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Development of a very sensitive electrochemical immunosensor for the determination of 17β-estradiol in bovine serum samples

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ABSTRACT

This study describes the development of a very sensitive electrochemical immunosensor (EI) for the determination of 17β-estradiol (17β-E). The novelty of this immunosensor is that the detection of 17β-E is carried out without sample pretreatment and unlabeled neither the antigen nor the antibody. Very good results in terms of sensitivity, kinetics, and working range are obtained. The immunosensor was constructed by immobilization of the anti-17β-E monoclonal antibody (mAbE) on a gold disk electrode modified with gold nanoparticles on a cysteamine self-assembled monolayer (AuNP-cys-Au disk). Bovine serum samples were spiked with known amounts of 17β-E and incubated on mAbE-AuNP-cys-Au disk electrodes. Then, the EI was transferred to pH 5.00 citrate buffer solutions, containing horseradish peroxidase (HRP), pyrocatechol (H_2Q), and H_2O_2 at given concentrations. The 17β-E and H_2Q, both enzyme co-substrates, react with HRP. The HRP, which did not react with 17β-E, in the presence of H_2O_2 catalyzes the oxidation of H_2Q to o-benzoquinone (Q). The back electrochemical reduction of Q to H_2Q was detected on the modified gold electrode surface by square wave voltammetry. The electrochemical signal was proportional to the amount of H_2Q that reacts with the enzyme, and inversely proportional to the amount of 17β-E presents in the bovine serum samples. The EI showed a linear range from 0.54 to 1.36×10^4 pg mL^{-1}. The limit of detection (LOD) was 0.84 pg mL^{-1}. Recovery percentages were very good, with values of 99.7, 106, and 105% for 10, 50 and 100 pg mL^{-1}, respectively. This EI is an attractive tool for the 17β-E determination in bovine serum samples.

1. Introduction

The natural estrogen with the highest estrogenic activity in mammals is 17β-estradiol (17β-E), followed by estrone and estriol. The importance of these hormones is that their concentration levels affect the health of mammals [1]. Estrogens are endogenous hormones that produce several physiological effects. These effects in women include actions related to the development, neuroendocrine problems in the control of ovulation, preparation for fertilization, and implantation in the reproduction. The main effects are those generated on carbohydrates, proteins, and lipids metabolism.

Monitoring the 17β-E levels in women is an indicator of the activity of ovaries. This monitoring allows to diagnose menstrual dysfunctions, and thus, to detect the states of hypoestrogenism and menopause. In the normal menstrual cycle, 17β-E levels are typically less than 50 pg mL^{-1} during menstruation, increase with follicular development (maximum 200 pg mL^{-1}), fall slightly during ovulation, and increase again during the luteal phase to a second maximum. During the menstrual cycle, at the end of the luteal phase, the 17β-E level decreases unless pregnancy is reached [2]. 17β-E is used in veterinary medicine to heal, and to prevent animal infections [3]. However, 17β-E is also used illegally in livestock production for growth promotion purposes because of its anabolic properties [4].

The traditional method for monitoring and routine screening of 17β-E is radioimmunoassays (RIA) [5–7]. Although this method is sensitive and reliable, it suffers from problems associated with the use of radioisotopes and the restriction to use it on the test site, i.e., it is not possible to use this technique outside specially equipped laboratories. Due to environmental and clinical importance of steroids, numerous strategies have been developed to achieve their analytical determinations. Usually, biological samples are analyzed by gas or HPLC chromatography's coupled to

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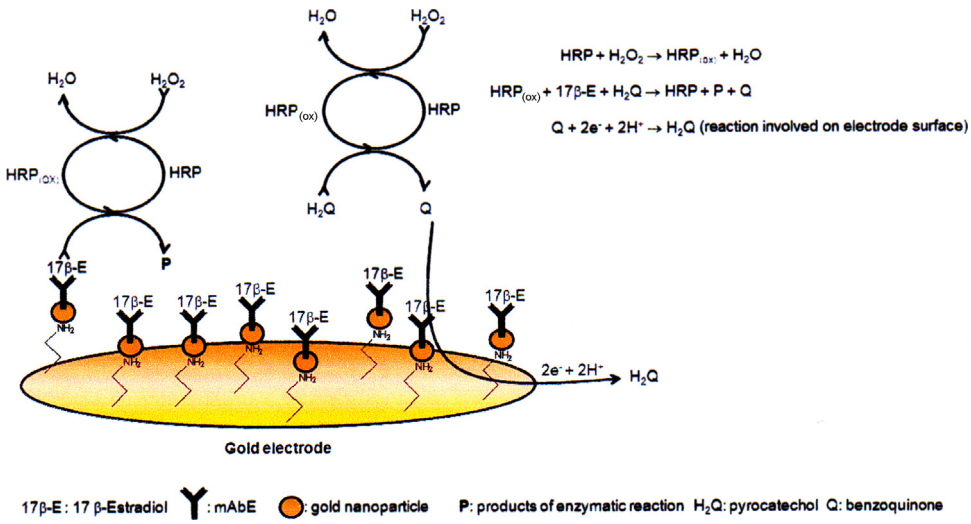

Scheme 1. Schematic representation of the 17β-E electrochemical immunosensor.

a mass spectrometer (GC-MS and HPLC-MS, respectively) [8–14]. These methods have the disadvantages that the samples require pretreatment and derivatization.

In recent years, the interest has focused on the development of electroanalytical techniques for steroid detections [15–18]. The selective and sensitive detection of steroids can be carried out by combining the advantages of electroanalytical techniques with the properties of nanomaterials, antibodies, enzymes, aptamers, etc. Yuan et al. [19] have developed an electrochemical sensor for the detection of 17β-E based on molecular imprinted polymer membranes.

Electrochemical immunosensors are one of the most powerful analytical tools that have been developed. These devices use electrodes modified with antibodies. Electrochemical immunoassays have been reported for the detection of 17β-E using an ELISA-style format [20,21]. Volpe et al. [21] found a limit of detection of 15 pg mL^{-1}, and a determination total time of 147 min. A disadvantage of this technique is that it requires an extensive pretreatment of the sample. Moreover, different immunosensors have been developed for the detection of 17β-E in human serum samples [22]. Liu and Wong [23] have developed an immunosensor for the detection of 17β-E with a limit of detection of 3.5 pg mL^{-1} and a determination total time of 120 min for each assay. Ojeda et al. [24] reported an electrochemical immunosensor for the detection of 17β-E using a carbon screen printed electrode, being the detection principle based on a competitive immunoassay, reaching a limit of detection of 0.77 pg mL^{-1}, and a total time of 175 min for each determination. Recently, a method based on anodic stripping differential pulse voltammetry has been reported for the determination of 17β-E using CdSe quantum dots, and an indirect competitive immunoassay, reaching a detection limit of 52.5 pg mL^{-1} [25]. In ELISA-type immunoassays of small molecules, the analyte competes for binding to a specific antibody with a tracer compound. The tracer typically consists of a structurally related molecule (competing hapten) that provides the binding site, and a catalytic molecule that generates the signal [26–28]. Synthesis or labeling of the hapten are time consuming, since the performance of the assay is greatly influenced by several factors related to the preparation of these conjugates, the final hapten/tracer ratio, the effect of the conjugation chemistry on the tracer enzyme activity, and the need for a careful purification of the conjugate from non-conjugated reactants [29]. Recently, Li et al. [30] have developed an electrochemical immunoassay for the 17β-E detection using graphene–polyaniline (GR-PANI) composites and carboxylated graphene oxide. Carboxylated graphene oxide was used as the carrier of the enzyme and antibody at a high ratio. GR–PANI composites were used to amplify responses of the immunosensor and a detection limit of 20 pg mL^{-1} was obtained. It has successfully been used in the detection of 17β-E in water and milk samples. In addition, a molecularly imprinted electrochemical sensor for the rapid detection of 17β-E in milk samples was reported using glassy carbon electrode (GCE) modified with gold nanoparticles (AuNP) and molecular imprinted polymer (MIP) [31]. AuNP were electrodeposited on the surface of GCE and used to increase the electrode surface area. Besides, the sensor signal was amplified with p-aminothiophenol combined with AuNP through Au-S bonds. The detection limit was 1.28 pg mL^{-1}.

In this work, we report a simple, and very sensitive electrochemical immunosensor (EI) to quantify 17β-E in bovine serum samples, without sample pretreatment and unlabeled neither the antigen nor the antibody. The EI was constructed by immobilization of the anti-17β-E monoclonal antibody (mAbE) on a gold disk electrode (Au disk) modified with AuNP on a cysteamine self assembled monolayer (AuNP-cys-Au disk). Bovine serum samples were spiked with known concentrations of 17β-E and incubated on mAbE-AuNP-cys-Au disk electrodes. Then, the EI was transferred to an electrochemical cell containing pH 5.00 citrate buffer solutions; where given amounts of horseradish peroxidase (HRP), pyrocatechol (H_2Q) and H_2O_2 were added. The 17β-E and H_2Q are both enzyme co-substrates. The HRP, in the presence of H_2O_2, catalyzes the oxidation of both the 17β-E to a given product, and the H_2Q to benzoquinone (Q). The electrochemical reduction of Q to H_2Q was detected on the modified gold electrode surface (mAbE-AuNP-cys-Au disk) by square wave voltammetry (SWV) (Scheme 1). The electrochemical response is proportional to the amount of H_2Q that reacts with the enzyme, and inversely proportional to the amount of 17β-E in bovine serum samples. Therefore, the maximum electrochemical response was obtained in the absence of 17β-E at the electrode surface for a given H_2Q concentration. This electrochemical immunosensor showed a very high sensitivity to determine trace levels of 17β-E in bovine serum samples, compared to other conventional techniques.

2. Materials and methods

2.1. Chemicals and immunochemicals

17β-Estradiol (17β-E), progesterone (P4), estrone (E1), estriol (E2), cysteamine (cys), anti 17β-estradiol sheep monoclonal

antibody (mAbE), horseradish peroxidase (HRP) (E.C:1.11.1.7, H_2O_2-oxide-reductase), and pyrocatechol (H_2Q) were purchased from SIGMA. Gold nanoparticles were synthesized using gold (III) chloride hydrate ($HAuCl_4$), and sodium borohydride ($NaBH_4$), both from SIGMA. All reagents were used as received. The following buffer solutions were prepared from their salts (Merck, p.a.): 1×10^{-2} mol L^{-1} phosphate, 0.137 mol L^{-1} NaCl and 2.7×10^{-3} mol L^{-1} KCl (pH 7.00, PBS); 5×10^{-2} mol L^{-1} citrate, 5×10^{-2} mol L^{-1} phosphate, (pH 5.00, CBS), and pH 7.00 PBS containing 0.05% Tween 20 (PBST). Ethanol, H_2O_2, and H_2SO_4 were Merck p.a. Toluene and water were Sintorgan, HPLC grade. Certified bovine serum samples containing 3.35 pg mL^{-1} of 17β-E, were gently supplied by the Facultad de Agronomía y Veterinaria, Universidad Nacional de Río Cuarto, and used without pretreatment.

2.2. Apparatus and electrodes

Electrochemical measurements were performed in a Teflon microcell. The cell operates with a volume of 200 μL. The working electrode was a polycrystalline gold disk (BAS, 1.6 mm diameter). Previous to perform the experiments, the electrode was successively polished on BAS™ cloth with diamond paste of 15, 3 and 1 μm and then, polished with wet alumina powder (0.3 and 0.05 μm, from Fischer), rinsed copiously with water and sonicated in a water bath for 2 min. Then, it was immersed in a solution of $H_2SO_4 + H_2O_2$ (3:1 v/v) during 5 min. Finally, the gold disk electrode was activated in 0.5 mol L^{-1} H_2SO_4 by cyclic voltammetry (CV) in a potential range from −0.2 to 1.6 V vs CSE, at a scan rate of 0.1 V s^{-1} until a typical voltammogram of a polycrystalline Au clean surface was obtained [32]. Then, it was rinsed with water and ethanol, and dried by a stream of N_2 before performing the thiol monolayer adsorption process. The counter electrode (CE) was a platinum foil. A calomel saturated electrode (CSE) or a silver (Ag) wire were used as reference or pseudo-reference electrodes, respectively.

The measuring system for performing SWV and CV was an Autolab PGSTAT 12 potentiostat run with the GPES software, version 4.9 (Eco-Chemie, Utrecht, The Netherlands). All SWV measurements were performed in the potential range from 0.1 to −0.2 V vs Ag wire, with square wave amplitude (ΔE_{SW}) of 0.025 V, a staircase step height (ΔE_S) of 0.005 V, and a frequency (f) of 25 Hz. These values of ΔE_{SW} and ΔE_S are commonly used for heterogeneous electronic transfers of $2e^-$ [33], because the oxidation of H_2Q to Q is a two-electron quasi-reversible redox process [34]. Atomic force microscopy (AFM) measurements were made with an Agilent 5420 AFM/STM microscope. A commercial Point Probe® Plus Non-Contact/Tapping Mode - Long Cantilever (PPP NCL) with a force constant 6 N/m and resonance frequency 156 Hz was used. Absorbance measurements were performed by a Hewlett-Packard spectrophotometer, Model 8452A, equipped with a temperature controller. pH measurements were carried out with a HANNA instruments, Bench Meters, model pH 211, Romania. Each stage of immunoassays was incubated to 37 °C using a NEO LINE stove, Argentina.

2.3. Gold nanoparticles preparation

Gold nanoparticles (AuNP) were prepared through a method developed by Bethel et al. [35], with minor modifications. An aqueous solution of 5.3×10^{-2} mol L^{-1} $NaBH_4$ was slowly added, under continuous stirring, to a solution containing 1.15×10^{-3} mol L^{-1} $HAuCl_4$ in toluene, and maintained for 2 h in the dark. Then, the organic phase was removed, and washed three times with a small portion of water. The aqueous phases were collected. They showed a wine-red color. The AuNP were stored in a dark glass bottle at 4 °C for further use. The AuNP solutions were very stable and did not show any sign of aggregation or other deterioration over periods of months. AFM and UV-Vis spectroscopy were used to determine the diameter of AuNP. The UV- Vis spectroscopy showed a plasmon resonance surface band at 538 nm indicating an average size of nanoparticles of 65 nm [36].

2.4. Cysteamine self-assembled monolayers on the gold electrode

Cysteamine self-assembled monolayers (SAMs) modified electrodes were prepared by immersing the clean gold disk electrode in 0.1 mol L^{-1} fresh thiol solutions in ethanol during 2 h. After adsorption, modified electrodes were thoroughly rinsed with ethanol and water.

2.5. 17β-E and AuNP immobilization onto cys-Au disk electrode

It is known that AuNP allow a better immobilization and orientation of proteins due to their interactions with amine and sulfhydryl groups present in protein chains, which makes the direct electron transfer more favorable [37]. For this reason, the construction of EI consisted in the immobilization the mAbE on the surface of the gold electrode modified with cysteamine SAMs and AuNP. Therefore, cys-Au disk electrode was immersed in an AuNP solution during 30 min at room temperature. AuNP were chemisorbed on cysteamine SAMs forming an AuNP-cys-Au disk electrode.

The AuNP-cys-Au disk electrode was washed three times with water and PBS. Then, 10 μL mAbE solutions (optimal dilution, see below) were dropped on AuNP-cys-Au disk electrode surface and incubated overnight at 4 °C in order to generate the EI (mAbE-AuNP-cys-Au disk electrode). Before use, the EI was washed with PBS to remove the weakly absorbed antibodies. Once used, the EI was stored in the PBS at 4 °C.

The morphology of EI was analyzed by in situ high-resolution AFM measurements, for each stage of assembly (see below).

2.6. Assays with the electrochemical immunosensor

Unspecific bindings at the mAbE-AuNP-cys-Au disk electrode were avoid by a treatment at 37 °C with 3% low-fat milk in PBS during 10 min and then washed with PBST. Thus, the AuNP free were prevented from interacting with sample components and the HRP added in the detection stage. Aliquots of 10 μL of solutions containing different 17β-E concentrations were dropped on the EI and incubate at 37 °C during 30 min and then, rinsed with PBS. Finally, the EI was transferred to the cell and 200 μL of solutions containing $HRP + H_2O_2 + H_2Q$ in CBS at different concentrations were added. After 10 min, the enzymatic reaction product (Q) was detected by SWV. The total time of immunoassay was 55 min. For next determination, the EI was reconditioned by desorption of 17β-E in a 0.1 mol L^{-1} glycine - pH 2.00 HCl solution during 2 min and, then, washed with PBS. The same electrode was used over about 100 determinations. Desorption efficiency was checked in a blank solution containing HRP, H_2O_2 and H_2Q, where a maximum was obtained for the Q reduction current.

2.7. Assays cross-reactivity

A study of the cross-reactivity between 17β-E and progesterone (P4), estrone (E1) and estriol (E2) was performed. Therefore, different solutions containing 1 ng mL^{-1} of P4, E1 and E2 were prepared in the absence and in the presence of 10 pg mL^{-1} of 17β-E. Then, 10 μL of each solution was dropped on the EI, incubated at 37 °C during 30 min and rinsed with PBS. Then, the EI was transferred to the electrochemical cell and 200 μL of the solution containing 7.8×10^{-11} M HRP + 5×10^{-3} M H_2O_2 + 2×10^{-3} M H_2Q in CBS was added. After 10 min, the enzymatic reaction product (Q) was detected by SWV. A similar procedure was carried

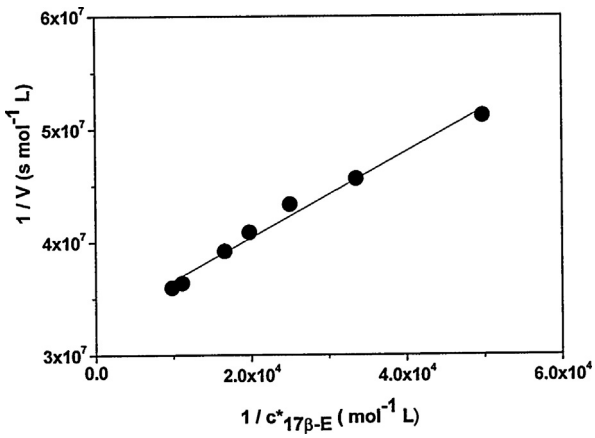

Fig. 1. Dependence of the reciprocal of the initial velocity with the reciprocal of the concentration of 17β-E. $c^*_{H_2O_2} = 2.26 \times 10^{-3}$ mol L^{-1}, $c^*_{HRP} = 3.26 \times 10^{-9}$ mol L^{-1}. Intercept: $(3.27 \pm 0.01) \times 10^7$ s mol^{-1} L, slope: (381.2 ± 0.2) s, $r = 0.9985$.

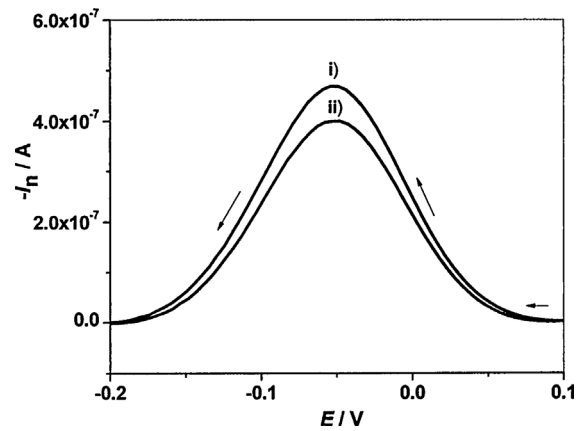

Fig. 2. Square wave voltammograms for the reduction of Q enzymatically generated in pH 5.00 CBS recorded at mAbE-AuNP-cys-Au electrode without (i), and with 1.36 ng mL^{-1} 17β-E incubated on the electrode surface (ii), obtained with the non-optimized EI parameters. $c^*_{H_2O_2} = 1 \times 10^{-3}$ mol L^{-1}, $c^*_{H_2Q} = 1 \times 10^{-3}$ mol L^{-1}, $c^*_{HRP} = 3 \times 10^{-9}$ mol L^{-1}. Reference electrode: Ag wire. $\Delta E_{SW} = 0.025$ V, $\Delta E_S = 0.005$ V, $f = 25$ Hz. The arrows indicate the direction of potential sweep.

out on the EI, which was dropped with a solution containing 10 pg mL^{-1} 17β-E + 1 ng mL^{-1} P4 + 1 ng mL^{-1} E1 + 1 ng mL^{-1} E2. All experiments were performed by triplicate.

3. Results and discussion

3.1. HRP activity toward 17β-E

17β-E is a phenolic compound with a hydroxyl group at carbon 3. It is well known that the phenolic compounds are co-substrates of HRP [36]. This behavior was studied by UV-Vis spectroscopy. Therefore, 17β-E UV–Vis spectra recorded in electrolytic solutions at different times of the enzymatic reaction were analyzed. Absorbance values at 280 nm increased as the reaction time was increased, which indicates a gradual apparition of product/s of the enzymatic reaction. From these experiments performed at different 17β-E concentrations ($c^*_{17\beta\text{-E}}$) and at a given H_2O_2 concentration, the Michaelis–Menten apparent constant (K_M) was calculated. Thus, from a plot of 1 V^{-1} vs $1/c^*_{17\beta\text{-E}}$ (Lineweaver–Burk plot [38], Fig. 1), where V is the enzymatic reaction rate, a value of $K_M = 1.14 \times 10^{-5}$ mol L^{-1} was obtained [38]. These results show that HRP is able to recognize 17β-E as co-substrate in homogeneous media. Then, it was necessary to check if 17β-E immobilized at the EI remains as enzyme co-substrate. Therefore, the affinity of HRP toward 17β-E was also evaluated by SWV. Fig. 2i shows a SW voltammogram recorded at the EI in a solution of HRP + H_2O_2 + H_2Q without 17β-E. Fig. 2ii shows a SW voltammogram recorded after incubating the EI in a solution with 17β-E. A higher net peak current ($I_{p,n}$) was observed in the absence of 17β-E (Fig. 2i), indicating that the HRP catalyses the oxidation of H_2Q to Q. However, the $I_{p,n}$ decreased when 17β-E formed the immunocomplex (Fig. 2ii), showing clearly that HRP reacts with both 17β-E and H_2Q. It was observed that the $I_{p,n}$ decreased as the 17β-E concentration increased (results no shown). Minor variations (about 5%) in $I_{p,n}$ for the same H_2Q concentration were obtained in the absence of HRP for different concentrations of 17β-E incubated at the EI surface, confirming that $I_{p,n}$ changes are due to the reaction of 17β-E with HRP.

3.2. Characterization of mAbE-cys-Au disk electrode

Cysteamine has been used as a platform suitable for the immobilization of antibodies [39–41]. We used CV to study each stage during the development of EI. Fig. 3 shows the cyclic voltammograms of 1×10^{-3} mol L^{-1} H_2Q in pH 5.00 CBS recorded in the

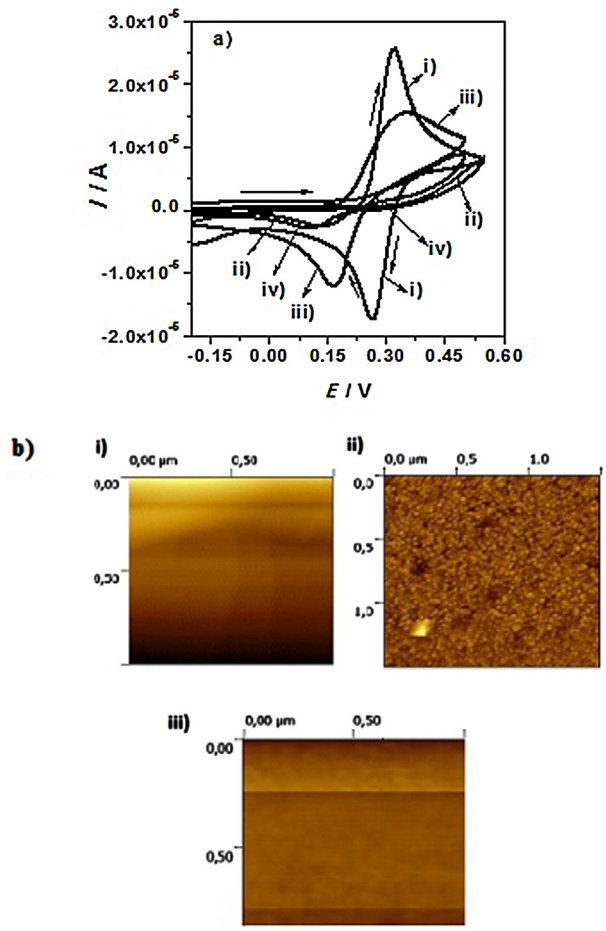

Fig. 3. (a) Cyclic voltammograms recorded for H_2Q in pH 5.00 CBS at: (i) the bare Au disk; (ii) the cys-Au disk; (iii) the AuNP-cys-Au disk, and (iv) the mAbE-AuNP-cys-Au disk electrodes. The arrows indicate the direction of potential sweep. Reference electrode: CSE. $v = 0.1$ V s^{-1}. (b) AFM images of (i) bare Au surface (ii) AuNP-cys-Au surface, and (iii) mAbE-AuNP-cys-Au surface.

potential range from −0.2 to 0.5 V vs CSE. At the bare gold disk electrode (Fig. 3a.i), the cyclic voltammogram showed a well-defined anodic peak and its corresponding cathodic peak, characteristic of a two-electron quasi-reversible redox couple [34]. The H_2Q electron transfer kinetics was disturbed at the cys-Au disk modified

electrode (Fig. 3a.ii). As can be observed, while the H_2Q oxidation peak practically disappears at the SAMs modified electrode, a small reduction peak is observed in the reverse scan. An increase in both the oxidation and reduction currents was obtained at the AuNP-cys-Au disk electrode (Fig. 3a.iii) in comparison with the SAMs modified electrode. This behavior suggests that the blockade of the electron transfer process by SAMs was clearly restored at the AuNP-cys-Au disk electrode. The reversibility of the H_2Q redox couple at the AuNP-cys-Au disk electrode was also improved. A significant decrease in the H_2Q oxidation current and an increase in the separation between the anodic and cathodic peak potentials were also observed when the mAbE was incubated at AuNP-cys-Au disk electrode (Fig. 3a.iv). However, a Q small reduction current is clearly observed at the EI during the reverse sweep potential (Fig. 3a.iv). This behavior enables the use of SWV in the detection step of the immunoassay.

On the other hand, the EI morphology was analyzed by in situ high-resolution AFM measurements during different stages in the modification process. Fig. 3b.i and b.ii show the bare Au surface and the cys-Au modified surface after depositing AuNP, respectively. It can be seen that the surface is covered with AuNP, which showed an average diameter of 65 nm, being this result in agreement with that obtained by UV-Vis spectroscopy [36]. Then, the Fig. 3b.iii shows clearly how the surface changes when the mAbE was immobilized at AuNP-cys-Au surface.

3.3. Optimization of the concentrations of species involved in the reaction of the immunosensor

The optimum concentration of mAbE was determinated by SWV for 1:100, 1:200, and 1:400 dilutions of commercial reagent in PBS. A great variation in $I_{p,n}$ was observed for different concentrations of 17β-E when the antibody concentration was higher (minor dilution factor) (data not shown). Therefore, a 1:100 dilution factor was chosen in order to achieve the best sensitivity. The enzymatic reaction conditions have been previously studied [42]. The maximum reaction rate was obtained in pH 5.00 CBS. On the other hand, as it is well known, 37 °C is the optimal temperature of immunoreaction for all IgG [26]. Therefore, all incubations were carried out at this temperature.

Fig. 4a shows the effect of varying the H_2O_2 concentration at given H_2Q (1.0×10^{-3} mol L^{-1}), HRP (1.5×10^{-10} mol L^{-1}) concentrations, and a concentration of 17β-E of 27 pg mL^{-1}. The optimal H_2O_2 concentration was 5×10^{-3} mol L^{-1}. We also studied the effect of H_2Q concentration at given concentrations of HRP, H_2O_2, and 17β-E. The optimal H_2Q concentration was 2×10^{-3} mol L^{-1} when HRP, H_2O_2, and 17β-E were 7.8×10^{-11} mol L^{-1}, 5×10^{-3} mol L^{-1} and 27 pg mL^{-1}, respectively (Fig. 4b). In addition, Fig. 4c shows the effect of varying the HRP concentration, at given concentrations of H_2Q, H_2O_2 and 17β-E (1×10^{-3} mol L^{-1}, 5×10^{-3} mol L^{-1} and 27 pg mL^{-1}, respectively). The optimal HRP concentration was 7.8×10^{-11} mol L^{-1}. These optimal concentrations of H_2O_2, H_2Q and HRP were then used for all next experiments.

3.4. Calibration curve for 17β-E. Analytical performance

A dose-response titration curve for 17β-E was carried out in the concentration range from 3×10^{-3} to 2.7×10^5 pg mL^{-1} (Fig. 5). Values of $I_{p,n}$ correspond to the reduction of Q enzymatically generated, which are indirectly proportional to the amount of 17β-E present. The calibration curve obtained under the optimal conditions showed a linear response over a wide range, i.e. from 0.54 to 1.36×10^4 pg mL^{-1}. The calibration curve was constructed as $I_{p,n}/I^o_{p,n}$ vs $c^*_{17\beta\text{-E}}$, where $I^o_{p,n}$ is the net peak current obtained in

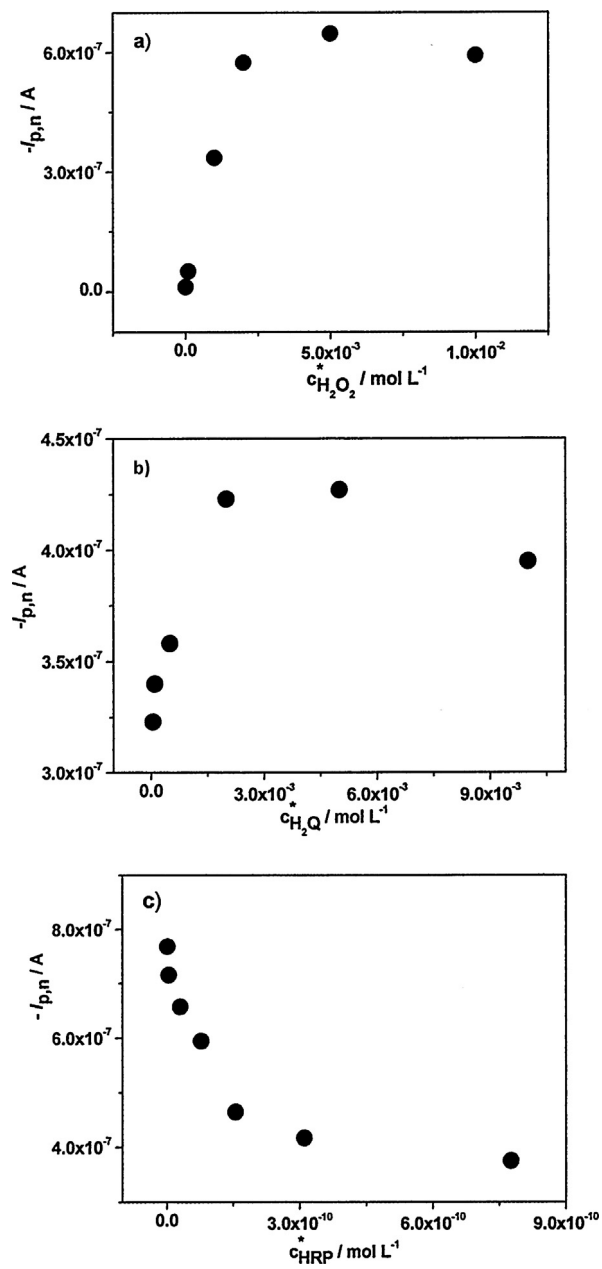

Fig. 4. Variation of the $I_{p,n}$ for a 27 pg mL^{-1} 17β-E solution at the EI as a function of:. (a) $c^*_{H_2O_2}$ ($c^*_{H_2Q} = 1.0 \times 10^{-3}$ mol L^{-1}, $c^*_{HRP} = 1.5 \times 10^{-10}$ mol L^{-1}); (b) $c^*_{H_2Q}$ ($c^*_{H_2O_2} = 5 \times 10^{-3}$ mol L^{-1}, $c^*_{HRP} = 7.8 \times 10^{-11}$ mol L^{-1}) and (c) c^*_{HRP} ($c^*_{H_2O_2} = 5 \times 10^{-3}$ mol L^{-1}, $c^*_{H_2Q} = 1 \times 10^{-3}$ mol L^{-1}). pH 5.00 CBS. SW parameters are the same as those in Fig. 2.

absence of 17β-E and $I_{p,n}$ is the net peak current obtained for different 17β-E concentrations. Experimental points are the average of three replicated measurements obtained with different biosensors. The error bars showed in Fig. 5 indicate a good reproducibility. The limit of detection (LOD), calculated as the concentration of 17β-E which produces a decrease in signal equal to three times the standard deviation of the blank was 0.84 pg mL^{-1} [43]. The LOD is lower than that of most the LOD found for other sensors for 17β-E in literature [21–24,28–31]. Besides, this EI has the advantage that the time of each assay (55 min) is shorter than those obtained with other sensors described in the literature [21–24,28].

The within-assay precision of EI was tested for 17β-E standard solutions of 50 pg mL^{-1} and 100 pg mL^{-1} in PBS measured by triplicate. Thus, percentage variation coefficients (VC %) were 2.0%

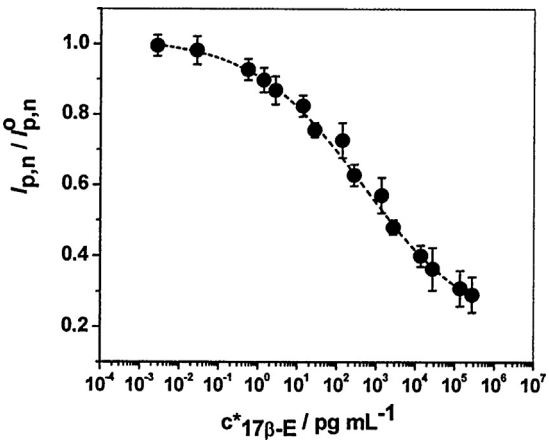

Fig. 5. Normalized calibration curves of 17β-E recorded using different EI for optimized parameters in pH 5.00 CBS. Each point is the average of three replicated measurements. $c^{*}_{H_2O_2} = 5 \times 10^{-3}$ mol L^{-1}, $c^{*}_{HRP} = 7.8 \times 10^{-11}$ mol L^{-1} and $c^{*}_{H_2Q} = 2 \times 10^{-3}$ mol L^{-1}. SW parameters are the same as those in Fig. 2.

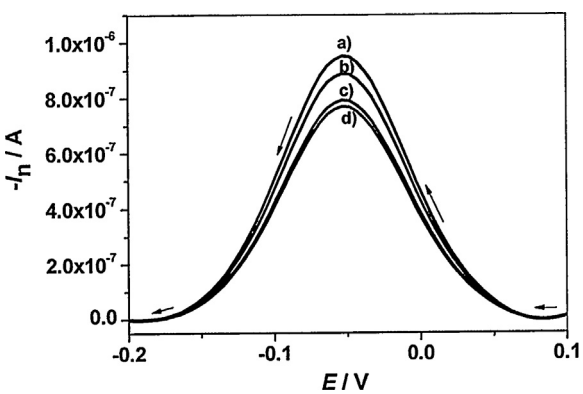

Fig. 6. Square wave voltammograms for spiked bovine serum samples in pH 5.00 CBS. The bovine serum sample contained 3.35 pg mL^{-1} of the 17β-E. Bovine serum (a) without 17β-E spiked; (b) 10 pg mL^{-1} 17β-E; (c) 50 pg mL^{-1} 17β-E; (d) 100 pg mL^{-1} 17β-E spiked. $c^{*}_{H_2O_2} = 5 \times 10^{-3}$ mol L^{-1}, $c^{*}_{HRP} = 7.8 \times 10^{-11}$ mol L^{-1} and $c^{*}_{H_2Q} = 2 \times 10^{-3}$ mol L^{-1}. Reference electrode: Ag wire. SW parameters are the same as those in Fig. 2. The arrows indicate the direction of potential sweep.

and 5.0%, respectively, showing a good repeatability. The precision inter-assays for the same concentrations did not exceed 5%.

Several tests were also carried out to assess the cross-reactivity of the 17β-E with other hormones structurally related such as P4, E1 and E2, as described in Section 2.7.

In the absence of 17β-E, an $I_{p,n}$ maximum was obtained due to the reduction of Q enzymatically generated, with a VC% of 2.5%.

In the presence of 10 pg mL^{-1} 17β-E + P4, 10 pg mL^{-1} 17β-E + E1 and 10 pg mL^{-1} 17β-E + E2 or a mixture thereof (17β-E with P4 + E1 + E2), lower $I_{p,n}$ was obtained (VC% of 4%) and its value was similar to that found when the solution contained only 17β-E on the EI. This demonstrates the high selectivity of EI to 17β-E. Therefore, if there is cross-reactivity, it would be within the percentage of variation of the detection method, which is less than 5%.

3.5. Determination of 17β-E in spiked bovine serum

The determination of 17β-E was carried out in bovine serum samples. As the samples originally contained 3.35 pg mL^{-1}, they were spiked with aliquots of a 17β-E solution prepared in PBS at a given concentration, minimizing dilution effects (≤1%). Therefore, three samples of bovine serum containing 10, 50, and 100 pg mL^{-1} of 17β-E were prepared (Fig. 6). It is important to emphasize that bovine serum samples were incubated in the immunosensor without any previous pretreatment. Recovery percentages were very good, with values of 99.7, 106, and 105% for 10, 50 and 100 pg mL^{-1}, respectively. The EI stability was tested for 25 days for a constant 17β-E concentration. It was found that current responses were constant during 21 days and then, they start to decrease gradually. The EI was regenerated as described in Section 2.6, which allowed us to use the biosensor over about 100 determinations. The regeneration was checked by measurements of net peak currents. They were reproducible, showing that the antibody activity loss is not appreciable. These measurements were performed using alternatively 17β-E standard solutions, and bovine serum samples.

4. Conclusion

An integrated electrochemical immunosensor was developed to determine 17β-E at trace levels in bovine serum samples. The determinations were performed without any pretreatment of samples. The immunosensor showed a high analytical performance in terms of an excellent limit of detection (0.84 pg mL^{-1}), high specificity, and an analytical range of interest, good reproducibility, and repeatability. The immunosensor developed can operate as a fast, selective, and sensitive detector. This device has several advantages over other methods for the determination of 17β-E in real samples, such as direct measurement without any pre-treatment, use of small volumes (harmful solvents and expensive reagents are avoided) and, mainly, without antigen or antibody labeled. The immunosensor also shows physical and chemical stability and a wide working concentration range. In addition, integrated approach makes it possible to consider a potential sensor miniaturization. Consequently, these features make this device an important analytical tool for the measurement of 17β-E in bovine serum, and potentially in other samples of interest.

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