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A multichannel electrochemical detector coupled with an ELISA microtiter plate for the immunoassay of 2,4-dichlorophenoxyacetic acid

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Abstract

A multichannel electrochemical detector (MED) consisting of eight sets of electrodes held on an ELISA microtiter plate for immunoassay of a herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D) was described. The eight-channel electrodes were characterized in terms of repeatibility, homogeneity and stability. The direct competitive immunoassay format was employed for the determination of 2,4-D. Monoclonal antibody (mAb) against 2,4-D, alkaline phosphatase-2,4-D (ALP-2,4-D) and *p*-aminophenyl phosphate (*p*-APP) were used as coating protein, enzyme labeled conjugate and substrate, respectively. The immuno-reaction and enzymatic reaction were carried out in an ELISA microtiter plate. The *p*-aminophenol (*p*-AP) produced was amperometrically detected by the pre-equilibrated eight-channel electrodes in another plate. The current responses measured were inversely proportional to the 2,4-D concentration. Under optimal conditions, the standard calibration curve was constructed in the dynamic range of 0.1–330 ng/ml and the detection limit of 0.072 ng/ml was achieved. The recoveries of 2,4-D in tap water samples spiked at the concentration of 0.1, 1.0, 10 and 100 ng/ml were found within 89.0–116.0%, and the relative standard derivation (R.S.D.) values of the intra-assay and inter-assay were less than 20%. The advantages of the presented multichannel electrochemical detector coupled with an ELISA plate for immunoassay were those with high stability and throughput, low detection limit, the ability of being repeatedly used and no need for regeneration.

Keywords: Multichannel electrochemical detector (MED); Amperometric immunosensor; 2,4-Dichlorophenoxyacetic acid (2,4-D); Enzyme linked immunosorbent assay (ELISA)

1. Introduction

Modern analysis for the samples in medical, biological and environmental requires very sensitive and fast techniques. In the last years, electrochemical immunosensors have gained growing attention because of their low detection limit, simplicity of the equipment required and the ability to analyze heterogeneous and colored samples [1–4].

Conventional electrochemical immunosensors are based on the immobilizaion of antibody or antigen on the surface of electrodes. Among different immunosensors, the most commonly used one is amperometric immunosensor, which integrates the high specificity owing to the immuno-reaction between antibody and antigen with the significant amplification of signal catalyzed by the labeled enzyme [5,6]. However the main problem associated with the conventional immunosensors is that after one signal measurement, the immunosensing surface on the electrode should be renewed for the next measurement. During the regeneration, a drastic solution is often used to break down the binding between antibody and antigen, which may cause the loss of the activity of antibody or antigen bound on the electrode, leading to irreproducible results [7–9]. Some attempts to avoid the regeneration step such as using disposable screen-printed electrodes or magnetic beads bound with antibody or antigen were made [10–14]. Another novel approach avoiding regeneration step was reported in which the immunoreactions were carried out in the wells of an ELISA microtiter plate and the generated electro-active species were detected one sample after another by the electrode in a flowing injection system (FIA) [15–17]. However the drawback of the approaches mentioned above [10–17] was their low throughput.

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2,4-D is one of the most used herbicides in agriculture. As phenoxyacetic acids and their metabolites may contaminate not only ground water and surface water, but also drinking water as well. The highly permissible concentration of a specific herbicide in drinking water set by European Union (EU) is 0.1 ng/ml. In the last years, besides ELISAs incorporating microtiter plates with a plate reader based on spectrophotometric technique for the analysis of 2,4-D [18–20], 2,4-D was often used as a model analyte for the development of immunosensors. Dozens of 2,4-D immunosensors using different labels, immobilization supports, and transduction systems were reported [5,21–29]. As the low throughput for signal channel immunosensors, Skladal and Kalab [30] developed a disposable multichannel immunochemical sensor consisting of the array of eight working electrodes and one reference electrode placed at a ceramic substrate for the determination of 2,4-D. Obviously the throughput of the disposable multichannel immunosensor was improved, but it was still difficult to construct calibration curve and analyze several samples simultaneously; in addition the repeatibility of the measurements was low and the electrodes could not be repeatedly used.

In order to develop a multichannel immunosensor with the properties of high throughput, repeated use and no need for regeneration, a multichannel electrochemical detector (MED) consisting of eight sets of electrodes held on an ELISA microtiter plate was developed and applied for the macromolecular compound, e.g. rabbit immunoglobin [31]. In this study, the purpose was to extent the application scope to small molecular compound, e.g. 2,4-D in environmental analysis. The MED was characterized in terms of repeatibility, homogeneity and stability, and was applied for the determination of a model analyte 2,4-D. The assays conditions were optimized. The immunoreaction and enzymatic reaction were carried out in the ELISA microtiter plate and the electrochemical species produced in the wells of the plate were amperometrically detected by the pre-equilibrated eight-channel electrodes.

2. Experimental

2.1. Instrumentation

Electrochemical analyzer (CHI 750A) used for cyclicvoltammetry study was purchased from CH Instruments, Inc. Polystyrene 96-well microtiter plates (MaxSorp and Polysorp) were obtained from Nunc Inc. Milli-Q equipment for water purification was from Millipore Inc.

2.2. Reagents and solutions

The mAb against 2,4-D (ascitic fluid, lyophilized, lot E2/G2, reconstituted with 0.45 ml pure water per vial before use) was a generous gift from Dr. Franek (Veterinary Research Institute, Brno, Czech Republic). 2,4-D standard, ALP (type VII-L, from bovine muvosa, 3020 units/mg), Tris [(hydroxymethyl)aminomethane], *N*-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), dimethyl formamide (DMF) and Sephadex G-25 were purchased from Sigma (USA). The *p*-aminophenol (*p*-AP) used for cyclicvoltammetry study was obtained from ACROS ORGANICS (USA). The *p*-aminophenyl phosphate (*p*-APP), used as the substrate, was prepared from hydrogenation of *p*-nitrophenyl phosphate (*p*-NPP) by following the procedures reported in the literature [32]. All chemicals and solvents used were of analytical grade and were used as received.

Solution of 0.05 mol l^{-1} Na_2CO_3–$NaHCO_3$, pH 9.6 was used as coating buffer. Assay buffer prepared from 0.1 mol l^{-1} phosphate buffer saline (PBS), pH 7.4, containing 0.5% casein and 0.15 mol l^{-1} NaCl, was used for preparing immuno-reagents and for blocking and washing microtiter plate. Solution of *p*-APP was prepared with 0.1 mol l^{-1} Tris buffer, containing 1 mg/ml $MgCl_2$, pH 9.0. The 2,4-D stock solution (500 μg/ml) was prepared by dissolving 2 mg of 2,4-D powder with 0.2 ml of DMF and then diluted with 0.1 mol l^{-1} PBS, pH 7.4, to a final volume of 4 ml. The standard solutions of 2,4-D with the concentrations at 0, 0.1, 1.0, 3.3, 10, 33, 100 and 330 ng/ml were prepared by diluting stock solution with assay buffer.

2.3. Preparation of eight-channel electrodes

The preparation of multichannel electrodes was adopted as Ref. [31]. A piece of Pt wire (1.0 mm in diameter and 1.0 cm in length) was sealed in a small glass tube and used as the working electrode. Another piece of Pt wire was used as the reference and counter electrode. As shown in Fig. 1, an electrode assembly consisted of eight sets of Pt electrodes fixed on the electrode holder in an arrangement fitted with a row of an ELISA microtiter wells. With the assembly, working electrodes were placed at the same depth (∼1 mm above the bottom) in microtiter wells that ensured the consistent performance of the electrodes. The working electrodes were polished with colloidal silica suspension and rinsed with pure water before use.

2.4. Synthesis of 2,4-D-ALP conjugate

The synthesis method was modified according to Ref. [33]. Briefly, 1 mg of 2,4-D, together with 1.7 mg of NHS and 6.2 mg of DCC were dissolved in 0.13 ml DMF and kept on stirring overnight at room temperature. After centrifugation, half supernatant with the activated 2,4-D was slowly added to 0.5 ml 0.13 mol l^{-1} $NaHCO_3$, pH 8.5, containing 2.2 mg of ALP. The reaction was carried out at room temperature for 3 h; then the mixture was desalted with a Sephadex G-25 column. Fractions containing the protein were pooled and dialyzed overnight at 4 °C against 0.013 mol l^{-1} $NaHCO_3$, pH 8.5. The 2,4-D-ALP conjugate was mixed with glycerol (1:1) and kept at −20 °C until use.

2.5. Cyclic voltammetry

The cyclic voltammetries (CV) for 1 mmol l^{-1} *p*-APP and 1 mmol l^{-1} *p*-AP were carried out at the scan rate of 0.05 V/s in 0.1 mol l^{-1} Tris buffer, containing 1 mg/ml $MgCl_2$, pH 9.0 using one set of electrodes in MED.