



Short communication

Biosensor based on acetylcholinesterase immobilized onto layered double hydroxides for flow injection/amperometric detection of organophosphate pesticides

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ABSTRACT

We developed a highly sensitive flow injection/amperometric biosensor for the detection of organophosphate pesticides (OPs) using layered double hydroxides (LDHs) as the immobilization matrix of acetylcholinesterase (AChE). LDHs provided a biocompatible microenvironment to keep the bioactivity of AChE, due to the intrinsic properties of LDHs (such as a regular structure, good mechanical, chemical and thermal stabilities, and swelling properties). By integrating the flow injection analysis (FIA) with amperometric detection, the resulting AChE-LDHs modified electrode greatly catalyzed the oxidation of the enzymatically generated thiocholine product, and facilitated the detection automation, thus increasing the detection sensitivity. The analytical conditions for the FIA/amperometric detection of OPs were optimized by using methyl parathion (MP) as a model. The inhibition of MP was proportional to its concentration ranging from 0.005 to 0.3 $\mu\text{g mL}^{-1}$ and 0.3 to 4.0 $\mu\text{g mL}^{-1}$ with a detection limit 0.6 ng mL^{-1} ($S/N=3$). The developed biosensor exhibited good reproducibility and acceptable stability.

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1. Introduction

Owing to the high toxicity, extensive use of OPs for pest control results in excessive pesticide residues in food, water as well as environment, posing a highly severe threat on human life (Pocai et al., 2006; Li et al., 2007a, 2007b). The inhibition of acetylcholinesterase (AChE) activity by OPs can cause respiratory paralysis and death (Kim et al., 2005). Therefore, rapid determination and reliable quantification of trace level of OPs have become increasingly important. Well established methods over the past decades for OPs monitoring include various chromatographic techniques coupled with various detectors (Albanis et al., 2004; Rotiroti et al., 2005; Leandro et al., 2006). These methods, however, require complicated pretreatment steps, extensive labor resources, and are not applicable for on-site determination. Over the past few years, inhibition biosensor systems based on the immobilization of AChE onto various electrochemical transducers, have shown satisfactory results for the pesticides analysis (Gong et al., 2009a, 2009b; Li et al., 2007a, 2007b; Bachmann and Schmid, 1999; Won et al., 2010; Chauhan and Pundir, 2012). Various materials, such as polymer, DNA film, CaCO_3 -chitosan composite, nanomaterials and mesoporous silicates have been used for this purpose. However, most of these reported carriers

are organic or semi-organic matrices, subjected to microbial attack in practical application. For the fabrication of biosensor, it still remains a great challenge by constructing a host matrix for effective immobilization of enzyme.

In recent years, as a class of two-dimensional nanostructured anionic clays, inorganic layered double hydroxides (LDHs) have been widely used in catalysis, separation science, drug delivery, and many other environmental-related fields (Wei et al., 2008; Scavetta et al., 2007; Choudary et al., 2002; Bruna et al., 2006; Wu et al., 2011). The positively charged layers contain edge-shared metal M(II) and M(III) hydroxide octahedral, with charges neutralized by A^{n-} anions located in the interlayer spacing or at the edges of the lamella (Braterman et al., 2004). On one hand, the open frameworks can provide substrates with convenient access to the immobilized enzymes. On the other hand, the protective environment of the interlayer gallery can effectively inhibit microbial degradation, extending the lifetime of the adsorbed proteins. Owing to the intrinsically excellent properties, LDHs have been demonstrated as a promising enzyme immobilization matrix (Shan et al., 2009; Zhu et al., 2010).

We describe herein the construction of an amperometric inhibition biosensor using LDHs as the immobilization matrix of AChE, coupled with a novel automatic FIA for the detection of OPs. By integrating a FIA system, the operation of a biosensor could be greatly facilitated for automation, and even realize the real-time on-line analysis. Several enzyme biosensor coupled with FIA have been reported (Marinov et al., 2011; Mishra et al., 2012; Valdés-Ramírez

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et al., 2009; Bucur et al., 2005; Crew et al., 2011). To the best of our knowledge, there has been no report on the combination of LDHs, AChE, and flow-injection/amperometric analysis for the construction of the highly sensitive OPs biosensor. The analytical characteristics of the resulting biosensor for FIA/amperometric detection of MP were investigated.

2. Experimental

2.1. Reagents

Acetylthiocholine chloride (ATCI) and AChE (Type C3389, 500 U mg⁻¹ from electric eel) were purchased from Sigma-Aldrich (St. Louis, USA) and used as received. Methyl parathion (MP) was obtained from Treechem Co. (Shanghai, China). The Mg/Al-LDHs were synthesized in pure methanol solvent by a co-precipitation method, according to a previous report (Gunawan and Xu, 2009). The detailed procedures were listed in supplementary data. All other chemicals were of analytical grade and used as received without further purification. Phosphate buffer solutions (PBS, 0.1 M) with various pH values were prepared by mixing stock standard solutions of K₂HPO₄ and KH₂PO₄. All aqueous solutions were prepared with double distilled water. All experiments were carried out at room temperature.

2.2. Apparatus

Electrochemical measurements were performed on a CHI 660D electrochemical workstation (CHI, USA) with a conventional three-electrode system comprising platinum wire as auxiliary electrode, saturated calomel electrode (SCE) as reference and the modified or unmodified glass carbon electrode (GCE) as working electrode. Tapping-mode atomic force microscopy (AFM) was conducted with a DI Nanoscope (Veeco Instruments, Inc., USA). The powder X-ray diffraction (XRD) patterns were recorded on a Rigaku Ultima III X-ray diffractometer with high-intensity Cu K_{α1} irradiation ($\lambda=1.5418$ nm). Flow injection analysis (FIA) was carried out at the IFIS-D computerized flow injector (Xian Ruimai, China). The sampling volume was 70 μ L. A home-made convenient thin-layer flow electrochemical cell adapted with the commercial available GCE or its modified electrode was employed as the electrochemical detector, as shown in Fig. 1. The thickness of the thin-layer chamber can be adjusted easily by screwing the cell cover for the optimized response.

2.3. Construction of the biosensor

Prior to modification, the basal GCE was successively polished by 1.0, 0.3 and 0.05 μ m alumina slurry, followed by sonication in

distilled water and ethanol for 5 min each. Then, to prepare the suspension of AChE-LDHs, a stock solution of AChE (20 mg mL⁻¹) was mixed with the colloidal solution of LDHs (10 mg mL⁻¹) in a 1:1 volume ratio. The resulting suspension was stored at 4 °C in a refrigerator. To obtain the AChE-LDHs modified GCE (labeled as AChE-LDHs/GCE), 10 μ L of the suspension was dropped onto the surface of a GCE and allowed to dry at room temperature. The modified electrodes were dipped into saturated glutaraldehyde for 15 min for the cross-linking of the membrane, and then rinsed by PBS (0.1 M, pH 8.0) to enhance the adhesive ability and the stability of the film. The modified electrodes were stored at 4 °C in a refrigerator when not in use.

2.4. Measurement procedure

For the measurements of MP, the obtained AChE-LDHs/GCE was first immersed in PBS solution containing different concentrations of standard MP for 20 min, and then transferred to a solution of 1.0 mL pH 8.0 PBS containing 3.0 mmol L⁻¹ ATCI to study the electrochemical response. The concentration of 3.0 mmol L⁻¹ ATCI is an optimized value. The inhibition of MP was calculated as follows:

$$\text{inhibition(\%)} = \frac{i_{p, \text{control}} - i_{p, \text{exp}}}{i_{p, \text{control}}} \times 100$$

where $i_{p, \text{control}}$ is the peak current of ATCI on AChE-LDHs/GCE, and $i_{p, \text{exp}}$ is the peak current of ATCI on AChE-LDHs/GCE with MP inhibition.

3. Results and discussion

3.1. Characterization of LDHs and AChE-LDHs complex

The as-prepared LDHs and AChE-LDHs complexes were characterized by XRD and AFM techniques, respectively (shown in Fig. 2). The XRD pattern of LDHs (Fig. 2A, curve a) displays characteristic diffraction peaks of layered structure at $2\theta=10.0^\circ$, 19.9° , and 60.7° , corresponding to the rhombohedra symmetry structure of LDHs (JCPDS 89-5434). Interestingly, it was found that the XRD pattern of AChE-LDHs (curve b) was similar to that of the pure LDHs, suggesting that AChE was limited to be intercalated into the interlayer space of LDHs. Obviously, the immobilization of the enzyme prevented the structural reconstruction of LDHs, and AChE was immobilized mainly on the external surface of LDHs.

Fig. 2B and C shows AFM images of the as-formed AChE-LDHs film under both air and liquid environment, respectively. The surface morphology of AChE-LDHs film obtained under air environment shows grain and a network-like structure (Fig. 2B). Compared with that obtained under air environment, the surface morphology of AChE-LDHs obtained under aqueous environment displays loose cotton-like structure with increased roughness and depth (Fig. 2C). It could be ascribed to the swelling properties of LDHs in aqueous environment (Shan et al., 2009). Rubio-Retama et al. (2005) demonstrated that the behavior of the enzyme immobilized in the swollen gel is similar to the enzyme in solution. Therefore, it is concluded that the bioactivity of the immobilized enzyme can be well maintained surrounded by the swollen LDHs.

3.2. Electrochemical behavior of AChE-LDHs/GCE

Fig. 3A shows the typical CVs of ATCI at 50 mV/s in 0.1 mol L⁻¹ PBS (pH 8.0). No peak was observed at bare GCE (curve a), and AChE-LDHs/GCE (curve b) in pH 8.0 PBS. With the addition of ATCI

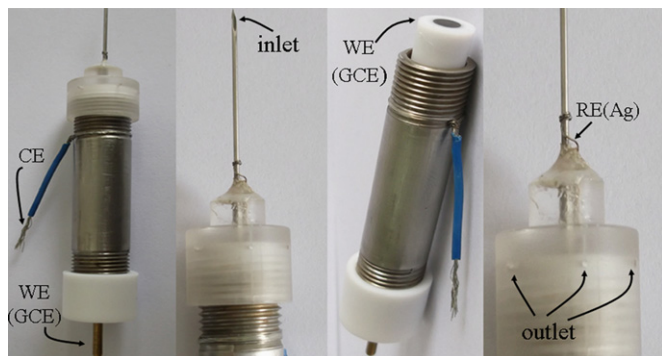


Fig. 1. Thin-layer flow electrochemical cell with the available GCE.

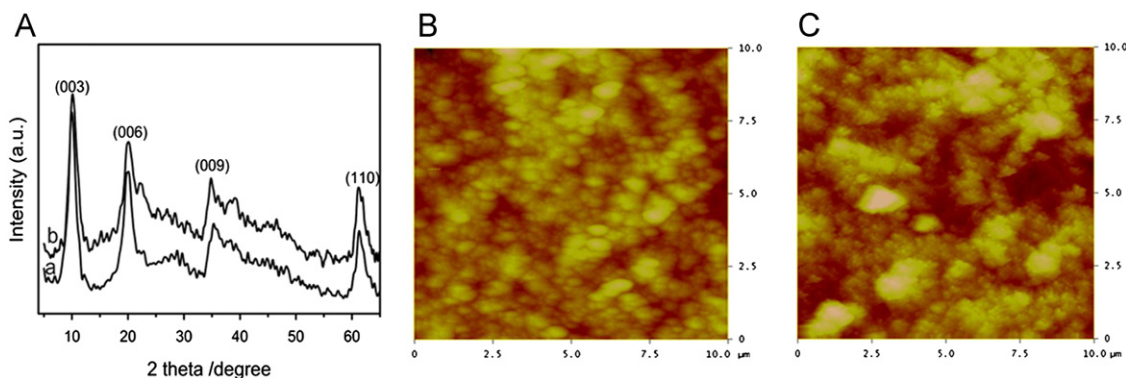


Fig. 2. (A) Powder X-ray diffraction pattern of (a) precursor LDHs and (b) AChE-LDHs film; AFM images of AChE-LDHs film under (B) air environment, and (C) liquid environment.

(3.0 mmol L⁻¹) into PBS, the CV of AChE-LDHs/GCE showed an obvious oxidation peak at 0.65 V (curve e), while no detectable signal was observed at LDHs/GCE (curve c). Obviously, this peak was attributed to the oxidation of thiocholine, hydrolysis product of ATCl, catalyzed by the immobilized AChE. The electrochemical response at AChE-LDHs/GCE was dramatically enhanced, with the oxidation overpotential reduced by 140 mV in comparison with that of ATCl at AChE/GCE (curve d). Undoubtedly, the presence of LDHs provides a high-performance immobilization platform for enzyme.

3.3. Effect of methyl parathion on response of AChE-LDHs/GCE

As shown in Fig. 3B, upon AChE-LDHs/GCE immersed in the standard solution of MP for 20 min, notably, the produced current decreased drastically. Moreover, the peak current continuously decreased with the further addition of MP. It could be attributed to the irreversible inhibition action of MP on AChE, thus leading to the reduced enzymatic activity. Due to the notable change in voltammetric signal of the AChE-LDHs/GCE, the simple method for determination of MP could be established.

3.4. Effects of pH, ATCl concentration and inhibition time on the response of AChE-LDHs/GCE

The bioactivity of the immobilized AChE depended on the solution pH. Fig. S1A shows the relation between the peak current of ATCl and solution pH. The maximum peak current was obtained at pH 8.0 in the pH range from 6.0 to 10.0. Therefore, pH 8.0 was used in the detection solution.

The effect of ATCl concentration on the response of the AChE-LDHs/GCE was observed in pH 8.0 PBS (shown in Fig. S1B). 3 mmol L⁻¹ of ATCl was selected as the detection condition.

Inhibition time is one of the influential parameters in pesticide analysis. As shown in Fig. S1C, with an increase of immersion time in the MP solution, the peak current of ATCl on the AChE-LDHs/GCE was decreased greatly. The curve tended to a stable value up to 20 min, indicating that the binding interactions with active target groups in the enzyme reached saturation.

3.5. Detection of methyl parathion

By integrating a FIA system, the operation of a biosensor could be greatly facilitated with the greatly enhanced sensitivity, and is particularly useful for the real-time analysis. Typical kinetic responses of AChE-LDHs/GCE are shown in Fig. 3C before and after exposure to MP. Before exposure of AChE-LDHs/GCE to MP, the group a shows the results of consecutive injection of

3 mmol L⁻¹ ATCl, indicating a satisfactory reproducibility. With increasing concentrations of MP in the immersion solution (from group b to e), the kinetic peaks at AChE-LDHs/GCE decreased gradually, agreeing well with the amperometric responses (Fig. 3B). Under the optimized experimental conditions, the MP inhibition to AChE-LDHs/GCE in FIA was proportional to its concentration in two ranges, from 0.002 to 0.3 μg mL⁻¹ and 0.3 to 4.0 μg mL⁻¹. The linear equations were inhibition (%) = 135.58c + 20.69 (%) and inhibition (%) = 5.32c + 59.18 (%), with the correlation coefficients of 0.9996 and 0.9990, respectively (inset of Fig. 3C). The detection limit was calculated to be about 0.6 ng mL⁻¹, comparable with that reported with flow injection detection (Gimenes et al., 2010; Jakmunee and Junsomboon, 2009) and also lower than that with an enzyme-based inhibitor electrochemical sensor (Wei et al., 2009; Gong et al., 2009a, 2009b).

The interferences from the other electroactive nitrophenyl derivatives, such as nitrobenzene, nitrophenol and other oxygen containing inorganic ions (PO₄³⁻, SO₄²⁻, NO₃⁻) were investigated (Fig. S2). No obvious inhibition behavior can be observed with the peak currents of ATCl varied slightly.

AChE inhibited irreversibly by OPs could be reactivated by use of nucleophilic compounds such as pralidoxime iodide. It was observed that AChE modified electrode inhibited by MP could recover 93% of the original activity after immersing in 5.0 mmol L⁻¹ pralidoxime iodide. Based on this reactivation procedure, the proposed biosensor could be used repeatedly with an acceptable reproducibility.

To further demonstrate the practicality of the present electrode, it was evaluated by processing real samples. We performed the recovery tests by adding different amounts of MP into real samples, including garlic, cabbage and apple, summarized in Table S1. From Table S1, the original MP concentrations in the cabbage and apple samples were tested to be 5.62 and 5.23 ng mL⁻¹, respectively. The recoveries of the spiked MP are from 97.2% to 104.6% for the real samples, indicating that the proposed method can be used for the direct analysis of relevant samples with high accuracy and reproducibility.

4. Conclusions

Herein we describe an effective flow amperometric inhibition biosensor for the detection of OPs using LDHs as the immobilization matrix of AChE. Coupled with FIA, such swollen LDHs could well retain the bioactivity of the entrapped enzyme, greatly facilitating the diffusion of substrates, and the function of the immobilized AChE for OPs determination. The resulting flow

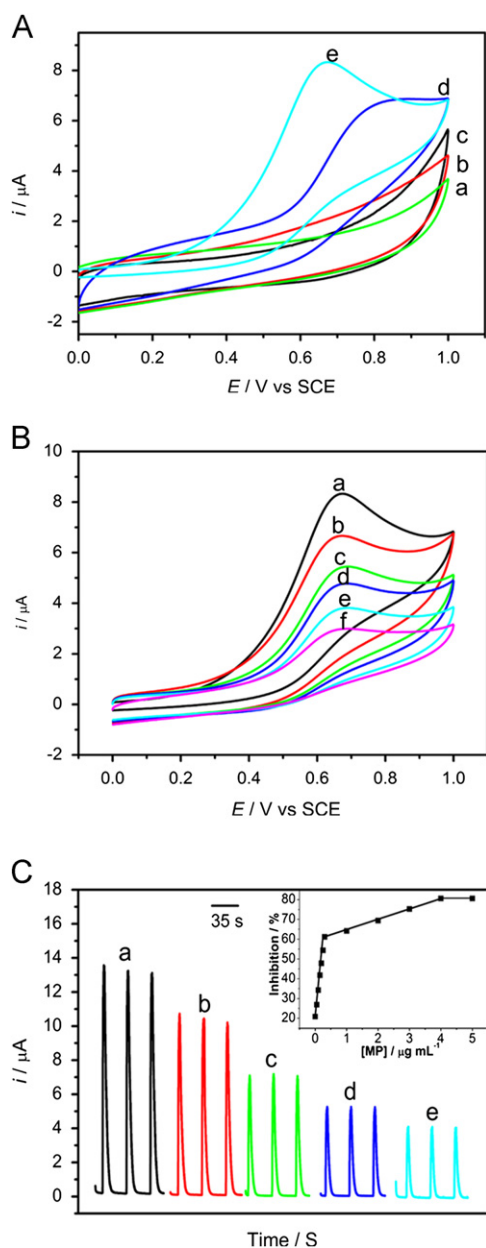


Fig. 3. (A) Cyclic voltammograms of (a) GCE, (b) AChE-LDHs/GCE in pH 8.0 PBS, (c) LDHs/GCE, (d) AChE/GCE and (e) AChE-LDHs/GCE in pH 8.0 PBS containing 3.0 mol L⁻¹ ATCI. Scan rate: 50 mV s⁻¹; (B) cyclic voltammograms of AChE-LDHs/GCE in pH 8.0 PBS containing 3.0 mol L⁻¹ ATCI after incubation in (a) 0, (b) 0.01, (c) 0.1, (d) 0.15, (e) 0.5, and (f) 2 μg mL⁻¹ MP solution, accordingly, for 20 min and (C) FIA kinetic peaks of AChE-LDHs/GCE in pH 8.0 PBS containing 3.0 mol L⁻¹ ATCI after incubation in (a) 0, (b) 0.005, (c) 0.02, (d) 0.3, and (e) 2 μg mL⁻¹ MP solution, accordingly for 20 min. The inset shows the calibration curve.

amperometric biosensor showed high sensitivity, good precision, reproducibility, and stability. We believe that the proposed system can be applied in online monitoring of OPs in real samples.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bios.2012.07.026.

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