrode surface that was modified with the cysteine film. Then, the unbound oligonucleotides were washed out from the working electrode with 0.1 M PBS.

2.7. DNA hybridization

The solution containing the HPV16 target was added onto a working electrode with the immobilized HPV16 probe and incubated at 55 °C for 10 min, at a stirring speed of 300 rpm to link the complementary sequence. The Tris–HCl buffer (20 mM, pH 7.0) was used to wash the electrode and remove the non-hybridized

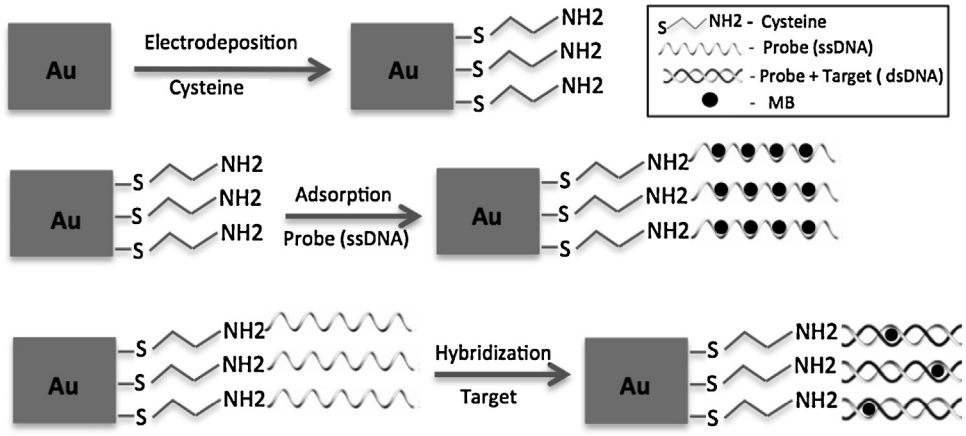


Fig. 1. Schematic drawing of the stepwise DNA biosensor fabrication process.

sequences. The same procedure was applied for interaction of HPV16 probe with non-complementary target.

For the hybridization of the genomic DNA with HPV16 probe on the modified working electrode, the extracted DNA was denatured by heating in a water bath (95 °C) for 5 min and was immediately chilled in ice to obtain denatured ss-DNA. Then, it was added to the modified working electrode surface. The formation of hybridization between the extracted DNA and HPV16 probe was also performed at 55 °C for 10 min at a stirring speed of 300 rpm. The electrode was washed with Tris–HCl buffer (20 mM, pH 7.0) to remove the non-hybridized sequences.

2.8. Electrochemical analysis

The differential pulse voltammetry (DPV) method was used for the electrochemical signal analysis. After the immobilization and hybridization process, 500 μM MB solution in Tris–HCl buffer (20 mM, pH 7.0) was added onto modified working electrode for 5 min and then washed with the Tris–HCl buffer. The DPV measurement of the MB electrochemical reduction was performed in the Tris–HCl buffer, under the following conditions: a potential sweep between −0.6 and 0 V with a scan rate of 20 mV s^{-1}.

3. Results and discussion

3.1. Preliminary investigations

The schematic representation of the fabrication procedure of DNA biosensor is shown in Fig. 1. The electrodeposited L-cysteine film (CYSFILM) was employed as the platforms for the probe immobilization onto gold working electrode surface. The L-cysteine amino acid was chosen because of the special functional end groups, such as —SH, —COOH and —NH_2 [17]. The electrodeposited L-cysteine film was formed by cyclic voltammetry (CV) technique. Electrochemical deposition has been reported as an effective method for immobilization of biomolecules when compared to the traditional process, the self-assembled monolayers method (SAMs) [18].

The ssDNA probe immobilization was achieved by adsorption method. Adsorption is the simplest immobilization method because it does not require special reagents or any nucleic acid modification [19,20]. In this work, the immobilization was based on ionic interactions occurring between the negatively charged phosphate groups of the DNA and the positively charged amine, present in the L-cysteine that covers the surface electrode [20,21].

The essence of the electrochemical DNA detection assay was based either on the electrochemical DPV of ssDNA or double-stranded DNA (dsDNA) using the reduction signals of MB. The MB is covalently attached to DNA and provides a sensitive redox reporter in DNA electrochemistry measurement. This is an aromatic heterocycle molecule that is often employed toward selective discrimination of ssDNA and dsDNA [22].

3.2. Characterization of the probe-modified electrode

The purpose of this experiment was to observe the behavior of probe concentration on the immobilization at the CYSFILM gold electrode through MB electrochemical reduction. The effect of probe concentration is shown in Fig. 2 and was carried out by DPV technique. The results showed that the concentration of 1000 nM had the highest current peaks. The lowest probe concentrations (250 nM and 500 nM) showed lower current signal due to a low amount of immobilized probe on the working electrode surface. The concentration of 2000 nM also showed lower current signal, however, in this case it can be explained by the massive probe accumulation on the electrode that causes probe overlapping [23], resulting in lower availability of the guanine bases and, consequently, a lower availability of MB electrochemical reduction

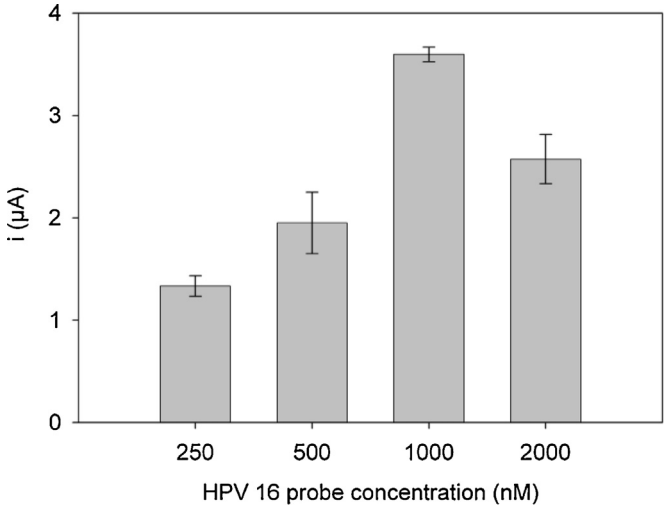


Fig. 2. Histogram of HPV16 probe concentration effect on the MB electrochemical reduction during the immobilization process. The differential pulse voltammetry (DPV) method was used to analyze the current signal in the following conditions: initial potential −0.7 V, end potential 0 V, modulation amplitude 50 mV and scan rate 20 mV s^{-1}. All results plotted were done in triplicates to different HPV16 probe concentrations.

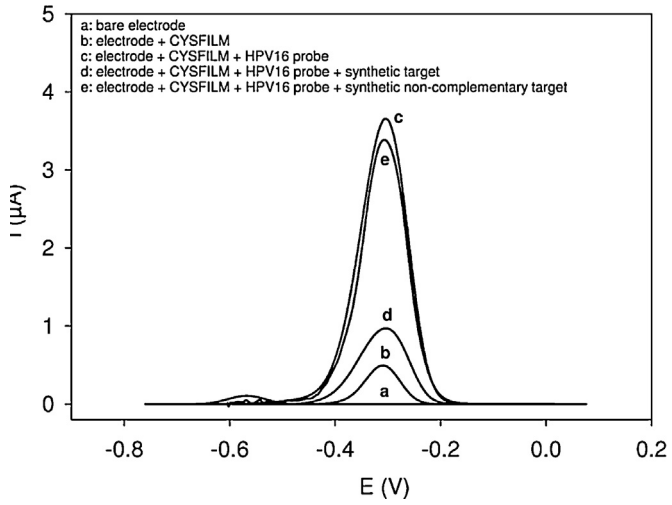


Fig. 3. The differential pulse voltammograms for the MB electrochemical reduction of (a) bare electrode, (b) modified L-cysteine film-gold electrode, (c) 1000 nM HPV16 probe immobilized on modified L-cysteine film-gold electrode before hybridization, (d) 1000 nM HPV16 probe immobilized on modified L-cysteine film-gold electrode after hybridization with 1000 nM HPV synthetic target and (e) 1000 nM HPV16 probe immobilized on modified L-cysteine film-gold electrode after hybridization with 1000 nM synthetic non-complementary target.

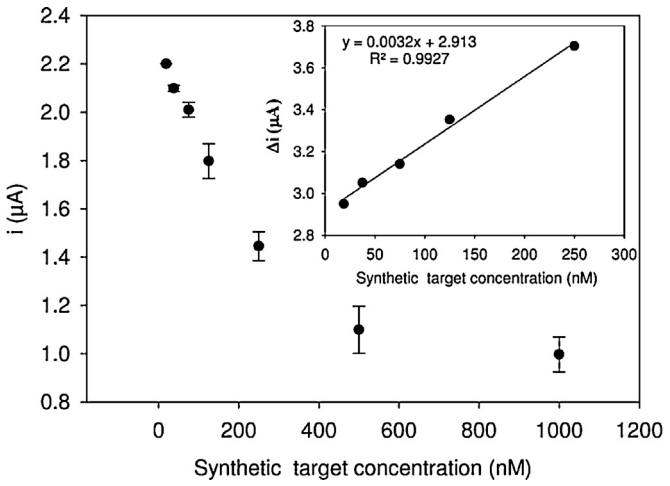


Fig. 4. The effect of the HPV16 target concentration on the MB electrochemical reduction during hybridization. Inset: Linear regression from difference between the DPV signals of MB on the probe modified-electrode in the presence and absence of target (Δi). The differential pulse voltametry (DPV) method was used to analyze the current signal in the following conditions: initial potential −0.7 V, end potential 0 V, modulation amplitude 50 mV and scan rate 20 mV s^{-1}. All results plotted were done in triplicates to different HPV16 target concentration.

[24,25]. Thus, the probe concentration of 1000 nM was selected for electrode modification.

3.3. Detection of hybridization for synthetic oligonucleotides

Detection of target DNA was monitored by means of DPV responses to MB electrochemical reduction on the probe modified-electrode in the absence and presence of target. Fig. 3 shows the DPV voltammograms for MB electrochemical reduction at bare gold electrode (a), CYSFILM electrode (b), probe-modified electrode before hybridization (c), probe-modified electrode after hybridization with synthetic complementary target (d) and probe-modified electrode after hybridization with synthetic non-complementary target (e).

As seen in Fig. 3 it was observed that the probe-modified electrode showed the highest current peak when compared to the other curves. This increase is attributed to strong affinity between MB and free guanine that are present in HPV16 probe. After hybridization of the probe-modified electrode with complementary target, the current signal of MB was decreased. This can be explained due to less MB accumulation on the ds-DNA caused by the inaccessibility of MB to the guanine bases [11,26] or may be due to a steric inhibition of the reducible groups of MB packed between the bulky double helix of the DNA hybrids [15,23,27,28]. Therefore, it is concluded that the decrease in the MB signal represents the extent of the hybridization at the electrode surface. However, the interaction between non-complementary target and probe-modified electrode did not lead to MB signal decrease, due to negligible hybridization. This result indicates that only a complementary target could hybridize on the probe modified-electrode and cause a significant decrease in the accumulation of MB.

3.4. Optimization of hybridization for synthetic oligonucleotides

To identify the conditions for optimal analytical performance of the biosensor proposed, the MB electrochemical reduction was measured after hybridization with synthetic target oligonucleotides at different concentrations (18.75–1000 nM).

Fig. 4 shows that the current signal decreases with the increase of target concentration of up to 250 nM and then it stabilizes at 1000 nM, indicating that all the available probe immobilized on the electrode surface have become involved in hybridization.

As seen on the inset of Fig. 4, the linear regression was obtained from the difference between the DPV signals of MB on the probe modified-electrode in the presence and absence of target (Δi). The calibration curve ($y = 0.0032x + 2.913$) was linear between 18.75 nM and 250 nM, with correlation coefficient of 0.99272. A detection limit of 18.13 nM could be estimated by equation $3\sigma/a$, where σ was the standard deviation of intercept and a was the line slope [15,29]. The relative standard deviation (RSD) over three independently probe modified-electrodes was 0.023% that was measured at 18.75 nM of the HPV16 target, which indicated a remarkable reproducibility of the detection method.

3.5. HPV sample analysis

In order to confirm of the presence of HPV in the extracted DNA from clinical samples, the PCR was carried out using consensus primers, directed at relatively conserved regions of the HPV genome. GP5+/GP6+ primer set, mediated by PCR is the most frequently used amplification system for the detection of HPV-DNA in clinical samples, amplifying DNA fragments in the conserved L1 region of approximately 150 bp [30–32]. In Fig. 5 it is possible to observe two amplification products of 150 bp in the electrophoresis gel that correspond to the HPV L1 gene. These results only confirm the presence of HPV-DNA in the sample.

After HPV detection by PCR, these extracted DNA were used to identify HPV types. The goal of this assay was also to compare the efficiency of the HPV type identification by biosensor proposed. The genotype results obtained by PapilloCheck® showed that the samples ESD1 (extracted sample DNA 1) and ESD2 (extracted sample DNA 2) were positive HPVs for 16 and 45, respectively (data not shown). This is a relatively novel test for the detection of HPV. This assay is a broad-spectrum PCR-based method that uses a consensus primer set that targets the E1 region of HPV DNA and that allows the simultaneous detection and genotyping of 24 different HPV types by DNA chip technology [30,33].

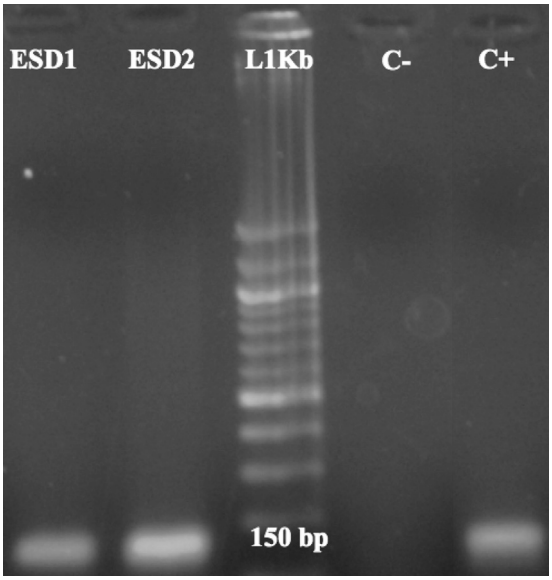


Fig. 5. The amplification of HPV L1 gene with the primer pair GP5+/GP6+ from DNA that was extracted from clinical samples. Lane ESD1: extracted sample DNA 1; Lane ESD2: extracted sample DNA 2; Lane L1Kb: 1 kb Plus DNA ladder (Invitrogen): Lane C−: negative control (ultrapure water) and Lane C+: positive control for the HPVL1 gene in pBR322 – HPV16 plasmid.

3.6. Performance of electrochemical DNA biosensor using real samples of HPV

The performance of this electrochemical DNA biosensor has been investigated by checking probe-modified electrode hybridization with extracted DNA from samples.

In this assay, we used the extracted sample DNA directly on the probe-modified electrode unlike other works that used PCR products [34,35]. Fig. 6 shows a DPV response of biosensor to positive sample of HPV16, positive sample of HPV45 (non-complementary sample) and mix-sample (HPV16 and HPV45). It is possible to observe that in the presence of positive sample of HPV16 the current peaks were decreased, which confirmed the occurrence

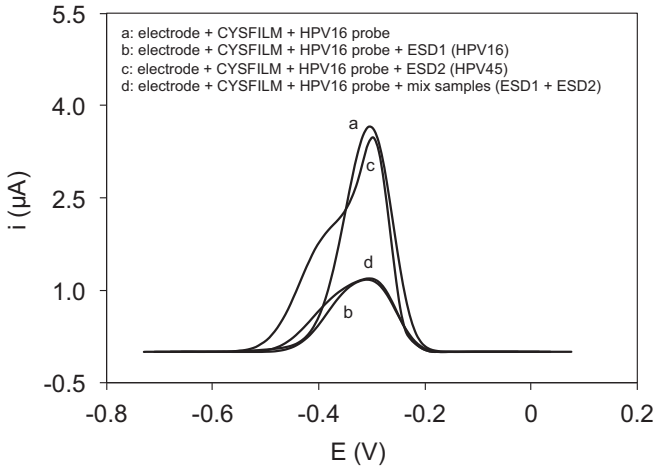


Fig. 6. The differential pulse voltammograms for the MB electrochemical reduction of (a) 1000 nM HPV16 probe immobilized on modified L-cysteine film-gold electrode before hybridization, (b) 1000 nM HPV16 probe immobilized on modified L-cysteine film-gold electrode after hybridization with 1000 nM ESD1 (HPV16); (c) 1000 nM HPV16 probe immobilized on modified L-cysteine film-gold electrode after hybridization with 1000 nM ESD2 (non-complementary target–HPV45) (d) 1000 nM HPV16 probe immobilized on modified L-cysteine film-gold electrode after hybridization with 1000 nM mix samples (ESD1 + ESD2).

of hybridization in the proposed detection system. It was also observed that the biosensor detects the biological samples within the concentration range shown in Fig. 4. Commercial methods, such as PapilloCheck®, also detect low concentrations of DNA, however, using PCR products [8]. Clinically, HPV diagnosis in this concentration range is a technological breakthrough when compared to miniaturized devices like biosensors.

The specificity of the biosensor was studied by the incubation of the non-complementary sample and mix-sample. Fig. 6 shows that the current peak after hybridization of the probe modified-electrode with non-complementary sample increased when compared to the hybrid formed. On the other hand, the interaction between the mix-sample and probe modified-electrode lead to a significant decrease in the current peak, indicating that there is hybridization when the HPV16 is present in the sample.

Another aspect observed in Fig. 6 was the presence of a poorly defined shoulder around −0.4 V, likely due to the chemical degradation of MB. This electrochemical behavior was a sporadic event that occurred only in the hybridization process of the probe modified-electrode with non-complementary sample (curve c). Eventually, several redox compounds, like methylene blue, can undergo irreversible electrochemical degradations and form chemical species that are represented by shoulders in voltammograms [36–41].

These data demonstrated that this electrochemical DNA biosensor able to detect the HPV16 presence in samples and non-complementary samples did not interfere in the specificity of the biosensor.

4. Conclusions

This paper reports the development of an electrochemical DNA biosensor for the detection of one of the most representative high-risk HPV types, the HPV16. The performance of the biosensor was checked using synthetic oligonucleotides and extracted DNA from the cervical swab of a confirmed positive patient. The biosensor was able to detect HPV16 using extracted DNA directly on the modified transducer unlike other works that used PCR products. Thus, results indicate that this electrochemical DNA biosensor is specific to HPV16 and it can be used to distinguish from other HPV types. With these data, it is possible to develop a new portable free-PCR detection system for HPVs, as well as to contribute to an effective diagnosis in the early stages of infection.

Acknowledgements

We are grateful for the financial support from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Unidade de saúde da família de Jardim Fragoso, Prefeitura de Olinda and Laboratório de Imunopatologia Keizo Asami (LIKA).

References

[1] C. Annunziata, L. Buonaguro, F.M. Buonaguro, M.L. Tornesello, Pathol. Oncol. Res. 18 (2012) 803–808.
[2] D.A. Machalek, M. Poynten, F. Jin, C.K. Fairley, A. Farnsworth, S.M. Garland, R.J. Hillman, K. Petoumenos, J. Roberts, S.N. Tabrizi, D.J. Templeton, A.E. Grulich, Lancet Oncol. 2045 (2012) 1–14.
[3] E. Tsimplaki, E. Argyri, L. Michala, M. Kouvousi, A. Apostolaki, G. Magiakos, I. Papassideri, E. Panotopoulou, J. Oncol. 2012 (2012) 1–7.
[4] S.S. Tungteakkhun, P.J. Duerksen-Hughes, Arch. Virol. 153 (2008) 397–408.
[5] C.A. Moody, L.A. Laimins, Nat. Rev. Cancer 10 (2010) 550–560.
[6] S.J. Hwang, K.R. Shroyer, J. Oncol. 2012 (2012) 1–9.
[7] M.-N. Didelot, N. Boulle, A. Damay, V. Costes, M. Segondy, J. Med. Virol. 83 (2011) 1377–1382.
[8] A.L.P. Abreu, R.P. Souza, F. Gimenes, M.E.L. Consolaro, J. Virol. 9 (2012) 1–9.
[9] N. Bhatla, N. Moda, Indian J. Med. Res. 130 (2009) 261–265.
[10] E.J. Cho, J.H. Do, Y.S. Kim, S. Bae, W.S. Ahn, J. Med. Microbiol. 60 (2011) 162–171.
[11] J.P. Tosar, G. Brañas, J. Laíz, Biosens. Bioelectron. 26 (2010) 1205–1217.

[12] B. Bohunicky, S.A. Mousa, Nanotechnol. Sci. Appl. 4 (2010) 1–10.
[13] B.J. Privett, J.H. Shin, M.H. Schoenfisch, Anal. Chem. 82 (2010) 4723–4741.
[14] N.J. Ronkainen, H.B. Halsall, W.R. Heineman, Chem. Soc. Rev. 39 (2010) 1747–1763.
[15] G.A. Nascimento, E.V.M. Souza, D.S. Campos-Ferreira, M.S. Arruda, C.H.M. Castelletti, M.S.O. Wanderley, M.H.F. Ekert, D. Bruneska, J.L. Lima-Filho, Biosens. Bioelectron. 38 (2012) 61–66.
[16] L.C. Umuhumuza, X. Sun, Eur. Food Res. Technol. 232 (2010) 425–431.
[17] W. Wang, J. Biophys. Chem. 03 (2012) 39–43.
[18] H. Niu, R. Yuan, Y. Chai, L. Mao, Y. Yuan, Y. Zhuo, S. Yuan, X. Yang, Biosens. Bioelectron. 26 (2011) 3175–3180.
[19] M.I. Pividori, A. Merkoçi, S. Alegret, Biosens. Bioelectron. 15 (2000) 291–303.
[20] A. Sassolas, B.D. Leca-Bouvier, L.J. Blum, Chem. Rev. 108 (2008) 109–139.
[21] R. Beutner, J. Michael, B. Schwenzer, D. Scharnweber, J.R. Soc. Interface 7 (Suppl. 1) (2010) S93–S105.
[22] D. Pan, X. Zuo, Y. Wan, L. Wang, J. Zhang, S. Song, C. Fan, Sensors 7 (2007) 2671–2680.
[23] M.H. Pournaghi-Azar, M.S. Hejazi, E. Alipour, Anal. Chim. Acta 570 (2006) 144–150.
[24] M. Tichoniuk, M. Ligaj, M. Filipiak, Sensors 8 (2008) 2118–2135.
[25] M. Ortiz, A. Fragoso, P.J. Ortiz, C.K. O'Sullivan, Photochem. Photobiol. J. A 218 (2011) 26–32.
[26] A. Erdem, K. Kerman, B. Meric, M. Ozsoz, Electroanalysis 13 (2001) 219–223.
[27] B. Meric, K. Kerman, D. Ozkan, P. Kara, S. Erensoy, U.S. Akarca, M. Mascini, M. Ozsoz, Talanta 56 (2002) 837–846.
[28] L.D. Tran, B.H. Nguyen, N. Van Hieu, H.V. Tran, H. Le Nguyen, P.X. Nguyen, Mater. Sci. Eng. C 31 (2011) 477–485.
[29] M. Gumustas, S. Ozkan, Open Anal. Chem. J. 5 (2011) 1–21.
[30] A.T. Hesselink, D.A.M. Heideman, J. Berkhof, F. Topal, R.P. Pol, C.J.L.M. Meijer, P.J.F. Snijders, J. Clin. Microbiol. 48 (2010) 797–801.
[31] M.G. De Lima Rocha, F.L. Faria, L. Gonçalves, M.D.C.M. Souza, P.Á. Fernandes, A.P. Fernandes, PLoS One 7 (2012) e40988.
[32] A. Asiaf, S.T. Ahmad, M.A. Zargar, S.M. Mufti, S.H. Mir, Genet. Test. Mol. Biomarkers 16 (2012) 904–909.
[33] J. Jones, N.G. Powell, A. Tristram, a N. Fiander, S. Hibbitts, J. Clin. Virol. 45 (2009) 100–104.
[34] H. Gao, X. Qi, Y. Chen, W. Sun, Anal. Chim. Acta 704 (2011) 133–138.
[35] G. Wu, N. Yang, T. Zhang, Z. Wang, X. Lu, J. Kang, Sens. Actuators B. 160 (2011) 598–603.
[36] T. Omata, K. Ono, S.O. Yao-Matsuo, Mater. Trans. 44 (2003) 1620–1623.
[37] M. Melle-Franco, M. Marcaccio, D. Paolucci, F. Paolucci, V. Georgakilas, D.M. Guldi, M. Prato, F. Zerbetto, Am. Chem. Soc. J. 126 (2004) 1646–1647.
[38] M. Panizza, a Barbucci, R. Ricotti, G. Cerisola, Sep. Purif. Technol. 54 (2007) 382–387.
[39] D.H. Evans, Chem. Rev. 108 (2008) 2113–2144.
[40] F. Manea, A. Pop, C. Radovan, P. Malchev, A. Bebeselea, G. Burtica, S. Picken, J. Schoonman, Sensors 8 (2008) 5806–5819.
[41] F. Scholz, Thermodynamics of electrochemical reactions, in: F. Scholz (Ed.), Electroanalytical Methods - Guide to Experiments and Applications, Springer, 2010, pp. 11–32.