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Surface plasmon resonance immunosensor for ErbB2 breast cancer biomarker determination in human serum and raw cancer cell lysates

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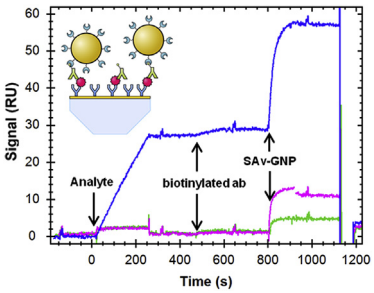
HIGHLIGHTS

- A SPR biosensor for ErbB2 breast cancer biomarker has been developed.
- Amplification with gold nanoparticles enhanced the sensitivity.
- A detection limit of 180 pg mL^{-1} has been achieved in human serum.
- Raw lysates from model breast cancer cell lines have been successfully assayed.

GRAPHICAL ABSTRACT



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ABSTRACT

A highly sensitive surface plasmon resonance (SPR) immunosensor for the important ErbB2 breast cancer biomarker has been developed. Optimization of the assay has been carried out, including signal enhancement employing gold nanoparticles (GNPs). The effect of the signal amplification of the GNPs has been also studied. The assay has been tested with clinically relevant matrices. Results in 50% human serum yielded a LOD of 180 pg mL^{-1} which is a concentration 83 times lower than the clinical cut-off. Raw lysates from model breast cancer cell lines (SK-BR-3, MCF-7 and MDA-MB-436) have been also assayed and higher quantities of the ErbB2 protein were clearly observed in the ErbB2 over-expressing case (SK-BR-3). The results confirmed that the simple and highly sensitive SPR immunosensor represents a feasible tool for ErbB2 detection.

1. Introduction

ErbB2 or HER2 is a tyrosine kinase receptor belonging to the epidermal growth factor receptor (EGFR) family and is involved in cellular signaling pathways, which lead to cell proliferation, growth, apoptosis and differentiation. These processes are essential

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to life, but loss of control within these pathways is frequently associated with several diseases, including cancer. Although the overexpression of ErbB2 is observed in some of breast cancer cases (thus cataloged as ErbB2 positive) and treatments using a monoclonal antibody to this molecule is now in use, it is only effective in patients with excess receptor levels. In fact, it is required to screen the patients before treatment to find those who are eligible for such administrations [1].

The ErbB2 protein is membrane-spanning, having both intracellular and extracellular domains. The extracellular domain (ECD) of ErbB2 can be cleaved by matrix metalloproteases and shed in the culture supernatant of certain breast cancer cell lines and in the serum of patients with breast cancer. This fragment of the intact ErbB2 protein is often termed p105, or soluble p105, as it represents the 105 kDa N-terminal fragment of the 185 kDa intact protein. The ECD is found at elevated levels in serum in approximately 50–60% of ErbB2 positive breast cancer patients. Further studies have shown increased levels of ErbB2 ECD in serum is associated with a poor prognosis, reduced therapeutic response and decreased survival chances [1,2]. All these findings have resulted in the use of ErbB2 or EDC as target analytes in the early detection of breast cancer. The blood ErbB2 ECD content of breast cancer patients is usually >15 ng mL^{-1}, which requires assay methods capable to reliably measure such low concentration levels in this complex biological sample.

Current diagnostic tests for ErbB2 involve the use of fluorescent in situ hybridization (FISH) or immunohistochemistry (IHC), which are optical semi-quantitative techniques. Moreover, both procedures are complicated and time consuming, demand elaborated sample preparations and require specially trained personnel to carry out the corresponding multi-step procedures [1].

A new generation of biosensors using optical, piezoelectric or electrochemical transduction principles are being developed [3]. Among them, surface plasmon resonance stands out as a highly sensitive optical detection method offering several advantages. SPR sensors sense very small changes in the refractive index on the gold surface of a chip [4,5]. The adequate and specific functionalization of this surface allows for quantification of analytes as the refractive index continuously changes upon binding. This permits a highly sensitive monitorization of the binding process in a real-time and label-free fashion. This technique is commonly used in many laboratories mainly for molecular recognition kinetics characterization or screening experiments with proteins, drugs or DNA. The use of SPR biosensors for protein determination is somehow less explored, mainly due to the problems arising from non-specific binding (NSB) when working with clinically-relevant complex samples such as serum, urine, saliva, etc. This problem can be overcome by employing different strategies such as the use of optimized sample dilution solutions to hinder NSB [6–10] or employing sandwich assays that provide the necessary selectivity for detection [7,8,11,12]. Despite these promising features only one SPR biosensor involving a direct assay and the use of recombinant Protein G to assist in the orientation of a specific capture antibody has been reported for the determination of ErbB2 with a limit of detection (LOD) of 11 ng mL^{-1} [2]. However, weak molecular interactions and an uncommon negative SPR shift complicated the quantification.

In the present work we report the development of an SPR immunosensor for the quantification of ErbB2 protein using signal enhancement with GNPs. Assay parameters have been optimized and both human serum and raw cell lysate samples have been tested. The results proved that the simple and highly sensitive SPR immunosensor developed represents a feasible tool for real-time ErbB2 detection in these clinically relevant matrices.

2. Materials and methods

2.1. Reagents and solutions

Human ErbB2/HER2 protein standards (10004-HCCH), capture mouse monoclonal antibodies (10004-MM03) and detection biotinylated antibodies (10004-H08H) were purchased from Sino Biological (Beijing, China). Mouse Immunoglobulin G (MIgG, ref. 015-000-003) and bovine serum albumin (BSA, ref. 001-000-161) were from Jackson Immunoresearch Europe (Newmarket, UK). N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC), N-hydroxysuccinimide (NHS) and ethanolamine hydrochloride (ETA) were from Sierra Sensors GmbH (Hamburg, Germany). 11-mercaptoundecanoic acid (MUA) and Tween 20 were from Sigma–Aldrich (Tres Cantos, Spain). For the tests in serum, pooled human serum was purchased also from Sigma–Aldrich (ref. H4522). Streptavidin decorated 20 and 40 nm gold nanoparticles (SAv-GNP, refs. BA.STP20 and BA.STP40, respectively) were obtained from British Biocell International (Cardiff, UK). The SAv-GNPs were used as received from the supplier (with an OD of 10.2 at 520 nm in the stock solution for both particle sizes).

The following buffer solutions were freshly prepared: phosphate-buffered saline pH 7.3 containing 0.05% (w/v) Tween 20 (PBST) and PBST supplemented with 0.2% (w/v) BSA (PBST-BSA), which was employed as running buffer throughout all experiments. The low detergent concentration was present to hinder the sticking of bubbles in the microfluidic channels, while BSA protein carried out a continuous blocking of all the surfaces. All other chemicals were of analytical grade and ultrapure water (Millipore Milli-Q) was used throughout.

2.2. Instrumentation

A SPR2 biosensor instrument from Sierra Sensors GmbH (Hamburg, Germany) was used for all experiment. The system is equipped with a continuous flow microfluidic sample delivery, an automated sample processing unit and accurate temperature control. The two spots in the microfluidic cell can be individually addressed via hydrodynamic focusing. By means of immobilizing a non-specific protein (MIgG) on the first spot (reference channel) and the specific capture antibody on the second one, real-time referencing was carried out. For all the experiments the temperature and flow rate of the SPR system were set to 25 °C and 25 μL min^{-1}, respectively. Glass prisms with a 50 nm-thick gold layer, also provided by Sierra Sensors, were used as sensor chips.

2.3. Ligand immobilization

A self-assembled monolayer (SAM) was created over the gold-coated sensor chips by immersion in 2.5 mM MUA ethanolic solution during 12 h. The chips were then gently rinsed with ethanol and water and dried under a nitrogen stream. Modified chips were stored at 4 °C until use.

The biofunctionalization of the sensor chips was made by means of standard amine coupling with PBST running buffer. After docking a previously modified sensor chip in the SPR system, the carboxyl groups present in the MUA SAM were activated by a 200 μL injection of a 200 mM EDC and 50 mM NHS 1:1 mixture solution. Subsequently, 200 μL of a 20 μg mL^{-1} mouse monoclonal anti-ErbB2 solution (prepared in 10 mM pH 5.5 sodium acetate) were injected in the active spot and 200 μL of 20 μg mL^{-1} MIgG (prepared in 10 mM pH 6.0 sodium acetate solution) in the reference spot. In order to block all the remaining unreacted NHS-ester groups and

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avoid any undesired non-specific binding (NSB), a 200 μL injection of 1 M ETA solution (pH 8.5) was carried over the two functionalized spots.

2.4. Sample preparation

The ErbB2 standard solutions were prepared in the SPR system running buffer (PBST-BSA). The pooled human serum was diluted to 50% in a special solution prepared to hinder NSB which, after optimization, consisted of running buffer supplemented with 4 M NaCl and 0.1% (w/v) Tween 20.

MCF-7, MDA-MB-436 and SK-BR-3 breast cancer cell lines were grown according to established protocols in DMEM (Dulbecco's modified Eagle's medium), supplemented with 10% fetal bovine serum, penicillin and streptomycin, and 2.5 mM L-glutamine (GIBCO-Invitrogen, Carlsbad, CA, USA) [13]. To lyse the cells, they were washed with cold PBS, incubated for 5 min with PBS 4 mM EDTA to detach them from the plates, centrifuged at 1200 rpm to remove PBS 4 mM EDTA, and disrupted by the addition of 1 mL of cold lysis buffer (25 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with 1X protease inhibitor cocktail, 1 mM phenylmethylsulfonyl fluoride, and 1 mM activated sodium orthovanadate. Then, cells were incubated on ice for 10 min, passed through a 25 gauge needle attached on a 1 mL syringe 10 times and transferred to a microcentrifuge tube. The cell lysate was then clarified by centrifuging at 13,200 rpm at 4 °C for 15 min. Total protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL) and the lysates stored at −80 °C until further use. For the SPR experiments, the lysates were further diluted in the mentioned lysis buffer.

2.5. ErbB2 SPR immunosensor

Two different approaches were used in the SPR immunosensor: a direct-binding label-free assay and a sandwich format including GNP amplification. 200 μL (8 min contact time) of the samples were injected over both the active and reference channels (functionalized with anti-ErbB2 and MIgG, respectively). This gave rise to measurable signals down to a point where signal amplification was necessary. Enhancement was achieved by subsequent 75 μL injections of a 4 μg mL^{-1} biotinylated anti-ErbB2 and a 20 times diluted SAv-GNPs (40 nm) solutions, respectively.

Surface regeneration was successfully carried out with a 25 μL injection of a 100 mM HCl solution which virtually removed all the bound protein without damaging the immobilized antibody layer. The activity of the chip was checked to be intact during at least 300 cycles.

In all cases, a minimum of two replicates were carried out for each sample. The samples were injected in the system in randomized order to avoid potential memory effects. Blank samples were also injected in order to perform double referencing [14]. Data was analyzed using Tracedrawer software (v. 1.3, Ridgeview Instruments AB, Uppsala, Sweden).

3. Results and discussion

3.1. Sensor surface biofunctionalization

Unlike for biomolecular binding kinetic studies, for pure detection purposes surfaces with very high ligand densities are usually preferred to obtain optimum analyte capture capacities. Typically, carboxymethylated dextran hydrogel modified chips are employed in SPR systems because of 3D-like structure and high binding capacity. However, these chips do not perform as well when assaying in complex matrices such as serum [15]. In this work the chip gold surfaces were modified with MUA SAMs and standard amine protein coupling [5]. The protein preconcentration solutions were prepared in sodium acetate buffers because of its low ionic strength. To maximize their electrostatic preconcentration over the surface during injection, pH scouting optimization experiments on non-activated SAM surfaces were carried out in the pH 5–6 range for both antibodies (anti-ErbB2 and MIgG). The optimal solution pH for each one of the proteins was determined by measuring the slope of 15 μL injections at each pH and resulted in pH 5.5 and 6.0 for anti-ErbB2 and MIgG, respectively. High antibody concentrations (20 μg mL^{-1}) were employed to maximize surface coverage. As it can be seen in Fig. S1, the immobilization signal, saturated on both spots, was ca. 2200 RU.

For the rest of experiments, the running buffer was changed from PBST (used during the previous amine coupling routine) to PBST supplemented with BSA (see section 2.2). The fluidics of the SPR system was primed with this new buffer and the baseline stabilized in less than 2 min. The activity of the anti-ErbB2 antibodies was checked by repeatedly injecting 25 μL of 200 ng mL^{-1} ErbB2 standard solutions (prepared in running buffer) and regenerating the surface. Several regeneration solutions were tested (10–100 mM pH 1.5–3 glycine and 10–100 mM HCl solutions). The best results were obtained with 100 mM HCl, which demonstrated full regeneration of the sensor surface without reducing the binding activity of the immobilized antibodies during more than 400 regeneration cycles. This fact was checked by periodically performing the mentioned activity tests during the lifetime of the sensor chip (see Fig. S2).

3.2. Immunosensor optimization

A label-free direct-binding immunosensor is the simplest and most logic first approach in any SPR immunosensor development. With this objective, simple 8 min-long (200 μL) injections of ErbB2 standard solutions with concentrations ranging from 500 down to 6.2 ng mL^{-1} (3-fold dilutions) and blank samples were carried out. Fig. 1a shows the obtained sensorgrams which display a strongly mass transport limited binding which only faded at the highest concentration. This proves the high binding capacity of the prepared chip surface and its suitability for determination experiments. In Fig. 1b the calibration curves obtained from the end-of-injection signal and from the initial slope of the binding (first 15 s) are shown. As expected, the behavior is highly linear. A LOD of 3.8 ng mL^{-1} was calculated as the concentration corresponding to a signal of 3 times the standard deviation of 3 blank samples. From the sensorgrams displayed in Fig. 1a it can be clearly stated that longer contact times would give rise to improved LODs, however a sample volume of 200 μL was considered as a top limit due to practical reasons of sample availability.

The obtained LOD is well below the clinical cut-off value of 15 ng mL^{-1} of this biomarker in human serum [16] so we proceeded to perform assays in real human serum. The use of pure or even diluted serum in label-free immunosensors is a difficult task because of the complex nature of the samples and the consequent high NSB. According to the literature, the use of special NSB hindering buffers has proved effective with SPR biosensors (see, e.g., [6–9,17]) For the ErbB2 immunosensor, we optimized a special solution using a cocktail approach [18] with varying concentrations of Tween 20 detergent, NaCl and BSA. The largest effect was attributed to the NaCl content that reduced the electrostatic interaction between proteins in the serum and the sensor surface. A solution of 4 M NaCl, 0.1% Tween 20 in PBS offered the best results,

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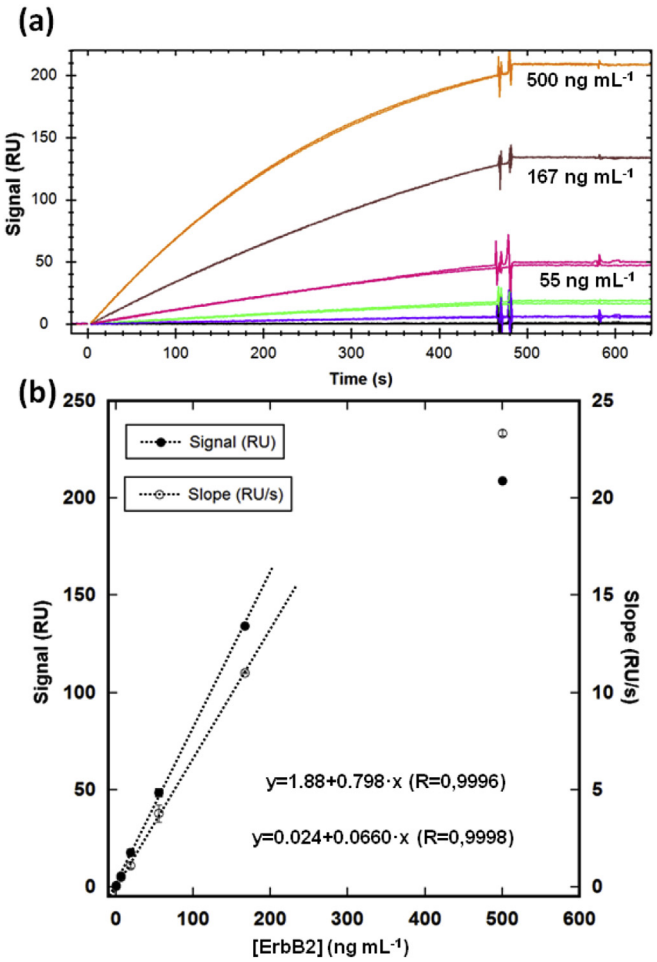

Fig. 1. a) Direct ErbB2 binding sensorgrams 8 min-long (200 μL) injections of ErbB2 standards with concentrations ranging from 500 down to 6.2 ng mL^{-1} (3-fold dilutions) and blank samples. The sensor surface was regenerated after each cycle with a 25 μL injection of 100 mM HCl. The sensorgrams have been double-referenced. b) Calibration curves obtained from the end-of-injection signal (t = 500 s) and from the initial slope of the binding (first 15 s). The linear fits have been obtained excluding the highest concentration point which suffered from surface saturation. Error bars are estimated as a triple of the standard deviation (n = 3). RU, response units.

with a ca. 85% reduction of the NSB. Using this special buffer, a qualitative detection of the ErbB2 analyte at very high concentrations (~>20 μg mL^{-1}) was observed when comparing the signals from the active and reference sensor spots. Unfortunately, quantification was not possible due to the non-negligible high remnant NSB signal.

In order to enhance the sensitivity of the assay in human serum a sandwich immunosensor was optimized utilizing a secondary anti-ErbB2 (biotinylated) antibody and SAv-GNPs. Although the label-free character of the assay was lost, this approach is expected to enhance the selectivity (due to sequential specific binding of high affinity antibodies and SAv-GNPs) as well as the sensitivity (due to signal amplification provided by the antibodies and the bulky GNP).

Optimization of the required steps to complete the sandwich assay was carried out. Fig. S3 shows the signal stemming from assays carried out at increasing concentrations of the secondary antibody (from 2 up to 16 μg mL^{-1}) with a fixed 4 ng mL^{-1} antigen concentration. As it can be seen, the largest increase occurs between 2 and 4 μg mL^{-1}, with a close to linear behavior at larger concentrations. It has to be mentioned that these secondary antibodies are the most expensive consumable of the assay and a compromise between cost and performance had to be reached. Taking this into account, injections of biotinylated anti-ErbB2 were carried out at 4 μg mL^{-1} (75 μL). The size and concentration of the SAv-GNP were also optimized. Fig. S4 shows the signals stemming from two folds dilutions of the 20 and 40 nm SAv-GNP stock solutions (from 1:5 down to 1:40). As it can be seen, the signals for both the 20 and 40 nm diameters saturated at a 1:20 dilution ratio while the signal of the largest nanoparticles (40 nm) doubled the signal of the 20 nm nanoparticles. In the view of this results, for GNP signal enhancement the 40 nm SAv-GNP were used.

Fig. 2a shows whole sensorgrams for the developed double sandwich amplification immunosensor in ErbB2 standard solutions of three different concentrations (0, 0.68 and 6.11 ng mL^{-1}). As it can be seen, at the highest concentration, the signal after the analyte injection was measurable and, for detection, amplification was not necessary (curve blue in Fig. 2a). However, when the analyte concentration was lowered to 0.68 ng mL^{-1}, the direct binding signal did not stand out of baseline (curve pink in Fig. 2a), making the following two amplification steps necessary (with secondary antibodies and GNP). The sensorgram at null analyte concentration proved the high specificity of the sandwich format. A full calibration curve in the 0.23–55 ng mL^{-1} concentration range was obtained as shown in Fig. 2b. It is worth noting that the binding information is in fact contained in the signal arising from the last amplification injection with GNPs which are logically proportional to the amount of bound ErbB2. This allows for quantification even in cases when there is non-negligible NSB during the sample injection. The sensorgrams for the GNP signals were referenced and are shown in Fig. S5. From these signals, a LOD of 180 pg mL^{-1} was calculated. This value is 20 times lower than that of the direct binding assay proving the high effectiveness of the amplification steps. A fit to a simple 1:1 biomolecular binding model was also carried with an effective equilibrium constant $K_D = 260 \pm 20$ pM which confirmed the high affinity of the employed antibodies.

At this point, it is worth comparing the results of the direct binding and the GNP-amplified assays. In Fig. 3, a comparison of the performance of both formats (at the concentrations where the direct binding assay gave measurable signals) is shown as well as data of the amplification factor provided by the GNPs amplification-based strategy. As it can be seen, the amplification factor increases with decreasing analyte concentrations (up to more than 10 at 6.11 ng mL^{-1}). Below this concentration a direct comparison cannot be established, however, as mentioned above, the LOD was reduced 20 times which could give an idea of signal enhancement achieved. It is also interesting to mention that in the absence of ErbB2, no signal was measured even after the amplification steps, which demonstrated the total absence of NSB in the enhanced assay.

Compared with the only SRP biosensor reported so far for ErbB2 determination [2], the LOD is about 3 and 60 times lower using the direct and the SAv-GNP-based sandwich strategies developed in this work (3.8 ng mL^{-1} and 190 pg mL^{-1}, respectively, *vs.* 11 ng mL^{-1}). This better sensitivity is particularly relevant when working with valuable but scarce biological samples.

3.3. Serum samples

The developed SPR ErbB2 sandwich immunosensor was applied to the determination of ErbB2 in spiked human serum samples using the special sample dilution buffer mentioned above. The samples were diluted to 50% in this buffer and injected in the SPR system (200 μL aliquots). The sensorgrams obtained for the GNP

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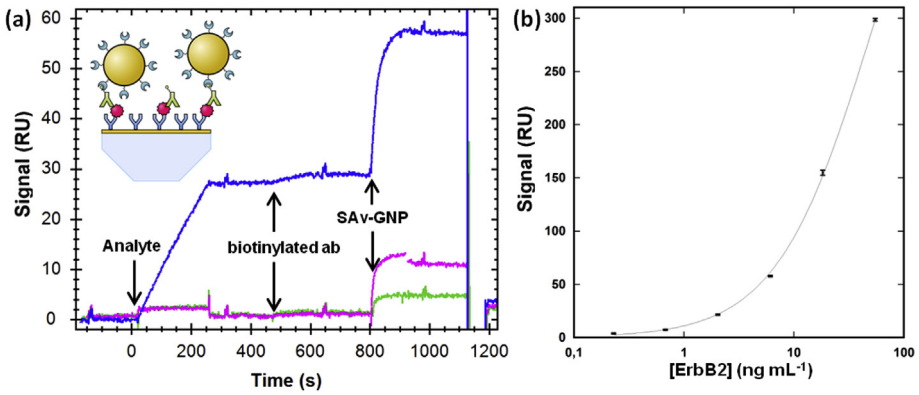

Fig. 2. a) Sensorgrams obtained using the double sandwich amplification immunosensor for three different standard ErbB2 solutions (0, 0.68 and 6.11 ng mL^{-1}) prepared in buffer. The inset shows a sketch of the GNPs-amplified sandwich immunoassay on the surface of the gold chip. b) Calibration curve obtained for ErbB2 standard solutions in the 0.23–55 ng mL^{-1} concentration range and fit to a 1:1 molecular interaction model. Error bars are estimated as a triple of the standard deviation (n = 3). RU, response units.

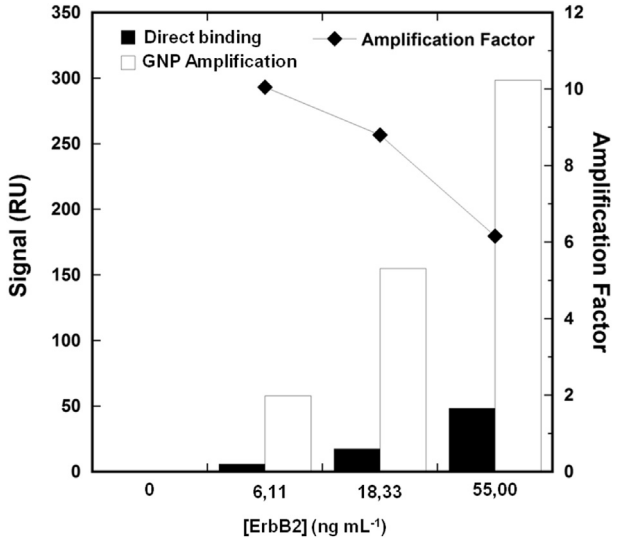

Fig. 3. Comparison of the performance of the direct binding and sandwich assays (enhanced with SAv-GNPs). The amplification factor (*i.e.* signal/blank ratio) is also shown at each concentration, except at null ErbB2 concentration.

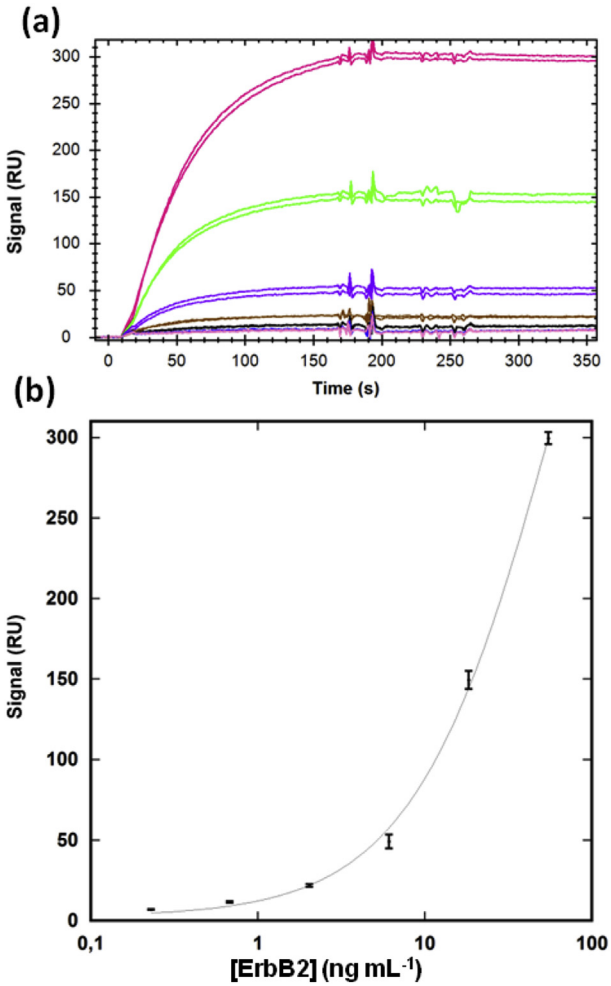

Fig. 4. a) Double-referenced sensorgrams obtained for the SAv-GNP amplification step with the sandwich immunosensor format for non-spiked and spiked human serum samples (from 55 down to 0.23 ng mL^{-1} ErbB2, 3-fold dilutions). b) Calibration curve obtained for 1:1 human serum samples spiked with increasing concentrations of ErbB2 and fit to a 1:1 molecular interaction model. Error bars are estimated as a triple of the standard deviation (n = 3). RU, response units.

amplification step with duplicates of decreasing concentrations of ErbB2 (from 55 down to 0.23 ng mL^{-1}) are shown in Fig. 4a. As it can be seen, non-spiked serum samples resulted in null signals which demonstrated the high selectivity of the developed assay. Again, using only the signal stemming from the last amplification step with SAv-GNPs, the calibration curve of Fig. 4b was constructed. A LOD of 180 pg mL^{-1} was obtained, which is highly similar to that obtained with standard solutions. As before, a fit to 1:1 biomolecular binding model was carried out with a resulting effective equilibrium constant $K_D = 340 \pm 70$ pM. Comparing these values in human serum with the ones obtained in standard solutions, it can be concluded that the matrix effects have been virtually removed. It has to be taken into account that, since a 50% dilution ratio was employed, the effective LOD for the serum samples is 360 pg mL^{-1}, still 42 times lower than the commonly accepted clinical cut-off value (15 ng mL^{-1}).

3.4. Cell lysate samples

The good results obtained in serum motivated us to test the performance of the developed methodology in relevant raw cell lysates. With this objective, three different metastatic breast cancer

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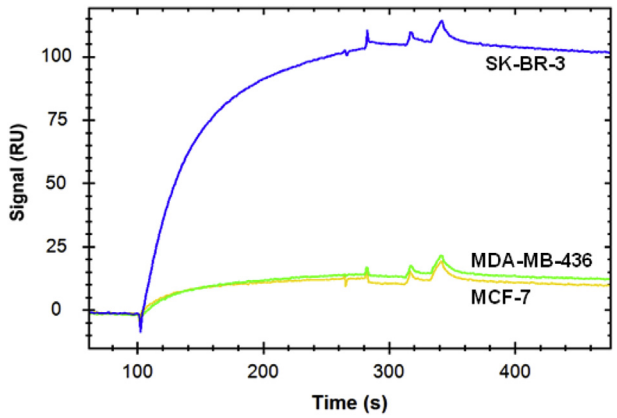

Fig. 5. Sensorgrams obtained for the SAv-GNP amplification step in the sandwich-type immunosensor format for different raw breast cancer cell lysate samples (at a final amount of 40 μg). RU, response units.

cell lines expressing varying levels of ErbB2 were selected: MDA-MB-436, SK-BR-3 and MCF-7. While MDA-MB-436 and MCF-7 breast carcinoma cell lines contain typical number of copies of the ErbB2 gene, SK-BR-3 cell line contains approximately 10-fold more copies of this gene, leading to a higher production of the ErbB2 protein [19].

To perform these experiments, 40 μg of each raw lysate were diluted to a total volume of 200 μL in lysis buffer and injected in triplicates over the functionalized gold chip. Fig. 5 shows the results obtained for the sandwich immunosensor (only SAv-GNP step shown). As it can be seen, ErbB2 protein was highly expressed in SK-BR-3 cells when compared to MCF-7 and MDA-MB-436 cells. The NSB binding of the raw cell lysates to the surface was low and, in any case, the matrix effect was overcome by the expected improved selectivity and demonstrated signal enhancement of the sandwich immunosensor. A calibration curve was simultaneously constructed with ErbB2 standard solutions (prepared in lysis buffer) to quantify the ErbB2 content of the lysates. The ErbB2 content of the positive SK-BR-3 cell line was estimated in 230 ± 10 ng μg^{-1} (ng of ErbB2 per μg of lysate). For the MCF-7 and MDA-MB-436 lysates the content, in both cases, was of 30 ± 10 ng μg^{-1}. These results are in the same order of magnitude than those determined by using an ErbB2 amperometric sandwich magnetoimmunosensor [13] and proved its potential applicability for ErbB2 testing not only in serum but also in other clinically relevant samples.

4. Conclusions

A sensitive and effective SPR approach that allows real-time biosensing of low concentrations of ErbB2 in serum samples and raw cell lysates has been presented. The combination of a sandwich format with the amplification power of GNPs significantly enhanced the sensitivity of the proposed immunosensor, which to the best of our knowledge is the most sensitive SPR-based biosensor for ErbB2 determination reported so far with a LOD of 180 pg mL^{-1} in 50% human serum. The results showed that the method is simple and sensitive enough for the determination of ErbB2 in serum samples (far below the clinical cut-off) and raw cell lysates from breast cancer cells with good reproducibility and accuracy.

This SPR-based immunosensor provides a convenient, sensitive and specific method for protein detection and represents a potential versatile tool for highly sensitive clinical analysis and other biotechnology applications and a viable alternative for use in clinical laboratories.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.aca.2015.12.020.

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