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Short communication

Electrochemical biosensor for the multiplexed detection of human papillomavirus genes

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ABSTRACT

A proof-of-concept of an electrochemical genosensor array for the individual and simultaneous detection of two high-risk human papillomavirus DNA sequences, HPV16E7p and HPV45E6 that exhibits high sensitivity and selectivity is reported. The optimum conditions for surface chemistry preparation and detection of hybridised target were investigated. The LOD obtained are in the pM range, which are sufficient for most real RNA/DNA samples obtained from PCR amplification, usually in the nanomolar range. In a multiplexed detection format, high selectivity was observed over the non-specific sequence, opening the way for the development of an electrochemical high throughput screening assay for multiple high-risk DNA sequences.

1. Introduction

The human papillomavirus (HPV) is one of the most common sexually transmitted infections, affecting the skin and mucous membranes. There are more than 40 HPV types that can infect the genital areas of men and women (Muñoz et al., 2003). HPV has been detected in virtually all invasive cervical cancers and has been confirmed as the major cause of this cancer. It is believed that most cervical cancers develop when various aggressive genetic HPV strains activate certain oncogenes. Oncogenes E6 and E7 are particularly important because they interfere with protective proteins p53 and pRb, respectively. These proteins limit cell growth under normal conditions, but once they are blocked, cell growth is not controlled, resulting in tumour development and cancer (Liu et al., 2006; Nomine et al., 2006).

Owing to the difficulties to perform serological assays and HPV cultures efficiently, some tools based on molecular recognition have been developed for the diagnosis of HPV infections. At the basis of molecular recognition, the detection of HPV DNA are in use, based on the extraction of genomic DNA from clinical samples with posterior PCR amplification and detection (Klaes et al., 1999; Nagao et al., 2002). However due to the high mutation rates of viruses, detection by PCR is complicated.

Another, less explored, possibility for HPV detection is the use of electrochemical biosensors with amperometric (labelled) and/or impedimetric (label-free) detection. Electrochemical biosensors have received considerable attention regarding the detection of DNA hybridisation due to the advantages of low cost, simplicity, high sensitivity, compatibility with mass manufacturing, possibility of microfabrication technologies and portability, making them excellent candidates for point-of-care DNA diagnostics. Electrochemical detection of HPV related sequences has been reported in the past by using methylene-blue as hybridisation indicator (Sabzi et al., 2008) or secondary probes labelled with ferrocene (Vernon et al., 2003). In the first case, a 20-mer probe sequence was adsorbed on the surface of a graphite electrode and used for the detection of a 20-mer target related to L1 gene of identical length by recording the variations in methylene-blue response before and after target recognition, achieving a limit of detection of 1.2 ng/μL (0.5 nM). The other example involved the use of a bioelectronic DNA detection platform formerly commercialised as eSensor™, for the detection of HPV sequences based on thiolated probes immobilised on the chip surface. After target immobilisation, a ferrocene-labelled probe was hybridised and the current response was measured. These chips were able to detect 86% of the HPV targets contained in clinical samples using a positive/negative type response. In a more recent report, detection of HPV was carried out by treating a captured dsDNA duplex with acid and directly measuring the released

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purine bases by square wave voltammetry (Zari et al., 2009). In the present work, we report an electrochemical sensor microarray based on DNA detection for the individual and simultaneous detection of specific high-risk HPV sequences, more specifically HPV16 and 45 and analytical parameters such as sensitivity, specificity and reproducibility have been studied.

2. Materials and methods

2.1. Electrochemical instrumentation

All electrochemical measurements were performed with a PGSTAT 12 potentiostat (Autolab, The Netherlands) controlled with the General Purpose Electrochemical System (GPES) software and equipped with a MUX module (Eco Chemie B.V., The Netherlands).

The electrode array consisted of a 16 gold working electrodes arranged in a four by four distribution on a borosilicate glass chip measuring 21 mm × 23 mm. Each working electrode (1 mm × 1 mm) was placed between a silver pseudo reference (0.2 mm × 1 mm) and a gold counter electrode of the same size in order to create 16 planar electrochemical cells. The electrode array was integrated within a microfluidic cell. The microfluidic channels were realised by mounting an electrode array onto a polycarbonate fluidic chip using double-sided medical grade adhesive foil of 50 μm thickness, which was previously laser machined to generate microchannel structures of 1 mm width. Connection of the assembled chip was realised via pogo-pin connectors to each of the 18 electrodes (16 working electrodes and 1 plus 1 reference and counter electrodes).

2.2. Materials

Dithiol 1 (DT1, 10-(3,5-bis((6-mercaptohexyl)oxy)phenyl)-3,6,9-trioxadecanol, CAS number 936115-52-5) was purchased from SensoPath Technologies (Bozeman, NT). Phosphate buffered saline with Tween 20 (pH 7.4) and 3,3′,5,5′-tetramethylbenzidine (TMB) liquid substrate system were from Sigma–Aldrich (Barcelona, Spain). Potassium dihydrogen phosphate and sodium hydroxide were purchased by Scharlau (Barcelona, Spain). All solutions were prepared with MilliQ water (18 MΩ) produced with a MilliQ RG system (Millipore Ibérica, Madrid, Spain).

Synthetic oligonucleotides were purchased from Biomers.net (Ulm, Germany). Sequences for HPV16E7p are listed below.

HPV16E7p thiolated capture probe (24-mer sequence): 5′-thiol C_6-GAG GAG GAG GAT GAA ATA GAT GGT-3′.

HPV16E7p HRP-labelled reporter probe (21-mer sequence): 5′-TTG GAA GAC CTG TTA ATG GGC-HRP-3′.

HPV16E7p target (159-mer sequence): 5′-GCC CAT TAA CAG GTC TTC CAA AGT ACG AAT GTC TAC GTG TGT GCT TTG TAC GCA CAA CCG AAG CGT AGA GTC ACA CTT GCA ACA AAA GGT TAC AAT ATT GTA ATG GGC TCT GTC CGG TTC TGC TTG TCC AGC TGG ACC ATC TAT TTC ATC CTC CTC CTC-3′.

2.3. Probe immobilisation on electrode array

Prior to modification, electrode arrays were cleaned following a two steps protocol. First, in order to remove the protective resist used during storage, the arrays were sequentially sonicated for 5 min in acetone and isopropanol and rinsed with water. In a second step, each electrode array was electrochemically cleaned in 0.5 M H_2SO_4 by application of a constant potential of 1.6 V for 10 s followed by 40 voltammetric cycles in the potential range −0.2 to 1.6 V at a scan rate of 0.5 V s^{-1}. Finally, electrodes were rinsed with MilliQ water and dried with nitrogen. The cleaned electrode arrays were modified via co-immobilisation of the thiolated probe (1 μM) and the backfiller DT1 (100 μM) in 1 M KH_2PO_4 aqueous solution (pH 3.5) for 3 h at room temperature under humid environment (minimum 90%). The electrode arrays were then washed in a stirring solution of PBS-Tween for 15 min, rinsed with water and dried with nitrogen.

2.4. DNA detection

DNA detection of both synthetic oligonucleotides (HPV16E7p and HPV45E6) was performed using a so-called sandwich type format (Fig. 1). First, 0.5 μL of HPV target of various concentrations ranged of 0.1–50 nM in PBS-Tween were cast on each of the oligonucleotide modified gold electrodes and were incubated for 1 h at room temperature. The sensors were then washed for 15 min in a stirring solution of PBS-Tween and dried with nitrogen. The second hybridisation was subsequently performed by spotting 0.5 μL of 10 nM reporter probe in PBS-Tween and incubating for 1 h at room temperature. The hybridised microarray was washed with PBS-Tween for 15 min and dried in nitrogen. The hybridisations of the target and detection probe were performed under a humid environment (minimum 90%).

2.5. Electrochemical detection

Modified electrode arrays were assembled on the microfluidic cell to perform the electrochemical detection. The detection process was carried out in the created microfluidic channels in the presence of TMB substrate. The reduction of TMB was detected using a steps and sweeps technique by applying two consecutive steps at 0 V for 1 ms and −0.2 V for 0.5 s.

3. Results and discussion

The hybridisation behaviour was detected by incorporating a HRP-labelled reporter probe that pairs with the target to form a "sandwich" structure at the electrode surface (Fig. 1). In this work, TMB was used as a chromogenic substrate for HRP. TMB is commonly used in ELISA assays and is oxidised to form a blue coloured product, which is known to be a single oxidised TMB complexed with a neutral TMB molecule (Kim et al., 2009). The oxidised TMB

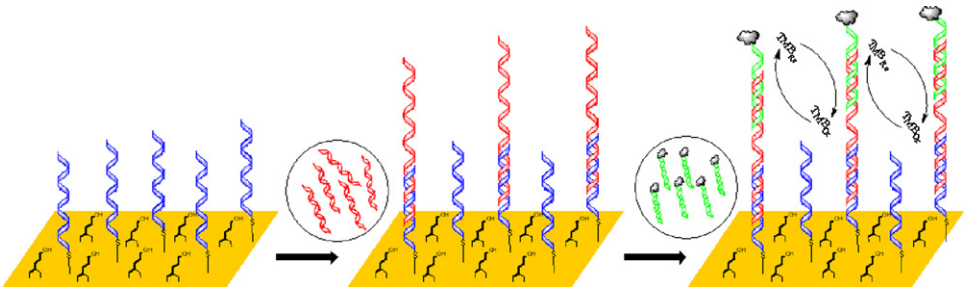

Fig. 1. Schematic description of the developed assay based on co-immobilisation of thiolated probe with backfiller, hybridisation process and electrochemical detection.

can be detected through its reduction at the electrode surface (Fanjul-Bolado et al., 2005).

3.1. Proof-of-concept of electrochemical detection of individual HPV sequences

The classic high-risk HPV types are 16 and 18 (Thomison Iii et al., 2008) although types 31 and 45 have also been found to be present in approximately the 80% of cases of cervical cancer together with types 16 and 18 (Stoler, 2000). For this reason HPV types 16 and 45 were chosen for this work.

To evaluate the analytical performance of the genosensor, two calibration curves were obtained using synthetic oligonucleotides of both specific high-risk HPV sequences. Fig. 2 shows the amperometric response of the reduction of oxidised TMB for a series of target concentrations ranging from 0.1 to 50 nM. First, a blank steps and sweep (SAS) measurement was carried out with PBS-Tween, followed by TMB measurement. The final amperometric response used for construction of the calibration curve resulted from the subtraction of the blank value from the TMB response. As can be seen, the signal tends to saturation for concentrations above 50 nM of target, as expected for the interaction of a solution analyte with an immobilised catching probe. Analysis of the data in terms of the Langmuir isotherm (Fragoso et al., 2008) afforded association constants of 2.3 and 0.17 nM^{-1} for HPV16E7p and HPV45E6, respectively. Assuming a linear behaviour at low target concentrations the electrochemical assays showed a sensitivity of $(0.15 \pm 0.02)\,\mu A\,nM^{-1}$ ($r^2 = 0.997$) in the range of 0.1–10 nM for HPV16E7p and $(1.02 \pm 0.09)\,\mu A\,nM^{-1}$ ($r^2 = 0.999$) in the range of 0.1–1 nM in the case of HPV45E6, with LOD of 490 and 110 pM, respectively. These differences in sensor parameters may be due to the fact that HPV16e7p amplicon is considerably longer (154-mer) than the HPV45E6 (78-mer) amplicon.

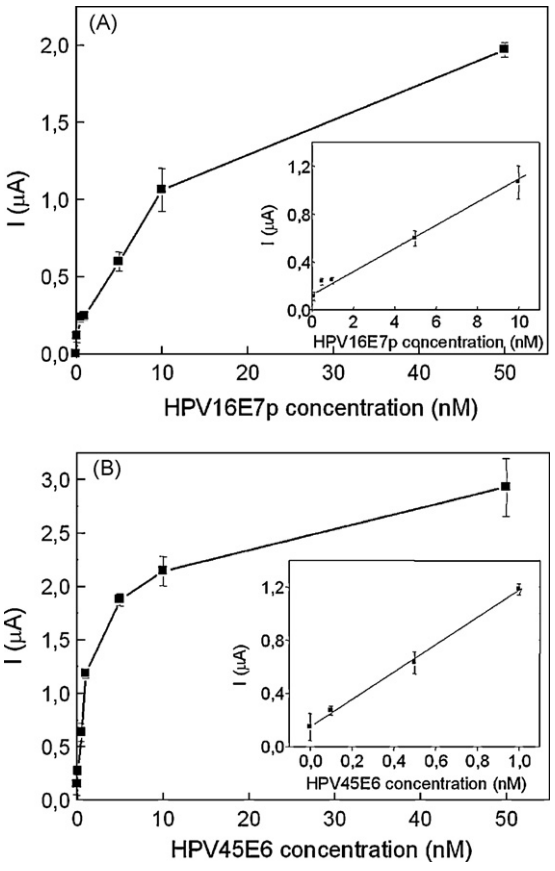

Fig. 2. Calibration curves obtained with: (A) HPV16E7p and (B) HPV45E6. Target concentration range: 0.1–50 nM.

3.2. Multiplexed detection of HPV sequences

Quantitative identification of biomarkers in a mixture without separation and at clinically relevant concentrations is a crucial requirement for the development of more effective and simpler diagnostic devices particularly when monitoring expression levels of disease associated RNA. Arrayed tests enable the use of pattern recognition approaches to assess disease changes, which is important in both diagnostics and monitoring. In the new and evolving paradigm of clinical diagnostics, the measurement of a single tumour marker does not give sufficient information for a complete clinical picture, and there is a growing requirement for low-density multiplex assays. The 16 gold working electrodes sensor array used in this work permits parallel detection of multiple targets.

For the simultaneous detection of several high-risk HPV sequences, a chip was modified with thiolated HPV16E7p and HPV45E6 probes in alternating electrode spots as depicted in Fig. 3.

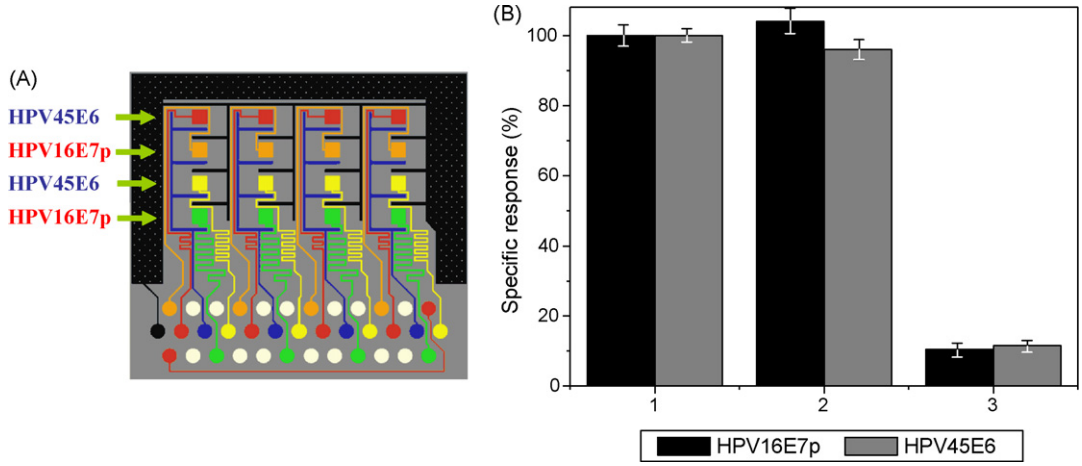

Fig. 3. (A) Modification map used for multiplexed electrochemical detection of HPV16E7p and HPV45E6. (B) Relative responses to the specific signal obtained for the simultaneous detection of HPV16E7p and HPV45E6: (1) with addition of 10 nM specific target; (2) with addition of a mixture of 10 nM specific + 100 nM non-specific targets, respectively; (3) with addition of 10 nM non-specific target.

Specific and non-specific targets (i.e. for a spot modified with thiolated HPV16E7p, specific target refers to HPV16E7p sequence and HPV45E6 refers to non-specific target) were then applied at a 10 nM concentration as well as a mixture of 10 nM specific and 100 nM non-specific target, respectively. The responses obtained ($n = 4$) are presented in Fig. 3 corresponding to different chips in order to test inter-array reproducibility. From the data obtained a high specificity of the sensor array was observed with negligible hybridisation signal with the non-specific target. The signal recovery upon addition of a high concentration of non-specific target (10 times) was $104 \pm 8\%$ for HPV45E6 and $96 \pm 5\%$ for HPV16E7p. These results demonstrate the applicability of the electrochemical genosensor array described here for multiplexed detection of DNA sequences. The application of this strategy for the detection of a panel of 18 HPV associated sequences is currently under evaluation.

4. Conclusions

This report demonstrates the proof-of-concept of an electrochemical genosensor array for the individual and simultaneous detection of two high-risk HPV types, HPV16E7p and HPV45E6 sequences that exhibits high sensitivity and selectivity. The optimum conditions for surface chemistry preparation and detection of hybridised target were investigated. The LOD obtained in the pM range are sufficient for most real RNA/DNA samples obtained from PCR amplification, usually in the nanomolar range. In a multiplexed detection format, high selectivity was observed over the non-specific sequence. Following this initial work, our attention is now focused on the introduction of additional high-risk HPV sequences in the multiplexed assay for the development of an electrochemical high throughput screening assay for multiple HPV markers.

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