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Development of a bio-electrochemical immunosensor based on the immobilization of SPINNTKPHEAR peptide derived from HPV-L1 protein on a gold electrode surface

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ABSTRACT

A novel bio-electrochemical immunosensor for sensitive and selective detection of anti-peptide antibodies of HPV was fabricated by immobilization of a peptide (SPINNTKPHEAR) (**1**) linked to a 6-aminohexanoic (*Ahx*) residue and ferrocene (Fc) on a gold electrode surface, used as sensing interface. The Fc–*Ahx*–peptide (Fc–*Ahx*–SPINNTKPHEAR) (**1AFc**) was obtained by solid phase peptide synthesis by using the Fmoc/tBu strategy. Peptides were purified by preparative HPLC, characterized by MS, MALDI-TOF, and Circular Dichroism and their purity was evaluated by using analytical HPLC. The electrochemical behavior of the modified electrode was examined by cyclic voltammetry in phosphate buffer solution (PBS) in order to evaluate the redox behavior of the ferrocene moiety. The influence of anti-peptide antibodies on the voltammetric response of the modified electrode was investigated by comparing results obtained with pre-immune (control) and post immune serum samples at similar dilution factor. Changes in such behavior upon addition of the serum samples to PBS suggested that the fabricated bioelectrochemical sensor was able to recognize the interaction between anti-peptide antibodies and the immobilized peptide (**1AFc**) with high selectivity and sensitivity. Such influence increased as the dilution decreased, but the effect was less pronounced at relatively high concentrated solutions owing to the effect of the sample matrix. Notwithstanding, the proposed biosensor can clearly detect the target anti-peptide antibodies at a significant large concentration range.

1. Introduction

Cervical cancer (CC) is the second most common cancer in women world-wide after breast cancer and the most common cause of death in women according to the World Health Organization [1]. The presence of human papilloma virus (HPV) infection has been related with the development of almost all cancer cases [1–4]. Despite this, a clinically useful screening or monitoring test for cervical cancer or HPV infection has not been fully developed [3]. Papanicolaou test (Pap smear) is the main form of detection of CC and HPV, but its repeatability is not satisfactory and it is not sufficiently predictive [1,5]. Other methods for detecting different types of HPV have also been developed, many of these are based on the detection of unique sequences of DNA in the HPV genome using Polymerase Chain reaction (PCR) to amplify and detect HPV DNA [1,3,5–8], one HPV type being identified at a time [1,3,6,7]. The procedures accept qualitative conditions which permit the incorporation of enzyme-linked immunosorbent assay (ELISA) [6]. Since these methods are in most cases slow, patented, expensive and require modern laboratory facilities and also due to variations related to environmental conditions, antigen lot, and operator, cutoff values generated by ELISA may vary from study to study [6]. For this reason, bioelectrochemical detection [9,10] is an excellent candidate as a method for HPV diagnostic. The high sensitivity, small sample volume requirement, low cost, simplicity and portability of electrochemical techniques represent advantages with respect to other analytical methodologies [9,10].

The use of ferrocene (Fc) probes in electrochemical biosensors has attracted large interest because ferrocene possesses unique redox behavior and it is quite stable in aqueous and non-aqueous solutions [11–13]. Moreover, ferrocene can be used as a building block for the incorporation of peptides and such conjugates can be then immobilized onto electrode surfaces to fabricate highly sensitive and specific platforms [13]. Such devices operate in such a way that the peptide acts as a recognition element by interacting specifically with the analyte and ferrocene is responsible for the electrochemical response. The specific binding of the target to the peptide induces a change in the ferrocene electrochemical behavior, hence the extent of such change can be correlated with the concentration of analyte in solution [9,10].

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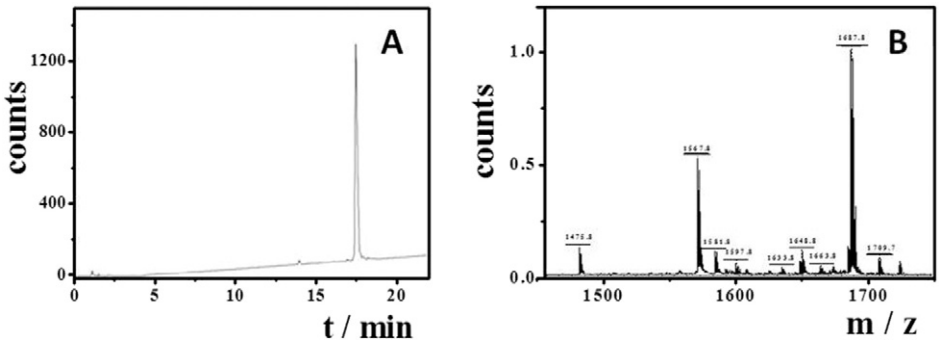

Fig. 1. Purified **1AFc** characterization: RP-HPLC profile (A) and MALDI-TOF spectrum (B).

Different serum samples from patients infected with HPV were detected specifically by sequences of fusion proteins as well as by synthetic peptides [2–4,14,15] whose sequences are derived from regions L1 and L2 of HPV proteins [3,4,14]. Synthetic peptides are valuable tools for the design and development of methods for the diagnosis of different diseases because they have the advantage of better safety in comparison to a virus itself, are innocuous, can be synthesized quickly and inexpensively, and possess higher operational stability and high specificity. Peptide residues usually retain its structure immunologically active, which is necessary for bond cleavage. Synthetic peptides can be specifically recognized by antibodies through antigen–antibody interaction owing to the high stability of the formed compounds (K_d 10^6–10^9 mol L^{-1}) [15–18], making peptide residues suitable as molecule probes for medical diagnostics [19]. Accordingly, the present paper aims at investigating the possibility of fabricating a peptide modified electrode as a sensing element for anti-peptide antibodies. The influence of different experimental parameters such as amount of mice serum on the biosensor response was investigated to optimize the response.

For the design of the biosensing layer, the SPINNTKPHEAR peptide (**1**) was used as a probe to detect the HPV antipeptide-antibody in pre-immune (control) and post-immune serum of mice immunized with a polymeric peptide CGSPINNTKPHEARGC (**2**). Peptides conjugated to polymers can be used in various applications with the advantage of being resistant to enzymatic cleavage [20,21]. Peptide **2** corresponds to peptide–synthetic polymer conjugates of peptide **1**. It consists of two CG — extra unities linked at each side of the peptide. Cysteine (C) residues play a valuable role by crosslinking in the peptide, which increases the rigidity of the peptide through the formation of disulfide bonds and confers proteolytic resistance and glycine (G) is used as spacer.

2. Material and methods

2.1. Chemical and apparatus

Rink amide resin (0.46 meq/g), Fmoc-Arg(Pbf)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Ala-OH, Fmoc-Ile-OH, Fmoc-Thr(tBu)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Pro-OH, Fmoc-His(Trt)-OH, 6-Fmoc-aminohexanoic acid (Fmoc-*Ahx*-OH), Fmoc-Ser(OtBu)-OH, 1-hydroxybenzotriazole (HOBt), and N,N-dicyclohexylcarbodiimide (DCC) were purchased from AAPPTec (Louisville, KY, USA). N,N-diisopropylethylamine (DIPEA), triisopropylsilane (TIPS), 1,2-ethanedithiol (EDT), piperidine, pyridine, ninhydrin, phenol, ferrocene carboxylic acid (Fc), KCN, and Nafion®, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, diethyl ether, N,N-dimethylformamide (DMF), absolute ethanol, dichloromethane (DCM), acetonitrile (ACN), isopropyl alcohol (IPA), and trifluoroacetic acid (TFA) were obtained from Honeywell-Burdick & Jackson (Muskegon, Michigan, USA). NaCl, KH_2PO_4, Na_2HPO_4 and KCl were purchased from Merck and used without further purification. Phosphate buffer saline (PBS), pH = 7.4, was prepared according to the literature [22].

All electrochemical measurements were performed by using a PGSTAT 128N potentiostat (Metrohm Siam Company Ltda.) controlled with NOVA software. The experiments were carried out in a Teflon homemade three-electrode cell (200 μL). The **1AFc** modified gold electrode was used as working electrode (2 mm diameter, 0.031 cm area) and adapted in one side of the cell. The counter (Pt wire) and reference ($Ag/AgCl_{(sat.\ KCl)}$) electrodes were positioned at the top of the cell. All electrochemical experiments were performed at 25 °C.

Morphologic images of the modified electrode were obtained by field emission scanning electron microscopy (FESEM, JSM-7401F from JEOL).

2.2. Peptide synthesis

The SPINNTKPHEAR (**1**) peptide was linked to a 6-aminohexanoic (*Ahx*) residue and ferrocene (Fc). SPINNTKPHEAR, CGSPINNTKPHEARGC (peptide **2**) and Fc–*Ahx*–SPINNTKPHEAR (**1AFc**) were synthesized by SPPS-Fmoc/tBu, which is a widely used methodology for obtaining synthetic peptides at room temperature [23–25]. Briefly, Rink amide resin (200 mg) was used as solid support. (i) The resin conditioning and Fmoc group removal were carried out by treatment with 20% piperidine in DMF for 10 min twice. Then, the resin was exhaustively washed with DMF, IPA, and DCM. (ii) For the coupling reaction, 0.42 mmol of Fmoc-amino acid were preactivated with DCC/HOBt (0.40/0.42 mmol) in DMF. The preactivation mixture was continuously shaken for 15 min. Then, the activated Fmoc-amino acid was added to a reactor containing deprotected resin; the reactor was shaken for 2 h, and the resin was subsequently washed with DMF and DCM. (iii) The Fmoc group elimination and the incorporation of each amino acid were confirmed by the

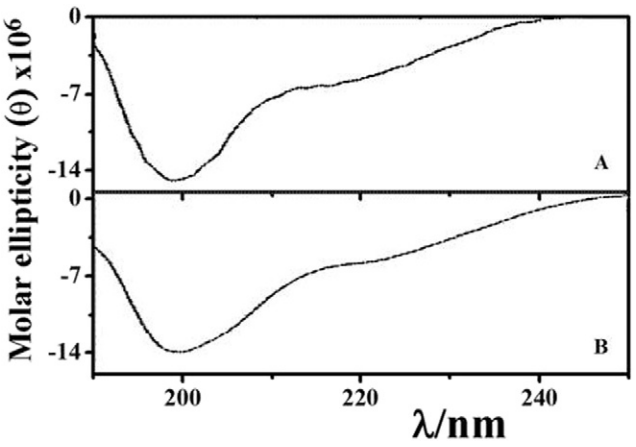

Fig. 2. CD characterization of purified peptides. *Ahx*–SPINNTKPHEAR peptide (A) and **1AFc** (B).

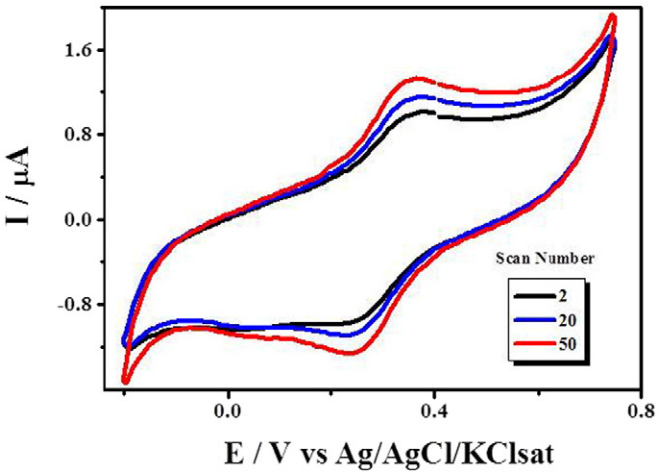

Fig. 3. Cyclic voltammograms (2nd, 20th and 50th) recorded with the **1AFc** modified electrode in 0.1 mol L^{-1} PBS, pH = 7.4, at 0.10 V s^{-1}.

ninhydrin test [4]. Side chain deprotection reactions and peptide separation from the solid support were carried out with a cleavage cocktail containing TFA/water/TIPS/EDT (93/2/2.5/2.5% v/v), and the reactor was shaken for 8 h. Then, the mixture was filtered and the solution was collected. Crude peptides were precipitated by treatment of the filtered solution with cool ethyl ether, and finally they were washed with ether five times.

2.3. Peptide purification

Crude peptides were purified by solid phase extraction (SPE) using a Supelclean LC-18 SPE column. The purified products were analyzed using HPLC–MS, (MALDI-TOF MS) and Circular Dichroism (CD). HPLC ferrocene peptide analyses were performed on an Agilent Eclipse XDB-C18 (4.6 × 150 mm, 3.5 μm) column using an Agilent 1200 liquid chromatograph (Omaha, Nebraska, USA). The mobile phase consisted of (A) 0.05% TFA (v/v) in HPLC-grade water and (B) 0.05% TFA in ACN. A linear gradient was applied from 5% to 50% of solvent B by 36 min using a flow rate of 1.0 mL min^{-1} and a diode array detection (DAD) detector at a specific wavelength of 210 nm for peptide detection. The MALDI-TOF MS analysis was performed on an Ultraflex III TOF–TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) in reflectron mode, using an MTP384 polished steel target (Bruker Daltonics), 2,5-dihydroxybenzoic acid, or sinapinic acid as a matrix; Laser: 500 shots and 25–30% power. The Circular Dichroism spectrum of peptide was recorded (0.1 mmol L^{-1} ferrocene peptide in 30% of TFE v/v aqueous solution) using a Jasco J-810 Circular Dichroism Spectrometer.

2.4. Immunization protocol, preparation of pre-immune and post-III immune serum samples

BALB/c mice (21 days) were immunized subcutaneously with an aqueous solution of peptide **2** (100 μg of peptide) in complete Freund's adjuvant for the first dose (day 1) and boosted twice with 100 μg of peptide **2** in incomplete Freund's adjuvant by the same route (days 21 and 41).

Animals were bled via the tail vein on days 0 (which corresponds to a pre-immune serum used as a control), 20, 40 and 60 (post-I, II, III immune serum samples, respectively). Blood samples were centrifuged and the serum was collected and stored at −20 °C before use.

Serum samples of mice were weighed and placed in homogenization buffer (0.1 mol L^{-1} PBS, pH = 7.4, 4 °C) at a ratio of 10 μg serum sample per milliliter (stock solution). Serum solutions were prepared by dilution of serum stock solution with PBS. Samples were homogenized in a vortex mixer and ultrasonic bath (10 min).

Electrochemical experiments were carried out with two different kinds of samples and both of them were obtained from serum extracted from mice: a sample corresponding to mice contaminated with the polypeptide CGSPINNTKPHEARGC and another one corresponding to the control (samples from mice which were not injected with the polypeptide CGSPINNTKPHEARGC).

2.5. Preparation of the *1AFc* modified electrode

9.0 mg of **1AFc** was added to 0.25 mL of a Nafion/DMF (1:1) mixture, and this solution was ultrasonicated and shaken in a vortex mixer for ~30 min to form a uniform yellow solution. Prior to the surface modification, the bare gold electrode was polished with 0.3 and 0.05 μm alumina slurries and washed with deionized water and alcohol several times. The drop-casting method was used to modify the gold electrode surface. Firstly, 4.5 μL (3 times × 1.5 μL aliquot) of **1AFc** solution (5.6%, m/v) was added to the gold surface. Then, the solvent was evaporated under a hot air flow in 10 min. After the solvent was dried and following the surface modification, the **1AFc** modified electrode was transferred to a desiccator for 1 day prior to use.

3. Results and discussion

3.1. Characterization of *1AFc*

The chromatographic profile of the purified **1AFc** shows a main peak at t_R = 17.60 min (Fig. 1A). The MALDI-TOF MS spectrum (Fig. 1B)

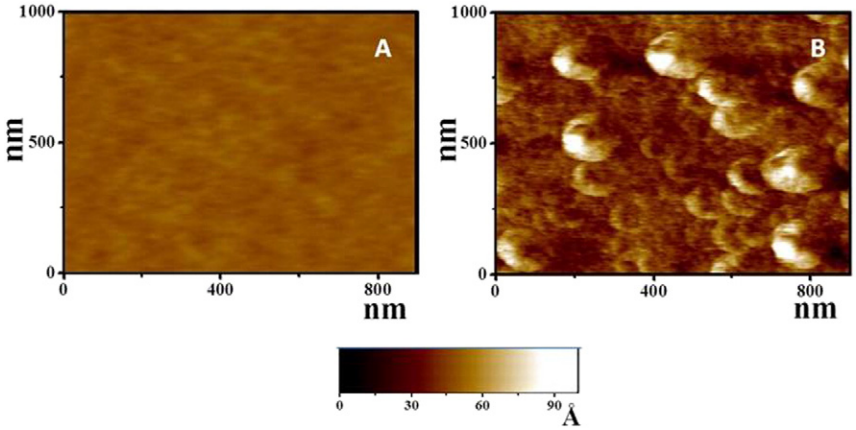

Fig. 4. SEM images of a bare gold surface before (A) and after modification with a film of **1AFc** (B).

shows a signal at m/z = 1687.8, corresponding to $[M + H]^+$ (theoretical value = 1687.79). Signal at m/z = 1567.8 corresponds to the product without a cyclopentadienyl ring and an iron atom. Signal at m/z = 1475.8 corresponds to the product without ferrocene. Circular Dichroism spectra (Fig. 2) of both the designed peptide and its control present a single negative band at 200 nm that is characteristic of unordered peptide structure (random coil). This analysis indicates that the ferrocene residue does not induce changes at the peptide secondary structure.

3.2. Electrochemical and morphological characterization of the **1AFc** modified electrode

The voltammetric behavior of the **1AFc** modified electrode was studied in 0.1 mol L^{-1} PBS, pH = 7.4. Fig. 3 shows voltammograms (2nd, 20th and 50th) recorded at 0.10 V s^{-1} and a pair of defined peaks corresponding to the redox behavior of the ferrocenyl group in the peptide can be noticed. The anodic (E_{pa}) and cathodic (E_{pc}) peak potential values are located at 0.35 and 0.24 V, respectively. Peak current values increased upon continuous potential cycling, but significant changes in the current (>5%) were not observed after the 50th cycle. The growth of the current between each cyclic voltammetry experiment is connected with the electron transfer between **1AFc** and the gold surface and can be understood as a possible charge-transport mechanism in the Nafion polymer and the physical diffusion of **1AFc** in the Nafion matrix, as proposed by White et al. [26].

The storage stability of the **1AFc** modified electrode was investigated by keeping the electrode at 25 °C in a desiccator under vacuum when the electrode was not in use. Cyclic voltammograms were daily recorded using the same electrode and the peak current decreased with the increase of storage time. However, the electrode could still retain about 72% of the initial response after storage for 12 days in the desiccator under vacuum. Such results indicate that the electrode has good stability.

SEM images of the sensor before and after surface functionalization are shown in Fig. 4A and B, respectively, and one can see that the gold surface changed from aligned strands (Fig. 4A) to ordered aggregates (Fig. 4B). In Fig. 4B, the spherical like structures that appear after functionalization correspond to the immobilized **1AFc** peptide.

3.3. Electrochemical behavior of the **1AFc** modified electrode in serum samples

Antibodies are made in animals as part of the immune response to various antigens by passive immunization or stimulation by exposure to small amounts of antigen [27]. The immune response resulting in levels of polyclonal antibodies is responsible for maintaining tolerance produced by injections of antigen (peptide **2**) in mice. Several and very low doses of peptide **2** induced the increase of the polyclonal antibody concentration in the mice. This suggested that the antigen itself might be useful for detecting an active immune response in mice by subcutaneous injections of doses of the antigen. The immobilization of peptide **1** on a gold electrode was used as a tool to identify the different responses between pre-immune (control) and post-III serum samples.

The **1AFc** modified electrode was allowed to interact with pre-immune serum (control) for around 20–30 min, typical times for a stable electrochemical response to be reached. Owing to the high stability of the antigen–peptide conjugate, the **1AFc** modified electrode is single use. Hence, in order to minimize problems arising from the different responses of the fabricated devices, all current measurements in serum sample solutions were normalized by using the expression I/I_P, where I is the peak current (at 0.35 V) in the serum solution and I_P is the peak current in PBS after 50 voltammetric cycles. Fig. 5 shows a plot of the normalized current (I/I_P) as a function of applied potential for different concentrations of the control, measured as micrograms of sample by liter of solution (μg L^{-1}). The red line in Fig. 5 corresponds to the electrochemical behavior of the biosensor in the absence of the control. As

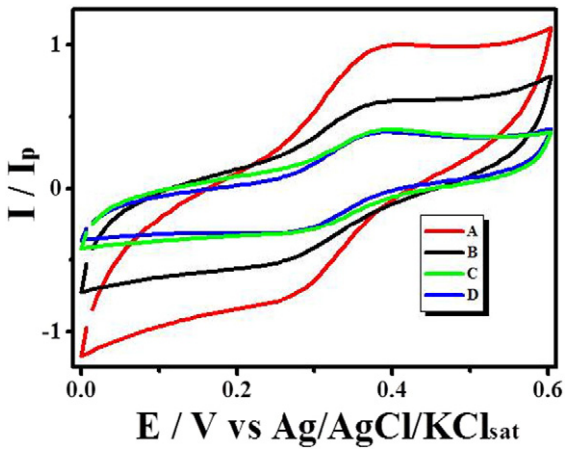

Fig. 5. Cyclic voltammograms recorded with the **1AFc** modified electrode in 0.1 mol L^{-1} PBS, pH 7.4, before (A) and after addition of pre-immune serum to give the following final concentrations in μg L^{-1}: 0.010 (B), 0.050 (C) and 0.10 (D). Each experiment was performed with a different modified electrode. Scan rate: 0.10 V s^{-1}.

it can be clearly seen in this figure, the presence of the serum samples in the PBS electrolyte at increased concentrations causes a decrease in the I/I_P value until a concentration of 0.10 μg L^{-1} is reached. No further reduction in the I/I_P value was noticed for more concentrated pre-immune serum solutions.

From cyclic voltammograms recorded in the presence of the control at different dilutions, a plot of normalized current peak response as a function of the concentration was constructed in order to assess the influence of amount of serum on the electrochemical response of the Fc-peptide modified electrode. The black points in Fig. 6 refer to data obtained with the control at different concentrations. Two main conclusions can be drawn: 1) there is a significant influence of serum components on the **1AFc** modified electrode response for concentrations below 0.01 μg L^{-1} and 2) a constant I/I_P value is obtained for concentrations up to 0.1 μg L^{-1}, which is an indication that the system is working under saturation conditions and the matrix reaches its maximum effect.

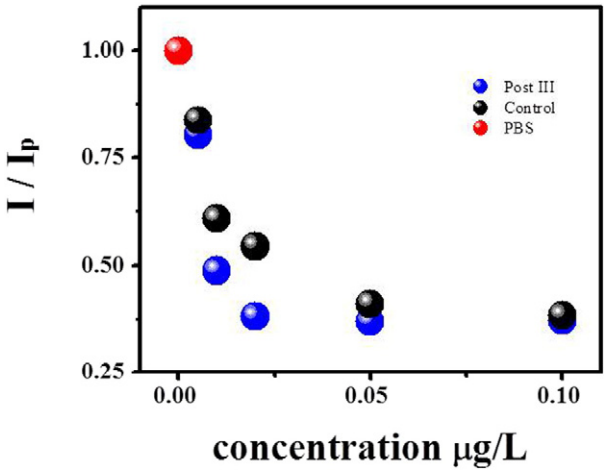

Fig. 6. Ratio of normalized peak current responses measured with the **1AFc** modified electrode in 0.1 mol L^{-1} (pH = 7.4), pre-immune (control) and post-III serum samples at different concentrations.

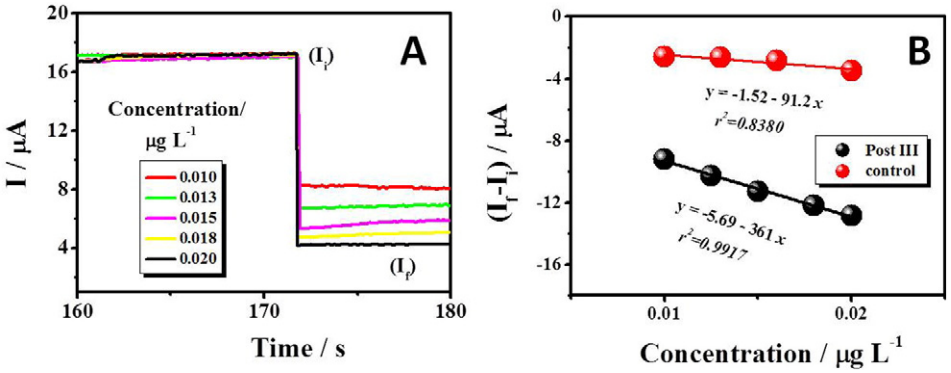

Fig. 7. A) Typical amperometric responses for the biosensor in the presence of different concentrations of post-III serum in PBS (pH 7.4). E = 0.55 V. B) Calibration plots are shown in the right for post-III (black) and pre-immune (red) serum (control).

In order to investigate the specificity of the proposed biosensor, the electrochemical behavior of the proposed **1AFc** modified electrode was investigated with post-III serum samples at different concentrations (voltammograms not shown). No significant changes in the voltammograms were observed after incubation of the fabricated biosensor in such serum samples, but a more significant decrease in the I/I_P value was noticed (blue points in Fig. 6) in comparison to data obtained with the control (pre-immune serum). The curve is not a linear one, in agreement with results commonly reported for immunoassay tests [28]. A similar trend was noticed in the previous experiment with pre-immune serum, i.e., the influence of the matrix is more significant at a certain dilution range and no significant changes were observed for more concentrated post-III serum samples. Notwithstanding, the unambiguous change in I/I_P values for concentrations between 0.01 and 0.02 μg L^{-1} confirms the specific response of the proposed electrochemical biosensor with respect to another antibodies or proteins that can react with the surface sensing (matrix effect).

From cyclic voltammetry results it has been shown that the proposed biosensor operates in the 0.01 and 0.02 μg L^{-1} concentration range, an experimental condition where electrochemical responses differ significantly for post-III and pre-immune samples. In a subsequent experiment, amperometry was also used as a tool for the determination of polyclonal antibodies under the same concentrations. Fig. 7A shows typical amperometric experiments carried out under optimal conditions and the initial current (I_i) response measured at a working potential of 0.55 V corresponds to the steady response involving the ferrocenyl group electron-transfer process. The I_i value was reproducible within 9% of relative error when a solution containing only 0.1 mol L^{-1} PBS was used. Then, post-serum III samples were added to the electrochemical cell, resulting in a prompt current change until a steady state was reached (I_f). The signal decrease noticed as the amount of post-III serum was increased represents the inhibition effect caused by the polyclonal antibodies. A linear relationship between the current difference ($I_f - I_i$) and the concentration of post-III serum sample in the range 0.010–0.020 μg L^{-1} was found, as shown in Fig. 7B.

To investigate the selectivity of the biosensor response, the amperometric experiment was repeated with pre-immune serum (control). The extent of the inhibition amperometric response obtained for pre-immune serum was compared to that of post-III serum and used as a criterion for the selectivity of the sensor [28,29]. By comparing slope values of both straight lines shown in Fig. 7B, one can clearly see that the response of polyclonal antibodies in post-III serum is almost four times higher than that for the control sample. Hence, the selectivity of the **1AFc** modified electrode based on its high specific antigen–antibody immune reaction can be considered satisfactory.

4. Conclusions

An electrochemical biosensor with ferrocene–amin–hexanoic–peptide (Fc–*Ahx*–SPINNTKPHEAR, **1AFc**) immobilized onto a gold electrode was developed. **1AFc** was obtained by solid phase peptide synthesis by using the Fmoc/tBu strategy. The proposed platform ensures minimization of interference effects when biological media are analyzed. The **1AFc** and (peptide–antibody) affinity reaction results in significant changes in the voltammetric and amperometric response of the developed Fc-peptide modified electrode, allowing ultralow and specific information on the amount of peptide–antibody in post-III serum samples to be obtained. This new approach envisages a great future for this type of HPV biosensor and the possibility of miniaturization and ease of integration with electronic instrumentation may lead to point of care testing platforms with wide applications.

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