Electrochemical immunosensor for the determination of 8-isoprostane aging biomarker using carbon nanohorns-modified disposable electrodes

E. Sánchez-Tirado, A. González-Cortés, M. Yudasaka, S. Iijima, F. Langa, P. Yáñez-Sedeño, J.M. Pingarrón

PII: S1572-6657(16)30607-5
DOI: doi:10.1016/j.jelechem.2016.11.003
Reference: JEAC 2932

To appear in: *Journal of Electroanalytical Chemistry*

Received date: 15 September 2016
Revised date: 24 October 2016
Accepted date: 2 November 2016

Please cite this article as: E. Sánchez-Tirado, A. González-Cortés, M. Yudasaka, S. Iijima, F. Langa, P. Yáñez-Sedeño, J.M. Pingarrón, Electrochemical immunosensor for the determination of 8-isoprostane aging biomarker using carbon nanohorns-modified disposable electrodes, *Journal of Electroanalytical Chemistry* (2016), doi:10.1016/j.jelechem.2016.11.003

Electrochemical immunosensor for the determination of 8-isoprostane aging biomarker using carbon nanohorns-modified disposable electrodes

E. Sánchez-Tirado[a], A. González-Cortés[a], M. Yudasaka[b], S. Iijima[c], F. Langa[d], P. Yáñez-Sedeño[a]*, J.M. Pingarrón[a]

[a] Department of Analytical Chemistry, Faculty of Chemistry, University Complutense of Madrid, Ciudad Universitaria s/n, 28040-Madrid. Spain

[b] Nanotube Research Center, National Institute of Advanced Industrial and Technology, Higashi, Tsukuba, Ibaraki 305-8565, Japan

[c] Department of Physics, Meijo University, Shiogamaguchi, Tenpaku-ku, Nagoya 468-8502, Japan

[d] Instituto de Nanociencia, Nanotecnología y Materiales Moleculares (INAMOL), Universidad de Castilla-La Mancha, 45071-Toledo, Spain

*Corresponding author: yseo@quim.ucm.es

Abstract

The first electrochemical immunosensor for the determination of 8-isoprostane (8-iso prostaglandin F2α, ISO), one of the most reliable biomarkers of lipid peroxidation in the human body and of aging related to Alzheimer´s disease or atherosclerosis is reported in this article. Disposable screen-printed carbon electrodes modified with carboxylated carbon nanohorns (CNHs) were employed as scaffolds for covalent immobilization of a specific anti-ISO antibody). A competitive immunoassay involving ISO and HRP-labeled antigen was designed and the determination of ISO was carried out by amperometry at -200 mV using the H_2O_2/hydroquinone (HQ) system. Under the optimized conditions, the immunosensor provides a linear response for ISO (r^2 = 0.998) extending up to 700 pg/mL, which is suitable for the determination of the target compound in human serum. The analytical performance of the immunosensor improves that claimed for ELISA kits in terms of linearity of the calibration plot, precision, with RSD values lower than 1 % , and assay time (1h 30min), and exhibits a low limit of detection, 12 pg/mL, a long storage stability (30 days), and an excellent selectivity against other proteins that may be found in human serum. The analytical utility of the developed immunosensor was demonstrated by determining ISO in two types of human serum samples: lyophilized spiked serum, and real human serum from healthy male and female individuals with good results.

Keywords: Aging biomarkers, electrochemical immunosensors, 8-isoprostane, prostaglandin

1. Introduction

Isoprostanes are prostaglandin–like compounds produced through the oxygen radical induced peroxidation of tissue phospholipids and lipoproteins [1]. Among them, 8-isoprostane (8-iso-prostaglandin F2α, ISO) (Figure 1), a stable product of oxidative stress, is one of the most reliable biomarkers of lipid peroxidation in the human body [2], and of aging related to Alzheimer´s disease or atherosclerosis [3]. Although detectable quantities of isoprostanes are usually present in tissues and biological fluids including plasma, saliva and urine, their levels increase as the oxidative damage increases [4]. Relatively high isoprostanes concentrations appear in smokers, and in patients suffering cardiovascular diseases, diabetes, obesity, hypercholesterolemia, metabolic syndrome [5], or cancer [6]. Although GC–MS and LC–MS/MS are the recommended techniques for the determination of 8–isoprostane in clinical samples [7,8], the methods, mainly applied to urine, are characterized by their high cost, extensive sample extraction and low throughput. As an alternative, ELISA immunoassays are also used. Table 1 summarizes the analytical characteristics claimed for various commercial ELISA kits for the determination of ISO. All configurations used involve competitive-type immunoassays and most of them use HRP-labeled immunoreagents and colorimetric detection upon H_2O_2 addition in the presence of 3,3´,5,5´-tetramethylbenzidine (TMB). Less common are those using acetylcholinesterase (AChE) or alkaline phosphatase-labeled ISO. The addition of Ellman´s reagent containing the substrate of AChE or p-nitrophenyl phosphate, respectively, allow the colorimetric detection to be performed. Despite the claimed advantages of immunosensors versus ELISA concerning higher sensitivity and lower detection limits, better precision, and shorter assay times [9,10], no immunosensors for the determination of ISO have been described in the literature so far.

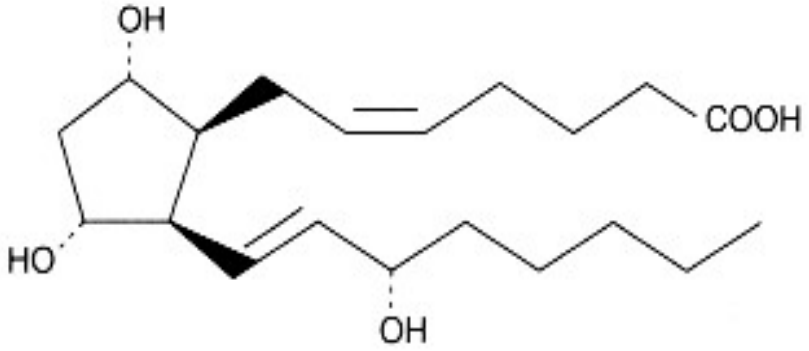

Figure 1. Structure of 8-isoprostane (8-iso prostaglandin F2α, ISO).

On the other hand, single-walled carbon nanohorns, SWCNHs (or simply CNHs), are a relatively new type of carbon allotrope consisting of a unique horn-shaped graphene with a diameter of 2-5 nm and a length of 40–50 nm [11]. CNHs assemble to form nanostructures shaped like dahlia flowers [12], with a diameter around 100 nm, that enables an easy dispersion in liquids [13]. Another important feature is that production of CNHs is performed in the absence of metal catalysts, so that they can be used directly without post treatment or purification [14]. Moreover, CNHs possess high conductivity, large surface area, and abundant oxidizable defects that provide a great number of oxygenated groups affordable for biomolecules immobilization [15]. Despite these interesting properties, scarce applications have been reported up to date related with the preparation of electrochemical immunosensors involving CNHs functionalized electrodes [16]. A competitive immunoassay design for the determination of microcystin-LR (MC-LR) involving covalent immobilization of the antigen to oxidized CNHs and the use of HRP-anti-MC-LR was reported by Zhang et al [17]. More recently, a sandwich-type impedimetric immunosensor for α-fetoprotein (AFP) was prepared by linking HRP and GOx enzymes to CNHs and biocatalyzed precipitation in the presence of 4-chloro-1-naphthol and H_2O_2 which led to a significant enhancement of the signal from $Fe(CN)_6^{3-/4-}$ redox couple [18]. Our group also used carboxylated CNHs

to construct electrochemical platform for the preparation of an immunosensor for fibrinogen (Fib) [19]. The approach involved the use of HOOC-CNHs deposited onto a SPCE to covalently immobilize Fib, and the establishment of an indirect competitive assay with horseradish peroxidase labeled Fib. A composite combining CNHs and alginic acid was also used to develop a sandwich-type enzyme-free immunosensor for carcinoembryonic antigen (CEA) [20]. The large concentration of immobilized antibodies provided a high sensitivity that was enhanced by using magnetic $NiCo_2O_4$ NPs decorated with hematin as labels for the secondary antibodies. A Chitosan/CNHs composite demonstrated to be also a suitable electrochemical platform to construct an electrochemical immunosensor for alpha-fetoprotein (AFP) [21].

In this work the first electrochemical immunosensor for the determination of ISO is reported. A simple and rapid strategy was developed involving the use of disposable screen-printed carbon electrodes modified with carboxylated CNHs as scaffolds for covalent immobilization of a specific antibody (anti-ISO). A competitive immunoassay involving the target antigen (ISO) and HRP-labeled antigen (HRP-ISO) was employed. ISO quantification was carried out by amperometry at -200 mV (vs. Ag pseudo-reference electrode) using the H_2O_2/hydroquinone (HQ) system. Therefore, the reduction current of the generated quinone at the SPCE surface was the analytical readout. All the steps involved in the preparation and performance of anti-ISO-CNHs/SPCE immunosensor were optimized and, under the optimized conditions, ISO could be determined with a high sensitivity. The analytical utility of the developed immunosensor was demonstrated by determining ISO in two types of human serum samples: lyophilized spiked serum, and real human serum from healthy male and female individuals with good results.

2. Experimental

2.1. Reagents and solutions

The rabbit anti-ISO capture antibody, the antigen (ISO), and the HRP-labeled antigen (HRP-ISO) used were the reagents included in the ELISA kit OxiSelect™ (STA-337) from Cell Biolabs. Working solutions from the antibody were prepared in a 25 mM 2-(N-morpholino) ethanesulfonic (MES, Gerbu) buffer solution of pH 5.0. Sample Diluent from Cell Biolabs was the solvent used to prepare ISO and HRP-ISO working solutions. CNHs were synthesized in the absence of metallic catalysts by graphite ablation with a CO_2 laser under argon atmosphere at 760 Torr and room temperature. The purity was higher than 90% and the 10% impurity was micrometer-sized graphitic balls [22]. CNHs oxidation was performed by treatment with a 30% H_2O_2 aqueous solution at ≈100ºC [23]. Activation of the carboxylic groups of CNHs for further immobilization of capture antibodies was made by using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (NHSS) (Acros Organics). Bovine serum albumin (BSA) from Gerbu was used as blocking agent. Compounds tested as potential interfering substances were: adiponectin (APN), ceruloplasmin (Cp), interleukin-6 (IL–6), interleukin-8 (IL–8) (Abcam), bilirubin (BR) (Aldrich), cholesterol (Chol), creatinin (CR), hemoglobin (HB) (Sigma), transforming growth factor β1 (TGF–β1), tumor necrosis factor α (TNF–α) (R&D Systems), and C reactive protein (CRP) (Fitzgerald). Hydrogen peroxide (Aldrich, 30% (w/w)) and hydroquinone (HQ) (Sigma) were used for the amperometric measurements. Deionized water from a Millipore Milli–Q (18.2 MΩ·cm at 25 ºC) purification system was also used.

Samples analyzed were lyophilized human serum (S2257, Sigma) spiked with 30, 35 and 40 pg/mL ISO, and real human serum samples from healthy male and female individuals (BBI Solutions, SG610–2 y SG609–2, respectively). Validation was

accomplished by comparing the results with those obtained by using the OxiSelect™ ELISA kit (STA-337) from Cell Biolabs.

2.2. Apparatus and electrodes

Amperometric measurements were made with an INBEA potentiostat using the IbGraph software (INBEA S.L., Madrid, Spain). 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. Electrochemical impedance spectrocopy was carried out with an Autolab type III potentiostat (Ecochemie) controlled by FRA2 software. 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, an MPW-65R centrifuge (MPW Med. Instruments), and a Vortex homogenizator from Heidolph were also used. Absorbance measurements in the ELISA method were made using a Sunrise™ Tecan microplate reader provided with the Magellan software. All experiments were performed at room temperature.

2.3. Procedures

2.3.1. Preparation of the immunosensor

Oxidized CNHs (1 mg) were suspended in 2 mL of deionized water in an Eppendorf tube and ultrasonicated for 90 min. Once homogenized, a 5-µL aliquot was transferred to the electrode surface and dried under IR radiation. This operation was repeated by depositing another 5-µL aliquot. Activation of CNHs carboxyl groups was made by addition of 5 µL of a 0.1 M each EDC/NHSS mixture solution, prepared in 25 mM MES buffer solution of pH 5.0, onto CNHs/SPCE, and let to stand during 30 min. Thereafter,

immobilization of the capture antibody was accomplished by adding 5 μL of a 1/600 diluted anti-ISO solution prepared in 25 mM MES buffer solution of pH 5.0, allowing incubation for 60 min at 25°C. Then, the anti-ISO-CNHs/SPCE immunosensor was washed with deionized water and allowed drying in air. Next, a blocking step was applied by adding 5 μL of a 2 % BSA solution and incubating for 30 minutes followed by washing with water. Finally, the resulting immunosensors were preserved by adding 50 μL of 50 mM phosphate buffer solution of pH 6.0 until use.

2.3.2. Immunoassay at anti-ISO-CNHs/SPCE

A competitive immunoassay scheme was employed for the determination of ISO. A mixture solution of the antigen (ISO) and the labeled antigen (HRP-ISO) was prepared in an Eppendorf tube with 5 μL of stardard ISO solutions of variable concentration or the sample, and 5 μL of a 1/250 diluted HRP-ISO solution. Then, 5 μL from this mixture solution were dropped onto the as prepared anti-ISO-CNHs/SPCE and incubated for 45 minutes. Amperometric measurements were obtained after addition of 45 μL of a 1 mM HQ solution prepared in a 50 mM phosphate buffer solution of pH 6.0, and by applying a detection potential of -200 mV. Once the background current was stabilized (200 s approximately), a 5-μL aliquot of 50 mM hydrogen peroxide solution was added and, after a period of 200 s for allowing the enzymatic reaction to take place, the reduction current of the formed quinone was measured.

2.3.3. Analysis of human serum

As indicated above, two types of human serum samples were analyzed. Lyophilized solid serum was reconstituted in deionized water and, once spiked, it was 1:1 diluted with HRP-ISO solution. No further treatment was necessary. A simple treatment was applied to real serum samples. It consisted of the addition of 100 μL of 10 M NaOH to 400 μL of sample and incubation for two hours at 45 ºC. This treatment was applied to

hydrolyze lipoprotein and phospholipid coupled ISO in order to measure both free and esterified ISO. Then, 100 µL of 10 M HCl were added to assist precipitation of lipoproteins and phospholipids. Centrifugation at 12,000 rpm for 5 min was carried out and the supernatant should be in the 6-8 pH range. In both cases, the determination of ISO was carried out by interpolation of the amperometric responses measured for the samples into the calibration plot constructed with ISO standards. Furthermore, validation of the results for real serum samples was accomplished by comparison with those provided by an ELISA method for human ISO from Cell Biolab. In this case, the protocol indicated in the suppliers directions was followed. In brief, this consisted of pipetting a mixture solution of ISO standards or samples and HRP-ISO into the anti-ISO coated-well plates and leaving an incubation time of 60 min. Then, colour development was achieved by addition of TMB substrate solution and after 20 min, an acid "stop solution" was added, and the absorbance was measured at 450 nm.

3. Results and discussion

The synthesis and characterization of CNHs were performed as described previously [19]. As indicated in the Experimental section, 30 % aqueous H_2O_2 was used for CNHs oxidation and generation of carboxylic acid groups [23] for further antibodies immobilization. The fundamentals of the different steps involved in the immunoreaction occurring on the activated functionalized CNHs-modified SPCE as well as of the amperometric transduction strategy are schematically depicted in Figure 2. Once carboxylated CNHs were adsorbed on the electrode surface, anti-ISO antibodies were covalently immobilized and the remaining active sites on the SPCE surface blocked with BSA. Thereafter, a competitive immunoassay strategy was designed by dropping onto the anti-ISO-CNHs/SPCE immunosensor a mixture solution of the target analyte

and HRP-ISO. The amperometric response for ISO quantification was measured upon addition of H_2O_2 as HRP substrate in the presence of HQ as the redox mediator.

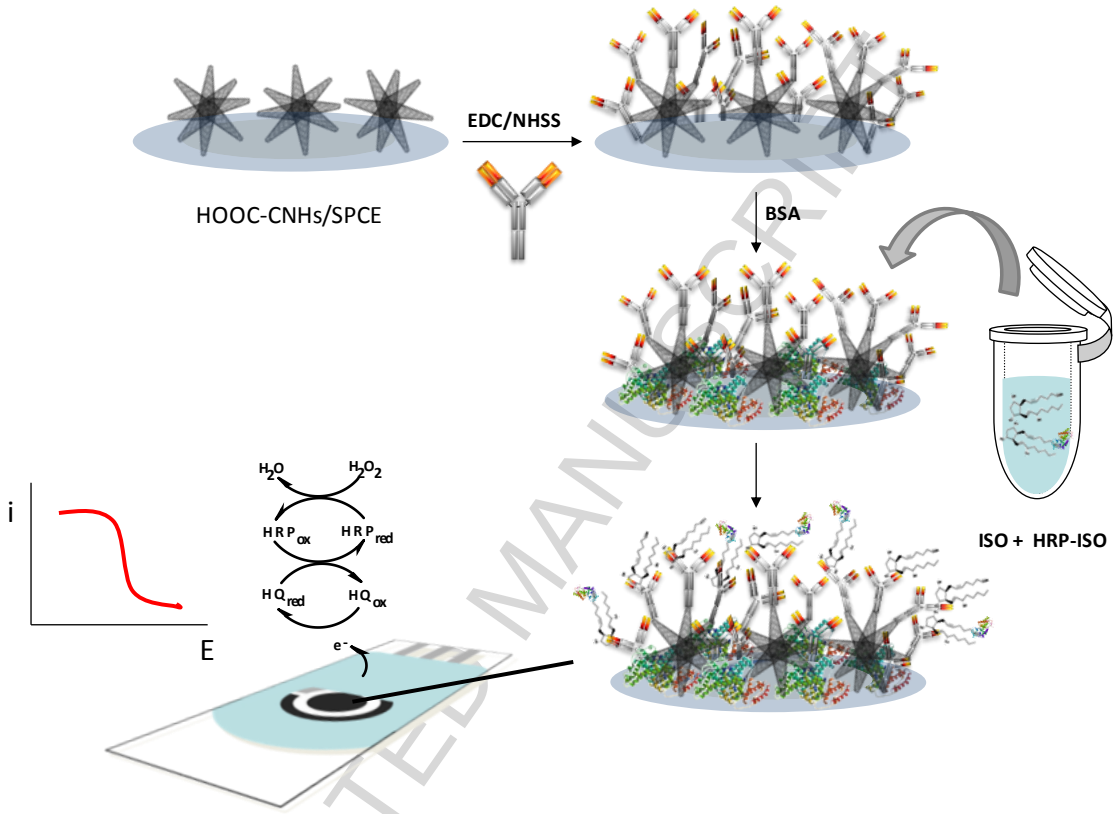

Figure 2. Schematic display of the different steps involved in the preparation and functioning of the anti-ISO-CNHs/SPCE immunosensor

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

Variables involved in the preparation of the immunosensor and its electrochemical performance were optimized. The evaluated variables were: a) loading of anti-ISO antibody immobilized onto CNHs/SPCE; b) concentration of HRP-ISO conjugate; c) time for competition between ISO and HRP-ISO for the binding sites of the capture antibody; d) reagents and incubation time used for blocking the remaining unmodified sites on the electrode surface. Details on the optimization studies are provided in the

Supporting Information and in Figures S1-S4. Other variables such as the amount of CNHs adsorbed onto the electrode surface (5 μg) or the composition of the H_2O_2/HQ system and the detection potential were optimized in previous works [19, 24].

3.2. Characterization of the immunosensor by electrochemical impedance spectroscopy and cyclic voltammetry

Electrochemical impedance spectroscopy (EIS) was employed to monitor the different steps involved in the preparation of the immunosensor. Figure 3a shows the Nyquist plots recorded for bare SPCE, HOOC-CNHs/SPCE, anti-ISO-CNHs/SPCE, and HRP-ISO-anti-ISO-CNHs/SPCE, using 5 mM $Fe(CN)_6^{3-/4-}$ in 100 mM KCl of pH 7.0 as the redox probe. As it can be seen, the charge transfer resistance at the bare SPCE (curve 1) was notably higher (R_{CT} = 284 ohm) than that measured for the CNHs//SPCE (curve 2, R_{CT} = 15 ohm) due to the expected electron transfer promotion occurring at carbon nanohorns-modified electrodes. The resistance increased to 45 ohm when the anti-ISO antibodies were deposited onto the CNHs/SPCE surface (curve 3), as a consequence of the isolating effect provoked by the biomolecules. It is interesting to remark that despite this isolating effect, the R_{CT} value is notably low which points out the electron transfer promotion caused by the presence of CNHs as electrode modifying material. Subsequent immobilization of the HRP-labeled antigen led to a further resistance increase (curve 4) (R_{CT} = 338 ohm), due to the lower conductivity of the resulting biosurface. Figure 3b shows cyclic voltammograms for the same redox probe solution recorded at SPCE. HOOC-CNHs/SPCE, anti-ISO-CNHs/SPCE, and HRP-ISO-anti-ISO-CNHs/SPCE. Results are consistent with those obtained by EIS. A noticeable increase in the oxidation and reduction peak current values for $Fe(CN)_6^{3-/4-}$ was observed when SPCEs were modified with HOOC-CNHs (voltammogram 2) which is

attributed to the improved electron transfer upon modification with CNHs. Surface immobilization of anti-iSO and formation of the immunocomplexes with HRP-ISO (voltammograms 3 and 4, respectively) produced slight decreases in the peak current values most likely due to the high conductivity of the modified electrode surface despite immobilization of biomolecules. These results confirmed the suitability of the used procedures for the electrode modification and immobilization of anti-ISO antibodies onto CNHs/SPCEs.

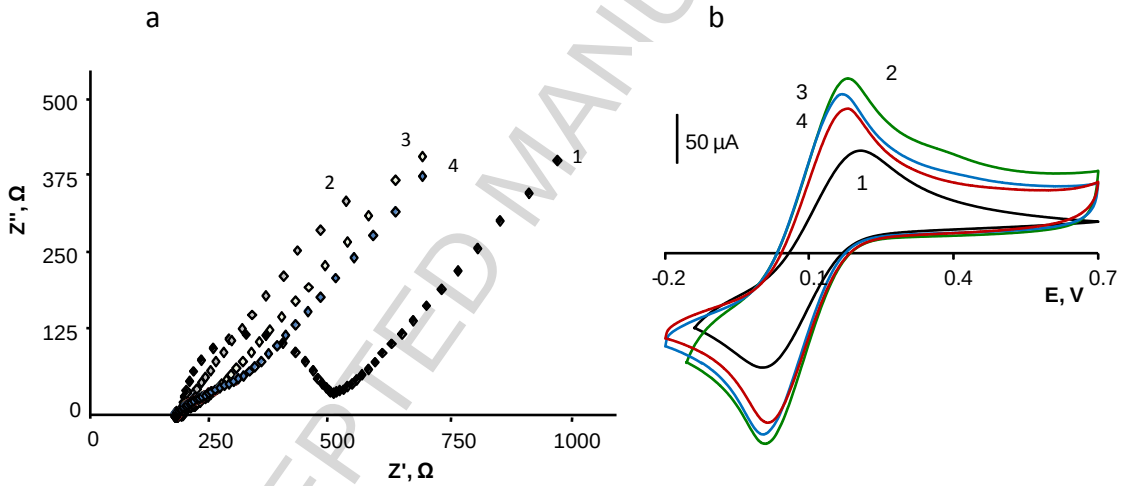

Figure 3. Nyquist plots (a) and cyclic voltammograms (b) recorded for (1) bare SPCE, (2) CNHs//SPCE, (3) anti-ISO-CNHs/SPCE, and (4) HRP-ISO-anti-ISO-CNHs/SPCE with 5 mM $Fe(CN)_6^{3-/4-}$ in 100 mM KCl of pH 7.0.

3.3. Analytical figures of merit of the immunosensor

Figure 4 shows the linear calibration plot (r^2 = 0.998) for ISO constructed by amperometry at -0.20 V with the anti-ISO-CNHs/SPCE immunosensor. The corresponding equation is: i_p, nA = (850 ± 8) [ISO, ng/mL] + (623 ± 3 nA), and the linear range extends up to 700 pg/mL ISO. Interestingly, this range is suitable for the determination of the target compound in human serum since the concentration levels found in healthy individuals are comprised between few tens and few hundreds of pg/mL [25, 26]. The limit of detection, 12 pg/mL, was calculated as the lowest concentration that can be statistically discriminated from zero, according to the $\bar{x} + 3 \cdot s$ criterion, where s was estimated as the standard deviation (n=10) for the blank (measurements in the absence of ISO) in concentration units (pg/mL). The limit of quantification achieved, $\bar{x} + 10 \cdot s$, was 19 pg/mL.

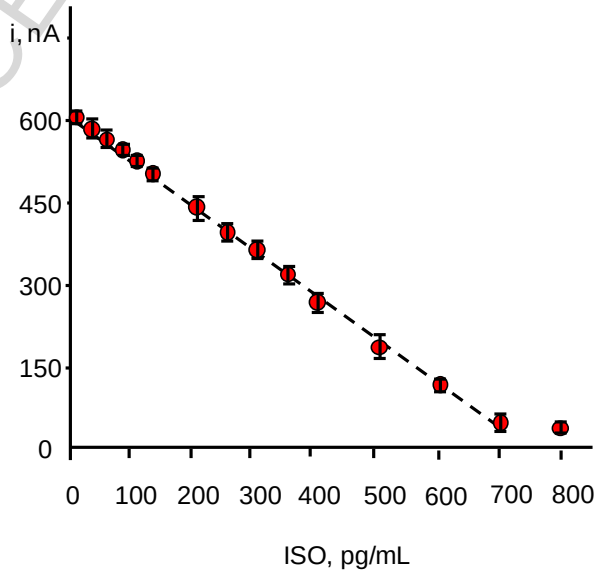

Figure 4. Calibration plot for ISO constructed with the anti-ISO-CNHs/SPCE immunosensor. See text for the experimental conditions.

The reproducibility of the amperometric measurements was tested by repetitive measurements carried out for 0 and 50 pg/mL ISO carried out with six different immunosensors. Relative standard deviations values, RSD, of 0.36 and 0.35 %, respectively were calculated for immunosensors prepared in the same day, while RSD values of 0.53 and 0.40 %, respectively, were found for immunosensors prepared in different days. These results demonstrated an excellent accuracy of the amperometric measurements and indicated that the proposed method for the immunosensor preparation was reliable and reproducible. When these analytical figures of merit are compared with data provided for commercial ELISA kits using similar immunoreagents, some noticeable differences become apparent. ELISA kits usually provide non-linear dynamic ranges covering from several tens to thousands of pg/mL with minimum detectable concentrations (MDC) of tens (or even one hundred) pg/mL. These parameters are calculated mostly from nonlinear logarithmic plots and the precision levels are around 10% or higher. It is important to note that the criteria used to calculate the MDC values for these kits are rarely given in the commercial protocols. Moreover, the time lasted for the assay is remarkably longer with ELISA kits which mostly require 2 h 30 min versus 1h 20 min needed when the immunosensor is used (counting, in both cases, since the immobilization of capture antibody). Therefore, it can be concluded that the analytical performance of the developed immunosensor, covering a wide linear range of clinically relevant ISO concentrations, improves, in general terms, the analytical performance claimed for ELISA kits.

The storage capability of the anti-ISO-CNHs/SPCE bioelectrode was also tested. Different immunosensors were prepared on the same day and stored at -20 ºC under dry conditions. Then, the immunosensors were used in different days to measure solutions without ISO. A control chart was constructed (Figure S5) 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 30 days, decreasing for longer storage periods probably as a consequence of the loose of activity of the antibody. These results suggested the possibility of preparing a set of anti-ISO-CNHs/SPCEs immunosensors and keeping them under storage until their use for the analysis of samples was requested.

Regarding the selectivity of the immunosensor, it is important to remind that anti-ISO antibodies used were the same that those employed in the ELISA kit OxiSelect^{TM} (STA-337) from Cell Biolabs. These antibodies exhibited a high specificity toward ISO (8-isoprostane, 8-iso-PGF2α), versus other types of similar compounds, with cross-reactivity factors of 4.6 % (PGF1α) or 1.8% (PGF2α), and much lower CR% for other derivatives [http://www.cellbiolabs.com/8-iso-prostaglandin-f2a-assay]. Therefore, we tested the additional selectivity of the capture antibody by measuring the amperometric responses of the immunosensor for 0 and 50 pg/mL ISO in the presence of other proteins: adiponectin (APN), bovine serum albumin (BSA), bilirubin (BR), cholesterol (Chol), ceruloplasmin (Cp), transforming growth factor β1 (TGF), tumoral necrosis factor alpha (TNF), hemoglobin (Hb), interleukin-6 (IL-6), and interleukin-8 (IL-8) at concentration levels that can be found in serum of healthy individuals. Figure 5 shows clearly as no significant differences were apparent 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 ISO.

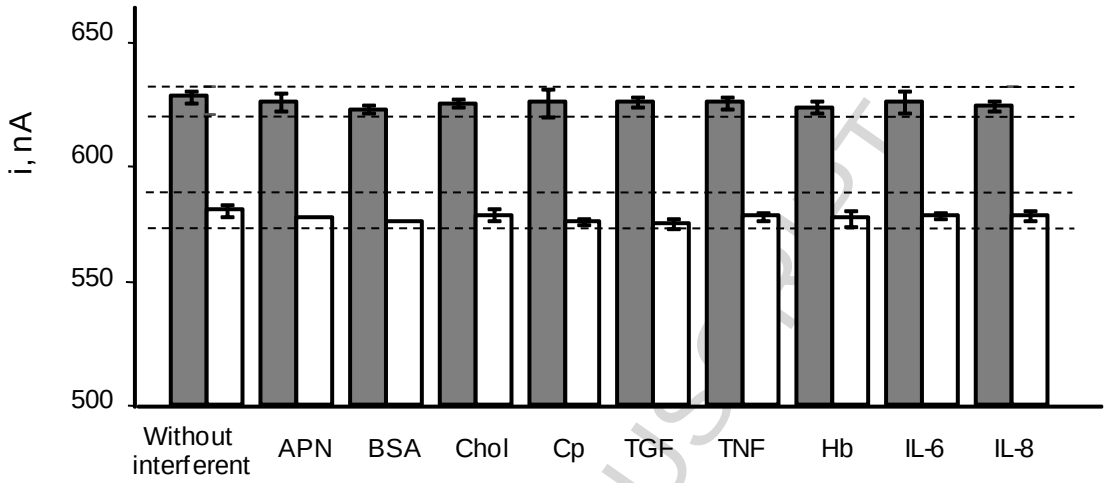

Figure 5. Interferences study: 0 (grey) and 50 (white) pg/mL ISO in the presence of 20 μg/mL adiponectin (APN), 5 mg/mL BSA, 20 μg/mL cholesterol (Chol), 500 μg/mL ceruloplasmin (Cp), 500 pg/mL transforming growth factor β1 (TGF), 100 pg/mL tumor necrosis factor α (TNF), 50 μg/mL hemoglobin (Hb), 50 pg/mL interleukin 6 (IL–6) , 30 pg/mL interleukin 8 (IL–8).

3.4. Determination of ISO in human serum

The developed immunosensor was applied to the determination of ISO in two types of human serum, lyophilized spiked serum, and real human serum from healthy male and female individuals by applying the procedures described in section 2.3.3. Regarding lyophilized serum, the possible existence of matrix effects was initially evaluated by constructing a calibration plot for reconstituted serum and spiked with ISO over the 0 to 40 pg/mL concentration range. The equation for the corresponding linear calibration graph was: i_p, nA = (896 ± 3) [ISO, ng/mL)] + (624 ± 1, nA). No statistically significant difference was found with the slope value of the calibration plot constructed with ISO standard solutions as the Student`s t_{exp} value, 1.247, was lower than the tabulated one,

t_{tab} = 4.303 for a 0.05 significance level. Therefore, no significant matrix effect was found and the determination of ISO could be carried out by interpolation of the current values measured for the serum samples into the calibration plot constructed with ISO standard solutions. Table 2 summarizes the results obtained by triplicate for lyophilized serum spiked at three different concentration levels. Recoveries ranged between 95 and 101%, indicating the reliability of the approach to determine low ISO concentrations in this kind of sample following a very simple working protocol.

Furthermore, two real human sera from male and female healthy individuals were also analyzed. As it was described in the Experimental section 2.3.3, a simple treatment was applied to these samples with the aim of hydrolizing lipoproteins and phospholipids to which ISO could be bound. This treatment was that recommended for the ELISA test for human ISO by Cell Biolab [http://www.cellbiolabs.com/8-iso-prostaglandin-f2a-assay]. Once verified the absence of matrix effects, the determination of ISO was carried out by interpolation of the amperometric responses for the samples into the calibration plot constructed with ISO standards. Validation of the obtained results was accomplished by comparing them with those provided by the OxiSelect^{TM} (STA–337) ELISA kit from Cell Biolabs following the procedure specified in Section 2.3.3. The results obtained by both methods are summarized in Table 2. RSD values (n=6) were in all cases lower than 2% exhibiting an excellent precision. The concentrations found were also statistically compared by means of the Struden´t *t* test, obtaining t_{exp} values of 1.714 (male) and 1.107 (female) which were lower than tabulated *t* (2.228). These results confirm that no significant differences were apparent between both methodologies at a significance level of 0.05. All these results demonstrate clearly the usefulness of the developed immunosensor to determine ISO at clinically relevant concentrations in human serum.

4. Conclusions

The first immunosensor for the determination of the aging biomarker 8-isoprostane is reported in this paper. In order to improve the analytical performance of the immunosensor an electrochemical platform consisting of disposable SPCE modified with carboxylated CNHs has been used as scaffold for the covalent immobilization of specific anti-ISO antibodies. Using a competitive immunoassay, ISO can be determined in human serum with a high sensitivity derived not only from the high conductivity of CNHs but also because of the large number of oxygenated moieties present on the CNHs-modified electrode surface allowing subsequent high loading of capture antibodies. The analytical performance exhibited by the electrochemical immunosensor is excellent, improving, in general terms, that provided by ELISA kits. The usefulness of the immunosensor was demonstrated by determining ISO in lyophilized spiked serum, and real human serum from healthy male and female individuals with results in statistic agreement with those found by applying an ELISA kit.

Acknowledgements

Financial support from the Spanish Ministerio de Economía y Competitividad, Research Projects CTQ2015-70023-R and CTQ2015-71955-REDT, and the NANOAVANSENS Program from Comunidad de Madrid (S2013/MT-3029) is gratefully acknowledged.

5. References

[1] T. Petrosino, M. Serafini, Antioxidant modulation of F2-Isoprostanes in humans: A systematic review, Crit. Rev. Food Sci. Nutr., 54 (2014)1202–1221.

[2] K. Mizuno, H. Kataoka, Analysis of urinary 8-isoprostane as an oxidative stress biomarker by stable isotope dilution using automated online in-tube solid-phase microextraction coupled with liquid chromatography–tandem mass spectrometry J. Pharm. Biomed. Anal. 112 (2015) 36–42.

[3] G.L. Milne, Q. Dai, L. J. Roberts II, The isoprostanes-25 years later, Biochim. Biophys. Acta, 1851 (2015) 433-445.

[4] T. A. McGowan, S. R. Dunn, B. Faulkner, K. Sharma, Stimulation of urinary TGF-β1 and isoprostanes in response to hyperglycemia in humans, Clin. J. Am. Soc. Nephrol. 1 (2006) 263–268.

[5] P. Montushi, P. Barnes, R.L. Jackson, II Insights into oxidative stress: The isoprostanes, Curr. Med. Chem., 14 (2007) 703-717.

[6] D.A. Barocas, S. Motley, M.S. Cookson, S.S. Chang, D.F, Penson, Q. Dai, G. Milne, L.J. Roberts II, J. Morrow, R.S. Concepcion, J.A. Smith Jr, J. H. Fowke, Oxidative stress measured by urine F2-isoprostane level is associated with prostate cancer, J. Urol., 185 (2011) 2102-2107.

[7] K.A. Smith, J. Shepherd, A. Wakil, E.S. Kilpatrick, A comparison of methods for the measurement of 8-isoPGF2α: a marker of oxidative stress, Ann. Clin.Biochem. 48 (2011) 147–154.

[8] J. Klawitter, M. Haschke, T. Shokati, J. Klawitter, U. Christians, Quantification of 15-F2α-isoprostane in human plasma and urine: results from enzyme-linked immunoassay and liquid chromatography / tandem mass spectrometry can not be compared, Rapid Commun. Mass Spectrom. 25 (2011) 463–468.

[9] I. Ojeda, M. Moreno-Guzmán, A. González-Cortés, P. Yáñez-Sedeño, J. M. Pingarrón, Electrochemical magnetoimmunosensor for the ultrasensitive determination of interleukin-6 in saliva and urine using poly-HRP streptavidin conjugates as labels for signal amplification, Anal. Bioanal. Chem. 406 (2014) 6363–6371.

[10] S. Guerrero, G. Martínez-García, V. Serafín, L. Agüí, P. Yáñez-Sedeño, J. M. Pingarrón, Electrochemical immunosensor for sensitive determination of the anorexigen peptide YY at grafted reduced graphene oxide electrode platforms, Analyst, 140 (2015) 7527- 7533.

[11] S. Zhu, J. Zhang, X. Zhao, H. Wang, G. Xu, J. You, Electrochemical behavior and voltammetric determination of L-tryptophan and L-tyrosine using a glassy carbon electrode modified with single-walled carbon nanohorns, Microchim. Acta, 181 (2014) 445-451.

[12] L. Shi, X. Liu, W. Niu, H. Li, S. Han, J. Chen, G. Xu, Hydrogen peroxide biosensor based on direct electrochemistry of soybean peroxidase immobilized on single-walled carbon nanohorn modified electrode, Biosens. Bioelectron., 24 (2009) 1159-1163.

[13] J. Miyawaki, M. Yudasaka, T. Azami, Y. Kubo, S. Iijima, Toxicity of single-walled carbon nanohorns, ACS Nano, 2 (2008) 213-226

[14] H. Tanaka, H. Kanoh, M. El-Merraoui, W. A. Steele, M. Yudasaka, S. Iijima, K. Kaneko, Quantum effects on hydrogen adsorption in internal nanospaces of single-wall carbon nanohorns, J. Phys. Chem. B, 108 (2004) 17457-17465.

[15] K. Ajima, M. Yudasaka, T. Murakami, A. Maigne, K. Shiba, S. Iijima, Carbon nanohorns as anticancer drug carriers, Mol. Pharm. 2 (2005) 475-480.

[16] P. Yáñez-Sedeño, A. González-Cortés, L. Agüí, J.M. Pingarrón, Uncommon carbon nanostructures for the preparation of electrochemical immunosensors, Electroanalysis, 28 (2016) 1679 – 1691.

[17] J. Zhang, J. Lei, C. Xu, L. Ding, H. Ju, Carbon nanohorn sensitized electrochemical immunosensor for rapid detection of microcystin-LR, Anal. Chem. 82 (2010) 1117-1122.

[18] F. Yang, J. Han, Y. Zhuo, Z. Yang, R. Yuan, Highly sensitive impedimetric immunosensor based on single-walled carbon nanohorns as labels and bienzyme biocatalyzed precipitation as enhancer for cancer biomarker detection, Biosens. Bioelectron., 55 (2014) 360-365.

[19] I. Ojeda, B. Garcinuño, M. Moreno-Guzmán, A. González-Cortés, M. Yudasaka, S. Iijima, F. Langa, P. Yáñez-Sedeño, J.M. Pingarrón, Carbon nanohorns as a scaffold for the construction of disposable electrochemical immunosensing platforms. Application to the determination of fibrinogen in human plasma and urine, Anal. Chem., 86 (2014) 7749-7756.

[20] H. Dai, L. Gong, S. Zhang, G. Xu, Y. Li, Z. Hong, Y. Lin, All-in-one bioprobe devised with hierarchical-ordered magnetic NiCo2O4 superstructure for ultrasensitive dual-readout immunosensor for logic diagnosis of tumor marker, Biosens. Bioelectron. 77 (2016) 928-935

[21] H. Dai, S. Zhang, L. Gong, Y. Li, G. Xu, Y. Lin, Z. Hong, The photoelectrochemical exploration of multifunctional TiO2 meso-crystals and its enzyme-assisted biosensing application, Biosens. Bioelectron. 72 (2015) 18 -24.

[22] S. Utsumi, M. Miyawaki, H. Tanaka, Y. Hattori, H. Itoi, N. Ichikuni, H. Kanoh, M. Yudasaka, S. Iijima, K. Kaneko, Opening mechanism of internal nanoporosity of single-wall carbon nanohorn, J. Phys. Chem. B, 109 (2005), 14319−14324.

[23] M. Zhang, M. Yudasaka, K. Ajima, J. Miyawaki, S. Iijima, Light-assisted oxidation of single-wall carbon nanohorns for abundant creation of oxygenated groups that enable chemical modifications with proteins to enhance biocompatibility, ACS Nano, 1 (2007) 265−272.

[24] M. Eguílaz, M. Moreno-Guzmán, S. Campuzano, A. González-Cortés, P. Yáñez-Sedeño, J.M. Pingarrón, An electrochemical immunosensor for testosterone using functionalized magnetic beads and screen-printed carbon electrodes, Biosens. Bioelectron., 26 (2010) 517-522.

[25] A.P. Cabral de Faria, V. Fontana, R. Modolo, N.R. Barbaro, A.R. Sabbatini, I.F. Pansani, S.E. Ferreira–Melo, H. Moreno, Plasma 8–isoprostane levels are associated with endothelial dysfunction in resistant hypertersion, Clin. Chim. Acta, 433 (2014) 179–183.

[26] C. Vassalle, N. Botto, M.G. Andreassi, S. Berli, A. Biagini, Evidence for enhanced 8–isoprostane plasma levels, as indez of oxidative stress in vivo, in patients with coronary artery disease, Coron. Artery Dis., 14 (2003) 213–218.

Table 1. Analytical characteristics reported for various ELISA immunoassays for ISO determination.

Type of immunoassay	ELISA kit	Dynamic range, pg/mL	MDC*, pg/mL	Precision, RSD % (inter/intra assays)	Assay time
Direct competitive between	Eagle Bioscience (8IS39-K01)	10 - 5000	10	-	2 h 30 min
ISO and HRP-ISO	Cayman Chemical (516351)	0.8 - 500	2.7	7.2% / 15.5%	2 h
Substrate: TMB	Saphire Bioscience (133-16456)	1.4 - 3000	18	≤10% / ≤12.6%	3 h 30 min
	Neogen Corporation (430110)	50 – 10000	30	≤10% / ≤10%	2 h 40 min
	Cusabio (CSB-E12100h)	78 - 5000	19.5	<15% / <15%	2 h 30 min
	EIAab (E0701h)	1.56 - 100	1	-	4 h 30 min
	Abcam (ab175819)	5 - 5000	1	-	2 h 30 min
	Oxford Biomed. Res. (EA84)	50-100	-	-	2 h 30 min
	Abbexa Ltd (abx055460)	16 - 1000	5	-	2 h 30 min
	US Biological (I9050)	100-1000	100	-	2 h 30 min
	Abnova (KA0443)	50-10000	100	-	2 h 40 min
	Invitrogen (KHP0041)	500-16000	100	3.8% / 4.6%	2 h 20 min
	Northwest (NWK-IS002)	50-10000	50	-	2 h 30 min
	Detroit R&D, Inc (8Iso1)	500-16000	100	-	2 h 30 min
	Cell Biolabs (STA-337)	50-200	50	-	2 h 30 min
Direct competitive between	IBL (CM500431)	23.4 - 3000	2.7	7.2% / 15.5%	2 h
SO and AP-ISO	CD Creative Diagnost. (DEIA6463)	40 - 100	40	12% / 20%	3 h 30 min
Substrate:	Enzo Life	3-	6.71	9.7-13.1%	< 3h

*p*NPP	Sciences ADI-900-069	50000			
Direct competitive between	CD Creative Diagnost. (DEIA5014)	40 - 100	10	12% / 20%	3 h 30 min
ISO and AChE-ISO. Substrate: thiocholine	CD Creative Diagnost. (DEIA6464)	40-100	16.3	12%/20%	3 h 30 min

Abbreviations: MDC, minimum detectable concentration; ISO, 8 isoprostane; HRP-ISO, peroxidase-labeled isoprostane; TMB, 3,3´5,5´-tetramethylbenzidine; AChE-ISO, acetilcholinesterase-labeled isoprostane; *p*NPP, 4-nitrophenyl phosphate.

Table 2. Determination of ISO in human serum samples with anti-ISO-CNHs/SPCE immunosensors

Lyophilized spiked serum

ISO, pg/mL	ISO found, pg/mL (n=3)	Recovery, %
30	30 ± 4	100 ± 1
35	33 ± 7	95 ± 6
40	42 ± 3	101 ± 4

Real human serum

Type	Immunosensor, pg/mL (n=6)	ELISA, pg/mL (n=6)
Healthy male (SG610-2, BBI)	403 ± 7	408 ± 5
Healthy female (SG609-2, BBI)	394 ± 4	398 ± 3