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Integrated electrochemical immunosensor with gold nanoparticles for the determination of progesterone

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ABSTRACT

Integrated immunosensors are based on the spatial integration of the sensing layer and the detection system. In this study, we report an integrated immunosensor to determine progesterone (P4) in bovine serum samples. P4 is a steroidal hormone with a vital role in the maintenance of human and animal health. Therefore, an integrated immunosensor for the determination of P4 based on direct attachment of anti-progesterone monoclonal antibody (mAbP4) on a modified gold disk (Au disk) electrode with gold nanoparticles (Au_{NP}) lodged on a cysteamine (cys) self-assembled monolayer (mAbP4-Au_{NP}-cys-Au disk) is reported. The immunosensor is based on a competitive assay involving P4 labeled with horseradish peroxidase (HRP), which oxidizes pyrocatechol (H_2Q), in the presence of hydrogen peroxide (H_2O_2), to benzoquinone (Q). Its back electrochemical reduction to H_2Q is detected on the surface of mAbP4-Au_{NP}-cys-Au disk electrode. Different experimental variables involved in the immunosensor preparation such as the gold nanoparticles loading, the amount of mAbP4 immobilized, and the cysteamine immobilization time were optimized. The calibration curve showed an IC_{50} of 0.54 ng mL^{-1}, and a detection limit (DL) of 0.08 ng mL^{-1}, with a linear range from 8×10^{-2} to 7 ng mL^{-1}. Good percent relative standard deviations (% RSD) values were obtained for bovine serum samples containing 1 and 10 ng mL^{-1}, i.e., about 6%. The recovery values were 109.6 and 110.1% for 1 and 10 ng mL^{-1}, respectively. The assays are fast, selective and very sensitive. Thus, the immunosensor shows to be a very useful tool to determine P4 in bovine serum samples without any pretreatment.

1. Introduction

Progesterone (P4) is an unsaturated α,β ketone, a steroid that is secreted by phase luteal ovarian cycle and during pregnancy. P4 participates in the regulation of the menstrual cycle and is especially important in preparing the uterus for implantation of the blastocyst and in the maintenance of pregnancy. Its disproportion can cause malformations in the reproductive system and infertility problems [1,2]. Thus, it is very important to know P4 levels in different reproductive systems, such as mare, cow, goat, sow, sheep and bitch [3] to diagnose their inadequacy, being this one of the main causes of reproductive flaw in species of interest in the productive system.

The methods to quantify P4 in animal serum are mainly based on enzyme-linked immunosorbet assays (ELISA) and radio immuno assays (RIA). These methods are applied using commercial kits [4–6]. HPLC is another technique used to quantify P4, where binary mixtures are employed as the mobile phase and the column must be thermostatized to 40 °C [7].

Recently, a fast and sensitive method based on liquid chromatography with mass detection (LC–MS) for the quantitative analysis of seven steroid hormones, includes P4, in human serum was developed and validated [8]. The sample preparation was performed by liquid–liquid extraction, followed by oxime derivatization to improve the ionization efficiency of substrates. Chromatographic separation was achieved on a reversed-phase column using a methanol–water gradient, which needs a considerable time of sample preparation.

On the other hand, the multi-walled carbon nanotubes (MWCNTs) based matrix solid phase dispersion (MSPD) was applied for the extraction of hormones, including P4 and norethisterone acetate in butter samples [9]. This method includes MSPD extraction of target substrates from butter samples, derivatization of hormones with a heptafluorobutyric acid anhydride–acetonitrile mixture, and determination by gas chromatography with mass detection (GC–MS). These methods provide quantitative and confirmatory results with high sensitivity and selectivity, but need expensive instrumentation and considerable time delays between sampling and obtaining results.

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Immunosensors are shown as an alternative tool for rapid, reliable and economic determinations of different substrates, particularly those for the detection of small substrates due to its high sensitivity and analytical range extended. Inmunosensors based on an enzymatic immunoassay for P4 determination in milk have been developed using anti-progesterone antibody adsorbed on the surface of carbon electrodes [10,11]. Claycomb et al. [12,13] have designed an immunosensor which uses the same enzymatic immunoassay method to detect P4 in milk. In addition, a rapid automated immunoassay using a surface plasmon resonance (SPR) biosensor to measure P4 in bovine milk was developed [14]. Two amperometric immunosensors have been reported, one of them is based on direct attachment of anti-progesterone on a gold nanoparticles-modified graphite–Teflon composite electrode and the other used a colloidal gold–graphite–Teflon–tyrosinase composite to determine P4 in milk [15,16].

An immunosensor coupled to a glassy carbon electrode in a microfluidic system to quantify P4 in bovine serum samples has been developed using a fast and sensitive method based on the use of an anti-progesterone monoclonal antibody [17]. However, the development of methodologies more sensitive, fast and the low cost to quantify P4 in bovine serum samples is still a challenge to promote their study.

A critical step in the construction of an immunosensor is the antibody immobilization, because it plays a fundamental role in what concerns the stability, reproducibility and sensibility of the measured signal [18,19]. Electrochemical biosensors involving the use of gold nanoparticles have demonstrated to possess interesting features [20]. Gold nanoparticles have the ability to adsorb biological molecules without loss of their activity as well as to promote electron transfer between redox proteins and electrodes [21]. On the other hand, the use of electrochemical techniques has won great consideration in the developing of biosensors, because they are simple and inexpensive to operate. Among the electrochemical techniques, the square wave voltammetry (SWV) is a versatile technique for electroanalytical purpose. It incorporates the best features of several voltammetric techniques. Therefore, the SWV has been established as a very reliable analytical technique widely recognized as one of the most sensitive electrochemical methods due to its ability to eliminate capacitive currents.

In this study, we report an integrated immunosensor to quantify P4 in a small volume of bovine serum samples without pretreatment. Integrated immunosensors are based on the spatial integration of the sensing layer and the detection/transduction system. The immobilization of the immunochemical reagents could, then, be performed on a support, brings in close contact with the transducer, or directly on the transducer. This last configuration, with a sensing layer localized at the surface of the transducer, allows new detection methods. The immunosensor was constructed by immobilization of the anti-progesterone monoclonal antibody (mAbP4) on a modified gold disk (Au disk) electrode with gold nanoparticles (Au_{NP}) lodged on a cysteamine (cys) self-assembled monolayer (Au_{NP}-cys-Au disk), and it is based on a heterogeneous competitive immunoassay. Bovine serum samples without P4 were spiked with known concentrations of P4 and enzyme-labeled P4 (i.e., the "conjugated"). Therefore, the labeled and unlabeled antigen competes with antibody binding sites bound at the modified electrode (Fig. 1). The enzyme used to label P4 was horseradish peroxidase (HRP) and pyrocatechol (H_2Q) was used as a redox mediator. HRP, in the presence of H_2O_2, catalyzes the oxidation of H_2Q to benzoquinone (Q). Its back electrochemical reduction to H_2Q was detected on the modified gold electrode surface (mAbP4-Au_{NP}-cys-Au disk) by SWV (Fig. 1). This response was proportional to the activity of the enzyme and inversely proportional to the amount of P4 in bovine serum samples.

2. Materials and methods

2.1. Chemicals and immunochemicals

All reagents used were of analytical grade. P4, progesterone-3-carboxymethyloxime (P4-3-CMO), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC), cysteamine (cys), 4-amino thiophenol (4-ATP), *N*-hydroxysuccinimide (NHS), antiprogesteron sheep monoclonal antibody (mAbP4), horseradish peroxidase (HRP), pyrocatechol (H_2Q) and 3,3′,5,5′-tetramethylbenzidine (TMB) were from Sigma Chemical Company. Anhydrus dimethylformamide was from Aldrich (DMF, HPLC grade). Glutaraldehyde (25% aqueous solution) was Merck p.a. 10 mM phosphate, 137 mM NaCl and 2.70 mM KCl buffer solution (pH 7.00 PBS), 50 mM citrate, 50 mM phosphate buffer solution (pH 5.00 CBS), and pH 7.00 PBS containing 0.05% Tween 20 (PBST) were prepared from their salts (Merck, p.a.). Ethanol (HPLC grade), H_2O_2, and H_2SO_4 were from Merck p.a. All reagents were used as received. Aqueous solutions were prepared using H_2O (HPLC grade) from Sintorgan. Bovine serum samples were gently supplied by the Facultad de Agronomía y Veterinaria, Universidad Nacional

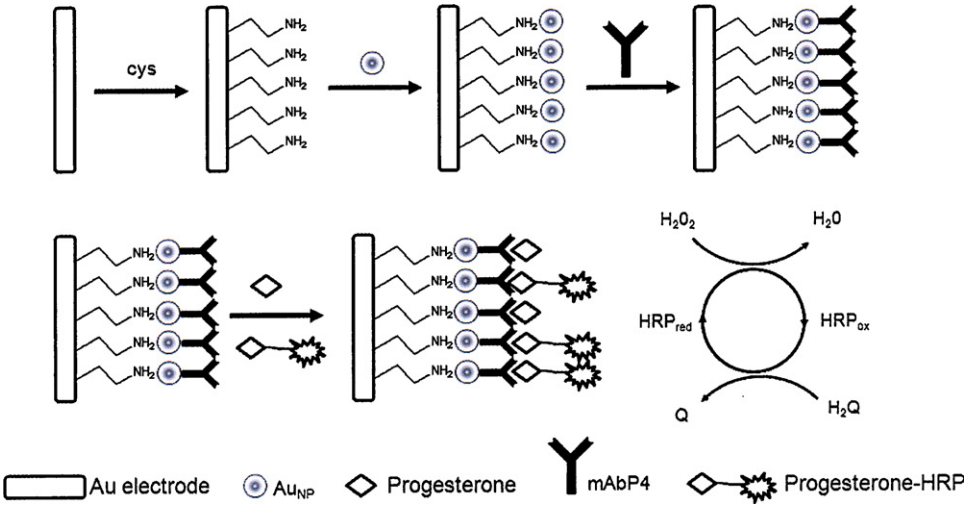

Fig. 1. Schematic representation of the progesterone immunosensor based on electrochemical detection using competitive assays.

de Río Cuarto and used without pretreatment. These samples did not contain P4.

2.2. Apparatus and electrodes

Electrochemical measurements were performed in a Teflon microcell. The cell operates with a volume of 100 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 distilled 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 1 M H_2SO_4, by cyclic voltammetry (CV) in a potential range from −0.2 to 1.6 V vs SCE, at a scan rate of 0.1 V s^{-1} until a typical voltammogram of a clean polycrystalline Au surface was obtained. Then, it was rinsed with double distilled water and ethanol before to perform the process of monolayer adsorption. The counter electrode (CE) was a platinum foil. A silver wire was used as pseudoreference electrode.

The measuring system for performing SWV and CV was an Autolab PGSTAT 30 potentiostat and run with the GPES software (Eco-Chemie, Utrecht, The Netherlands, version 4.9). All SWV measurements were performed in the potential range from 0.35 to 0 V, 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 $2e^-$ [22]. Atomic force microscopy (AFM) measurements were carried out 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 Bio-Rad Benchmark microplate readers (Japan) and a Hewlett-Packard spectrophotometer, Model 8452A, equipped with a temperature controller. pH measurements were carried out with a HANNA instrument, Bench Meters, model pH 211, Romania. The buffer solutions were thermostatized to 37 °C using a NEO LINE stove, Argentina.

2.3. P4-HRP conjugation

P4 was coupled to HRP by following the synthesis of the activated ester [23], with minor modifications, to generate the P4-HRP labeled. Thus, 7.9 mg P4-3-CMO were dissolved in 600 μL of DMF containing 400 μL distilled water and 18 mg NHS. Then, 33 mg EDAC were added. Reaction mixture was stirred at 4 °C in the darkness for 5 h to activate the steroid carboxy group. HRP (1 mg) was dissolved in 1 mL distilled water. Steroid activated solution was added to HRP aqueous solution and then, it was stored and shaken overnight at 4 °C. After the reaction was completed, the solution (P4-HRP labeled) was purified using a PD-10 column by means of gel filtration (packed with Sephadex™ 25). Then, it was dialyzed in pH 7.00 PBS for 5 days using a cellulose dialysis membrane (cut-off 10 kDa molecular weight) and finally, stored at 4 °C. The reaction product was confirmed by UV–vis spectrophotometry, where the appearance of new bands respect to P4 and HRP solutions were observed. P4-3-CMO presents a band of absorption with a peak at 252 nm in pH 7.00 PBS. HRP presents, among others, two absorption bands with peaks at 250 nm and 402 nm in the same reaction medium. Concentrations of synthesized P4-HRP conjugated (P4-HRP) were determined spectrophotometrically by measuring absorbance changes at 250 nm and 402 nm in pH 7.00 PBS. To check any loss of antibody recognition by P4, colorimetric checkerboards titrations were performed on a 96-well high binding microtiter plate.

2.4. Gold nanoparticles preparation

The gold nanoparticles (Au_{NP}) were prepared through a very simple method using a two phase-liquid system. The method used was that developed by Bethel et al. [24], with minor modifications. A solution containing 1.15 mM $HAuCl_4$ in toluene was prepared and 53.00 mM $NaBH_4$ aqueous solution was slowly added under continuous stirring, and maintained for 2 h. Then, the organic phase was removed and washed with small portions of water for three times. The aqueous phases were collected. They showed a wine-red color. The Au_{NP} were stored in a dark glass bottle at 4 °C for further use. The Au_{NP} solutions were very stable and did not show any sign of aggregation or other deterioration over periods of months. The presence of Au_{NP} adsorbed was checked by AFM in air at room temperature. The UV–vis spectroscopy was used to determine the diameter of Au_{NP}. The average size was 60 nm (Fig. 2a) [25].

2.5. Different self-assembled monolayers on the gold electrode

Different self-assembled monolayers (SAMs) modified electrodes were prepared by immersing the clean gold disk electrode in fresh thiol/ethanol solutions for 2 h. After adsorption, the different thiol modified gold electrodes were thoroughly rinsed with dried ethanol, and subsequently blown dried with high-purity nitrogen. The thiol solutions used were 0.1 M cysteamine, 0.1 M 4-aminothiophenol, and mixtures of 0.05 M cysteamine + 0.05 M 4-aminothiophenol and 0.075 M cysteamine + 0.025 M 4-aminothiophenol.

2.6. mAbP4 immobilization onto the modified gold electrode with thiol mixtures and gold nanoparticles

mAbP4 immobilization was carried out on Au_{NP}. For this purpose, a cysteamine modified gold disk electrode (cys-Au disk) was immersed in an Au_{NP} solution for 2 h at room temperature. Au_{NP} was chemisorbed on cysteamine forming an Au_{NP}-cys-Au disk. The morphology of the Au and Au_{NP}-cys-Au disk was analyzed by high-resolution in situ AFM imaging measurements, which clearly demonstrate the adsorption of Au_{NP} on the cys-Au disk (Fig. 2b-i and b-ii). Scan areas with a resolution of 512 × 512 pixels were obtained.

The Au_{NP}-cys-Au disk was washed three times with water and PBS. Then, 10 μL mAbP4 solution (optimal dilution, see below) was deposited on Au_{NP}-cys-Au disk surface and incubated overnight at 4 °C in order to generate the bioelectrode (mAbP4-Au_{NP}-cys-Au disk). Before use, the mAbP4-Au_{NP}-cys-Au disk was washed with pH 7.00 PBS to remove the weakly absorbed antibodies. Once used, the bioelectrode was stored in the PBS at 4 °C. mAbP4-Au_{NP}-cys-Au disk was stable for at least 21 days. Also, the morphology of the mAbP4-Au_{NP}-cys-Au disk was analyzed by high-resolution in situ AFM imaging measurements, which clearly demonstrate the adsorption of mAbP4 on the Au_{NP}-cys-Au disk (see Fig. 2b-iii). The fabrication sequence of the biosensor is shown in Fig. 1.

For comparative purposes, the mAbP4 also was attached on cys:4-ATP (1:1) mixture modified disk Au electrode using glutaraldehyde as cross linker for 2 h at room temperature.

2.7. Assay procedure for the electrochemical immunosensor

To avoid the unspecific bindings on mAbP4-Au_{NP}-cys-Au disk, the modified electrode was blocked by a treatment at 37 °C with 3% low-fat milk in pH 7.00 PBS for 10 min and washed with PBST. Then, 10 μL solution containing P4-HRP in 1:100 dilution factor

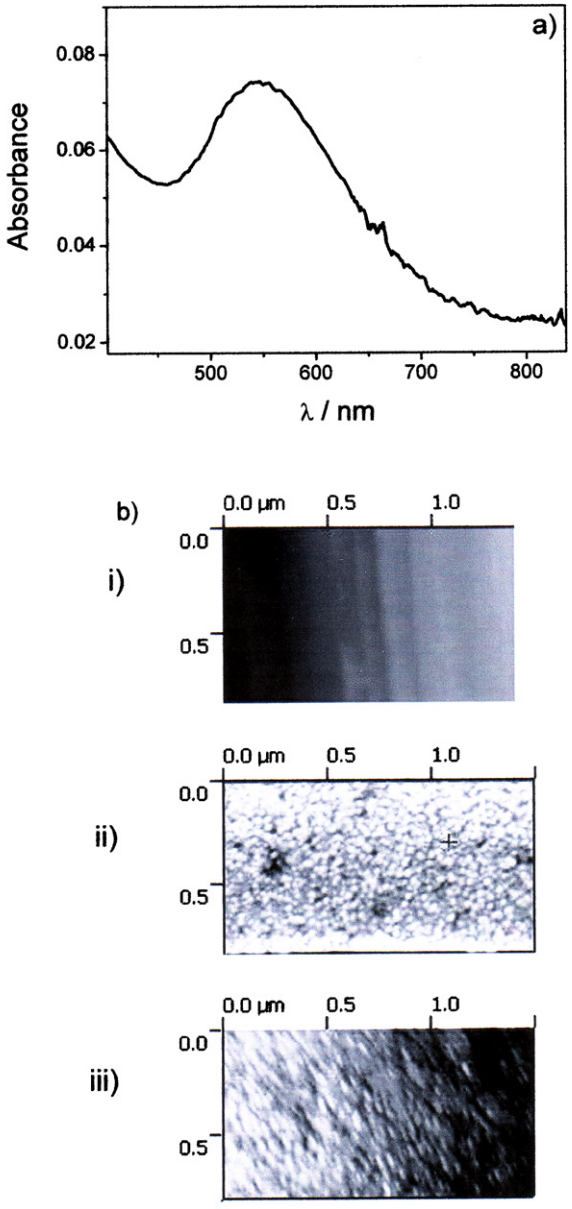

Fig. 2. (a) Absorption spectrum of Au_{NP} in solution. The diameter of Au_{NP} was 60 nm (λ_{max} = 538 nm). (b) AFM images of (i) the bare gold disk electrode; (ii) the Au_{NP}-cys-Au disk electrode and (iii) the mAb-AuNP-cys-Au disk electrode.

and P4 were dipped on the mAbP4-Au_{NP}-cys-Au disk and incubate at 37 °C for 30 min (competitive assay) and then, rinsed with pH 7.00 PBS. For incubation times of less than 30 min the results were not reproducible. Finally, 100 μL of the substrate solution (1×10^{-3} M H_2O_2 + 1×10^{-3} M H_2Q in pH 5.00 CBS) was placed in the cell, where the bioelectrode was introduced. After 10 min, the enzymatic reaction product (Q) was detected through SWV. The catalytic mechanism for Q generation is shown in Fig. 1.

For next detection, the immunosensor was reconditioned by desorption with 0.1 M glycine + pH 2.00 HCl solution for 2 min and, then, washed with PBS. Desorption efficiency was checked by the absence of any reduction current of enzymatic reaction product at the modified electrode after addition of H_2O_2 and H_2Q.

2.8. Optimization of P4-HRP and mAbP4 concentrations

Standard colorimetric checkerboards were performed in order to optimize P4-HRP and mAbP4 concentrations on a 96-well high binding microtiter plate. 100 μL of mAbP4 from twofold serial dilutions starting at 1:25 dilution factor were applied to the wells of A–H rows of the ELISA plate. After 1 h incubation at room temperature, the plates were washed with pH 7.00 PBS and blocked with 3% (w/v) skimmed milk in pH 7.00 PBS for 30 min, and washed with PBST. Then, 100 μL of twofold HRP-P4 serial dilutions starting at 1:10 dilution factor were dispensed to columns 1–11 of the microtiter plate and incubated for 1 h at room temperature with gentle rocking. Afterwards, the plates were washed with pH 7.00 PBS and 100 μL of the HRP substrate (0.4 mL of a solution formed by 6 mg mL^{-1} TMB in DMSO, 0.1 mL 1% H_2O_2 in water, in a total volume of 25 mL of 0.1 M pH 5.00 CBS) was dispensed into each well. The enzymatic reaction was stopped after 15–20 min by the addition of 50 μL 2 M H_2SO_4, and the absorbance at 450 nm (corrected at 600 nm) was read in a microtiter plate reader.

3. Results and discussion

3.1. Optimum conditions for the immune reactions and the determination of enzymatic products

The optimum concentrations of mAbP4 and P4-HRP were determinate by classical ELISA assays (see Section 2.8). For smaller mAbP4 dilution factor, an increase in absorbance values was observed. In addition, at a mAbP4 constant dilution factor, an increase of P4-HRP concentrations produced also an increase in absorbance values (results not shown). P4-HRP dilution factors lower than 1:10 produce the saturation of mAbP4 immobilized, since constant absorbance values were measured when the P4-HRP amount was greater (results not shown). The 1:100 dilution factor gave absorbance values close to one. Then this dilution factor was chosen in order to achieve the best sensitivity and a greater range of competition with the substrate. Competitive ELISA assays were performed with 1:100, 1:50 and 1:200 HRP-P4 dilution factors to determine which of them gave the highest concentration range of competition with the substrate. The widest linear range was obtained by using a 1:100 P4-HRP dilution factor, which includes the veterinary relevant range for P4 (1–20 ng mL^{-1}) (data not shown). Therefore, the optimal values for immune reaction were 1:50 mAbP4 and 1:100 HRP-P4 dilution factors, and they were then used in the preparation of the immunosensor.

3.2. Optimal conditions for the determination of the enzymatic products

The enzymatic reaction conditions have been previously studied [26]. The maximum reaction rate was obtained in pH 5.00 CBS. As it is well known, 37 °C is the optimal temperature of immunoreaction for all IgG [5]. Therefore, all measurements were carried out at this temperature. The effect of varying the H_2O_2 concentration from 7.0×10^{-4} M to 5.0×10^{-3} M at a given H_2Q concentration (1.0×10^{-3} M), and the effect of varying the H_2Q concentration from 1.0×10^{-4} M to 3.6×10^{-2} M at a given H_2O_2 concentration (1.0×10^{-3} M) were evaluated on immunosensor responses. The optimal concentrations for both H_2O_2 and H_2Q were 1.0×10^{-3} M (data not shown). These concentrations were chosen for all experiments. No signal was observed in absence of H_2O_2 (see Fig. S1 in Supplementary material), which indicates that the signal observed in presence of H_2O_2 is due to the reduction of benzoquinone (Q) enzymatically generated.

3.3. Electrochemical characteristics of thiol modified gold electrode in the presence and in the absence of gold nanoparticles

Cys and 4-ATP SAMs have been used as the base interface for antibodies immobilization [27–29]. A cyclic voltammogram of

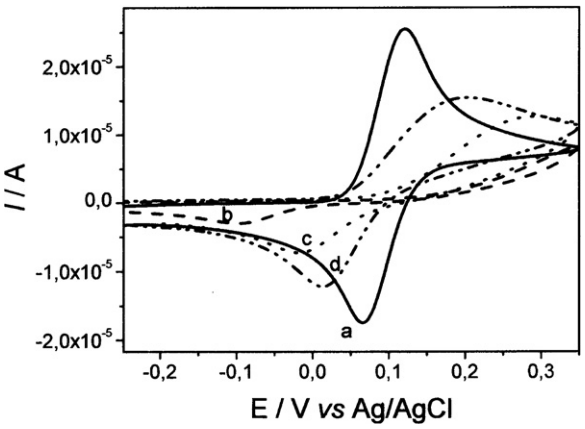

Fig. 3. Cyclic voltammograms of H_2Q 1×10^{-3} M in CBS (pH = 5.00) for (a) bare gold disk electrode, (b) cysteamine SAM on gold disk electrode (cys-Au disk electrode), (c) cysteamine:4-aminothiophenol (1:1) mixture SAM on gold disk electrode (cys-4-ATP-Au disk electrode) and d) Au_{NP} on cysteamine SAM on Au disk electrode (Au_{NP}-cys-Au disk electrode).

1×10^{-3} M H_2Q in pH 5.00 CBS was performed by scanning the potential from −0.250 to 0.350 V vs Ag/AgCl at both the bare gold disk and modified electrodes (Fig. 3). At the bare disk, the cyclic voltammogram showed a well-defined anodic peak and its corresponding cathodic peak, characteristic of a quasi-reversible redox couple (Fig. 3a). When the thiol modified electrode was used, the electron transfer kinetics of the H_2Q redox reaction was perturbed. Fig. 3b shows the cyclic voltammogram obtained for the oxidation of H_2Q to Q and the back reduction of Q to H_2Q on the cys-Au disk electrode. Fig. 3c shows the cyclic voltammogram obtained for the same redox couple on the cys:4-ATP (1:1) mixture-Au disk electrode. As can be seen, the SAMs on gold electrode leads to the decrease in the current values, and an increase in the separation between the cathodic and anodic peak potentials (ΔE_p) of the redox probe (Fig. 3, curves b and c), implying that SAMs are, in fact, immobilized on the electrode surface. The ΔE_p is smaller and current values are higher when the mixture of thiols is used instead of only cys. The higher conductivity when the mixture was used is probably due to the presence of pinholes in the disordered mixed monolayer due to the dipole moments of 4-ATP and cys, which generates an intermolecular repulsion [30].

When the SAM is formed only with 4-ATP, the cyclic voltammogram is similar to the one at the bare disk gold electrode. A small ΔE_p value is not appropriate for the immunoassay developed using SWV, due to the fact that at the beginning of the SWV

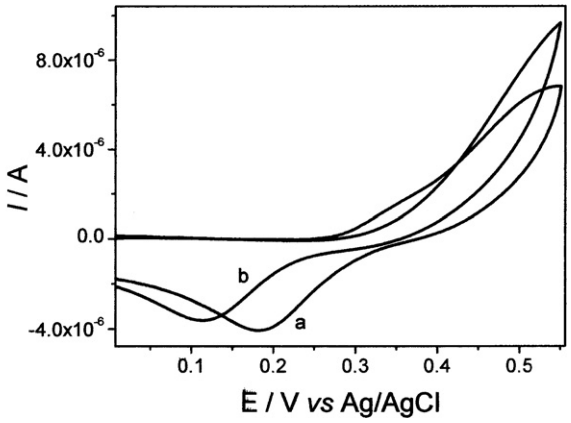

Fig. 4. Cyclic voltammograms of H_2Q 1×10^{-3} M in CBS (pH = 5.00) for mABP4 binding to: (a) Au_{NP} on cysteamine SAM on Au disk electrode and (b) cysteamine:4-aminothiophenol (1:1) mixture SAM on gold disk electrode.

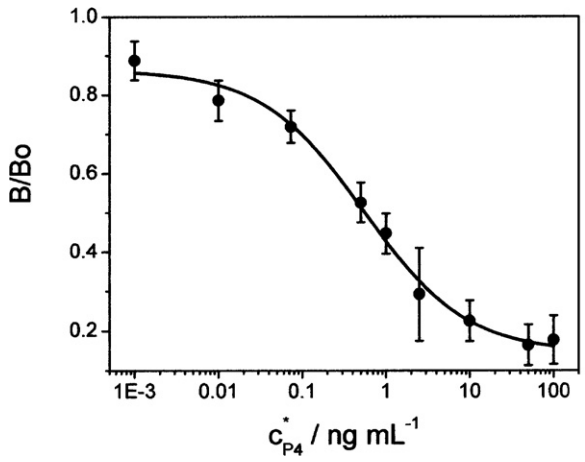

Fig. 5. Calibration curve for P4 obtained with the immunosensor. Each point is the average of three replicated measurements. Curve parameters for the immunosensor were: $IC_{50} = 0.54 \pm 0.02$ ng mL^{-1}, Hill Slope: -0.711 ± 0.193, $R = 0.946$.

sweep an electrooxidation of H_2Q occurs, producing Q. Thus, that detected signal, has two contributions, the reduction of Q generated by electrooxidation and Q enzymatically generated.

It is known that self-assembly of Au_{NP} onto the SAMs modified gold electrodes could result in a large electrode surface area and an easy attachment of antibodies [31,32]. Fig. 3d shows the electrochemical response of the H_2Q after the Au_{NP} chemisorption on cys-Au disk electrode (Au_{NP}-cys-Au disk). The peak current for the H_2Q oxidation increases with respect to that observed at the modified gold surface without Au_{NP}.

The mAbP4 immobilization leads to a considerable decrease of the peak current of H_2Q oxidation, and an increase in ΔE_p of the redox probe. The current recorded strongly depends on the time incubation of antibody on Au_{NP}. As a result, the immobilization of antibody insulates the electrode and perturbs considerably the interfacial electron transfer. Fig. 4a and b shows the cyclic voltammograms obtained when the mAbP4 is attached on the Au_{NP}-cys-disk Au electrode and the cys:4-ATP (1:1) mixture-Au disk electrode, respectively. The peak current for Q reduction is higher when Au_{NP} is used. Au_{NP} chemisorption on cys, 4-ATP, and the mixture of both SAMs were carried out. Results show no significant differences in peak current and ΔE_p values. For this reason, and in order to simplify the modification of the electrode in terms of preparation time and expenses, the Au_{NP}-cys-Au disk electrode was chosen for the immunosensor preparation.

3.4. Analytical characteristics for the progesterone determination

Using the optimized parameters, a dose–response titration for P4 was carried out in the range from 1×10^{-3} to 1×10^{2} ng mL^{-1} (Fig. 5). Net peak current values ($I_{p,n}$) obtained are due to the reduction of Q enzymatically generated. Therefore, $I_{p,n}$ are proportional to enzyme conjugated activity (P4-HRP) and, consequently, indirectly proportional to the amount of P4 bound to mAbP4. SW

Table 1
Statistical analysis of inmunoreactor responses for two samples of serum with different amounts of spiked P4.

c^*_{P4} (ng mL^{-1})[a]	c^*_{P4} (ng mL^{-1})[b]	% VC[c]	% Recovery
1	1.09	6.3	109.6
10	11.10	5.0	110.1

[a] P4 concentration in samples of bovine serum.
[b] Average value of P4 concentration determined by the immunosensor through three replicated $I_{p,n}$ measurements.
[c] Percentage variation coefficient.

Table 2
Comparison between statistical parameters obtained with our immunosensor and other immunosensors previously reported for P4 detection.

Samples	Linear range (ng mL^{-1})	Incubation time (min)	Stability	Detection limit (ng mL^{-1})	Reference
Bovine serum without pretreatmen	0.08–7	30	21 days	0.08	This work
Milk without pretreatment	Non linear regression	12	15–20 cycles	0.5	[13]
Milk without pretreatment	0–10	30	–	5	[11]
Milk without pretreatment	0.5–50	15	3600 cycles	3.56	[14]
Milk without pretreatment	0–30	120	–	0.84	[15]
Milk without pretreatment	0–40	70	14 days	0.37	[16]
Bovine serum without pretreatment	0.5–12.5	15	25 days	0.2	[17]

voltammograms recorded at different P4 concentration, are shown in Fig. S2 of Supplementary material. The calibration curve was constructed as binding percentage ($B/B_0 = I_{p,n}/I_{p,n}$ without inhibition) vs P4 concentration (c^*_{P4}). The calibration curve was fitted to a four-parameter logistic equation according to the following formula [33]:

$$y = D + \frac{A - D}{[1 + 10\ \exp((\log IC_{50} - \log c^*_{P4})(\text{Hill Slope}))]}$$

where A and D are the maximum and minimum B/B_0 values, respectively, while IC_{50} is the concentration of P4 which produces 50% inhibition, c^*_{P4} is the P4 concentration, and Hill Slope is the slope at the midpoint of the sigmoid curve. The reproducibility of the immunosensor was checked for three different mAbP4-AuNP-cys-Au disk electrodes using a P4 concentration of 1 ng mL^{-1}. The percent relative standard deviation (% RSD) was 9%. Experimental points shown are the average of three replicated measurements. The calibration curve exhibited a linear range from 0.08 to 7 ng mL^{-1}. The detection limit (DL), calculated as the concentration causing 20% inhibition of the A value [33,34] was $DL = 0.08$ ng mL^{-1}, and the sensitivity IC_{50} was 0.54 ± 0.02 ng mL^{-1}, with a Hill Slope of -0.711 ± 0.193. The within-assay precision of electrochemical measurements was tested by measurements of 0.5 ng mL^{-1} and 1 ng mL^{-1} P4 solutions in pH 7.00 PBS by triplicate, with % RSD of 2.0% and 13.0%, respectively, which shows a good precision.

3.5. Determination of progesterone in spiked bovine serum

The determination of P4 in serum bovine samples was carried out using samples that did not contain P4. Therefore, they were spiked with aliquots of a given P4 concentration prepared in pH 7.00 BPS, being the buffer concentration negligible in the serum ($\leq$1%) in order to avoid a dilution effect. Therefore, two samples of serum containing 1 and 10 ng mL^{-1} of P4 were prepared. An optimized amount of P4-HRP (1:100) was added at both serum samples, to carry out competitive assays. Note that bovine serum samples were incubated in the immunosensor without any previous pretreatment. Results obtained are depicted in Table 1.

Recovery percentages obtained were very good, with values of 109.6 and 110.1% for 1 and 10 ng mL^{-1}, respectively. The immunosensor stability was tested for 25 days for a constant P4 concentration. It was found that current responses were practically constant during 21 days and then, its value decreased gradually. The immunosensor was regenerated by desorption in buffer (0.1 M glycine + pH 2.00 HCl) for 2 min and then, washed with pH 7.00 PBS, which allowed to use the reactor over about 100 determinations. These measurements were performed using alternatively P4 standard solutions, and bovine serum samples. Current values were reproducible, showing that the antibody activity loss is not appreciable. Afterwards, to perform the next analysis set, the immunosensor had to be regenerated again.

Several important analytical advantages can be deduced when the performance of our immunosensor is compared with that other P4 immunosensors reported in literature. Table 2 summarizes more relevant immunosensors developed for P4 detection.

The determinations were performed without any pretreatment of samples and the detection limit is approximately one order of magnitude lower than that reported for an immunosensor coupled to a glassy carbon electrode in a microfluidic system to quantify P4 in bovine serum samples [17]. The response shows a good linearity at low P4 concentrations, in contrast to some of the P4 immunosensors previously described [13–16]. The immunosensor also shows physical and chemical stability, a wide working potential range and accuracy. On the other hand, the SWV detection technique is widely recognized as one of the most sensitive electrochemical methods, in contrast with the amperometric detection used in most of studies reported in Table 2.

4. Conclusion

In this study, an integrated electrochemical immunosensor was developed to determine progesterone at trace levels in bovine serum samples. These determinations were performed without any pretreatment of samples, which indicates the great selectivity of the antibody used. The immunosensor showed a high analytical performance in terms of an excellent detection limit (0.08 ng mL^{-1}), great sensitivity ($IC_{50} = 0.54 \pm 0.02$ ng mL^{-1}), high specificity, an important analytical range of interest, good reproducibility and an acceptable accuracy. The immunosensor developed can operate as a fast, selective, and sensitive detector. This device has several advantages over other methods for the determination of porgesterone in real samples, such as direct measurement without any pre-treatment, the use of small volumes (harmful solvents and expensive reagents are avoided), and the use of the mAbP4-Au_{NP}-cys-Au disk electrode, which minimizes the amount of mAbP4. The immunosensor also shows physical and chemical stability, a wide working potential range and accuracy. In addition, integrated approaches allow to consider a potential sensor miniaturization. Consequently, these features make this device a high potential tool for the measurement of progesterone in bovine serum samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.snb.2012.03.015.

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