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Alzheimer's disease: Development of a sensitive label-free electrochemical immunosensor for detection of amyloid beta peptide

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ABSTRACT

In this work, a highly sensitive label-free immunosensor for detection of the main biomarker of Alzheimer's disease (AD), amyloid beta 1–42 (Aβ (1–42)), is presented. A gold electrode was modified with a mercaptopropionic acid (MPA) self-assembled monolayer, electrodeposited gold nanoparticles (AuNPs) and a monoclonal antibody mAb DE2B4 to recognize Aβ; all the relevant experimental variables were optimized. Antibodies were functionalized through chemical modification (thiolation) to promote the antibody immobilization on the AuNPs surface with proper orientation which enabled the direct detection of Aβ(1–42). Scanning electron microscopy, square-wave voltammetry and electrochemical impedance spectroscopy were used to characterize the construction of the biosensor. Using the proposed immunosensor, Aβ(1–42) was specifically detected within the linear range of 10–1000 pg mL^{-1} with a 5.2 pg mL^{-1} and 17.4 pg mL^{-1} detection and quantification limit, respectively; recovery values for the tested spiking levels ranged from 90.3 to 93.6%. The immunosensor enables rapid, accurate, precise, reproducible and highly sensitive detection (14.6%$_{reduction}$ mL pg^{-1}) of Aβ with low-cost and opens the possibilities for diagnostic *ex vivo* applications and research-based *in vivo* studies.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative pathology that becomes increasingly common with aging, characterized by extracellular accumulation of amyloid β peptide (Aβ), intracellular appearance of neurofibrillary tangles and neuronal loss [1,2]. This degeneration leads to changes in behavior, personality and functional capacity, which hampers the daily life of the patient. Nowadays it affects 35 million individuals worldwide and it is estimated to affect 115 million people by 2050 [3].

The current diagnostic procedures of AD are difficult and made in an advanced stage of the disease. In addition, there are no treatments available that prevent this condition. Therefore, there is a continuing demand for fast and simple analytical methods for the determination of many clinical and biochemical parameters [4–6].

Research gives support to the "amyloid cascade hypothesis", which advocates that an imbalance between the production and clearance or degradation of Aβ in the brain is the initiating event in AD, leading to the formation of extracellular deposits of Aβ (senile plaques) [7]. The peptide with 42 amino acids, Aβ(1–42), is the major component of the senile plaques found in AD. Other pathological hallmarks of AD include intraneuronal inclusions of hyperphosphorylated tau protein in neurofibrillary tangles, together with downstream processes such as inflammation and oxidative stress. All of these steps contribute to the loss of synaptic integrity, effective neural network connectivity and progressive neurodegeneration [3,5,8,9].

Aβ(1–42) can be expressed in cerebrospinal fluid (CSF) and plasma. More specifically, CSF levels of Aβ(1–42) are lower in AD patients than in normal controls, reflecting amyloid pathology. Specifically, low CSF Aβ(1–42) levels are detected in preclinical disease stages and predict future cognitive decline and neurodegeneration [10]. An Aβ(1–42) concentration of < 500 pg mL^{-1} (0.1 nM) is indicative that Aβ(1–42) is accumulating in the brain and not circulating in the CSF [10].

Currently, most of the Aβ(1–42) assays rely on immunochemical detection such as the conventional enzyme linked immunoassay (ELISA) [10,11] which are methods that present high sensitivity, good specificity and less dependence on sample preparation [12]. Recently, an ELISA assay was optimized and validated present-

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ing a detection limit of 375 pg/mL [11]. In this work we present an electrochemical immunosensor which is particularly attractive in the field of diagnosis because biosensors unite the specificity and affinity of the antibody-antigen reaction with the inherent characteristics of electrochemical techniques such as high sensitivity, low cost, high efficiency and easy miniaturization [13–16]. A critical aspect in the development of an immunosensor is the antibody immobilization. The chosen method should be capable of immobilizing a high density of antibodies, maintaining the antibodies bioactivity and promoting the antibodies immobilization with proper orientation to interact favorably with its target antigens [12,17]. Nowadays mouse monoclonal antibody (mAb) are being used for targeting drugs, proteins and peptides to the brain as they are able to recognize a specific antigen [18,19]. In this work, the antibody used was the DE2B4 that is able to recognize the Aβ(1–42), once this anti-beta amyloid antibody bind at the region of 1–17 amino acid. The specificity of this mAb was previously studied [20–22].

Self-assembled monolayers (SAMs) have been used for years in electroanalytical chemistry as the basis for sensors, as they are a simple technique to modify and control the interface of certain materials allowing the immobilization of biomolecules, as for example, antibodies [23,24]. However, the available surface area for the binding of antibody with retention of their bioactivity is still a major issue [17]. For overcoming this problem, SAMs can be coupled with nanoscale materials, which present unique physical properties, ideal for the development of a sensing platform [17,25]. Gold nanoparticles (AuNPs) are chemically stable, non-toxic and easy to functionalize [26,27]. From an electroanalytical point of view, AuNPs are particularly interesting because of their high stability, good biological compatibility, excellent conducting capability and high surface-to-volume ratio [26,28,29]. Moreover, the use of AuNPs should not only enable the immobilization of a higher amount of antibody but also preserve the activity of the immobilized biomolecules, thus offering higher sensitivity and selectivity than conventional strategies. These features provide excellent prospects for interfacing biological recognition events with electronic signal transduction and make AuNPs extremely suitable for developing novel and improved electrochemical sensing and biosensing systems [29–31].

In this work, we present a simple and sensitive gold label free immunosensor for the quantification of AD main biomarker, Aβ(1–42), with the goal of performing a clinical diagnosis and monitor biochemical effects of AD treatments. The sensor's platform was composed of AuNPs which were electrodeposited on the previously mercaptopropionic acid (MPA) modified gold electrode (MPA/Au electrode). Then, the antibody mAb DE2B4 against Aβ(1–42) was functionalized through chemical modification (thiolation) to promote the antibody immobilization on the AuNPs surface with proper orientation which enabled the direct detection of Aβ(1–42). Square-wave voltammetry (SWV) and electrochemical impedance spectroscopy (EIS) were used to characterize the construction of the biosensor and assess the immune reaction between the modified electrode and Aβ(1–42).

2. Material and methods

2.1. Materials

Mouse monoclonal antibody DE2B4 (1 mg mL^{-1}) IgG (ab11132) was purchased from Abcam (UK). Human antigen β-amyloid peptide (1–42), purity >95%, was purchased from Genscript (USA). Goat anti-mouse IgG secondary antibody was purchased from Pierce antibodies/Thermo Scientific (USA). Sulfuric acid (H_2SO_4, 98%), hydrogen peroxide (H_2O_2, 30%), N-hydroxysuccinimide (NHS), citric acid, sodium citrate dihydrate, gold(III) chloride solution, ethylenediaminetetraacetic acid (EDTA), 2-imninothiolane hydrochloride, citrate buffer solution, albumin form bovine serum (BSA) N-(3, Dimethylaminopropyl)-N-ethyl-carbodiimide hydrochloride (EDC), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), cystamine and potassium ferrocyanide ($K_4[Fe(CN)_6]\cdot 3H_2O$), potassium ferricyanide ($K_3Fe(CN)_6$), potassium hydrogen phosphate (K_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4) were purchased form Sigma-Aldrich (Steinheim, Germany). 2-mercaptoethanol, 3-mercaptopropionic acid solution and glutaraldehyde were purchased from Fluka (Switzerland). Absolute ethanol was obtained from Panreac (Spain). Potassium nitrate (KNO_3) was purchased from Pronalab (Mexico). Milk powder was obtained from Molico Nestlé. Alumina solution (γ-Al_2O_3) 0.3 μm and 0.05 μm were purchased from Gravimeta. Ultrapure water (18.2 MΩcm^{-1} resistivity) was produced by a Milli-Q Simplicity 185 system (Millipore, Molsheim, France).

2.2. Electrochemical measurements

Electrochemical experiments were performed with a potentiostat/galvanostat, AUTOLAB model PGSTAT 30 (Metrohm-Eco Chemie, The Netherlands) controlled by a computer through the Model NOVA version 1.9 software. A conventional three-electrode cell was used for all electrochemical measurements: the developed biosensor (based on a polycrystalline gold electrode, BASi MF-2014, surface area 2.0 mm^2 and diameter of 1.6 mm) as a working electrode, platinum as counter-electrode and a Ag|AgCl|3 M KClsat reference electrode to which all potentials are referred.

Square-wave voltammetry (SWV) and electrochemical impedance spectroscopy (EIS) were performed using $Fe(CN)_6^{3-/4-}$ as electroactive indicator at a concentration of 2.5 mM in 0.1 M PBS solution (pH = 7.4). SWV results were obtained by varying the potential from 0.00 to 0.60 V at 0.400 V/s scan rate. The optimal SWV parameters were a frequency of 100 Hz, amplitude of 40 mV and scan increment of 4 mV. EIS measurements were performed using a frequency range from 10^{-1} to 10^5 Hz with an amplitude perturbation of 5 mV.

2.3. Immunosensor development

The surface of the bare gold electrode was cleaned using piranha solution (30:70, v/v, H_2O_2 and H_2SO_4) at room temperature (RT) for 5 min. Then the electrode was polished repeatedly with 0.3 and 0.05 μm alumina powder on microfiber cloth. Subsequently the surface was rinsed with ultrapure water and ultrasonically cleaned in absolute ethanol. After that, the electrode was cycled from 0.0 to 1.6 V in 0.5 M H_2SO_4 solution at a scan rate of 100 mV/s. The process was repeated until typical gold cyclic voltammograms were obtained. Finally, the electrode was washed with ultrapure water and modified (Fig. 1).

2.3.1. Self-assembled monolayer

Four different SAMs were studied by immersing the gold electrode, for a 12 h period at RT, in ethanol solutions of cystamine (CYS) (20 mM), cystamine + mercaptoethanol (CYS + ME) (20 mM), mercaptopropionic acid (MPA) (1 mM) and mercaptopropionic acid + mercaptoethanol (MPA + ME) (1 mM). After washing with ultrapure water, the CYS modified electrodes were immersed for a period of 30 min in a 3% glutaraldehyde solution, while the other modified (MPA) electrodes were immersed in an EDC/NHS solution.

After selection of the optimum SAM, the incubation time (2–12 h) and concentration of the solution (1–20 mM) for the SAM formation were optimized.