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# Implementation of a SPR immunosensor for the simultaneous detection of the 22K and 20K hGH isoforms in human serum samples

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ABSTRACT

We have implemented a Surface Plasmon Resonance (SPR) immunosensor based on a sandwich assay for the simultaneous detection of the two main hGH isoforms, of 22 kDa (22K) and 20 kDa (20K). An oriented-antibody sensor surface specific for both hormone isoforms was assembled by using the biotin–streptavidin system. The immunosensor functionality was checked for the direct detection of the 22K hGH isoform in buffer, which gave high specificity and reproducibility (intra and inter-assay mean coefficients of variation of 8.23% and 9% respectively). The selective determination of the 22K and 20K hGH isoforms in human serum samples in a single assay was possible by using two specific anti-hGH monoclonal antibodies. The detection limit for both hormone isoforms was 0.9 ng $mL^{-1}$ and the mean coefficient of variation was below 7.2%. The excellent reproducibility and sensitivity obtained indicate the high performance of this immunosensor for implementing an anti-doping test.



## 1. Introduction

Human growth hormone (hGH) is synthesized by the pituitary gland and directly secreted into the bloodstream. The release of the hormone is mainly controlled by the hypothalamic peptides growth hormone releasing hormone (GHRH) and somatotropin releasing inhibiting factor (SRIF). Both factors interact to generate pulsatile waves of GH release, resulting in widely fluctuating levels in blood with peaks of 50–100 ng $mL^{-1}$ and minimum levels of 0.03 ng $mL^{-1}$. Recently, ghrelin, a small peptide predominantly produced by the stomach, is also known to be involved in the control of GH pulsatility [1]. In addition, the hormone secretion is regulated by many factors and conditions such as age, gender, ethnicity, sleep, physical activity, diet and stress [2,3]. The human growth hormone exists as a complex combination of multiple isoforms, derived from the GH-N and GH-V genes. The GH-N gene expresses the main pituitary isoform of the GH family, including the major 22K form (65–80%), the second most abundant isoform 20K (5–10%) resulting from alternative mRNA splicing, and other minor isoforms resulting from post-translational modifications [4]. Determination of 22K hGH in serum is essential for the diagnosis of disorders in the hormone secretion. It is widely accepted, in the determination of hGH deficiency, a cut-off value of 10 ng $mL^{-1}$ in response to an appropriate provocative test [5].

The biosynthetic recombinant human Growth Hormone (rhGH), identical to the native 22K isoform, is widely used in clinical practice since the last eighties to treat hGH deficiency as growth failure and short stature in children and lean body mass or poor bone density in adults [6,7]. It is also well known that the rhGH is widespread used in the sporting area, often in combination with anabolic steroids, as a performance-enhancing agent. Although some of its supposed effects remain the subject of scientific controversy, the rhGH has been widely promoted in the athletic community as an ergogenic aid that can increase muscle hypertrophy and strength, and reduce body fat, conferring a competitive advantage to athletes [8]. The rhGH abuse was firstly reported in Dan Duchaine's "Underground Steroid handbook" published in 1982, where the author, known as an expert in the body building community, described the rhGH as a new athletic drug. Since then many cases of hGH abuse have been reported, including that of elite athletes. The anti-doping authority, the International Olympic Committee (IOC), included the biosynthetic hormone in its prohibited substance list in 1989. Nowadays rhGH is still believed to be one of the most widely abused performance-enhancing agents, and remains banned in sport, as established in the list of prohibited substances of the actual anti-doping authority, the World Anti-Doping Agency (WADA). However, a test for the detection of the fraudulent use of rhGH is not routinely included in anti-doping controls, and it is still the objective of many studies [9–11].

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The similarity between rhGH (represented by the 22K isoform) and the naturally occurring hormone, analytically indistinguishable, the wide physiological fluctuations in the circulating concentrations and the marked interindividual variability makes the development of a robust test the major challenge in the fight against doping. Two alternatives strategies have been mainly investigated to overcome the problems of the doping detection with rhGH in sport, the isoform approach, which allows the direct detection of rhGH misuse, and the marker approach, an indirect method used for the same purpose [12,13]. The isoform approach is based on the relative abundance of the 22K isoform as compared to the rest of isoforms, which circulating levels decrease following the administration of the recombinant 22K, due to a negative feedback mechanism. The method, initially described by Strasburger et all. [14], is based on differential immunoassays using a monoclonal antibody (mAb) that preferentially recognizes the 22K hGH (Rec hGH), and a permissive mAb that recognizes all pituitary isoforms (Pit hGH). By calculating the Rec hGH:Pit hGH ratio the relative abundance of 22K can be determined, indicating the rhGH abuse. However, due to the short half-life of rhGH in circulation, this method is limited to 24–36 h after hormone administration. The maker approach or indirect method has been investigated as an alternative strategy, since it is based on the quantification of hGH-dependent proteins directly affected by GH intake [15].

Recently, the availability of specific anti-20K hGH antibodies has allowed the development of other isoform-based method for the detection of exogenous hGH, using the quantification of the 22K hGH form, together with the determination of the 20K hGH, which relative circulating concentration is expected to be reduced by negative feedback after rhGH administration. The circulation ratio of both isoforms in the blood is typically 9:1 (22 versus 20K), which seems to be independent of sex, age, exercise, and even certain pathologies [16–18]. The marked increase of this ratio following rhGH intake has been reported in several studies of doping, suggesting this parameter as the most suitable indicator for the detection of rhGH abuse [19,20].

Different antibody-based assays have been employed for hormones detection in the clinical field, such as radioimmunoassays (RIA), immunoradiometric assay (IRMA), immunochemiluminometric assay (ICMA) or enzyme-linked immunosorbent assays (ELISA). All these techniques reach very low limits of detection, in the range of pM–fM. However, they require the use of labels and several incubation steps rendering in time-consuming analysis that have to be done in conventional laboratory settings. The Surface Plasmon Resonance (SPR) immunosensing technique, which monitors biomolecular interactions in real time without the need for labeling and revelation steps, has proved to be a promising alternative to conventional methods. Sensitive, fast and high performance immunoassays are achieved by SPR biosensor technology [21,22], which in addition can provide a portable, automated and user-friendly analytical equipment, especially useful for routine tests and in-situ analysis [23,24]. Some publications have reported the study of the binding profile of different monoclonal antibodies against hGH isoforms using a SPR biosensor [25,26]. We showed the validation of a SPR immunoassay for the direct detection of rhGH in serum samples at physiological concentrations, without sample pre-treatment [27] and by using a home-made portable SPR sensor.

In this work, we have implemented a sandwich SPR immunoassay for the simultaneous determination of the 22K and 20K hGH isoforms directly in human serum samples, providing a powerful method for differentiation between both isoforms and opening the route for a fast anti-doping test. A schematic representation of the employed methodology is shown in Fig. 1. The detection and differentiation was possible through a previous optimization of a biofunctionalization protocol based on the use of the biotin-streptavidin system for the site-directed immobilization of the capture antibody, and thanks to the use of non-commercial monoclonal antibodies of high quality and specificity. Although previous publications have shown SPR analysis of hGH isoforms, we report here how an optimized method is able to identify both 22 and 20K hGH isoforms contained in the same serum sample by using only one sensor chip. In addition, we have optimized the immobilization method to control the density and the orientation of the receptor antibody layer in order to achieve the same sensitivity level for both isoforms detection, despite their different concentrations.

## 2. Materials and methods

### 2.1. Reagents

Three anti-hGH monoclonal antibodies were employed, hGH-12 which recognizes all isoforms, and hGH-25 and hGH-33 which specifically recognize the 22K and 20K hGH isoforms, respectively [28]. All antibodies, purified by ammonium sulfate precipitation from ascites fluid, were supplied by the Centro Nacional de Biotecnología (CNB-CSIC, Madrid, Spain). Recombinant human Growth Hormones 22K (lot#AFP8990) and 20K (lot#AFP4286) were supplied by Dr. Parlow, National Hormone and Peptide Program (NHPP), National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK, CA, USA). Serum sample from human male AB plasma was purchased from Sigma-Aldrich (Steinheim, Germany). HiTrap NHS-activated 1 mL column and PD-10 desalting column were purchased from GE Healthcare (Uppsala, Sweden). Biotin-LC-hydrazide, sulfo-NHS-LC-biotin, streptavidin, N-hydroxysuccinimide (NHS), 1-ethyl-3 (3-dimethyl-amino-propyl) carbodiimide hydrochloride (EDC) and ethanolamine hydrochloride were obtained from Sigma-Aldrich (Steinheim, Germany). The reagents for the preparation of buffers as well as the alkanethiols 16-mercaptohexadecanoic acid and 11-mercaptoundecanol were also provided by Sigma-Aldrich (Steinheim, Germany). Organic solvents for sensor chip cleansing process, trichloroethylene, acetone and ethanol, and piranha solution components $H_2SO_4$ and $H_2O_2$, were supplied by Panreac (Barcelona, Spain). PBS buffer (10 mM phosphate pH 7.4 with 137 mM NaCl and 2.7 mM KCl) was prepared with potassium chloride, sodium chloride, disodium hydrogen phosphate and potassium dihydrogen phosphate. 10 mM acetate buffer (pH 4.5) was prepared with acetic acid and sodium acetate. MES buffer, 2-(N-morpholino) ethanesulphonic acid, was prepared at a concentration of 0.1 M and carbonate buffer (0.2 M $NaHCO_3$ pH 8.3 with 0.5 M NaCl) was prepared with sodium bicarbonate, sodium carbonate and sodium chloride.

### 2.2. SPR sensor platform

A home-made Surface Plasmon Resonance sensor, based on Kretschmann configuration, was employed for monitoring the binding events in real time by carrying out the detection at a fixed angle of excitation. A polarized light emitted by a 3 mW laser diode, operating at 670 nm, is divided in two equal beams and made incident on the gold-coated sensing surface where two flow cells with the same volume (300 nL) are placed. The use of two flow cells allows the measurement of two independent samples or the use of a reference channel. All experiments were done by the injection of the sample using an automatic flow delivery system incorporated in the platform, which ensures the injection of accurate sample volumes of 220 μL, while maintaining a continuous flow between 10 and 40 μL $min^{-1}$. The total size and low weight of the SPR instrumentation permits its use as a portable platform [29]. SPR sensors chips 10 mm square were obtained from Ssens (Enschede, The Netherlands). The sensing

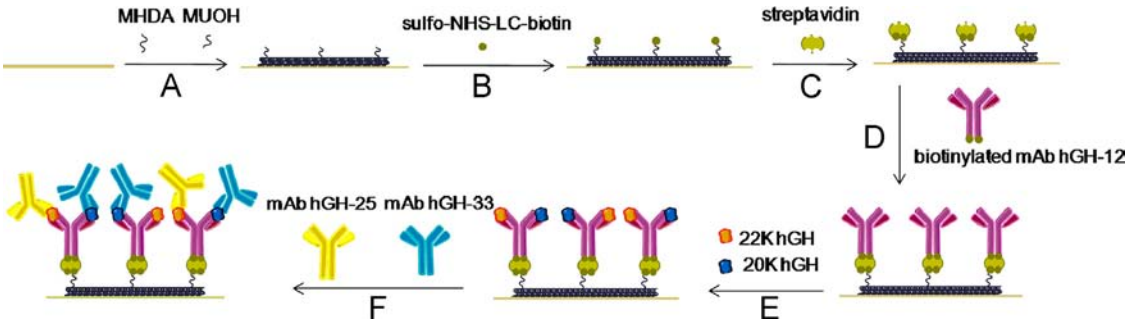


**Fig. 1.** Scheme of the sandwich immunoassay methodology used to detect the hGH isoforms: (A) formation of the mixed SAM, (B) biotinylation of the SAM surface, (C) streptavidin immobilization, (D) coupling of the capture antibody, (E) binding of the 22 and 20K hGH isoforms to the antibody surface and (F) specific recognition of each isoform by the corresponding selective antibody.

surface is a cover glass slide ($10 \times 10 \times 0.3$ mm) coated with 45 nm of gold and 2 nm of Ti.

Prior to immobilization, sensor chips were firstly cleaned in successive steps with organic solvents (trichloroethylene, acetone and ethanol). Following, the chips were immersed in a freshly prepared piranha solution ($H_2SO_4/H_2O_2$, 3:1), rinsed with water, ultrasonicated for 5 min and dried under nitrogen flux. This cleaning procedure was carried out using the appropriate protective equipment.

### 2.3. Preparation of biotin labeled antibody

MAb hGH-12 was affinity-purified using a hGH 22K-coupled Sepharose column of 1 mL, after dialyzing from ammonium sulfate by circulating through a desalting column pre-equilibrated with PBS pH 7.4. According to the manufacturer's instructions all samples and solutions were loaded onto the column by using a syringe and at a maximum flow rate of 1 mL $min^{-1}$. Firstly, the coupling of the hGH to the column was carried with 1 mL of the hormone at 1.7 mg $mL^{-1}$ in carbonate buffer pH 8.3. Then, a washing and deactivation procedure was performed by alternating injections of the solutions 0.1 M ethanolamine/0.5 M NaCl pH 8.3 and 0.1 M acetate/0.5 M NaCl pH 4. Finally, the column pH was neutralized with PBS pH 7.4. After preparing the hGH column, the purification of the mAb hGH-12 was carried out. A solution of 1 mL of antibody at 4 mg $mL^{-1}$ in PBS pH 7.4 was injected. Then, a washing step was performed with 10 mL of the same buffer followed by the injection of a solution of 0.1 M glycine pH 2.5 for the elution of the antibody. Immediately after, a buffer exchange into PBS was performed by using a desalting column.

Once purified, the mAb hGH-12 was biotinylated by incubation with sulfo-NHS-LC-biotin varying the ratio of biotin to antibody concentration. Two solutions of 1 mL of antibody in PBS at pH 8 and at a concentration of 1 mg $mL^{-1}$ were incubated for 30 min at room temperature, with 33.4 and 66.7 μL respectively, of 20 mM sulfo-NHS-LC-biotin solution, in order to obtain a 50 and 100 M excess of biotin, in the modified antibody. The excess of non-reacted biotin was removed by a buffer exchange into PBS using a desalting column.

### 2.4. Assembly of the streptavidin-modified surface

A mixed self-assembled monolayer (SAM) was assembled on the gold surface by covering the sensor chip overnight with an ethanolic mix solution of 11-mercapundecanol (11-MUOH) and 16-mercaptohexadecanoic acid (16-MHDA) in a molar proportion of 50:1. This ratio was optimized in a previous work (data not shown). The SAM surface was then biotinylated by covalent linking of biotin-LC-hydrazide using the EDC/NHS chemistry. The amino coupling of the biotin reagent was achieved by the injection of 0.2 M EDC/0.05 M NHS solution in MES buffer (pH 5.6), followed by the injection of biotin-LC-hydrazide prepared at a concentration of 5 mM in acetate buffer (10 mM, pH 4.5). Then, a solution of ethanolamine (1 M, pH 8.5) was injected to deactivate residual unreacted groups on the surface. After biotinylation of the SAM coated surface, the running buffer was changed from milliQ water to PBST (pH 7.4) that was employed in the rest of the immobilization procedure. Solutions of 5 and 50 μg $mL^{-1}$ of streptavidin, respectively, were prepared in PBST (pH 7.4) and injected separately in each channel of the sensor.

Additional studies were done to discard non-specific adsorption of biotin or streptavidin onto the SAM coated surface. A sensor chip was functionalized with the same mixed SAM, then, a 5 mM of biotin-LC-hydrazide solution in acetate (10 mM, pH 4.5) was flowed over the SAM surface in one of the flow cells, omitting the EDC/NHS mediated activation step. After that, 50 μg $mL^{-1}$ of streptavidin in PBST was injected in both channels to check that there was no attachment of streptavidin and this attachment can be only achieved via the previous assembled biotinylated alkanethiols.

### 2.5. Immobilization of biotinylated hGH-12 monoclonal antibody

Both mAb hGH-12 samples, labeled with 50 and 100 M excess of biotin, respectively, were prepared at a concentration of 10 μg $mL^{-1}$ in PBST. In order to compare the effect of the streptavidin density on the immunoassay performance, the solution of antibody modified with 50 M excess of biotin was injected over streptavidin surfaces prepared with 5 and 50 μg $mL^{-1}$, respectively, followed by the injection of a solution of 1 μg $mL^{-1}$ of 22K hGH in PBST (pH 7.4). To check the influence of the degree of antibody biotinylation on the immunoassay, solutions of Abs labeled with the two amounts of biotin were injected in each channel of the SPR device, over the streptavidin surface prepared with 50 μg $mL^{-1}$ of the protein. Then, a solution of 1 μg $mL^{-1}$ of 22K hGH was injected in PBST (pH 7.4).

The antibody modified with a 50 M excess of biotin was also flowed over both non biotinylated and biotinylated SAM coated surface, prepared exactly as described above, to ensure that no antibody was adsorbed without the presence of streptavidin on the surface.

### 2.6. SPR Immunoassay formats

After the mAb hGH-12 surface development a direct immunoassay was carried out for the detection of the 22K hGH isoform in a concentration range from 50 ng $mL^{-1}$ to 5 μg $mL^{-1}$, in PBST. The mean values of triplicate measurements were plotted versus

hGH concentration and fitted to a four-parameter Hill equation:

$$y = A + (B-A)x^n/k^n + x^n \quad (1)$$

where $y$ is the response, $x$ is the concentration, $A$ is the minimum signal, $B$ is the maximum signal, $k$ is the concentration at which the response is 50% of the maximum signal and $n$ is the Hill coefficient.

Once the antibody surface functionality was assessed a sandwich immunoassay was developed for the simultaneuos determination of the 22 and 20K hGH isoforms in serum. Solutions of both isoforms mixed in different proportions were prepared in 110 μL of commercial human serum which was diluted 1:1 in a PBST buffer containing 500 mM NaCl, 0.1% Tween 20 and pH 8 (PBST-S). This buffer was selected as we have previously demonstrated that it is the most suitable one to diminish non-specific adsorptions when analyzing human serum samples [27]. After establishing a fixed flow of PBST-S, the serum samples containing the mixtures of 22 and 20K hGH were flowed over the mAb hGH-12 surface, for the binding of the isoforms to the antibody. Then, the monoclonal antibodies hGH-25 and hGH-33, which selectively binds to the 22K and 20K, respectively, were injected in PBST-S, for the determination of both isoforms concentration. The averaged responses of triplicate measurements of each isoform were plotted versus the isoforms concentration and the calibration curves were fitted to a Hill function (1) in the case of the 22K hGH, and to a linear function $y=A+Bx$, in the case of the 20K hGH.

## 3. Results and discussion

### 3.1. Assembly of the antibody layer

The first step in the development of the sandwich assay was the assembly of a reliable and robust antibody layer which, as it is well-known, would present more limited reusability than when the antigen is used as the bioreceptor molecule [30]. For that a site-directed antibody immobilization using the streptavidin–biotin system was employed. The optimization of this process was carried out by using the 22K hGH isoform. The reduced commercial availability and the high cost of the 20K hGH, as compared to the 22K isoform, were the main reasons for such choice. The modification of the sensor surface with a streptavidin monolayer was done by using a biotinylated alkylthiol self-assembled monolayer. Since mixed SAMs, particularly those formed by long-chain alkanethiols diluted with shorter hydroxythiols, have been shown to prevent denaturation of proteins and non-specific interactions while minimizing steric hindrance, in comparison with pure SAMs [31,32], a mixture of 11-MUOH and 16-MHDA was employed for coating the sensor surface. Once formed, the SAM surface was biotinylated via their carboxyl terminal groups. Streptavidin's four biotin binding sites and their dyad symmetry enable this protein to attach to this surface via either one or two biotin links, while leaving accessible the other two biotin sites for binding a second layer of biotinylated molecules [33].

The streptavidin concentration chosen for modifying the sensor surface was 50 μg mL$^{-1}$, according with previous works [34,27]. The efficiency of the streptavidin surface was evaluated by immobilizing mAb hGH-12 modified with 50 M excess of biotin and subsequent hGH immunodetection. As observed in Fig. 2, high signals of antibody coupling and hormone detection were achieved with this streptavidin concentration.

As the immobilization of streptavidin gave rise to a large SPR signal, we considered the possibility of improving the immunoassay performance by decreasing the protein concentration. This could contribute to reduce the steric hindrance between neighboring antibodies that could occur when the mAb hGH-12 is immobilized on a surface with high density of streptavidin molecules. For this purpose, 5 μg mL$^{-1}$ of streptavidin, a significantly lower concentration, was assayed. The SPR signal showed much lower level using 5 rather than 50 μg mL$^{-1}$ of streptavidin, as expected, which indicates that 5 μg mL$^{-1}$ is far from being a saturating concentration (Fig. 2A). The level of antibody immobilization achieved with the streptavidin surface prepared with 50 μg mL$^{-1}$ was much higher than that obtained with the surface modified with 5 μg mL$^{-1}$ (Fig. 2B). The SPR response to the hGH detection had the same tendency, obtaining much higher signal with the surface modified with the highest streptavidin concentration (Fig. 2C). Therefore, we selected 50 μg mL$^{-1}$ as the optimal concentration.

Two different molar ratio of biotin reagent to antibody were tested, in order to study the influence of the biotinylation degree on the efficiency of the antibody immobilization. Molar ratios of 100:1 and 50:1 (biotin:antibody) were chosen as the modification of antibodies with less amount of biotin has shown worst results in previous experiments (data not shown). The two antibodies modified with different amount of biotin were immobilized on the streptavidin surface at a concentration of 10 μg mL$^{-1}$. The SPR responses to the immobilization of mAb hGH-12 labeled with 100 M excess of biotin and that of 50 M excess were similar, obtaining a slightly higher signal in the attachment of the antibody tagged with greater amount of biotin (Fig. 3A). However, the two antibody surfaces led to very different results for the detection of 1 μg mL$^{-1}$ of hGH. The surface coated with mAb hGH-12 modified with 50 M excess of biotin gave much higher level than the one coated with the antibody modified with 100 M excess (Fig. 3B). A loss of binding affinity of the antibody to the hGH, due to an excessive biotin labeling, might explain the reduced sensitivity obtained by employing mAb hGH-12 biotinylated in a molar ratio of 100:1.

As the best immunoassay performance was achieved with the immune-surface developed using streptavidin at a concentration of 50 μg mL$^{-1}$ and mAb hGH-12 biotinylated in a molar ratio of 50:1, this surface was selected as the optimal one for the detection of the hGH isoforms.

### 3.2. Evaluation of the hGH-12 antibody surface efficiency by SPR

The efficiency of the receptor hGH-12 antibody surface was evaluated by performing a direct immunoassay for the detection of the 22K hGH isoform, in a concentration range from 50 ng mL$^{-1}$ to 5 μg mL$^{-1}$. The immunoassay was carried out in PBST, which was used to prepare six hGH solutions of different concentrations. Each hormone solution was injected sequentially three times over the same hGH-12 antibody surface and the disruption of the immune interaction after recognition was achieved by the injection of a HCl 5 mM solution at 30 mL min$^{-1}$. Despite the difficulty of preserving the antibody-based sensor stability after using regeneration buffers, the HCl solution employed for the disruption of the mAb hGH-12–hGH 22K interaction allowed the sensor reutilization during several cycles as the SPR signal returned to its initial value after regeneration (Fig. 4). The sensorgram of Fig. 5A shows the SPR response for the hormone determination in the concentration range assayed, and Fig. 5B shows the calibration curve calculated from triplicate measurements. The reproducibility of the immunoassay was evaluated by the calculation of the coefficient of variation (CV) of the signal response. The results show low intra-assay variability with a mean CV of 8.23%. The inter-assay variability was evaluated by carrying out the immunoassay with three different hGH-12 antibodies sensor chips, and the results show a CV of 9%. The immunoassay detection limit (LOD) calculated as the analyte concentration equivalent to three times the standard deviation of the SPR signal of the running buffer was 5 ng mL$^{-1}$. The assay exhibited a working range 0.037 to 1 μg mL$^{-1}$,

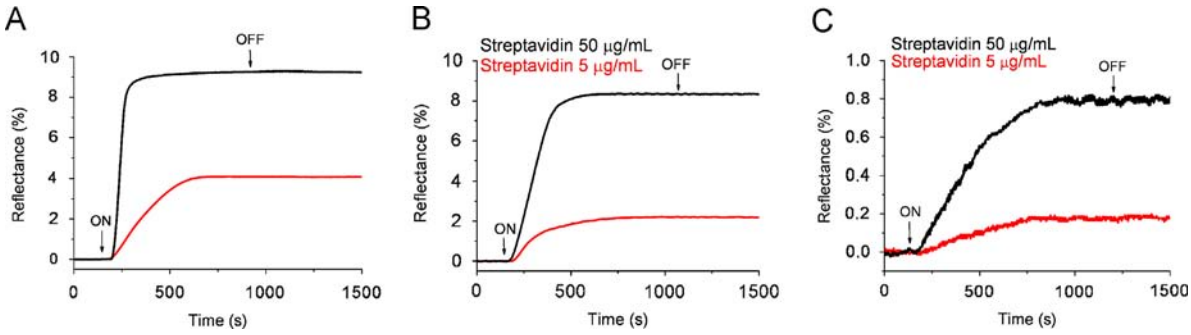


**Fig. 2.** Influence of the streptavidin concentration on the immunoassay performance: (A) immobilization of 50 (black line) and 5 (red line) $\mu g\,mL^{-1}$ of streptavidin, (B) coupling of 10 $\mu g\,mL^{-1}$ of biotinylated mAb hGH-12 to each streptavidin surface and (C) corresponding binding of 1 $\mu g\,mL^{-1}$ of 22K hGH. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

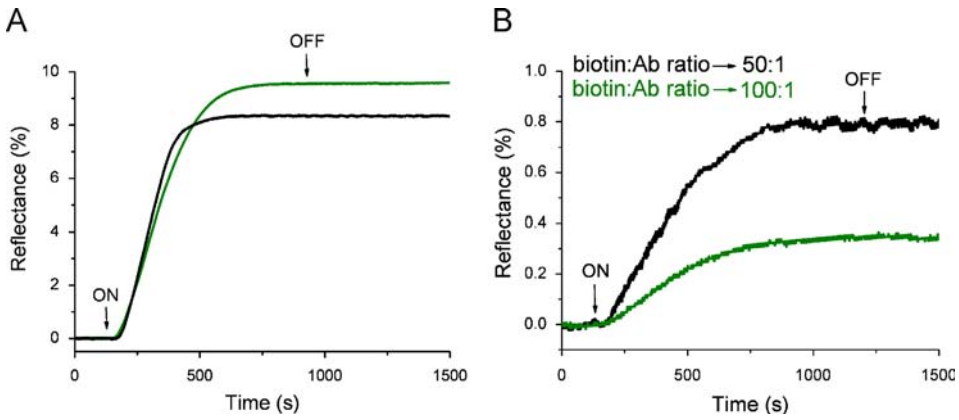


**Fig. 3.** Influence of antibody biotinylation degree on the immunoassay performance: (A) immobilization of 10 $\mu g\,mL^{-1}$ of antibody biotinylated in a biotin:Ab molar ratio 100:1 (green line) and 50:1 (black line) and (B) corresponding binding of 1 $\mu g\,mL^{-1}$ of 22K hGH. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

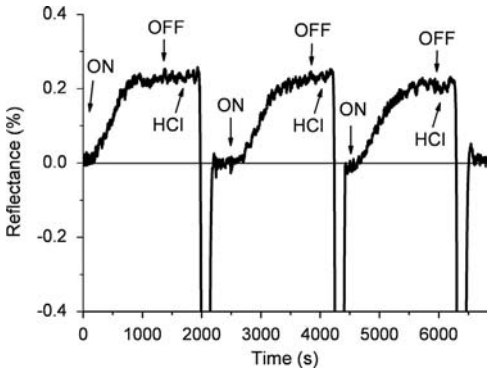


**Fig. 4.** SPR response for successive detections of 0.25 $\mu g\,mL^{-1}$ of 22K hGH with regeneration cycles in between.

which was calculated as the sensing range where the $R^2$ value of the calibration curve fitted by linear regression was higher than 0.98. The specificity of the immunosensor was evaluated by assaying 1 $\mu g\,mL^{-1}$ of a human Thyroid Stimulating Hormone (hTSH) solution that did not lead to any signal (data not shown).

Since a single-use sensor is desirable for clinical applications, and taking into account the inevitable loss of activity of an immobilized antibody after several regeneration cycles [35–37], the reusability of the antibody sensor chip was not an important aim in this work. Nevertheless, the success of our methodology for the fabrication of the antibody surface was demonstrated by the high stability of the sensor chip, as the functionality of the hGH-binding surface was preserved under regeneration conditions, allowing its reutilization at least for three evaluation cycles. In addition, the excellent intra-assay and chip to chip reproducibility, together with the high immunoassay sensitivity and specificity, demonstrated the high efficiency of the hGH-12 antibody sensor chip.

### 3.3. *Determination of the 22K and 20K hGH isoforms in human serum*

Once the sensor chip was functionalized with the mAb hGH-12, which recognizes the hGH of 22 kDa and the hGH of 20 kDa with similar affinity [28], a SPR immunoassay based on a sandwich format was carried out for the determination of the two hormone isoforms in human serum samples containing a mix of both isoforms. hGH-25 and hGH-33 monoclonal antibodies were used for the specific detection of the isoforms. In order to guarantee the selectivity of the sandwich assay for the determination of the hormone isoforms from mixtures of both analytes, the hGH-12 antibody chip was firstly used to carry out an immunoassay using mAb hGH-25 and mAb hGH-33 against the non-specific isoform in PBST. For this purpose, 1 $\mu g\,mL^{-1}$ of each isoform prepared in PBST was flowed over an hGH-12 antibody surface (using PBST as running buffer) followed by the successive injections, first, of the non-specific detection antibody, and later the specific one, both prepared in PBST at a concentration of 25 $\mu g\,mL^{-1}$. As observed in the sensorgrams of Fig. 6, following the high SPR signals obtained in the binding of the isoforms to the hGH-12 antibody surface, the injection of the non-specific antibody did not led to any SPR response. On the contrary, when the specific antibody to each hormone isoform was used, high signals were obtained. This experiment proved the specificity of the detection of the 22 and

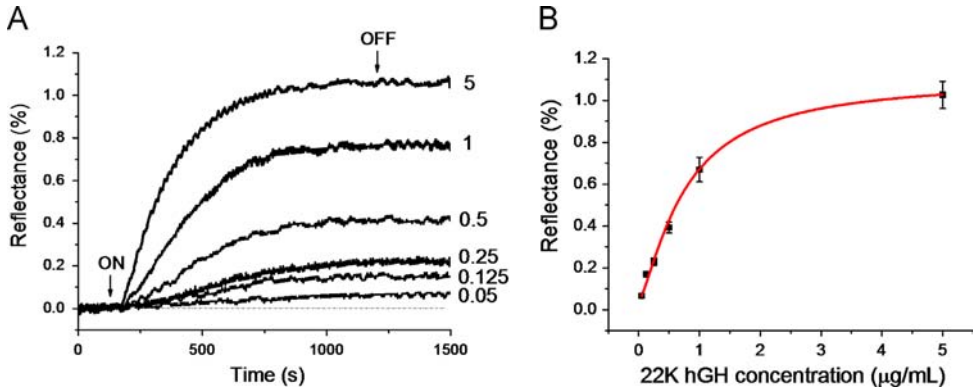


**Fig. 5.** Immunoassay for the determination of the 22K hGH isoform in PBST: (A) hormone detection signals in a concentration range between 0.05 and 5 μg mL$^{-1}$ and (B) calibration curve obtained from triplicate measurements with the same antibody surface. Each point represents the mean value ± SD (mean CV=8.23%). A LOD of 5 ng mL$^{-1}$ is achieved.

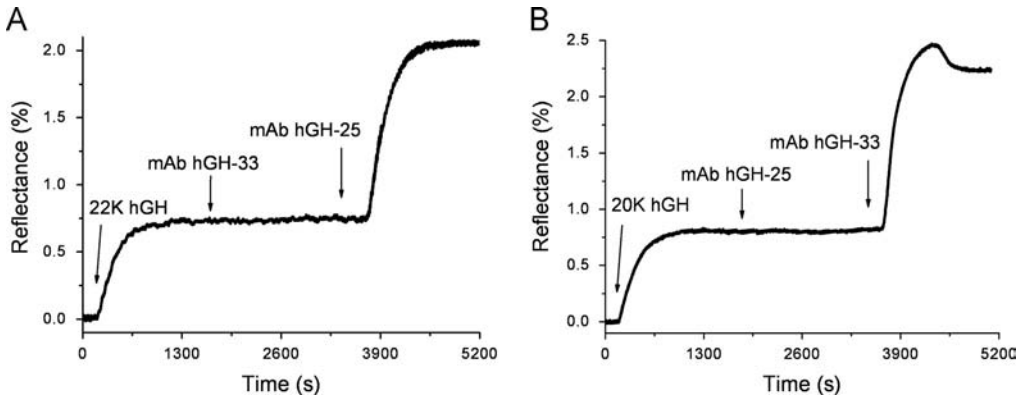


**Fig. 6.** Evaluation of the immunoassay specificity: (A) specific mAb hGH-25 binds only to 22K hGH isoform and (B) specific mAb hGH-33 only recognizes the 20K isoform.

20K hGH, bonded to the same surface, by employing the hGH-25 and 33 monoclonal antibodies. At the same time the lack of non-specific adsorption of these antibodies on the sensor surface was demonstrated.

Once ensured that no cross-reactivity would take place in the sandwich assay, the use of lower concentration of the detection antibodies was tested, in order to improve the immunoassay performance. As similar behavior had been observed between these antibodies, the pair 22K hGH/mAb hGH-25 was only used for this purpose. 50 ng mL$^{-1}$ of 22K hGH, the lowest concentration used in the direct assay above described, was flowed over an hGH-12 antibody surface, followed by the injection of 25 and 10 μg mL$^{-1}$, on independent flow cells, of the corresponding detection antibody. As observed in Fig. 7, decreasing the mAb hGH-25 concentration to more than half of the previously assayed, led to a non-significant loss of the SPR signal. Therefore, 10 μg mL$^{-1}$ was selected as the concentration of the detection antibodies to carry out the sandwich assay.

Since blood circulation level of the 20K isoform is nearly one order below that of 22K, the isoforms were assayed in different concentration ranges, 0.5–50 ng mL$^{-1}$ and $1 \times 10^{-3}$ to 1.5 μg mL$^{-1}$ respectively, in order to work as much as possible closer to the real conditions. Mixtures of 22K and 20K hGH were prepared in human serum diluted 1:1 in PBST-S. The 22K:20K (ng mL$^{-1}$) proportions used were 1:0.5, 1:1, 10:1, 20:1, 40:10, 100:10, 200:20, 500:20, $10^3$:50 and $1.5 \times 10^3$:50. A continuous flow of PBST-S was established and then each solution of isoforms mixture was flowed over the hGH-12 antibody surface, followed by a solution of mAb hGH-25 and other of mAb hGH-33, both sequentially injected in this order at 10 μg mL$^{-1}$ in PBST-S, for the specific detection of 22K and 20K hGH, respectively. All samples were run in triplicates and the averaged responses were plotted obtaining the calibrations curves

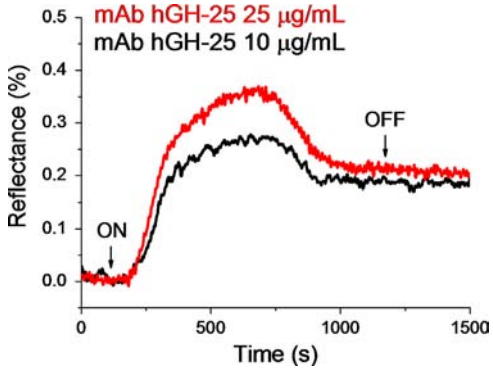


**Fig. 7.** Optimization of the Ab concentration. Specific binding of mAb hGH-25 to 50 ng mL$^{-1}$ of 22K hGH.

shown in Fig. 8. As observed, the 20K concentration range assayed, which gives rise to a linear SPR response, corresponds to a linear section in the 22K calibration curve, which indicates that the difference between both curve models, linear versus non-linear, is due to the different isoform concentration range used to fit with the isoforms ratio in real samples. Since the reutilization of the immunosensor surface is not possible after performing a sandwich assay, two measurements of the triplicates were obtained using the same chip but on independent antibody surfaces (using the two SPR cells), and for the third replica a different sensor chip was used, which gave rise to very similar results. The CV (of the signal response) obtained, with mean values of 6.7% and 7.16% for the 22K hGH and the 20K hGH, respectively, showed the high

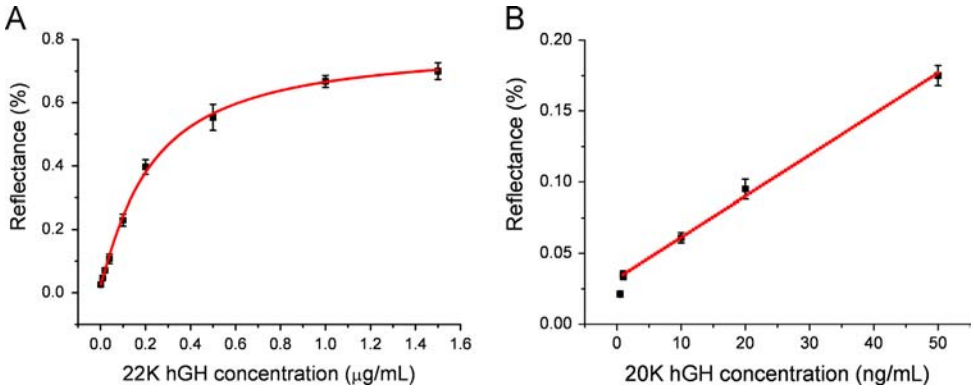


**Fig. 8.** Calibration curves of the determination of 22K (A) and 20K (B) hGH from serum samples containing both isoforms in the concentration ranges 0.5–50 ng $mL^{-1}$ and $1 \times 10^{-3}$ to 1.5 μg $mL^{-1}$, respectively. Each point represents the mean value (±SD) of triplicate measurements. A LOD of 0.9 ng $mL^{-1}$ was achieved for both isoforms. The mean CV of the immunoassay for 22K and 20K hGH were 6.7% and 7.16% respectively.

reproducibility of the immunoassay. The limit of detection for both isoforms was 0.9 ng $mL^{-1}$. A linear sensing response to the 20K hGH isoform was achieved in the concentration range assayed ($R^2$=0.9967). Since a linear calibration was obtained for the 20K isoform, only the lower limit of quantification of the working range could be calculated. This value is 0.11 ng/mL and therefore, the 20K hGH isoform circulating levels can be accurately determined in a concentration which ranges from 0.11 ng/mL to at least 50 ng/mL (the highest concentration assayed). However, this assay accurately determines the 20K hGH isoform physiological circulating levels, which range from 0.1 to 10 ng/mL. On the other hand the limit of detection obtained for both isoforms is lower than the mean physiological serum levels of the 22K isoform and higher than that of the 20K isoform, approximately 1.1 and 0.1 ng $mL^{-1}$, respectively [26]. Since the LOD value for the 20K hGH determination is not far from its physiological levels (it is within the same order of magnitude), the sensitivity as well as the reproducibility of the assay were excellent. The results demonstrate the successful implementation of a SPR immunosensor for the determination of the 22 and 20K hGH isoforms in human serum by performing a single assay, providing a very promising tool for doping analysis, which still requires a well-defined cut-off [11]. In addition, the biosensing methodology employed in this work could be carried out using highly sensitive nanophotonic platforms, such as bimodal interferometers recently developed in our group, which greatly improve the sensitivity of the SPR system [38,39].

## 4. Conclusions

We have developed the first SPR immunosensor for the simultaneous determination of 22K and 20K hGH isoforms in human serum, aimed for developing an efficient and fast hGH-doping test. The biosensing methodology based on an anti-hGH surface for detecting both hormones by a sandwich assay configuration, renders in a useful technique for the development of single-use sensor chips. The strategy followed for the preparation of the immunosensor surface, based on the use of the biotin–streptavidin system for the successful immobilization of the capture anti-hGH antibody, together with the high efficiency and selectivity of the detection antibodies employed, led to a highly sensitive, reproducible and reliable immunoassay. Although the application of this SPR immunosensor in anti-doping test would be limited by the extremely low concentration reached by the 20K isoform after hGH administration (below 50 pg $mL^{-1}$), the methodology could be easily transferred to other sensor platforms, such as nanophotonic devices based on interferometric bimodal waveguides, in order to improve the sensitivity of the immunoassay to the required limits of detection.

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