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A disposable electrochemical immunosensor for the determination of leptin in serum and breast milk

Irene Ojeda, María Moreno-Guzmán, Araceli González-Cortés, Paloma Yáñez-Sedeño* and José M. Pingarrón

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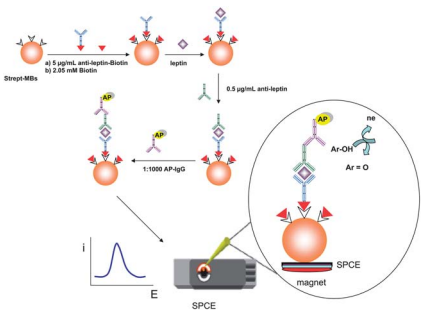

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A disposable electrochemical immunosensor for the determination of leptin in serum and breast milk

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Irene Ojeda, María Moreno-Guzmán, Araceli González-Cortés, Paloma Yáñez-Sedeño* and José M. Pingarrón

The preparation of a disposable electrochemical immunosensor for the quantification of the hormone leptin is described in this work. The preparation approach involved immobilization of a specific biotinylated anti-leptin antibody on the surface of streptavidin-functionalized magnetic beads (Strept-MBs) and a sandwich-type immunoassay involving the target analyte, monoclonal anti-leptin, and IgG labeled with alkaline phosphatase (AP-IgG). The electrochemical transduction step was accomplished by trapping the MBs bearing the immunoconjugates onto screen-printed carbon electrodes (SPCEs) by means of an Nd magnet and measuring the electrochemical oxidation of the 1-naphthol generated in the AP enzyme reaction upon 1-naphthyl phosphate (1-NPP) additions by differential pulse voltammetry (DPV). A calibration plot with a linear range between 5 and 100 pg mL^{-1} as well as a detection limit of 0.5 pg mL^{-1} ($3s_b/m$) were achieved. This value is more than 27 times lower than that reported in the only voltammetric immunosensor for leptin described in the literature until now. The usefulness of the immunosensor was demonstrated by analyzing different types of real samples: human serum, infant powdered milk, and breast milk from a nursing mother with two months of breastfeeding.

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1 Introduction

Leptin is a 16 kDa peptide hormone that plays an important role in regulating food intake and body composition in mammals.[1] It is mainly released from adipose tissue and thought to act as an adiposity signal to the brain, promoting anorexia.[2,3] The level of this hormone directly reflects energy status[4] and appears as a central link between adiposity, appetite and weight. It seems to contribute to body weight regulation by controlling food intake and energy expenditure at the hypothalamic level.[5,6] In humans, leptin is associated with obesity, appetite regulation, energy expenditure, and reproduction. Normal levels are 3.7–11.1 ng mL^{-1} in women and 2.0–5.6 ng mL^{-1} in men, whereas noticeably higher leptin levels have been observed in obese individuals.[7–9] In mammals, leptin is expressed primarily in white adipose tissue, although other tissues such as gastric epithelial lining, placenta, muscle, brain, pituitary, and hypothalamus have also been found to be sites of leptin expression. The leptin function in the regulation of appetite, energy metabolism and fertility, and in the maintenance of body weight by acting as a peripheral hormone 'sensor' for the size of adipocyte energy (lipid) stores has been reported.[10] Human mammary epithelial cells are able to synthesize the hormone, which is also present in breast milk suggesting that leptin in human milk might be involved in the regulation of postnatal nutrition and growth.[11] In fact, it has been claimed that breast milk leptin provides protective effects against overweight in adulthood and it has been proposed as an important factor that could partially explain the increased risk of obesity of formula-fed infants with respect to breastfed infants.[12] Moreover, although no infant formula contains leptin, this compound could be added to the formula and delivered through various feeding methods without loss.[13]

Analytical methods involving capillary electrophoresis,[14,15] immunofunctional assay,[16] enzyme-linked immunosorbent assay (ELISA),[17] radioimmunoassay[18–22] and western blotting[23–25] techniques have been used to determine human leptin. Various ELISA kits based on the use of peroxidase-labelled immunoreagents and colorimetric detection by addition of hydrogen peroxide in the presence of 3,3′,5,5′-tetramethylbenzidine (TMB) are commercially available. The analytical characteristics of some representative examples of these methods are summarized in Table 1. Apart from these, a nanoarray protein chip involving a sandwich fluorescence immunoassay monitored by total internal reflection fluorescence microscopy (TIRFM) was also reported.[4] Moreover, a chemiluminescence enzyme immunoassay (CLEIA) using anti-leptin polyclonal antibody and alkaline phosphatase, achieving a detection limit of 0.1 pg mL^{-1}, has been reported more recently.[26]

Regarding electrochemical immunosensors, to the best of our knowledge, only two configurations have been proposed. One of the approaches involved the use of a tri(ethylene glycol)-terminated alkanethiol (TEGCnSH)-modified gold electrode on which the capture antibody was covalently immobilized. Leptin and

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Table 1 Analytical characteristics reported for various immunoassays available for the determination of leptin

Immunoassay	Procedure	LOD[a], ng mL^{-1}	Dynamic range, ng mL^{-1}	Sample volume, μL	Assay time	Precision, RSD %	Sample
Millipore EZHL-80SK sandwich ELISA colorimetric	Biotin-anti-leptin + leptin + avidin-HRP + substrate (H_2O_2/TMB)	0.5	0.5–100	25	3 h 30 min	2.6–4.6 (intra-assay); 2.6–6.2 (inter-assay)	Human serum, plasma
R&D systems Quantikine sandwich ELISA colorimetric	Anti-leptin + leptin + anti-leptin-HRP + substrate (H_2O_2/TMB)	0.078	0.0156–1.0	100	4 h	3.0–3.3 (intra-assay); 3.5–5.4 (inter-assay)	Human serum, plasma
GenWay Biotech Inc. sandwich ELISA colorimetric	Anti-leptin + leptin + anti-leptin-HRP + substrate (H_2O_2/TMB)	0.2	1–50	50	2 h 30 min	4.2–7.6 (intra-assay); 4.4–6.7 (inter-assay)	Human serum; plasma
Alpco sandwich ELISA colorimetric	Anti-leptin + leptin + anti-leptin-Biotin + Strept-HRP + substrate (H_2O_2/TMB)	0.50	up to 100	20	2 h 5 min	3.7–5.5 (intra-assay); 5.8–6.8 (inter-assay)	Human serum; plasma
Cayman Chem sandwich ELISA colorimetric	Anti-leptin + leptin + anti-leptin-Biotin + Strept-HRP + substrate (H_2O_2/TMB)	0.5	1–50	100	2 h 25 min	3.0–7.5 (intra-assay); 3.2–9.2 (inter-assay)	Human serum; plasma
Diagnostics Autom., Inc., sandwich ELISA colorimetric	Anti-leptin + leptin + anti-leptin-Biotin + Strept-HRP + substrate (H_2O_2/TMB)	1.0	0–100	15	3 h 25 min	6.0–6.9 (intra-assay); 8.7–11.5 (inter-assay)	Human serum; plasma
Invitrogen sandwich ELISA colorimetric	Anti-leptin + leptin + anti-leptin-Biotin + Strept-HRP + substrate (H_2O_2/TMB)	<0.035	0–1	100	3 h 25 min	3.0–3.8 (intra-assay); 3.9–5.3 (inter-assay)	Human serum; plasma
Assay Pro Max human leptin ELISA kit EL2001-1	Anti-leptin + leptin + anti-leptin-Biotin + Strept-HRP + substrate (H_2O_2/TMB)	0.12	0.125–32	50	5 h	4.5 (intra-assay) 7.2 (inter-assay)	Human serum; plasma
Alpha diagnostic human leptin ELISA kit 0010	Anti-leptin + leptin + anti-leptin-Biotin + Strept-HRP + substrate (H_2O_2/TMB)	0.5	0–100	20	2 h	7.4–12 (intra-assay) 8–9 (inter-assay)	Human serum; plasma
Millipore human leptin ELISA kit EZHL-80SK	Anti-leptin + leptin + anti-leptin-Biotin + Strept-HRP + substrate (H_2O_2/TMB)	0.2	0.78–100	100	3 h 10 min	1.6–4.6 (intra-assay); 2.6–6.2 (inter-assay)	Human serum; Plasma
Enzo leptin (human) ELISA kit ADI 900-028A	Anti-leptin + leptin + anti-leptin-Biotin + Strept-HRP + substrate (H_2O_2/TMB)	0.0234	0.0031–2	100	3 h	4.4–13.4 (intra-assay); 3.7–15.2 (inter-assay)	Human serum; plasma
Abnova leptin (human) ELISA kit KA 0649	Anti-leptin + leptin + anti-leptin-Biotin + Strept-HRP + substrate (H_2O_2/TMB)	0.0625	0–4	150	2 h 20 min	<9 (intra-assay); <12 (inter-assay)	Human serum; plasma, breast milk

[a] As the lowest protein concentration that could be differentiated from zero.

anti-leptin antibody–alkaline phosphatase were also used and the square-wave voltammetric oxidation current for *p*-aminophenol as the enzyme product was employed to monitor the antigen concentration. A linear range for leptin between 100 pg mL^{-1} and 10 ng mL^{-1}, and a detection limit of 13.6 pg mL^{-1} ($S/N = 3$) were reported.[27] The second described configuration consisted of a label-free impedimetric immunosensor. A pyrrole and pyrrole propylic acid composite was prepared by co-electropolymerization in the presence of gold nanoparticles, followed by the covalent attachment of protein G to capture anti-leptin IgG. A dynamic range of 10–10^5 ng mL^{-1} and a detection limit of 10 ng mL^{-1} in 0.01 M PBS-1% serum solutions were obtained.[28]

In this work, the development of a disposable electrochemical immunosensor for the quantification of leptin in human serum and milk, including breast milk, is reported. The immnunosensor design involves the use of screen-printed carbon electrodes (SPCEs) and functionalized magnetic beads (MBs), which have been demonstrated to be powerful and versatile tools for the development of efficient immunosensing platforms.[29] Biotinylated polyclonal anti-leptin antibodies were immobilized on streptavidin-modified MBs and a sandwich type immunoassay involving leptin, monoclonal anti-leptin, and alkaline phosphatase (AP)-labeled IgG was carried out. The MBs bearing the resulting bioconjugates were trapped on the surface of the SPCE with a small magnet. This simple methodology allowed measurements to be carried out with small solution volumes giving rise to a high concentration of reagents on the transducer surface and, therefore, enabling the achievement of low detection limits. The quantification of leptin was accomplished by DPV monitoring of 1-naphthol formed in the enzyme reaction catalyzed by AP upon 1-naphthyl phosphate addition. The packing of MBs on the SPCE surface also implied that the enzyme reaction product was generated very close to the electrode surface, thus allowing the steady-state to be reached rapidly (which implies faster measurements), and minimizing diffusion limitations of the electroactive species.

2 Experimental

2.1 Apparatus and electrodes

The electrochemical measurements were performed with an Autolab PGSTAT 12 potentiostat equipped with the General Purpose Electrochemical System (GPES) 4.7 software (EcoChemie B.V.). An Optic Ivymen System provided with stirring and temperature control, a magnetic separator (DynaMagn®, Invitrogen Dynal), a Vortex homogenizator (Heidolph), and a magnetic stirrer from P-Selecta, Agimatic, were also used. Screen-printed carbon electrodes (SPCEs) consisting of a carbon working electrode 4 mm in diameter, a Ag pseudo-reference electrode, and a carbon counter electrode were purchased from DropSens. All the electrochemical measurements were performed at room temperature.

2.2 Reagents and solutions

Mouse monoclonal anti-leptin (anti-leptin, clone 3G1-1C9, 500 μg mL^{-1}, Sigma), rabbit polyclonal human anti-leptin biotinylated (anti-leptin-Biotin, 1000 μg mL^{-1}, LifeSpan), human leptin (1 mg mL^{-1}, Sigma), and anti-mouse IgG (Fc specific, from goat) labeled with alkaline phosphatase (AP-IgG, Sigma) were the immunoreagents used. Magnetic microbeads functionalized with streptavidin (Strept-MBs, 10 mg mL^{-1}, Dinabeads streptavidin, Invitrogen Dynal, 10 mg mL^{-1}), bovine serum albumin (BSA, Type VH, Gerbu), and D-Biotin (Gerbu) were also employed. Adrenocorticotropin (ACTH, Genway Biotech), human growth hormone (hGH, Sigma), progesterone (Sigma), prolactin (Immunometrics), cortisol (Sigma) and 17 β-estradiol (Sigma) were other hormones tested in the interference study. 1-Naphthyl phosphate monosodium salt monohydrate (1-NPP, Sigma), Trizma (tris(hydroxymethyl) aminomethane, Sigma), $MgCl_2$ (Panreac), EDTA (Panreac), and Tris–HCl (Scharlau) were also utilized. From these, a 10 mM Tris–HCl buffer solution of pH 7.4, containing also 2 mM NaCl and 1 mM EDTA (Tris-washing), and a 5 mM Tris–HCl buffer solution of pH 7.4, containing 1 M NaCl and 0.5 mM EDTA (Tris-coating), were prepared. A 50 mM Tris–HCl buffer solution of pH 7.4, containing 140 mM NaCl and 1 mM $MgCl_2$ (Tris-binding), was also employed to prepare biotin solutions. Tris-binding buffer solutions containing 5% or 2% BSA were used to prepare leptin, anti-leptin or AP-IgG solutions (5, 5, and 2% BSA, respectively). 1-NPP solutions and the AP enzyme reaction working medium were prepared with 0.1 M Trizma buffer solution of pH 9 containing 1 mM $MgCl_2$ (Trizma buffer). Three different types of samples were analyzed: human serum from Sigma (S-7397) spiked with leptin at 5.0, 25, 50 and 100 ng mL^{-1} concentration levels; an infant formula consisted of powdered milk from Blemil (Blemil Plus 1 Forte) which was spiked at the 1.0 and 7.0 ng mL^{-1} leptin concentration levels; and breast milk containing an unknown leptin concentration which was obtained from a nursing mother of two months of breastfeeding with BMI = 27.3. Water used was obtained from a purification Millipore Milli-Q system.

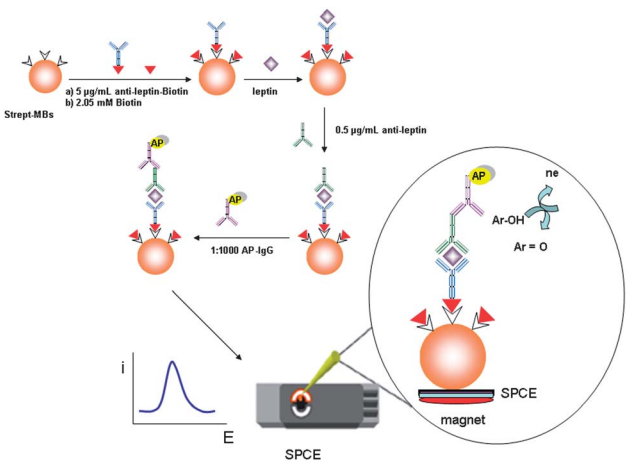

Fig. 1 Schematic display of the different steps involved in the preparation and functioning of the AP-IgG/anti-leptin/leptin/anti-leptin-Biotin/Strept-MB immunosensor.

2.3 Procedures

2.3.1 Preparation of the AP-IgG/anti-leptin/leptin/anti-leptin-Biotin/Strept-MB conjugates and determination of leptin. A scheme of the different steps involved in the preparation and functioning of the AP-IgG/anti-leptin/leptin/anti-leptin-Biotin/Strept-MB immunosensor is depicted in Fig. 1. In detail, 2 μL of the Strept-MB suspension were transferred into a 1.5 mL Eppendorf tube and washed twice with the washing buffer. Each washing step consisted of a re-suspension of the MBs in the washing solution and gentle stirring for 1 min (up to homogenization) followed by separation with the magnet for 2 min and removal of the solution. Next, 50 μL of a 5 μg mL^{-1} anti-leptin-Biotin solution were added and incubated for 45 min. All the incubation steps were performed in an incubator at 37 °C and 950 rpm and two washing steps were applied in all cases after the incubation period. Thereafter, 50 μL of a 2050 μM Biotin solution were added allowing incubation for 30 min. Further, 50 μL of leptin solution of variable concentration, 50 μL of a 0.5 μg mL^{-1} anti-leptin solution and 50 μL of a 1 : 1000 AP-IgG solution were sequentially added with a 45 min incubation period after each addition. After the preparation of the immunoconjugates, the AP-IgG/anti-leptin/leptin/anti-leptin-Biotin/Strept-MBs were re-suspended in 45 μL of Trizma buffer of pH 9, and transferred onto the surface of the SPCE. This was done by keeping the SPCE horizontal and placing a neodymium magnet on the bottom part of the electrode to localize in a reproducible way the MBs onto the working surface, thus avoiding variations in the bead layer thickness or spreading area of the electrode surface between different measurements. Finally, 5 μL of 0.05 M 1-naphthyl phosphate solution were deposited on the modified electrode and allowed to stand for 5 min. Differential pulse voltammograms (step potential = 50 mV; modulation time = 50 ms; scan rate = 50 mV s^{-1}) were then recorded in the −0.15 to +0.75 V potential window.

2.3.2 Analysis of the different samples. As mentioned above, solid lyophilized human serum was spiked with leptin at 5, 25, 50 and 100 ng mL^{-1} concentration levels. The solid serum was reconstituted in 1 mL of Tris buffer solution of pH 7.2 also containing 5% BSA by mixing up total dissolution. Thereafter, a 1 : 1000 dilution with the same buffer plus 5% BSA solution was performed. On the other hand, Blemil plus 1 forte infant formula for newborn from birth to 6 months of age consisting of powdered milk and spiked with 1 or 7 ng mL^{-1} leptin was also analyzed. 150 mg of the sample were dissolved in 1 mL of distilled water at 40 °C and the resulting solution was treated similarly to breast milk. Thereafter, 1 : 100 or 3 : 1000 dilution, respectively, was made with Tris buffer of pH 7.2 also containing 5% BSA. All the prepared sample solutions were analyzed by following the protocol described in Section 2.3.1. Finally, breast milk from a nursing mother in the second month of lactation was also analyzed. The sample was homogenized by manual and ultrasonic stirring and centrifuged at 2000 rpm for 20 min at 4 °C to separate cream. A 1 : 100 dilution with Tris buffer of pH 7.2 containing 5% BSA was carried out. Validation of the obtained results in the analysis of leptin in breast milk was accomplished by comparing with those provided by an ELISA kit for human leptin from Abnova (Catalog Number KA 0649 Version:02). The protocol indicated in the suppliers directions consisted, in brief, of pipetting standards or samples into the anti-leptin coated-well plates and leaving an incubation time of 1 h. Then, biotinylated anti-human leptin was added and allowed to stand for 30 min. After this, HRP–streptavidin was also pipetted into the well plates, leaving 30 min, and colour development was achieved by addition of TMB substrate solution. Finally, after 20 min, an acid "stop solution" was added, and the absorbance was measured at 450 nm.

3 Results and discussion

The fundamentals of the different steps involved in the immunoreaction occurring on the functionalized MBs, the transfer of MBs bearing the immunoconjugates on the SPCE surface and the voltammetric transduction strategy employed were schematically depicted in Fig. 1. Anti-leptin-Biotin antibodies were attached to Strept-MBs and a blocking step with biotin of the remaining active sites in the functionalized MBs was accomplished. Thereafter, the anti-leptin-Biotin/Strept-MBs were incubated in the analyte solution and the sandwich immunoassay was completed by attachment of anti-leptin to the MB-conjugate. Then, anti-leptin/leptin/anti-leptin-Biotin/Strept-MBs were incubated in a AP-IgG solution in order to link the detection antibody to the sandwich immunoconjugates. The modified MBs were subsequently transferred onto the SPCE surface where they were magnetically entrapped and, upon addition of 1-NPP as the AP-substrate, the generated 1-naphthol was electrochemically oxidized at the SPCE and the DPV response was used for leptin quantification.

3.1 Optimization of experimental variables

All the variables concerning the preparation of the immunosensor and affecting its electrochemical performance were optimized. The analytical signals were the peak currents measured by differential pulse voltammetry between −0.15 and +0.75 V for the 1-naphthol generated in the enzyme reaction with alkaline phosphatase. The amount of anti-leptin-Biotin immobilized on the Strept-MBs was evaluated by preparing different immunosensors incubated with MBs in solutions containing 2 to 20 μg mL^{-1} anti-leptin-Biotin. 2 μL MBs, 2050 μM Biotin, 1 μg mL^{-1} anti-leptin, 0, 0.05 and 5 ng mL^{-1} leptin concentrations, 1 : 5000 AP-IgG and 5 mM 1-NPP were used in these assays. Fig. 2a shows that the difference between the currents measured for 5 and 0.05 ng mL^{-1} was larger than that measured for a 5 μg mL^{-1} anti-leptin-Biotin loading which implies a larger sensitivity in the subsequent determination of leptin. Moreover, it is also apparent that for this anti-leptin-Biotin concentration the unspecific adsorption (the signal measured for 0 ng mL^{-1} leptin) was very low. Therefore, this loading was selected for further work. The influence of the anti-leptin antibody concentration used to build the sandwich configuration was checked over the 0.25–2 μg mL^{-1} range (Fig. 2b). The measured peak current values for 5 ng mL^{-1} leptin were similar to those for 0.5 and 1 μg mL^{-1} anti-leptin indicating that saturation was reached for 0.5 μg mL^{-1} anti-leptin.

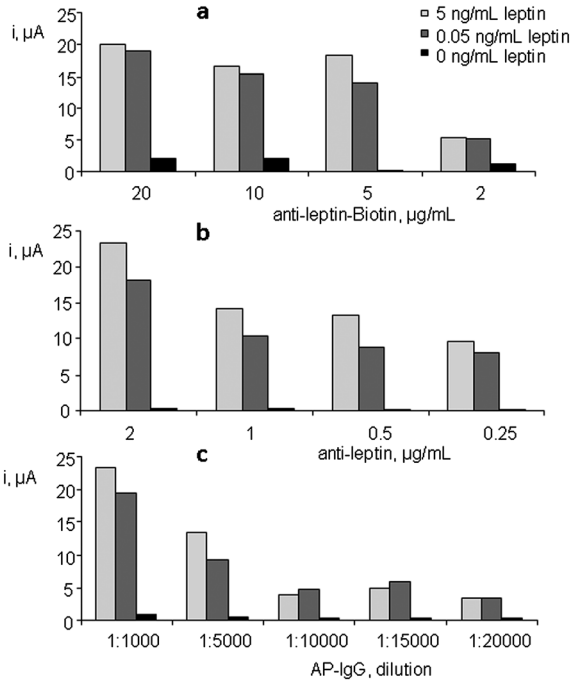

Fig. 2 Effect of anti-leptin-Biotin (a) and anti-leptin (b) loadings and AP-IgG dilution factor (c) on the DPV responses obtained for 0, 0.05 and 5 ng mL^{-1} leptin. See text for the other experimental conditions.

Probably, the big increase in the current observed for 2 μg mL^{-1} anti-leptin, which is a much higher concentration than that required to saturate the antigen, was due to the formation of an antibody-double layer. According to these observations, 0.5 μg mL^{-1} anti-leptin was selected for further work. Larger peak currents were obtained for the lower AP-IgG dilution factors assayed (Fig. 2c) and no significant differences in current were observed when smaller dilution factors were applied. Therefore, a 1 : 1000 AP-IgG dilution was used for preparation of the immunosensor.

As it is described in the Experimental section, Biotin and BSA were used as the blocking agents to minimize unspecific adsorptions. The concentration of these reagents used in the different steps of the immunosensor preparation was also optimized. Firstly, blocking of the remaining free sites on the Strept-MBs after anti-leptin-Biotin binding was performed using a Biotin solution. The results obtained (not shown) revealed that effective blocking was reached from Biotin concentrations higher than 1500 μM and, accordingly, a 2050 μM Biotin solution was employed for this purpose. Moreover, as it is described in Section 2.2, the leptin, anti-leptin and AP-IgG solutions were prepared in buffer solutions containing optimized BSA percentages (5, 5, and 2% BSA, respectively). This procedure allowed the smallest current values for control solutions without analytes to be obtained. Under these conditions unspecific adsorptions represented only 7.5% of the peak current obtained for 5 ng mL^{-1} leptin.

Table 2 Optimization of the working variables involved in the preparation of AP-IgG/anti-leptin/leptin/anti-leptin-Biotin/Strept-MB immunosensors

Variable	Tested range	Selected value
Anti-leptin-Biotin, μg mL^{-1}	2–20	5
Anti-leptin, μg mL^{-1}	0.25–2	0.5
AP-IgG, dilution factor	1000–20 000	1000
1-Naphthyl phosphate, mM	0.05–50	5
Time for hydrolysis	0–8	5

The influence of 1-naphthyl phosphate concentration on the DPV responses for 5 ng mL^{-1} leptin was also evaluated. Fig. 3a shows that the i_p values increased with the substrate concentration up to 5 mM exhibiting a sharp decrease for larger concentrations most likely due to the electrode fouling as a consequence of the electrochemical reaction product electropolymerization. Accordingly, 5 mM 1-NPP was selected to guarantee that the enzyme reaction rate depended only on the enzyme concentration. This enzyme reaction was performed at pH 9.0, which corresponded to the optimum AP activity.[30] Finally, the period of time that the enzyme hydrolysis reaction was let to proceed was also checked (Fig. 3b). As can be seen, the DPV response levelled off for 5 min.

All the optimized working variables and the corresponding selected values are summarized in Table 2.

3.2 Analytical figures of merit for the determination of leptin with the AP-IgG/anti-leptin/leptin/anti-leptin-Biotin/Strept-MB immunosensor

Fig. 4 shows the calibration plot constructed for leptin determination by DPV under the optimized conditions stated above. The

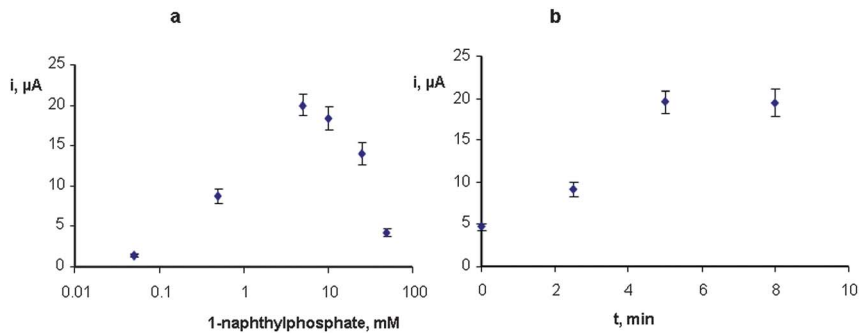

Fig. 3 Effect of the 1-NPP concentration (a) and the time elapsed to allow hydrolysis (b) on the DPV response for 5 ng mL^{-1} leptin at the AP-IgG/anti-leptin/leptin/anti-leptin-Biotin/Strept-MB immunosensor.

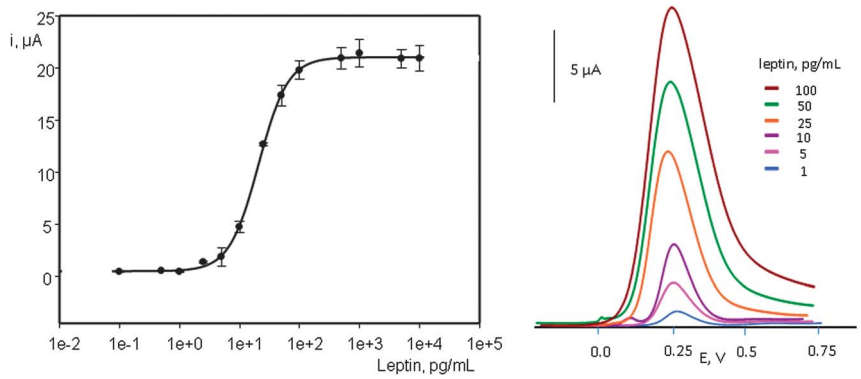

Fig. 4 Calibration plot constructed for leptin by DPV at the AP-IgG/anti-leptin/leptin/anti-leptin-Biotin/Strept-MB immunosensor. Differential pulse voltammograms recorded for 1–100 pg mL^{-1} leptin.

linear dynamic range extended between 5 and 100 pg mL^{-1} ($r^2 = 0.982$). Taking into account both normal and abnormally high levels of leptin in serum, this range allowed dilution of real samples to be performed in a factor of approximately 1000 which was necessary to maintain matrix effects negligible. The slope value of the linear portion in the calibration plot was 14.7 ± 1.2 μA pg^{-1} mL^{-1}. The limit of detection was calculated according to the $3s_b/m$ criterion, where s_b was estimated as the standard deviation from ten successive measurements of the peak current measured for the lowest leptin concentration of the linear part in the calibration plot and m was the slope of such a linear portion mentioned above. The calculated value was 0.5 pg mL^{-1}, which is remarkably lower than those reported previously with all the immunoassays listed in Table 1. Furthermore, it is more than 27 times lower than that obtained with the only voltammetric immunosensor for leptin described in the literature until now,[27] and twenty thousand times lower than that achieved with the label-free impedimetric immunosensor.[28]

The reproducibility of the DPV responses was evaluated by carrying out measurements with a different immunosensor per assay for: (a) 5, 25 and 100 pg mL^{-1} leptin on the same day, and (b) 100 pg mL^{-1} on different days. The relative standard deviation (RSD) values obtained were: (a) 6.0% ($n = 10$), 7.4% ($n = 6$) and 6.7% ($n = 6$), respectively, and (b) 4.8% ($n = 7$). These results demonstrated the suitability of the immunosensor preparation procedure. On the other hand, the storage stability of the anti-leptin-Biotin/Strept-MB bioconjugates was checked by placing the same amount of conjugate, prepared on the same day, in different Eppendorf tubes containing 50 μL of binding buffer and 0.01% Tween 20 and stored in a refrigerator at 8 °C. The corresponding AP-IgG/anti-leptin/leptin/anti-leptin-Biotin/Strept-MB immunosensors were then prepared from each conjugate and used to measure the DPV response for 100 pg mL^{-1} leptin. Fig. 5 shows the control chart constructed setting the central value as the mean value of ten different measurements carried out on the first working day, and the upper and lower limits of control as three times the standard deviation of the central value (±3 s). As it can be seen, the DPV responses obtained with the immunosensors prepared with the stored immunoconjugates remained inside the control limits for more than one month (no longer periods were checked). These results indicated a high storage stability of the antibody-modified MB conjugates leading to the possibility of their preparation and storage until the immunoassays have to be carried out.

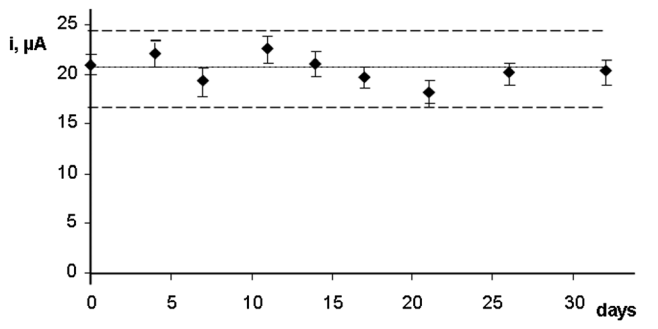

Fig. 5 Control chart constructed to evaluate the storage stability of anti-leptin-Biotin/Strept-MB conjugates. Each point corresponds to the mean value of three successive measurements for 100 pg mL^{-1} leptin.

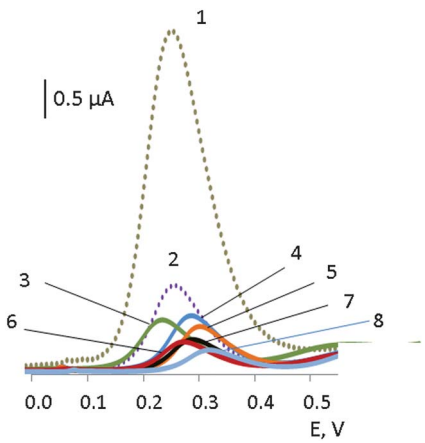

Fig. 6 Differential pulse voltammograms recorded at the AP-IgG/anti-leptin/leptin/anti-leptin-Biotin/Strept-MB immunosensor for 10 pg mL^{-1} leptin (1), 0 pg mL^{-1} leptin (2), and for physiological concentrations of potential interfering compounds: 250 ng mL^{-1} cortisol (3); 60 pg mL^{-1} ACTH (4); 1.5 ng mL^{-1} estradiol (5); 200 ng mL^{-1} progesterone (6); 50 ng mL^{-1} hGH (7), and 200 ng mL^{-1} prolactin (8).

3.3 Cross-reactivity

The determination of leptin in biological fluids or in breast milk implies that other hormones may be present in these real samples. Therefore, various compounds were tested as potential interferences for the developed immunosensor response to leptin. These compounds were: ACTH, hGH, progesterone, prolactin, cortisol and estradiol. Fig. 6 compares the DP voltammograms recorded for 10 pg mL^{-1} leptin (voltammogram 1) and in the absence of leptin (voltammogram 2) with those obtained for the potential interfering compounds at the concentration values corresponding to physiological levels for each hormone. As it can be seen, all the tested compounds exhibited a much smaller voltammetric signal compared to that of the analyte, which was similar to that obtained for the blank solution. These results suggested that the measured signals corresponded to non-specific adsorptions on the Strept-MBs and that none of the checked compounds produced interference on the voltammetric response for leptin.

3.4 Determination of leptin in human serum, an infant formula and breast milk samples

The analytical usefulness of the immunosensor was demonstrated and evaluated by determining leptin in three different samples: a human serum spiked with leptin at 5.0, 25, 50 and 100 ng mL^{-1} concentration levels, a commercial infant formula (powdered milk) spiked with 1.0 and 7.0 ng mL^{-1} leptin, and breast milk from a nursing mother in the second month of lactation. The employed protocols are described in Section 2.3.2. Firstly, in order to evaluate the influence of a possible matrix effect in serum, samples spiked with 100 ng mL^{-1} leptin were reconstituted in Tris buffer solution of pH 7.2 containing 5% BSA and diluted with the same buffer solution. The results obtained (not shown) revealed that the peak current measured from a 1 : 10 diluted serum was 24% lower than that recorded for a leptin standard solution, thus revealing the existence of a significant matrix effect from the sample components. However, no significant differences between the i_p values for the sample and the standard solution were apparent from a 1 : 100 dilution factor. Considering the sample spiking concentration levels and the linear dynamic range of the calibration plot, a 1 : 1000 sample dilution factor, where the matrix effect was certainly negligible, was used for the analyses. Under these conditions, a calibration graph for leptin, similar to that obtained for leptin standard solutions, was constructed with a slope value for its linear portion of 13.5 ± 0.4 μA pg^{-1} mL^{-1}, which was not statistically different (calculated Student's *t*-value $1.057 < t_{tab}$ 2.015 for a significance level of 0.05) than that the slope value obtained with leptin standards given above (14.7 ± 1.2 μA pg^{-1} mL^{-1}). Therefore, the quantification of leptin in serum samples was accomplished by interpolation of the measured peak current into the calibration plot constructed with leptin standard solutions. The results obtained are summarized in Table 3. Recoveries ranged between 96 and 102% with RSD values of 2.9, 2.7, 3.0 and 1.8 ($n = 3$) for 5.0, 25, 50 and 100 ng mL^{-1} leptin, respectively.

Moreover, the infant formula Blemil plus 1 forte for newborn from birth to 6 months old spiked with 1.0 and 7.0 ng mL^{-1} leptin was also analyzed. The experimental procedure was described in Section 2.3.2 and, in this case, involved a sample dilution of 1 : 100 or 3 : 1000, respectively, with the Tris buffer of pH 7.2 containing 5% BSA. These dilution factors guaranteed a negligible matrix effect and that the measured current fell within the linear range of the calibration plot constructed with leptin standard solutions. Table 3 shows that recoveries of 98% and 102% with RSD values of 5.9% and 2.9% ($n = 4$) were obtained for 1 and 7 ng mL^{-1} leptin, respectively. Finally, breast milk from a nursing mother in the second month of lactation was analyzed. The experimental procedure described in Section 2.3.2 implies a 1 : 100 dilution factor with the same buffer solution (considering that the content of leptin in this kind of milk is between 0.11 and 4.97 ng mL^{-1}).[31] In this case, in order to avoid the matrix effect of this very complex sample, the standard additions method was used for the analyte quantification (using a different immunosensor for each point of the standard additions plot). The mean leptin ($n = 3$) concentration found was 2.6 ± 0.1 ng mL^{-1}, which falls within the above mentioned concentration range. This result was compared with that obtained by using the Abnova ELISA kit for human leptin following the protocol described in Section 2.3.2. The mean leptin concentration obtained ($n = 3$) was 2.9 ± 0.5 ng mL^{-1}. Both mean values were statistically compared by applying Student's *t*-test. As the t_{exp} value, 2.548, was lower than the tabulated *t* value (2.776), no significant differences were found between both results at a significance level of 0.05.

According to all the obtained results, it can be concluded that the developed immunosensor is useful for the determination of leptin at the required concentration levels in different types of relevant samples such as human serum and breast milk.

Table 3 Determination of leptin in spiked human serum and infant formula with the AP-IgG/anti-leptin/leptin/anti-leptin-Biotin/Strept-MB immunosensor

Leptin, ng mL^{-1}	Leptin found[a], ng mL^{-1}	Recovery, %
Human serum		
5.0	4.8 ± 0.2	96 ± 5
25	25.5 ± 1.3	102 ± 5
50	51 ± 3	101 ± 6
100	96 ± 2	96 ± 2
Infant formula		
1.0	0.98 ± 0.08	98 ± 8
7.0	7.2 ± 0.3	102 ± 4

[a] Mean value ± $ts/\sqrt{n}$.

4 Conclusions

A novel magnetoimmunosensor for the determination of the hormone leptin, involving the use of functionalized magnetic beads, a sandwich-type immunoassay and differential pulse voltammetric transduction at screen-printed carbon electrodes, was developed. The magnetoimmunosensor exhibits an excellent analytical performance in terms of sensitivity, with a much lower detection limit than those reported for different available

immunoassays, selectivity, reproducibility of the measurements and storage stability of the prepared immunoconjugates. Furthermore, the immunosensor demonstrated to possess a high analytical versatility because it is suitable to quantify leptin in a variety of real samples such as human serum, infant formula or breast milk. Therefore, the immunosensor appears to be a useful analytical tool to face up relevant problems regarding obesity and related aspects with it.

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