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A highly stable acetylcholinesterase biosensor based on chitosan-TiO_2-graphene nanocomposites for detection of organophosphate pesticides

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ABSTRACT

A highly stable electrochemical acetylcholinesterase (AChE) biosensor for detection of organophosphorus pesticides (OPs) was developed simply by adsorption of AChE on chitosan (CS), TiO_2 sol-gel, and reduced graphene oxide (rGO) based multi-layered immobilization matrix (denoted as CS@TiO_2-CS/rGO). The biosensor fabrication conditions were optimized, and the fabrication process was probed and confirmed by scanning electron microscopy and electrochemical techniques. The matrix has a mesoporous nanostructure. Incorporation of CS and electrodeposition of a CS layer into/on the TiO_2 sol-gel makes the gel become mechanically strong. The catalytic activity of the AChE immobilized CS@TiO_2-CS/rGO/glassy carbon electrode to acetylthiocholine is significantly higher than those missing any one of the component in the matrix. The detection linear range of the biosensor to dichlorvos, a model OP compound, is from 0.036 μM (7.9 ppb) to 22.6 μM, with a limit of detection of 29 nM (6.4 ppb) and a total detection time of about 25 min. The biosensor is very reproducibly and stable both in detection and in storage, and can accurately detect the dichlorvos levels in cabbage juice samples, providing an efficient platform for immobilization of AChE, and a promisingly applicable OPs biosensor with high reliability, simplicity, and rapidness.

1. Introduction

Pesticides, especially organophosphorus pesticides (OPs) have been widely used in the past several decades to protect crops from insects. However, OP pesticides are toxic to human and most animals through inhibition of the enzymes in neurosynapses—acetylcholinesterases (AChE), resulting in accumulation of acetylcholine in human body, over stimulating its receptors in synapses and eventually damaging the nervous system (Patocka et al., 2004; Long et al., 2015; Costa, 2006; Eddleston et al., 2008; Fukuto, 1990). Detection of OPs in various samples with high reliability, simplicity, and rapidness has become increasingly necessary.

In the past decades, great efforts have been made to develop efficient and simple methods to determine OPs levels (Long et al., 2015; Lu and Xia, 2015; Qian and Lin, 2015; Yu et al., 2015). Among them, electrochemical methods have many advantages such as high reliability, simple instruments, fast result acquirement, easiness for operation, high sensitivity, and compatible to complex samples. The detection mechanism usually depends on the AChE catalyzed reaction of acetylthiocholine (ATCl) to produce the electro-active thiocholine (TCl) (Wei and Wang, 2015; Yu et al., 2015). Inhibition of the AChE activity by OPs would result in reduced TCl production of the biosensor. In development of practically applicable electrochemical biosensors, effective immobilization of AChE while maintaining its native catalytic activity is one of the key considerations; the electrocatalytic activity of the electrode to TCl, and the transport of the enzymatic reaction related molecules through the biofilm are also the main factors; the stability of the biosensor is the last key factor. Recently, the advances in nanotechnology have provided various nanomaterials with special properties, such as high specific surface area and high electrocatalytic activity, for fabrication of AChE electrochemical biosensors (Shi et al., 2006; Sundarmurugasan et al., 2016; Wei and Wang, 2015; Yu et al., 2015). However, most of the reported AChE electrochemical biosensors still face serious instability problems.

TiO_2 porous sol-gel film as a protein immobilization matrix has attracted great attention due to its large specific surface area, low cost, non-toxicity, good thermal and chemical stability, semi-conductivity, and excellent biocompatibility (Cui et al., 2013; Yu and Ju, 2002; Zhu et al., 2009). However, the TiO_2 gel, when fabricated on smooth solid surfaces as a monolithic part, suffers from the drawbacks of low adhesion and high fragility, resulting in the loss of sensor stability upon immersion in water. Blending polymer with nanomaterials to form nanocomposites has been an effective strategy to reinforcing the mechanical strength of each component (Behera et al., 2017; Tang

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et al., 2016; Yang et al., 2017).

Graphene as a carbon nanomaterial has attracted tremendous research interests in various applications due to its large surface area, excellent thermal/chemical stability, high electronic/thermal conductivity, and superior mechanical flexibility (Bai et al., 2014; Balandin et al., 2008; Feng et al., 2013; Ge et al., 2017a, 2017b; Geim, 2009; Novoselov et al., 2004; Tang et al., 2011), and has contributed greatly as a component of AChE immobilization matrices in electrochemical biosensor fabrication, mainly by improving the matrix electro-conductivity and surface area (Chen et al., 2016; Li et al., 2017; Wang et al., 2016).

In this study, by taking dichlorvos (DDVP) as a model OP, we investigated the usage of TiO_2 sol-gel film as an immobilization matrix, and chitosan (CS), a well-known biocompatible and positively charged polymer as an incorporation polymer in the TiO_2 sol-gel, for AChE biosensor fabrication on glassy carbon (GC) electrode. To further improve the matrix mechanical stability and the immobilization efficiency for the negatively charged AChE, a CS layer was electrodeposited onto the TiO_2-CS blending gel, resulting in a multi-layered matrix (denoted as CS@TiO_2-CS). Reduced graphene oxide (rGO) was introduced as an rGO film onto the GC electrode to improve the sensor sensitivity. The AChE biosensor fabrication procedure was characterized and optimized, and the performance of the biosensor in DDVP detection was evaluated. Finally, the sensitive, simple, and highly stable AChE biosensor was evaluated for applications in DDVP detection in vegetable samples.

2. Experimental

2.1. Materials and chemicals

AChE (from electric eel) and ATCl were from Sigma-Aldrich. CS (viscosity > 400 mPa s) and DDVP (1 mg mL^{-1} in methanol) were from Aladdin Bio-Chem Technology (Shanghai, China). Graphene oxide (GO) (thickness: 0.8~1.2 nm, lateral size: 1~5 μm, purity: > 99%) was from Nanjing XFNANO Materials Tech. (China). Other remaining chemicals were obtained from Sinopharm Chemical Reagent (Shanghai, China). All chemicals were of analytical grade and used as received. Purified nitrogen with a purity of 99.99% was obtained from Zhengzhou Keyi Industrial Gas (China). Deionized water obtained from a Millipore water system was used throughout the experiment.

2.2. Apparatus and measurement

The modified GC electrode was probed by scanning electron microscopy (SEM, JEOL JSM-7500F), and also characterized by cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) techniques. Fourier transform infrared (FTIR) spectra were collected using PerkinElmer Spectrum 2 FTIR spectrophotometer. The CVs were performed in 0.01 M phosphate buffer (PBS, pH 7.4), or in 5 mM $K_3[Fe(CN)_6]$ supported by 1 M KCl. The EIS measurement was performed in 0.1 M KCl containing equimolar $[Fe(CN)_6]^{3-/4-}$ (10/10 mM) with AC frequency from 0.1 Hz to 100 kHz. The effective surface area (A, cm^2) of the electrodes was obtained by running CVs at various scan rates in the 5 mM $K_3[Fe(CN)_6]$ (detailed in Supplementary Material). All electrochemical measurements were performed at room temperature (~25 °C) using a CHI-660E electrochemical workstation (Shanghai Chenhua Instrument, China) in a three-electrode arrangement consisting of a working electrode (modified GC electrode), a Pt counter electrode and an Ag|AgCl|KCl (3 M) reference electrode.

2.3. Preparation of rGO

The rGO as a black suspension (0.3 mg mL^{-1}) was prepared from GO as described by Park and Ruoff (2009), in our previous paper (Bai et al., 2014), and also in the Supplementary Material.

2.4. Preparation of TiO_2 sol-gel, CS solution and TiO_2-CS sol-gel

The TiO_2 and the TiO_2-CS sol-gel can be formed in minutes from the respective precursor solution upon exposure a thin layer of the solution to air. The TiO_2 precursor solution was synthesized as described in the Supplementary Material. The TiO_2-CS precursor solutions were prepared by mixing the TiO_2 precursor solution with a CS solution of various concentrations, at different volume ratios. The CS solutions were prepared by dissolving 1 g CS in 100 mL 1% (vol%) acetic acid solution, and then diluting the 1% CS solution to various concentrations with water.

2.5. Fabrication of the biosensor

The GC electrode was cleaned by being polished consecutively with 0.3 and 0.05 μm alumina powder, followed by sonication in water bath. The as-prepared rGO suspension was diluted to 0.2 mg mL^{-1} with ethanol, and 4 μL of the diluted rGO was cast on the dried GC electrode. After air-dried, the rGO/GC electrode was coated with 4 μL of TiO_2-CS solution, and then left in the air for gelation. The volume content of the TiO_2 sol-gel, and the weight content of CS in the TiO_2-CS mixture was varied from 90.0% to 99.5% (taken the as-synthesized TiO_2 gel as 100%), and from 0.002% to 0.020%, respectively, to obtain an optimum biosensor. The resulted TiO_2-CS composites are denoted as $TiO_2^{90.0}$-$CS^{0.002}$ etc., accordingly. For simplification, the optimized TiO_2-CS nanocomposite, $TiO_2^{99.0}$-$CS^{0.005}$ (shown in Section 3.3), is also named as TiO_2^{m}-CS^{m}. Afterwards, a CS layer was electrodeposited on the TiO_2-CS/rGO/GC surface by holding the potential at −2.5 V for seconds in 0.2% CS. Finally, AChE was immobilized on the CS@TiO_2-CS/rGO/GC electrode by dropping 4 μL AChE solution (in PBS, containing 1% bovine serum albumin) onto the electrode surface. The as-fabricated enzyme electrode was dried in the air and kept at 4 °C, ready for used.

2.6. Electrocatalytic and sensing performance

The electrocatalytic performance to ATCl was investigated by differential pulse voltammetry (DPV) technique (from 0.2 to 1.0 V; amplitude, 0.05 V; pulse width, 0.005 s; pulse period, 0.02 s) in PBS. Before the DPV measurement, the electrode was cleaned by running CVs in PBS until a stable CV curve was obtained, to remove loosely adsorbed AChE. The sensing performance to DDVP was evaluated by recording DPVs in PBS containing 1 mM ATCl before and after incubating the electrode in a DDVP solution for 10 min. The inhibition ratio (Inhibit%) of DDVP to the enzyme electrode, which was taken as the sensing signal towards OPs, was determined from Eq. (1), where I_{cat}^{0} and I_{cat}' represents the DPV peak current in response to 1 mM ATCl before and after, respectively, the DDVP incubation.

$$\text{Inhibit\%} = (1 - I_{cat}'/I_{cat}^{0}) \times 100\% \quad (1)$$

The detection stability was evaluated by repetitively recording the DPVs in 1 mM ATCl. The storage stability was evaluated by monitoring the DPVs in 1 mM ATCl during a wet storage in sterilized PBS at 4 °C, and a dry storage at −20 °C, respectively, for 30 days. The electrodes were taken out for the ATCl test every 10 days during the storage period.

2.7. Detection of vegetable samples

The applicability of the biosensor was evaluated by detecting DDVP levels in DDVP spiked cabbage juice samples. For preparation of the juice samples, cabbage was firstly cleaned with water and then dried in the air. Then 100 g cabbage pieces were mixed with 100 mL PBS (0.02 M, pH 7.4) in a homogenizer at room temperature. The cabbage homogenate was then centrifuged at 5000 rpm for 10 min, and the