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Electrochemical immunosensor based on gold nanoparticles deposited on a conductive polymer to determine estrone in water samples

Melisa Jimena Monerris, Fabiana D´Eramo, Fernando Javier Arévalo, Héctor Fernández, María Alicia Zon, Patricia Gabriela Molina *

Departamento de Química. Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Agencia Postal N° 3, 5800 Río Cuarto, Argentina

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ABSTRACT

This paper describes the development of a simple and sensitive electrochemical immunosensor (EI) to quantify estrone (E) in water samples. This EI does not require the sample pre-treatment, to label neither the antigen nor the antibody, and its detection format is based on the fact that E is co-substrate of the horse radish peroxidase (HRP). Therefore, the EI was constructed by immobilization of the anti-E monoclonal antibody (mAbE) on a glassy carbon electrode (GCE) modified with gold nanoparticles (AuNPs) electro-synthesized on a 1-naphtylamine polymer (pNap) film. This format reduced significantly the time of EI preparation. Water samples were spiked with known E concentrations, and then incubated on mAbE-AuNPs-pNap-GCE disk electrode. The electrochemical response was proportional to the amount of pyrocatechol (H_2Q), another enzyme co-substrate, and inversely proportional to the amount of E presents in water samples. The immunosensor showed a linear range from 8×10^{-2} to 2×10^{4} pg mL^{-1}. The limit of detection (LOD) was 0.061 pg mL^{-1}. Recovery percentages obtained were very good, with values of 98.20, 105.50, and 100.85% for 50, 100 and 200 pg mL^{-1}, respectively. Tests were also conducted to evaluate the cross-reactive of E with other hormones of similar structure such as 17β-estradiol, progesterone and estriol. The EI showed a high selectivity to determine E in the presence of these hormones. Thus, this EI is an attractive tool to determine E in water samples.

1. Introduction

Both natural and synthetic estrogens are well known to be endocrine disrupting compounds (EDC), which constitute interfering compounds in the endogenous hormonal system of mammals, producing adverse effects.

Estrone (E) is a natural estrogenic hormone secreted by the ovary, and adipose tissue. It is the predominant hormone in post-menopausal women. In recent years, new biochemical functions have been found for E in different tissues, including the pituitary gland, breast, vascular and the colon [1].

The presence of hormones in the aquatic environment began to be important when a connection was established between a synthetic contraceptive drug and its impact on fish [2]. Steroid hormones can get into environment through discharge of wastewater, as they are excreted by humans and animals [3], and in different amounts [4], depending on gender, health status, age, diet or pregnancy [5].

Estrogens are released into the urine as a glucuronide conjugate complexes or as sulfates [6], which may be converted quickly to potent hormones by excision and/or during transport and treating wastewater [7].

In this sense, the first estrogenic contamination of aquatic environments was detected by the appearance of hermaphroditic fish in British rivers [8]. Therefore, exposure of aquatic life in polluted waters with EDC has important consequences [9].

Based on these results, there is a growing need for a continuous and fast monitoring of pollution levels. Therefore, different monitoring techniques have been developed; including capillary electrophoresis [10], receptor assays [11] and chromatographic techniques [12–18]. Chandra Bose et al. [19] have developed an assay for the simultaneous determination of dexamethasone, testosterone, and E using reverse phase HPLC chromatography. Recoveries were in the range from 98 to 102%. The disadvantages of chromatographic methods are the high cost of instrumentation and maintenance, high consumption of solvents and time, and the difficulty of conducting experiments outside the laboratory.

Pre-concentration methodologies such as the solid phase extraction (SPE) and the liquid-liquid extraction (LLE) may be used to identify and quantify hormones in water samples with limits of quantification (LOQ) between 0.02 and 1.02 ng L^{-1} [20]. Although the LOQ were good, SPE also involves a large consumption of organic solvents. In this sense, the solid phase micro extraction (SPME) has the advantage of

* Corresponding author.
E-mail address: pmolina@exa.unrc.edu.ar (P.G. Molina).

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consuming small amounts of solvents, but it also presents difficulties such as high cost, fragility, and a time useful limited of the fibers [21]. Regarding the extraction in liquid phase, the main weakness of the method is that it consumes a long period of time as well as large volumes of organic solvents. Thus, the liquid phase micro-extraction (LPME) appears as a good alternative to other methodologies [22]. The hollow fiber LPME (HF-LPME) was used in combination with GC–MS to detect steroid hormones in tap and sewage water samples, with limits of detection (LOD) from 1.6 to 10 ng L^{-1} [23].

In recent years it has become important the development of immunosensors to detect and quantify different analytes at very small concentrations. The importance of these devices is that they can be miniaturized and portable, making them a fast, sensitive and inexpensive technique.

In addition, the immunoassays based on the ELISA method have been developed to determine E in humans and animals fluids [24,25], and in environmental samples [26,27].

Li et al. [28] developed an ELISA method to analyze E in water samples. The LOD was 0.14 μg L^{-1}. However, the LOD was 1.25 ng L^{-1} when this methodology was combined with the SPE method.

On the other hand, the E electrochemical detection was reported by Brocenschi et al. [29]. The anodic oxidation of E was investigated at glassy carbon, nitrogen-incorporated tetrahedral amorphous carbon and boron-doped diamond electrodes.

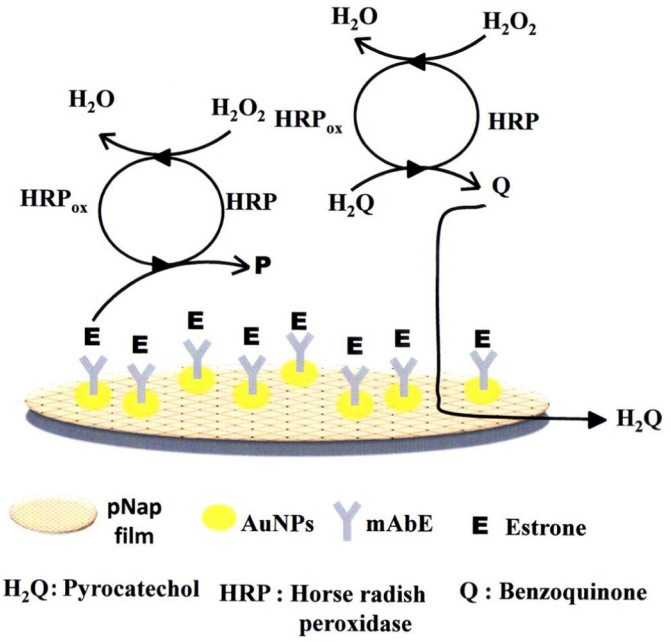
Yang et al. [30] detected E by linear sweep voltammetry (LSV) using surfactants and multiple-walled carbon nanotubes. The LOD was 1.35 μg L^{-1}, and the method was used to determine E in commercial pills.

Gao et al. [31] studied the interaction between E and the polyclonal antibody anti-E using an electrode modified with polypyrrole, doped with polyclonal antibody anti-E. The interaction between E and antibody-polypyrrole film was evaluated by voltammetric measurements based on the response of a probe redox couple (ferricyanide), being the response in current inversely proportional to the concentration of E.

Sun et al. [32] developed a method to detect E using an electrochemical detection platform based on bio-assembled nano-circuits covalently bound to the antibody anti E. The detection of E was performed using $[\mathbf{Ru}(\mathbf{NH_3})_6]^{+3/+2}$ as the probe redox couple to sense the antigen-antibody interactions. The LOD was 1.4 pg mL^{-1}.

We have recently developed an immunosensor to detect 17β-estradiol (17β-E) in bovine serum samples. The immunosensor showed a high analytical performance, and the LOD was 0.84 pg mL^{-1} [33].

In this work, we report a simple, and sensitive electrochemical immunosensor (EI) to quantify E in water samples. The EI does not require the sample pre-treatment, and to label neither the antigen nor the antibody. The EI was constructed by immobilization of the anti-E monoclonal antibody (mAbE) on a glassy carbon electrode (GCE) modified with AuNPs electro-synthesized on a 1-Naphtylamine (pNap) polymer film (AuNPs-pNap-GCE). These composite structures possess interesting properties arising from both the size effects of AuNPs as well as the exceptional properties of the polymer, which allows stacking of the particles and increase their stabilization [34–36]. Indeed, the (pNap) polymer can provide a large specific surface and a compact matrix for the incorporation of AuNPs, and leads to the improved stability of the resultant AuNP-pNap-GCE. Water samples were spiked with known concentrations of E and, then, incubated on mAbE-AuNPs-pNap-GCE. Then, the EI was transferred to an electrochemical cell containing pH 5.00 citrate buffer solutions (CBS), where given concentrations of HRP, pyrocatechol (H_2Q) and H_2O_2 were added. The E and H_2Q, both enzyme co-substrates, react with HRP. The HRP, in the presence of H_2O_2, catalyzes the oxidation of both the E to a given product and the H_2Q to benzoquinone (Q). The back electrochemical reduction of Q to H_2Q was performed on the modified electrode surface (mAbE-AuNPs-pNap-GCE) 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 E in water samples. Therefore, the maximum electrochemical response is obtained in the absence of E at the electrode surface for a given H_2Q concentration. This EI showed a very high sensitivity to determine E at trace levels in water samples, compared to other conventional techniques.



Scheme 1. Schematic representation of the electrochemical immunosensor developed to determine estrone in water samples.

2. Materials and methods

2.1. Reagents and solutions

E, 17β-E, progesterone (P4), estriol (E1), anti estrone sheep monoclonal antibody (mAbE), HRP (E.C:1.11.1.7, H_2O_2-oxido-reductase), H_2Q, pNap and gold (III) chloride hydrate ($HAuCl_4$) were purchased from SIGMA, USA. AuNPs were synthesized using $HAuCl_4$. 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 buffer solutions, 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 buffer solution, (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. Real samples (tap water) free of E were used without pre-treatment.

2.2. Instruments

Electrochemical measurements were performed in a Teflon microcell. The cell operates with a volume of 200 μL. The working electrode was a glassy carbon disk electrode (GCE) (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 during 2 min. 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 cyclic voltammetry (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 in heterogeneous electronic transfers of 2 e^- [37], because the oxidation of H_2Q to Q is a two-electron quasi-reversible redox process [38]. Scanning electron microscopy (SEM) images were obtained using a Zeiss LEO 1450VP. Absorbance measurements were performed by a Hewlett-Packard spectrophotometer, Model 8452A, equipped with a temperature controller. The pH measurements were carried out with a HANNA instruments, Bench Meters, model pH 211, Romania. Each stage of immunoassay was incubated to 37 °C using a NEO LINE stove, Argentina.

2.3. Fabrication of the immunosensor

pNap films were obtained as described previously for platinum and glassy carbon electrodes [39,40]. We have applied the technique of CV as a method of synthesis due to the fact that this technique has the advantage of exercising control over the thickness and homogeneity film. Thus, the working electrode potential was continuously scanned at 0.025 V s^{-1} between 0 and 0.8 V during 15 cycles in a 6×10^{-4} mol L^{-1} pNap in a pH 1 $HClO_4$ solution. These experimental conditions led to the formation of an adherent, mechanically stable film in a short time, which allowed obtaining highly reproducible electrochemical responses.

The electro-deposition of the AuNPs on the pNap film was obtained by immersing the modified glassy carbon electrode in an electrochemical cell of two compartments. The cell contained a 0.1 mol L^{-1} H_2SO_4 + 1×10^{-3} mol L^{-1} $HAuCl_4$ solution and the electrode was subjected to10 continuous voltammetric cycles between −0.1 V and 1.3 V at 0.050 V s^{-1} [41].

For comparison, studies were conducted between the responses obtained at AuNPs-pNap-GCE and at AuNPs-GCE. The morphology of both electrodes was analyzed by SEM (see below).

Finally, the mAbE is immobilized on the AuNPs-pNap-GCE. Thus, the AuNPs-pNap-GCE was washed three times with water and PBS. Then, 10 μL of a mAbE solution (optimal dilution, see below) was dropped on AuNPs-pNap-GCE surface and incubated overnight at 4 °C in order to generate the EI (mAbE-AuNPs-pNap-GCE). 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.

2.4. Assay procedure for the electrochemical immunosensor

The unspecific bindings at the mAbE-AuNPs-pNap-GCE were avoid by a treatment at 37 °C with 3% low-fat milk in PBS during 10 min and washed with PBST. Thus, the free AuNPs were prevented from interacting with sample components and the HRP added in the detection stage. Then, aliquots of 10 μL of solutions containing different E concentrations were dropped on the EI and incubated at 37 °C during 30 min and then, rinsed with PBS. Finally, the EI was immersed to the cell and 200 μL of a solution containing given concentrations of HRP + H_2O_2 + H_2Q in CBS. After 10 min, the enzymatic reaction product (Q) was detected through SWV. The total time of immunoassay was 45 min.

For next determination, the EI was reconditioned by desorption of E with 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 40 determinations. Then, the current gradually decreases and dropped to 70% of its initial value in determining number fifty. This could be due to the progressive denaturation with time and successive determinations of mAbE immobilized. We verify the efficiency of E desorption by checking that the maximum reduction current was obtained for the enzymatic reaction product after addition of HRP, H_2O_2 and H_2Q (Fig. A in Supplementary material).

2.5. Assay cross-reactivity procedure

After the EI was treated to prevent nonspecific adsorption (see Section 2.4), 10 μL of a solution containing 1 ng mL^{-1} of the P4, 17β-E and E1 separately in the absence and in the presence of 10 pg mL^{-1} of E were dropped on the EI and incubated at 37 °C during 30 min and then, rinsed with PBS. Finally, the EI was immersed to the cell and 200 μL of 8×10^{-11} mol L^{-1} HRP + 6×10^{-3} mol L^{-1} H_2O_2 + 3×10^{-3} mol L^{-1} H_2Q solution in CBS was added. After 10 min, the enzymatic reaction product (Q) was detected through SWV. The same procedure was carried out by dropping on the EI a solution containing 10 pg mL^{-1} E + 1 ng mL^{-1} P4 + 1 ng mL^{-1} 17β-E + 1 ng mL^{-1} E1. Each experiment was performed by triplicate.

3. Results and discussion

3.1. Characterization of AuNPs-pNap–GCE

It is known that the AuNPs allow a best orientation of immobilized proteins, which makes the direct electron transfer more favourable [42]. Also, polymers have been used as a platform suitable for the immobilization of AuNPs [35,36]. We used CV to study each stage during the development of EI. Fig. 1a shows cyclic voltammograms of 1×10^{-3} mol L^{-1} H_2Q in pH 5.00 CBS recorded in the potential range from −0.1 to 0.7 V vs. CSE. At the bare GCE (Fig. 1a.i), the cyclic voltammogram showed a well-defined anodic peak and its cathodic peak, corresponding to well known two-electron (H_2Q/Q) quasi-reversible redox couple [38]. The H_2Q electron transfer kinetics was disturbed at the modified pNap-GCE (Fig. 1a.ii). As it can be observed, a marked decrease in oxidation and reduction peaks was found. Then, an increase in both the oxidation and reduction currents was obtained at the AuNPs-pNap-GCE (Fig. 1a.iii) in comparison with the modified pNap-GCE. This behaviour suggests that the blockade of the electron transfer process by pNap film was clearly restored at the AuNPs-pNap-GCE. A probable explanation for this behaviour would be the increase in the electrode area by the presence of AuNPs. The H_2Q redox couple at the AuNPs-GCE was also studied for comparison. A significant increase in H_2Q oxidation and reduction currents was also observed when the AuNPs was electro-deposited on bare GCE in the absence of the film (Fig. 1a.iv).

On the other hand, the modified GCE morphology was analyzed by SEM measurements for the different modification processes. Fig. 1b shows the bare GCE surface (Fig. 1b.i–ii) and the modified pNap-GCE surface (Fig. 1b.iii–iv) after depositing AuNPs. It can be observed that both surfaces are covered with AuNPs. The electrochemical deposition of AuNPs on the bare GCE surface was not homogeneous and the aggregation of AuNPs was observed (Fig. 1b.i–ii), where the particle size of AuNPs was determined to be 20–80 nm. When the AuNPs were deposited on the modified pNap-GCE surface an increased coverage, no aggregation and a homogenous distribution on the surface was observed. The AuNPs size was determined to be 30–60 nm (Fig. 1b.iii–iv). These sizes of AuNPs are consistent with those obtained by other authors using this methodology [41]. A different morphology is clearly obtained from deposition of AuNPs on bare and modified GCE (Fig. 1b). On the modified GC surface, AuNPs are uniformly distributed and no aggregation is observed while agglomerates are present at the bare CGE. We assume that this can occur because of the polymer probably has a lesser rough surface than the bare GCE, favouring a more homogeneous distribution of electro-deposited AuNPs. In addition, as it will be discussed later the presence of film could facilitate the binding and/or orientation of the antibody in the design of EI.

3.2. HRP activity towards E

The enzymatic reaction between HRP and E was studied by UV–visible spectroscopy and SWV techniques.

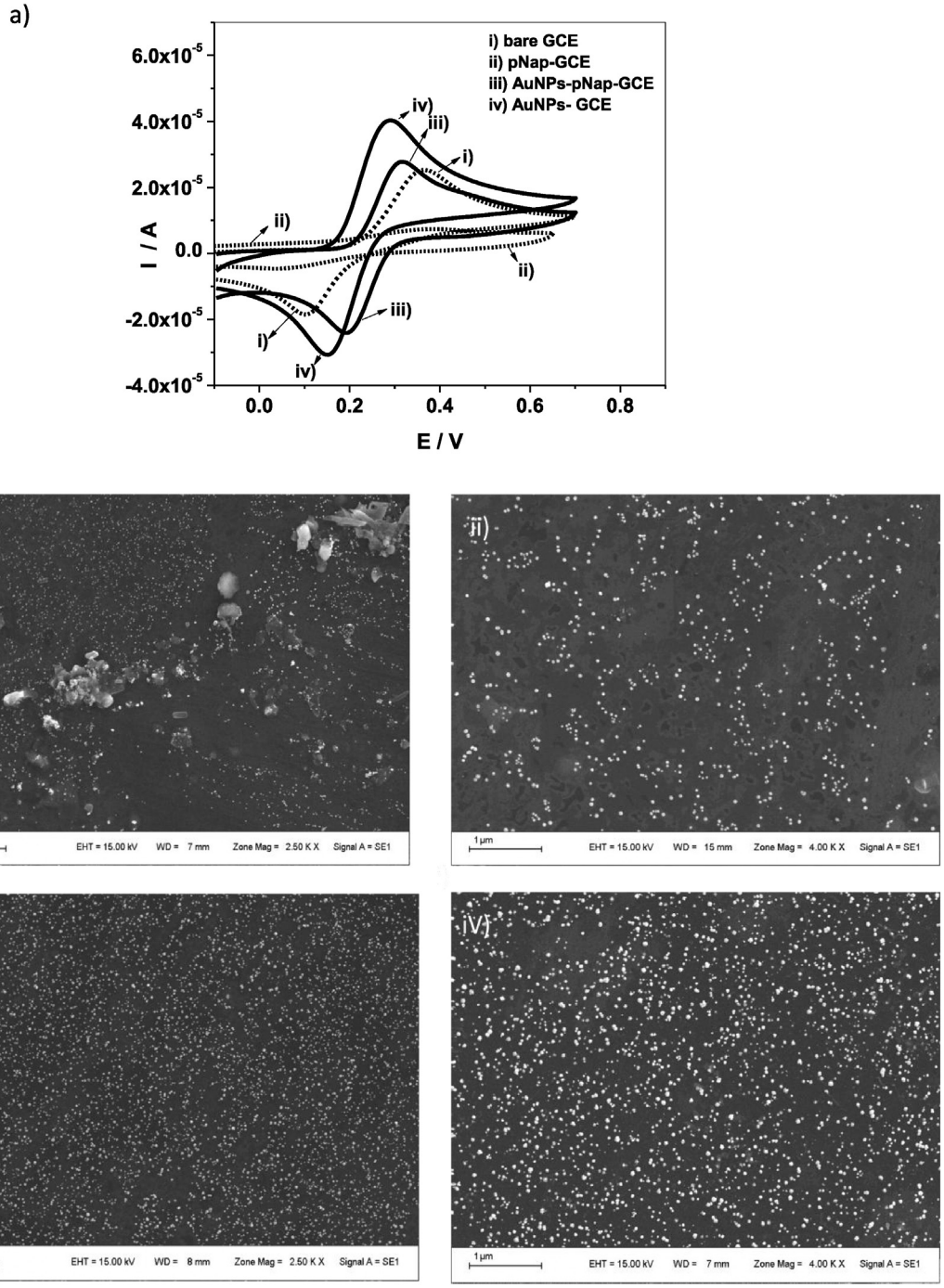


Fig. 1. a) Cyclic voltammograms recorded for H_2Q in pH 5.00 CBS of bare and modified GCE electrodes as it indicated in the figure insert; b) SEM images of AuNPs on bare GCE disk electrode at different scale (i and ii) and on pNap-GCE at different scale (iii and iv).

UV–Vis spectra of E in electrolytic solutions at different times of the enzymatic reaction were analyzed by monitoring the absorbance at 294 nm by triplicate. From these experiments, the Michaelis–Menten apparent constant (K_M) was calculated. From a plot of $1/V_i$ vs. $1/c_E^*$ (Lineweaver-Burk plot, Fig. 2) [43], where V_i is the initial enzymatic reaction rate and $c_E^*$is the E bulk concentration, a value of $K_M = 8.7 \times 10^{-5}$ mol L^{-1} was obtained. This result shows that HRP can recognize E as co-substrate in homogeneous media, as occurs with other phenolic compounds [33,38].

Then, the affinity of HRP towards E immobilized on the electrode was evaluated by SWV for the following modified electrodes: mAbE-AuNPs-GCE and EI. Fig. 3a shows a SW voltammogram recorded in the solution of HRP + H_2O_2 + H_2Q at the mAbE-AuNPs-GCE in the absence of E. Fig. 3b shows a SW voltammogram recorded after incubating the mAbE-AuNPs-GCE in a solution with E. No change in current was observed, so that this analytical platform cannot be used to detect E. This is probably because the antigen-antibody complex is not formed due to the orientation of the antibody is not appropriate.

Fig. 3c shows a SW voltammogram recorded in the solution of HRP + H_2O_2 + H_2Q at the EI in the absence of E. Fig. 3d shows a SW voltammogram recorded after incubating the EI in a solution with E. A higher net peak current ($I_{p,n}$) was observed in the absence of E (Fig. 3c), indicating that all HRP is consumed by H_2Q generating Q.

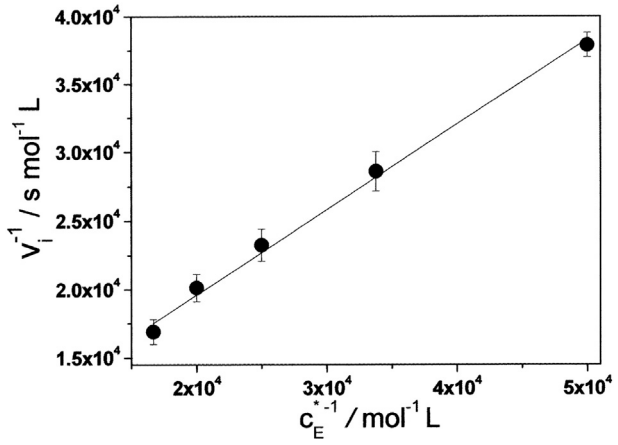


Fig. 2. Dependence between the reciprocal of the initial velocity and the reciprocal of the E concentration. $c^*_{H_2O_2} = 2.26 \times 10^{-3}$ mol L^{-1}; $c^*_{HRP} = 3.26 \times 10^{-9}$ mol L^{-1}. Intercept: $(7.12 \pm 0.01) \times 10^3$ s mol^{-1} L, slope: (0.6 ± 0.1) s, r = 0.9980.

However, the $I_{p,n}$ decreased for the SW voltammogram recorded when E formed the immune-complex (Fig. 3d), showing clearly that HRP reacts with both E and H_2Q. It was observed that the $I_{p,n}$ decreased as the E concentration increased (results no shown). $I_{p,n}$ variations below 5% were obtained in the absence of HRP for different concentrations of E incubated at the EI surface, and the same H_2Q concentration. These results indicate that changes in the $I_{p,n}$ found in the presence of HRP are due to the reaction of E with the enzyme.

The presence of pNap film favors the distribution of AuNPs and probably also the immobilization and/or orientation of mAbE, favoring the formation of the antigen-antibody complex (see Fig. 1b.iii–iv), considering that in the absence of the film no change in $I_{p,n}$ was observed in both the presence and the absence de E as was previously discussed.

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

The enzymatic reaction conditions have been previously studied [44] and the maximum reaction rate was obtained in pH 5.00 CBS. Incubations were carried out at 37 °C since it is the optimum temperature of immunoreaction for all IgG [45].

Once all the variables were optimized, the influence of the mAbE concentration on the EI response was also evaluated. This was performed for 1:100, 1:200, and 1:400 dilution factors. A major change in the $I_{p,n}$ ($\Delta I_{p,n}$) values for different E concentrations was obtained when the mAbE dilution factor decreased (Fig. B in Supplementary material). $\Delta I_{p,n}$ increases because a larger amount of E can form the antigen-antibody complex with the mAbE immobilized. Thus, a 1:100 dilution factor was selected for further experiments.

Other optimized variables were the concentration of H_2O_2, H_2Q and HRP. The results are shown in Fig. 4. All determinations were performed by triplicate. Fig. 4a.i 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 given E incubated concentration (50 pg mL^{-1}). The influence of H_2Q concentration when HRP, H_2O_2, and E incubated were 1.5×10^{-10} mol L^{-1}, 6×0^{-3} mol L^{-1} and 50 pg mL^{-1}, respectively is displayed in Fig. 4a.ii. Finally, Fig. 4b shows the effect of varying the HRP concentration, at given concentrations of H_2Q, H_2O_2 and E incubated (2×10^{-3} mol L^{-1}, 6×10^{-3} mol L^{-1} and 50 pg mL^{-1}, respectively). From these studies, the optimal concentrations were 6×10^{-3} mol L^{-1}; 3×10^{-3} mol L^{-1} and 8×10^{-11} mol $L^{-1}$for H_2O_2; H_2Q and HRP, respectively. These values were used in the following experiments.

3.4. Calibration curve

A typical calibration curve constructed for E under the optimized working conditions is displayed in Fig. 5. The tested concentration range ~~tested~~ was from 1×10^{-3} to 2 x 10^5 pg mL^{-1}, and the linear range was between 8×10^{-2} and 2×10^4 pg mL^{-1}. As previously mentioned, the $I_{p,n}$ correspond to the reduction of Q enzymatically generated, which is indirectly proportional to the amount of E incubated on the EI. The results were normalized to $I^o_{p,n}$ (the net peak current obtained in the absence of E) to correct for slight variations in the fabrication of the EI. Every point corresponds to the average of three replicated measurements obtained with different biosensors.

Error bars in Fig. 5 show a good reproducibility. The LOD, calculated as the concentration of E which produces a decrease in signal equal to three times the standard deviation of the blank was 0.061 pg mL^{-1} [46]. This value is notably better than those achieved with other

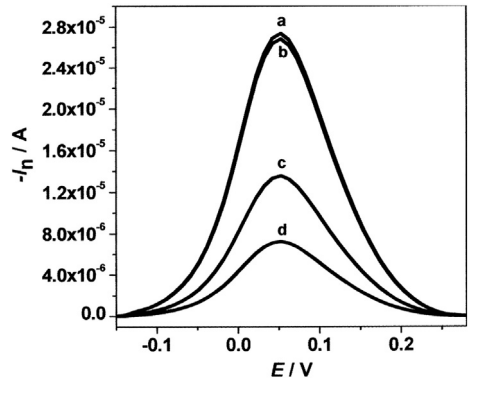


Fig. 3. Square wave voltammograms for two analytic platforms obtained in pH 5.00 CBS: a) mAbE-AuNPs-GCE in the absence of E; b) mAbE-AuNPs-GCE with 500 pg mL^{-1} of E, previously incubated. c) mAbE-AuNPs-pNap-GCE in the absence of E. d) mAbE-AuNPs-pNap-GCE with 500 pg mL^{-1} of E, previously incubated. $c^*_{H_2O_2} = 5 \times 10^{-3}$ mol L^{-1}, $c^*_{HRP} = 8 \times 10^{-11}$ mol L^{-1}, $c^*_{H_2Q} = 3 \times 10^{-3}$ mol L^{-1}, $\Delta E_{SW} = 0.025$ V, $\Delta E_s = 0.005$ V, and f = 25 Hz.

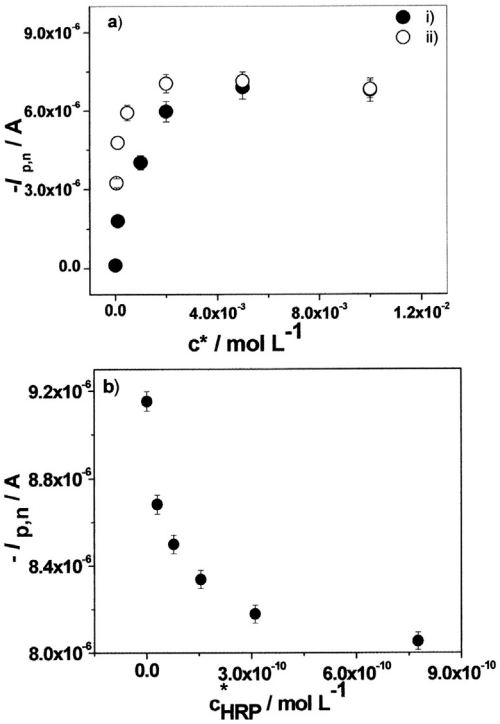


Fig. 4. Variations of $I_{p,n}$ for 50 pg mL^{-1} E solution on the mAbE-AuNPs-pNap-GCE with: a) $c^*_{H_2O_2}$ i) ($c^*_{H_2Q} = 1.0 \times 10^{-3}$ mol L^{-1}, $c^*_{HRP} = 1.5 \times 10^{-10}$ mol L^{-1}); $c^*_{H_2Q}$ ii) ($c^*_{H_2O_2} = 6 \times 10^{-3}$ mol L^{-1}, $c^*_{HRP} = 8 \times 10^{-11}$ mol L^{-1}; b) c^*_{HRP}, ($c^*_{H_2O_2} = 6 \times 10^{-3}$ mol L^{-1}; $c^*_{H_2Q} = 3 \times 10^{-3}$ mol L^{-1}. pH 5.00 CBS. SW parameters are the same that those in Fig. 3.

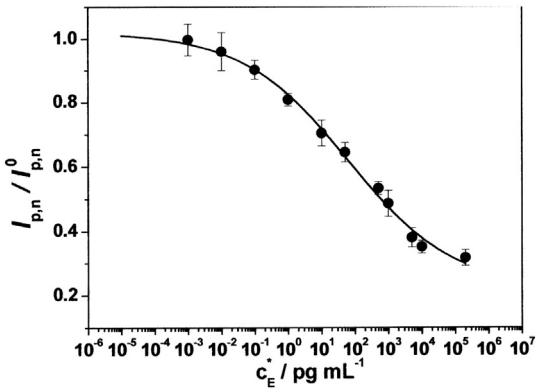


Fig. 5. Normalized calibration curve for E recorded using different EI, and optimized parameters in pH 5.00 CBS. Each point is the average of three replicated measurements. $c^*_{H_2O_2} = 6 \times 10^{-3}$ mol L^{-1}, $c^*_{HRP} = 8 \times 10^{-11}$ mol L^{-1}, $c^*_{H_2Q} = 3 \times 10^{-3}$ mol L^{-1}. SW parameters are the same that those in Fig. 3.

immunosensors previously reported: 1.25 pg mL^{-1} [28], 1.35 ng mL^{-1} [30] and 1.4 pg mL^{-1} [32] and 10 times lower than that obtained for 17β-E with a similar detection process [33].

In addition, the time to modify the electrode surface with AuNPs is reduced considerably (from 150 min to 15 min) compared with the immunosensor developed to detect 17β-E [33]. The within-assay precision of electrochemical measurements was tested for E standard solutions of 50 pg mL^{-1} and 100 pg mL^{-1} in PBS by triplicate. Thus, percentage variation coefficients (VC %) were 1.0% and 4.0%, respectively, showing a good repeatability. The precision inter-assays for the same concentrations did not exceed 5%.

Tests were also conducted to evaluate the cross-reactivity of E with other hormones of similar structure such as 17β-E, P4 and E1 (Fig. 6). Fig. 6a shows the $I_{p,n}$ obtained for EI in the absence of E. Afterwards, 10 μL of 1 ng mL^{-1} 17β-E solution was dropped on the EI in the absence of E (Fig. 6b). Then, the EI was incubated for 30 min at 37 °C, rinsed and immersed to the detection cell. In the absence of E and in the presence of the 17β-E, a $I_{p,n}$ maximum (VC% 4%) is obtained due to the discharge of Q enzymatically generated indicating that 17β-E was not immobilized by mAbE.

In the presence of 10 pg mL^{-1} E (Fig. 6c) and 10 pg mL^{-1} E with 1 ng mL^{-1} of 17β-E, P4, and E1 (Fig. 6d), similar $I_{p,n}$ was obtained (VC% 4%) on the EI. This result demonstrates the high selectivity for E on the EI. Therefore, if any cross-reactivity would exist, it is within the

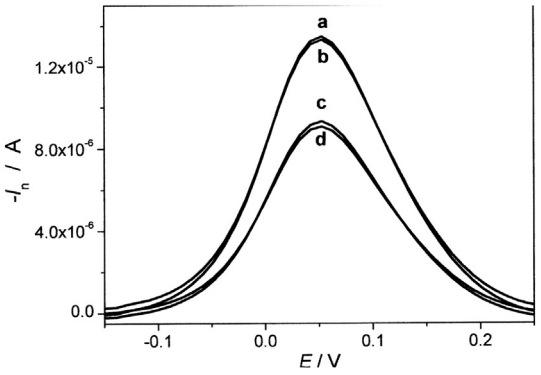


Fig. 6. Square wave voltammograms in pH 5.00 CBS for the electrochemical immunosensor in the absence of E (a); with 1 ng mL^{-1} 17β-E (b); 10 pg mL^{-1} E (c); 10 pg mL^{-1} E + 1 ng mL^{-1} 17β-E + 1 ng mL^{-1} P4 + 1 ng mL^{-1} E1 (d). $c^*_{H_2O_2} = 6 \times 10^{-3}$ mol L^{-1}, $c^*_{HRP} = 8 \times 10^{-11}$ mol L^{-1}, $c^*_{H_2Q} = 3 \times 10^{-3}$ mol L^{-1}. SW parameters are the same that those in Fig. 3.

Table 1
Statistical analysis of immunosensor responses for three water samples with different amounts of spiked E.

[a]c^*_E (pg mL^{-1})	[b]c^*_E (pg mL^{-1})	% Recovery
50	49,0	98,2
100	100,5	105,5
200	201,3	100,8

[a] E concentration in water samples.
[b] Average value of E concentration determined with the immunosensor.

percentage of variation of the detection method that is <5% (see error bars in Fig. 5).

3.5. Real sample analysis

The usefulness of the mAbE-AuNPs-pNap-GCE immunosensor for the analysis of real samples was demonstrated by analyzing water samples spiked with E at different concentrations: 50, 100 and 200 pg mL^{-1}. It is important to emphasize that water samples were incubated in the immunosensor without any previous pretreatment. The results obtained are given in Table 1 with mean recoveries of 98 and 105%. They are considered to be good recoveries. The storage stability of EI was evaluated during twenty five days for a constant E concentration. The initial immunosensor response was constant during ten days and then, it started to decrease gradually, reaching a value of 84% at twenty five days.

The regeneration of the EI surface is a key factor for developing a practical immunosensor. The EI surface was regenerated by simply immersing it in a 0.1 mol L^{-1} glycine - HCl, pH 2.00 during 2 min followed by washing with PBST, which allowed us to use the biosensor over about forty determinations. It was checked by measurements of net peak currents and using alternatively E standard solutions and water samples. Between each experiment set, the immunosensor was regenerated again. The results were reproducible, showing that the antibody activity loss is not appreciable.

4. Conclusions

In this study, an integrated electrochemical immunosensor was developed to determine estrone at trace levels in water 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 limit of detection (0.061 pg mL^{-1}), great sensitivity, high specificity, an important analytical range of interest, good reproducibility and an acceptable accuracy. This performance is notably better than those achieved with other immunosensors previously reported [28,30,32]. The detection limit was reduced tenfold. In addition, the modification time of the modified electrode surface with AuNPs was considerably reduced compared to other immunosensors with a similar process of detection.

The immunosensor developed can operate as a fast, selective, and sensitive detector. This device has several advantages over other methods for the determination of estrone in real samples, such as direct measurement without any pre-treatment, use of small volumes (harmful solvents and expensive reagents are avoided), without antigen or antibody labeled and mainly with the advantage that the electrochemical immunosensor platform is formed quickly by using cyclic voltammetry, which has the advantage of exercising control over the polymer film thickness and homogeneity. The EI also shows physical and chemical stability and accuracy. In addition, integrated approaches allow considering a potential sensor miniaturization.

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