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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