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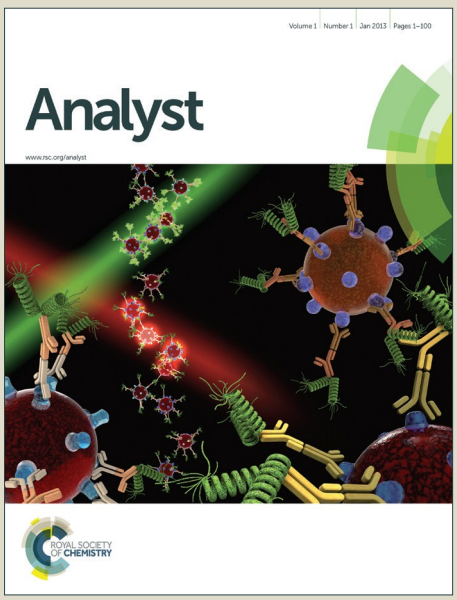

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Carbon nanotubes functionalized by click chemistry as scaffolds for the preparation of electrochemical immunosensors. Application to the determination of TGF-beta 1 cytokine

E. Sánchez-Tirado, A. González-Cortés, P. Yáñez-Sedeño*, J.M. Pingarrón
Department of Analytical Chemistry, Faculty of Chemistry, University Complutense of Madrid, 28040- Madrid, Spain. yseo@quim.ucm.es

Abstract

An electrochemical immunosensor for the determination of the multifunctional Transforming Growth Factor β1 cytokine (TGF-β1) has been prepared using multi-walled carbon nanotubes (MWCNTs)-modified screen-printed carbon electrodes. MWCNTs were functionalized by means of copper(I) catalyzed azide-alkyne cycloaddition ("click" chemistry) as an efficient strategy for the covalent immobilization of immunoreagents without altering their configurations and preserving the biological activity. Alkyne-functionalized IgGs were also prepared and used to assemble IgG-alkyne-azide-MWCNTs conjugates used as scaffold for the immunosensor preparation. After a blocking step with casein, anti–TGF were immobilized and the target cytokine was sandwiched with biotinylated anti-TGF labeled with poly–HRP-labeled streptavidin. The affinity reaction was monitored amperometrically at -0.20 V using the hydroquinone (HQ) / H_2O_2 system. The calibration plot for TGF-β1 exhibited a range of linearity (r^2 = 0.995) extending between 5 and 200 pg/mL which is suitable for the determination of the target cytokine in human serum. A limit of detection of 1.3 pg/mL was achieved. The analytical performance of the immunosensor can be advantageously compared with that of claimed for ELISA kits. The immunosensor was applied to the analysis of spiked human serum samples at different concentration levels with excellent recoveries.

Keywords: "click" chemistry, azide-alkyne cycloaddition, Transforming Growth Factor β1 cytokine (TGF-β1), carbon nanotubes, electrochemical immunosensors.

1. Introduction

Recently, copper(I) catalyzed azide-alkyne cycloaddition ("click" chemistry) has emerged as a powerful tool for functionalization of different materials as well as an efficient strategy for immobilization of biomolecules. This high interest is due to the high yields, no by-products, and moderate reaction conditions of this environmentally friendly process that can be applied in aqueous medium and in physiological conditions[1]. The methodology, involving azide and alkyne complementary functions, can be used for the functionalization of electrode surfaces[2] which can be used as platforms where biomolecules can be covalently coupled to different substrates without altering their configurations and preserving the biological activity.

Furthermore, carbon nanotubes (CNTs) have demonstrated largely the ability to promote the electron transfer reaction of a wide number of molecules[3] thus making CNTs an attractive material for the development of electrochemical sensors and biosensors. A large number of enzyme biosensors using CNTs-modified electrodes as convenient scaffolds have been described in the literature[4]. However, CNTs-modified electrodes based immunosensors have been reported in a much smaller extent. This is probably due to biomolecules immobilization methods on this type of modified electrodes rely mostly on direct adsorption or covalent approaches which can reduce the stability of immunoreagents and the reproducibility of bioelectrodes[5]. In the efforts made to find a method for achieving stable and oriented immobilization of antigens or antibodies onto CNTs, "click" synthesis represents a promising strategy. However, despite the high interest of this methodology, only one electrochemical immunosensor

has been reported so far using the Cu (I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction to prepare CNTs-based platforms for immobilization of immunoreagents. Alkyne-IgG was coupled on single-walled carbon nanotubes (SWCNTs) functionalized with azide groups and the bioconjugate was casted onto a glassy carbon surface to develop a competitive electrochemical immunoassay for the determination of anti-IgG as the target compound with HRP as enzyme label[6].

On the other hand, there is also a recent growing interest in the determination of cytokines[7] due to their demonstrated relation with inflammation or disease progression. Among cytokines, the multifunctional TGF-β1 (Transforming Growth Factor β1) is involved in various immune and inflammatory diseases[8], and is considered as a good biomarker of liver fibrosis, bladder carcinoma, or renal diseases. TGF-β1 concentrations ranging between 0.1 and 25 ng/mL in plasma have been reported for healthy individuals[9], while circulating levels of this cytokine have been shown to be increased in patients suffering various types of diseases such as various cancers[10,11] or autoimmune disorders[12], and severely depressed in advanced atherosclerosis[13].

Current method to determine this protein is by ELISA tests. There are a variety of commercial kits using colorimetric detection and sandwich-type immunoassay configurations with peroxidase-labeled or biotinylated anti-TGF-β1 detection antibodies. The range in which TGF-β1 can be determined is between several tens to thousands of pg/mL with minimum detectable concentrations of a few pg/mL. However, the time required for this type of assays is rather long usually lasting over four hours. Regarding immunosensors, only one very recent impedimetric immunosensor for the determination of TGF-β1 in human serum has appeared in the literature. A self-assembled monolayer of polyethylene glycol (PEG) prepared onto interdigitated electrodes was used for the covalent immobilization of antibodies. A linear impedance

vs log [TGF-β1] range between 1 and 1000 ng/mL with a detection limit of 0.570 ng/mL was reported[14]. However, this sensitivity is not sufficient for applications in clinical samples containing low TGF-β1 concentrations.

In this work, multi-walled carbon nanotubes (MWCNTs) were used to prepare scaffolds as electrode modifiers for the construction of a TGF-β1 immunosensor involving CuAAC reaction to synthesize the alkyne-azide conjugates. MWCNTs were selected for such purpose due to their well-known excellent conducting and electrocatalytic properties[15]. The steps involved in the oxidation of carbon nanotubes and functionalization with an azide group as well as alkyne-functionalization of IgG and preparation of IgG-alkyne-azide-MWCNTs conjugates were optimized. In addition, variables involved in the preparation and performance of the immunosensor were also addressed and optimized. Under the optimized conditions, TGF-β1 could be determined with the developed immunosensor with a high sensitivity and low detection limit exhibiting suitability for the analysis of clinical samples.

2. Experimental

2.1. Reagents and solutions

An anti-mouse IgG Fc specific from Sigma (SAB3700848) was used as primary antibody. The capture antibody was a mouse anti–TGF, reconstituted with 500 µL of 0.1 M phosphate buffer solution of pH 7.4 (PBS) up to a 240 µg/mL concentration. A chicken biotinylated antibody (Biotin–anti–TGF) reconstituted up to a 18 µg/mL concentration with 1 mL of Reagent Diluent 1 (R&D Systems) was also used. Both anti-TGF antibodies and human TGF–*β*1 were the same than those used in the DuoSet® ELISA Development System (DY240–05) from R&D Systems. HRP-labeled streptavidin (Roche) and poly–HRP–Strept (65R-S105PHRP) (Fitzgerald) solutions were prepared in 0.1 M PBS of pH 7.4. Buffer solutions used were 0.1 M phosphate

buffer solution (PBS) of pH 7.4; 10 mM PBS of pH 7.0; 0.1 M PBS 0.15 M NaCl of pH 7.2 (Coupling Buffer), and 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer of pH 6.5. Reagent Diluent 1 and Washing Buffer from R&D Systems included in the Duoset® Ancillary Reagent Kit 1 (DY007) were also used. Multi-walled carbon nanotubes (MWCNTs, PD30L5–20) were from NanoLabs. 1% (w/v) casein solution (Blocker™ Casein, Thermo Fisher) in 0.1 M PBS of pH 7 was used as the blocking agent. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysulfo-succinimide (NHSS) were from Across. N,N′-dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide (NHS) were from Sigma. Other reagents used were 11-azide-3,6,9-trioxaundecan-1-amine (Aldrich), and ethynyl hydrazide (Chem Space). All other reagents and solvents used were of analytical grade. De-ionized water was obtained from a Millipore Milli-Q purification system (18.2 MΩ cm). The analyzed samples were a lyophilized human serum from clotted whole blood (Sigma, S2257) spiked with TGF-β1 at different concentrations, and two real serum samples from one healthy (SG609-2, BBI Solutions) and one hypercholesterolemic (SG376-2, BBI Solutions) female.

2.2. Apparatus and electrodes

Amperometric measurements were performed with an INBEA potentiostat using 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 carried out at 25 °C using an Optic Ivymen System constant temperature incubator shaker (Comecta S.A.). Electrochemical impedance spectroscopy was used with a μAutolab type III potentiostat (Ecochemie) controlled by FRA2

software. A Crison Basic 20+ pHmeter, an Elmasonic S-60 ultrasonic bath (Elma), and a Vortex homogenizator from Velp Scientifica were also employed.

2.3. Procedures

Azide-functionalized MWCNTs and alkyne-functionalized IgG were prepared by following the procedures described in the Supplementary Information (Procedures S1 - S3) and schematized in Figures S1 and S2, respectively.

2.3.1. Preparation of the electrochemical immunosensor

Figure 1 shows schematically the steps involved in the preparation of the amperometric immunosensor for TGF-β1. Step 1 depitcs preparation of IgG-alkyne-azide-MWCNTs conjugates by mixing 67.5 μL of a 1/100 diluted alkyne-functionalized-IgG solution with 45 μL of 5 mM ascorbic acid and 45 μL of 5 mM copper sulfate. Then, 157.5 μL of a 0.5 mg/mL azide-functionalized-MWCNTs dispersion were added and magnetically stirred at 4 ºC overnight. Step 2 implies 5-μL dropping of the IgG-alkyne-azide-MWCNTs conjugates dispersion onto the SPCE surface allowing drying, and a further blocking step by addition of 5 μL of 1% casein solution in 0.1 M PBS of pH 7.4 and allowing incubation for 30 min. Step 3 involved 5-μL addition of a 10 μg/mL anti–TGF solution and incubation for 60 min. Thereafter, 5 μL of a TGF–β1 standard solution (or the sample), and 5 μL of a 1 μg/mL Biotin-anti-TGF were added (step 4) allowing incubation for 60 min. Finally, step 5 involved labeling with 5 μL of a 1/500 diluted poly–HRP–Strept in 0.1 M PBS of pH 7.4 incubating for 20 min.

2.3.2. Determination of TGF–β1

Determination of TGF-β1 was accomplished by dropping 45 μL of a 1 mM hydroquinone (HQ) solution in 0.5 M PBS of pH 6.0 on the surface of the poly - HRP -

Strept -Biotin -anti -TGF -TGF -β1 -anti -TGF - IgG - MWCNTs / SPCE immunosensor horizontally positioned. A detection potential of -0.20 V was applied. Once the background current was stabilized (aprox. 100 s), 5 μL of a 50 mM H_2O_2 solution were added and allowed standing for 200 s. The oxidation of hydrogen peroxide is catalyzed by peroxidase. The oxidized form of peroxidase oxidizes hydroquinone, and the product of the oxidation is reduced electrochemically at the electrode. The steady state current corresponding to the electrochemical reduction of benzoquinone was used as the analytical readout.

TGF-β1 determination was performed by applying this procedure to both serum samples spiked at 25, 75 and 125 pg/mL protein levels, which were 100 times diluted with PBS, and real serum samples from female individuals. The measured steady-state currents were interpolated into the linear calibration plot constructed with TGF-β1 standard solutions. In addition, the results obtained for the real samples were compared with those provided by a commercial DuoSet® ELISA Development System (DY240–05) kit.

3. Results and discussion

As it is described in section 2.3.1 and depicted in Figure 1, azide-functionalyzed MWCNTs and alkyne-functionalized IgG were synthesized and used to prepare IgG-alkyne-azide-MWCNTs conjugates by Cu (I) catalyzed azide-alkyne-cycloaddition (CuAAC). Supplementary Information (Procedure S1) describes the employed method to prepare azide-MWCNTs by covalent immobilization of 11-azide-3,6,9-trioxaundecan-1-amine onto oxidized MWCNTs. Figure S3 in Supplementary information shows IR absorption spectra obtained for azide-functionalized carbon nanotubes as well as for a commercial azide. A characteristic band at 2106 cm^{-1}

corresponding to the stretching vibration of the -N3 group appeared in both cases indicating the successful functionalization of MWCNTs.

Furthermore, alkyne-IgGs were prepared using two different protocols (Procedures S2 and S3) involving the use of succinimidyl-3-propiolate[16] or sodium periodate[17], respectively. These protocols were compared by testing the responses obtained with TGF-β1 immunosensors prepared according to procedure 2.3.1 and using each alkyne-IgG derivative. Figure 2 shows the amperometric measurements obtained for 0 (unspecific) and 200 pg/mL TGF-β1 with the immunosensors constructed with alkyne-IgG prepared by both methods. As it can be seen, the specific-to-unspecific current ratio was about 25% larger when periodate was used to get alkyne-IgG. This finding is in agreement with the claimed properties of this reagent able to oxidize 1,2-diols of carbohydrates existing in the Fc region of antibodies thus leading to the oriented modification of IgG. Conversely, this oriented modification is not produced when succinimidyl-3-propiolate is used[17]. Accordingly, the protocol involving the use of periodate for the preparation of alkyne-functionalized IgG was selected for further work. Figure S3 in Supplementary information shows also the IR absorption spectrum obtained after click reaction of the azide group IgG-alkyne-azide-MWCNTs. As it can be seen, the band at 2106 cm^{-1} did not appear in this case suggesting the successful binding of alkyne-anti-IgG.

3.1. Optimization of the variables involved in the preparation and performance of the immunosensor

Once IgG-alkyne-azide-MWCNTs conjugates were prepared according to that described in section 2.3.1, an aliquot of this conjugate dispersion was dropped on the SPCE to construct the scaffolds employed for immobilization of the specific anti-TGF-

β1 antibodies. The different variables affecting the performance of the developed immunosensor were optimized. These optimization studies involved evaluation of: a) IgG-alkyne-azide-MWCNTs loading on the SPCE; b) type of blocking agent; c) type of enzymatic labeling of the detection antibody; d) concentration of poly-HRP-Strept; e) anti-TGF loading on IgG-alkyne-azide-MWCNTs/SPCE; f) Biotin-anti-TGF loading on TGF β1-anti-TGF- IgG-alkyne-azide-MWCNTs/SPCE. Details on these optimization studies are given in Supplementary information (Figures S4-S7).

Electrochemical impedance spectroscopy (EIS) was employed to monitor the steps involved in the electrode modification. Figure 3 shows the Nyquist plots recorded at SPCE, azide-MWCNTs/SPCE, IgG-alkyne-azide-MWCNTs/SPCE, and anti-TGF-IgG-alkyne-azide-MWCNTs/SPCE, using 5 mM $Fe(CN)_6^{3-/4-}$ as the redox probe in 0.1 M PBS of pH 7.4, as well as the equivalent circuits used to fit spectra. Spectra 1, 2 and 3 fitted well to a Randles circuit whereas spectrum 4 fitted better to the equivalent circuit depicted on the right since some parts of the electrode are coated by the antibody while others remain exposed to the solution. The nesting parallel RC circuits mean that there is a film with defects such as pinholes or with a non-uniform thickness throughout the substrate[18]. As expected, the charge transfer resistance at the bare SPCE (curve 1) is notably higher (R_{CT}= 1247 ohm) than that measured at azide-MWCNTs/SPCE (curve 2, R_{CT} = 240 ohm) due to the well-known electron transfer promotion observed at carbon nanotubes-modified electrodes. Furthermore, the resistance increased to a value of 658 ohm when the IgG-alkyne-azide-MWCNTs conjugate was deposited onto the SPCE surface (curve 3), as a consequence of the isolating effect provoked by the biomolecules. Subsequent immobilization of the capture antibody led to a further resistance increase (curve 4) (R_{CT} = 865 ohm), due to the lower conductivity of the

resulting biosurface. These results confirmed the suitability of the procedure used for the modification of the electrode involving immobilization of anti-TGF antibodies on IgG-alkyne-azide-MWCNTs.

3.2. Analytical figures of merit of the immunosensor

Figure 4 shows the calibration plot for TGF-β1 constructed by amperometry at -0.20 V with the poly - HRP - Strept - Biotin - anti - TGF - TGF - β1 - anti - TGF - IgG - MWCNTs / SPCE immunosensor together with some typical amperometric responses recorded for different TGF-β1 concentrations. The range of linearity (r^2 = 0.995) extended between 5 and 200 pg/mL according to the equation Δi, nA = 5.60 [TGF-β1], pg/mL + 32.5. In this equation, Δi is the current obtained by substracting the background current measured in the absence of antigen from the amperometric response of the immunosensor[19]. Interestingly, this range is suitable for the determination of the target cytokine in human serum since the concentration levels found in healthy individuals are comprised between 0.1 and 25 ng/mL[9].

The limit of detection, 1.3 pg/mL, was calculated according to the 3 s_b criterion, where s_b was estimated as the standard deviation (n=10) for the blank (measurements in the absence of TGF β1) in concentration units (pg/mL). Moreover, the limit of determination was calculated as $10s_b$ and the value was 4.3 pg/mL. When these analytical figures of merit are compared with data provided for commercial ELISA kits using similar immunoreagents, some noticeable differences are apparent. ELISA kits claim for dynamic ranges usually covering from several tens to thousands of pg/mL with minimum detectable concentrations of a few pg/mL. However, these parameters are calculated mostly from nonlinear logarithmic ranges and the precision levels are around 10% or higher. It is important to note that the criteria used to calculate the LOD

values for these kits are rarely given in the commercial protocols. Moreover, the time lasted for the assay is remarkably longer with ELISA kits extending even over 4 h versus 1h 20min required for the immunosensor counting, in both cases, since the immobilization of capture antibody. Therefore, it could be concluded that the analytical performance of the developed immunosensor, covering a wide linear range of TGF-β1 concentrations within the clinically relevant interval, improved, in general terms, the performance claimed for ELISA kits. On the other hand, when the comparison is made versus the reported impedimetric immunosensor[14], the reported LOD was 0.570 ng/mL in this latter case, which is more than four hundred times higher than that calculated with the developed immunosensor.

The reproducibility of the amperometric measurements was tested for both 0 and 125 pg/mL TGF-β1 with five different immunosensors prepared on the same day. Relative standard deviations values, RSD, of 1.5 and 2.7 %, respectively, were obtained. Furthermore, RSD values of 1.9 and 2.5 % were obtained using five different immunosensors prepared in different days. Thus, these results demonstrated the good accuracy of the amperometric measurements and indicated that the proposed method for the immunosensor preparation is reliable and reproducible.

The storage capability of the anti-TGF-IgG-MWCNTs/SPCE bioelectrode was also tested. In order to do that, different immunosensors were prepared on the same day and stored at 8 ºC in humid environment with 20 µL of Reagent Diluent 1. Then the immunosensors were used to measure 125 pg/mL TGF-β1 on different days according to the procedure described in Sections 2.3.1 and 2.3.2. A control chart was constructed (Figure S7) by setting as control limits ± 3s, where s was the standard deviation of the measurements (n=10) carried out on the first day. The immunosensor responses

remained inside the control limits for at least 40 days (no longer storage times were tested) demonstrating a great stability of the prepared anti-TGF-IgG-MWCNTs/SPCE.

Regarding the selectivity of the immunosensor, it is important to note that anti-TGF antibodies were the same that those used in the DuoSet® ELISA Development System (DY240–05) from R&D Systems (Section 2.1). These antibodies exhibited a high selectivity toward other kinds of TGFs, with cross-reactivity factors of 0.15, 0.96 and 1.8 %, for TGFβ2, TGFβ3, and TGFβ5, respectively. Moreover, the selectivity of the capture antibody was also evaluated by measuring the amperometric responses in the absence and in the presence of other proteins, adiponectin (APN), bovine serum albumin (BSA), bilirubin (BR), ceruloplasmin (Cp), tumoral necrosis factor alpha (TNF), ghrelin (GHRL), hemoglobin (Hb), interleukin-8 (IL-6), interleukin-6 (IL-8), C-reactive protein (CRP), as well as cholesterol (Chl). All of them were checked at concentrations that can be found in serum of healthy individuals. Figure 4 shows clearly as there were no significant differences between the currents measured in the absence or in the presence of these compounds, thus demonstrating the practical specificity of the immunosensor for the determination of TGF-β1.

3.3. Determination of TGF-β1 in human serum

The possible existence of matrix effect in human serum was firstly evaluated. Figure 6 compares the amperometric measurements recorded with the poly-HRP-Strept-Biotin-anti-TGF-TGFβ1-anti-TGF-IgG-MWCNTs/SPCE immunosensor in 0.1 M PBS of pH 7.4 and in human serum containing no TGF-β1 or spiked with 125 pg/mL TGF-β1 (final concentration) upon dilution with 0.1 M PBS of pH 7.4 at different ratios. As it can be deduced, measurements at a 1/10 serum dilution ratio (bars 1) showed a slight increase in mean current value (n=3) for the blank (light grey), together with a slight

decrease of the mean current value for 125 pg/mL TGF-β1 (dark grey), with respect to the responses obtained in 0.1 M PBS (bars 0). These small differences could be attributed to a weak matrix effect provoked by the serum components. However, it is clear that no apparent matrix effect occurred from a 1/50 sample dilution ratio (bars 2). Taking into account the high sensitivity of the developed method and with the aim of ensuring the complete removal of any matrix effect, a 1/100 dilution ratio was selected to accomplish the protocol for the analysis of human serum. Under these conditions, a comparison of the calculated slope values of the calibration plots constructed with TGF-β1 standard solutions in 0.1 M PBS and in serum samples spiked with TGF-β1, at concentrations ranging between 0 and 125 pg/mL, was carried out. The slope value of the linear calibration graph obtained for spiked serum samples was 5.57 ± 0.01 nA mL pg^{-1} which is not statistically different from the slope of the calibration plot constructed in 0.1 M PBS with TGF-β1 standard solutions (5.60 nA mL pg^{-1}), thus demonstrating the complete removal of matrix effect. Accordingly, the determination of TGF-β1 in serum could be accomplished by interpolation of the current measured with the immunosensor in 1/100 diluted samples into the calibration plot prepared with TGF-β1 standard solutions. No further sample preparation was needed. Table 1 summarizes the results obtained in the analysis of both spiked serum and real serum samples from female individuals. Excellent recoveries ranging between 98 ± 1 and 101 ± 4 % were found in serum spiked with 25, 75 and 125 pg/mL TFG-β1. In addition, the results obtained with sera from one healthy and one hypercholesterolemic female were compared with those provided by using the DuoSet® ELISA Development System (DY240–05). As it can be seen in Table 1, mean TFG-β1 concentrations of 1.38 ± 0.05 ng/mL and 2.11 ± 0.06 ng/mL were found with the immunosensor (n=8) for the healthy and hypercholesterolic patients, respectively. These mean values are in excellent

agreement with those provided by the ELISA kit. Therefore, these results demonstrated clearly the suitability of the approach to determine low TGF-β1 concentrations in complex biological samples.

4. Conclusions

A novel amperometric immunosensor for the cytokine TGF-β1 has been developed in this work. The immunosensor preparation strategy involves building up of electrode scaffolds composed of MWCNTs functionalized by copper(I) catalyzed azide-alkyne cycloaddition for binding of alkyne-functionalized IgGs and formation of IgG-alkyne-azide-MWCNTs conjugates. This strategy allows oriented immobilization of capture antibodies preserving their biological activity. A sandwich-type configuration using poly–HRP-streptavidin labeling to achieve signal amplification led to the preparation of immunosensors exhibiting an excellent analytical performance in terms of sensitivity, clinically relevant linear range, reproducibility, storage stability and selectivity. The achieved analytical characteristics improve, in general, the performance claimed for ELISA kits and the reported impedimetric immunosensor. Moreover, the immunosensor shows an excellent suitability for the analysis of human serum with a minimal sample treatment consisting only in a 1/100 dilution with 0.1 M PBS of pH 7.4**.**

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Figures

Figure 1. Schematic display of the different steps involved in the preparation and functioning of the poly-HRP-Strept-Biotin-anti-TGF-TGFβ1-anti-TGF-IgG-MWCNTs/SPCE immunosensor.

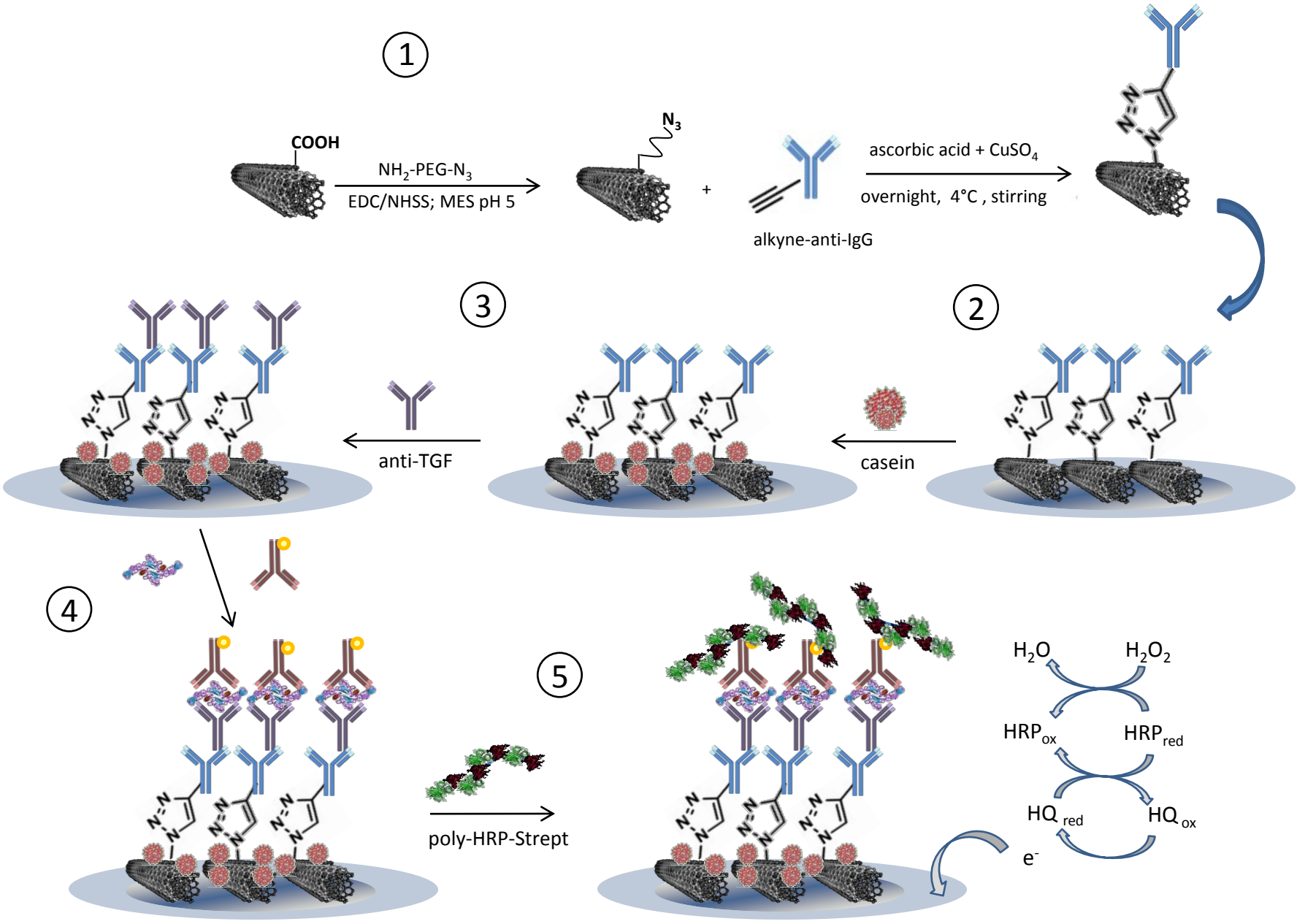
Figure 2. Effect of the protocol applied for IgG-alkyne functionalization on the amperometric response measured with poly-HRP-Strept-Biotin-anti-TGF-TGF-β1-anti-TGF-IgG-MWCNTs/SPCE using succinimidyl-3-propionate (1) or sodium peryodate (2). 5 µL 10 µg/mL IgG-alkyne-azide-MWCNTs; 5 µL casein, 30 min; 5 µL 10 µg/mL anti-TGF, 60 min; 0 (white) or 200 (grey) pg/mL TGF-β1; 5 µL 1 µg/mL Biotin-anti-TGF, 60 min; 5 µL 1/500 poly-HRP-Strept, 20 min.

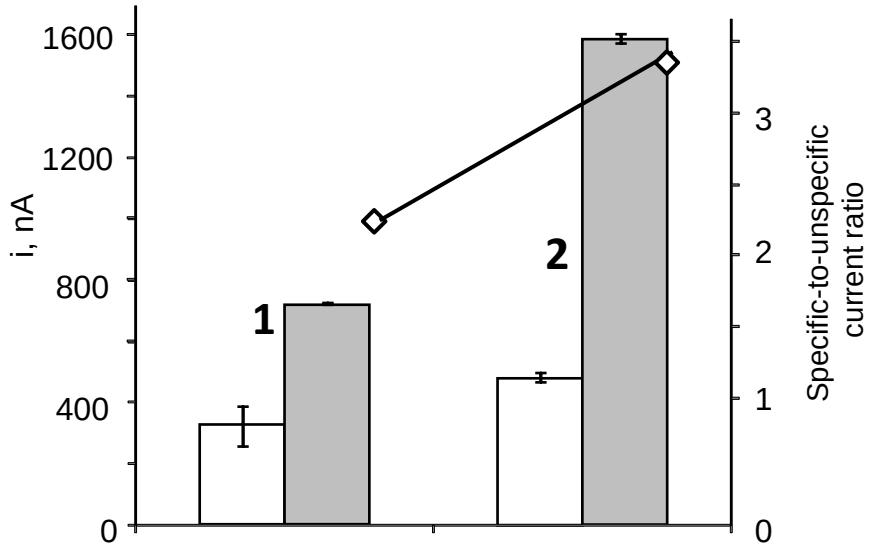
Figure 3. Nyquist plots recorded at SPCE (1), azide-MWCNTs/SPCE (2), IgG-alkyne-azide-MWCNTs/SPCE (3), and anti-TGF-IgG-alkyne-azide-MWCNTs/SPCE (4), and equivalent circuits. 5 mM $Fe(CN)_6^{3-/4-}$ as the redox probe in 0.1 M PBS of pH 7.4.

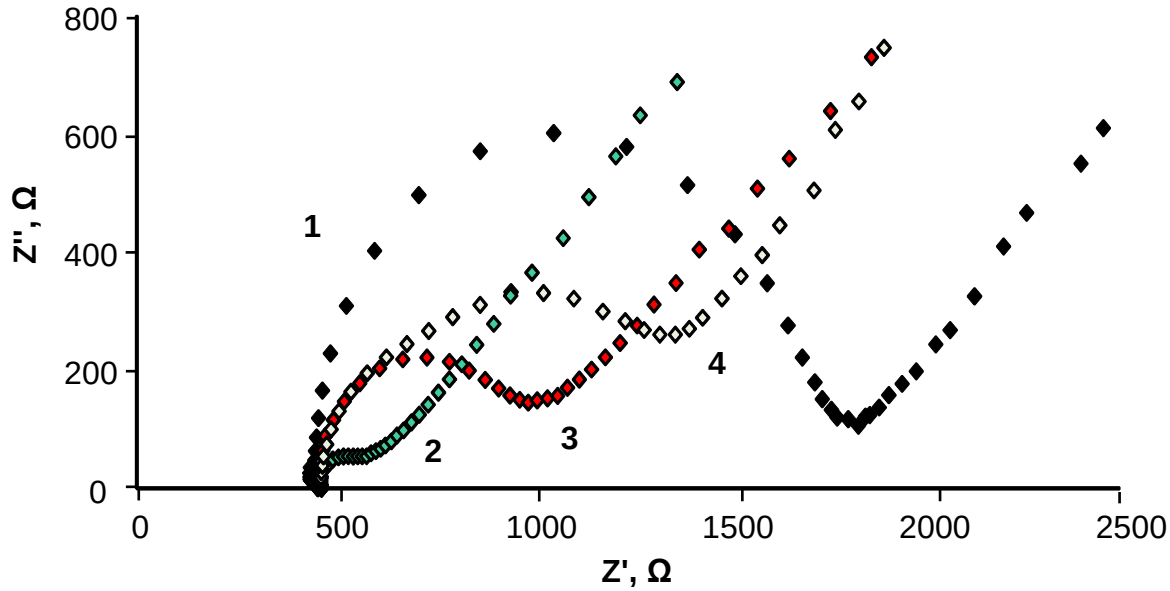
Figure 4. Calibration plot and typìcal amperometric responses for TGF-β1 recorded with the poly-HRP-Strept-Biotin-anti-TGF-TGF-β1-anti-TGF-IgG-MWCNTs/SPCE: a) 0 pg/mL; b) 5 pg/mL; c) 10 pg/mL; d) 15 pg/mL; e) 30 pg/mL; f) 50 pg/mL; g) 100 pg/mL; h) 125 pg/mL; i) 200 pg/mL TFG-β1. See the text for more information.

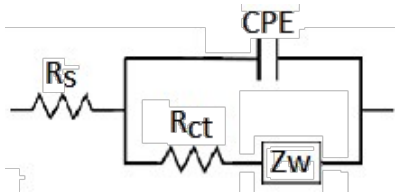
Figure 5. Amperometric responses measured with the poly-HRP-Strept-Biotin-anti-TGF-TGF-β1-anti-TGF-IgG-MWCNTs/SPCE immunosensor for 0 (white) and 125 (grey) pg/mL TGF-β1 in the presence of 20 μg/mL adiponectin (APN), 5 μg/mL BSA, 190 ng/mL bilirubin (BR), 500 μg/mL ceruloplasmin (CP), 100 pg/mL tumor necrosis factor alpha (TNF), 500 pg/mL ghrelin (GHRL), 50 μg/mL hemoglobin (Hb), 50 μg/mL interleukin 6 (IL-6), 30 pg/mL interleukin 8 (IL-8), 1 μg/mL C-reactive protein (CRP), and 20 μg/mL cholesterol (Chl).

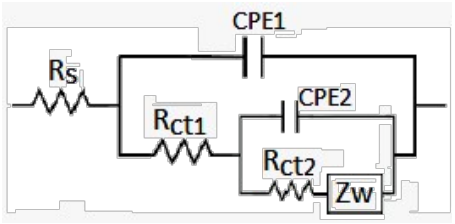
Figure 6. Amperometric responses obtained with the poly-HRP-Strept-Biotin-anti-TGF-TGF-β1-anti-TGF-IgG-MWCNTs/SPCE for 0 (light grey) and 125 pg/mL TGF-β1 (dark grey) in 0.1 M PBS of pH 7.4 (0), and in human serum diluted with 0.1 M PBS pH 7.4 at the 1/10 (1); 1/50 (2) and 1/100 (3) ratios.

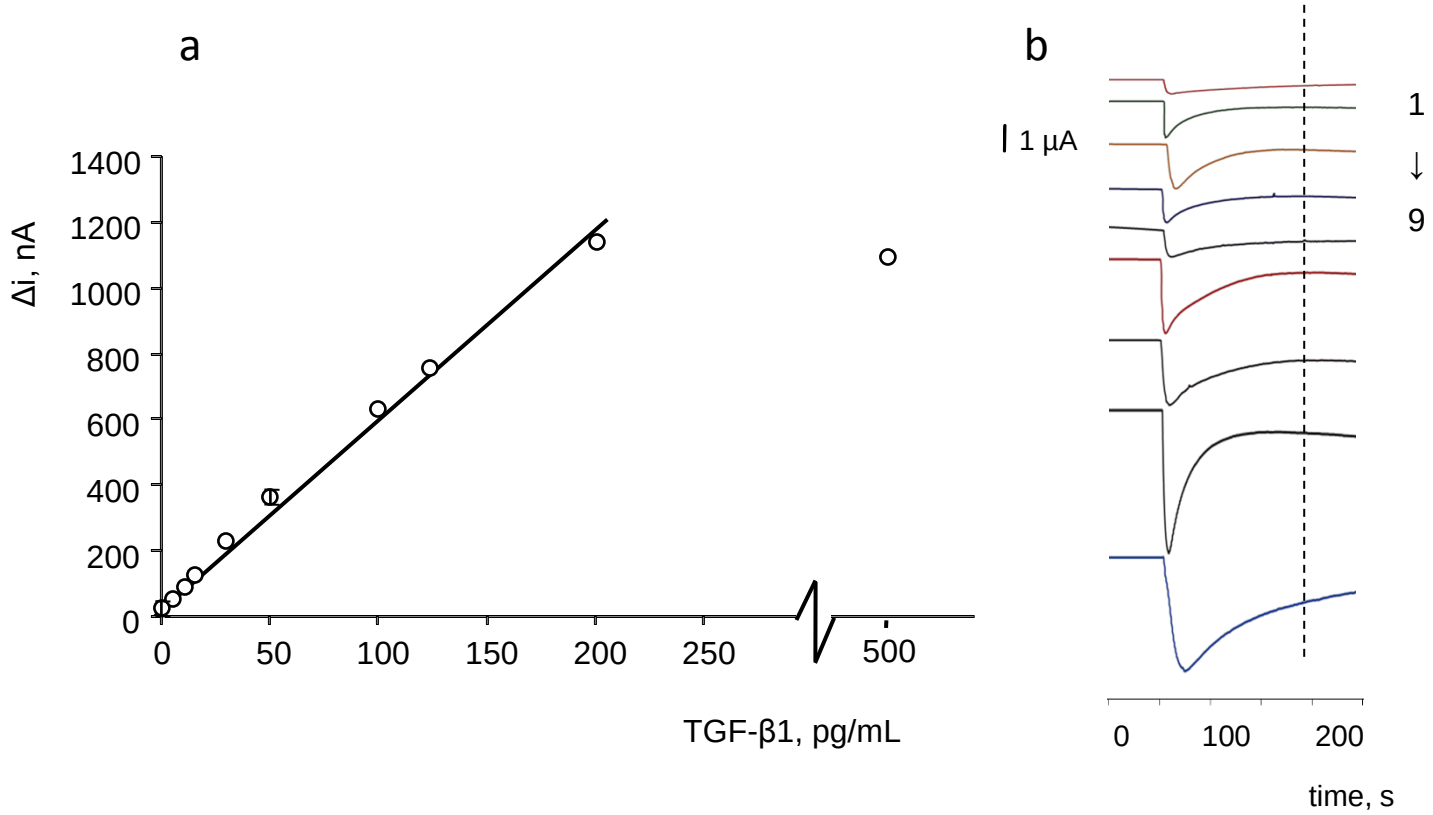


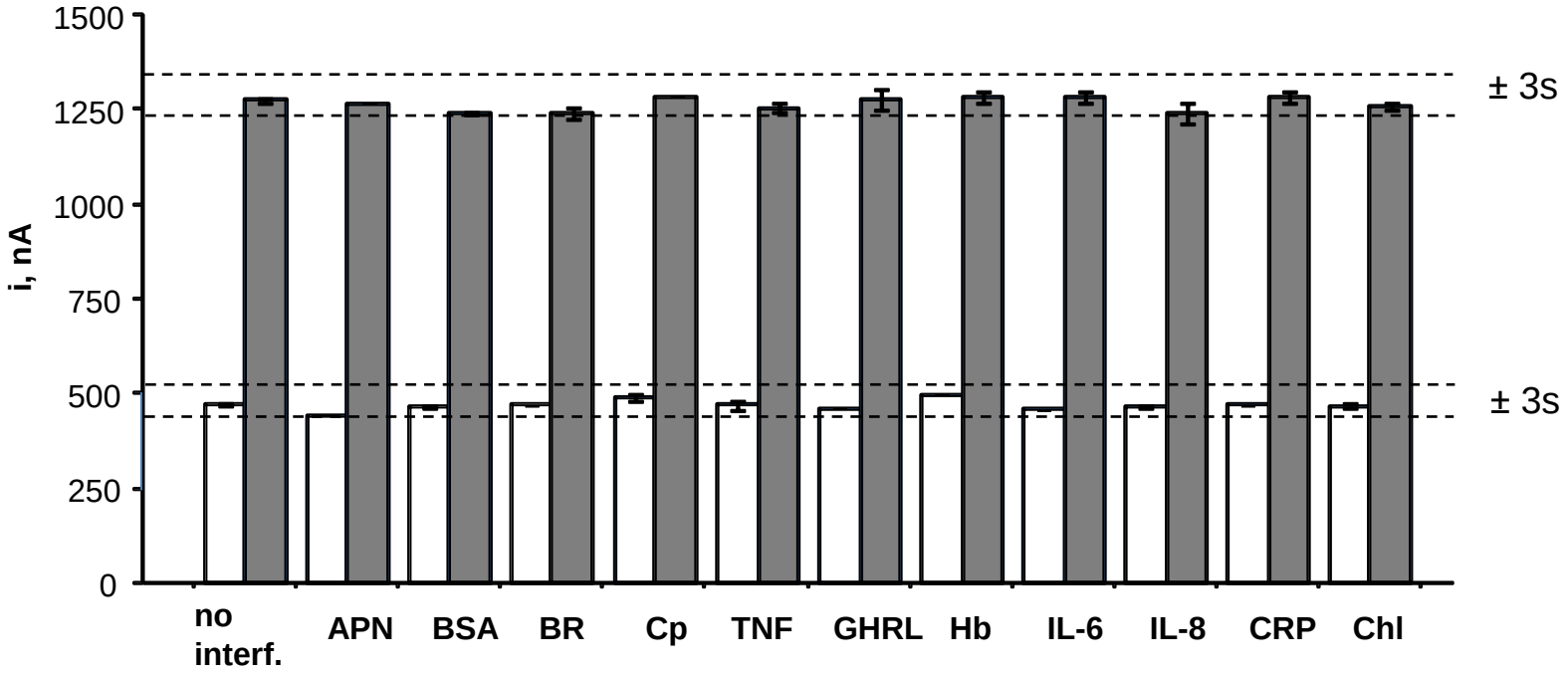












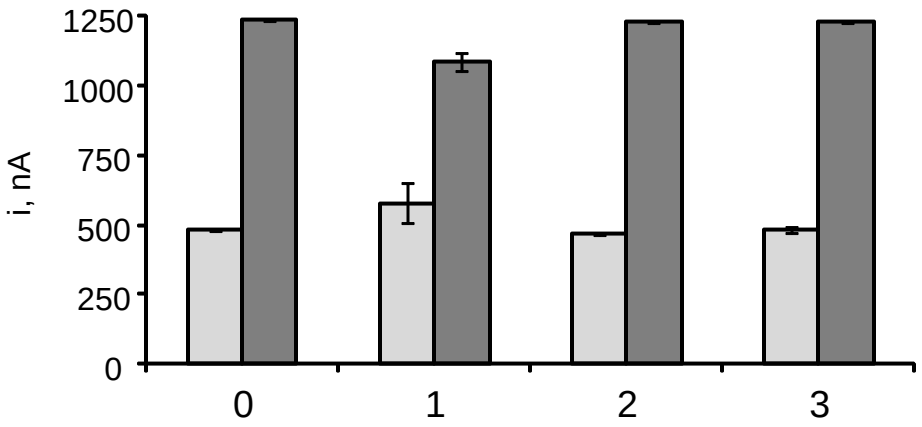


Table 1. Determination of TGF-β1 in human serum with the polyHRP-Strept-Biotin-anti-TGF-TGF-β1-anti-TGF-IgG-MWCNTs/SPCE

Spiked serum	TGF–β1, pg/mL	TGF–β1 found, pg/mL	Recovery, %
	25	25 ± 2	100 ± 9
	75	76 ± 3	101 ± 4
	125	123 ± 1	98 ± 1
Real serum	**Immunosensor, ng/mL**	**ELISA, ng/mL[a]**	
(SG609-2, BBI Solutions)*	1.38 ± 0.05	1.40 ± 0.02	
(SG376-2, BBI Solutions)**	2.11 ± 0.06	2.10 ± 0.04	

[a]Mean value ± ts (n=8, α=0.05)

*From a health female; **From a hypercholesterolemic female.