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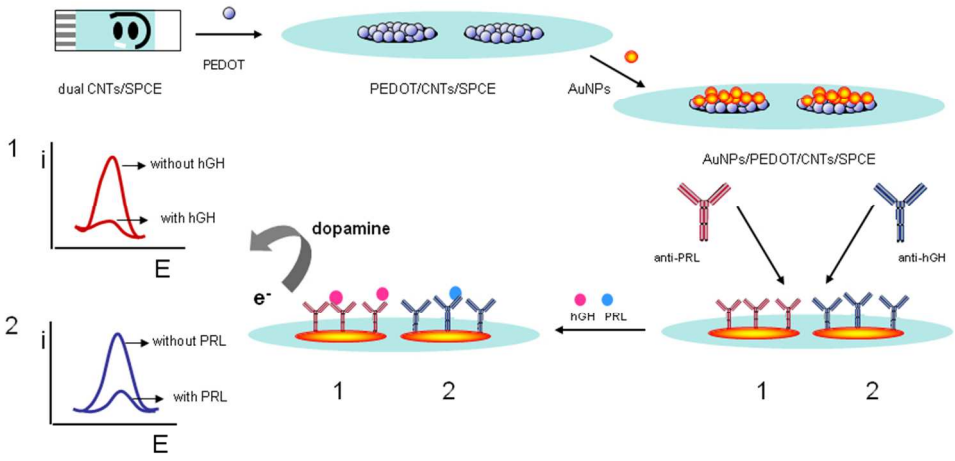

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A label-free dual electrochemical immunosensor for simultaneous determination of human growth and prolactin hormones was prepared for the first time

Multiplexed determination of human growth hormone and prolactin at a label free electrochemical immunosensor using dual carbon nanotubes-screen printed electrodes modified with gold and PEDOT nanoparticles

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Abstract

A label-free dual electrochemical immunosensor was constructed for the multiplexed determination of human growth (hGH) and prolactin (PRL) hormones. The immunosensor used an electrochemical platform composed of carbon nanotubes-screen printed carbon electrodes (CNTs/SPCEs) modified with poly(ethylene-dioxythiophene) (PEDOT) and gold nanoparticles, on which the corresponding hGH and PRL antibodies were immobilized. The affinity reactions were monitored by measuring the decrease in the differential pulse voltammetric oxidation response of the redox probe dopamine.The experimental variables involved in the preparation of both AuNPs/PEDOT/CNTs/SPCE modified electrodes and the dual immunosensor were optimized. The immunosensor exhibited an improved analytical performance for hGH and PRL with respect to other electrochemical immunosensor designs, showing wide ranges of linearity and low detection limits of 4.4 and 0.22 pg/mL, respectively. An excellent selectivity against other hormones and in the presence of ascorbic and uric acids was found. The usefulness of the dual immunosensor for the simultaneous analysis of hGH and PRL was demonstrated by analyzing human serum and saliva samples spiked with the hormones at different concentration levels.

Keywords: Human growth hormone, prolactin, electrochemical immunosensor, carbon nanotubes, poly(ethylene-dioxythiophene), colloidal gold, screen printed carbon electrodes, simultaneous determination.

1. Introduction

Human growth hormone (hGH) and prolactin (PRL) are naturally occurring peptide hormones produced by the anterior pituitary gland[1]. hGH is essential for body growth since it stimulates the production of insulin growth factor (IGF-1), which in turn stimulates the production of cartilage cells, resulting in bone growth. Also, hGH plays important roles in the metabolism of proteins, lipids and carbohydrates[2]. Typical levels of hGH in blood range between 1 and 9 ng/mL (male), and 1 and 16 ng/mL (female), although this hormone is released into the circulation in a pulse manner, i.e. during sleep[3], with peaks between 50 and 100 ng/mL, and minimum levels of 0.03 ng/mL[1].

Prolactin (PRL) is involved in various important biological processes such as stimulation of lactation, regulatory roles in the growth and differentiation of the mammary glands, and in reproduction[4]. PRL human hormone circulates in serum in multiple forms of different molecular sizes, the monomeric prolactin being the most abundant (85–95%). Typical levels of PRL in blood are in the range of 2 to 18 ng/mL (male) and 2 to 25 ng/mL (female), although this last can increase up to more than two hundred ng/mL in pregnancy. PRL concentrations higher than 35 ng/mL also warns about the presence of a prolactinoma[5]. Alterations in hGH and/or PRL secretion are associated with health disorders frequently related to the existence of pituitary adenomas. Much attention has been paid on hGH/PRL circulating levels in relation to mammary tumour formation[6]. On the other hand, PRL and hGH have also a significant role in regulating neuroprotective and/or neurorestorative mechanisms in brain[7]. The

effects of both hormones on adipose tissue development and function[8], and the role of PRL and hGH as insulin-antagonists, are other relevant biological roles of these hormones[9].

All these effects clearly highlight the importance of the determination of hGH and PRL in clinical medicine. Therefore, the development of analytical methods able to provide multiplexed determination of both hormones can provide tools for a faster and easier diagnosis of relevant diseases. While blood serum is the main biological sample used for the determination of hGH and PRL, saliva has attracted an increasing interest for hGH/PRL analysis[10,11] since it provides an easily available non-invasive biological sample for the diagnostic of a rapidly widening range of diseases and clinical situations.

Various immunochemical methods have been proposed for the individual determinationof hGH and PRL. A time-resolved immunofluorimetric assay for hGH involving monoclonal antibody-coated microtiter strip wells and a europium-chelate labelled antibody was described[12]. A surface plasmon resonance immunoassay (SPR) was also developed to determine hGH without pre-treatment in human serum samples[13]. The same methodology was also used for the determination of various pituitary hormones including hGH (but not PRL) in serum and urine[14]. Moreover, fluorometric detection was employed in a multianalyte device for the determination of follitropin, human chorionic gonadotropin and PRL in human serum, which was provided with different antibodies reacting specifically with the analytes[15].

Because of their high sensitivity, rapid response and usability, electrochemical immunosensors have demonstrated to be excellent alternatives in clinical analysis of selected analytes[16,17]. The first voltammetric immunosensors for the detection of hGH[18] and PRL[19] were developed recently by our group. These consisted of electrochemical magnetoimmunosensors using tosyl-activated magnetic microparticles to immobilize

covalently monoclonal hGH antibody[18], or streptavidin-functionalized magnetic particles to immobilize biotinylated anti-PRL antibodies[19]. In both cases, sandwich-type immunoassays using alkaline phosphatase labelled antibodies were performed. Poly(o-phenylenediamine)-carried nanogold particles (GPPDs) functionalized with HRP-anti-PRLconjugates were also used for the development of a sandwich-type electrochemical immunoassay for PRL on a glassy carbon electrode[20]. Furthermore, an impedimetric immunosensor for hGH was reported involving the immobilization of hGH monoclonal antibody onto gold nanoparticle-coated 1,6-hexanedithiol-modified gold electrode[21].

Label-free electrochemical immunosensors combine the relevant advantages commented above with the simplification of the sensing protocols[22]. An immunoreagents label-free strategy was employed in this work to construct a dual electrochemical immunosensor for the multiplexed determination of hGH and PRL which fulfils the requirements of sensitivity, selectivity and reproducibility needed in clinical applications. In order to do that, an electrochemical platform composed of carbon nanotubes-screen printed carbon electrodes (CNTs/SPCEs) modified with poly(ethylene-dioxythiophene) (PEDOT) and gold nanoparticles, on which the corresponding hGH and PRL antibodies were immobilized, was implemented. The decrease in the voltammetric responses of dopamine, used as a redox probe in the label-free approach, recorded at the modified electrodes upon addition of hGH and/or PRL, was employed to monitor the affinity reactions. The analytical performance of the dual configuration was evaluated by determining hGH and PRL in human serum and saliva with a minimum sample treatment.

2. Experimental

2.1. Apparatus and electrodes

A BAS (West Lafayette, IN, USA) 100 B potentiostat provided with a BAS C2 EF-1080 cell stand and a CHI1030 multi-potentiostat (CH Instruments, Inc.) were used for the electrochemical measurements with the single screen-printed carbon electrodes (SPCEs, DropSens, 4 mm ø), and dual SPCEs (DropSens, consisted of two elliptic carbon electrodes (DropSens with surface area 5.6 mm^2), respectively. The electrodes also incorporated an Ag pseudo-reference electrode and a carbon counter electrode. They were modified with multiwalled carbon nanotubes (CNTs/SPCE) and used as working electrodes. All electrochemical experiments were performed at room temperature.

2.2. Reagents and solutions

Multiwalled carbon nanotubes (CNTs, 30±15 nm ø, 95% purity) were supplied by NanoLab, Brighton, MA. Before use, CNTs were chemically shortened and carboxylated by treatment with 2.6 M nitric acid under reflux for 24 h. The resulting product was centrifuged at 4,000 rpm for 10 min, washed repeatedly with distilled water up to pH 7, and dried under nitrogen. CNTs/SPCEs were prepared by casting 2 μL of a 0.5 mg CNTs per mL suspension in dimethyl formamide onto the SPCE surface and drying under IR radiation.

Mouse monoclonal anti-hGH antibody (mAbhGH; Western blot analysis predicted band size at 22-24 kDa) was from Abcam. Antibody solutions were prepared in 0.1 M Tris buffer solution of pH 7.2. hGH from human pituitary, recombinant, expressed in *Escherichia coli*, molecular weight 22 kDa, was obtained from Sigma-Aldrich. Mouse monoclonal anti-PRL antibody and PRL antigen were purchased from Immunometrics.

The solutions of anti-PRL and PRL were prepared in 0.1 M Tris buffer solution of pH 7.2. A 0.01 M dopamine (Sigma) stock solution was prepared in 0.1 M PBS of pH 7.4. More diluted solutions were prepared by dilution with the same buffer. Poly(3,4-ethylenedioxythiophene) (PEDOT) nanoparticles, 1 wt. % dispersion in water, were purchased from Aldrich. Sodium citrate (Panreac, 98%) and $HAuCl_4.3H_2O$ (Sigma, >49% as gold) were used for the preparation of gold nanoparticles following the procedure described earlier[23]. Briefly, 2.5 mL of a 1 % sodium citrate solution were added to 100 mL of a boiling aqueous solution containing 1 mL 1 % $HAuCl_4$. The diameter of the Au_{coll} particles was 16 ± 2 nm and they were stored in dark glass bottles at 4 ºC for further use.

2.3. Procedures

2.3.1. Modification of CNTs/SPC electrodes

Once the CNTs/SPCEs were prepared, 2 µL of the 1 wt. % PEDOT nanoparticles dispersion (in the case of single SPCE) or 0.7 µL (when working with the dual SPCE) were dropped onto the electrodes and allowed drying in the dark. The difference in the used volumes reflects the differences in the active surface area of the corresponding working electrodes. Then, the PEDOT/CNTs/SPCEs were washed with deionized water and immersed in 1 mL of the colloidal gold suspension at 4 ºC for 30 minutes. The modified AuNPs/PEDOT/ CNTs/SPCEs were washed with 0.1 M PBS of pH 7.4 and dried at room temperature.

2.3.2. Preparation of the immunosensors

The dual hGH and PRL immunosensor was prepared on AuNPs/PEDOT/CNTs/SPCE1 and AuNPs/PEDOT/CNTs/SPCE2 modified electrodes, respectively. 3-µL aliquots of

30 µg/mL anti-hGH or anti-PRL solutions in 0.1 M Tris buffer solution of pH 7.2 were dropped onto the respective electrodes and left overnight in humid ambient at 4ºC. Then, each resulting antibody-AuNPs/PEDOT/CNTsSPCE was washed with 0.1 M Tris buffer solution of pH 7.2 and the dual immunosensor was immersed in 1 mL of a 0.25 % BSA blocking solution for 30 min at room temperature.After washing with the same buffer solution, 3 µL of each antigen were deposited onto anti-hGH/AuNPs/ PEDOT/ CNTs/SPCE1 or anti-PRL/AuNPs/PEDOT/CNTs/SPCE2, respectively, allowing incubation for 1 h in humid ambient at room temperature. The determination of hGH and PRL was performed by immersion of the dual immunosensor in a 0.5 mM dopamine solution prepared in 0.1 M Tris PBS pH 7.4, and recording differential pulse voltammograms over the -250 to + 600 mV potential range. The analytical outputs were obtained from the change in the DPV peak current measurements before and after the antigen-antibody reaction. Between both measurements, the immunosensors were washed with the buffer solution and three cyclic voltammetric scans in the -200 - +500 mV potential range were made in order to remove any dopamine or oxidation product from the immunosensor surface.

2.3.3. Multiplexed determination of hGH and PRL in human serum and saliva

Human serum (Sigma, S-7394) and saliva collected from a lab researcher, were analyzed. Both were spiked with hGH and PRL at different concentration levels. The lyophilized serum was reconstituted in 1.0 mL 0.1 M PBS, pH 7.4, by mixing up to total dissolution. Thereafter, 2- µL aliquots of spiked serum were diluted with 100 µL of 0.1 M Tris buffer of pH 7.2 and the determination of the hormones was carried out directly by applying the procedure described in Section 2.3.2 using 3-µL aliquots of the diluted serum. The simultaneous determination of hGH and PRL were performed by measuring

the peak currents from DP voltammograms at each electrode, and interpolation of the analytical signals (Δi_p) into the linear portion of the corresponding calibration plots obtained with hGH or PRL standard solutions.

Regarding saliva, the samples were collected using a Salivette® collection device (Sarstedt). Briefly, the volunteer rinsed the mouth thoroughly with water and then inserted the cotton swab into the mouth and chewed for 1 min. Then, the swap saturated with saliva was inserted into the vial, sealed with the cap and centrifuged for 5 min at 5000 x g. The determination of hGH and PRL was performed immediately by diluting 1 mL of spiked saliva with 1 mL 0.1 M Tris buffer of pH 7.2 and applying the procedure described in Section 2.3.2 using 3-µL aliquots of the diluted saliva.

3. Results and Discussion

Figure 1 illustrates the different steps involved in the dual CNTs/SPCE modification as well in the design of the immunosensing strategy. CNTs-modified SPCE were selected as electrode platform to support further modifications due to the well known behavior of these electrodes in terms of achievable high current response and excellent reproducibility of the measurements[24]. The label-free immunoplatform design involved modification of CNTs/SPCEs with PEDOT and gold nanoparticles and the use of the voltammetric oxidation of dopamine to dopamine o-quinone as an adequate transduction signal[25]. This modification was justified by the improved voltammetric response of dopamine at the AuNPs/PEDOT/CNTs/SPCE as well as the stable immobilization of the biomolecules. Figure 2 shows cyclic voltammograms recorded for 0.5 mM dopamine at electrodes with different modification steps. As it can be seen, CVs obtained at electrodes prepared with no PEDOT, AuNPs/CNTs/SPCE (curve 3) and CNTs/SPCE (curve 4), exhibited a less reversible behavior than that obtained with polymer nanoparticles, AuNPs/PEDOT/CNTs/SPCE (curve 1) and PEDOT/CNTs/

SPCE (curve 2).This improved electron transfer can be attributed to the ability of PEDOT to act as an electronic mediator as a consequence of its rich electronic cloud[26]. Moreover, the polymer provided a hydrophobic environment which favored a more reversible oxidation of dopamine[27]. The subsequent modification of PEDOT/ CNTs/SPCEs with gold nanoparticles provoked a slight shift of dopamine anodic and cathodic peaks towards less positive potential values and an increase in the oxidation peak current (voltammograms 1 and 2 in Figure 2). The peak potential values were 153 and 64 mV at the AuNPs/PEDOT/CNTs/SPCE, and 200 and 109 mV at PEDOT/CNTs/SPCE, with a similar $E_{pa} - E_{pc} \approx$ 90 mV for both modified electrodes. Conversely, the anodic peak current values were 108 µA at the AuNPs/PEDOT/ CNTs/SPCE and 92 µA at the PEDOT/CNTs/SPCE. This behavior can be explained taking into account that, at pH 7.4, cationic dopamine can be adsorbed on negative charged gold nanoparticles prepared from citrate-colloidal gold. The apparent narrower oxidation peak shape observed with the AuNPs/PEDOT/CNTs/SPCE supports this assumption.

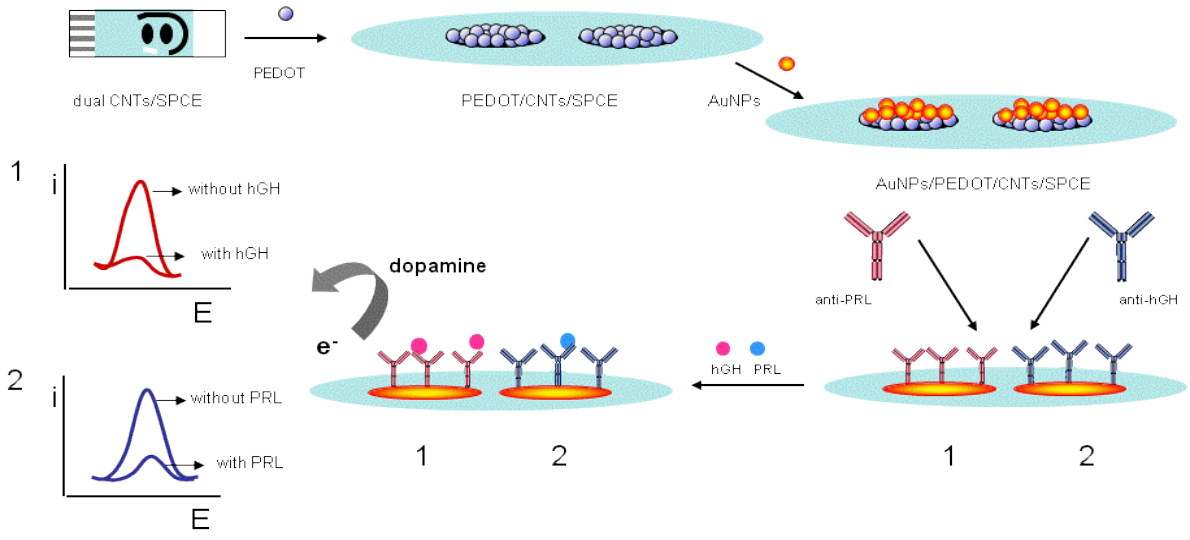

Figure 1. Schematic display of the different steps involved in the preparation and functioning of the dual electrochemical immunosensor for multiplexed determination of hGH and PRL.

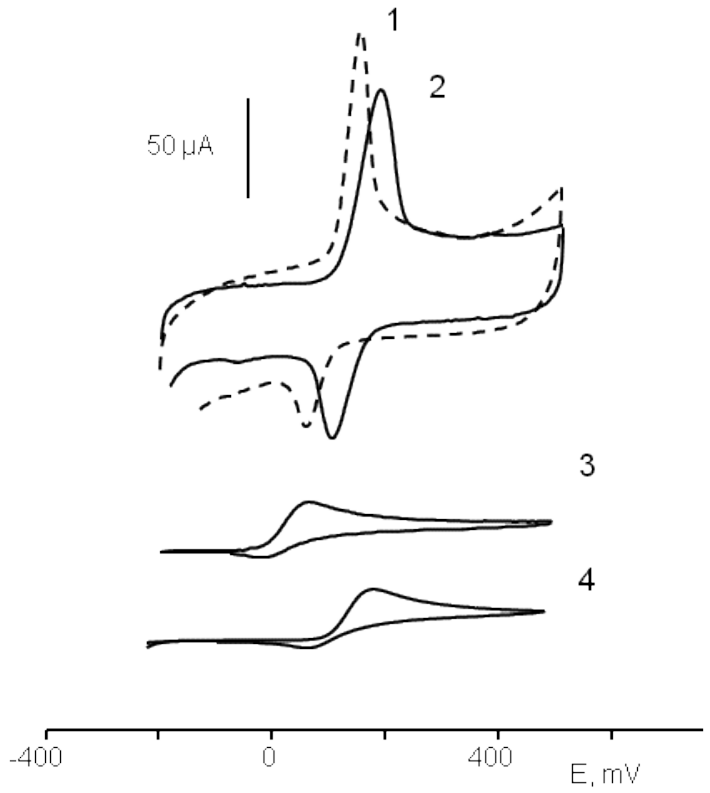

Figure 2. Cyclic voltammograms of 0.5 mM dopamine at AuNPs/PEDOT/CNTs/SPCE (1); PEDOT/CNTs/SPCE (2) AuNPs/CNTs/SPCE (3); CNTs/SPCE (4) in 0.1 M PBS pH 7.4; v= 50 mV/s.

As it is illustrated in Figure 1, once the modified electrode platforms were prepared, the anti-hGH and anti-PRL antibodies were immobilized on the AuNPs/PEDOT/CNTs/SPCE1 and AuNPs/ PEDOT/CNTs/SPCE2, respectively, by profiting the adsorption ability of negatively charged AuNPs towards the capture antibodies. After a blocking step with BSA, hGH and PRL standard solutions (or the corresponding samples) were dropped onto the respective immunoelectrode and the label-free monitoring of the affinity reactions was accomplished by recording the DP voltammetric oxidation read outs of a dopamine solution.

3.1. Optimization of the experimental variables involved in the preparation of AuNPs/PEDOT/CNTs/SPCE modified electrode

These optimization studies were performed by using single CNTs/SPC electrodes. The loading of PEDOT nanoparticles deposited on the surface of CNTs/SPCE was

optimized by measuring the i_p values from 0.5 mM dopamine solutions at electrodes prepared with different volumes of the 1 wt. % PEDOT nanoparticles dispersion. Figure 3a shows as the largest current values were obtained for the smallest PEDOT loading, i.e. 1 µL. This is probably due to the decrease in conductivity of the modified CNTs/SPCE surface as the PEDOT loading increased as a consequence of the lower polymer conductivity compared with that of CNTs. Accordingly, a slightly compact PEDOT coating was prepared in order to achieve a highly sensitive dopamine response. However, as it is observed in Fig. 3a, the reproducibility of the voltammetric measurements was much better when the CNTs/SPCE was modified with 2 µL of polymer nanoparticles (RSD values of 2.8 % (n=3) vs. 16 % (n=3), respectively). Therefore, a PEDOT nanoparticles volume of 2 µL was selected for further optimization studies. However, a volume of 0.7 µL was used when the dual SPCEs were employed due to the smaller surface area of these elliptic electrodes.

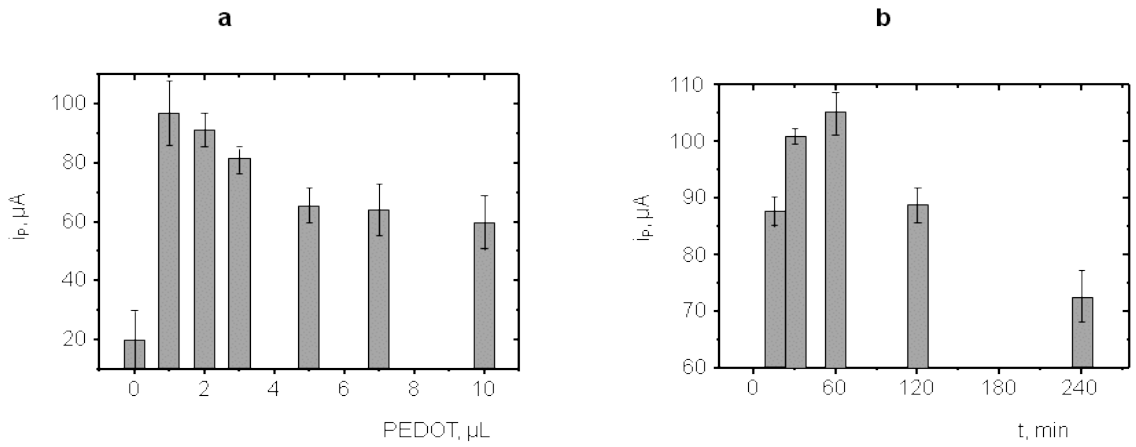

Figure 3. Effect of (a) PEDOT nanoparticles loading on CNTs/SPCE (a) and the incubation time of PEDOT/CNTs/SPCE in the colloidal gold suspension (b) on the DPV response of 0.5 mM dopamine.

The time for incubation of the PEDOT/CNTs/SPCE into the gold nanoparticles suspension was also optimized. Figure 3b shows as the current increased notably up to 60 min with an important decrease for longer periods. This behavior is most likely due

to a moderate AuNPs loading favors the already mentioned adsorption of positively charged dopamine and enhances the conductivity of the resulting AuNPs/PEDOT/CNTs/SPCE. However, a large amount of AuNPs probably provoked a partial blocking of the electrode surface as well as an increase in the double layer capacitance. As a compromise between high sensitivity and short analysis time, an incubation period of 30 min was selected for further work.

3.2. Optimization of the experimental variables involved in the preparation of hGH and PRL dual immunosensor

The variables affecting the functioning of the hGH and PRL immunosensors were optimized using AuNPs/PEDOT/CNTs/SPCEs prepared as indicated in section 3.1.

Non-specific adsorption of hGH and PRL to the electrodes surfaces was observed by recording cyclic voltammograms for the hormones with AuNPs/PEDOT/CNTs/SPCEs without immobilized antibodies (results not shown). Therefore, a blocking step with BSA was accomplished to minimize such unwanted effect. Figure 4a shows the changes in current measured at the immunosensors for 0.25 µg/mL hGH or 1 µg/mL PRL with respect to that obtained at AuNPs/PEDOT/CNTs/SPCEs blocked with different BSA concentrations. As it can be observed, the largest differences in the i_p values measured for 0.5 mM dopamine were found using 0.3 % or 0.2 % BSA for PRL (white bars) and hGH (grey bars), respectively, these blocking agent percentages being used in further work. Moreover, an immersion time of 30 min at room temperature of the immunosensor in the BSA solution was shown to provide an efficient minimization of the unspecific binding.

Furthermore, the respective antibody loadings on the modified electrodes were optimized by checking the DP voltammetric responses obtained for 0.5 mM dopamine

with different immunosensors prepared by casting on the electrode surface 10 µL of anti-hGH or anti-PRL solutions in the 10 to 50 µg/mL concentration range. Figure 4b shows the changes in current measured at the immunosensors for 0.25 µg/mL hGH or 1 µg/mL PRL with respect to that obtained at AuNPs/PEDOT/CNTs/SPCEs. In the case of the hGH immunosensor (grey bars), the absolute Δi_p values increased with the anti-hGH loading up to 30 µg/mL and decreased for larger loadings probably as a consequence of a less efficient immobilization process leading to higher i_p values in the presence of target hGH. Regarding the PRL immunosensor, a less sharp effect of the anti-PRL loading was observed with the largest Δi_p values occurring for 20 and 30 µg/mL antibody. According to these results, a 30 µg/mL concentration of both antibodies was selected for further work.

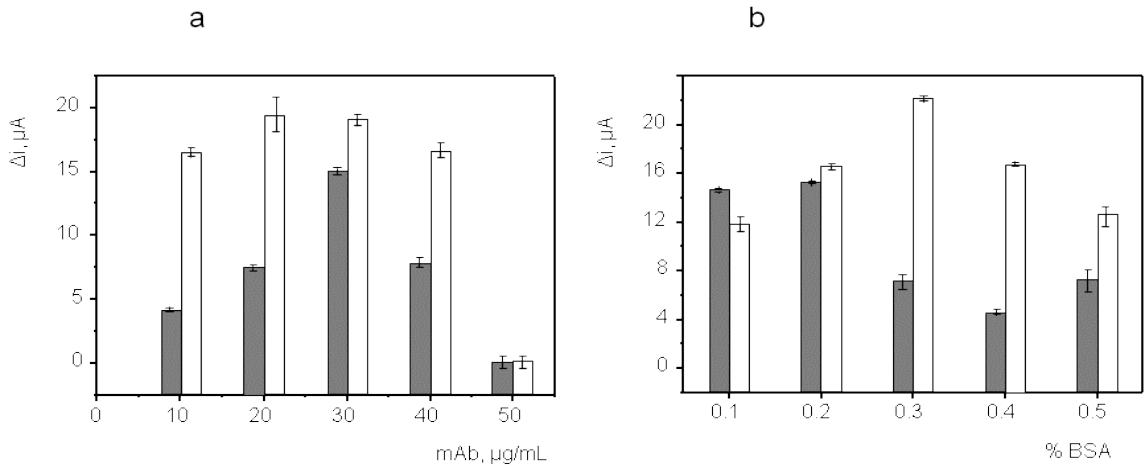

Figure 4. Effect of the BSA percentage used in the blocking step (a) and the antibody loading (b) on the changes in the DPV peak current measured for 0.5 mM dopamine with anti-hGH/AuNPs/PEDOT/CNTs/SPCE (grey) or anti-PRL/AuNPs/PEDOT/CNTs/SPCE (white) immunosensors; 0.25 µg/mL hGH, 1 µg/mL PRL.

3.3. Analytical characteristics for the determination of hGH and PRL with the dual immunosensor

Calibration plots for hGH and PRL were constructed with the dual immunosensor under the optimized conditions (Figure 5). Linear ranges between 0.05 - 1000 ng/mL hGH (r = 0.991) and 0.001 – 1000 ng/mL PRL (r = 0.992) were much wider than those reported for other electrochemical immunosensors (see Table 1). As an example of the voltammetric responses obtained, Figure 5b displays differential pulse voltammograms for 0, 1, 10, 100 and 1000 pg/mL PRL. As we mentioned above, the analytical outputs used to construct the calibration plots shown in Fig. 5a were the changes in the DPV peak currents, electronically measured, between the absence of analyte and those measured for the respective analyte concentrations. The respective limits of detection were calculated according to the $3s_b/m$ criterion, where s_b was estimated as the standard deviation (n = 10) of the zero value (the i_p value measured in the absence of hGH or PRL). As it can deduced from Table 1, the LOD value for hGH, 4.4 pg/mL, is slightly lower than that obtained using the electrochemical magnetoimmunosensor with a sandwich configuration and AP-labeling[18] but much better that the reported with a SPR immunoassay (4 ng/mL)[13]. Regarding PRL, the achieved LOD, 0.22 pg/mL, was more than 450 times lower than that obtained using an electrochemical anti-PRL/AuNPs/ poly-L-lysine/GCE immunosensor in a sandwich configuration with poly(o-phenylene-diamine)-carried AuNPs functionalized with HRP-anti-PRL, as signal tags[20], and much better than the LOD value found with a magnetoimmunosensor using anti-PRL-biotin-strept-MBs,3.74 ng/mL[19]. Probably, this enhanced analytical performance of the dual immunosensor was related with the excellent electrochemical behavior of dopamine at the screen printed electrodes modified with PEDOT/CNTs hybrids together with the efficient immobilization of the antibodies on the surface confined AuNPs.

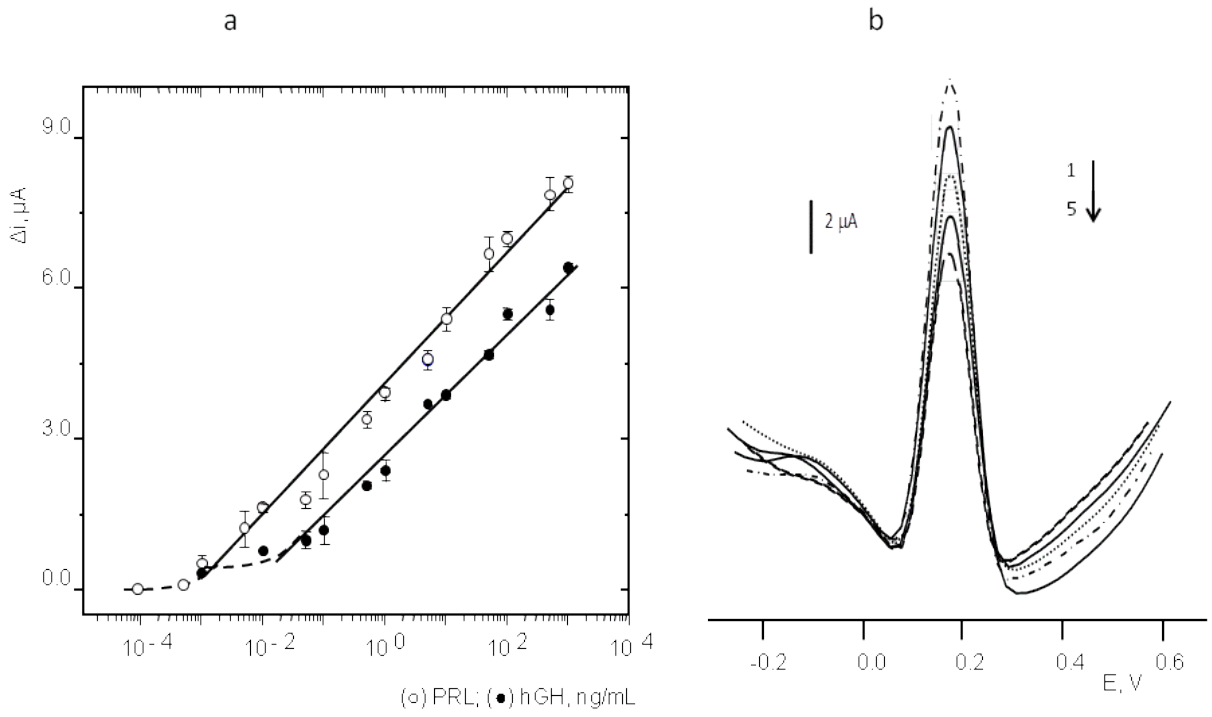

Figure 5. (a) Calibration plots for hGH (●) and PRL(○) obtained with the anti-hGH/AuNPs/PEDOT/CNTs/SPCE and anti-PRL/AuNPs/PEDOT/CNTs/SPCE dual immunosensor. (b) DP voltammograms of 0.5 mM dopamine at anti-PRL/AuNPs/PEDOT/CNTs/SPCE for 0 (-·-·- 1), 1 (—— 2), 10 (····· 3), 100 (— 4) and 1000 (——5) pg/mL PRL. See the text for other conditions.

The reproducibility of the DP voltammetric measurements was evaluated by carrying out repetitive assays on the same day and on different days for solutions containing 1 ng/mL hGH or PRL. Relative standard deviation (RSD) values of 3.5 and 2.7%, respectively, were obtained for the measurements performed on the same day, whereas these values were 4.0 and 4.8% when the measurements were made on different days. Also, we tested the storage stability of the anti-hGH/AuNPs/PEDOT/CNTs/SPCE and anti-PRL/AuNPs/PEDOT/CNTs/SPCE immunoconjugates. Several immunosensors were prepared on the same day and stored in a refrigerator at 4º C. Then, they were used in different days to measure the voltammetric responses of hGH and PRL in the 1 - 500 ng/mL concentration range. The results obtained (not shown) revealed that these responses remained inside the control limits set at ± 3 times the standard deviation of five initial measurements for 4 days, in the case of hGH, and 7 days for PRL.

Table 1. Analytical characteristics reported for the determination of hGH and PRL using different electrochemical immunosensors

Antigen	hGH	hGH	hGH	PRL	PRL	PRL
Electrode	AuNPs/PEDOT/ CNTs/SPCE (this work)	AuNPs/HDT/ AuE[21]	SPAuE[18]	AuNPs/PEDOT/ CNTs/SPCE (this work)	SPCE[19]	AuNPs/pLL/ GCE[20]
Bioconjugate	anti-hGH/AuNPs	anti-hGH/ AuNPs/HDT	anti-hGH-Ts-MBs	anti-PRL/AuNPs	anti-PRL-biotin-strept-MBs	anti-PRL-AuNPs/pLL
Detection	label free; dopamine oxidn.	label free; imped. $Fe(CN)_6^{3-/4-}$	anti-IgG-AP with 4-APP	label free; dopamine oxidn.	anti-PRL-AP with 1-NPP	anti-PRL-GPPD-HRP with H_2O_2
Technique	DPV	EIS	SWV	DPV	DPV	DPV
Linear range, ng/mL	0.05-1000	0.003 - 0.1	0.01-100	0.001-1000	10 -2000	0.5-180
r	0.991	0.997	0.992	0.992	0.993	0.993
Equation	Δi, μA = 5.71+ +1.01 log C (ng/mL)	R, Ω = 0.2988 +18.5 C (ng/mL)	i, μA = 11.6+ +5.7 log C (ng/mL)	Δi, μA = 8.0 +1.35 log C (ng/mL)	i, μA = $7x10^{-3}$C (ng/mL)	i, μA = -4.047- -0.664 log C (ng/mL)
LOD, pg/mL	4.4 ($3s_b$/m; n=10)	0.64 (unknown criterion)	5 (3s; n=10)	0.22 ($3s_b$/m; n=10)	3.74 x 10^3 ($3s_{y/x}$/m; n=10)	100 (3s; n=11)
RSD, % (same day)	3.5 (1ng/mL; n=5)	1.77 (0.1ng/mL;n=8)	3.0 (1ng/mL; n=10)	2.7 (1ng/mL; n=5)	3 - 8 (10- 2000 ng/mL; n=5)	4.7 (25ng/mL; n=3)
RSD, % (different days)	4.0 (1ng/mL; n=5)	-	7.6 (1ng/mL; n=10)	4.8 (1ng/mL; n=5)	7 - 9 (10- 2000 ng/mL; n=5)	9.6 (25ng/mL; n=3)
Stability, days	4	-	6	7	34	14 (92.1%)
Sample	spiked human serum and saliva	spiked human serum	spiked human serum	spiked human serum and saliva	spiked human serum	spiked cattle serum
Assay time, min	60	120	135	60	60	60

Key: HDT, 1,6-hexanedithiol; pLL, poly-L-lysine; Ts-MBs, tosylated magnetic beads; AP, alkaline phosphatase; 4-APP, 4-aminophenyl-phosphate; 4-NPP, 4-naphthylphosphate; GPPD, poly(o-phenylenediamine)-carried nanogold particles

3.4. Selectivity of the dual immunosensor

The determination of hGH and PRL in biological fluids implies that other different hormones may be also present. Therefore, various potentially interfering substances, progesterone, cortisol, testosterone and follicle stimulating hormone (FSH) were tested.The DP voltammetric responses from 0.5 mM dopamine solutions were measured with the dual immunosensor in the presence of each compound at a 1 μg/mL concentration level and in the absence of target analyte.In all cases, the differences in current measured were similar to those obtained for solutions with no hGH or PRL indicating that no apparent interference from the tested hormones occurred. Moreover, other electroactive species such as uric acid (UA) and ascorbic acid (AA), which are

able to be oxidized at relatively low potential values, are also present in biological fluids and may lead to false responses on the electrochemical immunosensors. Therefore, in order to test the behavior of these compounds at the AuNPs/PEDOT/CNTs/SPCE, DP voltammograms were recorded for 0.5 mM solutions of each compound (84 µg/mL UA; 88 µg/mL AA). Figure 6a shows that no significant oxidation signals were obtained for AA and UA in the potential values range where the oxidation peak of dopamine appeared and, therefore, no interference can be expected from these substances in the determination of hGH and PRL at the label-free immunosensor. This is most likely due to the effect of PEDOT nanoparticles through hydrophobic/non-specific interactions that favours the separation between voltammetric signals of the more hydrophobic dopamine and those from the anionic AA and UA[28]. Furthermore, the possible crosstalk between the two adjacent working modified SPCEs in the dual configuration and reaction products was evaluated by comparing DP voltammograms for 0.5 mM dopamine recorded at the anti-PRL/AuNPs/PEDOT/CNTs /SPCE2 where 100 ng/mL hGH were deposited, and at the anti-hGH-AuNPs/PEDOT/CNTs/SPCE1 with 100 ng/mL PRL, with those obtained with no analyte at both immunosensors. Figure 6b shows that no significant differences in the dopamine voltammetric responses at each immunosensor were found in the presence and in the absence of the non-target antigen, thus demonstrating that no appreciable cross-talking occurred with the developed dual immunosensor.

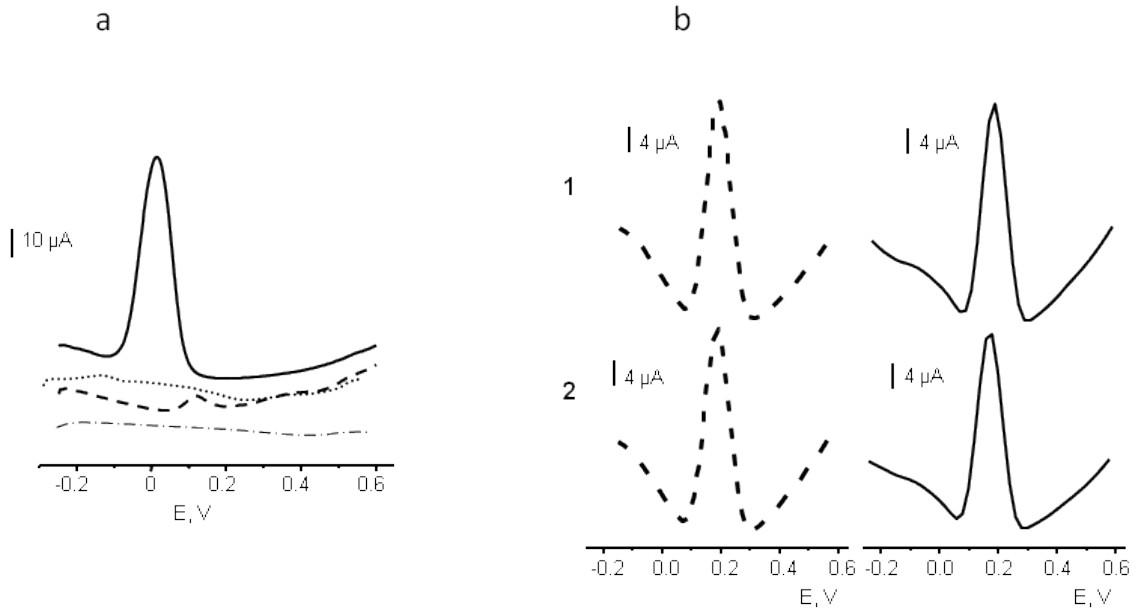

Figure 6. (a) DPV responses recorded at AuNPs/PEDOT/CNTs/SPCEfor 0.5 mM solutions of: dopamine (──),uric acid (- - -)and ascorbic acid (┈┈); background current (-·-·-) 0.1 M PBS pH 7.4. (b) DP voltammograms recorded for 0.5 mM dopamine at (1) anti-PRL/AuNPs/PEDOT/CNTs/SPCE + 100 ng/mL hGH (- - -); anti-PRL/AuNPs/ PEDOT/CNTs/SPCE + 0 ng/mL hGH (──); (2) anti-hGH/AuNPs/PEDOT/CNTs/ SPCE+100 ng/mL PRL (- - -) ; anti-hGH/AuNPs/PEDOT/CNTs/SPCE + 0 ng/mL PRL (──); v = 50 mV/s.

3.5. Simultaneous determination of hGH and PRL in spiked human serum and saliva.

The usefulness of the dual immunosensor for the multiplexed analysis of hGH and PRL in real samples was firstly evaluated by analyzing human serum spiked with the hormones at three concentration levels, 1, 20 and 50 ng/mL hGH, and 1, 20 and 200 ng/mL PRL. These concentration values can be typically found in real samples[3,5]. In order to verify a possible matrix effect, calibration graphs for hGH and PRL were constructed by appropriate dilution of the reconstituted spiked serum. The linear ranges of these calibration plots provided slope values of 1.0±0.3 µA (hGH) and 1.37±0.01 µA (PRL) which were very similar to those obtained with hGH and PRL standards: 1.01 ± 0.01 and µA 1.35 ± 0.05 µA, respectively. The application of the Student´s t-test for a

significance level of 0.05 demonstrated that no significant differences existed between the slope values and, therefore, the determination of hGH and PRL could be accomplished by interpolation of changes observed in the i_p values into the calibration plots obtained with hGH and PRL standard solutions (Figure 5a). Table 2 summarizes the results obtained in the analysis by triplicate of the serum samples. Recoveries ranged between 98 ± 2 and 102 ± 1 % for hGH and 99 ± 1 and 103 ± 3 % for PRL. Similar results were obtained in the analysis of spiked saliva. The slope values of the linear calibration plots in saliva were 0.9 ± 0.3 µA (hGH) and 1.3 ± 0.1 (PRL). As no matrix effect was apparent, the determination of both hormones could be also carried out by interpolation of the analytical readouts into the calibration plots displayed in Figure 5a. The recovery values (Table 2) were between 97 ± 3 and 101 ± 4 (hGH), and 96 ± 1 and 102 ± 4 (PRL) for this biological fluid.

All these results demonstrated clearly the usefulness of the developed dual immunosensor for the simultaneous analysis of hGH and PRL hormones at low concentration levels both in human serum and saliva with practically no sample treatment.

Table 2. Simultaneous determination of hGH and PRL in spiked human serum and saliva with the dual immunosensor.

Added, ng/mL		Found, ng/mL		Mean recovery, %	
Serum					
hGH	PRL	hGH	PRL	hGH	PRL
1	1	1.0; 0.96; 0.98	1.0; 1.0; 1.1	98 ± 2	103 ± 3
20	20	19.7; 20.2; 19.9	19.9; 19.6; 19.6	100 ± 1	99 ± 1
50	200	50.4; 51.1; 51.0	198; 196; 197	102 ± 1	99 ± 1
Saliva					
hGH	PRL	hGH	PRL	hGH	PRL
0.5	0.5	0.48; 0.52; 0.51	0.53: 0.49; 0.51	101 ± 4	102 ± 4
2.5	2.5	2.49; 2.42: 2.35	2.38; 2.41; 2.45	97 ± 3	96 ± 1
5.0	5.0	4.85; 5.25; 5.05	4.85; 5.25; 5.05	100 ± 3	101 ± 4

4. CONCLUSIONS

The simultaneous determination of human growth hormone and prolactin can be accomplished at a label-free dual electrochemical immunosensor constructed by using electrode platforms consisting of CNTs/SPCEs modified with PEDOT and gold nanoparticles and monitoring the differential pulse voltammetric oxidation response of the redox probe dopamine. The dual immunosensor exhibited an excellent analytical performance both in terms of sensitivity, with limits of detection of even sub pg/mL, selectivity against other hormones and applicability to different biological matrices such as human serum and saliva. Moreover, the absence of labelled immunoreagents makes the developed methodology simpler, faster and of lower cost which paves the way for a potential use of the dual electrochemical immunosensor in point-of-care analytical devices.

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