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Electrochemical immunosensor for sensitive determination of transforming growth factor (TGF) - β1 in urine

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ABSTRACT

The first amperometric immunosensor for the quantification of TGF-β1, a cytokine proposed as a biomarker for patients having or at risk for renal disease, is described in this work. The immunosensor design involves disposable devices using carboxylic acid-functionalized magnetic microparticles supported onto screen-printed carbon electrodes and covalent immobilization of the specific antibody for TGF-β1 using Mix&Go polymer. A sandwich-type immunoassay was performed using biotin-anti-TGF and conjugation with peroxidase-labeled streptavidin (poly-HRP-Strept) polymer. Amperometric measurements were carried out at −0.20 V by adding hydrogen peroxide solution onto the electrode surface in the presence of hydroquinone as the redox mediator. The calibration plot allowed a range of linearity extending between 15 and 3000 pg/mL TGF-β1 which is adequate for the determination of the cytokine in plasma and urine. The limit of detection, 10 pg/mL, is notably improved with respect to those obtained with ELISA kits. The usefulness of the immunosensor for the determination of low TGF-β1 concentrations in real samples was evaluated by analyzing spiked urine at different pg/mL concentration levels.

1. Introduction

Cytokines are low molecular weight bioactive proteins produced by many different cells and strongly associated with the immune system (Stenken and Poschenrieder, 2015) (Liu et al., 2016). There is enormous clinical interest in cytokines determination as elevated concentrations of these proteins are associated with inflammation or disease progression and, therefore, various types of cytokines are widely used as biomarkers to characterize the immune function, predict diseases and monitor their evolution and treatments. These applications require the availability of highly sensitive analytical methods because cytokines appear into the extracellular milieu at pM concentration range.

The transforming growth factor-β (TGF-β) family is a collection of structurally related multi-functional cytokines which regulates a wide range of physiological and pathological processes. They are involved in cell-growth, rate of proliferation, differentiation and production of extracellular matrix proteins (Grainger et al., 2000). Three isoforms (TGF-β1, -β2, and -β3) are present in mammals with some differences in biological activities and also in their potencies. Particularly, TGF-β1 is involved in immune and inflammatory responses showing a hundred times more potent behavior as growth inhibitor of hematopoeitic stem cells than the others. This cytokine has been considered as a good biomarker of liver fibrosis (Fallatah, 2014) or bladder carcinoma (Eder et al., 1996). Increasing evidence also links TGF-β1 to the progression of renal fibrosis and scarring associated with diabetic nephropathy or hypertensive nephrosclerosis (Tsapenko et al., 2013) and glomerulonephritis (Grainger et al., 1995). TGF-β1 concentration levels between 0.1 and 25 ng/mL have been reported in plasma from healthy individuals (Grainger et al., 2000). The variability observed depends to some extent on the assay type used for the determination. Circulating levels of TGF-β1 increase in patients suffering various types of cancer in addition to the aforementioned kidney diseases, and are severely depressed in advanced atherosclerosis (Matharu et al., 2014).

Despite its importance, relatively few methods are available for the determination of this cytokine. Immunoassay strategies based on sandwich-type configurations with peroxidase-labeled or biotinylated anti-TGF-β1 as detection antibodies are employed in commercial ELISA colorimetric kits. These methods are valid for determining TGF-β1 in the range from several tens to thousands of pg/mL with minimum detectable concentrations that can drop to a few units of pg/mL. In the particular case of biosensors for TGF-β1 determination, only two configurations have been found in the literature. An aptasensor involving aptamer-modified Au electrodes integrated with microfluidics was reported. Thiolated aptamers labeled with methylene blue were self-assembled on gold

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surfaces. The linear range covered up to 250 ng/mL with a detection limit of 1 ng/mL. This device was used to monitor TGF-β1 release from hepatic cells (Matharu, et al., 2014). Very recently, an impedimetric immunosensor was developed for the determination of TGF-β1 in human serum. A self-assembled monolayer of polyethylene glycol (PEG) prepared onto interdigitated electrodes was used for the covalent immobilization of the antibodies. A linear impedance vs log [TGF-β1] range between 1 and 1000 ng/mL was found with a detection limit of 0.570 ng/mL (Yao et al., 2016). However, biosensors exhibiting higher sensitivity are needed to be applied to clinical samples containing very low TGF-β1 concentrations. For example, in urine this cytokine ranges typically between 10 and 50 pg/mL and it has been proposed as a biomarker for patients having or at risk for renal disease (Tsapenko et al., 2013) (Grainger et al., 1995) (Honkanen et al., 1997). Obviously, it is also mostly important the noninvasive nature of collecting urine sample and therefore its usefulness to be employed with point of care devices.

In this work, the first amperometric immunosensor for the quantification of TGF-β1 is described, with the objective of developing a sensitive, reliable, and robust analytical tool for the determination of this cytokine in complex clinical samples. The immunosensor implies a disposable device using carboxylic acid-functionalized magnetic microparticles supported onto screen-printed carbon electrodes. These magnetic beads have demonstrated to be powerful tools for the preparation of electrochemical immunosensors enabling minimization of matrix effects in the analysis of complex samples (Zacco et al., 2006), (Ruiz-Valdepeñas Montiel et al., 2015). Covalent immobilization of the specific antibody for TGF-β1 (anti-TGF) was performed using Mix&Go, a polymer containing several metallic complexes selected for their efficiency to bind proteins (Ojeda et al., 2015). A sandwich-type immunoassay was designed using biotin-anti-TGF, and conjugation with peroxidase-labeled streptavidin (poly-HRP-Strept) polymer was used as to amplify the electrochemical detection. Amperometric measurements were performed by adding hydrogen peroxide solution onto the electrode surface in the presence of hydroquinone as the redox mediator. The analytical usefulness of the immunosensor was demonstrated by application to urine samples containing different TGF-β1 concentrations at the pg/mL level.

2. Experimental

2.1. Reagents and solutions

Human TGF–β1, mouse capture antibody (anti-TGF), and chicken biotinylated antibody (Biotin-anti-TGF) were from R&D Systems and included in the DuoSet® ELISA Development System (DY240-05). Horseradish peroxidase-labeled streptavidin (HRP-Strept) (Roche), poly-HRP-Strept (85-R200) (Fitzgerald), carboxylic acid-functionalized magnetic beads (HOOC-MBs) (Dynabeads® M–270 Carboxylic Acid, 2.8 μm diameter, 30 mg/mL), and Mix&Go™ polymer from Anteo Diagnostics, were also used. Buffer solutions used were: 0.1 M phosphate buffer solution of pH 8.0 and 0.05 M phosphate buffer solution of pH 6.0 prepared from Na_2HPO_4 and NaH_2PO_4; saline phosphate buffer of pH 7.2 (PBS) containing 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl; washing buffer solution (WBS) prepared from the latter PBS by dissolving 0.05% Tween 20; 25 mM MES buffer solution of pH 5.0 prepared from *2*–(*N*–morpholine) ethanosulfonic acid (Gerbu). Hydrogen peroxide (Aldrich, 30% (w/w) and hydroquinone (Sigma) were also used. 2 M ethanolamine (Sigma) prepared in 0.1 M phosphate buffer solution of pH 8.0 was used as blocking solution. Ascorbic acid (AA) (Fluka), uric acid (UA) and creatinine (CR) (Sigma), adiponectin (APN), interleukin 6 (IL–6) and interleukin 8 (IL–8) (Abcam), and tumor necrosis factor alpha (TNF–α) (BD Pharmingen) were tested as potential interferents. Deionized water was obtained from a Millipore Milli-Q purification system (18.2 M Ω cm at 25 °C).

2.2. Apparatus and electrodes

Amperometric measurements were performed using an INBEA potentiostat provided by the IbGraph software. Screen-printed carbon electrodes (SPCEs, 110 DRP, ϕ 4 mm) from DropSens (Oviedo, Spain) were used as working electrodes. These electrodes are provided with a silver pseudo-reference electrode and a carbon counter electrode. Incubation steps were performed at 25 °C using an Optic Ivymen System constant temperature incubator shaker (Comecta S. A.) and pH measurements were made using a Crison Basic 20+ pHmeter. A P-Selecta ultrasonic bath, a magnetic separator (DynaMagn®, Invitrogen Dynal), and a Vortex homogenizator from Heidolph were also used. All experiments were performed at room temperature.

2.3. Procedures

2.3.1. Preparation of the poly-HRP-Strept/Biotin-anti-TGF/TGF-β1/anti-TGF-MBs-conjugates

3 μL of the commercial HOOC-MBs suspension were transferred into a 1.5 mL Eppendorf tube and washed twice with 50 μL of MES buffer solution at 25 °C. Each washing step consisted of a re-suspension of the functionalized MBs in the buffer solution and stirring at 600 rpm for 10 min (up to homogenization) followed by magnetic separation for 4 min and removal of the solution. Next, 25 μL of Mix&Go were added and incubated for 60 min at 25 °C under stirring at 600 rpm. Thereafter, two washing steps with WBS were carried out and, further, 25 μL of a 5 μg/mL anti–TGF solution prepared in 25 mM MES buffer solution of pH 5.0 were added, allowing incubation for 60 min at 25 °C under stirring at 600 rpm. Then, the immunoconjugates were washed firstly with 50 μL of 25 mM MES buffer solution of pH 5.0 and, secondly, with 50 μL of 100 mM PBS of pH 8.0. Next, a blocking step by incubation of the anti–TGF-MBs conjugate with 50 μL of 2 M ethanolamine solution prepared in 0.1 M PBS of pH 8.0 was applied for 1 h. Thereafter, the excess of ethanolamine was removed by washing successively with 50 μL of the same PBS buffer and with 50 μL of the WBS. Bioconjugation of the target cytokine was carried out by adding 25 μL of a TGF-β1 standard solution or the sample and incubating for 60 min at 25 °C. Two washings with 50 μL WBS were performed and 25 μL of a 2 μg/mL Biotin–anti–TGF-β1 solution containing 1% BSA were added and incubated for 60 min at 25 °C under stirring at 600 rpm. Then, two washing steps with 50 μL WBS were applied followed by the addition of 25 μL of 1/500 diluted poly–HRP–Strept in PBS, and incubation for 20 min. Finally, two more washing steps with 50 μL WBS were applied. All buffer solutions used were those recommended by the supplier of DuoSet® ELISA Development System (DY240–05).

2.3.2. Determination of TGF-β1

The as prepared poly-HRP-Strept/Biotin-anti-TGF/TGF-β1/anti-TGF-MBs were re-suspended in 45 μL of 1 mM hydroquinone and transferred onto the surface of the SPCE. This was done by keeping the SPCE horizontal and placing a neodymium magnet on the bottom part of the electrode to locate in a reproducible way the biofunctionalized MBs onto the working electrode surface area. A detection potential of −200 mV was applied and the background current was recorded until stabilization (100 s approximately). Further, a 5 μL aliquot of 50 mM hydrogen peroxide solution was added and, after a period of 200 s for the enzymatic reaction to

take place, the reduction current of the formed quinone was measured.

2.3.3. Analysis of spiked urine

The procedure described above was applied to urine samples which were spiked with TGF-β1 at final concentrations of 25, 45 or 100 pg/mL. No pretreatment was required except a 1:3 dilution with 0.1 M PBS of pH 7.2. The determination of TGF-β1 was carried out by interpolation of the amperometric responses for the samples into the calibration plot constructed with TGF-β1 standards.

3. Results and discussion

Fig. 1 shows schematically the different steps involved in the preparation and functioning of the poly-HRP-Strept/Biotin-anti-TGF/TGF-β1/anti-TGF-MBs/SPCE immunosensor. Covalent immobilization of capture antibodies onto carboxylic acid-functionalized magnetic microparticles (step 1) was carried out by using the polymer Mix&Go. This polymer uses ligands to bind Fc domains that mimic those binding domains from proteins A and G (Ooi et al., 2014), and can also interact strongly with electron donating moieties such as dissociated carboxyl groups. Therefore, combination of HOOC-MBs with the use of Mix&Go for antibody immobilization resulted in a convenient methodology to be applied as a general route for the preparation of electrochemical immunosensors (Ojeda et al., 2015). Capture antibodies immobilization was followed by a blocking step with ethanolamine (step 2) and the implementation of a sandwich-type immunoassay employing a biotinylated secondary antibody and poly-HRP-Strept for signal amplification (step 3). The use of poly-HRP-Strept instead of conventional HRP-Strept conjugate has demonstrated to be advantageous for the design of sensitive electrochemical immunosensors as multiple HRP molecules are available to biocatalyze H_2O_2 reduction (Ojeda et al., 2014). Once the MBs bearing the immuno-conjugates were transferred to the surface of SPCE, hydrogen peroxide was added (step 4) and the amperometric response at −200 mV in the presence of hydroquinone was measured according to the reactions displayed in Fig. 1.

3.1. Optimization of the experimental variables involved in the preparation of the immunosensor

The effect of the different variables involved in the preparation of the TGF-β1 immunosensor on the corresponding analytical responses was studied. The amount of Mix&Go used was that optimized previously in our group (Ojeda et al., 2015) and, in these studies, conventional HRP-Strep was used to label the detection antibody since the effect of the other variables should not depend on the type of label employed.

The anti-TGF loading on the carboxylic acid-functionalized MBs was optimized by measuring the specific and unspecific responses (without sandwiched antigen) with antibody concentrations ranging between 2.5 and 20 μg/mL. Fig. 2(a) shows as the largest specific-to-unspecific current ratio was obtained for a 5 μg/mL antibody concentration. Higher concentrations produced a significant decrease of the specific response most likely due to hindering of the electrochemical reaction in the presence of a large biomolecule loading. Accordingly, such a concentration was selected to construct the immunosensor. Optimization of the incubation time for this step (results not shown) led us to select 60 min for the covalent binding of the antibody.

Ethanolamine was chosen as the blocking agent to minimize unspecific adsorptions onto HOOC-MBs after anti-TGF binding due to its proved efficiency for this purpose (Ojeda et al., 2014). In order to optimize this blocking step, 1 and 2 M ethanolamine solutions and different incubation times over 30–90 min range were tested. Fig. 2(b) and (c) show as an effective blocking was reached using a 2 M ethanolamine solution for 60 min. Under these conditions, the current due to unspecific adsorption was less than 25% than that measured for such a low TGF-β1 concentration as 125 pg/mL.

The Biotin-anti-TGF concentration was also optimized by testing the electrochemical responses measured with different

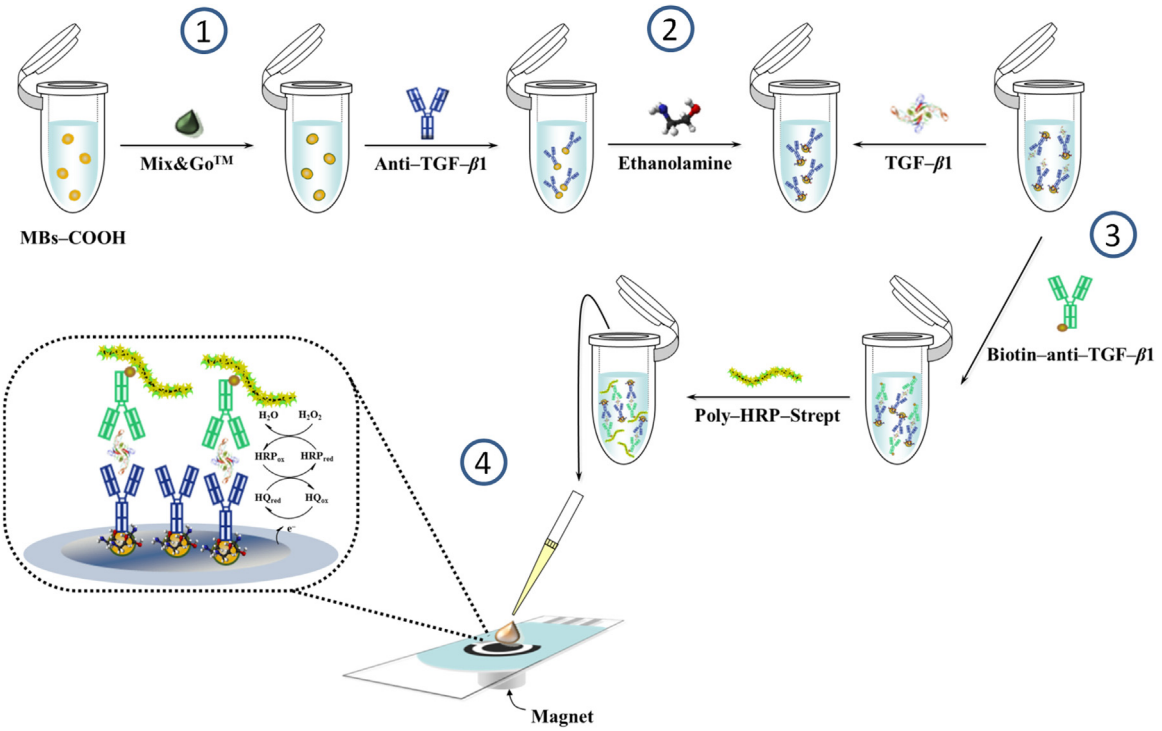

Fig. 1. Schematic display of the different steps involved in the preparation of the amperometric immunosensor for TGF-β1.

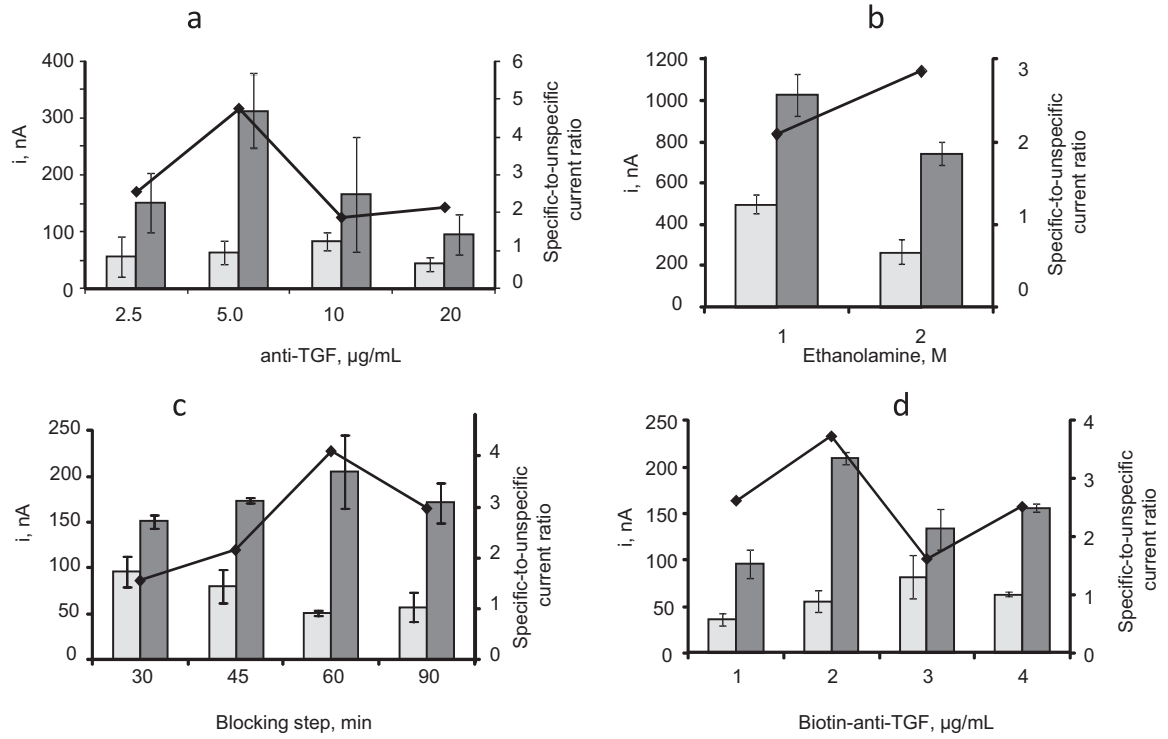

Fig. 2. Effect of the anti-TGF loading (a), the blocking agent concentration (b), the incubation time in the blocking step (c) and the concentration of Biotin-anti-TGF (d) on the amperometric response obtained with the TGF-β1immunosensor. 3 μL HOOC-MBs; 25 μL Mix&Go, 60 min; (a) 2.5–20 μg/mL anti-TGF, 60 min; 50 μL 1 M ethanolamine, 90 min; 25 μL 2 μg/mL Biotin–anti–TGF, 60 min; 25 μL 1/2000 dilution HRP-Strept; 20 min; (b) 5 μg/mL anti-TGF, 60 min; 50 μL 1 or 2 M ethanolamine, 90 min; (c) 50 μL 1 M ethanolamine, 30–90 min; 25 μL 2 μg/mL Biotin–anti–TGF, 60 min; 25 μL 1/2000 dilution HRP-Strept, 20 min; (d) 5 μg/mL anti-TGF, 60 min; 50 μL 1 M ethanolamine, 60 min; 25 μL 1–4 μg/mL Biotin-anti-TGF, 60 min; 25 μL 1/2000 dilution HRP-Strept, 20 min 25 μL 125 pg/mL TGF-β1 (dark grey) and 0 pg/mL TGF-β1 (light grey), 60 min; $E_{app} = -200$ mV. See the text for more information. Triplicate measurements with error bars at ± s values.

immunosensors prepared with conjugate loadings over the 1-4 μg/mL range. Results obtained (Fig. 2(d)) showed an increase in the amperometric response with increasing the Biotin-anti-TGF concentration between 1 and 2 μg/mL. Larger concentrations produced smaller and rather similar responses suggesting saturation of the antibodies binding sites. Accordingly, 2 μg/mL was

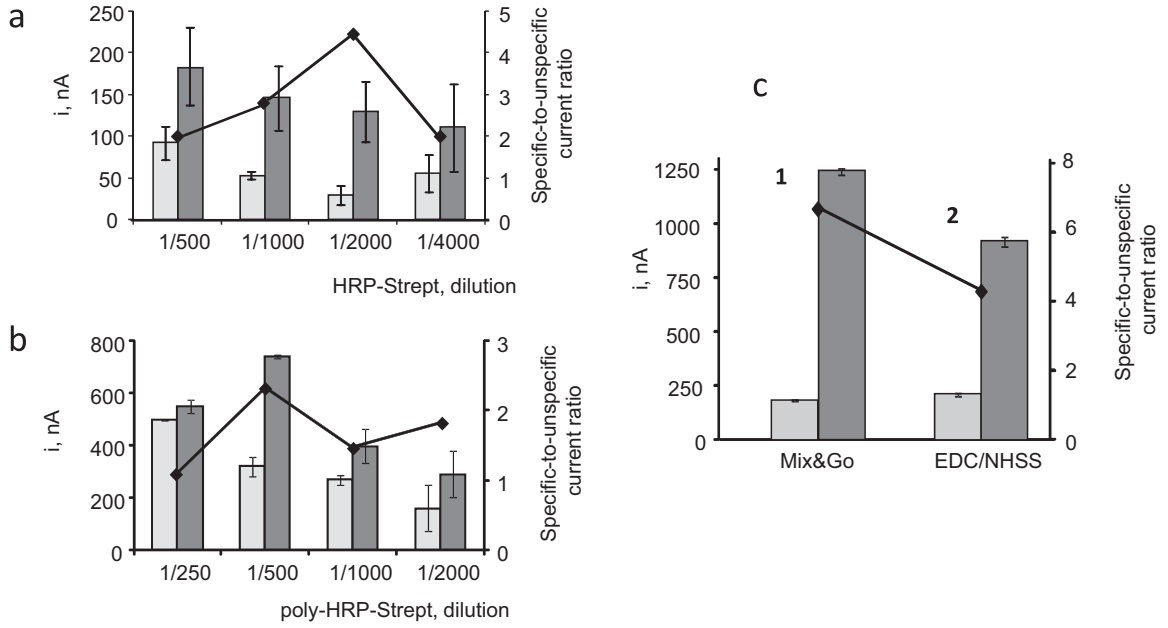

Fig. 3. Comparative responses of the TGF-β1 immunosensor when different loadings of HRP-Strept (a) and poly-HRP-Strept (b) were used to label the biotinylated anti-TGF detection antibody: 25 μL 125 pg/mL TGF-β1 (dark grey) or 0 pg/mL TGF-β1 (light grey), 60 min; 25 μL HRP-Strept, 20 min (a); 25 μL 60 pg/mL TGF–β1 (dark grey) or 0 pg/mL TGF-β1 (light grey), 60 min; 25 μL poly. HRP-Strept, 20 min (b); 3 μL HOOC-MBs; 25 μL Mix&Go, 60 min; 25 μL 5 μg/mL anti-TGF, 60 min; 50 μL 1 M ethanolamine, 90 min; 25 μL 2 μg/mL Biotin-anti-TGF, 60 min (c) amperometric currents for 25 μL of 125 pg/mL (dark grey) or 0 (light grey) TGF–β1, 60 min, measured with immunosensors prepared by covalent immobilization of anti-TGF onto HOOC-MBs using Mix&Go (1) or by activation with EDC/NHSS (2). 3 μL HOOC-MBs; 25 μL Mix&Go, 60 min or 25 μL 25 mg/mL EDC/NHSS, 60 min; 50 μL 2 M ethanolamine, 60 min; 25 μL 2 μg/mL Biotin–anti–TGF–β1, 60 min; 25 μL, 1/500 poly–HRP–Strept, 20 min; $E_{app} = -200$ mV. See the text for more information. Results for triplicate analysis with error bars at ± s values.

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chosen as the optimal conjugate concentration. Regarding the incubation time for this step (results not shown), a period of 60 min was shown be enough to allow binding of all biotinylated antibodies to TGF-β1 antigen.

In order to get the highest possible sensitivity to achieve the goal mentioned in the Introduction for this work, an electrochemical signal amplification strategy was implemented by labeling the detection antibody with a peroxidase-streptavidin polymer instead of conventional HRP-Strept. This strategy has demonstrated to enhance sensitivity in the preparation of electrochemical immunosensors since multiple HRP molecules are available to be used in the biocatalysis of the enzyme substrate (Ojeda et al., 2014). The foreseen amplification effect provoked by the use of poly-HRP-Strept was confirmed by comparing the responses obtained with both conjugates. Fig. 3(a) shows the results when HRP-Strept was employed with dilution factors ranging from 1/500 to 1/4000. The specific response for a TGF-β1 concentration of 125 pg/mL slightly decreased when the conjugate concentration was lower while the best specific-to-unspecific current ratio was obtained for a 1/2000 HRP-Strept dilution. When poly-HRP-Strept was employed, a remarkable larger current was measured (Fig. 3(b)) for a TGF-β1 concentration of 60 pg/mL, thus demonstrating the achieved amplification in the electrochemical response. In this case, the largest specific-to-unspecific current ratio occurred for a 1/500 dilution factor and, as it could be expected, it was significantly smaller than that occurring with HRP-Strept. In both cases, an incubation time of 20 min was enough for binding with the biotinylated secondary antibody.

As commented above, the strategy for binding capture antibodies to carboxylic acid-functionalized MBs implied the use of the Mix&Go polymer. This was claimed as an efficient strategy for the stable and oriented binding of antibodies (Ojeda et al., 2015) and, in this case, it was demonstrated by comparing the immunosensor response when anti-TGF antibodies were covalently immobilized using Mix&Go, with that obtained when conventional EDC/NHSS chemistry was employed for the antibody binding. As Fig. 3(c) shows, a remarkably enhanced specific-to-background current was apparent when anti-TGF was immobilized using Mix&Go, probably as a consequence of the suitable orientation of the antibody provided by this polymer.

3.2. Analytical characteristics of the immunosensor

Fig. 4 shows the calibration plot for TGF-β1 constructed with the developed immunosensor under the optimized working conditions. Error bars were calculated from measurements carried out with three different immunosensors in each case. The steady state current vs. logarithm of TGF-β1 concentration followed the adjusted equation I(nA)=978 log C(pg/mL)−734 (r^2=0.991), with a range of linearity extending between 15 and 3000 pg/mL TGF-β1. This range covers more than two orders of magnitude and it is adequate for the determination of the cytokine in real samples taking into account the expected concentrations, at ng/mL level in plasma (Grainger et al., 1995), or tens of pg/mL in urine (Tsapenko et al., 2013). The limit of detection, 10 pg/mL, was calculated by applying the 3 s_b criterion, where s_b was estimated as the standard deviation in concentration units (n=10) of measured blank currents (0 ng/mL TGF-β1). The analytical characteristics achieved with the proposed immunosensor improve notably those reported for the commercial ELISA kits. For example, RayBio® Human TGF-beta1 ELISA kit (ELH-TGFb1) claims (⟨www.raybiotech.com/files/manual/ELISA/ELH-TGFb1.pdf⟩) for a minimun detectable dose (analyte concentration resulting in an absorbance equal to 2 s higher than that of the blank) of 80 pg/mL, which is eight times higher than that achieved in this work. The immunosensor exhibits also remarkably higher sensitivity than that reported for both the aptasensor (LOD of 1 ng/mL) (Honkanen et al., 1997) and the impedimetric imunosensor (LOD =0.570 ng/mL) (Yao et al., 2016).

The reproducibility of the amperometric responses obtained with different immunosensors was evaluated. Sets of immunosensors were prepared on the same day and on different days using a new anti-TGF-MBs/SPCE in each case. The relative standard deviation (RSD) (n=5) values were 2.9% and 3.9% for the assays performed on the same day in the absence and in the presence of 250 pg/mL TGF-β1, respectively, whereas RSD (n=5) values were 3.7% and 4.2%, respectively, for the measurements made on different days. These results revealed the good level of precision achieved in the fabrication and functioning of the proposed immunosensing platform. Moreover, the storage ability of anti-TGF-MBs/SPCE conjugates was also tested. Different anti-TGF-MBs/SPCE were prepared on the same day, stored under humidity conditions at 4 °C, and employed to prepare immunosensors to measure 250 pg/mL TGF-β1 on different days. The results obtained (not shown) indicated that the immunosensor responses remained within the control limits, located at ±3 s, were s was the standard deviation of the measurements (n =10) carried out on the first working day, for at least 30 days (no longer storage times were tested) demonstrating the good stability of the anti-TGF-MBs/SPCE conjugates.

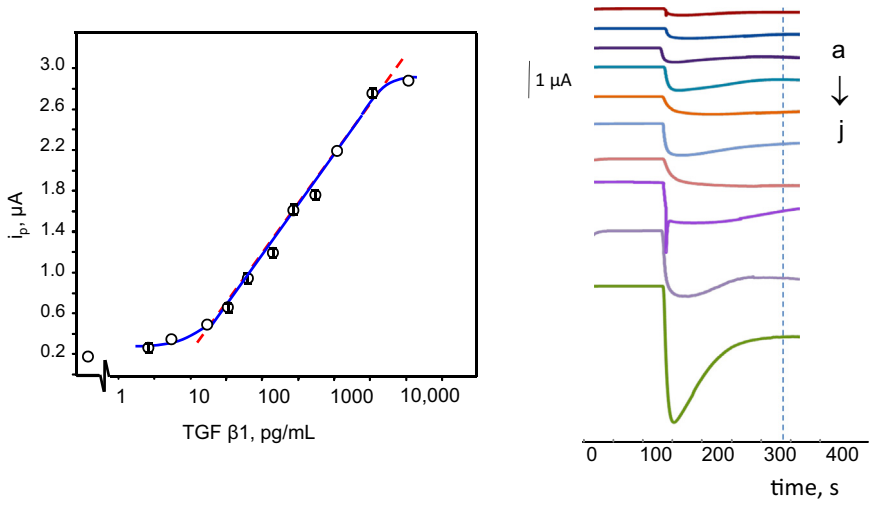

Fig. 4. Calibration plot construted for TGF-β1 by amperometry at the poly-HRP-Strept/Biotin-anti-TGF/TGF-β1/anti-TGF-MBs/SPCE immunosensor. Amperograms recorded for a) 0; b) 2.5; c) 15; d) 30; e) 60; f) 125; g) 250; h) 500; i) 1000; j) 3000 pg/mL TGF-β1. Dotted line indicates the time of measurement.

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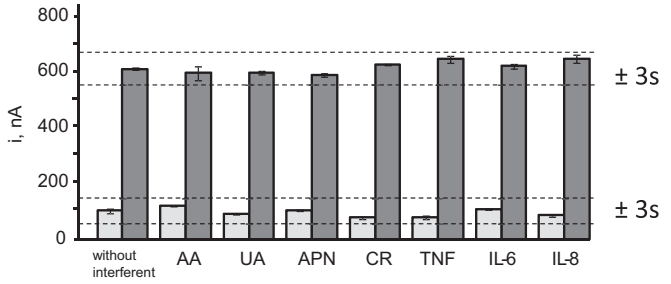

Fig. 5. Amperometric responses measured with the poly-HRP-Strept/Biotin-anti-TGF/TGF-β1/anti-TGF-MBs/SPCE immunosensor for 0 (light grey) and 25 pg/mL TGF-β1 (dark grey) in the presence of 370 μg/mL ascorbic acid (AA), 50 μg/mL uric acid (AU), 200 pg/mL adiponectin (APN), 10 pg/mL creatinine (CR), 200 pg/mL tumor necrosis factor alpha (TNF), 500 pg/mL interleukin 6 (IL-6), and 10 ng/mL interleukin 8 (IL-8).

Table 1
Determination of TGF-β1 in spiked urine with the poly-HRP-Strept/Biotin-anti-TGF/TGF-β1/anti-TGF-MBs/SPCE immunosensor.

TGF-β1, pg/mL	TGF-β1found, pg/mL	Recovery, %
25	25.9 ± 0.5	103 ± 8
45	44.9 ± 0.3	100 ± 3
100	96 ± 1	97 ± 5

The used antibody also exhibited a great selectivity against other proteins. Fig. 5 displays the immunosensor responses in the absence and in the presence of 25 pg/mL TGF-β1, and in the presence of ascorbic acid (AA), uric acid (UA), adiponectin (APN), creatinine (CR), tumor necrosis factor alpha (TNF), interleukin 6 (IL-6) or interleukin 8 (IL-8) at the expected concentrations in healthy patients. As it is clearly seen, no significant differences were apparent in any case. Interestingly, due to the detection potential value used, no interference from electroactive substances such as ascorbic and uric acids was observed.

3.3. *Determination of TGF-β1 in spiked urine*

The usefulness of the immunosensor for the determination of low TGF-β1 concentrations in real samples was evaluated by analyzing spiked urine following the procedure described in section 2.3.3. The sample used was Liquichek™ Urine Chemistry Control (BioRad) containing uric acid, amilase, calcium, chloride, cortisol, creatinine, phosphorous, glucose, magnesium, albumin, potassium, sodium, and urea, and it was spiked with TGF-β1 at 25, 45 or 100 pg/mL concentration levels.

The possibility of a matrix effect was evaluated by constructing calibration graphs in urine by spiking it with TGF-β1 concentrations ranging between 25 and 250 pg/mL and applying 0, 1:2 and 1:3 dilution ratios with 0.1 M PBS of pH 7.2. The results obtained revealed that a 1:3 dilution was enough to avoid significant matrix effects since the slope of the calibration plot, 911 nA per decade of concentration, was not statistically different from that obtained with TGF-β1 standards. Accordingly, the determination of TGF-β1 in urine could be accomplished by interpolation of the current measured with the immunosensor in the 1:3 diluted samples into calibration plot prepared with standards. No other sample pretreatment was needed in any case. Table 1 summarizes the results obtained in the analysis of spiked urine with recoveries near to 100% in all cases, thus demonstrating the suitability of the approach to determine low TGF-β1 concentrations in a complex biological fluid such urine is.

4. Conclusions

The first amperometric immunosensor for the quantification of the cytokine TGF-β1, proposed as a biomarker for patients having or at risk for renal disease, is described in this work. The rational design of the immunosensor involving stable and oriented immobilization of the specific antibodies on carboxylic acid-functionalized MBs by using the polymer Mix&Go, and an electrochemical signal amplification strategy by labeling the detection antibody with a peroxidase-streptavidin polymer, allowed remarkably improved analytical characteristics to be obtained with respect to ELISA kits or previous methods. Combining these benefits, a calibration plot suitable for the determination of the cytokine in plasma and urine is achieved. This allowed the determination of TGF-β1 concentrations in urine at the pg/mL concentration level with no sample treatment except a 1:3 dilution with buffer solution. The obtained results show that the initial objective of developing a sensitive, reliable, and robust analytical tool for the determination of this cytokine in complex clinical samples, and, therefore, suitable to develop point-of-care devices is reasonably achieved.

Acknowledgments

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