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Surface plasmon resonance immunoassay for the detection of the TNFα biomarker in human serum

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ABSTRACT

A simple method for the detection of TNF-alpha protein biomarker in human serum with great sensitivity has been developed using a surface plasmon resonance biosensor. Signal amplification based on a sandwich immunoassay including gold nanoparticles was used. Detection in serum proved to be challenging due to high undesirable non-specific binding to the sensor surface stemming from the matrix nature of the sample. After optimization of the assay parameters and, in the case of serum, of a sample dilution buffer to minimize the non-specific binding, very low limits of detection were achieved: 11.6 pg/mL (211 fM) and 54.4 pg/mL (989 fM) for spiked buffer and human serum respectively. The amplification steps with high affinity biotinylated antibodies and streptavidin-fuctionalized nanoparticles greatly enhanced the signal with the advantage of additional specificity. Due to its simplicity and sensitivity, the immunoassay has proved feasible to be used for detection of low concentration biomarkers in real samples.

1. Introduction

Since the first commercial devices were made available two decades ago, biosensors based on surface plasmon resonance (SPR) have become a common tool in many laboratories. SPR is an optical non-destructive method than can detect very small changes in the refractive index (RI) on a typically gold sensor surface. With adequate surface biofunctionalization, the binding of a given analyte results in a change of the RI on the surface which, in turn, is measured as a change in the SPR angle or reflected light intensity. This technique allows to measure molecular interactions in a real-time and label-free fashion and it is being widely used to extract affinity and kinetic constants of, for example, protein–protein, DNA–DNA or protein–DNA interactions [1,2].

Despite the high sensitivity offered by SPR biosensors, their use for the determination of concentrations of analytes is much less extended. The usual necessity of assaying in clinically relevant matrices such as blood, serum or saliva adds the difficulty of large uncontrolled background signals due to non-specific binding (NSB). This can mask the analyte signal, therefore, reducing the sensitivity and resulting in inadequate limits of detection (LOD) or false readings. This effect is especially important when the analyte is a small molecule and/or its concentration is low. NBS can be minimized using surface blocking agents such as protein-resistant self-assembled monolayers (SAMs) [3–6], polymers [7] or, more commonly, non-specific proteins such as bovine serum albumin (BSA). Additives in the buffer have also been successfully used in several works (e.g. [8–11]) to reduce protein NBS on gold surfaces.

Many relevant protein biomarkers are present at very low levels in serum or other physiological fluids and, if a SPR biosensor is to be used, signal enhancement is necessary for their detection. Different approaches involve amplification steps using sandwich assays which produce a signal dependent on the initial analyte concentration. Despite the loss of the real-time and label-free format that SPR boasts, the high sensitivity and ease of use that this type of optical biosensors offer can still be a great asset in assay development. In the case of immunoassays, the simplest option is the use of specific secondary antibodies [4,10,12,13]. A slightly more complex approach involves functionalized nanoparticles (NP), which due to their great mass, can amplify the initial signal further. Due to their widespread use in other fields, gold NPs have been applied in a variety of works, e.g. [11,14–19]. Nonetheless, the use of latex particles [20], magnetic NP [21,22] or quantum dots [23] has also been reported. In exchange of the loss of the label-free format, amplification steps can provide, in many cases, the necessary sensitivity and specificity to work with analytes present at low concentrations in complex matrices in which NSB and signal noise make direct detection impossible.

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Tumor necrosis factor-α (TNFα) is a well-known pleitropic pro-inflammatory cytokine produced by activated macrophages that acts as a mediator in many immune and inflammatory processes [24]. The biologically active TNFα molecule is a 55 kDa homo-trimer found in serum at very low concentrations (in the pg/mL range) and at elevated levels when an inflammatory cascade is present. Assaying TNFα in serum is interesting for many immunological studies. For instance, the binding inhibition of circulating TNFα to its receptors by biological drugs has been seen to alleviate the symptoms of certain diseases such as rheumatoid arthritis or Crohn's disease [25]. In both clinical investigations and research, the concentration of TNFα in serum is measured using conventional enzyme-linked immunosorbent assays (ELISA) which can be costly and slow.

Different biosensor systems have been used for assaying TNFα. For example, Qureshi et al. used a capacitive biosensor obtaining a LOD of 25 pg/mL in buffer [26]; Yin et al. reached, also in buffer, a LOD of 10 pg/mL with an electrochemical immunosensor based on alkaline phosphatase functionalized nanospheres and proved good performance in human serum but did not estimate the LOD [27]; Liu et al. employed electrochemical aptasensor obtaining a LOD of 10,000 pg/mL in whole blood [28]; and Bettazi et al. reported a LOD of 44 pg/mL in a serum-like buffer employing the reagents of an ELISA kit in a magnetoimmunoassay [29]. Bahk et al. used a quartz crystal microbalance and magnetic field amplification to reach a LOD of 25,000 pg/ml in buffer [30]. Using SPR technology and gold nanorods for plasmonic enhancement, Law et al. reported a LOD for TNFα in buffer of 500 pg/mL but did not carry out assays in serum [19]. By means of SPR as well, Battaglia et al. reached a LOD of 770 pg/mL in a cell culture medium [31]. All these reports have been summarized and compared to the present work in Table S1. Due to the clear importance of TNFα in many immunological studies, this protein was chosen as an analyte in this research.

Apart from the TNFα protein, LODs reported for clinical or complex samples using SPR technology are usually in the ng/mL range. For example, Treviño et al. reported a 6 ng/mL LOD for a human growth hormone binding inhibition immunoassay in serum [32]; in this same medium, down to 28 ng/mL of ferritin was detected using a sandwich method by Cui et al. [12]; Chou et al. assayed the interleukin-6 cytokine in cell culture supernatant with a c. 4 ng/mL LOD [4]; and Uludag et al. assayed the total prostate-specific antigen (tPSA) in human serum samples using nanoparticle signal amplification and an optimized matrix buffer down to 390 pg/mL [11]. However, relevant concentrations for many biomarkers, such as the TNFα protein, are well below these figures.

In the present work, a simple yet very sensitive immunoassay using gold nanoparticles for signal amplification in a SPR biosensor system has been developed. Optimization of the immunoassay parameters has been carried out with the aim of maximizing the signal and minimizing NSB to obtain relevant LODs in human serum. As a result, the inflammation TNFα protein biomarker has been assayed in both spiked buffer and human serum at clinically relevant levels in the pM–fM range.

2. Material and methods

2.1. Reagents and solutions

Recombinant Human TNFα, monoclonal anti-human TNFα (anti-TNFα) and biotinylated rabbit polyclonal anti-human TNFα antibodies were purchased from Peprotech Inc. (London, UK). Mouse Immunoglobulin G (Mouse IgG) and bovine serum albumin (BSA) were obtained from Jackson Immunoresearch Europe (London, UK). 20 and 40 nm gold nanoparticles functionalized with streptavidin (SAv-GNP) were from British Biocell International (Cardiff, UK). N-hydroxysuccinimide (NHS), ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and ethanolamine HCl pH 8.5 were from Sierra Sensors GmbH (Hamburg, Germany). 11-Mercaptoundecanoic acid (MUA), Dulbecco's phosphate-buffered saline (PBS) and Tween 20 were from Sigma-Aldrich (Tres Cantos, Spain). All other chemicals were of analytical grade. The Biodonostia Hospital (San Sebastian, Spain) provided the pooled human serum used in this study.

2.2. Instrumentation

A SPR2 biosensor system and gold-coated sensor chips were from Sierra Sensors GmbH (Hamburg, Germany). The instrument makes use of SPR imaging technology to measure the absorbed mass over two independently addressable spots inside a microfluidic cell. One spot is used as active spot for specific protein capturing and the other is used as real-time reference. It boasts an autosampler to automate the measurements, temperature control (which was set at 25 °C throughout) and flow rate control (25 μL/min unless otherwise stated).

2.3. Ligand immobilization

A SAM was created over the gold sensor chips by overnight immersion in a 2.5 mM ethanol solution of MUA. The chips were then rinsed thoroughly with ethanol and water, dried with N_2 and stored at 4 °C until use.

The chips were docked in the system to perform the biofunctionalization via the protein primary amines. The running buffer was phosphate buffer saline pH 7.3 added with 0.01% Tween 20 (PBST). 200 μL of a 1:1 mixture of EDC 200 mM and NHS 50 mM was injected over the two addressable sensor spots to activate the carboxyl residues in the SAM. 300 μL of anti-TNFα 20 μg/mL (pH 5.5) and Mouse IgG 20 μg/mL (pH 6) and 10 mM sodium acetate solutions were injected over what will be referred as the active and reference spots respectively to covalently attach the antibodies via their primary amines. These solutions were prepared in the sodium acetate buffer because of its low ionic strength and injected at 50 μL/min to maximize the amount of immobilized protein. The pH for each solution was chosen after a pH scouting experiment to enhance the electrostatic preconcentration of the protein over the surface [2]. Finally, a 200 μL injection of 1 M ethanolamine hydrochloride pH 8.5 was performed to block all the remaining unreacted NHS-ester groups. The saturated immobilization level on both spots was c. 2200 RU (see Fig. S1) and the stability were proved to be very good.

2.4. Sample preparation

PBST added with 0.02% of BSA (PBST–BSA) was used for the TNFα spiked buffer samples. Pooled human serum was obtained by allowing the blood to clot at room temperature for 30 min and centrifuging at 1500*g* for 15 min. Negligible concentration of TNFα (below 5 pg/mL) in the pooled serum samples was confirmed using an enzyme immunoassay (Immunotech SAS, Marseille, France). Storage prior to use was done at −80 °C. For the spiked serum samples, a sample dilution buffer with different additives was optimized to prevent non-specific binding to the sensor surface (see Section 3.3). Spiking and dilution of the serum samples were carried out in this special solution. In this study, because sample dilution is a work-around due to the NSB, the concentrations shown for the diluted serum samples always correspond to the original undiluted sample concentrations.

2.5. SPR TNFα immunoassay

200 μL of the sample solutions was injected over the active and reference spots for TNFα binding. At low concentrations, the amount of mass captured during the 8 min-long injection was not enough to provide a measurable signal and amplification was necessary. Biotinylated rabbit anti-TNFα was immediately injected as a secondary antibody (75 μL). For further amplification, 100 μL of SAv-GNP was injected for the double-sandwich assay. The SAv-GNPs were 20-fold diluted in running buffer but otherwise was used as received from the manufacturer (OD 10.2 at 520 nm). The signal was extracted as a 10 s average after the SAv-GNP injection on both spots. The effective signals were taken as the difference between the active and reference signals.

A surface regeneration scouting study was performed and it was concluded that the surface was best regenerated with a 25 μL injection of 100 mM pH 1.8 glycine–HCl solution, which removed the bound analyte and maintained the ligand activity intact. In fact, the chip stability was checked periodically throughout the life-span of the chip with known positive TNFα spiked samples. Prepared chips showed no decrease in activity after more than 400 regeneration cycles.

When obtaining the calibration curves, duplicates of each sample were tested in a random order to ensure reproducibility and to avoid memory effects. Samples with null concentrations were also prepared and run for double referencing [33]. Data was processed and analyzed using the Tracedrawer software (Ridgeview Instruments AB, Uppsala, Sweden).

3. Results and discussion

3.1. Sensor surface biofunctionalization

For protein concentration determination purposes, surfaces with maximum protein binding capacity are desired. In this work high density ligand surfaces were achieved using gold surfaces modified with MUA SAMs and standard amine protein coupling [2]. Sodium acetate buffer pH and concentration optimizations were performed for efficient surface preconcentration of the anti-TNFα and Mouse IgG antibodies on the surface during the coupling process [2]. Concentrations higher than 20 μg/mL did not result in higher immobilization levels at the optimum pHs (5.5 and 6) and saturation was reached for both the active and reference spots at c. 2200 RUs (Fig. S1). Higher densities can be achieved using carboxymethylated dextran hydrogel modified surfaces, but these tend to show larger NSB than carboxylated SAMs when working with complex matrices like blood or serum [6].

After the protein immobilization, the system was primed with what was to be the running buffer throughout all the experiments, PBST–BSA, and the baseline completely stabilized after two regeneration solution injections. The activity of anti-TNFα antibodies and the NSB of TNFα to the reference spot were checked carrying out injections of TNFα. The binding signal of TNFα to the reference spot was null and the activity of the monoclonal anti-TNFα was proved to be good. After each binding experiment, the surface was completely regenerated using a 25 μL injection of 100 mM pH 1.8 glycine–HCl solution. The activity of the surface was checked periodically with known positive TNFα spiked buffer samples (25 μL at 500 ng/mL), and no signal decrease was observed after more than 400 cycles.

The affinity of anti-TNFα antibodies was checked calculating the kinetic constants (see Fig. S2) using a low density surface (150 RU immobilization level) and a global fitting to a 1:1 binding model. A rather low K_D of 76 pM (see the inset in Fig. S2) confirmed a very high affinity and the suitability of the monoclonal anti-TNFα antibodies to perform concentration assays.

3.2. Immunoassay optimization

A direct binding immunoassay was run on the prepared surface with 4 min-long (100 μL) injections of TNFα in running buffer solutions with concentrations ranging from 333 to 0 ng/mL (3-fold dilutions). The sensorgrams are shown in Fig. 1a where it can be seen how at the lowest concentrations the binding is linear, as expected due to mass transport limited interactions. This effect is due to the high binding capacity of the surface, which maximizes the capture of TNFα. The calibration curve extracted from the data is shown in Fig. 1b. A LOD of 1.2 ng/mL (21.8 pM) was calculated as the mean signal of 3 blank injections plus 3 times the standard deviation. This LOD could be slightly improved with longer contact times; however, from the practical point of view, it has to be taken into account that volumes of real samples are limited as well as that assaying time should also be minimized.

As mentioned, the clinically relevant concentration range of the TNFα biomarker lies well below this LOD so a sandwich immunoassay using secondary rabbit anti-TNFα antibodies was employed to further amplify the TNFα binding signal. Using this strategy a 10-fold signal enhancement was achieved, which resulted in a LOD of around 54.5 pg/mL (see Fig. S3 and the discussion below). For further amplification, a double sandwich immunoassay using biotinylated rabbit anti-TNFα antibodies and SAv-GNPs was designed to improve the sensitivity.

The inset of Fig. 2a shows a schematic representation of the TNFα double sandwich immunoassay. The use of modified gold colloids in optical biosensors has been reported in the literatures [11,14–19] and GNP size-dependence of the signal enhancement has been studied. Uludag et at. reported a direct correlation of the

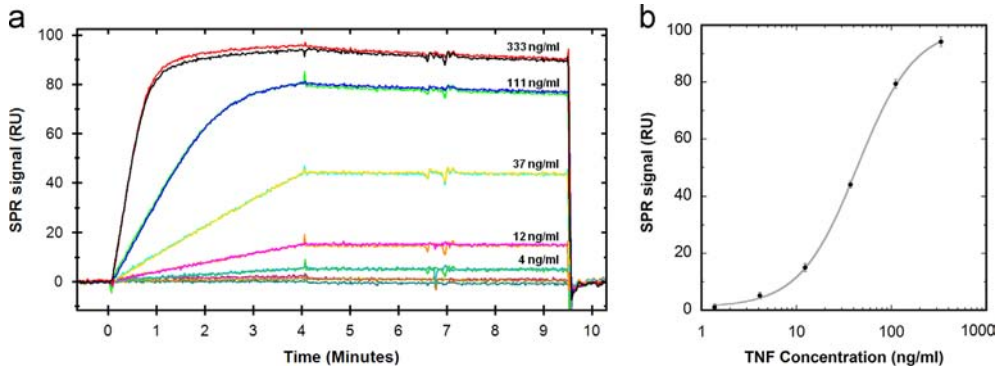

Fig. 1. (a) Direct TNFα binding sensorgrams. 100 μL duplicates of TNFα spiked buffer with concentrations ranging from 333 ng/mL down to 1.4 ng/mL and running buffer blanks were injected. The sensor surface was, after every concentration cycle, regenerated with a 1 min injection of 100 mM glycine–HCl pH 1.8. The sensorgrams shown have been double referenced. (b) Calibration curve obtained for TNFα binding using a label-free real-time detection SPR immunoassay. The calculated LOD was 1.2 ng/mL.

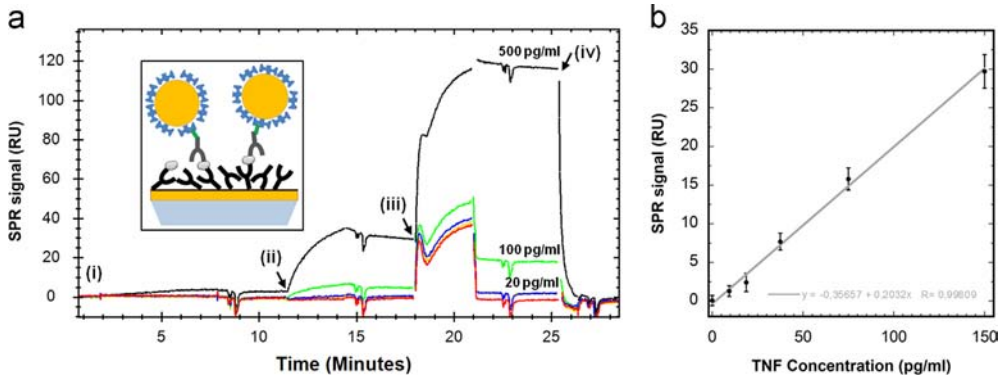

Fig. 2. (a) Sensorgrams obtained for the TNFα double sandwich immunoassay using biotinylated antibodies and SAv-GNPs for signal amplification in spiked buffer (scheme shown in inset). (i) 200 μL duplicates of each concentration were injected and the signal was sequentially amplified with (ii) biotinylated rabbit anti-TNFα (20 μg/mL and 100 μL) and (iii) SAv-GNPs (75 μL). After each cycle, the surface was regenerated with a (iv) glycine–HCl injection (100 mM, pH 1.8, and 25 μL). The sensorgrams are referenced to the reference spot of the system. Bulk shifts present in the injection (iii) were due to the high refractive index of the GNPs solutions and non-perfect referencing. The start and end of injection spikes have been removed for the sake of clarity. (b) Calibration curve obtained for TNFα double sandwich immunoassay. The calculated LOD was 11.6 pg/mL.

GNP volume to the amplification with 20 and 40 nm GNPs [11], thus attributing signal change to a bulk size effect. Previously, Mitchell et al. had found no amplification size-dependence with colloids ranging from 25 to 50 nm [17]. For the present study, the effect of GNPs with 20 and 40 nm diameters was assessed and no size-dependent signal enhancement was observed. In the light of this result, in this study 20 nm GNPs were used for the immunoassay to avoid possible steric hindrances.

Optimization of contact times and concentrations of the two amplifications steps (rabbit anti-TNFα and SAv-GNPs) was carried out. In the case of the biotinylated polyclonal antibodies, a compromise between cost and sensitivity had to be found and the injection was performed with 75 μL at 15 μg/mL. The final amplification step with SAv-GNPs was performed during 4 min (100 μL). Fig. 2a shows the whole sensorgrams for different TNFα sample concentrations, including the final regeneration steps with 100 mM glycine–HCl pH 1.8. The NSB of both rabbit anti-TNFα antibodies and SAv-GNPs to the active and reference spots was almost null as can be seen in the blank injections. This double sandwich immunoassay resulted in a LOD of 11.6 pg/mL (211 fM) with a wide linear detection range (see calibration curve in Fig. 2b). This concentration is, to the best of our knowledge, the lowest LOD obtained for TNFα protein using an SPR biosensor and actually clinically relevant to the case of the TNFα biomarker [25]. Thus, the developed immunoassay was considered a good starting point to check the detection performance in real serum.

We consider that it is worth carrying out an estimation of the amplification factors reached after each enhancement step. To do so, the sensorgram at 500 pg/mL of Fig. 2a, where the signals for all steps, including the sample injection, are measurable was chosen (see Fig S4). The amplification factor of the secondary antibodies with respect to the initial sample signal was 10.5. Sequentially, SAv-GNPs further enhanced this signal by a 3.1 factor. The accumulation of the two amplification steps resulted in an effective signal amplification factor of 45. In contrast to this figure, the LOD was reduced by a factor of 103 (1.2 ng/mL for the direct assay vs. 11.6 pg/mL for the GNP amplified assay). This difference can be attributed to the fact that sequential specific binding of high affinity biotinylated antibodies and SAv-GNP did not only amplify the initial signal, but also the specificity of the assay. This can be of great importance in the presence of NSB as it was found while assaying real samples.

3.3. Serum samples and NSB

Serum is a complex matrix which is known to have a negative effect on the LODs due to large NSB to sensor surfaces. In our case, running the assay in pure serum made it useless and even diluting it in running buffer down to 10% resulted in very large LODs. For this reason, we optimized a buffer containing complements to hinder this undesirable effect. This same approach has been used previously in SPR serum assays. Treviño el al. optimized a PBS based solution added with 500 mM NaCl and 0.1% Tween 20 at pH 8 for human growth hormone detection in 50% serum obtaining an 88% NSB reduction [32]; Situ et al. used a NSB buffer based on HEPES added with 500 mM NaCl and 0.005% carboxymethylated dextran at pH 9 for assaying in bovine serum [8]; Ayela et al. added 0.5% sarkosyl surfactant to a HEPES based buffer in 1% human serum for the detection of IA-1 autoantibodies [9]; and Uludag et al. prepared a special PBS buffer solution with 200 μg/mL BSA, 500 mM NaCl, 500 μg/mL dextran and 0.5% Tween 20 for assaying tPSA in 75% human serum and reported a 98% NSB reduction [11].

In the present work, the sample dilution buffer was optimized and eventually found to be consisted of a cocktail of 1 M NaCl, 0.5% BSA and 2% Tween 20 in PBS pH 7.3. The use of higher salt concentrations in the buffer reduces the electrostatic interaction between proteins in the serum and the surface. In the same way, detergents such as the Tween 20 can hinder the NSB by disrupting ionic and hydrophobic protein–surface interactions. The NSB was virtually removed in 50% serum with only a small remnant signal of around 40 RU (see gray sensorgram in Fig. 3a), which corresponds to a 97% reduction of the NSB when compared to a NSB signal of c. 1300 RU measured after the injection of 50% serum in PBST–BSA (dark sensorgram in Fig. 3a). The large bulk shifts (c. 4000 RUs) during the injections are mainly due to increased RI of the samples and, unfortunately, completely mask the real-time monitoring of any binding process. However, a subsequent injection of specific secondary antibodies can quantify the antigen captured by the primary antibody immobilized on the surface with the additional advantage of enhanced specificity.

It is of upmost importance that the activity and affinity of the TNFα protein and capture antibodies are maintained in all the buffer solutions. Using a direct capture approach the activity of TNFα at 50 ng/mL in PBST–BSA and in 25% serum dissolved in the prepared sample dilution buffer was compared (see Fig. 3b). The serum injection left a small constant remnant signal of 12 RUs that compares very well with the increased signal of the positive sample when pondered with injections in PBST–BSA. Therefore, it was concluded that the activity of the proteins and affinity of the interaction were unaltered in the prepared sample buffer.

3.4. Detection in human serum

Using the prepared sample dilution buffer, an immunoassay in 25% spiked human serum was carried out. The sensorgrams of

Fig. 4a show duplicates of serum positive (300 pg/mL) and negative samples. Although a small amount of NSB was present after the serum injections (see Fig. 3b), the subsequent sandwich injections clearly enhanced the specificity and null signals were obtained for negative serum samples. As mentioned in Section 3.2, the specificity, as well as the sensitivity, was also enhanced in the signal amplification steps. Using only the signal stemming from the last amplification step with SAv-GNPs, which is proportional to the amount of bound TNFα, the calibration curve of Fig. 4b was constructed. As it can be seen, the response is linear with the concentration and a LOD of 54.4 pg/mL (989 fM) was calculated using the aforementioned criterion.

It has to be taken into account that the serum was diluted in the sample dilution buffer down to 25%, therefore, so was the TNFα concentration presented to the biosensor surface. As a consequence, the assayed solution concentration was a four-fold dilution of that of the sample. In order to assess the serum matrix effect, the 54.4 pg/mL LOD obtained in 25% serum corresponds to an effective LOD of 13.6 pg/mL on the whole sample, which compares very well with the 11.6 pg/mL obtained for spiked buffer. This fact clearly shows that the matrix effect has been almost completely hindered and that very low analyte concentrations are subject of being assayed even in real human serum.

It is worth mentioning that apart from the relevant LOD achieved in serum, the characteristics of the developed immunoassay such as its simplicity, short sample assaying time, very good surface regeneration and longevity of the prepared chips can make it cost-effective and a good contender for real clinical application. Interestingly, it can be expected that the present immunoassay can be readily applied to other protein biomarkers. The main requirements are that high-affinity antibodies for the target biomarker are available and that the binding activity is maintained in the special buffer solution so that the NSB can be effectively hindered. If so, the approach presented in this work which makes use of SAv-GNPs for signal (and specificity) enhancement could be extended and applied for the detection in serum of a wide variety of biomarkers at low concentrations in a simple way. In addition, the format presented here might be feasible for extrapolation to DNA-assays or be used with other biosensing techniques, such as electrochemical, optical or piezoelectric (see e.g., [11]).

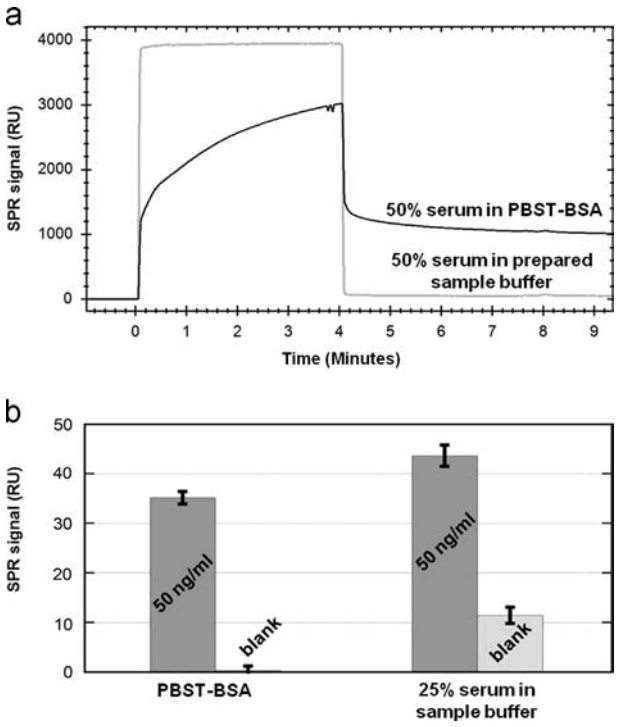

Fig. 3. (a) NSB of serum to the chip surface. The dark sensorgram represents a 100 μL injection of 50% human serum diluted in PBST–BSA which, after the bulk shift, left a slowly dissociating NSB signal of c. 1300 RU. The gray sensorgram represents a 100 μL injection of 50% human serum diluted in the prepared sample buffer solution containing 1 M NaCl, 0.5% BSA and 2% Tween 20 in PBS pH 7.3. After a c. 4000 RU bulk shift, a greatly reduced NSB signal of around 40 RU remained. The NSB suffered a 97% reduction in the prepared sample buffer. (b) Activity of the TNFα biomarker at 50 ng/mL in PBST–BSA compared to the activity in 25% serum in the prepared sample buffer. Signals arising from blank injections are also shown. The increased signal of the TNFα in 25% serum diluted in the prepared sample buffer (43 RU) when compared to that in PBST–BSA (35 RU) can be almost completely attributed to the small remnant constant NSB signal left by the serum (11 RU). This proves an almost perfect activity of the TNFα–anti-TNFα pair in the presence of the sample buffer prepared to hinder the NSB.

4. Conclusions

In this work, a very sensitive yet simple immunoassay for the detection of the inflammation biomarker TNFα was developed for its use in an SPR biosensor. Using a sandwich immunoassay including gold nanoparticles for signal amplification a LOD of 11.6 pg/mL (211 fM) was obtained in spiked buffer. In addition to the enhanced sensitivity and specificity provided by the amplification steps a sample dilution buffer solution was prepared to reduce the NSB in real samples. 25% human serum was assayed and, due to an almost complete hindering of the matrix effects, a LOD of 54.4 pg/mL (989 fM) was achieved. This low limit of detection,

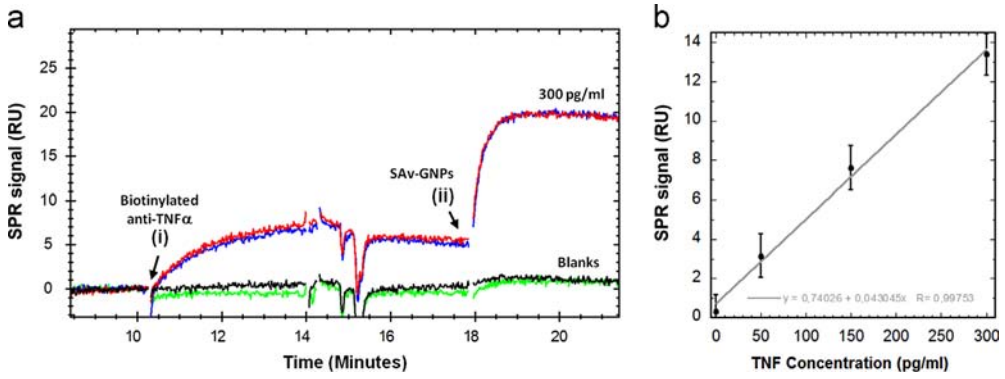

Fig. 4. (a) Sensorgrams showing the amplifications for TNFα in 25% spiked human serum with null and 300 pg/mL concentrations. Duplicates of each concentration were injected during 8 min (not shown in the graph because of the huge bulk shifts; see gray sensorgram of Fig. 3a) and the signal sequentially amplified with (i) biotinylated rabbit anti-TNFα (20 μg/mL) and (ii) SAv-GNPs. After each amplification cycle, the surface was regenerated with a 1 min injection of 100 mM glycine–HCl pH 1.8. The sensorgrams shown are double referenced. (b) SPR signal TNFα concentration in spiked human serum. The calculated LOD was 54.4 pg/mL.

together with good repeatability and longevity of the chips, suggests the validity of the presented immunoassay for detection of low concentration biomarkers in real samples.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.11.063.

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