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Amperometric immunosensor for the determination of ceruloplasmin in human serum and urine based on covalent binding to carbon nanotubes-modified screen-printed electrodes

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ABSTRACT

A novel electrochemical immunosensor for the determination of ceruloplasmin (Cp) in human serum and urine is reported. The immunosensor configuration involves an indirect competitive immunoassay implying covalent immobilization of Cp on activated carboxylic groups at carbon nanotubes-modified screen-printed electrodes (CNTs/SPE). After Cp immobilization and reaction between the target analyte and anti-ceruloplasmin antibodies in solution, the remaining non-conjugated antibody is attached on the Cp-CNTs modified electrode. Monitoring of Cp is performed by means of a secondary antibody labeled with peroxidase (HRP-anti-IgG) and measurement of the amperometric current resulting from the addition of hydrogen peroxide in the presence of hydroquinone as the redox mediator. The experimental variables affecting the analytical performance of the immunosensor were optimized. Calibration curves for Cp provided a linear range between 0.07 and 250 μg/mL (r=0.997). The limit of detection achieved was 21 ng/mL. These analytical characteristics allow the immunosensor to be successfully used for the determination of Cp in spiked human serum and urine at various concentration levels.

1. Introduction

The efficient, reliable, rapid and low cost monitoring of disease biomarkers is an analytical challenge involving several technological fields. A relevant example in this field regards with the development of electrochemical immunosensors, an area exhibiting an enormous evolution in recent years which has been possible mainly due to the irruption of nanomaterials. The choice of a suitable electrode platform capable of providing the best conditions for a stable and oriented immobilization of immunoreagents while giving rise to appropriate electroanalytical signals, is a crucial step in the design of electrochemical immunosensors useful for applications to real sample analysis. Carbon nanotubes (CNTs) have been widely used in the design of electrochemical sensors and enzyme biosensors, although not to the same extent in the case of immunosensors [1,2]. Nevertheless, in recent years various strategies for antibodies immobilization on CNTs have been proposed [3]. Hydrophobic adsorption, the simplest alternative, has various drawbacks related with the scarce stability of protein coating and the fast decay in biological activity of biomolecules. Furthermore, lack of orientation also results in a reduced capacity for antigen binding. Efforts have been made to find methods capable of achieving stable and oriented immobilization of antibodies. Among them, those involving carboxylation of carbon nanotubes followed by covalent binding with amine groups of biomolecule have demonstrated to be particularly useful [3,4]. This is because it does not require antibody chemical modification or derivatization and the simple methodology used not need either the application of several steps, which implies a reduction in the consumption of time and reagents.

Ceruloplasmin (Cp) is an α2-glycoprotein containing more than 95% copper present in blood. It belongs to the group of inflammation-sensitive proteins (ISPs) and is considered as an important cardiovascular diseases risk factor. It can be used as a biomarker for obesity and correlated positively with body fat mass or weight gains [5]. High serum levels of Cp have been found in patients with central obesity, being also related with serum triglyceride and cholesterol levels [6]. Increased urinary excretion of Cp also predicts future development of microalbuminuria [7]. On the other hand, low levels of Cp ($<$20 μg/mL in adults) are associated with Wilson's disease, an infrequent cause of chronic liver disease [8,9] produced by an autosomal recessive disorder of copper accumulation.

Various colorimetric methods based on the oxidase activity of Cp using p-phenylenediamine (PPD) or dianisidine (3,3′-dimethoxy benzidine) as substrates [10–12] were reported to determine Cp. Kinetic methods using Fe^{2+} were also described [13,14]. Another

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proposed analytical strategies involve the use of techniques such as turbidimetry [9,15], inductively-coupled plasma mass spectrometry (ICPMS) [16] and stripping potentiometry [17]. Furthermore, there also available commercial ELISA kits for Cp, which are mainly based on the use of biotinylated immunoreagents and avidin or streptavidin labeled with peroxidase and colorimetric detection after addition of hydrogen peroxide and tetramethyl benzidine (TMB). The limits of detection of these assays, expressed as the minimum detectable concentration of Cp, are between units and hundreds of ng/mL.

A very small number of immunosensors for Cp has been reported in the literature. A piezo-immunosensor in which anti-Cp was immobilized by electrostatic adsorption onto a polymer-modified crystal and polyethylene glycol was added to enhance the immunosensor response was reported. This immunosensors determined Cp in a range between 0.31 and 27.0 μg/mL with a detection limit of 0.15 μg/mL [18]. Recently, our group reported a comparative study between two configurations of magnetoimmunosensors for Cp [19]. The described designs utilized magnetic microparticles functionalized with protein-A or streptavidin allowing the immobilization of anti-Cp or biotinylated anti-Cp, respectively.

In this work, a simpler and lower cost electrochemical immunosensor configuration for the determination of Cp in human serum and urine is described. This configuration implied the covalent binding of Cp to activated carboxylated multiwalled carbon nanotubes and an indirect competitive immunoassay. Monitoring of Cp was carried out amperometrically using a secondary antibody labeled with peroxidase (HRP-anti-IgG), upon addition of hydrogen peroxide in the presence of hydroquinone as the redox mediator and with disposable CNT/SPEs as transducers. This strategy allowed the construction of an immunosensing platform with excellent capabilities for the rapid, sensitive and low cost electrochemical transduction of the affinity event with ability for portable screening.

2. Experimental

2.1. Reagents and solutions

Human ceruloplasmin (Cp) was from Abcam. Stock 1 mg/mL Cp solutions were prepared by dissolving the product in two different buffered media. Solutions of Cp to be immobilized on the modified electrode surface were prepared in 25 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer of pH 5.0 while standard Cp solutions were prepared in 0.1 M phosphate buffer solution (PBS) of pH 7.4 (the presence of sodium ion in this solution was avoided according to the recommendation of the Cp supplier due to the possible precipitation of this protein). Solutions of anti-ceruloplasmin (anti-Cp, Abcam) and anti-IgG labeled with HRP (HRP-anti-IgG, Sigma) were prepared in 0.1 M PBS of pH 7.4. N-ethyl-N-dimethyl-aminopropyl-carbodiimide (EDC) and N-hydroxysulfo-succinimide sulfate (NHSS) were both from Acros. 0.1 M solutions of each compound were prepared in 25 mM MES buffer of pH 5.0. Stock 5% casein solutions were prepared in 0.1 M KOH. More diluted solutions were prepared by dilution with 0.1 M PBS of pH 7.4. 1 mM hydroquinone and 50 mM hydrogen peroxide solutions were prepared from the products (Sigma) and dilution with 0.05 M PBS of pH 6.0. Buffer solutions used were prepared as follows: 25 mM MES buffer solution of pH 5.0 was prepared by dissolving 244 mg of the product (Gerbu) in 50 mL of deionized water and adjusting the pH value with 2 M KOH. 0.1 M PBS solutions of pH 7.4 and 0.05 M PBS solutions of pH 6.0 were prepared by dissolving the adequate amounts of K_3PO_4 and K_2HPO_4 (Panreac) in deionized water and adjusting pH values with 2 M KOH. PBST buffer solution was also prepared from a 10 mM PBS solution of pH 7.4 also containing 0.05% Tween 20 (Aldrich).

All other chemicals used were of analytical reagent grade, and the water used was obtained from a Milli-Q purification system (Millipore, Bedford, MA).

2.1.1. Samples

Lyophilized human serum S-7394 from Sigma spiked with Cp at 1.0, 5.0, 10.0 and 20.0 μg/mL concentration levels was analyzed. The solid serum was reconstituted in 1 mL of 0.1 M PBS solution of pH 7.4 by mixing up to total dissolution. Liquichek urine Chemistry Control (Level 1, BioRad 63221) spiked with 0.08, 0.5 and 1.0 μg/mL Cp was also analyzed by adjusting to pH 7.4 with PBS.

2.2. Apparatus and electrodes

All electrochemical measurements were carried out using a PGSTAT 12 potentiostat from Autolab. The electrochemical software was the general-purpose electrochemical system (GPES 4.9) (EcoChemie B.V.). Carbon nanotubes screen-printed electrodes (CNTs/SPE, 4 mm diameter) were purchased from DropSens (Oviedo, Spain) and used as the working electrodes. These electrodes include a silver pseudo-reference electrode and a carbon counter electrode. All experiments were performed at ambient temperature. A P-Selecta ultrasonic bath, an Optic Ivymen System constant temperature incubator shaker (Comecta S.A.), a P-Selecta Agimatic magnetic stirrer, and a Vortex (Heidolph) stirrer, all distributed by Scharlab, were also used. pH measurements were made with a precision Metrohm Herisau E-510 pH-meter.

2.3. Procedures

2.3.1. Preparation of the HRP-anti-IgG-anti-Cp-Cp-CNTs/SPE immunosensors

Carboxylic groups of CNTs/SPE were activated by reaction with EDC/NHSS. In brief, 10 μL of a 100 mM EDC/NHSS mixture prepared in 25 mM MES buffer solution of pH 5.0 were dropped onto the electrode surface allowing incubation for 30 min. Thereafter, 10 μL of a 30 μg/mL Cp solution prepared in the same buffer were also dropped onto the electrode allowing to react at 25 °C to dryness (30–40 min, approximately). After this, the unmodified electrode surface was blocked by adding 10 μL of 2% casein and incubating for 45 min. In order to perform the indirect competitive immunoassay, 20 μL of a mixture solution containing 2.5 μg/mL anti-Cp and the standard Cp solution or the sample, in 0.1 M PBS of pH 7.4, were introduced into an eppendorf tube and stirred at 4 °C for 20 min. After the occurrence of the affinity reaction, 10 μL of the solution containing the remaining non-conjugated anti-Cp were deposited on the activated CNTs/SPE surface and incubation was proceeded 30 min. As a final step, 10 μL of HRP-labeled anti-IgG antibody 1:750 diluted in 0.1 PBS of pH 7.4 were dropped onto the anti-Cp-Cp-CNTs/SPE and incubated for 30 min. After each modification step, the modified electrode was washed with PBST buffer solution and deionized water. Cp determination was accomplished by dropping 45 μL of 1 mM hydroquinone solution prepared in 0.05 M PBS of pH 6.0 onto the surface of the immunosensor horizontally positioned and applying a detection potential of −0.2 V. When the background current was stabilized, 5 μL of 50 mM H_2O_2 solution prepared in the same buffer were added and the steady-state current was measured (see Fig. 1).

2.3.2. Determination of Cp in human serum and urine

The reconstituted spiked serum was analyzed by applying the same procedure described in Section 2.3.1. Spiked urine samples were also analyzed in the same manner. In both cases, the

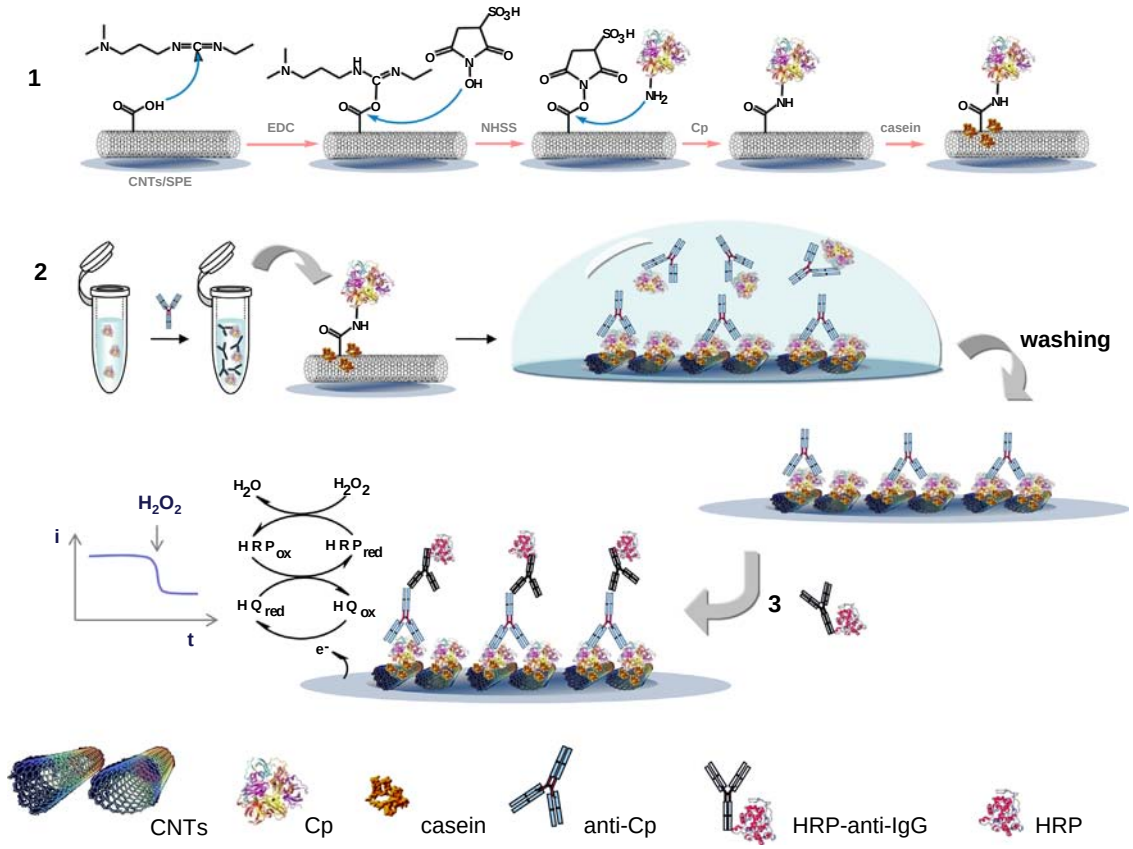

Fig. 1. Schematic display of the reactions and protocols involved in the preparation of the electrochemical immunosensor for Cp using carbon nanotubes-screen printed electrodes.

determination of Cp was carried out by interpolation of the steady-state current measured with the HRP-anti-IgG-anti-Cp-Cp-CNTs/SPE immunosensor into the calibration curve constructed with Cp standard solutions.

3. Results and discussion

The schematic illustration of the CNTs/SPE activation, the immunosensor fabrication and immunoassay fundamental as well as the electrochemical basis of the affinity reaction monitoring are presented in Fig. 1. The protocol involved covalent immobilization of Cp on EDC/NHSS activated carboxylic groups existing at the CNTs/GCE, followed by a blocking step with casein (Fig. 1.1). Then, anti-Cp was mixed with a Cp standard solution or the sample solution and the reaction between the anti-ceruloplasmin antibody and the target analyte was allowed to proceed. After this step, an aliquot of the solution containing the remaining non-conjugated antibody was dropped onto the Cp-CNTs/GCE to attach the free anti-Cp to the immobilized Cp (Fig. 1.2). Finally, HRP-anti-IgG was incubated on the anti-Cp-Cp-CNTs/GCE immunosensor (Fig. 1.3), and Cp was determined amperometrically after addition on hydrogen peroxide in the presence of hydroquinone.

3.1. Optimization of the variables affecting the performance of HRP-anti-IgG-anti-Cp-Cp-CNTs/SPE.

The experimental variables affecting the amperometric response of the immunosensors were optimized. These studies involved the preparation of different immunosensors and the evaluation of: (a) the pH value used for Cp immobilization onto CNTs/SPE; (b) the Cp loading immobilized onto the activated CNTs/SPE; (c) the blocking step; (d) the concentration of anti-Cp used to react with Cp in solution; (e) the time and temperature used for this reaction; (f) the incubation time of the non-conjugated anti-Cp onto Cp-CNTs/SPE, and (g) the concentration of HRP-anti-IgG and the corresponding incubation time. Details on these optimization studies can be found in the text and Figs. S1 and S2 of Supplementary material. The ranges of the variables checked and the corresponding selected values are summarized in Table 1.

3.2. Analytical characteristics of the immunosensor

The calibration plot for Cp constructed with the HRP-anti-IgG-anti-Cp-Cp-CNTs/SPE immunosensor prepared under the optimized working conditions stated in Table 1 is shown in Fig. 2. Error bars were calculated from the measurements carried out with three different immunosensors in each case. The i_p vs. Cp concentration curve was fitted by non-linear regression using the Sigma Plot data analysis software. The adjusted equation (r=0.996) was:

$$y = \frac{i_{max} - i_{min}}{1 + \left(\frac{EC_{50}}{x}\right)^h} + i_{min}$$

where i_{max}=2.8 ± 0.1 μA, and i_{min}=0.4 ± 0.2 μA are the maximum and minimum current values in the calibration graph. The EC_{50} value, which corresponded to the Cp concentration producing 50% competition, was 8 ± 2 μg/mL, with a Hill slope, h, of −0.52 ± 0.09. The range of linearity extended between 0.07 and 250 μg/mL (r=0.996). The achieved limit of detection, 21 ng/mL, was

Table 1
Optimization of the performance conditions of the HRP-anti-IgG-anti-Cp-Cp-CNTs/SPE immunosensor.

Variable	Tested range	Selected value
Working medium and pH value for Cp immobilization onto CNTs/SPE	PBS, 7.4; 6.0; MES, 5.0	MES, 5.0
Cp loading onto CNTs/SPE, μg/mL	20–50	30
Blocking of Cp/CNTs/SPE	2%, 5% BSA; 1%, 2% casein	2% casein
Anti-Cp concentration, μg/mL	0.75–10.0	2.5
Time for the reaction between Cp and anti-Cp, min	0-45	20
Temperature for the reaction between Cp and anti-Cp, °C	4; 25; 37	4
Incubation time of anti-Cp onto Cp-CNTs/SPE, min	15–60	30
HRP-anti-IgG concentration, dilution factor	1/200–1/750	1/750
Incubation time of HRP-anti-IgG onto anti-Cp-Cp-CNTs/SPE, min	15–60	30

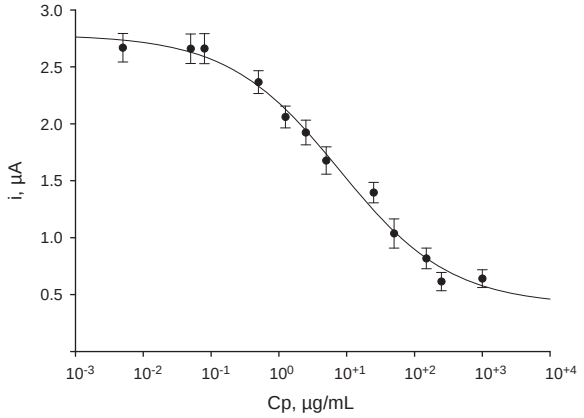

Fig. 2. Calibration plot for Cp constructed with the HRP-anti-IgG-anti-Cp-Cp-CNTs/SPE immunosensor.

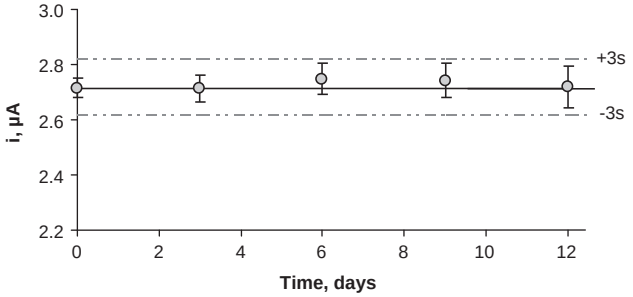

Fig. 3. Control chart constructed to test the stability of Cp-CNTs/SPE conjugates upon storage at 4 °C under dry conditions. The central value was the mean amperometric current obtained for the measurements ($n=3$) carried out on the first day for different solutions containing no Cp. The upper and lower limits were calculated from such measurements as ± 3 s.

calculated from the equation:

$$LOD = EC_{50}\left(\frac{i_{max} - i_{min}}{i_{max} - i_{min} - 3s} - 1\right)^{-1/h}$$

where s is the standard deviation ($n=5$) of the zero value (the i_p value measured in the absence of Cp), ± 34 nA. Furthermore, the limit of quantification, estimated as the lowest analyte concentration that can be measured in the linear portion of the corresponding calibration plot, was 70 μg/mL.

Reproducibility studies were made by performing different assays in the absence of Cp analyte and in the presence of 8 μg/mL Cp. Different immunosensors were prepared both on the same day and on different days for these measurements using a new CNTs/SPE in each case. The relative standard deviation (RSD) values obtained ($n=5$) were 1.2% and 7%, for the assays performed on the same day in the absence and in the presence of Cp, respectively, whereas RSD ($n=5$) values were 6.8% and 8.9%, respectively for the measurements made on different days. These results revealed the good level of precision achieved in the fabrication and functioning of the proposed immunosensing device.

The storage stability of the Cp-CNTs/SPE conjugates at 4 °C under dry conditions was also evaluated. Various bioconjugates were prepared on the same day, stored and used to construct the corresponding HRP-anti-IgG-anti-Cp-Cp-CNTs/SPE immunosensors in different days. Fig. 3 shows the control chart for the currents measured for solutions containing no Cp. As it can be seen, the immunosensor response remained within the limits of control, set at ± 3 times the standard deviation of the measurements ($n=3$) carried out on the first day, for at least 12 days (no longer period was checked) which can be considered as an acceptable storage stability for the Cp-CNTs/SPE conjugates thus allowing their preparation and storage under the above specified conditions and their use for the fabrication of the immunosensors on request.

Table 2 compares the analytical characteristics of the developed immunosensor with those reported for other Cp immunosensors and ELISA kits [19]. In particular, when the comparison is made against the reported piezo-immunosensor [18], it can be seen as the range of linearity covers from a Cp concentration more than four times lower up to a Cp concentration more than nine times higher than the range given for the piezo-immunosensor. Moreover, the limit of detection was 7 times lower. On the other hand, the analytical performance of the HRP-anti-IgG-anti-Cp-Cp-CNTs/SPE immunosensor is similar to that achieved using magnetoimmunosensor configurations (MBs) [19], although the linear range of the calibration plot obtained using the HRP-anti-IgG-anti-Cp-Cp-CNTs/SPE immunosensor extends up to a Cp concentration 12.5 times higher than that reported for anti-Cp-Biotin-Strept-MBs conjugates. Regarding ELISA kits, it can be concluded as a general assessment that the developed immunosensor provides wider analytical linear range, similar or even better sensitivity, higher precision and shorter time of analysis than those achieved with most of the reported assays.

3.3. Selectivity

Various species which may be present in biological fluids together with Cp were tested as potential interfering compounds regarding the amperometric response the of HRP-anti-IgG-anti-Cp-Cp-CNTs/SPE immunosensor. Some of these potential interferences such as bilirubin, hemoglobin and cholesterol are recommended in a commercial kit for Cp in human serum (Konelab™).

Table 2
Comparison of the analytical characteristics of HRP-anti-IgG-anti-Cp-Cp-CNTs/SPE immunosensor with various immunoassay systems for ceruloplasmin (Cp).

Immunoassay system	Characteristics	Range, μg/mL	LOD, ng/mL	Precision, RSD %	Samples	Assay time
HRP-anti-IgG-anti-Cp-Cp-CNTs/SPE	Competitive indirect immunoassay with Cp immobilized. Amperometric detection after immobilization of HRP-anti-IgG and addition of H_2O_2 in the presence of hydroquinone	0.07–250	21[a]	1.2	Human serum, urine	1 h 50 min
Piezoimmunosensor based on PPF-modified crystal [18]	Immobilization of Ab onto alginate – PPF QCM. Immuno-assay in the presence of PEG	0.31–27	150 (3σ)	6.2	Human serum	–
Anti-Cp-Protein A-MBs/SPCE electro-chemical magnetoimmunosensor [19]	Competitive immunoassay with anti-Cp-Protein A-MBs between Cp and Cp-AP. DPV detection by addition of 1-NPP	0.1–1000	40[a]	5.3	Human serum	50 min
Anti-Cp-Biotin-Strept-MBs/SPCE electro-chemical magnetoimmunosensor [19]	Competitive immunoassay with anti-Cp-Biotin-Strept-MBs between Cp and Cp-AP. DPV detection by addition of 1-NPP	0.025–20	18[a]	3.2	Human serum	50 min
Assaypro Assay Max Human Ceruloplasmin ELISA kit EC4001-1	Competitive immunoassay with anti-Cp immobilized between Cp and Biotin-Cp. Colorimetric setection with Strept-HRP and TMB/H_2O_2	0.63–40	100[b]	4	Human plasma,	2 h 47 min
Uscn E90909Hu96 Tests ELISA kit (human)	Sandwich immunoassay with anti-Cp immobilized and Biotin-anti-Cp. Colorimetric detection with Avidin-HRP and TMB/H_2O_2	0.0094–0.6	<3.4[b]	<10	Human serum, plasma, tissue,	3 h 45 min
Mybiosource MBS701656	Sandwich immunoassay with anti-Cp immobilized and HRP-anti-Cp. Colorimetric detection with TMB/H_2O_2	0.015–0.5	7.5[b]	No data	Human serum, plasma	1 h 45 min
Abnova KA0469	Competitive immunoassay with pAb between Cp and Biotin-Cp. Colorimetric detection with Strept-HRP and TMB/H_2O_2	0.63–40	100[b]	4.1	Human plasma,	2 h 47 min
Abnova, KA0470	Sandwich immunoassay with anti-Cp immobilized and Biotin-anti-Cp. Colorimetric detection with Strept-HRP and TMB/H_2O_2	0.00125–0.32	~1[b]	4.1	Human urine, saliva, milk, cell culture	3 h 50 min
Pro Alt Human Ceruloplasmin ELISA Quantitation Kit PRO-20074 F	Sandwich immunoassay with Ab and HRP-Ab. Colorimetric detection with Avidin-HRP and TMB/H_2O_2	0.0039–0.25	No data	No data	Human serum, other biolog. samples	4 h
MDbioproducts Human ceruloplasmin MO46029	Competitive immunoassay with anti-Cp immobilized between Cp and Biotin-Cp. Colorimetric detection with Strept-HRP and TMB/H_2O_2	0.63–20	<60[b]	4.2	Human serum, plasma	2 h 45 min
Abcam Ab108817 Ceruloplasmin ELISA kit	Competitive immunoassay with anti-Cp immobilized between Co and Biotin-Cp. Colorimetric detection with Strept-HRP and TMB/H_2O_2	0.625–40	600[b]	4.2	Plasma, serum	2 h 40 min
Assaypro Assay Max Human Ceruloplasmin ELISA kit EC4101–1	Sandwich immunoassay with anti-Cp immobilized and Biotin-anti-Cp. Colorimetric detection with Strept-HRP and TMB/H_2O_2.	0.00125–0.32	~1[b]	4.1	Human urine, saliva, milk, cell culture	4 h

[a] See used equation in the text.
[b] Minimum detectable concentration. Abbreviations: TMB, 3,3′5,5′-tetramethylbenzidine; PPF, plasma-polymerized film; PEG, poly(ethyleneglycol); 1-NPP, 1-naphthylphosphate

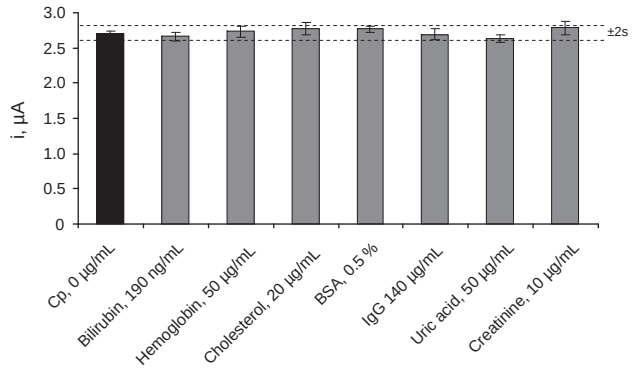

Fig. 4. Selectivity of the HRP-anti-IgG-anti-Cp-Cp-CNTs/SPE immunosensor against uric acid, creatinine, albumin, bilirubin, human serum immunoglobulin, hemoglobin and cholesterol. The amperometric responses were obtained in absence of Cp. See the text for more information.

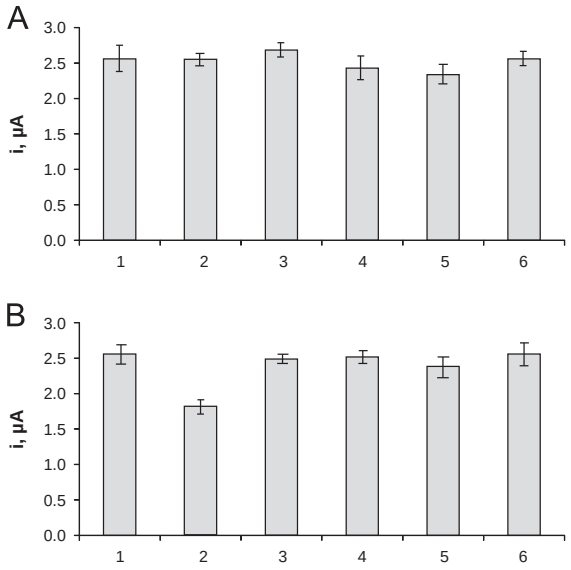

Fig. 5. Effect of human serum (A) and urine (B) dilution with 0.1 M PBS of pH 7.4 on the amperometric response measured with the HRP-anti-IgG-anti-Cp-Cp-CNTs/SPE immunosensor in the absence of Cp: (A) buffer solution (1); undiluted human serum (2) and 1/10 (3), 1/100 (4), 1/300 (5) and 1/1000 (6) PBS diluted human serum. (B) buffer solution (1); undiluted urine, pH 5.0 (2), undiluted urine pH 7.4 (3) and 1/2 (4); 1/10 (5) and 1/500 PBS diluted urine. For more information, see the text.

Moreover, human serum immunoglobulin was tested as an example of glycoprotein and uric acid, creatinine and albumin as potential interfering compounds in urine. All these compounds were checked at the respective concentration levels that can be found in serum and urine. Fig. 4 shows the amperometric responses obtained with the immunosensor in the absence of Cp and in the presence of each of these interfering compounds. As it can be observed, there was no significant differences between the currents obtained in absence or in the presence of these compounds, which demonstrated the excellent selectivity of the immunosensor for the determination of Cp.

3.4. Determination of Cp in human serum and urine

As a first step, the possible existence of matrix effect in these biological samples was investigated. Fig. 5 shows the results obtained with the HRP-anti-IgG-anti-Cp-Cp-CNTs/SPE immunosensor in serum and in urine containing no Cp which were diluted to different ratios with 0.1 M PBS of pH 7.4. Fig. 5A shows as the measured currents for buffer solution and the undiluted serum were similar, with mean values of 2.55 ± 0.13 μA and 2.55 ± 0.11 μA. Interestingly, the responses obtained for diluted serum at different ratios were also similar giving a mean current value, calculated from the results of the four diluted samples, of 2.50 ± 0.14 μA. A statistical comparison between this value with those obtained for the buffer solution and the non-diluted serum by applying the Student *t* test confirmed that no significant differences existed between both results. Therefore, it could be concluded that the determination of Cp in serum did not require any sample treatment. The absence of matrix effect was confirmed by constructing a calibration plot from the reconstituted serum between 1 and 20 μg/mL Cp. Aliquots from the reconstituted

Table 3
Determination of ceruloplasmin in human serum and urine with the HRP-anti-IgG-anti-Cp-Cp-CNTs/SPE immunosensor.

	Cp, μg/mL	Found, μg/mL[a]	Recovery, %
Human serum	0.50	0.51 ± 0.04	102 ± 8
	2.5	2.59 ± 0.16	103 ± 7
	5.0	5.2 ± 0.3	104 ± 6
	10	10.1 ± 0.9	101 ± 9
Urine	0.080	0.079 ± 0.006	99 ± 7
	0.50	0.52 ± 0.04	103 ± 8
	1.0	0.99 ± 0.10	100 ± 10

[a] Mean value $\pm ts/\sqrt{n}$.

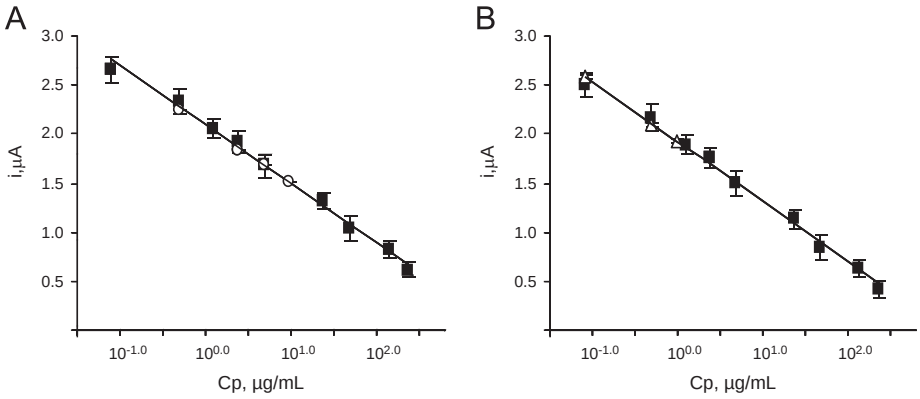

Fig. 6. Calibration plots constructed for Cp with the HRP-anti-IgG-anti-Cp-Cp-CNTs/SPE immunosensor: standard Cp solutions (black squares), and in human serum (white points) (a), and urine (white triangles) (b).

serum spiked with the different Cp concentrations were 1:1 (v/v) mixed with anti-Cp and incubated for 20 min in an eppendorf tube. Thereafter, 10 μL of the solution were dropped on the Cp-CNTs/SPE and the procedure described in section 2.3.2 was followed. Fig. 6a shows the points of this calibration (white points) superimposed to the calibration plot constructed with Cp standard solutions. As it can be observed, the matching was perfect. In fact, the equation describing the linear calibration in serum, i, $nA = [-570 \pm 20] \log C + [2070 \pm 40]$, was statistically compared with the one obtained for Cp standard solutions, i, $nA = [-597 \pm 17] \log C + [2100 \pm 20]$. Both the slopes and intercepts values provided Student t_{exp} values lower than the tabulated ones.

In the case of urine, the initial pH value of the samples was 5. The measurements in this undiluted urine (Fig. 5B, bar 2) gave rise to a signal decrease with respect to that obtained in the buffer solution (bar 1). However, this effect could be avoided by adjusting the sample pH to 7.4 by adding solid phosphate salts (bar 3) or simply by making a 1:2 PBS dilution (bar 4). A similar comparison to that performed with serum, between a calibration graph constructed with spiked urine samples and Cp standard solutions was also carried out (Fig. 6b). In this case, urine was spiked with Cp concentrations ranging between 0.08 and 1.0 μg/mL, a 1:100 (v/v) anti-Cp/spiked urine mixture was incubated and the same procedure described for serum samples was applied. As it can be seen, similar conclusions to those raised in serum could be deduced with the equation describing the linear calibration in urine being i, $nA = [-590 \pm 20] \log C + [2090 \pm 20]$.

All these results led us to accomplish the determination of Cp in the biological samples by simple interpolation of the measured amperometric currents into the calibration plot constructed with Cp standard solutions.

These results constitute a relevant practical advance with respect to the behavior of the previously reported magnetoimmunosensors [19]. A strong matrix effect in the analysis of human serum sample (the only biological sample analyzed in that case) was observed when using the magnetoimmunosensor. The complete removal of the matrix effect was only possible upon a 1:1000 sample dilution using the configuration involving Protein A-MBs.

The HRP-anti-IgG-anti-Cp-Cp-CNTs/SPE immunosensor was applied to the Cp determination in spiked samples and the results obtained by triplicate for each sample are summarized in Table 3. As it can be observed, recoveries ranged between 101% and 104% for serum, with RSD values ranging between 6% and 9%, whereas the recoveries for urine were between 99% and 103% with RSD values between 7% and 10%. These results demonstrated the usefulness of the developed immunosensor for the analysis of Cp at the physiological levels that can be expected in clinical samples with practically no sample treatment.

4. Conclusions

A simple and low cost electrochemical immunosensor suitable for the determination of Cp in human serum and urine is reported in this work. The novel configuration developed implies the covalent binding of Cp to activated carboxylated multiwalled carbon nanotubes, an indirect competitive immunoassay and amperometric monitoring at disposable SPEs of the affinity event using a secondary antibody labeled with peroxidase. The developed configuration exhibits excellent analytical performance for the rapid, low cost and treatment-less analysis of real biological samples with ability for portable screening.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.10.008.

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