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Surface plasmon resonance immunoassay analysis of pituitary hormones in urine and serum samples

Juan Treviño [a,b,*], Ana Calle [c], José Miguel Rodríguez-Frade [d], Mario Mellado [d], Laura M. Lechuga [a,b]

[a] Grupo de Nanobiosensores y Biofísica Molecular, Centro de Investigación en Nanociencia y Nanotecnología (CIN2: CSIC-ICN), ETSE, Campus UAB, Bellaterra, Barcelona, Spain
[b] Centro de Investigación Biomédica en Red en Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN), Barcelona, Spain
[c] Instituto de Microelectrónica de Madrid (CNM-CSIC), Madrid, Spain
[d] Departamento de Inmunología y Oncología, Centro Nacional de Biotecnología (CNB-CSIC), Madrid, Spain

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ABSTRACT

Background: Direct determination of four pituitary peptide hormones: human thyroid stimulating hormone (hTSH), growth hormone (hGH), follicle stimulating hormone (hFSH), and luteinizing hormone (hLH) has been carried out using a portable surface plasmon resonance (SPR) immunosensor.
Methods: A commercial SPR biosensor was employed. The immobilization of the hormones was optimized and monoclonal antibodies were selected in order to obtain the best sensor performance. Assay parameters as running buffer and regeneration solution composition or antibody concentration were adjusted to achieve a sensitive analyte detection.
Results: The performance of the assays was assessed in buffer solution, serum and urine, showing sensitivity in the range from 1 to 6 ng/mL. The covalent attachment of the hormones ensured the stability of the SPR signal through repeated use in up to 100 consecutive assay cycles. Mean intra- and inter-day coefficients of variation were all <7%, while batch-assay variability using different sensor surfaces was <5%.
Conclusions: Taking account both the excellent reutilization performance and the outstanding reproducibility, this SPR immunoassay method turns on a highly reliable tool for endocrine monitoring in laboratory and point-of-care (POC) settings.

1. Introduction

The pituitary gland is part of the endocrine system which regulates many physiological processes including growth, reproduction and metabolism. The anterior pituitary produces and secretes thyroid stimulating hormone (hTSH), growth hormone (hGH), follicle stimulating hormone (hFSH), luteinizing hormone (hLH), and other peptide hormones such as the adrenocorticotropic hormone (ACTH), prolactin (PRL) and endorphins. Among them, we have chosen the evaluation of the hormones hTSH, hGH, hFSH and hLH due to their high biological interest and diagnostic value. hTSH, hLH and hFSH are heterodimeric glycoprotein hormones, with molecular weights about 30 kDa, formed by an alpha chain and a beta chain [1]. The alpha chain is identical in these three two hormones as well as in the placental hormone human chorionic gonadotropin (hCG). The beta chain is unique for each hormone and is the responsible for the bioactivity and the immunological differentiation. hGH is a non-glycosylated polypeptide hormone with a molecular weight of 22 kDa [2].

hTSH regulates the endocrine function of the thyroid gland, stimulating the secretion of the thyroid hormones thyroxine (T_4) and triiodothyronine (T_3), which are fundamental for normal metabolism. Normal hTSH values range from 0.4 to 4 μIU/mL [3,4]. Increased values are associated to hypothyroidism and pituitary tumors, while drops in its concentration reveal hyperthyroidism or hypopituitarysm. hTSH is measured as well in newborn screening for congenital hypothyroidism [3,5], and those patients with a hTSH value >20 μIU/mL are recalled for further testing.

hGH is essential for normal growth and development. Besides height growth in children, it regulates the metabolism throughout all adult life. It has a pulsatile secretion which results in widely fluctuating levels that can reach 50–100 ng/mL peaks, while basal concentration falls below 0.03 ng/mL [6,7]. Disorders in hGH secretion produce excess or deficiency problems. hGH excess, generally due to benign pituitary tumours, causes acromegaly. hGH deficiency produces different problems at various ages, but its effects are more severe in children as produces growth failure. A cut-off value of 10 ng/mL after stimulation is widely accepted for hGH deficiency diagnostic.

Gonadotropic hormones hFSH and hLH play a key role in the development and function of the reproductive system. hFSH stimulates spermatogenesis in men while it controls ovarian follicle growth

* Corresponding author. Centro de Investigación en Nanociencia y Nanotecnología (CIN2: CSIC-ICN), Edificio Q - ETSE, 3ª Planta, Campus UAB, 08193, Bellaterra, Barcelona, Spain. Tel.: +34 93 586 80 12; fax: +34 93 586 80 20.
E-mail address: juan.trevino@cin2.es (J. Treviño).

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and estrogens production in females. Normal hFSH levels vary from 2 to 10 mIU/mL. Concentration increases in females just before ovulation to 5–20 mIU/mL and reaches up to 100 mIU/mL in post menopause. Determination of hFSH concentration is used for the diagnosis of reproduction and development disorders as infertility and premature or delayed puberty [8]. hFSH tests are used as well to check ovarian reserve and menopausal status. In men, hLH stimulates testosterone production while in females it regulates androgen and progesterone production and activates ovulation. Normal hLH values range from 2 to 10 mIU/mL, with a marked increase to 20–100 mIU/mL in females previous to ovulation and 20–70 mIU/mL in post menopause. Abnormally increased hLH levels are related with primary gonadal dysfunction, polycystic ovary syndrome, post menopause and pituitary adenoma in females. High hLH levels are present in men with hypogonadism, and primary testicular failure [9]. hFSH and hLH are simultaneously determinate to check ovulation, for the diagnosis of reproductive disorders and for monitoring endocrine therapy.

Different immunoassay methods as radioimmunoassays (RIA), immunometric assays (IMA) or enzyme-linked immunosorbent assays (ELISA) have been developed for the determination of those hormones [3,4,6–9]. Immunoassay techniques have been extensively used for fast diagnostic and monitoring in point-of-care settings because of its simplicity, sensitivity and specificity [10]. Routine measurement for the determination of these hormones is generally carried out using automated high throughput systems based mostly in immunometric assays with chemiluminiscent labels (ICMA). These assays reach very low limits of detection, in the range of pM–fM, but they show heterogeneous results and sensitivities, mainly due to the heterogeneity of the hormones and the reference preparations, and are affected by matrix effects [3,4,6–9]. Furthermore, the complexity of the equipment make impossible to use them near the patient, enlarging the testing time due to logistics of sample transport and results management. It would be a benefit for the treatment of the diseases associated to these hormones having a diagnostic method that can produce immediate results in point-of-care (POC) settings. Biosensors can achieve similar analytical characteristics to immunoassays but they offer some additional advantages as a rapid response, portability, simple use and automation [11,12]. Surface Plasmon Resonance (SPR) biosensors [13] allow the real-time monitoring of biomolecular interactions as increases in refractive index caused by mass changes at the sensor surface. SPR devices have shown the necessary performance and in addition they provide a real-time analysis avoiding labelling steps. However, the employment of optical biosensors for real clinical applications is hampered due to the matrix effects produced by the non-specific binding, onto the sensor surface, of the components of biological fluids. Applications of SPR biosensors that measure directly clinical samples are still unusual. Some methods have developed using serum [14,15], plasma [16], saliva [17] and urine [18,19], but generally such analyses require sample pre-treatment, the employment of extremely dilute samples and the use of signal amplification methods.

The aim of the present study is to develop an SPR immunosensor capable to detect these pituitary hormones, at physiological concentration levels, directly in urine and serum, with minimal sample treatment and handling. To our knowledge, this is the first example of a biosensor method for the analytical determination of hGH and hTSH and the first SPR biosensor method for the analytical determination of hFSH and hLH. The simple operation and fast response turns the SPR device in a useful equipment for clinical laboratories and POC settings.

2. Materials and methods

2.1. Reagents

Recombinant hGH was obtained from Pfizer (Spain). hFSH (NIDDK-hFSH-I-SIAFP-2, AFP-7220C, 14,296 IU/mg in terms of WHO 2nd IRP-HMG), hLH (NIDDK-hLH-I-SIAFP-2, AFP-4395A, 8444 IU/mg in terms of WHO 1st IRP 68/40) and hTSH (NIDDK-hTSH-SIAFP-B-2, AFP-3951A, 7.54 IU/mg in terms of highly purified hTSH) were obtained from Dr. Parlow, National Hormone & Peptide Program (NHPP), National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), Torrance, CA (US). Human serum was purchased from Sigma-Aldrich (Steinheim, Germany). Urine was obtained from healthy laboratory volunteers and was used without any further treatment. Monoclonal antibodies (mAb) were obtained and characterized at the National Centre for Biotechnology (CNB-CSIC, Madrid, Spain). Ammonium sulphate purified mAbs were employed. Three mAbs were chosen as candidates to carry the hTSH assay: hTSH-1, hTSH-2 and hTSH-3. mAbs hFSH-1, hFSH-2, hFSH-9 and hFSH-14 were chosen for the hFSH assay and mAbs hLH-6 and hLH-7 for the hLH assay. mAb-hGH-12 was selected for the hGH assay using previous data [20].

Mercaptoundecanoic acid, N-hydroxysuccinimide (NHS) and 1-ethyl-3-(-3 dimethyl-amino-propyl) carbodiimide hydrochloride (EDC) were purchased from Sigma-Aldrich (Steinheim, Germany). Organic solvents used in gold chip cleansing process: tricholoroethylene, acetone and ethanol, and piranha solution components H_2SO_4 and H_2O_2 were supplied by Merck (Darmstadt, Germany). Potassium chloride, sodium chloride, disodium hydrogen phosphate and potassium dihydrogen phosphate, used for the preparation of PBST buffer (10 mM phosphate pH 7.4 with 137 mM NaCl, 2.7 mM KCl and 0.05% Tween 20), and PBST-S buffer (10 mM phosphate pH 8 with 500 mM NaCl, 2.7 mM KCl and 0.1% Tween 20), acetic acid and sodium acetate for the preparation of acetate buffers were provided by Panreac (Barcelona, Spain). Ethanolamine hydrochloride blocking agent was obtained from Acros Organics (Geel, Belgium). Tween-20 was purchased from Quantum Appligene (Heidelberg, Germany).

2.2. Instrumentation

A commercial Surface Plasmon Resonance biosensor, from Sensia SL (Spain) was employed for SPR measurements. The sensor has two flow cells with a volume of 300 nL each, allowing the measurement of two independent samples or the use of a reference channel. The device incorporates optics and upgradeable electronic modules as well as computer controlled pumps, valves and injection fluidics. All the measurements were performed by sample injection using the flow delivery system incorporated in the platform that assures the injection for analysis of precise volumes of 220 µL while maintaining a continuous flow of buffer between 10 and 40 µL/min. Further description of this system can be found elsewhere [21].

2.3. Preparation of the sensor surface. Immobilization procedure

A monomolecular layer of the ligand was achieved by the formation of a self-assembled monolayer (SAM). The different hormones were covalently attached to the sensor surface using amino coupling between carboxyl group end in the SAM and amino groups in the protein. For this purpose, SPR gold chips were cleaned using trichloroethylene, acetone and ethanol. Chips were then submerged in fresh piranha solution (H_2SO_4/H_2O_2, 3:1), rinsed, ultrasonicated in distilled water and dried with N_2. Finally, gold chip was pushed over the flow cells and the prism was adhered using matching refractive index oil. The protein covalent immobilization process was carried at a constant flow of 10 µL/min. An alkanethiol SAM was formed by adsorption of a solution 0.05 mM of mercaptoundecanoic acid on the gold surface. Then, ethanol was flowed to remove alkanethiol excess and a continuous flow of water was maintained for the following steps of the immobilization process.

For protein amino coupling, the surface was activated using a mixture of EDC 0.2 M and NHS 0.05 M. Immediately after, the appropriate hormone was immobilized via its amino groups. Then, an ethanolamine 1 M and pH 8.5 solution was injected to deactivate residual unreacted groups on the surface. Finally, potential non-covalent bound biomolecules remaining on the surface were removed by an injection of 100 mM HCl at 30 µL/min.

2.4. SPR immunoassay format

A binding inhibition immunoassay format was used for the detection of the hormones. For calibration curves, triplicate standard concentrations of the correspondent hormone in the 10^{-4} µg/mL–100 µg/mL range in PBST and blank controls were mixed (1:1) with the antibody in PBST during 10 min. Afterwards, solutions were injected sequentially over the sensor surface at 20 µL/min and SPR signal was monitored in real-time using the two flow cells of the device in parallel. Reutilization of the sensor surface was achieved by an injection of HCl 5 mM regeneration solution at a flow speed of 30 µL/min. SPR signal of each standard was expressed as the percentage of the maximum response [100 × (SPRsignal/SPRsignal,max)]. The averaged responses of the three standards measured for each concentration were plotted versus the logarithm of the hormone concentration and fitted to a four-parameter logistic equation:

$$y = \left\{D + (A - D) / \left[1 + (x/C)^B\right]\right\}$$

were x refers to hormone concentration, y is the response, A is the asymptotic maximum, corresponding to the signal in absence of analyte, B is the slope at the inflection point, C is the inflection point, equivalent to the half inhibitory concentration I_{50} and D is the asymptotic minimum, corresponding to the background signal.

2.5. Antibody selection

The candidate antibodies were tested in order to choose the ones which could provide the best assay performance. Calibration curves in PBST using different

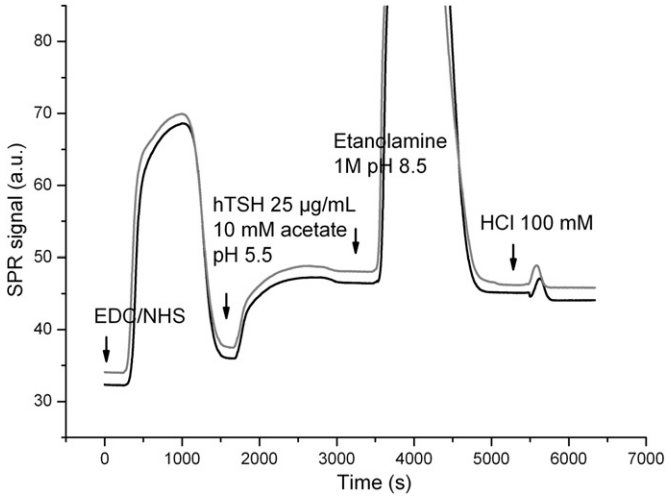

Fig. 1. Representative sensorgram of the hTSH immobilization process carried simultaneously in the two flow cells of the device (flow cell 1: black, flow cell 2: grey).

antibodies were measured, using the procedure described above, in order to compare the performance of the inhibition assay for each antibody. Calibration curves for hTSH were prepared using 0.1 µg/mL of hTSH-1, hTSH-2 or hTSH-3. In the case of hFSH sensor, 0.5 µg/mL solutions of the antibodies hFSH-1, hFSH-2, hFSH-9 and hFSH-14 were used. Finally, for hLH sensor, concentrations of 0.05 of the antibodies hLH-6 and hLH-7 were assayed. As mentioned, hGH-12 was used at 0.25 µg/mL for hGH sensor.

2.6. hGH immunoassay in serum

Calibration curve standards, including blank samples, were triplicate prepared in human serum (110 µL) in the $4 \cdot 10^{-3}$–40 µg/mL range and mixed (1:1) with mAb hGH-12 to a final concentration of 2 µg/mL in PBST-S. Standards were then injected sequentially over the sensor surface at 20 µL/min using the two flow cells of the device in parallel. Regeneration of the sensor surface after each serum measurement was performed using 2 M NaCl, 0.1% Tween 20 and pH 11 at 30 µL/min instead of the HCl 5 mM solution used for the rest of the matrices. SPR immunosensor signals were analysed and calibration curves were calculated as described above.

2.7. hFSH/hLH immunoassay in urine

Triplicate calibration standards of hFSH, hLH and blank samples were prepared in urine in the 10^{-4} µg/mL–100 µg/mL range. mAb-hFSH-14 concentration was diminished from 0.5 µg/mL used in the PBST assay to 0.3 µg/mL for urine. Then, hFSH calibration standards were mixed (1:1) to a final concentration of 0.3 µg/mL of mAb-hFSH-14 and hLH calibration standards were mixed (1:1) to a final concentration of 0.05 µg/mL of mAb-hLH-7. Solutions were injected on the sensor surface at 20 µL/min using the two flow cells of the device in parallel and SPR signals were analysed to obtain calibration curves as described above.

3. Results

3.1. Optimization of the hormone immobilization

The inhibition immunoassay format allows a direct measure avoiding secondary reagents or labelling procedures. This assay is based on the immobilization of the corresponding hormone onto the sensor surface and it was mainly chosen in order to assure immunosensor stability during continuous measurements and regeneration cycles. The process of immobilization leads to the formation of a monomolecular film of the ligand in a controlled and stable manner.

Dextran hydrogel surfaces are widely used because they provide an adequate environment for protein immobilization, but they have some disadvantages associated with the non-specific binding and steric effects due to the lack of control over surface distribution of the ligands [22]. Dextran surfaces did not reduce the non-specific binding to a level low enough to detect the analytes in untreated serum [23]. Furthermore, dextran surfaces presented a 3D structure with a large amount of points for biomolecule immobilization, but the immobilization densities were similar to that obtained using 2D self-assembled monolayers [24]. In this work we used an alkanethiol self-assembled monolayer (SAM) since, in our opinion, they provide an excellent immobilization method due to the simplicity, reproducibility and reliability of the process and their flexibility for the immobilization of different biomolecules [25]. The choice was also motivated by the further potential of using mixed self-assembled monolayers to control the ligand surface distribution and to incorporate different groups which could reduce the non-specific adsorption [26]. Further work in this way could produce an additional reduction of the matrix effects in our assays.

Hormone immobilization was optimized to obtain the best sensor performance. Coupling between amino groups in the protein and carboxyl terminal groups on the SAM was carried out using carbodiimide coupling [27]. The hormone concentration and the pH of the immobilization solution were tested. Concentrations of the different hormones of 10, 25, 50 and 100 µg/mL were employed for immobilization in 10 mM acetate buffer which pH was varied between 4, 4.5, 5, 5.5 and 6. SPR signals corresponding to the interactions between the immobilized hormones and their specific antibodies were registered. A representative immobilization sensorgram is shown in Fig. 1. Optimal hormone concentrations were 25 µg/mL for hTSH, hFSH and hLH and 50 µg/mL for hGH. Although hTSH was immobilized at all the pH values tested, the optimal immobilization was obtained at 5.5. hFSH immobilization occurs in the range 4.5–5.5, but it was optimal at pH 4.5. For hLH we obtained good immobilization

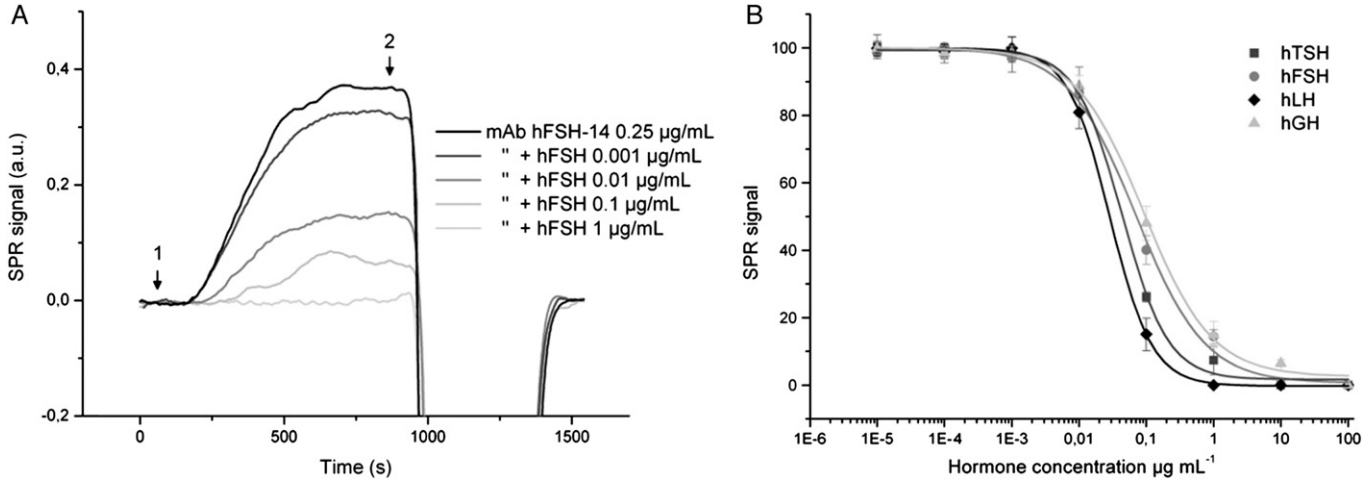

Fig. 2. (A) SPR response to the binding of different hFSH concentrations and subsequent regeneration cycles during the performance of binding inhibition tests in PBST. 1: sample injection. 2: regeneration solution injection (B) Standard calibration curves of hTSH, hFSH, hLH and hGH in PBST. Each point represents the mean value of triplicate measurements.

Table 1
Analytical characteristics of the SPR immunoassays for hTSH, hFSH, hLH and hGH using different antibodies.

	hTSH			hLH	
Analytical characteristics	mAb-hTSH-1	mAb-hTSH-2	mAb-hTSH-3	mAb-hLH-6	mAb-hLH-7
LOD (ng mL^{-1})	4	15	3	9	3
I_{50} (ng mL^{-1})	44	159	81	45	29
Working range (ng mL^{-1})	14–138	51–488	18–367	17–123	10–78
	hFSH				hGH
Analytical characteristics	mAb-hFSH-1	mAb-hFSH-2	mAb-hFSH-9	mAb-hFSH-14	mAb-hGH-12
LOD (ng mL^{-1})	32	19	24	2	4
I_{50} (ng mL^{-1})	212	213	408	70	91
Working range (ng mL^{-1})	88–560	68–735	108–1547	14–370	18–542

results at all the pH values analyzed, but it was optimal at pH 6. Finally, hGH immobilization occurs between 4.5 and 5, but it was optimal at pH 5. These differences in the pH range to obtain a correct hormone immobilization could be due to differences in the origin of the hormones used. Whereas hTSH, hFSH and hLH, are from pituitary origin and could contain a mixture of several isoforms, which behave different during the immobilization process, hGH is a recombinant product, with a uniform structure, properties and immobilization performance.

3.2. Immunoassays performance

The inhibition immunoassay format is based on the addition of a constant amount of the antibody to all the hormone samples. After incubation with the antibody, the samples are injected in the SPR sensor, but the binding of the antibody to the immobilized hormone is inhibited by the hormone in solution, generating lower SPR signals when the hormone concentration in the sample increases (Fig. 2A). The calibration curves allowed us to choose the antibodies with the best performance for the assays. Finally, the injection of HCl 5 mM after each measurement disrupts completely the biointeraction allowing the sensor to be reused. The entire assay cycle, including measurement and regeneration steps, takes around 20 min. Calibration curves were calculated using mean triplicate measurements of each standard. The limit of detection (LOD) was established as the analyte concentration needed to inhibit the antibody SPR signal three times the standard deviation of the blank. The linear working range comprises the concentrations producing an inhibition from 20% to 80% of the maximum SPR signal and the I_{50} is the concentration producing 50% of the maximum SPR signal. The analytical characteristics of the assays performed with all the antibodies are shown in Table 1. Based on their better analytical performance the mAbs selected were: hTSH-1, hFSH-14, hLH-7 and hGH-12 for the determination of: hTSH, hFSH, hLH and hGH, respectively. Representative calibration curves in PBST for the evaluation of the four hormones using the selected antibodies are shown in Fig. 2B.

The hTSH assay reached a detection limit of 3 ng/mL, which is equivalent to 23 μIU/mL. Although the sensitivity of this assay for hTSH detection is comparable to those obtained for the other hormones, the hTSH physiological concentrations are lower and, in consequence, its detection requires better sensitivity. Further optimization of assay conditions is necessary for the accurately detection of the cut-off level for congenital hypothyroidism (20 μIU/mL) and to reach the physiological range (0.4–4 μIU/mL). For the hFSH assay, the detection limit obtained was 2 ng/mL (29 mIU/mL) and in the case of hLH the detection limit was 3 ng/mL (26 mIU/mL) indicating that the SPR immunoanalysis is useful to detect hFSH and hLH biomarkers. Finally, the limit of detection for hGH was 4 ng/mL achieving the levels required to determine hGH in human samples.

3.3. Selectivity of the immunoassays

The specificity of each hormone sensor was evaluated flowing solutions of the antibodies (1 μg/mL) specific for the rest of the hormones. hFSH-14, hLH-7 and hGH-12 were injected in the hTSH sensor, hTSH-1, hLH-7 and hGH-12 in the hFSH sensor, hTSH-1, hFSH-14 and hGH-12 in the hLH sensor and hTSH-1, hFSH-14 and hLH-7 in the hGH sensor. The specificity of each sensor to recognize only the

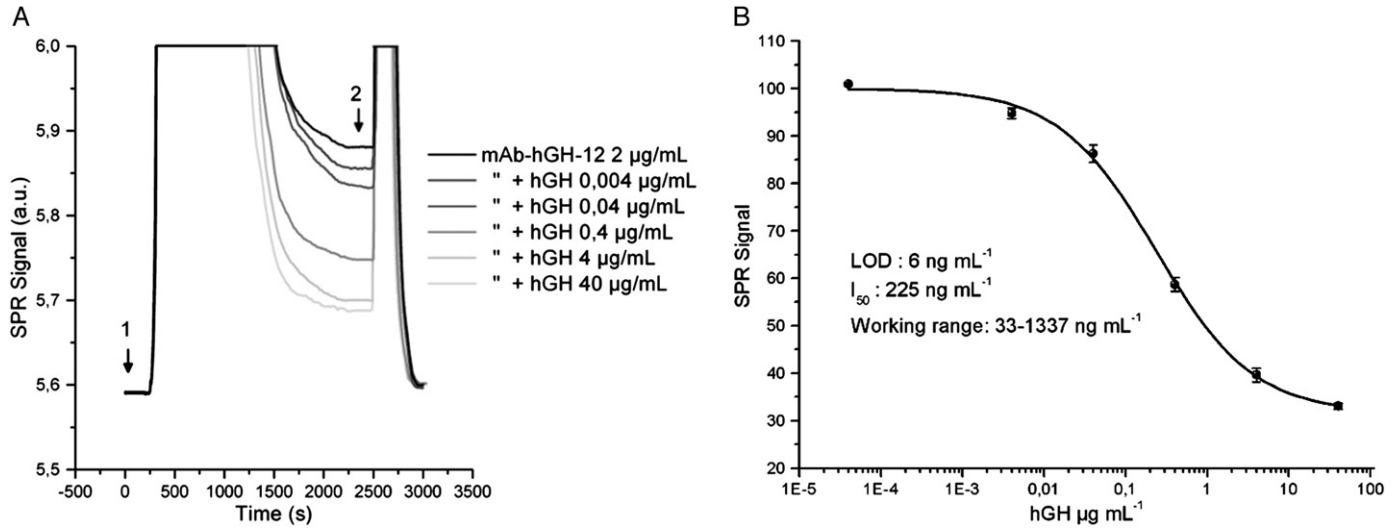

Fig. 3. (A) SPR response to the binding of different hGH concentrations and subsequent regeneration cycles during the performance of binding inhibition tests in serum. 1: sample injection. 2: regeneration solution injection (B) Standard calibration curve of hGH in serum. Each point represents the mean value of triplicate measurements.

complementary sample was demonstrated by the negligible SPR signals obtained in all other cases. Cross-reactivity of the antibodies employed in the different assays was assessed as well using mixtures containing the specific antibody and non-specific analytes. Mixtures of hTSH-1 at 0.1 μg/mL and hFSH, hLH or hGH at 10 μg/mL were tested in the hTSH sensor, mixtures of hFSH-14 at 0.5 μg/mL and hTSH, hLH or hGH at 10 μg/mL were injected in the case of hFSH sensor, mixtures of hLH-7 at 0.05 μg/mL and hTSH, hFSH or hGH at 10 μg/mL in the hLH sensor and mixtures of hGH-12 at 0.25 μg/mL and hTSH, hFSH or hLH at 10 μg/mL in the hGH sensor. In all cases, the antibody SPR signal was not affected by the presence of any of the unspecific analytes.

3.4. hGH SPR immunoassay in serum

Once the sensitivity of the different assays was established, we employed the hGH assay as a model to demonstrate the determination in serum. Measurements of hGH in serum at the concentration levels reached by SPR immunoassay would provide a valuable field application for the diagnostic of hGH deficiency. The analyte should be measured directly in serum samples without any pretreatment. For this purpose, a modified PBST assay buffer, called PBST-S, was used, as its high ionic strength and slightly basic pH, reduces dramatically the non-specific binding of serum proteins to the sensor surface. Regeneration after each measurement was not achieved using HCl 5 mM as in the case of the PBST assay. Somewhat harsher regeneration conditions were needed for breaking the specific interaction and for desorbing residual non-specific bound species. A regeneration solution of NaCl 2 M, 0.1% Tween 20 and pH 11 which combines an elevated ionic strength and a basic pH, completes the regeneration of the sensor surface. In order to enhance the specific SPR response over the non-specific residual background signal, the fixed antibody concentration used in the inhibition immunoassay was increased to 2 μg/mL. An increment in antibody concentration could diminish the assay sensitivity but a compromise between assay sensitivity and a high SPR signal was required to determine hGH in serum.

Standards of hGH in serum were measured, and calibration curves were calculated as described above. The measurements in serum samples were carried out with the same flow parameters for the PBST assay (sample: 20 μL/min, regeneration: 30 μL/min), but after each interaction, the running buffer was flown to wash the surface until reaching a stable signal, enlarging the measurement and regeneration cycle to 45 min. Fig. 3A shows the SPR response to different standards of hGH and the complete regeneration of the sensor surface. The injection of serum samples produced an elevated SPR response due to high bulk refractive index of serum regarding to PBST-S and, in a lower extent, to non-specific binding of serum components. Once all the injected serum volume has passed through the flow cell, PBST-S flowed again, lowering the refractive index and removing non-specific serum components to a large extent, reaching a stable SPR signal that consists mostly on the interaction of the antibody and a background signal due to residual adsorption of serum components. SPR signal was always measured 2500 s after the sample injection, once the signal had reached a stable value, just before regeneration.

A calibration curve and the analytical characteristics are shown in Fig. 3B. The hGH SPR immunoassay allows the determination of hGH in serum in the range between 6 ng/mL and 1.3 μg/mL which makes feasible a fast clinical diagnostic of hGH deficiency directly and without sample pre-treatment, using a small sample volume of 110 μL.

Reproducibility of the assay was examined by the evaluation of intra-, inter-day and chip-to-chip variability. Calibration curves were measured in three different days under the same experimental conditions used previously to assess inter-day variation. The reproducibility of the assay was excellent, with mean coefficients of variation of

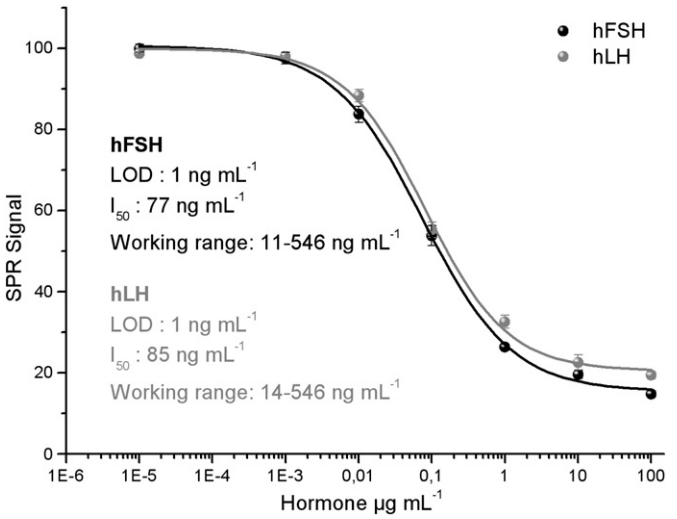

Fig. 5. Standard calibration curves for hFSH and hLH in urine. Each point represents the mean value of triplicate measurements.

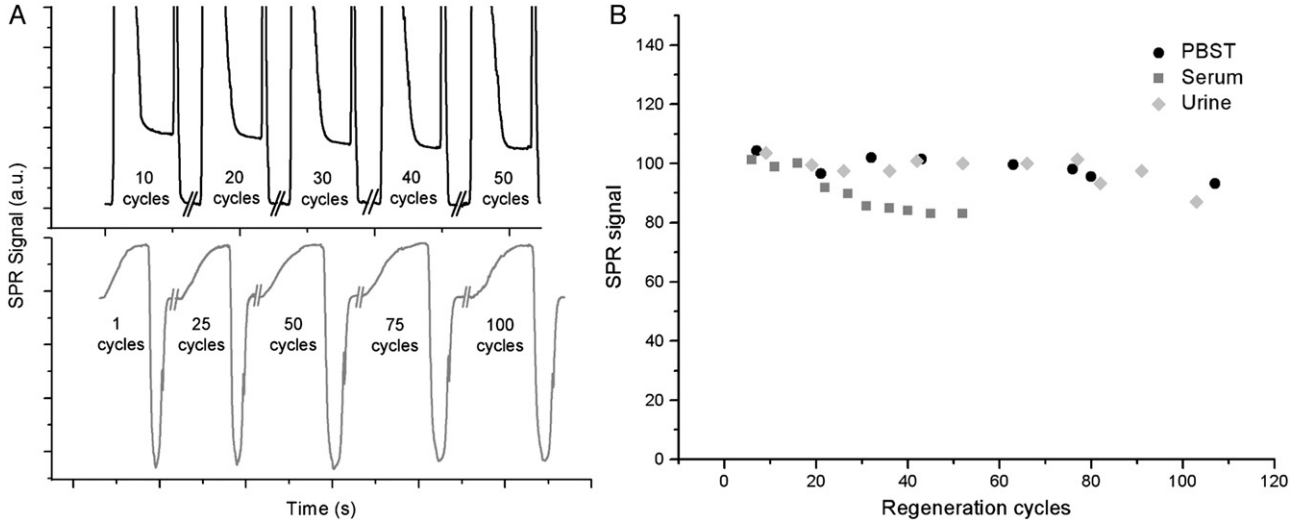

Fig. 4. (A) Sensorgrams showing the reproducibility of the SPR response throughout successive measurement and regeneration cycles in PBST and serum samples. (B) Representation of the SPR blank signal drop throughout measurement and regeneration cycles.

2.59% for intra-assay and 1.87% for inter-assay variability, respectively. Chip-to-chip variability was tested by the evaluation of calibration curves using three different sensor surfaces, prepared at different times. The coefficient of variation, 2.19%, indicates an excellent chip-to-chip reproducibility of the assay. Reusability of a biosensor is, in addition to reproducibility, one of the most important features that affect the robustness of the device. This parameter is related to the stability of the sensor surface throughout continuous measurement cycles. Reutilization of an immunosensor is achieved by the regeneration of the sensor surface, which means the complete dissociation of the antigen–antibody complex while the damage of the immobilized biomolecule is minimized. The variation in the SPR signal of blank samples is the parameter used to check the stability of the immunosensor. The stability was maintained throughout more than 50 measurement cycles during 5 consecutive days with a maximum SPR signal drop of 17% of the initial value (Fig. 4).

3.5. hFSH/hLH SPR immunoassay in urine

Determination of the gonadotropic hormones hFSH and hLH was chosen to test the convenience of the SPR sensor for the analysis of untreated urine samples. The use of urine as diagnostic fluid is advantageous due to the non-invasive and easy sample collection, particularly for long-term monitoring studies. The protein content in urine is minimal as compared to the content in serum, in such way that non-specific binding to the sensor surface is much lower in this case. Urine assay could then be carried out in the same conditions used for the PBST assay, without any modification of assay buffer or regeneration solution. The only change in assay conditions was the reduction in mAb-hFSH-14 concentration from 0.5 μg/mL to 0.3 μg/mL, as this last is enough to carry the hFSH assay.

Standards of hFSH and hLH in urine were measured and calibration curves were calculated as described above. Measurements were carried out with the same flow parameters for PBST, but after each interaction, the buffer was flown to wash the surface until a stable signal was reached, increasing the measurement and regeneration cycle to 30 min. Calibration curves and analytical characteristics of the assay are shown in Fig. 5. The use of the two flow cells of the SPR biosensor allows us to determine simultaneously both hormones in urine at concentrations as low as 1 ng/mL. This value is equivalent to 8 mIU/mL of hLH and 14 mIU/mL of hFSH. Those data indicate that SPR immunoassay provides a fast method to detect hFSH and hLH in urine samples, allowing its use for reproductive monitoring in a small urine volume and without sample pre-treatment.

Reproducibility and reusability of the assay were evaluated as described before for the hGH serum assay. The assay showed an excellent reproducibility with mean coefficients of variation of 6.47% and 2.41% for intra-assay and 6.44% and 2.14% for inter-assay variability for hFSH and hLH, respectively. Chip-to-chip variability was also tested rendering in mean coefficients of variation of 4.20% and 3.70% for hFSH and hLH, respectively. The reusability of the surface was confirmed by its stability throughout more than 100 measurement cycles carried out in six days with a maximum drop of the SPR signal of 13% from the initial value (Fig. 4).

4. Conclusions

The SPR immunosensor represents a novel and valuable instrument for the determination of biomarkers in human samples such as the pituitary hormones hTSH, hFSH, hLH and hGH. The optimization of the immobilization process and the immunoassay parameters, together with the selection of the best antibodies for each hormone, determinates the excellent performance of all the assays. The sensitivity levels of the assays allowed the determination of these hormones at concentrations down to 1 ng/mL equivalent to 28 pM of hFSH and 35 pM of hLH. Although these sensitivities are lower that the ones obtained using routine immunometric methods, the values are adequate for several diagnostic applications. The SPR immunoassay method brings some advantages as a fast and simple determination could be carry out near the patient for a better treatment of these diseases.

Two applications were chosen to demonstrate the usefulness of the SPR immunoassay evaluation in biological fluids: the measurement of hGH in serum for the evaluation of hGH deficiency and the determination of the gonadotropic hormones hFSH and hLH in urine for the determination of the fertile period, the onset of menopause, the monitoring of endocrine therapy and for the diagnostic of several associated diseases.

The developed methodology allows a fast, label-free and real-time determination of these pituitary hormones in a direct way, using a reduced volume of serum or urine samples, and without any pre-treatment. The robustness of the method is confirmed by an excellent reproducibility and an outstanding reutilization performance maintaining the stability during up to 100 measurement cycles.

The SPR biosensor used in this work could be easily applied for the determination of similar analytes in biological fluids. It could be a portable instrument for fast and simple determination of clinical analytes in biological samples and for monitoring protein disease biomarkers.

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