Materials Science and Engineering C 35 (2014) 253–258

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

A novel acetylcholinesterase biosensor based on carboxylic graphene coated with silver nanoparticles for pesticide detection

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ARTICLE INFO

Article history:
Received 4 May 2013
Received in revised form 12 September 2013
Accepted 31 October 2013
Available online 14 November 2013

Keywords:
Silver nanoparticles
Carboxylic graphene
Nafion
Acetylcholinesterase biosensor

ABSTRACT

A novel acetylcholinesterase (AChE) biosensor based on Ag NPs, carboxylic graphene (CGR) and Nafion (NF) hybrid modified glass carbon electrode (GCE) has been successfully developed. Ag NPs–CGR–NF possessed predominant conductivity, catalysis and biocompatibility and provided a hydrophilic surface for AChE adhesion. Chitosan (CS) was used to immobilize AChE on the surface of Ag NPs–CGR–NF/GCE to keep the AChE activities. The AChE biosensor showed favorable affinity to acetylthiocholine chloride (ATCl) and could catalyze the hydrolysis of ATCl with an apparent Michaelis–Menten constant value of 133 μM, which was then oxidized to produce a detectable and fast response. Under optimum conditions, the biosensor detected chlorpyrifos and carbaryl at concentrations ranging from 1.0×10^{-13} to 1×10^{-8} M and from 1.0×10^{-12} to 1×10^{-8} M. The detection limits for chlorpyrifos and carbaryl were 5.3×10^{-14} M and 5.45×10^{-13} M, respectively. The developed biosensor exhibited good sensitivity, stability, reproducibility and low cost, thus providing a promising tool for analysis of enzyme inhibitors. This study could provide a simple and effective immobilization platform for meeting the demand of the effective immobilization enzyme on the electrode surface.

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1. Introduction

Organophosphate and carbamate pesticides are widely used as insecticides in order advance crop yield. Unfortunately, these pesticides exhibit high acute toxicity, with the majority being hazardous to human and animal health. Indeed, the inhibition of acetylcholinesterase (AChE) activity by pesticides can lead to a disturbance of normal neuronal function and possibly death [1,2]. The exact and speedy measurement of pesticides in water and food is of great importance. Biosensors based on AChE have emerged as a promising technique for toxicity analysis, environmental monitoring, foodstuff quality and military investigations in recent years [3,4]. The main application of AChE biosensors is for the detection of pesticides based on enzyme inhibition. The biosensors are designed to complement or replace the existing reference analytical methods such as HPLC, GC, and GC/MS by simplifying sample preparation and analytic process, thus decreasing the analysis time and cost. Our research purpose is to develop a sensitive and stable AChE biosensor for detection of pesticides to reach the same level of these analytical instruments.

Graphene (GR), a two-dimensional sheet of sp2-bonded carbon atoms arranged in a honeycomb lattice, has attracted increasing attention since it was first isolated from three-dimensional graphite by mechanical exfoliation [5]. Due to its extraordinary thermal, mechanical, electrical and electrochemical properties, GR is usually considered as a competitive candidate for next-generation electronic and electrochemical applications such as super-capacitors [6,7], batteries [8,9], fuel cells [10,11], solar cells [12,13], catalysts [14,15], sensors [16,17], and biosensors [18,19]. However, many researches have reported that the pure GR actually exhibits unsatisfactory electrical conductivity and electrochemical catalytic activity because of the inevitable aggregation. Some of the useful and unique properties of GR such as conductivity and catalysis can only be realized after it is functionalized with organic groups such as hydroxyl, carboxyl, amino and the like. Functionalized GR sheets are easier to disperse in organic solvents, which can improve the dispersion and conductivity [20,21].

It is well known that Ag NPs possess high conductivity, surface area, excellent catalytic activity, and biocompatibility. Ag NPs exhibit high catalytic activity for hydrogen peroxide reduction [22,23], and Ag NPs could provide a suitable microenvironment to retain biological activity for biomolecule immobilization [24]. Ag NPs facilitate more efficient electron transfer between the immobilized biomolecules and electrode substrates. This has led to the construction of electrochemical biosensors with enhanced analytical performance using Ag NPs [25,26].

Chitosan (CS) is an abundant natural biopolymer with excellent film forming ability, biocompatibility and nontoxicity, provides natural microenvironment to the enzyme and also gives sufficient accessibility to electrons to shuttle between the enzyme and the electrode [27]. For example, an amperometric glucose biosensor based on immobilization of glucose oxidase in CS on a glassy carbon electrode modified with gold–platinum alloy NPs/multiwall carbon nanotubes with a high sensitivity, good reproducibility, stability and selectivity [28]. Recently, an

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http://dx.doi.org/10.1016/j.msec.2013.10.036

amperometric biosensor was developed for ethanol detection by co-immobilizing multiwalled carbon nanotubes (MWCNTs) and alcohol dehydrogenase (ADH) within a CS matrix on a glassy carbon electrode [29]. And also, an optical biosensor based on glutamate dehydrogenase immobilized in a CS film was developed for the determination of ammonium in water samples [30].

Based on the above research results, Ag NPs and CGR have been synthesized which Ag NPs can be equably separated and anchored on carboxylic graphene (CGR) and dispersed in Nafion (NF). In this work, a novel AChE biosensor has been developed based on Ag NPs–CGR–NF modified GCE. The Ag NPs–CGR nanocomposites were homogeneously dispersed in NF then modified on the surface of GCE and formed symmetrical membrane which possessed excellent conductivity, catalytic activity, and biocompatibility which were attributed to the synergistic effects of Ag NPs, CGR and NF and provided a hydrophilic surface for AChE adhesion. Furthermore, CS was used to immobilize AChE on the surface of Ag NPs–CGR–NF/GCE to keep the AChE activities and assist electrons to shuttle between the enzyme and CGR–NF/GCE. Finally, NF was used as a protective membrane of the AChE biosensors to improve the stability of the biosensor. The biosensor exhibited excellent affinity to its substrate and the catalytic effect on the hydrolysis of ATCl. The biosensor has been demonstrated as a device with high sensitivity, acceptable stability and reproducibility for the analysis of ATCl and pesticides. More importantly, this study provides a universal and effective platform for meeting the demand of the effective immobilization enzyme on the Ag NPs–CGR–NF/GCE surface.

2. Experimental

2.1. Chemicals

Acetylcholinesterase (AChE Type C3389, 500 U/mg from electric eel), Acetylthiocholine chloride (ATCl), Chitosan (CS 85% deacetylation) and Nafion (NF 5% in lower aliphatic alcohols and water) were purchased from Sigma-Aldrich (St. Louis, USA). Chlorpyrifos and carbaryl (99.99%) were obtained from AccuStandard (USA). Graphite powder was purchased from Sinopharm Chemical Reagent Company (China). $AgNO_3$ was obtained from Shanghai Chemical Reagent Co. Ltd. (China). All other reagents were of analytical grade and obtained from Shanghai Chemical Reagent Co. Ltd. (China). Aqueous solutions were prepared with deionized (DI) water (18 MΩ cm).

2.2. Preparation of CGR

Graphite oxide prepared by Hummers' method [31] was suspended in water and exfoliated through ultrasonication for 2 h to obtain graphene oxide (GO) solution. GO solution was centrifuged at 3000 rpm to remove unexfoliated graphite oxide. CGR was prepared as reported [32] with the modification of replacing the method of drying in an oven with vacuum freeze-drying. Briefly, GO aqueous suspension (5 ml) was diluted to give a concentration of 2.0 mg/ml, and then sonicated for 1 h to give a clear solution. 1.2 g of NaOH and 1.0 g chloroacetic acid (Cl–CH_2–COOH) were added to the suspension and sonicated for 3 h to convert the −OH groups to −COOH via conjugation of acetic acid moieties. Sequentially the suspension was separated by centrifuging at a speed of 15,000 rpm, washed with DI water for several cycles. After vacuum freeze-drying, CGR was obtained.

2.3. Synthesis of Ag NPs–CGR nanocomposites

The Ag NPs–CGR nanocomposites were prepared as follows: briefly, 2.0 mg CGR was suspended in 2.0 ml of 0.46 mM $AgNO_3$ by sonicating for 10 min to disperse CGR equably. Then 1.0 ml of 0.01 M sodium citrate and 4.0 ml ethanol were added to the above suspension. Ice-cold, freshly prepared 1.0 ml of 0.01 M $NaBH_4$ solution was added to the above mixture while stirring until the color of the solution did not change. After stirring for an additional 10 h, the suspension was separated by centrifuging at a speed of 12,000 rpm, washed with DI water for several cycles. After vacuum freeze-drying, Ag NPs–CGR nanocomposites were obtained.

2.4. Preparation of biosensors

NF solution (0.125%, Wt/V) was prepared by diluting 5% of NF with ethanol and DI water (V/V, 1/1). The Ag NPs–CGR (0.5 mg) were added to 1.0 ml of the NF solution and sonicated thoroughly until a homogeneous suspension of Ag NPs–CGR–NF obtained. Similarly 0.5 mg/ml CGR–NF and GO–NF homogeneous suspension was obtained, respectively. The suspensions were stored under refrigeration at 4 °C. A GCE was polished carefully to a mirror-like with 0.3 and 0.05 μm alumina slurry and sequentially sonicated for 3 min in nitric acid (V/V, 1/1), ethanol and water. Before the experiment, the electrode was scanned from −0.1 to +1.1 V until a steady-state current–voltage curve was obtained. The Ag NPs–CGR–NF/GCE was prepared by dropping 5 μl of 0.5 mg/ml Ag NPs–CGR–NF suspension onto the surface of GCE and drying at room temperature. A similar method was used to prepare CGR–NF/GCE and GO–NF/GCE. The enzyme solution was mixed with 0.05 U AChE and 0.2% CS (Wt/V, 50 mM acetic acids). The modified electrodes were each coated 4.5 μl of AChE–CS (V/V, 2/1) and dried at 4 °C. The AChE–CS/GO–NF/GCE, AChE–CS/CGR–NF/GCE and AChE–CS/Ag NPs–CGR/GCE biosensors were obtained and washed with 0.1 M PBS to remove the unbound AChE. Finally, three types of biosensor were each covered with 3 μl 0.1% (Wt/V) NF as the protective membrane. Thus, three types of biosensor structure were NF/AChE–CS/GO–NF/GCE, NF/AChE–CS/CGR–NF/GCE and NF/AChE–CS/Ag NPs–CGR/GCE. Similarly, NF/AChE–CS/GCE was produced as a control.

2.5. Material characterization

Scanning electron microscopy (SEM, QUNT200 USA), scanning probe microscopy (SPM, SPA400) and transmission electron microscopy (TEM, Tecnai G F30 USA) were used to characterize CGR and Ag NPs–CGR morphologies. Raman spectra (Raman Station 400F PERKINELMER USA) and Fourier transform infrared spectra (FTIR, Thermo Fisher SCIENTIFIC Nicolet IS10 USA) were used to study the GO and CGR. An X-ray diffractometer (XRD, Rigaku TTR III Japan) was used to identify the phase of Ag NPs on CGR sheets. The solution CGR and Ag NPs–CGR 0.5 mg/ml of DI water respectively were used for SEM, AFM and TEM detection. GO and CGR which were not treated were used for Raman spectra detection. GO and CGR which were mixed with a fix quantity of potassium bromide were used for Fourier transform infrared spectrometry detection.

2.6. Measurements

Electrochemical analysis of the bioelectrodes was performed using an IM6ex (Zahner Elektrik instruments, Germany) electrochemical work station. A conventional three-electrode system was employed with a saturated calomel electrode (SCE) as the reference electrode, platinum foil as the counter electrode, and the modified GCE (diameter = 3 mm) as the working electrodes. Cyclic voltammetry (CV) measurements were performed in 0.1 M phosphate buffer solution (PBS, pH 7.4) between 0.0 and 1.0 V for characteristic investigations of NF/AChE–CS/Ag NPs–CGR–NF/GCE biosensors. The apparent Michaelis–Menten constant of the biosensor was calculated from the Lineweaver–Burk equation:

$$\frac{1}{i_{ss}} = \left(\frac{K_m^{app}}{i_{max}}\right) \cdot \left(\frac{1}{C}\right) + \left(\frac{1}{i_{max}}\right) \quad (1)$$

where i_{ss} is the steady-state current after the addition of substrate, i_{max} is the maximum current measured under saturated substrate condition and C is the concentration of the substrate. The K_m^{app} is apparent

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