



Flexible sensor based on conducting polymer and gold nanoparticles for electrochemical screening of HPV families in cervical specimens

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ABSTRACT

Considering the low sensitivity of cytological exams and high costs of the molecular methods, the development of diagnostic tests for effective diagnosis of HPV infections is a priority. In this work, biosensor composed of polypyrrole (PPy) films and gold nanoparticles (AuNPs) was obtained for specific detection of HPV genotypes. The biosensor was developed by using flexible electrodes based on polyethylene terephthalate (PET) strips coated with indium tin oxide (ITO). Polymeric films and AuNPs were obtained by electrosynthesis. Oligonucleotides sequences modified with functional amino groups were designed to recognize HPV gene families strictly. The modified oligonucleotides were chemically immobilized on the nanostructured platform. Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) were used for the analysis of the electrode modification and monitoring of molecular hybridization. Electrochemical changes were observed after exposure of the biosensors to plasmid samples and cervical specimens. The biosensor based on the BSH16 probe showed a linear concentration range for target HPV16 gene detection of $100 \text{ pg } \mu\text{L}^{-1}$ to $1 \text{ fg } \mu\text{L}^{-1}$. A limit of detection (LOD) of $0.89 \text{ pg } \mu\text{L}^{-1}$ and limit of quantification (LOQ) of $2.70 \text{ pg } \mu\text{L}^{-1}$ were obtained, with a regression coefficient of 0.98. Screening tests on cervical specimens were performed to evaluate the sensibility and specificity for HPV and its viral family. The expression of a biomarker for tumorigenesis (p53 gene) was also monitored. In this work, a flexible system has been successfully developed for label-free detection of HPV families and p53 gene monitoring with high specificity, selectivity, and sensitivity.

1. Introduction

The human papillomavirus is a non-enveloped DNA virus and the oncogenic infectious agent responsible for causing infections in the mucosal and anogenital epithelium [1]. The HPV can lead to a variety of clinical conditions, like warts caused by viral types of low oncogenic risk (HPV6, 11, 40, 42, 43, 44, 53, 54, 61, 72, and 81). In addition, neoplasms can result from cytogenetic changes caused by viral types of high oncogenic risk (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82). Of note, HPV16 and 18 are responsible globally for ~70% of the cases of cervical carcinoma [2]. Co-infections between HPV16 and 18 with other viral types, such as HPV45 and 31, can cause serious clinical conditions. Furthermore, persistent HPV infections are intrinsically

related to the development of cancers, such as cervical, penile, vaginal, vulvar, and anal cancer [3].

Neoplastic disorders are mainly associated with the expression of two HPV genes (E6 and E7). The products of the E6 and E7 genes interfere with the cell cycle control through the degradation of the p53 and Rb (retinoblastoma) proteins. The p53 tumor suppressor gene regulates the growth arrest and apoptosis after DNA damage. The transcription of the p53 gene can be intensified in HPV infections to maintain cellular homeostasis [4].

Early diagnosis of HPV infections and identification of viral serotypes in clinical samples can prevent about 93% of cervical cancers [5]. The main techniques used for molecular diagnosis of HPV include hybrid capture assays and nucleic acid amplification assays, such as

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quantitative real-time polymerase chain reaction (RT-qPCR). Hybrid capture assays have become the standard method in many countries and are widely used in FDA-approved clinical trials. On the other hand, hybrid capture assays do not allow specific identification of HPV genotypes, identifying only high-risk and low-risk groups. Hybrid capture assays have lower sensitivity than PCR techniques. Also, positive results can be obtained from cross-hybridization with other HPV types [6]. The PCR method has a laborious procedure and needs to perform multiple type-specific reactions for detecting HPV genotype in a single sample. Besides, expensive reagents/equipment, appropriate laboratories, and qualified professionals are required [7]. Thus, we can highlight the importance of the development of new effective molecular methods for HPV diagnosis.

Genosensors allow a rapid analysis of the analyte using small sample volumes, low cost, simple operation, miniaturization, reusability, and stability in adverse environmental conditions [8]. Genosensors present broad applicability for laboratory HPV diagnosis. Nanomaterials have been used to avoid loss of the recognition capacity after DNA anchoring, reproducibility of the analytical signal, manufacturing scalability, and long-term stability [9]. Also, AuNPs present a high contact area, electrical conductivity, quantum size effect, biological compatibility, non-toxicity, molecular adsorption ability, and others. Therefore, the incorporation of AuNPs into detection systems can increase the analytical effectiveness and robustness of the sensor devices [10].

Conducting polymers are considered functional materials for the development of sensor platforms [11]. The PPy polymer has been investigated because of its potential for the manufacturing of ultrasensitive biosensors. Among the properties that contribute significantly to the improvement of the analytical performance and robustness of the biodetection systems are the high conductivity, high electron transfer kinetics at the electrode/solution interface, charge storage capacity, redox reversibility, relative biological compatibility, and presence of functional chemical groups for directed anchoring of biomolecules [12]. In addition, this organic polymer exhibits structural flexibility, environmental stability, low cost and easy synthetic protocols, favoring new biotechnological applications. Previous studies have shown that PPy is an excellent material to be used as a substrate or matrix for the electrodeposition of AuNPs with reduced molecular aggregation and increased surface area [13,14].

Flexible plastic substrates such as polyethylene naphthalene, polytetrafluoroethylene, polyimide, and polyethylene terephthalate (PET) have been investigated as supports of biosensing platforms [15]. In particular, conductive ITO layers are oxide films used for the development of transparent electrodes, liquid crystal displays, solar cells, optoelectronic, and bioanalytical devices [16]. This high-performance plastic substrate is used in biosensors due to their high electrical conductivity, low capacitive current, electrochemical activity over a wide range of potential, and a possibility for surface modifications [17].

Flexible electrochemical biosensors allow a high selectivity, sensitivity, low power requirement, and miniaturization [15]. Flexible electrochemical devices have been developed for the diagnosis of different diseases [18,19]. However, flexible electrochemical biosensors for HPV are still limited [20]. The approaches employed are based on gold electrode [21], pencil graphite surface [22], paper [20], Si substrate [23], screen-printed carbon electrode [24], glassy carbon electrode [25], and interdigitated platinum electrodes [26].

The present study described a methodological process for obtaining label-free flexible electrochemical biosensors used to identify HPV families, correlating positive diagnoses for viral infection with the potential development of cervical cancer. The flexible ITO electrode was modified with PPy, AuNPs, and cysteamine (Cys). Furthermore, a novel specific oligonucleotide sequences for papillomavirus families were designed. DNA probes were anchored covalently on the nanostructured platform. The proposed flexible system may also be used for purposes other than HPV detection, varying the nature of the bioreceptor.

2. Materials and methods

2.1. Materials

ITO, PPy, tetrachlorouronic acid (HAuCl_4), Cys, and bovine serum albumin (BSA), hydrochloric acid (HCl), potassium ferricyanide ($\text{K}_3[\text{Fe}(\text{CN})_6]$), potassium ferrocyanide ($\text{K}_4[\text{Fe}(\text{CN})_6]$), glutaraldehyde, sodium phosphate monobasic and dibasic were purchased from Sigma Aldrich (St. Louis, MO, USA). Trizol reagent was purchased from Invitrogen Co. Ltd. (Carlsbad, CA, USA). Milli-Q plus purification system was used to obtain deionized water (Billerica, MA, USA).

2.2. DNA probes and biological samples

Probes specific for the detection of HPV families and DNA probe for detection of the p53 gene were purchased from Invitrogen Co. Ltd. (Carlsbad, CA, USA). Methodology for obtaining the biorecognition probes and the access numbers downloaded from the site: <http://www.ncbi.nlm.nih.gov/nucleotide> to be used in sequence alignment were presented in supplementary information and listed in Table S1.

Samples of recombinant plasmids containing L1 gene of different HPV types (HPV6, 11, 16, 18, 33, 45, 51, 53, 58, 61, 62, 66, 72, 81, and 84) were obtained from subcloning in a pTA vector. Oligonucleotide primers (complementary to linker region of the plasmid vector) were used for the sequencing of the recombinant plasmids. cDNA samples were obtained from the reverse transcription of mRNA segments present in cervical specimens of women infected with HPV. The mRNA (total RNA) segments were isolated from cervical cells using Trizol reagent. DNA probes specific for HPV families and the genotypes that each oligonucleotide can recognize are described in Table S2.

The clinical samples were collected under the consent of the patients, and the corresponding protocol was approved by the local ethics committee (process n° CAAE 23698513.0.0000.5190). All samples and biorecognition probes were validated with RT-PCR and agarose gel electrophoresis stained with ethidium bromide (Fig. S1). RT-PCR previously characterized cDNA samples as positive samples (with genotype identification) or negative samples for HPV. Dilutions of the biological samples were prepared with 10 mM phosphate-buffered saline (PBS) at pH 7.4 and kept frozen.

2.3. Fabrication of the flexible electrodes and assembly of the oligonucleotides

The prototypes were obtained from flexible ITO strips measuring 3 cm in height by 8 mm in width. To standardize the electroactive work area, a polyvinyl chloride (PVC) adhesive sheet with a circular hole ($\varphi = 5$ mm) was superimposed over the ITO surface. Thus, a specific area for mounting the biosensor (0.2 cm^2) and an area for electrical contact (0.8 cm^2) were obtained.

The first stage of the sensor assembly consists of the electropolymerization of pyrrole. First, the flexible working electrodes were immersed in a solution of 0.5 M HCl containing the pyrrole monomer at a concentration of 30 mM. Six cycles of polymerization were applied in a potential range of -0.2 to $+0.7$ V at a scan rate of 100 mV s^{-1} . The second step comprises the synthesis and electrochemical deposition of AuNPs on the PPy film. After, ten voltammetric cycles were performed using a 0.25 mM solution of HAuCl_4 under a potential range of $+0.2$ to -1 V and a scan rate of 50 mV s^{-1} to obtain AuNPs [27]. The third step refers to the chemisorption of Cys molecules on the AuNPs. This step, 2 μL of 2 mg mL^{-1} Cys hydroalcoholic solution (ethanol:water, 3:1) was dropped on the electrode surface for 30 min.

The chemical immobilization of the DNA probes was performed after obtaining the PPy-Cys-AuNPs nanostructured platform. Specific probes for the detection of HPV families and probe for detection of the p53 gene were used. Initially, 2 μL of 0.5% glutaraldehyde was added onto the nanostructured platform for 10 min. Subsequently, 2 μL of a 10 mM

oligonucleotide solution was added for 15 min. Nonspecific sites of the DNA flexible biosensors were blocked with BSA molecules. In this step, 2 μL of 1% BSA solution (pH 7.4) was added over the surface of the modified electrodes for 30 s. The assembly process is schematically shown in Fig. 1.

2.4. Evaluation of the bioactivity and monitoring of p53 gene

Initially, the biological samples were heated to 94 °C for DNA denaturation. The genosensors were exposed to 2 μL of the biological samples for 15 min to hybridization. The specificity, sensitivity, and selectivity of the genosensors were evaluated by using a) recombinant plasmids containing the L1 gene nucleotide sequence for specific types of HPV; and b) mixtures of plasmids containing the HPV L1 gene not belonging to the probe recognition family (mixed samples used as negative control). Besides, cervical specimens (cDNA samples) were used to explore the detection capacity of the genosensors and to monitor the p53 gene.

2.5. Electrochemical measurements

The voltammetric and impedimetric analyses were performed on a PGSTAT 128 N potentiostat/galvanostat interfaced with NOVA 1.11 software (Metrohm Autolab, The Netherlands). The experiments were

performed in a three-electrode electrochemical cell system. The electrochemical analysis was conducted in 10 mM $\text{K}_4[\text{Fe}(\text{CN})_6]/\text{K}_3[\text{Fe}(\text{CN})_6]$ (1:1, v/v) in PBS (10 mM, pH 7.4). The flexible ITO substrate was used as a working electrode. A platinum electrode and Ag/AgCl electrode saturated with KCl (3 M) were used as counter and reference electrodes, respectively. Cyclic voltammograms were obtained in a potential range of -0.2 to $+0.7$ V with a scan rate of 50 mV s^{-1} . The electrochemical spectra were recorded in a frequency range between 100 mHz and 100 kHz with an alternating amplitude potential of 10 mV. All electrochemical analyses were performed in triplicates at room temperature and inside a Faraday cage.

2.6. Atomic force microscopy measurements

The molecular images with topographic mapping were recorded from an SPM-9500 atomic force microscope (Shimadzu Corporation, Japan). Cantilevers with aluminum-coated silicon probe (Nanoworld, Japan, resonant frequency = 300 kHz, spring constant = 42 N m^{-1}) were used to obtain the AFM images in a non-contact mode in air. Topographic images were acquired with a lateral resolution of 512×512 pixels and a scan area of $5 \times 5 \mu\text{m}$. Three macroscopically separated areas were analyzed in each sample by the AFM Gwyddion software to obtain the multiparametric images.

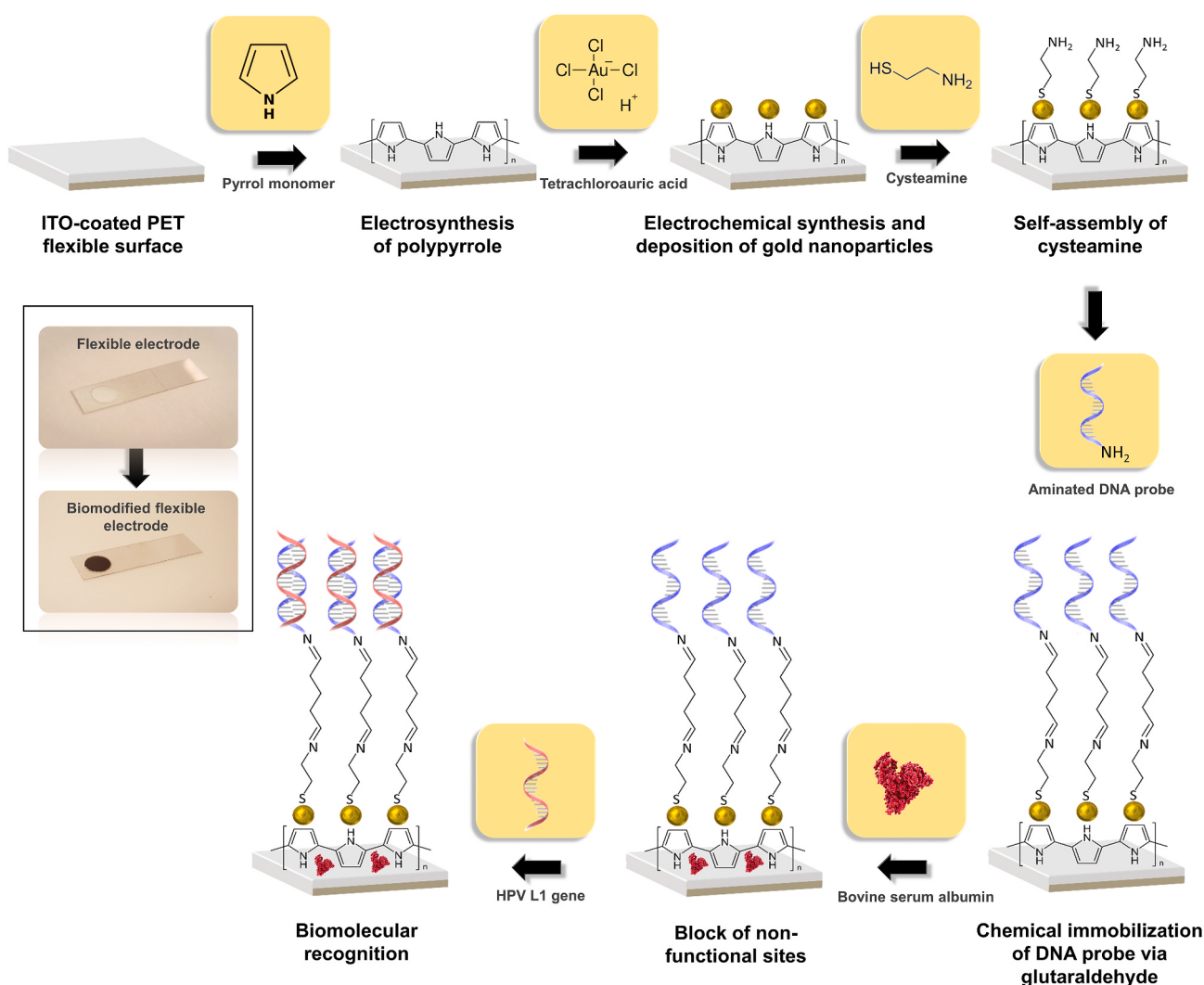


Fig. 1. Schematic representation of the fabrication stages of the electrochemical biosensor and images of the flexible miniaturized electrode before and after the biofunctionalization process.

3. Results and discussion

3.1. Topographic analyses

The functionalization of the flexible electrode and hybridization process were evaluated by the AFM technique (Fig. 2a–f). Fig. 2a shows 2D and 3D topographic images for the PPy film electrochemically deposited on the electrode surface. The morphology of the PPy film is globular, with a maximum height of 308 nm. The polymer particles synthesized on the electrode surface are globular-shaped. Our results are according to the literature [28,29]. Metallic nanoparticles were homogeneously deposited on the PPy polymer, where the PPy-AuNPs film has a maximum height of 372 nm (Fig. 2b). The height difference of the PPy and PPy-AuNPs films suggests that the AuNPs have a maximum diameter of 64 nm. Chen et al. [30] related that the PPy is a facilitating material for the nucleation and growth process of AuNPs during electrosynthesis. For this reason, the electrodeposited AuNPs exhibit a high affinity for modified electrodes with PPy.

We observed the presence of peaks up to 494 nm after Cys immobilization on the PPy-AuNPs film (Fig. 2c). The Cys chemisorption occurs through a thermodynamically favorable process between the thiols groups of the Cys and the gold atoms [31]. The morphology for the PPy-AuNP-Cys-Probe_{BSH16} film is shown in Fig. 2d. The topographical analysis shows the presence of structures on the electrode surface with a maximum height of 603 nm. A rougher surface with increased morphological heterogeneity is observed after the addition of BSA

(Fig. 2e). To prevent non-specific adsorption, BSA molecules were used. Fig. 2f presents the topographic characterization of the biosensor after its exposure to clinical samples from a patient infected with HPV. An increase in the density of molecules immobilized on the biomodified electrode was observed after hybridization with the complementary DNA target. The roughness increased to 892 nm after the sensor captured HPV L1 gene, suggesting the biorecognition capability.

3.2. Electrochemical characterization of the biosensor

Fig. 3a shows cyclic voltammograms for each step of assembly of the flexible biosensor. The cyclic voltammogram of the ITO electrode after the pyrrole electropolymerization presents a high current density with a significant amperometric response ($I_{pa} = 194.36 \pm 10.27 \mu A$) (Fig. 3a). Accordingly, the PPy film exhibited a low charge transfer resistance ($R_{CT} = 0.02 \pm 0.007 k\Omega$) (Fig. S2).

In this work, we employed the Randles circuit composed of R_{CT} , constant-phase element (CPE), Warburg impedance (Z_W), and ohmic resistance (R_s). The R_{CT} is related to the faradaic process of charge transfer between the redox species $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$, whose values can be calculated from a modified Randles circuit (inset of Fig. S2). A CPE, a non-faradaic capacitance, represents the accumulation of charge on the flexible electrode surface. Warburg impedance is related to mass diffusion of electroactive species in the bulk solution to the electrode surface. The ohmic resistance of the electrolyte solution is mentioned as R_s . The values of the equivalent circuit elements are

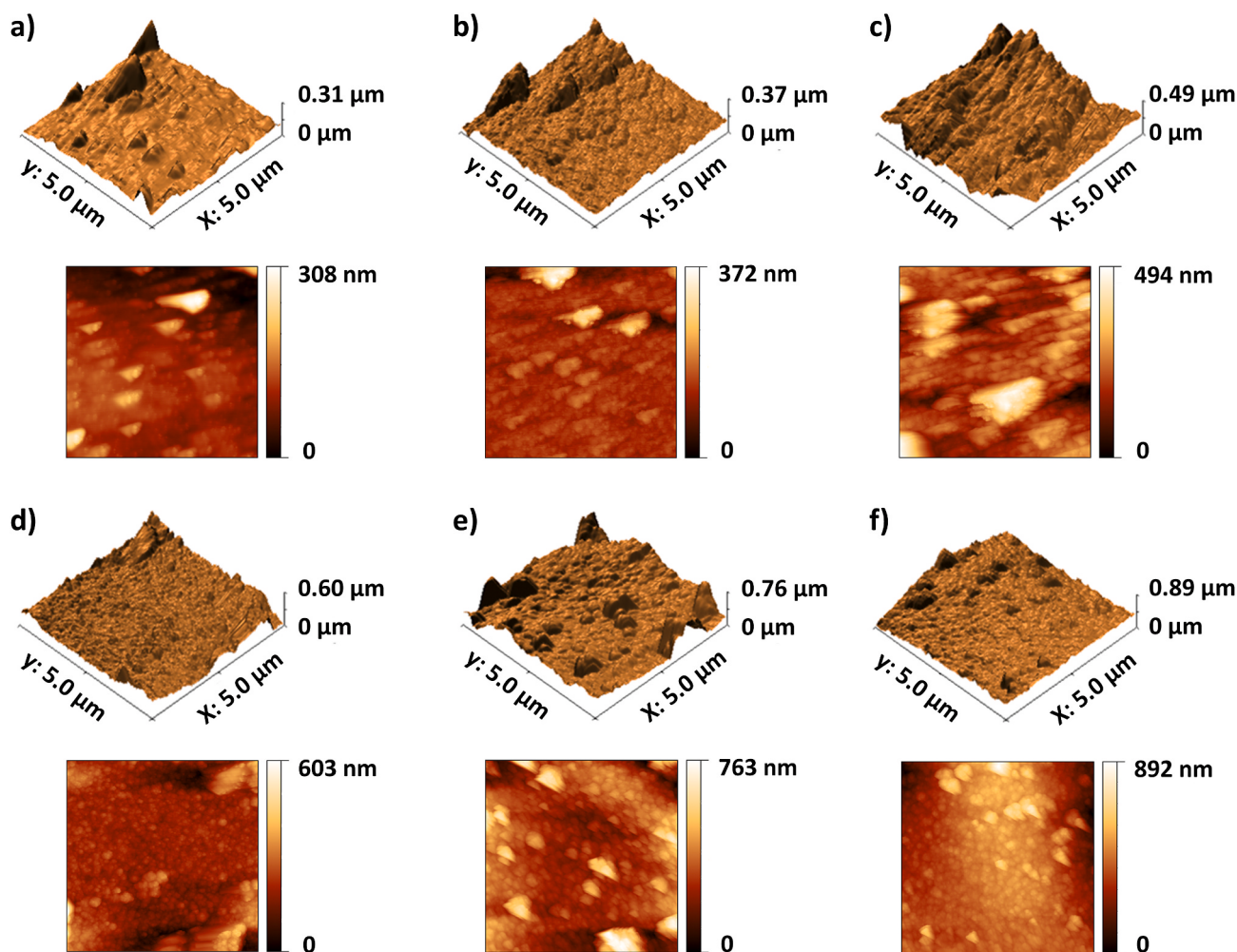


Fig. 2. 2D and 3D AFM images of the PPy-AuNP-Cys-ProbeBSH16 film (a), PPy-AuNP-Cys-ProbeBSH16-BSA film (b) PPy-AuNP-Cys-ProbeBSH16-BSA-HPV film, with the corresponding cross-section. Scan area of $5 \mu m \times 5 \mu m$.

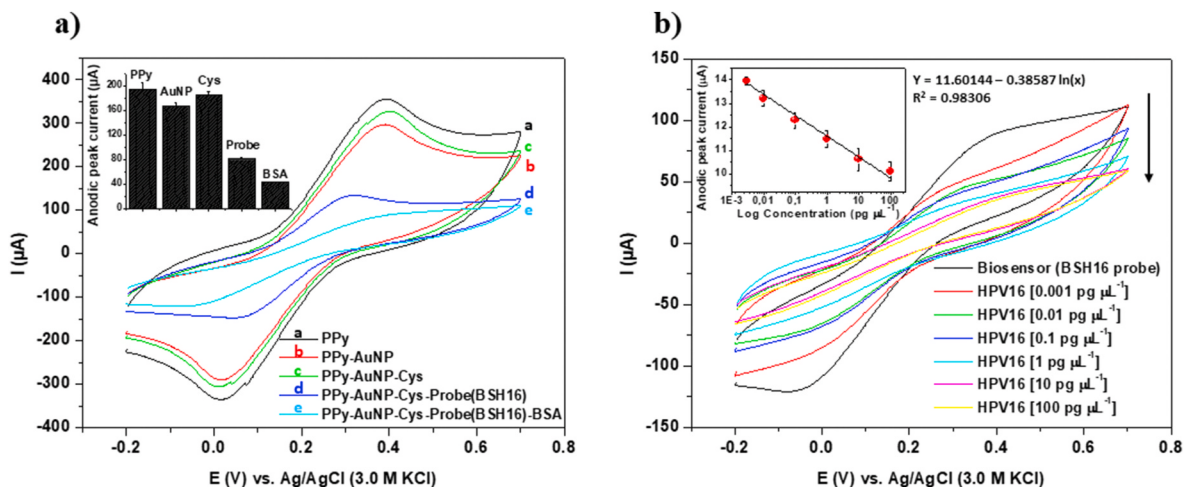


Fig. 3. Voltammetric characterization (a) of each step of assembly of the flexible biosensor. Cyclic voltammograms for the biosensor BSH16 exposed to different concentrations of recombinant plasmids containing the HPV16 L1 gene (DNA target: 0.001, 0.01, 0.1, 1, 10, and 100 $\text{pg } \mu\text{L}^{-1}$) (b). Inset: anodic peak currents obtained during the biosensor construction (a) and calibration plot of the biosensor and histograms for anodic peak current (b). Three replicates for each experimental condition were used. Experimental values are described as the mean values $\pm$ their half-deviation.

reported in the supplementary material (Table S3). Of note, the oxidation process on the working electrode surface results in the formation of a stable polymeric film with controllable thickness and high electrical conductivity.

A decrease in the anodic oxidation ($I_{pa} = 166.84 \pm 5.74 \mu\text{A}$) (Fig. 3a) and an increase in the resistive properties ($R_{CT} = 0.05 \pm 0.003 \text{ k}\Omega$) (Fig. S2) were obtained after electrosynthesis of AuNPs. The PPy films provide a significant number of nucleation sites for the formation of nanostructures. Thus, AuNPs are synthesized with nanometric dimensions and in a state of non-aggregation [13]. Organosulfur Cys molecules were chemisorbed on the AuNPs surface resulting in a higher conductivity response ($I_{pa} = 185.50 \pm 4.70 \mu\text{A}$) (Fig. 3a) and lower interfacial resistance ($R_{CT} = 0.03 \pm 0.01 \text{ k}\Omega$) (Fig. S2). The covalent binding occurs due to the thiol functional group of the Cys molecule. Besides, Cys acts as a molecular spacer in the nanostructured platforms that contribute to the increase of the sensitivity [10].

The immobilization of the DNA probe (BSH16 probe) on the PPy-AuNP-Cys-modified electrode results in a reduction of the current signal (Fig. 3a) ($I_{pa} = 80.78 \pm 3.07 \mu\text{A}$). Also, an increase in the impedimetric response ($R_{CT} = 0.66 \pm 0.07 \text{ k}\Omega$) is obtained (Fig. S2). The blockage of the redox process in the electrical double layer is due to electrostatic repulsion between the oligonucleotide phosphate groups and the $[\text{Fe}(\text{CN})_6]^{3-}/[\text{Fe}(\text{CN})_6]^{4-}$ anions [32]. For blocking of nonfunctional regions were used BSA molecules. These protein molecules caused a decrease in the voltammetric measurement ($I_{pa} = 42.95 \pm 0.11 \mu\text{A}$) (Fig. 3a) and an increase in the resistivity ($R_{CT} = 0.68 \pm 0.007 \text{ k}\Omega$) (Fig. S2).

3.3. Analytical performance of the biosensor

Cyclic voltammograms of the biosensor exposed to different concentrations of recombinant plasmids containing the L1 gene of the HPV16 genotype are shown in Fig. 3b. The concentrations evaluated of DNA target were 0.001, 0.01, 0.1, 1, 10, and 100 $\text{pg } \mu\text{L}^{-1}$. The hybridization process results in a decrease in the oxidation and reduction peaks due to lower electron transfer. The amperometric current is inversely proportional to the sample concentration. Also, alterations in voltammetric signals suggest the presence of dielectric elements or negatively charged molecules, such as DNA molecules [33].

The detection degree of an electrochemical biosensor can be defined according to the percentage of the relative deviation of the anodic current variation (ΔI), as follow:

$$\Delta I(\%) = \frac{I_b - I_a}{I_b} \times 100 \quad (1)$$

where I_b and I_a correspond to the anodic peak current before and after the hybridization process, respectively. The amperometric anodic shift for the biosensor BSH16 after its interaction with different concentrations of plasmid samples is shown in Fig. S3. A gradual increase in the magnitude of the ΔI (%) values was observed that correlate with the rise of the concentrations of the analyzed samples. A linear variation of ΔI (%) ranging from $208.04 \pm 3.92\%$ to $325.34 \pm 18.69\%$ was obtained after HPV16 specific recognition (Table S4).

The mathematical models were based on the confidence limits of the straight regression line applied to a linear portion of the calibration curve [34]. The LOD, LOQ, and sensitivity were expressed as $3.3 \sigma/\text{slope}$, $10\sigma/\text{slope}$, and $\text{slope}/(\text{area of the electrode})$, respectively, where σ is the standard deviation of the blank measurement [35]. The biosensor BSH16 presents a sensitivity of $1.93 \mu\text{A}/\text{pg } \mu\text{L}^{-1} \text{ cm}^2$ with a detection range varying from $100 \text{ pg } \mu\text{L}^{-1}$ to $1 \text{ fg } \mu\text{L}^{-1}$. The LOD and LOQ of the proposed biosensor are found to be $0.89 \text{ pg } \mu\text{L}^{-1}$ and $2.70 \text{ pg } \mu\text{L}^{-1}$, respectively, with a regression coefficient of 0.98 (inset of Fig. 3b).

3.4. Voltammetric characterization of the bioactivity of DNA probes against HPV families

We obtained different biosensors based on DNA probes specific for HPV families, as follow: biosensor BSH6 (Fig. 4a), biosensor BSH16 (Fig. 4b), biosensor BSH18 (Fig. 4c), biosensor BSH26 (Fig. 4d), biosensor BSH53 (Fig. 4e), and biosensor BSH61 (Fig. 4f). The specificity of the DNA probes for each genotype is detailed in Table S2. Biosensors were exposed to plasmids containing the L1 viral gene. The presence of double-stranded DNA (dsDNA) on the sensor surface causes a significant reduction of the amperometric response [36]. The anodic peak was reduced due to partial blockage of the oxide-reduction processes (see insets of Fig. 4).

Also, electrochemical biosensors were exposed to plasmid mixtures containing the HPV L1 gene not belonging to the probe recognition family (mixed samples used as negative control). The HPV types present in the mixed samples are described in Table S5. Changes in the ΔI values ($0.33 \pm 0.01\%$ to $22.14 \pm 0.80\%$) were observed after exposure of the biodevices to negative samples. These voltammetric variations indicate the physical adsorption of non-complementary DNA sequences. Of note, the ΔI values for the biosensor exposed to negative samples are

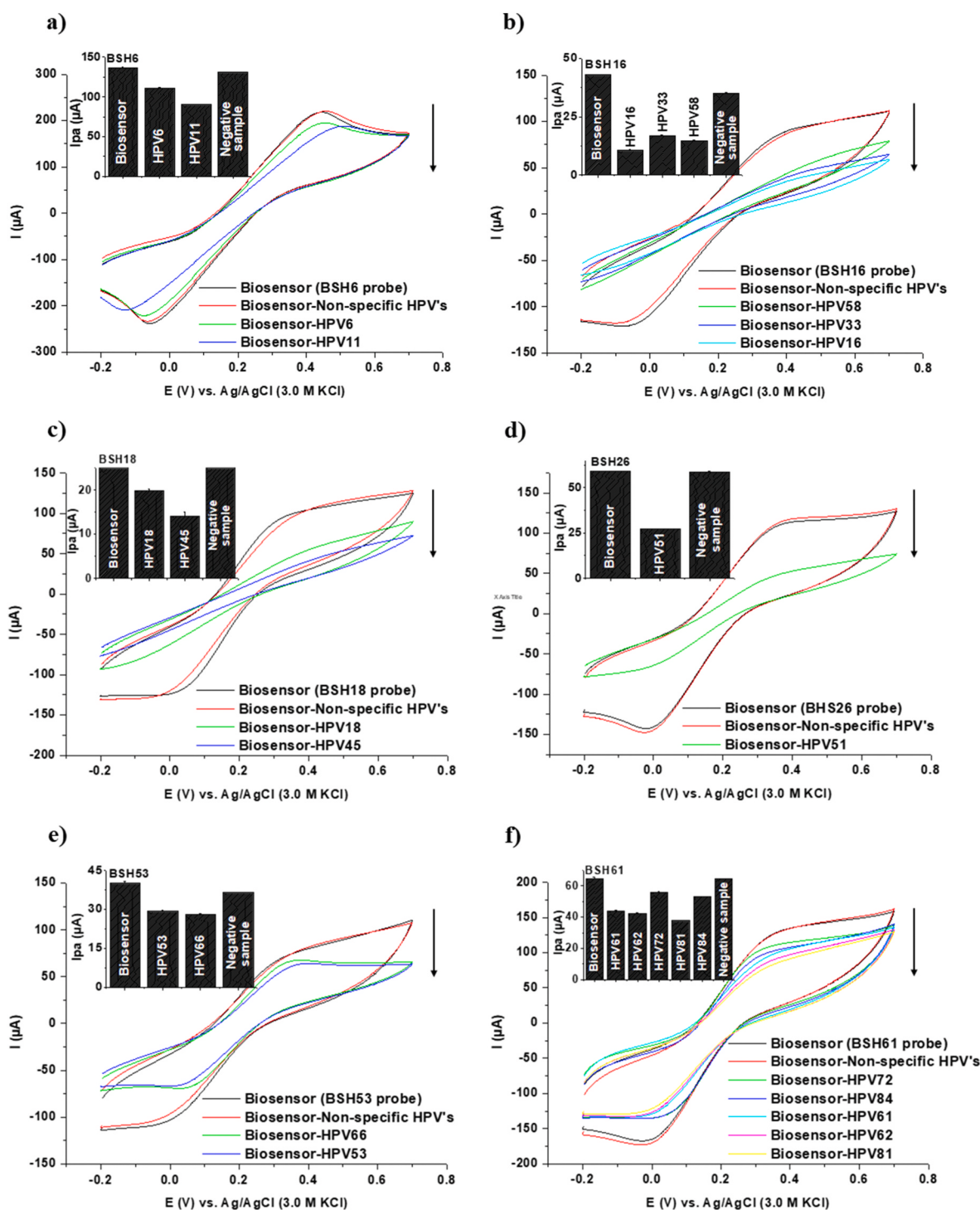


Fig. 4. Voltammetric characterization of the flexible biosensors based on the use of DNA probes specific for HPV families: BSH6 (a), BSH16 (b), BSH18 (c), BSH26 (d), BSH53 (e) and BSH61 probe (f). Each biosensor was exposed to plasmids containing the L1 viral gene whose HPV type is included in the biorecognition family and plasmid mixtures containing the HPV L1 gene (used as negative control). Inset: histograms for anodic peak current. The concentration of all samples is 100 μ L⁻¹.

significantly lower than the ΔI values obtained during assays with complementary DNA sequences (see Table S4).

3.5. p53 gene monitoring and HPV families identification in cervical specimens

The existence of the relationship between high-risk HPV infection and cervical carcinogenesis highlights the importance of early cancer

diagnosis. The p53 gene is a biomarker for carcinogenicity, and its increased expression may be associated with the establishment of malignant lesions [4]. Thus, a DNA probe for p53 gene was used in biosensing assays.

Fig. 5a shows the voltammetric characterization for the biosensor_{p53} after bioactivity tests with cervical specimens obtained from women infected with different types of HPV (HPV6, 11, 16, 31, 33, 45, and 58). After the exposure of the sensor to clinical samples occurs a reduction in

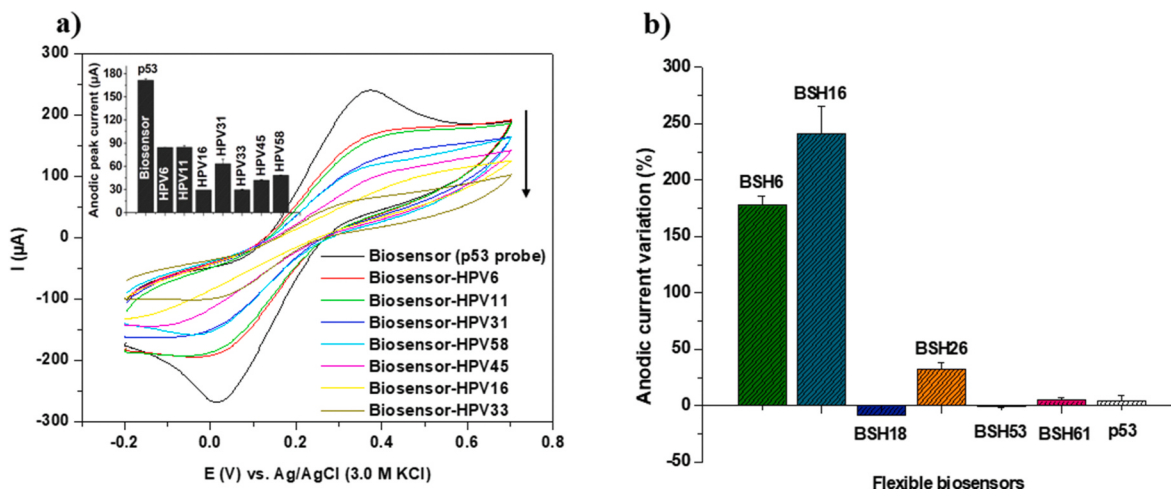


Fig. 5. Voltammetric characterization for the biosensor p53 exposed to cervical specimens obtained from women infected with different types of HPV (HPV6, 11, 16, 31, 33, 45, and 58) (a). Screening test in the clinical sample using flexible biosensors (b). Inset: histograms for anodic peak current. The concentration of all samples is $100 \text{ pg } \mu\text{L}^{-1}$.

the voltammetric areas (inset of Fig. 5a), indicating the capture of the target gene on the flexible transducer. A detailed analysis reveals that the biosensor_{p53} is sensitive to detect variations in the p53 gene expression in women with high-risk HPV (oncogenic) (HPV16, 31, 33, 45, and 58). A decrease in voltammetric current was obtained (Table S6), with ΔI values ranging from $171.72 \pm 25.46\%$ to $491.02 \pm 3.52\%$. This fact may be related to a supposed overexpression of p53 gene (associated with predisposition to cervical cancer).

Specimens with low-risk HPV (HPV6 and 11) presented ΔI values ranging from $102.68 \pm 5.33\%$ to $103.22 \pm 0.40\%$. These data suggest that samples with low-risk HPV may not have overexpression of the p53 gene. This molecular profile is expected because oncogenic proteins (E6 and E7 proteins) from low-risk genotypes cause incompetent degradation of the p53 protein [37]. Thus, the expression of the p53 gene is not intensified.

The electrochemical results for a screening test in a cervical specimen using flexible biosensors are shown in Fig. 5b. Seven electrodes differentiated by the nature of the biorecognition probes (BSH6, BSH16, BSH18, BSH26, BSH53, BSH61, and p53 probes) were used to evaluate

the clinical samples. The biosensor results were associated with current variation after exposure to clinical samples.

We used HPV mixed samples as a negative control. The HPV mixed samples contain the HPV L1 gene not belonging to the probe recognition family. The screening test exhibited positive results for HPV families associated with the BSH6 and 16 probes. A positive response was observed for the BSH26 probe. Of note, irrelevant amperometric results for BSH18, BSH53, BSH61, and p53 probes were obtained.

The specificity of the biosensing system was evaluated with oligonucleotide sequences not complementary to the biorecognition probes (plasmid sample), clinical sample from a non-HPV infected patient, and clinical samples containing the genome of one of the following external infecting agents; hepatitis C virus (HCV), *Candida albicans*, *Mycobacterium tuberculosis*. In Fig. S4 can be observed that the variation of the electrochemical responses after the biorecognition assays is insignificant compared to clinical sample infected with HPV. Therefore, the high specificity of the biosensor and the low capacity to produce false positive results are suggested. A comparison of the proposed biosensor with other reported biosensors developed for the identification of HPV is

Table 1

Analytical comparison between the biosensor presented in this work and the other sensor systems reported in the literature for the oligonucleotide detection.

Biosensitive platform	DNA target	Analytical technique	Hybridization marker	Detection time	Detection range	Limit of detection	Limit of quantification	Reference
ITO surface/PPy/AuNP/Cys/Probe _{BSH16} /BSA	HPV16 HPV31 HPV33 HPV35 HPV52 HPV58	CV and EIS	Label-free	15 min	6.42 pM to 64.2 aM	57.14 fM	173.34 fM	This work
Screen-printed carbon electrode/nanocomposite of reduced graphene oxide and multiwalled carbon nanotubes/AuNPs/cysteine/HPV probe	HPV18	DPV	anthraquinone-2-sulfonic acid monohydrate sodium salt	15 min	0.01 nM to 0.01 fM	0.05 fM	–	[39]
Polycarbonate surface/gold nanotubes/HPV probe	HPV16	EIS	Label-free	–	0.01 pM to 1 μM	1 fM	–	[5]
Gold surface/L-cysteine/HPV probe	HPV16	DPV	Methylene blue	10 min	18.75 nM to 1 pM	18.13 nM	–	[21]
IDE chip/ZnO-Nanorods/AuNP/HPV probe	HPV16	EIS	Label-free	10 min	0.1 μM to 1 fM	1 fM	–	[40]
Electrokinetic membrane sensor associated with a microfluidic detection chip	microRNAs	Piezoelectric detection based on surface acoustic wave	Label-free	30 min	–	1 pM	–	[41]

DPV - differential pulse voltammetry.

shown in Table 1.

The PCR and hybrid capture techniques can be considered gold standard methods for the molecular diagnosis of HPV [7]. Noteworthy, the PCR technique exhibits a superior performance in terms of sensitivity, specificity and quantification capacity compared to other molecular methods [38]. However, these methods require expensive instrumentation, qualified professionals, and a long time for multiple analyses [6]. Electrochemical biosensors have been developed to detect the HPV genome (Table 1) since the virus identification is essential for the treatment and early diagnosis of cervical cancer. The proposed sensor was able to identify HPV families at minimal concentrations with LOD of $0.89 \text{ pg } \mu\text{L}^{-1}$ and LOQ of $2.70 \text{ pg } \mu\text{L}^{-1}$. The analytical results were obtained in a fast time of analysis (15 min) with high sensitivity, specificity, and selectivity. The developed degenerate probes were able to recognize the HPV L1 gene and discriminate strains in viral families. Also, the specific identification of the HPV genotypes can be performed through a decision tree between positive and negative results for different probes used simultaneously (Table S7). The proposed biosensor is a useful alternative for the rapid screening of HPV in clinical specimens.

4. Conclusions

The present study provides a simple process for obtaining flexible electrodes constructed from the electrochemical polymerization of PPy, followed by AuNPs electrosynthesis. Conductive PPy film is a promising substrate for the synthesis of metallic nanoparticles in a non-aggregate state. The association of these functional materials allowed obtaining a platform with improved electrochemical properties and high surface area. Oligonucleotide probes were developed for the specific recognition of HPV families. Thus, it was possible to construct label-free biosensors for the differential diagnosis of papillomavirus. The electrochemical results reveal the ability of the biosensor to identify specific types of HPV at minimal concentrations. The innovative aspect of this study is the combination of HPV screening with p53 gene monitoring. To the best of our knowledge is the first time that this approach is reported in electrochemical biosensors for HPV. The suggested analysis strategy is essential to evaluate the oncogenic potential of HPV infections and prevent cervical cancer. In particular, the proposed electrode is an economically viable model with the simplicity of construction. These characteristics enable its use in the sensitive, selective, and rapid diagnosis of clinical diseases.

CRediT authorship contribution statement

Karen Y.P.S. Avelino: Methodology, Investigation, Formal analysis, Writing - Original Draft. **Léony S. Oliveira:** Investigation, Formal analysis. **Norma Lucena-Silva:** Validation, Formal analysis, Funding acquisition. **César A.S. Andrade:** Conceptualization, Formal analysis, Writing - Review & Editing, Supervision, Funding acquisition. **Maria D. L. Oliveira:** Conceptualization, Formal analysis, Writing - Review & Editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.talanta.2021.122118>.

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