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Development of a novel label-free amperometric immunosensor for the detection of okadaic acid

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ABSTRACT

Okadaic acid (OA), a lipophilic phycotoxin is mainly produced by toxigenic dinoflagellates. The need to develop high performing methods for OA analysis able to improve the traditional ones is evident. In this work, a novel experimental methodology for label-free detection of OA was developed. Protein G magnetic beads (protein-G-MBs) modified gold electrode was used to immobilize anti-OA monoclonal antibody (anti-OA-MAb). Preliminary, colorimetric tests were performed in order to validate protein-G-MBs and anti-OA-MAb reaction. Electrochemical detection was carried out by differential pulse voltammetry in ferri/ferrocyanide solution. The limit of detection value obtained (0.5 $\mu g\,L^{-1}$) validated the developed electrochemical immunosensor as a promising tool for routine use. The matrix effect and the recovery rate were also assessed with real samples, showing a good percentage of recovery.

1. Introduction

In recent years, different types of bioassays have been emerged as cheap and quick alternative to traditional chromatographic methods. One of the attractive candidatures is immunoassays, likely due to the high affinity interactions between antigens and antibodies, often involving higher sensitivity and lower limits of detection. Several immunoassay formats and immobilization techniques have been used to develop improved biosensor devices [1,2]. The conventional enzyme-linked immunosorbent assay (ELISA), however, is a time consuming, expensive and multistep process. As an alternative, electrochemical immunosensors have been appeared a promising bio-tool due to their low limit of detection and high sensitivity [3–5]. As antibody and antigen are chemically inert, a label such as enzyme is required to generate an electrochemical signal [6–9]. Although these developed methods are very sensitive, however, there are several challenges in the construction of amperometric immunosensors. Labeling of either antigen or antibody makes the assays more complex, time consuming and laborious. Moreover, labeling process is costly and often results in the denaturation of the modified biomolecules [10]. In an effort to overcome these drawbacks, there is an increasing interest in the development of label free immunosensors. Various transducer techniques such as electrochemistry, surface plasmon resonance, piezoelectric and cantilever have been used to fabricate label-free immunosensors [11–13]. Among these methods, label-free electrochemical immunosensors have attained growing attention because of their high sensitivity, low cost, fast response time and simple equipment. The label-free approach with electrochemical detection simplifies the sensing protocol [14–16]. In these experiments, the current response of redox probe is studied on the electrode surface. There is change in mass and thickness on electrode surface due to the formation of antibody–antigen complex, which in turn blocks the electron transfer of the redox probe on electrode surface [17,18].

Based on the above observations, we investigated a label-free amperometric immunosensor for okadaic acid (OA) detection. OA, a lipophilic marine biotoxin, is mainly produced by algae of the genera *Dinophysis* and *Prorocentrum* [19]. This toxin causes diarrheic shellfish poisoning (DSP) to humans after its ingestion, although it is harmless for shellfish. DSP is responsible for gastrointestinal disturbance such as diarrhea, vomiting and abdominal pain in addition to its cancerogenic, mutagenic and immunotoxic effects [20,21]. This contamination of bivalves has been increasing economic concern for shellfish industry worldwide [20]. The maximum level of OA allowed by the commission of the European Community is 160 μg of OA kg^{-1} of mussels (EC No. 853/2004 15). The European Food Safety Authority (EFSA) has suggested to reduce the maximum permitted level from 160 $\mu g\,kg^{-1}$ to 45 $\mu g\,kg^{-1}$ of mussels [22]. Mouse bioassay was adopted as the reference method until the 1st January 2011 for OA analysis [23]. This method, however, is under consideration due to poor selectivity and accuracy in addition to ethical problems. Due to these reasons, some alternative methods, such as liquid chromatography

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coupled to fluorescence, liquid chromatography combined with mass spectrometry have been proposed by European Union (EC No. 2074/2005 17). Immunosensors based on different formats and immobilization methods have been proposed for OA detection [24–27]. To our knowledge, so far there is no report on the label-free amperometric immunosensor for OA detection.

Recently, MBs have been emerged as powerful immobilization support in biosensing technologies. MBs are a class of nanoparticles that are available with a variety of surface functional groups and can be manipulated under influence of an external magnetic field [28,29]. The use of MBs offers advantages in term of large surface, stability of the surface-bound antibodies, enhancement of the assay kinetics and improvement in orientation of the immobilized antibody. Another important advantage of the MBs is that they re-disperse upon removal of the external magnet and such a behavior is very useful for easy and fast renewal of the biosensing surface [30,31]. In the present work, a new immunosensor combining the MBs advantages with label-free detection was developed, and conventional direct competitive ELISA (dc-ELISA) was used to investigate the affinity interaction between anti-OA-MAb modified protein-G-MBs and OA. The characterization of electrochemical immunosensors during different fabrication stages was investigated by cyclic voltammetry (CV) in $[Fe(CN)_6]^{4-/3-}$ solution for two type of immobilization strategies. Differential pulse voltammetry (DPV) in $[Fe(CN)_6]^{4-/3-}$ solution was used for the label-free detection of OA. In a word, our developed method provided a low cost, fast response time and ease of preparation technique for the detection of OA in mussel samples.

2. Experimental

2.1. Materials and reagents

OA potassium salt, purchased from Sigma, was firstly dissolved in ethanol ($1\,g\,L^{-1}$) and then diluted in phosphate buffer saline (PBS 1×). Buffer components, Tween 20, bovine serum albumin (BSA), *N*-hydroxysuccinimide (NHS), *N*-(3-dimethylaminopropyle)-*N*′-ethyl-carbodiimide hydrochloride (EDC), potassium ferrocyanide ($K_4Fe(CN)_6$), potassium ferricyanide ($K_3Fe(CN)_6$) and horseradish peroxidase (HRP) were purchased from Sigma (France). Monoclonal antibody (anti OA-MAb, developed in mouse) against OA was obtained from Novus Biologicals (UK). Protein G magnetic beads (protein-G-MBs) (Invitrogen, USA) were used as support to immobilize anti-OA-MAb. 96 well microplates, PS, U-bottom were obtained from Greiner Bio-One (France). Adem-Mag 96 (adapted for 96-well micro titer plates) and Adem-Mag SV (single magnet position adapted for 1.5 mL micro tubes) were from Ademtech S.A (France). 3,3′,5,5′-tetramethylbenzidine (TMB) from Sigma was used as received.

2.2. Apparatus

The electrochemical measurements were performed with an AUTOLAB PGSTAT100 potentiostat/galvanostat equipped with general purpose electrochemical system (4.9) for voltammetry (Eco Chimie, The Netherlands). Colorimetric measurements were performed with a lab systems Multiskan EX micro titer plate reader (Thermo Life Sciences, France). Screen-printed gold electrodes were purchased from DropSens (Spain). The electrode consists of conventional three electrode configuration with a disk-shaped Au working electrode (1.66 mm diameter, $0.02\,cm^2$ geometrical area), Au counter and silver pseudo-reference electrode. A small 4 mm-diameter magnet was taped on the back side of the working electrode to immobilize the MBs to the electrode surface. A horizontal shaker (IKA, Vibrax-VXR) was also used.

2.3. Anti-OA-MAb immobilization via protein-G-MBs

2.3.1. dc-ELISA protocol

2.3.1.1. Modification of OA with HRP. 30 μL of OA ($1\,g\,L^{-1}$) were added in 10 μL of 10 mM sodium acetate buffer at pH 4.5. 60 mg of EDC were dissolved in 1 mL of 10 mM sodium acetate buffer and 30 mg of NHS were dissolved in 1 mL of sodium acetate buffer. 12 μL of EDC and NHS solutions (1:1) were added to OA solution and the mixture was stirred at room temperature for 1 h. 58 μL of HRP ($5\,mg\,mL^{-1}$) prepared in PBS (pH 7.2) were added to this mixture and was stirred overnight at room temperature. The OA-HRP conjugate was purified by dialysis in saline solution ($9\,g\,L^{-1}$ NaCl) with solution changed 3 times.

2.3.1.2. Modification of protein-G-MBs with anti-OA-MAb. 50 μL of protein-G-MBs ($10\,mg\,mL^{-1}$) were taken in 1.5 mL microtube; then the particles were collected using the Adem-Mag SV and washed three times with 50 μL of PBS (1×) and resuspended in 50 μL of anti-OA-MAb at dilution of 1/2 for 1 h at room temperature; then washed three times with 50 μL of PBS (1×). The modified MBs were recovered in 50 μL of PBS (1×) and stored at 4 °C.

2.3.1.3. Colorimetric measurements. In the dc-ELISA, 100 μL of the modified MBs at dilution of 1.5/100 were added inside the wells and the buffer was removed; the nanoparticles were collected onto the inner wall of the well by using the Adem-Mag 96. Blocking step was carried out by adding 250 μL of PBS (1×) containing 1% BSA for 1 h. Afterwards, the competition step was performed for 1 h min using 10 μL of OA standard solutions at different concentrations and 40 μL of OA-HRP solution at dilution of 0.75/100 in sodium acetate buffer. 100 μL of TMB solution were incubated for 20 min. All the process was carried out at room temperature with constant shaking. Washing steps were performed by adding 200 μL of PBS (1×) containing 0.05% Tween 20 between each step and when the different solutions were discarded, the MBs were collected by using the Adem-Mag 96. Absorbance values were measured at 620 nm and assays were carried out in triplicate.

2.3.2. Assembling the electrochemical immunosensor

Prior to immobilization, polycrystalline Au electrodes were subjected to electrochemical pretreatment by 2 cyclic potential scans between 0.0 and 1.5 V with $100\,mV\,s^{-1}$ in 0.5 M H_2SO_4. To prepare an electrochemical immunosensor, a small magnet was taped under the working electrode surface and a 10 μL aliquote of protein-G-MBs at dilution of 1/10 was immobilized on the electrode surface. The electrode surface was incubated with 20 μL of 0.5% BSA for 30 min to prevent non specific adsorption of antibody. Next, 20 μL of anti-OA-MAb at dilution of 1/100 was added to electrode surface for 1 h. The electrode was rinsed with washing buffer and distilled water between each step. Then, 20 μL of OA standard solutions at different concentrations or 20 μL of spiked samples was applied to electrode surface for 1 h at room temperature prior to rinsing electrode with distilled water.

2.4. Anti-OA-MAb immobilization via direct adsorption at electrode surface

Prior to immobilization, each Au electrodes was subjected to electrochemical pretreatment by 2 cyclic potential scans between 0.0 and 1.5 V with $100\,mV\,s^{-1}$ in 0.5 M H_2SO_4. To fabricate electrochemical immunosensor, each Au electrode was incubated with 20 μL of anti-OA-MAb at dilution of 1/100 in PBS (pH 7.4) overnight at 4 °C under water saturated atmosphere. After washing with distilled water, 20 μL of OA standard solutions at different