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The first electrochemical immunosensor for TGF-β1 cytokine in human serum based on carbon nanotubes functionalized by click chemistry is reported.

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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* and J. M. Pingarrón

An electrochemical immunosensor for the determination of the multifunctional Transforming Growth Factor β1 (TGF-β1) cytokine has been prepared using multi-walled carbon nanotube (MWCNT)-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 their biological activity. Alkyne-functionalized IgGs were also prepared and used to assemble IgG-azide–MWCNT conjugates used as scaffolds for the immunosensor preparation. After a blocking step with casein, anti-TGF was 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 hydrogen peroxide (H2O2) system. The calibration plot for TGF-β1 exhibited a range of linearity (r² = 0.995) extending between 5 and 200 pg mL⁻¹ which is suitable for the determination of the target cytokine in human serum. A limit of detection of 1.3 pg mL⁻¹ was achieved. The analytical performance of the immunosensor can be advantageously compared with that claimed for ELISA kits. The immunosensor was applied to the analysis of spiked human serum samples at different concentration levels with excellent recoveries.

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 under physiological conditions.¹ The methodology, involving azide and alkyne complementary functions, can be used for the functionalization of electrode surfaces² which can be used as platforms where biomolecules can be covalently coupled to different substrates without altering their configurations and preserving their biological activity.

Furthermore, carbon nanotubes (CNTs) have demonstrated largely the ability to promote the electron transfer reaction of a large number of molecules³ thus making CNTs an attractive material for the development of electrochemical sensors and biosensors. A large number of enzyme biosensors using CNT-modified electrodes as convenient scaffolds have been described in the literature.⁴ However, CNT-modified electrode based immunosensors have been reported to a much smaller extent. This is probably because biomolecule 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 in this methodology, only one electrochemical immunosensor has been reported so far using the Cu(i)-catalyzed azide–alkyne cycloaddition (CuAAC) reaction to prepare CNT-based platforms for immobilization of immunoreagents. Alkyne-IgG was coupled on single-walled carbon nanotubes (SWCNTs) functionalized with azide groups.
and the bioconjugate was cast onto a glassy carbon surface to develop a competitive electrochemical immunoassay for the determination of anti-IgG as the target compound with HRP as an enzyme label.\textsuperscript{6}

On the other hand, there is also recent growing interest in the determination of cytokines\textsuperscript{7} due to their demonstrated relationship with inflammation or disease progression. Among cytokines, the multifunctional TGF-β1 (Transforming Growth Factor β1) is involved in various immune and inflammatory diseases,\textsuperscript{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\textsuperscript{-1} in plasma have been reported for healthy individuals,\textsuperscript{9} while circulating levels of this cytokine have been shown to be increased in patients suffering from various types of diseases such as various cancers\textsuperscript{10,11} or autoimmune disorders,\textsuperscript{12} and severely depressed in advanced atherosclerosis.\textsuperscript{13}

The current method to determine this protein is by ELISA. There is 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\textsuperscript{-1} with minimum detectable concentrations of a few pg mL\textsuperscript{-1}. However, the time required for this type of assay 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] ranging between 1 and 1000 ng mL\textsuperscript{-1} with a detection limit of 0.570 ng mL\textsuperscript{-1} was reported.\textsuperscript{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 the CuAAC reaction to synthesize alkyn-azole conjugates. MWCNTs were selected for such a purpose due to their well-known excellent conducting and electrocatalytic properties.\textsuperscript{15} The steps involved in the oxidation of carbon nanotubes and functionalization with an azide group as well as alkyn-functionalization of IgG and preparation of IgG-alkyne-azole–MWCNT 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 a 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\textsuperscript{-1} concentration. A chicken biotinylated antibody (Biotin-anti-TGF) reconstituted up to a 18 μg mL\textsuperscript{-1} concentration with 1 mL of Reagent Diluent 1 (R&D Systems) was also used. Both anti-TGF antibodies and human TGF-β1 were the same as 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 and 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 (BlockerTM 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-hydroxysuccinimide (NHS) were from Acros. N,N’-Dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide (NHS) were from Sigma. Other reagents used were 11-azide-1,6,9-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 IgGraph 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 Iśmyen System constant temperature incubator shaker (Comecta S.A.). Electrochemical impedance spectroscopy was carried out 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 homogenizer from Velp Scientifica were also employed.

2.3. Procedures

Azide-functionalized MWCNTs and alkyn-functionalized IgG were prepared by following the procedures described in the ESF1 (Procedures S1–S3) and are schematized in Fig. S1 and S2,\textsuperscript{†} respectively.

2.3.1. Preparation of the electrochemical immunosensor.

Fig. 1 shows schematically the steps involved in the preparation of the amperometric immunosensor for TGF-β1. Step 1
Fig. 1  Schematic display of the different steps involved in the preparation and functioning of the poly-HRP-Strept-Biotin-anti-TGF-TGFB1-anti-TGF-IgG-MWCNT/SPCE immunosensor.

depicts the preparation of IgG-alkyne-azide-MWCNT 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⁻¹ azide-functionalized-MWCNT dispersion were added and magnetically stirred at 4 °C overnight. Step 2 involves 5 µL dropping of the IgG-alkyne-azide-MWCNT conjugate dispersion onto the SPCE surface and allowing it to dry, 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⁻¹ anti-TGF solution and incubation for 60 min. Thereafter, 5 µL of a TGFB1 standard solution (or the sample), and 5 µL of a 1 µg mL⁻¹ 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 TGFB1. Determination of TGFB1 was accomplished by dropping 45 µL of a 1 mM hydroquinone (HQ) solution in 0.5 M PBS of pH 6.0 onto the surface of the poly-HRP-Strept-Biotin-anti-TGF-TGFB1-anti-TGF-IgG-MWCNT/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 H₂O₂ solution were added and allowed to stand 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.

TGFB1 determination was performed by applying this procedure to both serum samples spiked at 25, 75 and 125 pg mL⁻¹ 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 TGFB1 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 is described in section 2.3.1 and depicted in Fig. 1, azide-functionalized MWCNTs and alkyne-functionalized IgG were
3.1. Optimization of the variables involved in the preparation and performance of the immunosensor

Once IgG-alkyne–azide–MWCNT conjugates were prepared according to the procedure described in section 2.3.1, an aliquot of this conjugate dispersion was dropped onto the SPE 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 the evaluation of: (a) the IgG-alkyne–azide–MWCNT loading on the SPE; (b) the type of blocking agent; (c) the type of enzymatic labeling of the detection antibody; (d) the concentration of poly-HRP-Strept; (e) the anti-TGF loading on the IgG-alkyne–azide–MWCNT/SPE; (f) the Biotin-anti-TGF loading on the TGF β1-anti-TGF-IgG-alkyne–azide–MWCNT/SPE. Details on these optimization studies are given in the ESI (Fig. S4–S7).

Electrochemical impedance spectroscopy (EIS) was employed to monitor the steps involved in the electrode modification. Fig. 3 shows the Nyquist plots recorded at the SPE, azide–MWCNT/SPE, IgG-alkyne–azide–MWCNT/SPE, and anti-TGF–IgG-alkyne–azide–MWCNT/SPE, using 5 mM Fe(CN)₆³⁻/⁴⁻ as the redox probe in 0.1 M PBS of pH 7.4, as well as the equivalent circuits used to fit the 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 nested parallel RC circuits mean that there is a film with defects such as pinholes or with a non-uniform thickness throughout the substrate. As expected, the charge transfer resistance at the bare SPE (curve 1) is notably higher ($R_{CT} = 1247$ ohm) than that measured at the azide–MWCNT/SPE (curve 2, $R_{CT} = 240$ ohm) due to the well-known electron transfer promotion observed at carbon nanotube-modified electrodes. Furthermore, the resistance increased to a value of 658 ohm when the IgG-alkyne–azide–MWCNT conjugate was deposited onto the SPE 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

Fig. 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–MWCNT/SPE 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⁻¹ according to the equation $\Delta i, nA = 0.50 [TGF-β1] pg mL^{-1} + 3.25$. In this equation, $\Delta i$ is the current obtained by subtracting the background current measured in the absence of an antigen from the amperometric response of the immunosensor. Interest-

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

synthesized and used to prepare IgG-alkyne–azide–MWCNT conjugates by Cu(ii) catalyzed azide-alkyne-cycloaddition (CuAAC). The ESI† (Procedure S1) describes the employed method to prepare azide-MWCNTs by covalent immobilization of 11-azide-3,6,9-trioxadecan-1-amine onto oxidized MWCNTs. Fig. S3 in the ESI† 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, azlkyne-IgGs were prepared using two different protocols (Procedures S2 and S3) involving the use of succinimidyl-3-propionate or sodium periodate, respectively. These protocols were compared by testing the responses obtained with TGF-β1 immunosensors prepared according to procedure 2.3.1 and using each azlkyne-IgG derivative. Fig. 2 shows the amperometric measurements obtained for 0 (unspecific) and 200 pg ml⁻¹ TGF-β1 with the immunosensors constructed with azlkyne-IgG prepared by both methods. As can be seen, the specific-to-unspecific current ratio was about 25% larger when periodate was used to get alkynl-IgG. This finding is in agreement with the claimed properties of this reagent able to oxidize 1,2-diols of carbohydrates existing in the Pc region of antibodies thus leading to the oriented modification of IgG. Conversely, this oriented modification is not produced when succinimidyl-3-propionate is used. Accordingly, the protocol involving the use of periodate for the preparation of azlkyne-functionalized IgG was selected for further work. Fig. S3 in the ESI† also shows the IR absorption spectrum obtained after the click reaction of the azide group in IgG-alkyne–azide–MWCNTs. As can be seen, the band at 2106 cm⁻¹ did not appear in this case suggesting the successful binding of azlkyne-anti-IgG.
Fig. 3 Nyquist plots recorded at the SPCE (1), azide–MWCNT/SPCE (2), IgG-alkyne–azide–MWCNT/SPCE (3), and anti-TGF-IgG-alkyne–azide–MWCNT/SPCE (4), and equivalent circuits using 5 mM Fe(CN)$_6^{3-/4-}$ as the redox probe in 0.1 M PBS of pH 7.4.

Fig. 4 Calibration plot and typical amperometric responses for TGF-β1 recorded with the poly-HRP-Strept–Biotin-anti-TGF–TGF-β1–anti-TGF–IgG–MWCNT/SPCE: (a) 0 pg mL$^{-1}$; (b) 5 pg mL$^{-1}$; (c) 10 pg mL$^{-1}$; (d) 15 pg mL$^{-1}$; (e) 30 pg mL$^{-1}$; (f) 50 pg mL$^{-1}$; (g) 100 pg mL$^{-1}$; (h) 125 pg mL$^{-1}$; (i) 200 pg mL$^{-1}$ TGF-β1. See the text for more information.

ingly, 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$^{-1}$.

The limit of detection, 1.3 pg mL$^{-1}$, 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$^{-1}$). Moreover, the limit of determination was calculated as 10$s_b$ and the value was 4.3 pg mL$^{-1}$. 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$^{-1}$ with minimum detectable concentrations of a few pg mL$^{-1}$. 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 1 h 20 min required for the immunosensor counting, in both cases, because of 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, the reported LOD was 0.570 ng mL⁻¹ 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⁻¹ 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-MWCNT/SPCE bioelectrode was also tested. In order to do that, different immunosensors were prepared on the same day and stored at 8 °C in a humid environment with 20 μL of Reagent Diluent 1. Then the immunosensors were used to measure 125 pg mL⁻¹ TGF-β1 on different days according to the procedure described in sections 2.3.1 and 2.3.2. A control chart was constructed (Fig. S7†) by setting ±3s as control limits, where s is the standard deviation of the measurements (n = 10) carried out on the first day. The immunosensor responses remained within the control limits for at least 40 days (no longer storage times were tested) demonstrating a good stability of the prepared anti-TGF-IgG-MWCNT/SPCE.

Regarding the selectivity of the immunosensor, it is important to note that anti-TGF antibodies were the same as 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 such as adiponectin (APN), bovine serum albumin (BSA), bilirubin (BR), ceruloplasmin (Cp), tumor 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 the serum of healthy individuals. Fig. 5 shows clearly that 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 effects in human serum was firstly evaluated. Fig. 6 compares the amperometric measurements recorded with the poly-HRP-Strept-Biotin-anti-TGF-β1-anti-TGF-β1-IgG-MWCNT/SPCE in 0.1 M
PBS of pH 7.4 and in human serum containing no TGF-β1 or spiked with 125 pg mL\(^{-1}\) TGF-β1 (final concentration) upon dilution with 0.1 M PBS of pH 7.4 at different ratios. As can be deduced, measurements at a 1/10 serum dilution ratio (bars 1) showed a slight increase in the mean current value (n = 3) for the blank (light grey), together with a slight decrease of the mean current value for 125 pg mL\(^{-1}\) 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\(^{-1}\), was carried out. The slope value of the linear calibration graph obtained for spiked serum samples was \(5.57 \pm 0.01\) 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 effects. 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\(^{-1}\) TGF-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 can be seen in Table 1, mean TGF-β1 concentrations of 1.38 ± 0.05 ng mL\(^{-1}\) and 2.11 ± 0.06 ng mL\(^{-1}\) were found with the immunosensor (n = 8) for the healthy and hypercholesterolemic 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 with copper(II) catalyzed azide–alkyne cycloaddition for binding of alkyne-functionalized IgGs and formation of IgG-alkyne–azide–MWCNT 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 of only a 1/100 dilution with 0.1 M PBS of pH 7.4.

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