COMPETITIVE ELECTROCHEMICAL IMMUNOSENSOR FOR AMYLOID-BETA 1-42 DETECTION BASED ON GOLD NANOSTRUCTURATED SCREEN-PRINTED CARBON ELECTRODES

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Abstract

Alzheimer's disease is the most common form of dementia, characterized by the progressive accumulation of plaques with amyloid-beta peptide of 42 amino acids as one of primary constituents. A disposable electrochemical immunosensor for the detection of amyloid-beta 1-42 is developed. Screen-Printed Carbon Electrodes nanostructured with gold nanoparticles generated "in situ" are used as the transducer surface. The immunosensing strategy consists in a competitive immunoassay: biotin-amyloid-beta 1-42 immobilised on the electrode surface and the analyte (amyloid-beta 1-42) compete for the anti-amyloid-beta 1-42 antibody. The electrochemical detection is carried out using an alkaline phosphatase labelled anti-rabbit IgG antibody. The analytical signal is based on the anodic stripping of enzymatically generated silver by cyclic voltammetry. The immunosensor achieved shows a low limit of detection (0.1 ng/mL) and a wide linear range (0.5-500 ng/mL).

Keywords

Amyloid-beta 1-42; Alzheimer's disease; Screen-Printed Carbon Electrode; electrochemical immunosensor; gold nanoparticles.

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid-beta; SPCE, Screen-Printed Carbon Electrode; anti-IgG-AP, anti-rabbit IgG antibody labeled with alkaline phosphatase; biotin-Aβ1-42, amyloid-beta 1-42 labelled with biotin; anti-Aβ1-42, amyloid-beta 1-42 monoclonal antibody recombinant rabbit IgG; NPAu, gold nanoparticles; B-AP, biotin conjugated to alkaline phosphatase.

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1. Introduction

Today, over 35 million people worldwide currently live with dementia, and this number is expected to double by 2030 [1]. Alzheimer's disease (AD) represents 50-75% of all dementias [2]. The major histopathological hallmarks of AD are the progressive accumulation of plaques with amyloid-β (Aβ), and neurofibrillary tangles containing microtubuli-associated tau protein [3]. Aβ peptide comprising of 39–42 amino acids is the primary constituent of these plaques that hinder the communication between neurons causing cell death, cognitive dysfunction, and behavioral abnormalities [4,5]. Among these Aβ peptides, Aβ1-40 is the most abundant, but Aβ1-42 appears to be essential for initiating Aβ aggregation and is considered central to the amyloid cascade hypothesis of AD [6]. This hypothesis postulates a central initiating role for Aβ1-42 in the subsequent pathological features of AD, such as neuroinflammation, synapse and neuritic dysfunction, tau hyperphosphorylation and development of intraneuronal neurofibrillary tangles. Due to their roles in the pathogenesis of AD, Aβ1-42 seems to be a more useful biomarker for AD than Aβ1-40 [6].

Cerebrospinal fluid (CSF) is in direct contact with the extracellular space of the brain and biochemical changes in the brain are thought to be reflected in CSF [7].

Nowadays, there are few works described about devices for Aβ1-42 detection and these works are very recently (from 2010 to now) [8-13] There is one previously reported (2008), that, so far, is the only one based on Screen-Printed Electrodes [14]. But, for this sensor the Aβ peptides recognition is based on the saccharide-protein interactions, and the analytical signal is the oxidation peak of tyrosine that Aβ peptides have. So, this sensor cannot discriminate between Aβ1-40 and Aβ1-40.

In this work, the first electrochemical immunosensor based on Screen-Printed Electrode for Aβ1-42 detection is described. The biosensor consists of a competitive immunoassay carried out on a Screen-Printed Carbon Electrode nanostructured with gold nanoparticles. Concentration of labelled antigen, antibody and secondary labelled antibody are optimized, and non-specific binding is also studied. Label used is alkaline phosphatase and a mixture of 3-indoxyl phosphate with silver ions (3-IP/Ag^{+}) is used as substrate. The analytical signal is based on the anodic stripping of enzymatically generated silver by cyclic voltammetry. The linear range of the immunosensor developed allow the diagnosis of AD because, although the values are not well

established, several authors consider 500 pg/mL as an optimum cut-off value to differentiate between patients with dementia and healthy patients [13,15-18].

2. Experimental

2.1 Apparatus and electrodes

SPCE gold nanostructuration is performed with a μStat 8000 potentiostat (DropSens, Spain) interfaced to a Pentium 4 2.4 GHz computer system and controlled by DropView 8400 1.0 software.

The Voltammetric measurements are carried out using an ECO Chemie μAutolab type II potentiostat/galvanostat interfaced to a Pentium 4 2.4 GHz computer system and controlled by the Autolab GPES software version 4.9. All measurements are performed at room temperature.

Disposable Screen-Printed Carbon Electrodes (SPCEs) are purchased from DropSens (Spain). These electrodes incorporate a conventional three-electrode cell configuration, printed on ceramic substrates (3.4 x 1.0 cm). Both the working- (disk-shaped 4 mm diameter) and counter electrodes are made of carbon inks, whereas the pseudoreference electrode and the electric contacts are made of silver. An insulating layer delimits the electrochemical cell (50 μL) and the electric contacts. The SPCEs are easily connected to the μStat 8000 potentiostat and to the μAutolab potentiostat through the specific DropSens connector (Spain) in each case.

2.2. Reagents and solutions

Tris(hydroxymethyl)aminomethane (Tris), magnesium nitrate, bovine serum albumin fraction V (BSA), β-casein from bovine milk (casein), streptavidin (molecular weight, 66 kDa) and biotin conjugated to alkaline phosphatase (B-AP; dimmer, four units of B per molecule of AP, molecular weight, 160 kDa) are purchased from Sigma (Spain). Standard gold (III) tetrachloro complex ($AuCl_4^-$), silver nitrate, hydrochloric acid (37%) and nitric acid (HNO_3) are obtained from Merck (Germany). Biosynth (Switzerland) supplied the 3-indoxyl phosphate disodium salt.

Aβ1-42 monoclonal antibody recombinant rabbit IgG (clone H31L21) specific to amino acids 707-713 is purchased from Life Technologies (Spain). Anti-rabbit IgG

(whole molecule) labelled with alkaline phosphatase (anti-IgG-AP) is provided by Sigma (Spain). Aβ1-42 and Aβ1-42 labelled with biotin (Biotin-LC-β-Amyloid 1-42) are purchased from Anaspec (USA).

Ultrapure water obtained from a Millipore Direct-Q^{TM} 5 purification system from Millipore Ibérica S.A. (Spain) is used throughout the work. All chemicals employed are of analytical reagent grade. Working solutions of streptavidin, Aβ1-42 monoclonal antibody, Aβ1-42 and Aβ1-42 labelled with biotin (biotin-Aβ1-42) are prepared in a 0.1 M Tris-HNO_3 pH 7.2 buffer (buffer 1). Working solutions of secondary alkaline phosphatase labelled antibody are prepared in a 0.1 M Tris-HNO_3 pH 7.2 buffer containing 2 mM $Mg(NO_3)_2$ (buffer 2). A solution containing 1.0 mM 3-IP and 0.4 mM silver nitrate is prepared daily in 0.1 M Tris-HNO_3 pH 9.8 buffer containing 20 mM $Mg(NO_3)_2$ (buffer 3), and store in opaque tubes at 4 ºC. Casein and albumin lyophilized powder are reconstituted in buffer 1.

2.3. Procedures

2.3.1. SPCEs nanostructuration

Gold nanoparticles are generated “in situ” over SPCEs (SPCEs-NPAu) following a method previously reported by Martínez-Paredes et al. [19], using μStat 8000 potentiostat. The procedure consists in applying a constant current intensity of -100 μA for 240 s in an acidic solution of 0.1 mM $AuCl_4^-$. Then a potential of +0.1 V for 120 s is applied. Finally, the nanostructured electrodes are rinsed with water and are ready to use. NPAu generation is performed at room temperature. Using the μStat 8000 potentiostat, gold nanoparticles can be generated over eight different Screen-Printed Carbon Electrodes at the same time.

2.3.2. Evaluation of the analytical signal improvement using SPCEs-NPAu

The reaction streptavidin-biotin is used to evaluate the effect of the NPAus generated over the SPCE. A drop of 10 μL of 0.1 μM streptavidin [20] solution is placed on the nanostructurated surface of the SPCE solution and incubated overnight at 4 ºC. The immobilization of the streptavidin on the electrode surface is achieved by physical adsorption. Then, the electrode is washed with buffer 1, and the free surface

sites are blocked with 40 μL BSA solution (2%) during 30 min. The electrode is washed again using buffer 2, and a drop of 40 μL B-AP solution (0.1 nM) is dropped on the streptavidin modified electrode for an hour reaction. After a washing step with buffer 3, the enzymatic reaction is carried out dropping 40 μL of a mixture of 1.0 mM 3-IP/0.4 mM silver nitrate solution on the electrode. The enzymatically silver deposition catalyzed by alkaline phosphatase has been already reported [21]. AP works as the enzymatic label and a mixture of 3-IP with silver ions (Ag^{+}) as the substrate. AP hydrolyzes 3-IP resulting a indoxyl intermediate. This intermediate reduces the silver ions presents in solution resulting in metallic silver (Ag^{0}) and indigo blue (I) [21]. Thus, the silver enzymatically deposited on the electrode surface can be detected through the redissolution peak when an anodic stripping scan is carried out. After 20 min of enzymatic reaction, an anodic stripping cyclic voltammetric scan is recorded from 0.0 V to +0.4 V at a scan rate of 50 mV/s.

2.3.3. Immunosensor for the detection of Aβ1-42

The following procedure (Fig. 1) describes an optimized assay. The working area of SPCE-NPAu is coated with 10 μL of 0.1 μM streptavidin [20] solution and incubated overnight at 4 ºC. After the overnight incubation step, the electrode is washed with buffer 1. Free surface sites of the streptavidin-modified SPCE-NPAu are blocked with 40 μL casein solution (2%) during 30 min. After another washing step with buffer 1, an aliquot of 40 μL of 300 ng/mL biotin-Aβ1-42 solution is dropped on the streptavidin modified electrode for an hour reaction. After a washing step with buffer 1, the sensing part of the immunosensor is completed. Then, 40 μL of a solution of Aβ1-42 and antibody anti-Aβ1-42 (0.5 μg/mL) is dropped for an hour to carry out the competitive reaction. The competition was established via the binding between analyte (Aβ1-42) and the biotin-Aβ1-42 previously immobilised in the electrode surface, for the limited binding sites of the anti-Aβ1-42. Finally, after a washing step with buffer 2, the immunosensor is incubated with 40 μL of an anti-IgG-AP (1:15,000) solution for 60 min and washed with buffer 3. The enzymatic reaction is performed as is explained in Section 2.3.2.: placing a 40 μL aliquot of the 1.0 mM 3-IP/0.4 mM silver nitrate solution on the sensor, and after 20 min, recording an anodic stripping cyclic voltammetric scan from 0.0 V to +0.4 V at a scan rate of 50 mV/s. Buffers employed

have been chosen because of the satisfactory results in immunosensors with similar procedure [22,23].

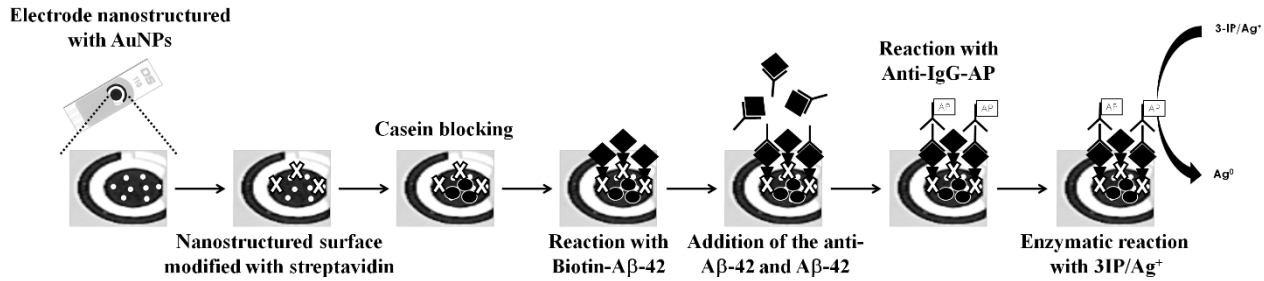

Figure 1. Schematic representation of the immunosensing strategy for the detection of Aβ1-42.

3. Results and discussion

3.1. Analytical signal improvement using SPCEs-NPAu

The use of this gold nanoestructuration is based in works previously reported [24]. It is well known that gold nanostructured surfaces as electrochemical transducers show better sensitivities than non-nanostructured surfaces [25]. To corroborate this improvement of sensitivity, the assay described in Section 2.3.2 is carried out using an SPCE-NPAu and a SPCE non-nanostructurated. The SPCE non-nanostructurated is pretreated applying the same procedure than to achieve a SPCE-NPAu but using an acidic solution without $AuCl_4^-$. As Fig. 2 shows, the capacitive current is slightly higher using a SPCE-NPAu than using a SPCE non-nanostructurated, but the intensity of the peak is much higher using the SPCE-NPAu. So, a SPCE-NPAus is better transducer to develop the immunosensor than a SPCE non-nanostructurated.

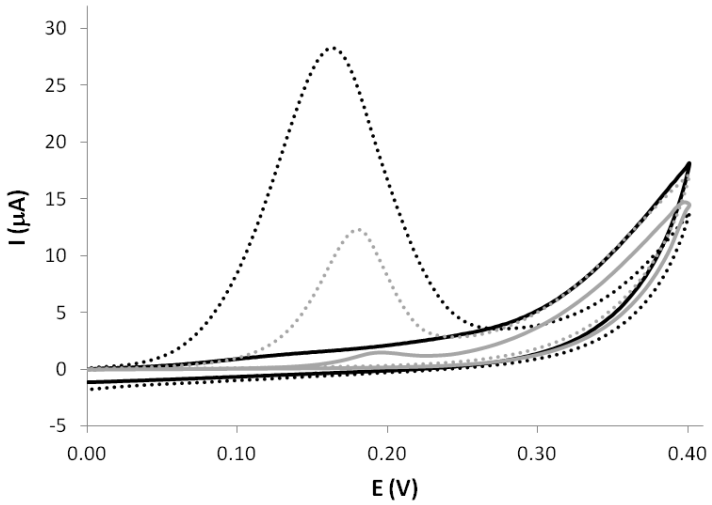

Figure 2. Cyclic voltammograms for redissolution peak of metallic silver enzymatically deposited on the electrode surface using a SPCE-NPAu (black lines) and a SPCE pretreated (grey lines). Analytical signal (dashed lines) and background signal (solid lines).

3.2. Optimization of the experimental conditions

The variables involved in the construction of the immunosensor can influence the analytical response, therefore an optimization study was carried out.

Different anti-IgG-AP antibody dilutions were tested: 1:10000, 1:15000 and 1:20000. The dilution chosen for further studies was 1:15000 because the best compromise between analytical signal and non-specific is achieved (data non shown).

Non-specific binding of anti-IgG-AP over the electrode surface are avoid adding BSA in the solution of this antibody. If BSA is not present in anti-IgG-AP solution, the signal in absence biotin-Aβ1-42 is about 14 µA, due to non-specific adsorptions. But, adding 1% BSA concentration to the solution of anti-IgG-AP, these undesirable adsorptions are avoided without an important loss of signal given by the sensor (Fig. 3).

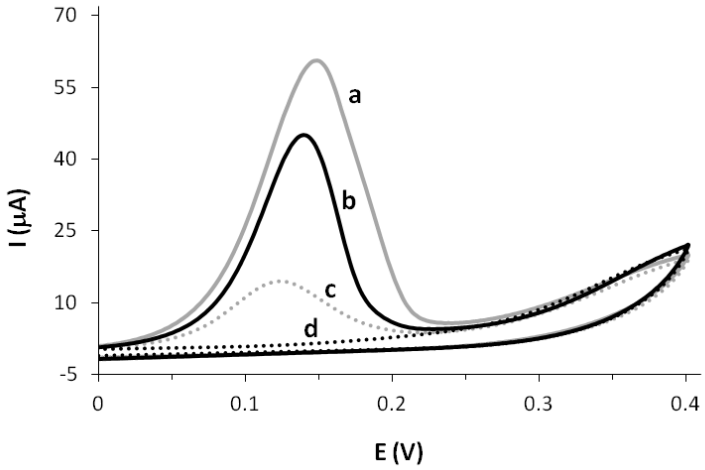

Figure 3. Cyclic voltammograms given by the immunosensor: in absence of BSA, with biotin-Aβ1-42 500 ng/mL (a) or without biotin-Aβ1-42 (c). When BSA is added in anti-IgG-AP solution, with biotin-Aβ1-42 500 ng/mL (b) or without biotin-Aβ1-42 (d). Experimental conditions: anti-Aβ1-42 0.3 µg/mL; anti-IgG-AP 1:15,000; 3-IP 1.0 mM; Ag^+ 0.4 mM.

The adequate concentration of the biotin-Aβ1-42 is evaluated in order to achieve the best performance of the immunosensor. Although biotin-Aβ1-42 concentration of 500 ng/mL shows the highest analytical signal, a biotin-Aβ1-42 concentration of 300 ng/mL is chosen because the analytical signal achieved is similar and the reproducibility is better (Fig. 4). Moreover, higher concentration of biotin-Aβ1-42 than 300 ng/mL saturates the electrode surface and shows worse reproducibility.

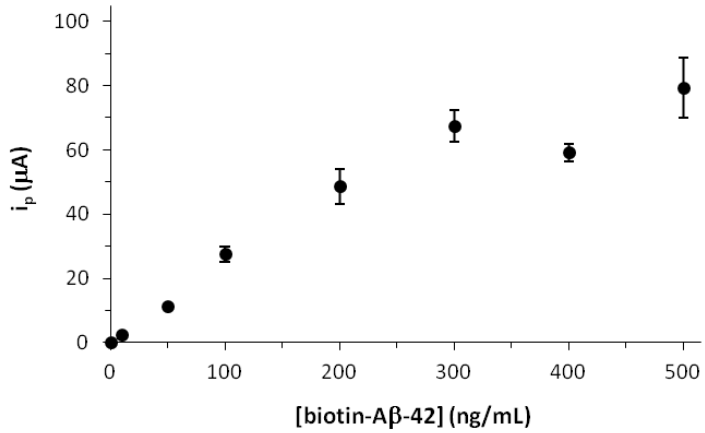

Figure 4. Peak current intensities obtained for different concentration of biotin-Aβ1-42. Experimental conditions: Casein 2%; anti-Aβ1-42 1 µg/mL; anti-IgG-AP 1:15,000; 3-IP 1.0 mM; Ag^+ 0.4 mM. Data are given as average ±SD (n = 3).

The concentration of the antibody anti-Aβ1-42 is a crucial parameter. If it is too high, when the amount Aβ1-42 in the sample is low, the competitive reaction could not be detected, due to there is amount of antibody enough to react with the Aβ1-42 in the sample and with the biotin-Aβ1-42 immobilized on the electrode surface. Fig. 5 shows the results of the optimization of anti-Aβ1-42 antibody concentration. The higher analytical signal is obtained for a concentration of 1 μg/mL, but in order to assure a lack of amount of antibody, a concentration of 0.5 mg/mL is chosen for further studies.

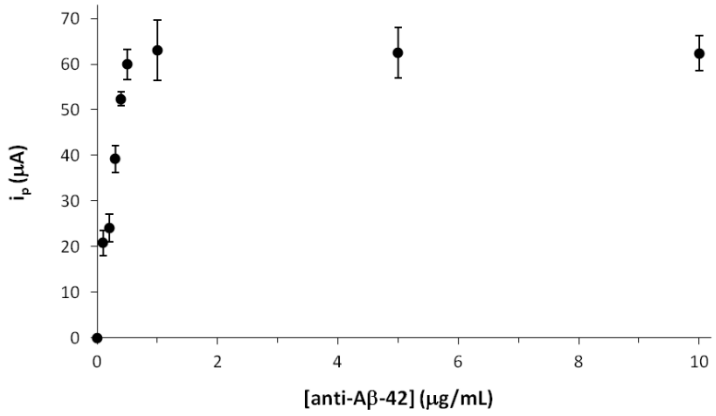

Figure 5. Peak current intensities obtained for different concentration of anti-Aβ1-42. Experimental conditions: Casein 2%; biotin-Aβ1-42 300 ng/mL; anti-IgG-AP 1:15,000; 3-IP 1.0 mM; Ag^+ 0.4 mM. Data are given as average ±SD (n = 3).

3.2. Analytical characteristics of the immunosensor

After the optimization of these analytical parameters, a calibration plot for Aβ1-42 with the equation $(i_0\text{-}i)/i_0$ (μA) = 18 *Log[Aβ1-42]* (ng/mL) + 13, R^2 = 0.991, and a linear range between 0.5 and 500 ng/mL is obtained (Fig. 6). The limit of detection (LOD) and the limit of quantification (LOQ) is calculated from the calibration plot using the equations: LOD = $3s_b/m$ and LOQ = $10s_b/m$ (where s_b is the standard deviation of the intercept and m is the slope of the calibration plot). The LOD value thus obtained is 0.1 ng/mL and the LOQ is 0.4 ng/mL.

In order to evaluate the reproducibility of the immunosensors, several sensors are prepared in different days. Each sensor is used for only one measurement (single use). The maximum signal obtained with these sensors is 61 ± 3 μA (n = 6).

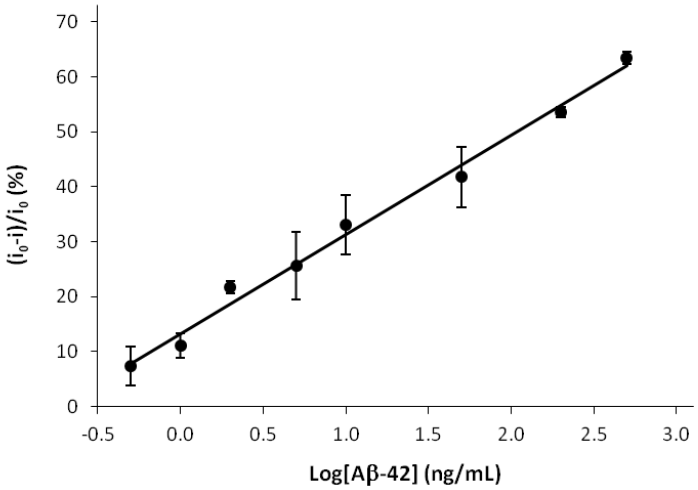

Figure 6. Calibration plot for the immunosensor in the presence of different concentrations of Aβ1-42: 500, 200, 50, 10, 5, 2, 1, 0.5 ng/mL. Experimental conditions: Casein 2%; biotin-Aβ1-42 300 ng/mL; anti-Aβ1-42 0.5 μg/mL; anti-IgG-AP 1:15,000; 3-IP 1.0 mM; Ag^+ 0.4 mM. Data are given as average ±SD (n = 3).

This immunosensor shows good analytical characteristics when is compared with others earlier reported [9-12]. The linear range obtained is wider than the achieved by other methods much more laborious and that required more steps and time of fabrication, for example liquid chromatography tandem mass spectrometry [9] or an immunoassay using carbon fiber microelectrodes [12]. Moreover, so far, there is only one sensor for Aβ peptides (Aβ1-40 and Aβ1-42) detection based on Screen-Printed Electrode, and the LOD of this device for both peptides is ≈ 4.5 μg/mL [14].

4. Conclusions

An electrochemical immunosensor based on Screen-Printed Electrodes for Aβ1-42 detection is developed. Several parameters involved in the immunosensing strategy, such as the biotin-Aβ1-42 and the anti-Aβ1-42 antibody concentrations, were optimized leading to an analytical performance without non-specific adsorptions. The sensor developed shows a very low LOD of 0.1 ng/mL and a wide linear range between 0.5 and 500 ng/mL. This linear range allows the diagnosis of AD considering 500 pg/mL as an optimum cut-off value. On the other hand, the fabrication of this immunosensor is

simple and it can be a portable and ready-to-use device because of the use of SPCE as transducer.

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