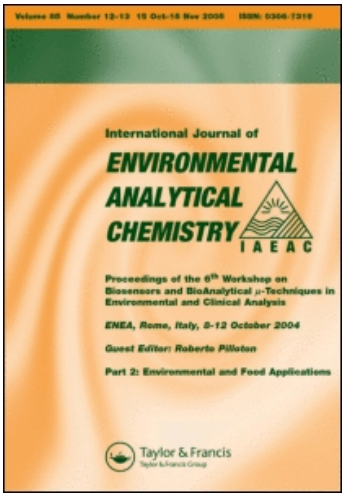

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IMMUNOSENSOR FOR THE DETERMINATION OF OKADAIC ACID BASED ON SCREEN-PRINTED ELECTRODE

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A disposable immunosensor for okadaic acid (OA), using a screen-printed electrode (SPE), was developed and characterised. Detection of the product, *p*-aminophenol, resulting from the reaction catalysed by alkaline phosphatase (AP), was carried out using an amperometric three-electrode system poised at a voltage of +300 mV *versus* Ag/AgCl. Alkaline phosphatase was used as a label for the antigen, OA, and two kinds of alkaline phosphatase preparation were studied for the conjugation of okadaic acid. The calibration curve for okadaic acid obtained from the conjugate created from low-activity AP, 969 units/mg, was unsatisfactory in terms of sensitivity, but a high-activity conjugate delivered the required sensitivity and limit of detection. Studies on the stability of the sensor with α-OA antibody and OA–AP conjugate showed that the current response decreased drastically after one day. Stabilisation strategies have been formulated to overcome this problem. The calibration curve obtained with the high activity conjugate was linear up to 40 ng/ml of okadaic acid with a minimum concentration of analyte detected of 5 ng/ml and a detection limit of 2 ng/ml.

Keywords: Screen-printed electrode; Okadaic acid; Immunosensor; Alkaline phosphatase; Amperometric detection

INTRODUCTION

Algae occur in ponds, ditches, wells, lakes, rivers and seas, and they may produce chemicals that are harmful to fish and invertebrates. They may produce potent toxins that can find their way through the food chain to humans, causing a variety of gastrointestinal and neurological illnesses. As a result, we are faced with the problems of public health and economic impact of harmful algal blooms. Consequently, more and more scientists over the world are now investigating this field. Okadaic acid is a diarrhoeic shellfish toxin and causes diarrhoea, nausea, vomiting and abdominal pain. Long-term exposure may promote tumour growth.

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Various methods such as chemiluminescent immunoassay, HPLC and commercial immunoassay kits for the determination of OA have been developed [1–3]. A method for detecting okadaic acid was also developed using ELISA in this lab and the OA system has been extensively investigated [4]. HPLC is laborious, time-consuming and suffers from some interferences at low detection levels. Immunoassay kits appeared to be more sensitive, specific and faster than HPLC.

The objective of the work was to develop a disposable and rapid immunosensor using a screen-printed electrode for the determination of seafood toxins. Screen-printing is a promising technique, because it offers the advantages of cheaper and reproducible electrode production, a decrease in the amount of reagents used and the opportunity for single use. Electroanalytical detection in combination with a disposable electrode has many inherent advantages [5,6] and there has been increasing interest in the development of biosensors for industrial, clinical and environmental analysis [7–9].

EXPERIMENTAL

Apparatus

ELISA was performed in 96-well microtiter plates (Nalge Nunc International, NUNC-Immuno^{TM} Plate, MaxiSorp^{TM} Surface). Bio-Tek Instruments supplied the reader (model EL 311SX). An electrochemical analyser (BAS 100B, Bioanalytical Systems, USA) was used for amperometry. A DEK 247 screen-printer was used to produce SPEs. A Sephadex PD-10 column (Supelco, Bellefonte, PA) was used for the purification of OA–AP conjugates and the prepared conjugates were analysed for protein content using a UV/VIS spectrophotometer (Hewlett-Packard).

Chemicals

Anti-okadaic acid monoclonal antibody was purchased from Iatron laboratories, INC (Tokyo, Japan). Okadaic acid (*Prorocentrum concavum*) was purchased from Calbiochem (Nottingham, UK). Alkaline phosphatase (AP, from calf intestine), *p*-nitrophenyl phosphate (*p*-NPP), bovine serum albumin (BSA), 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), and other chemicals were obtained from Sigma Chemical LTD (Dublin, Ireland), and *p*-aminophenyl phosphate (*p*-APP) was synthesized in-house [10].

Conjugation of Okadaic Acid to Alkaline Phosphatase

One hundred microgram of OA (1.24×10^{-7} mol in 60 µl of ethanol) was added to the mixture of 10 µl EDC and 20 µl of NHS (20 mg/ml each). The mixture was allowed to react for 1 h at room temperature. The molar ratio of OA to AP was 10 : 1. 100 µl of AP solution (2.1 mg) was dissolved in 400 µl, 0.1 M, pH 7.4 Tris–HCl buffer and it was added drop-wise to activated-OA. The reaction was allowed to proceed for 4 h at room temperature. Purification was achieved using Sephadex PD-10 column. Ten fractions (1 ml of each) were collected and they were analysed by a spectrophotometer at 280 nm and the third fraction was found to contain most of the conjugate. The desired OA–AP conjugate was stored at 4°C.

ELISA Procedures

The immobilisation of anti-okadaic acid antibody was attained by adding antibody solution to a microtiter plate and incubation, followed by blocking using 100 µl, 1% BSA in 50 mM, pH 7.4 Tris buffer. The OA–AP binding was then carried out. Colour development was achieved by the addition of 100 µl, 1 mg/ml of *p*-NPP in 0.1 M, pH 9.5 diethanolamine containing 1 mM $MgCl_2$ and 50 mM KCl and incubation. The yellow product, *p*-nitrophenol, was monitored at 405 nm. The incubation volume was 50 µl and all incubations proceeded at 37°C for 1 h. Between incubations, the ELISA plate was washed three times with Tris buffer.

Construction of Screen-printed Electrode

SPEs consisted of a conducting silver layer, an insulation ink and a carbon ink working area (0.16 cm^2). They were used as disposable electrodes produced by the controlled deposition of ink in a manner that formed reproducible structures. The layers of ink, constituting a particular electrode, were deposited onto a PVC substrate.

Preparation of Screen-printed Electrode for Measurement

Five microlitre of α-OA antibody solution was added onto the carbon area and left at room temperature for 1 h. The subsequent steps were performed in a microtiter plate and the SPE was immersed into incubation solutions. All incubations proceeded at 37°C for 1 h. After blocking using 300 µl, 1% BSA in 50 mM, pH 7.4 Tris buffer, the immobilization of OA–AP conjugate (180 µl) was performed. Between incubations, the electrodes were washed using Tris buffer. For calibration, the procedures for the preparation of the SPEs were the same as mentioned above, except the last step, in which the SPEs were immersed into the mixture of free OA and OA–AP conjugate (90 µl of each).

Amperometric Measurement System

Signal detection was carried out by placing a SPE into a stirred electrochemical three-electrode cell containing 4.95 ml of 50 mM, pH 9 Tris buffer with 100 mM NaCl, 1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$. A platinum wire in the cell served as an auxiliary electrode. The cell was connected to a amperometric detector (BAS 100 B) and a voltage of 300 mV *versus* Ag/AgCl reference electrode was applied to the working electrode. Fifty microlitre (25 mg/ml) of *p*-APP was injected after a steady-state current was attainted. Each electrode was used for one single measurement.

RESULTS AND DISCUSSION

Competitive Format Combined with Amperometric Detection

As shown in Fig. 1, since okadaic acid is a small molecule, it is unlikely that direct capture will yield a good detection limit [11]. Competition and displacement assays would have potential for detecting small molecules. Our earlier studies [4] have shown that the competitive format with ELISA yielded the best results in terms of

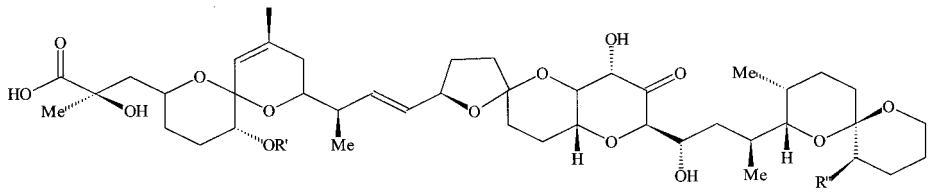

FIGURE 1 Structure of okadaic acid.

linear range and detection limit. With this in mind it was decided to apply the competitive format using alkaline phosphatase as a label for okadaic acid in our studies. Labelled okadaic acid and free okadaic acid were added simultaneously and competed for the binding sites of antibodies bound on the carbon area of SPE.

p-APP was used as the substrate of alkaline phosphatase because it provides a relatively low oxidation potential at a carbon electrode and low propensity for electrode surface fouling [12]. The current produced is directly proportional to the amount of alkaline phosphatase labelled okadaic acid bound on the electrode, but is inversely proportional to the concentration of free okadaic acid in solution.

In order to obtain a high sensitivity enzyme immunosensor, the working conditions such as buffer type, concentration and pH must be considered. In previous work [13], alkaline phosphatase as a label for immunoassay with amperometric detection for a variety of substrates has been extensively studied. Tris–HCl buffer has shown to be an optimum buffer offering optimum Michaelis–Menten characteristics, a high oxidation limit and a low background. Therefore, Tris–HCl buffer (0.1 M, pH 9.0) was used in our work.

Conjugation of Okadaic Acid to Alkaline Phosphatase

Alkaline phosphatase and its conjugates maintain good immunological and enzymatic activity and the variety of substrates available for this enzyme using amperometric detection makes this enzyme the preferred choice [14]. Activity losses may occur after coupling to an antibody or antigen molecule. Using a high-activity alkaline phosphatase preparation helps to maintain good resultant activity in the conjugate [15]. Two kinds of AP were studied for the conjugation of okadaic acid. The high activity alkaline phosphatase (5238 units/mg protein) resulted in a high activity of OA–AP conjugates as expected, which gave almost the same current response at the dilution factor of 1 : 20 as the low activity alkaline phosphatase (969 units/mg protein) at a dilution factor of 1 : 2. The calibration for okadaic acid obtained from the conjugate with the low activity AP was unsatisfactory in terms of the sensitivity, but a good result was obtained by the high activity AP.

The efficiency of conjugation of OA to AP was difficult to assess. The conjugate density (the ratio of moles of OA/AP) was impossible to measure since OA had no detectable parameter. The evidence for successful coupling was inferred from the result of ELISA.

Optimisation of the Working Conditions

Initially, the amount of anti-okadaic acid antibody for the saturation of the surface of electrode was investigated. Figure 2 shows the response of electrodes increased with increasing amount of antibody. 0.5 μg of antibody sufficed for saturation of electrode's surface and was used for the further studies. Each point on the curve was the average of the response of two electrodes.

The dilution factor of stock OA–AP conjugate was optimised. The influence of three dilutions of stock OA–AP conjugate, 1 : 5, 1 : 10 and 1 : 20, to the response of sensors were investigated and the results obtained are shown in Fig. 3. The dilution factor of 1 : 10 gave adequate signal and the higher concentration of conjugate did not increase the response of the sensor significantly. As a consequence, a dilution of 1 : 10 was used for the calibration of okadaic acid. Each points on the curve was the average of the response of two electrodes.

The incubation time of the labelled antibody on SPE had previously been investigated in the previous work by us [14]. Signals with amperometric detection increased dramatically with the incubation time of labelled antibody up to 30 min and then

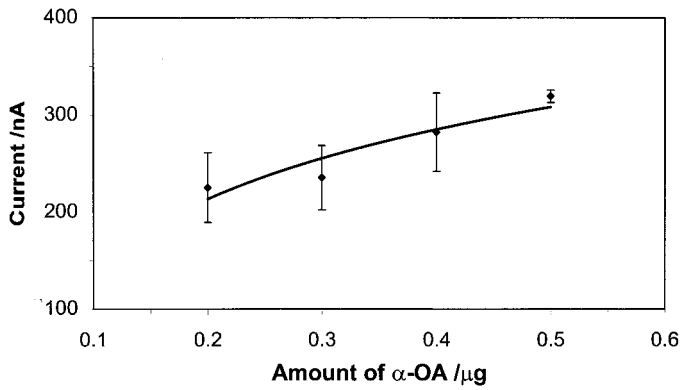

FIGURE 2 Variation of current response of SPEs with the concentration of anti-okadaic acid antibody. The dilution factor of stock OA–AP was 1 : 2. AP = 969 units/mg.

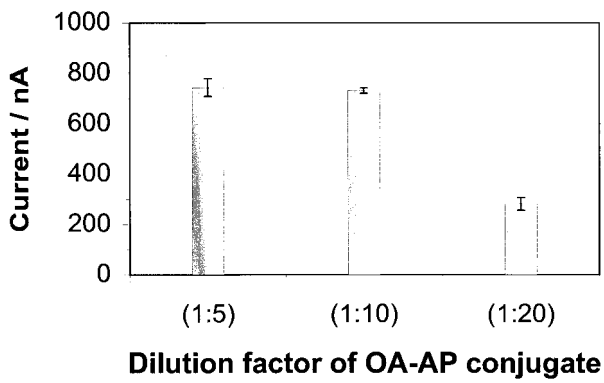

FIGURE 3 Current response of SPEs with dilution factor of stock okadaic acid–alkaline phosphatase conjugate. [α-OA] = 0.5 μg. AP = 5238 units/mg.

TABLE I The variation of response of SPE with α-OA and OA–AP over time

Day	1*st*	2*nd*	7*th*
Current ± RSD (nA, %)	607 ± 11	56 ± 28	39 ± 20

increased slightly until 60 min. With this in mind, an incubation time of 60 min was chosen for labelled antigen immobilization onto the surface of the electrode.

Life-time of SPE with α-OA and OA–AP

The operational stability of the prepared SPE for amperometric detection was found to be a problem during a long periods of measurements. This prompted us to examine the life-time of SPE with α-OA and OA–AP. Twenty-four SPE were bound with anti-OA antibody (0.5 μg) and OA–AP (1 : 10 dilution of stock conjugate), and offer blocking using 1% BSA. The immobilized SPEs were then washed with 0.03% (w/v) sodium azide solution to prevent bacteria growth. After air-drying they were stored dried at 4°C. The current response of the SPEs (four SPE were examined each time) had decreased by about 90% after one day, as shown in Table I. The two minimal signals were the background current and the electrodes tested produced a current equivalent to no response after one day.

The short life-time of the immobilized SPE can be explained by the fact that the alkaline phosphatase lost its activity over time and/or the antibody came off the surface of the electrode. The following suggestions might help with the improvement of the operational and storage stability of prepared SPEs. Previous studies [16] have shown that DEAE-dextran and Gafquat 755N (is a proprietary cationic additive and is a polyquarternary ammonium polymer) had a stabilizing effect on dissolved horseradish peroxidase in all used buffer systems. Polyethylenimine was used to improve the storage stability of reticulated vitreous carbon electrodes with immobilized glycerol dehydrogenase and diaphorase for the determination of NADH [17] as well as the stability of a cholinesterase biosensor for the detection of pesticides in water-miscible organic solvents [18]. Other polymer additives provided for the better operational stability of amperometric sensors for lysine and hypoxantine sensors with the enzymes incorporated with Teflon into the carbon paste [19,20]. Assuming immobilized antibody loss from the electrode's surface was the cause, covalent binding by using EDC and NHS might be a strategy to improve the storage stability of the sensors.

Calibration Curve for Okadaic Acid

The preparation of the calibration curve was performed in the concentration range from 0 to 320 ng/ml in an amperometric system and the linear portion of the calibration curve is shown in Fig. 4. As can been seen, it was linear up to 40 ng/ml of OA with a detection limit of 2 ng/ml of OA ($R^2 = 0.999$). Spectrophotometric detection was set up as a reference method to compare the calibration of okadaic acid and the result showed the same linear range.

The amount of labelled antigen plays an important role in a competitive immunoassay and it must suffice for saturation of the surface of the electrode, which means free antigen could compete with labelled antigen and be bound onto the antibody layer immobilized on the electrode's surface.

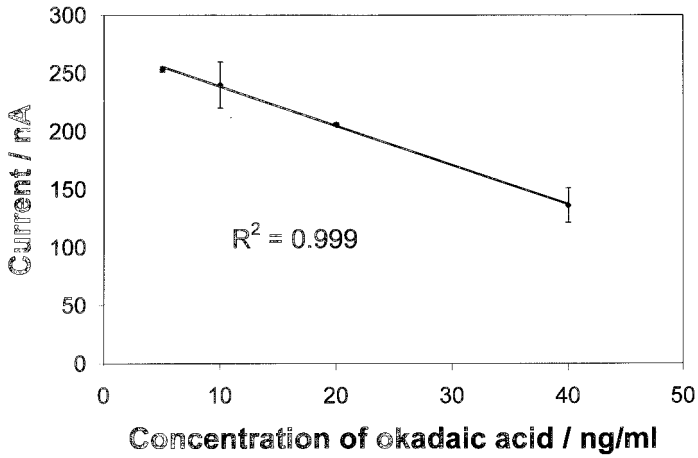

FIGURE 4 Calibration curve for okadaic acid with amperometric detection. The dilution factor of stock OA–AP was 1 : 10. AP = 5238 units/mg. [α-OA] = 0.5 µg.

CONCLUSIONS

The screen-printed immunosensor was able to quantify okadaic acid in standard solution. High-activity alkaline phosphatase was the preferred enzyme label for the creation of conjugate and this led to the saturation of the sensor surface with product. Displacement of the high activity conjugate by free antigen generated the required sensitivity and a detection limit of 2 ng/ml. The operational and the storage stability of the sensors may be improved by the addition of a polymer. Work is continuing to optimise the characteristics of the immunosensor in order to furnish a rapid seafood toxin monitoring.

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