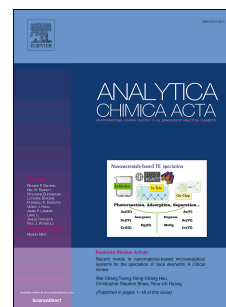


Accepted Manuscript

Palladium-copper nanowires-based biosensor for the ultrasensitive detection of organophosphate pesticides

Dandan Song, Yan Li, Xiong Lu, Muxue Sun, Hui Liu, Guangming Yu, Faming Gao



PII: S0003-2670(17)30705-5

DOI: [10.1016/j.aca.2017.06.004](https://doi.org/10.1016/j.aca.2017.06.004)

Reference: ACA 235247

To appear in: *Analytica Chimica Acta*

Received Date: 22 October 2016

Revised Date: 15 May 2017

Accepted Date: 13 June 2017

Please cite this article as: D. Song, Y. Li, X. Lu, M. Sun, H. Liu, G. Yu, F. Gao, Palladium-copper nanowires-based biosensor for the ultrasensitive detection of organophosphate pesticides, *Analytica Chimica Acta* (2017), doi: 10.1016/j.aca.2017.06.004.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

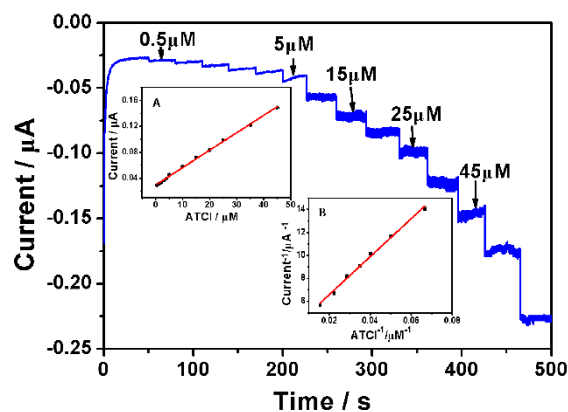
Highlights

A simple and low cost biosensor was developed for detection of organophosphate pesticides.

AChE-Chitosan/Pd-Cu NWs/GCE was utilized as an effective sensing platform.

The biosensor displayed sensitive detection of malathion and the LOD was 1.5 ppt.

Palladium-copper nanowires were employed to construct a highly sensitive acetylcholinesterase electrochemical biosensor for the quantitative determination of organophosphate pesticides in vegetables and fruits.



Palladium-copper nanowires-based biosensor for the ultrasensitive detection of organophosphate pesticides

Dandan Song[‡], Yan Li[‡], Xiong Lu, Muxue Sun, Hui Liu, Guangming Yu, Faming Gao*

Key Laboratory of Applied Chemistry, Department of Applied Chemistry, Yanshan University, Qinhuangdao 066004, P. R. China.

* Corresponding Author E-mail: fmgao@ysu.edu.cn. Phone: 86 335 8387552. Fax: 86 335

8061569

[‡] These authors contributed equally to this work.

ABSTRACT

A highly sensitive acetylcholinesterase (AChE) electrochemical biosensor for the quantitative determination of organophosphate pesticides (OPs) in vegetables and fruits based on palladium-copper nanowires (Pd-Cu NWs) was reported. AChE immobilized on the modified electrode could catalyze hydrolysis of acetylthiocholine chloride (ATCl), generating an irreversible oxidation peak. When exposed to the OPs, the activity of the AChE was inhibited and the current significantly decreased. The detection mechanism is based on the inhibition of AChE. The Pd-Cu NWs not only provide a large active surface area (0.268 ± 0.01) cm² for the immobilization of AChE, which was approximately 3.8 times higher than the bare glass carbon electrode, but also exhibit excellent electro-catalytic activity and remarkable electron mobility. The biosensor modified with Pd-Cu NWs displayed a good affinity to ATCl and catalyzed hydrolysis of ATCl, with a low Michaelis–Menten constant (K_M) of 50.56 μ M. Under optimized conditions, the AChE-Cs/Pd-Cu NWs/GCE biosensor detected malathion with wide linear ranges of 5 ~ 1000 ppt and 500 ~ 3000 ppb, and the low detection limit was 1.5 ppt (4.5 pM). In addition, the OPs biosensor has been applied to the analysis of malathion in commercial vegetable and fruit samples, with excellent recoveries in the range of 98.5% ~ 113.5%. This work provides a simple, sensitive and effective platform for biosensors and exhibits future potential in practical application for the OPs assay.

Keywords: Acetylcholinesterase biosensor; Pd-Cu nanowires; Organophosphate pesticides; Malathion.

1. Introduction

Organophosphate pesticides (OPs) play a significant role in modern agriculture all over the world owing to their high efficiency for insect eradication. However, pesticide residues pose an overwhelming threat to both human health and environment owing to their high toxicity and bioaccumulation effect [1-3]. Therefore, the establishment of a rapid, accurate, sensitive and reliable analytical technique for monitoring OP compounds is very necessary to protect humans and environmental safety. Compared with conventional analytical methods, such as liquid/gas chromatography-mass spectrometry, high-pressure liquid chromatography and enzyme-linked immunosorbent assays, electrochemical sensors have drawn increasing attention attributing to their potential in miniaturization and portability, high sensitivity, rapid response, inexpensive instrumentation and simplicity of construction [4-6]. AChE biosensors have emerged as a promising alternative for rapid detection of pesticide residue [7-10]. However, there is still an urgent demand for fabricating an efficient and sensitive AChE biosensor with a low detection limit.

Various nanomaterials have been synthesized and extensively utilised as electrochemical sensing platform to improve the conductivity, stability, biocompatibility, sensitivity and catalytic activity, as well as increase the loading of biomolecules, for example, enzymes, proteins and antibodies [11-14]. Recently, the applications of bimetallic alloy nanomaterials exhibit great potentials for constructing sensitive electrochemical sensing platforms [15-19]. Compared to the single-component nanostructures, the bimetallic nanomaterials not only generate a

synergic effect between catalytic activity and conductivity to facilitate the electron transfer, but also show the chemical stability and easy functionalization, resulting in a highly sensitive biosensing platform [20-23]. These good performances are related to the electronic interactions, change of atomistic arrangements and surface strain [24]. Bimetallic nanomaterials involving Pd are generally regarded as ideal candidates for modification of biosensors owing to the relatively low cost, earth abundant and versatility of Pd [25,26]. In these nanomaterials, Cu is often a second metal employed to combine with Pt or Pd to further enhance the catalytic activity of the noble metals [27]. Pd-Cu alloys were synthesised and then applied to develop sensors for electrochemical assay of melamine [28], glucose [29], hydrogen [21] and carcinoembryonic antigen [30]. However, there is little report about the application of Pd-Cu alloy for organophosphate pesticides.

More recently, considerable attention has been devoted to nanowire structures due to their fascinating properties including high surface-to-volume ratio, excellent electrical conductivity, no particle aggregation and enhanced catalytic capacity. Multi-component wire-like nanostructures were prepared, such as Pd-Ni nanowires [31], Pt-Ag nanowires [32], Pd-Au nanowires [23]. The nanowires exhibited considerable stability, improved mass transfer efficiency, large surface area, all contributing to their favorable electrochemical performance. However, to the best of our knowledge, there is no report on the employment of Pd-Cu NWs for AChE biosensor.

In this study, an ultrasensitive, simple and cost-effective biosensor was constructed for the determination of pesticides on the basis of Pd-Cu NWs, coupled with Chitosan (Cs) to immobilize AChE. As a natural hydrophilic polysaccharide, Chitosan was employed to entangle with enzyme and nanomaterials due to its excellent biocompatibility, good adhesion, film forming capacity, nontoxicity and a susceptibility to chemical modifications [7]. Owing to its network-like nanostructure and synergistic electronic effect between Pd and Cu, Pd-Cu NWs are expected to immobilize more enzyme molecules and accelerate the electron transfer. The proposed biosensor showed high performance for monitoring malathion and the limit of detection (LOD) is 1.5 ppt (4.5 pM), which is much lower than those reported in previous researches [7], [34], [35], [36], [38], [39], [40], [44], [45], [46] and [47]. The simplicity, high accuracy at low cost and ease-to-operate of the proposed approach offers a promising strategy to develop OPs biosensor that can be applied to on-site detection of pesticides in vegetables and fruits.

2. Experimental

2.1. Reagents and Materials

Acetylcholinesterase (Type VI-S, E.C.3.1.1.7, 500 U/mg from electric eels), acetylthiocholine chloride (ATCl) ($\geq 99\%$ purity), chitosan, $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$ and Triton X-114 were purchased from Sigma-Aldrich, USA. Malathion, $\geq 95\%$ purity, was obtained from LGC Promochem (Wesel, Germany). Sodium borohydride (NaBH_4 , 98%, powder) and Na_2PdCl_4 were purchased from Aladdin. The AChE stock solution (0.2 U/ μL) was prepared by dissolving AChE in 0.1 M pH 7.4 phosphate buffered

saline. Phosphate buffered saline (PBS) was prepared by mixing the stock solutions of NaH_2PO_4 and Na_2HPO_4 . A 1.25% (w/v) chitosan solution in 0.1 M acetic acid was prepared as reported previously [33]. A 5 mM $\text{K}_3[\text{Fe}(\text{CN})_6]/\text{K}_4[\text{Fe}(\text{CN})_6]$ in 1 M KCl solution was used in electrochemical impedance spectroscopy experiments. All aqueous solutions were freshly prepared with high purity water.

2.2. Apparatus

Electrochemical measurements were performed using a CHI 750E electrochemical working station (Shanghai Chen hua Co., China) with a standard three-electrode cell consisting of a platinum wire auxiliary electrode, a KCl saturated calomel reference electrode and a Pd-Cu NW coated 3-mm diameter glassy carbon working electrode. The electrochemical impedance spectroscopy experiments were carried out in 5 mM $\text{Fe}(\text{CN})_6^{4-/3-}$ in 0.1 M KCl solution with the frequency ranging from 10^{-2} to 10^5 Hz. All other electrochemical measurements were performed in 0.1 M (pH 7.4) PBS. All measurements were performed at a constant temperature of $20 \pm 1^\circ\text{C}$.

The morphology and structure of the Pd-Cu NWs were investigated using a transmission electron microscope (JEOL JEM-200EX, Japan) and X-ray diffraction (XRD) patterns. X-ray diffraction (XRD) patterns of Pd-Cu NWs were obtained on a D/max-2500/PC X-ray diffractometer with $\text{Cu-K}\alpha$. Detection of malathion in samples was performed using a 1290-6460 LC-MS/MS system.

2.3. Synthesis of Pd-Cu NWs

Pd-Cu nanowires were synthesized using a surfactant-directed aqueous method as reported previously [41]. Briefly, 2 mL of metal precursor Na_2PdCl_4 and $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$ with a total metal ions concentration of 1 M containing 0.5%wt Triton X-114 were prepared. Then, the freshly prepared NaBH_4 (0.1 M, 5 mL) solution was quickly added into this solution under vigorous stirring. After 30 min, the resulting solution was centrifuged by deionized water and ethanol, respectively. The Pd-Cu nanowires were re-dispersed in water before further characterization.

2.4. Preparation of the AChE-Cs/Pd-Cu NWs/GCE biosensor

Firstly, the glass carbon electrode (GCE) was sequentially polished in 0.3 μm and 0.05 μm alumina slurry, ultrasonically rinsed for 1 min each in ethanol, nitric acid and high purity water. Secondly, the electrode was electrochemically cleaned in 0.5 M H_2SO_4 solution by scanning in the potential range from 0 V to 1.2 V vs. SCE at a scanning rate of 100 mV/s. After being rinsed with high purity water and dried in an argon atmosphere, Pd-Cu NWs (20 μL , 1 mg/mL) were loaded onto the electrode surface. Finally, 11 μL of a mixture containing AChE and chitosan (AChE: Chitosan ratio= 1:10, 1.25%wt chitosan) was further immobilized on Pd-Cu NWs /GCE, which was then kept at 4°C for 8 h. The AChE-Cs/Pd-Cu NWs/GCE biosensor was stored at 4 °C for future use.

2.5. Sample pre-treatment for LC-MS/MS analysis

The samples (courgettes, carrots, lettuces and orange) used in spiked experiment were prepared according to a Chinese Official Standard Method [42]. Briefly, 20 g of a homogenised sample was dissolved in 40 mL acetonitrile and 5 g sodium chloride.

Then, 20 mL of the supernatant was evaporated to 1-2 mL on a vacuum rotary evaporator at 40 °C. The concentrate solution obtained was purified using a carbon/NH₂ cartridge and eluted with acetonitrile: toluene (3:1, v/v). Finally, the eluate was concentrated to dryness before reconstituted with acetonitrile: water (1:1, v/v) containing 0.1% formic acid. Prior to injection into the LC-MS/MS system, each sample was filtered through a 0.22 µm polytetrafluoroethylene filter.

After the pretreatment, the prepared samples were detected by the proposed biosensor and LC-MS/MS method. The concentration of malathion in the sample was evaluated based on a calibration plot.

2.6. Electrochemical analysis

Malathion is an AChE inhibitor and chosen as a model because it is the most commonly used organophosphate insecticide. Therefore, in the present work it was chosen as standard pesticide for the AChE inhibition. Differential pulse voltammetry (DPV) measurements at 0.55 V were carried out in 0.1 M (pH 7.4) PBS containing 3.0 mM ATCl. The original DPV response was recorded before incubation in malathion. Then, the electrode was slightly rinsed with PBS and immersed in a given concentration of malathion for 20 min, subsequently transferred into another cell of 0.1 M PBS containing 3.0 mM ATCl for DPV measurements. The inhibitions of malathion were calculated as follows:

$$Inhibition(\%) = [(I_0 - I_i) / I_0] \times 100$$

Here, I_0 and I_i were the peak currents of 3.0 mM ATCl on AChE-Cs/Pd-Cu NWs/GCE without and with malathion inhibition, respectively.

3. Results and discussion

3.1. Characterization of the Pd-Cu NWs

3.1.1. Transmission electron microscope characterization

The morphology of Pd-Cu NWs was investigated by transmission electron microscope (TEM). As shown in Fig. 1A, the nanomaterials contain large frameworks and consist of high-quality intertwining nanowires. In the enlarged TEM images (Fig. 1B), Pd-Cu NWs showed ultrathin characteristics and large surface area with an average diameter of 3 nm. Furthermore, these nanowires display no significant aggregation and there were no byproducts such as nanoparticles in the entire micrograph.

The crystal structure of the PdCu NWs was further investigated by X-ray diffraction (XRD) (Fig. S1). The PdCu NWs present a classic face-centred cubic (FCC) structure, where the (111), (200), (220) and (311) planes derived from the Pd FCC structure are apparent without any obvious crystalline peaks corresponding to Cu or Cu oxide. The strongest (111) plane diffraction peak of the PdCu NWs was located between those of pure Pd (JCPDS. No.46-1043) and Cu (JCPDS. No. 04-0836), demonstrating the formation of the alloyed structure.

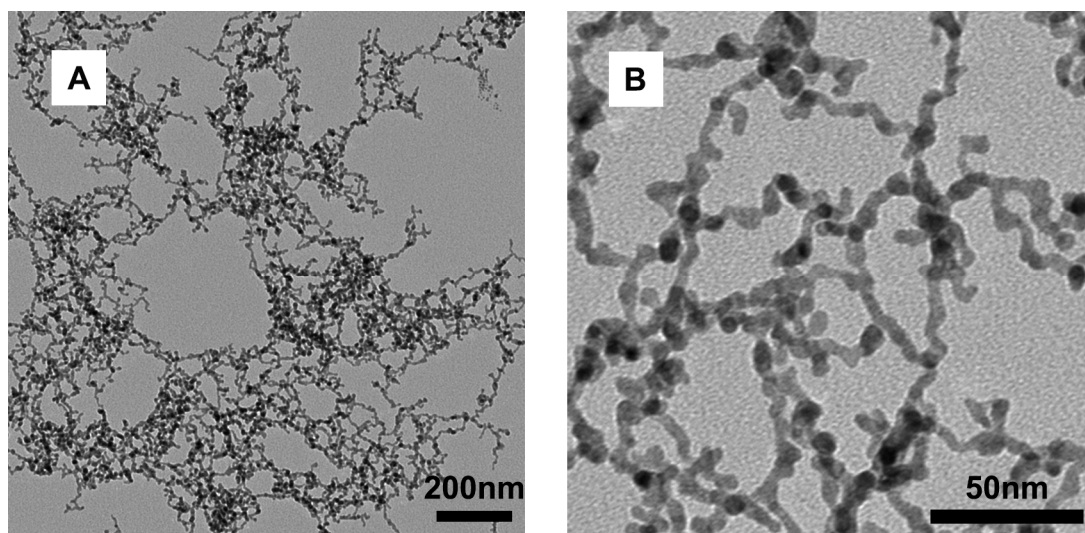


Fig. 1. TEM images of (A, B) Pd-Cu NWs.

3.1.2. Electrochemical characterization of modified electrode

To study the electroactive surface area, cyclic voltammetry (CV) was carried out at a Pd-Cu NWs modified electrode using 0.1 M KCl as supporting electrolyte. Fig. 2A shows the voltammogram of Pd-Cu NWs modified GCE over a range of scan rate. As shown in the Fig. 2A, a pair of stable and well-defined reversible redox peaks due to $\text{Fe}^{3+}/\text{Fe}^{2+}$ redox couple for Pd-Cu NWs/GCE is observed. In Fig. 2B, the plots of anodic peak current (a) and cathodic peak current (b) are proportional to the square root of scan rate with $R^2 = 0.998$ ($N=3$) for $i_{pa} \sim v^{1/2}$ and $R^2 = 0.997$ ($N=3$) for $i_{pc} \sim v^{1/2}$, respectively. According to the Randles–Sevcik equation [6] and [23], the electroactive surface area of Pd-Cu NWs/GCE had been calculated as $(0.268 \pm 0.01) \text{ cm}^2$ from the slope of i_p versus $v^{1/2}$, which was about 3.8 times larger than the surface area of the bare glass carbon electrode 0.0707 cm^2 (GCE, $\phi=3 \text{ mm}$) and it was about 1.25 times larger than 0.214 cm^2 (GCE, $\phi=4 \text{ mm}$) [23]. The increase of the active surface area was advantageous to the adsorption and enrichment of the substance being detected.

The results indicated that Pd-Cu NWs featured by large surface area could be utilized as immobilization platform.

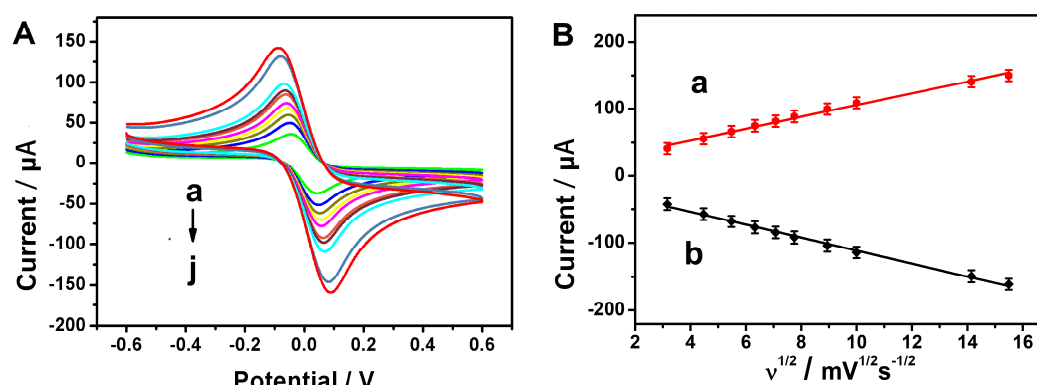


Fig. 2. (A) Cyclic voltammetry curves of Pd-Cu NWs/GCE in 5.0 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1 : 1) solution containing 0.1 M KCl at scan rate of (a) 10, (b) 20, (c) 30, (d) 40, (e) 50, (f) 60, (g) 80, (h) 100, (i) 200 and (j) 240 $mV \cdot s^{-1}$. (B) Plots of anodic peak current (a) and cathodic peak current (b) versus $v^{1/2}$, respectively.

3.2. Electrochemical behavior of the biosensor

3.2.1. Electrochemical impedance spectroscopy (EIS)

EIS is a powerful electrochemical measurement that has become increasingly popular in biosensing due to its good performance in probing interfacial properties of different modified electrodes [6]. A typical Nyquist plot is composed of a semicircle at high frequencies that is related to the electron-transfer-limited process and a linear part at low frequencies that is correlated with diffusion-limited process. In the semicircle, its diameter is often used to estimate the electron transfer resistance (R_{ct}). In Fig. 3, curve a depicts the EIS plot at a bare GCE with an electron-transfer resistance about 310 Ω . At the Pd-Cu NWs modified electrode, the semicircle domain has dramatically decreased (the R_{ct} is approximately 70 Ω) and the plot appears to be nearly linear, indicating the excellent electrical conductivity of the Pd-Cu NWs (curve

b). After the immobilization of AChE, the EIS of the AChE-Cs/Pd-Cu NWs/GCE (curve c) resulted in an apparent increase in the electron transfer resistance to 1250 Ω , which was attributed to the macro-biomolecule obstructing electron transfer reaction. The results of the Nyquist plots indicated that the Pd-Cu NWs could either enhance the conductivity of the electrode interface or facilitate the electron transfer.

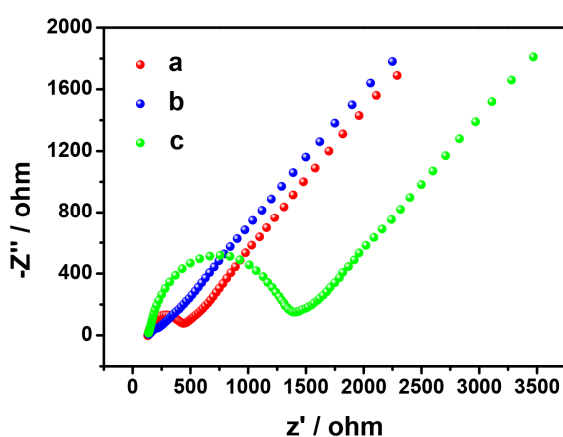


Fig. 3. EIS of (a) bare GCE (b) Pb-Cu NWs/GCE (c) AChE-Cs/Pd-Cu NWs/GCE bare and modified electrodes in 1.0 M KCl containing 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ (1:1) with a frequency range from 0.01 Hz to 100 kHz, with an amplitude of 5 mV.

3.2.2. Cyclic Voltammograms (CV)

The CV measurements of 3 mM ATCl was conducted at the AChE biosensor in 0.1 M (pH 7.4) PBS over the 30-150 $\text{mV} \cdot \text{s}^{-1}$ range are shown in Fig. 4A (curves a-f). These voltammograms showed an oxidation peak arising from 3.716 μA to 6.154 μA as the scan rate increased from 30 to 150 $\text{mV} \cdot \text{s}^{-1}$. Fig. 4B demonstrated a good linear relationship between the oxidation peak current and the square root of scan rate ($R^2=0.997$, $N=3$, for $i \sim v^{1/2}$). According to the diffusion reaction, we deduce that the enzyme retained its biological activity and was not desorbed in process of the

employment of the biosensor. Consequently, the Cs was made use of immobilizing AChE, successfully prevented the loss of the AChE enzyme molecules, established a biocompatible microenvironment for enzyme and effectively improved the stability of the biosensor.

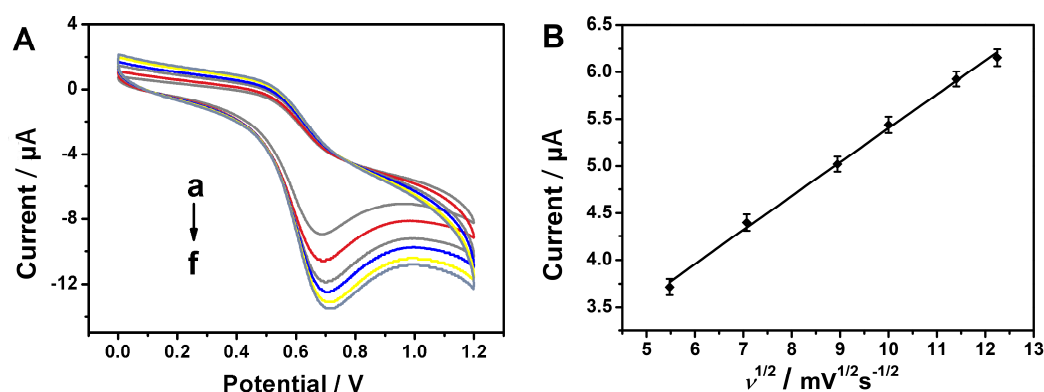


Fig. 4. (A) CV of AChE-Cs/Pd-Cu NWs/GCE in 0.1 M pH 7.4 PBS containing 3mM ATCl at scan rate of (a) 30, (b) 50, (c) 80, (d) 100, (e) 130 and (f) 150 $\text{mV}\cdot\text{s}^{-1}$. (B) Plot of oxidation peak current versus the square root of scan rate.

3.3. Optimization of experimental parameters

To further improve the performance of the biosensor, we optimized critical experimental parameters, such as the quantity of Pd-Cu NWs, the chitosan to AChE ratio, the PH of the PBS, the incubation time in the pesticide and the amount of AChE. To optimize each parameter, all other parameters are maintained at their optimal conditions.

As shown in Fig. S2A, the quantity of Pd-Cu NWs has a great effect on the current response of the constructed biosensor. The response increased with increasing the quantity of Pd-Cu NWs and decreased after 20 μL (1 mg/mL) which may be

attributed to an increase in electrode resistance. Therefore, 20 μ L Pd-Cu NWs were used to prepare the biosensor.

The bioactivity of the immobilized AChE depends on the pH of the PBS (0.1 M). The pH effect on the performance of AChE-Cs/Pd-Cu NWs/GCE was studied in 0.1 M PBS (pH from 5 to 10) containing 3 mM ATCl by DPV. According to the results shown in Fig. S2B, the current response increased in the range of 5–7 and decreased with the increasing pH value of PBS. When pH reached 7.4, the maximum current can be obtained. As a result, this pH value was used in further electrochemical detections.

The influence of the chitosan to AChE ratio on the response of biosensor was also investigated. In Fig. S2C, the current response reached a maximum when the ratio of chitosan to AChE was 10:1. Hence, 10:1 was chosen as the optimal ratio of chitosan to AChE.

Another important parameter was the incubation time in the pesticide. In Fig. S2D, the current showed significant decrease with the increase of inhibition time within 20 min. When the incubation time in pesticides exceeded 20 min, the current signal did not obviously change. The result maybe reveal that the binding interaction between OPs and active target groups in the AChE was saturated. Thus, the optimum inhibition time was selected as 20 min.

The amount of enzyme also plays an important role on the response of biosensor. In Fig. S2E, with increasing the amount of immobilized AChE enzyme from 0.05 U to 0.2 U, the current obviously increased, and then reached the maximum value at 0.2 U. However, to further increase the amount of enzyme, the current response decreased,

as a result of the over-thickness of immobilized AChE layer obstructing electron transfer reaction. In summary, pH 7.4, 11 μL of mixture of chitosan and AChE (10:1), 20 μL (1 mg/mL) Pd-Cu NWs, 20 min incubation time and 0.2 U AChE were selected as the optimum conditions in the subsequent measurements (see the Supplemental Information, Fig. S2).

3.4. Amperometric response toward ATCl

The current-time curve of AChE-Cs/Pd-Cu NWs/GCE biosensor was investigated by successively adding ATCl into a stirred 0.1 M (pH 7.4) PBS and the results obtained are shown in Fig. 5A. The biosensor displayed a good linear correlation between the oxidation current (I) and the concentration of ATCl (C) in the range from 5 μM to 175 μM . The equation was $I = 0.00269C + 0.02924$ ($R^2 = 0.998$) (Fig. 5B), with a sensitivity of 2.69 $\mu\text{A}/\text{mM}$. In order to value the enzymatic affinity and the ratio of microscopic kinetic constants, K_M was calculated from the electrochemical version of Lineweaver–Burk equation [34] (Fig. 5C). The K_M value for the proposed biosensor was 50.56 μM , which is lower than those of 106 μM [35], 67.4 μM [36] and 131 μM [37] reported by previous papers, implying the immobilized AChE had a higher affinity towards ATCl. In addition, as shown in Fig. 5A, the detection limit of ATCl was as low as 0.5 μM , illustrating the AChE has been effectively immobilized and the catalytic activity of AChE was successfully retained.

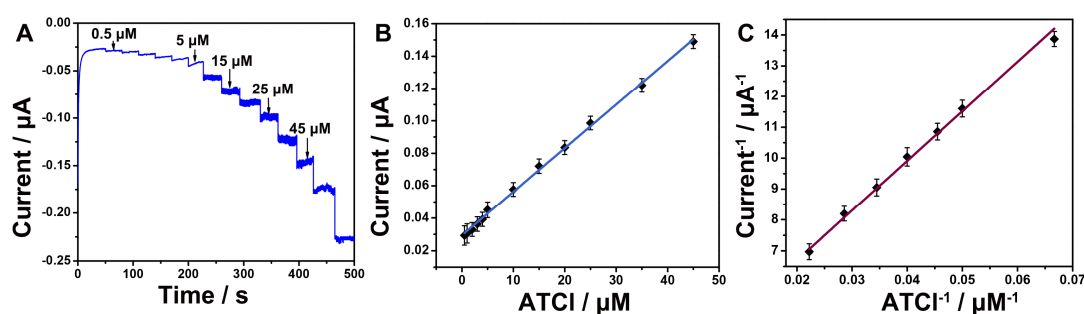


Fig. 5. (A) Amperometric responses for the sensor on successive addition of ATCl (0.5 μM , 5 μM , 15 μM , 25 μM , 45 μM) to pH 7.4 PBS, at 0.55 V, constantly stirring. (B) The calibration curve for ATCl determination. (C) The Lineweaver–Burk plot of $1/I_{ss}$ vs. $1/C$.

3.5. Pesticide determination

Fig. 6A shows the DPV responses of malathion at an AChE-Cs/Pd-Cu NWs/GCE before and after being incubated in malathion for 20 min. No peak was observed, without the addition of ATCl in the PBS (curve i). After injecting 3.0 mM ATCl in the cell, an oxidation peak appeared at 0.564 V (curve a). This peak has most likely resulted from the oxidation of thiocholine, which was generated by the catalysed hydrolysis of ATCl by AChE. In the presence of increasing malathion, the corresponding peak current (curve b-h) decreased. It suggests that the activity of AChE was reduced, which could be ascribed to the interaction between pesticides and serine hydroxyl groups of AChE.

Fig. 6B shows that the relationship between inhibition percentages of AChE-Cs/Pd-Cu NWs/GCE biosensor and malathion. Linear calibration plots (Fig. 6C, Fig. 6D) were obtained in two ranges, from 5 ppt to 1000 ppt, ($I(\%) = 14.34 + 0.029C(\text{ppb})$, $R^2 = 0.999$, $N = 3$) and from 500 ppb to 3000 ppb

($I(\%)=65.19+4.624C(\text{ppm})$, $R^2=0.997$, $N=3$). The limit of detection was estimated to be 1.5 ppt (signal-to-noise ratio of 3). Compared with the previous studies [7], [34], [35], [36], [38], [39], [40], [44], [45], [46] and [47], it displayed either much lower detection limit or wider linearity ranges. The results are summarized in Table 1. It suggests that the Pd-Cu NWs-based biosensors could satisfy the need for the fast and sensitive detection of malathion.

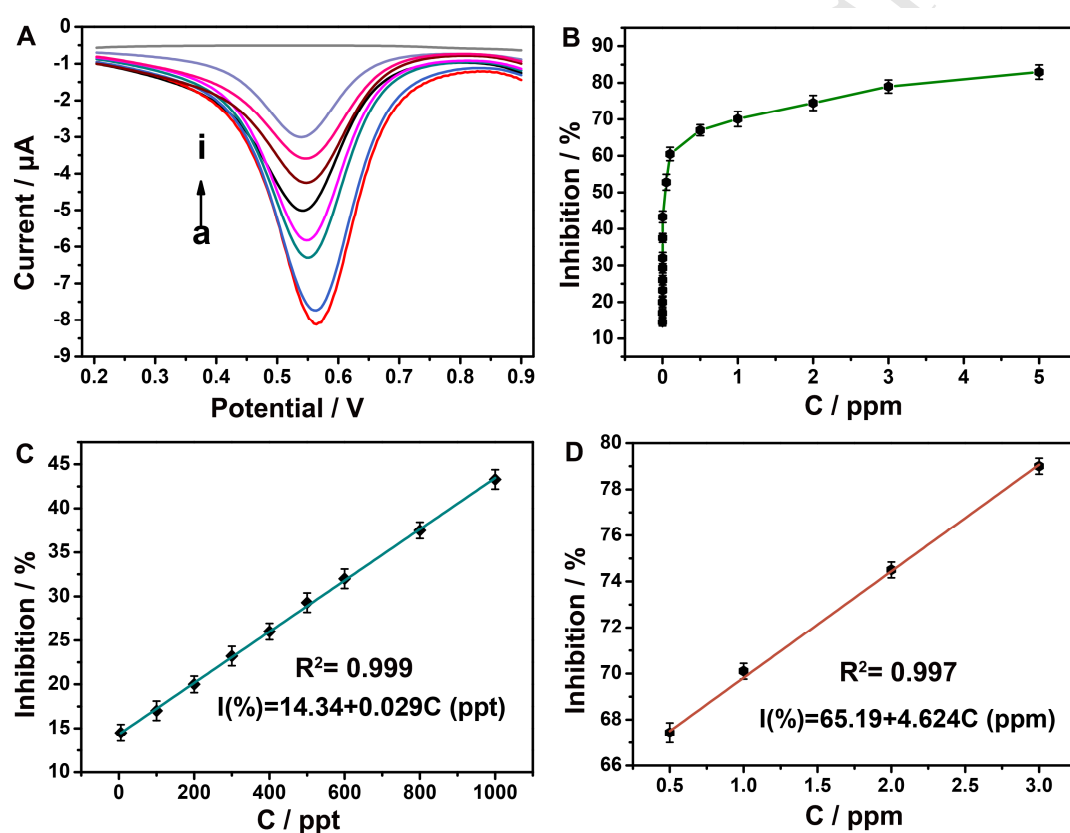


Fig. 6. (A) DPV responses of AChE-Cs/Pd-Cu NWs/GCE in 0.1 M pH 7.4 PBS solution with (a-h) or without (i) 3.0 mM ATCl after incubation with malathion for 20 min. Malathion concentration (a)–(h): 0 ppt, 1 ppt, 50 ppt, 0.3 ppb, 0.5 ppb, 1 ppb, 5 ppb, 10 ppb. (B) Plot of the inhibition rate versus malathion concentration. (C) and (D) show the calibration curves for malathion determination.

Table 1 Comparisons of this proposed AChE biosensor with other biosensors for the detection of malathion.

AChE biosensors	Linear range	K_M (mM)	Detection limit	References
AChE/Chit-PB-MWNTs -HGNs/Au	0.05-75 nM	0.21	0.05 nM	[7]
AChE-MWCNTs-Au-CHIT/GCE	3-3000 nM and 6-45 μ M	0.268	1.8 nM	[34]
CS/AChE/PB-CS/ERGO -AuNPs- β -CD/GCE	24-6000 pM	0.106	12.4 pM	[35]
AChE/CNT-NH ₂ /GCE	0.2-1 and 1-30 nM	0.067	0.08 nM	[36]
AChE/PAn-PPy-MWCNTs/GCE	0.03-1.5 and 3-75 μ M	-----	3 nM	[38]
AChE-AuNPs-CaCO ₃ /silica sol-gel/AuE	0.1-100 nM	-----	0.1 nM	[39]
AChE/CPBA/AuNPs/RGO-CS/GC E	1.5-30 and 60-300 nM	0.16	1.5 nM	[40]
AChE/sol-gel/SPE	10-500 nM	-----	3.6 nM	[44]
Fe ₃ O ₄ NP/c-MWNT/Au	0.1-40 nM	-----	0.1 nM	[45]
AChE/MWCNT/CA/NPG	3-1500 nM	-----	1.5 nM	[46]
AChE-Fe ₃ O ₄ NPs/c-MWCNTs/ITO	0.1-70 nM	-----	0.1 nM	[47]
AChE-Cs/Pb-Cu NWs/GCE	5-1000 ppt and 500-3000 ppb (15-3000 pM and 1500-9000 nM)	0.05	1.5 ppt (4.5 pM)	This issue

SPE: screen printed carbon electrode

NPG: nanoporous gold film

Au/AuE: gold electrode

3.6. Reproducibility and stability

To evaluate the reproducibility of the as-prepared biosensor, DPV measurements were carried out by six different AChE-Cs/Pd-Cu NWs/GCE electrodes in pH 7.4 PBS containing 3.0 mM ATCl after being incubated in 10 ppb malathion for 20 min. The RSD was obtained to be 1.8%, indicating an acceptable reproducibility. Similarly, the repeatability was investigated with six independent DPV measurements and the RSD was 2.2%.

To investigate long-term stability, the current responses of the biosensor were tested for 30 consecutive days. The fabricated bioelectrode was stored at 4 °C. After a week, the response current of the biosensor decreased to 96% of the initial signal. After a storage period of 30 days, the biosensor maintained 91% of its original current signal, showing a favorable stability of the electrochemical sensor.

3.7. Interference study

In order to demonstrate the selectivity of the AChE-Cs/Pd-Cu NWs/GCE biosensor, the effect of the most common interfering electroactive substances was investigated. The amperometric responses of 3.0 mM ATCl in the absence and presence of interfering species including ascorbic acid, citric acid, glucose, metal ions (Cu^{2+} , Pb^{2+}), p-nitroaniline, toluene, oxygen-containing inorganic ions (NO_3^- , SO_4^{2-} and PO_4^{3-}) in the PH 7.4 PBS were presented in Fig S3 (in Supplemental Information). The results revealed that no significant changes in the signals were detected in the presence of ascorbic acid, citric acid, glucose, Cu^{2+} , Pb^{2+} , NO_3^- , SO_4^{2-} and PO_4^{3-} . However, p-nitroaniline and toluene severely interfered with the determination.

3.8. Method validation

Method trueness and precision were evaluated by recovery studies. Samples confirmed as not containing the malathion by our laboratory were used as blank and spike aliquots for recovery determination. Recovery studies were carried out at the concentration of 0.2 ppb and three replicates were performed. The samples were spiked with appropriate volumes of malathion and afterwards shaken for uniform

distribution, then held for 30 min before prepared following the procedure describes as sample pre-treatment. Recovery results and the RSDs were listed in Table 2.

The recoveries of malathion from spiked vegetable and fruit samples ranged from 98.5% to 113.5% and the RSDs were below 10%, complying with the requirements of SANCO 2007/3131 [43]. This demonstrated that the method has relatively good accuracy and precision.

3.9. Application to real samples

To further assess the applicability of the prepared biosensor, the AChE-Cs/Pd-Cu NWs/GCE was employed to detect malathion in courgettes, carrots, lettuces and orange samples, obtained from a local market. For comparison, the same samples were also analyzed by LC-MS/MS and the results obtained are tabulated in Table 2. As shown in Table 2, the LOD of the sensors is lower than those of LC-MS/MS method, in which the pesticides with concentration lower than 1.4 ppb cannot be detected. Moreover, the concentration levels detected by the biosensor were lower than the European maximum residue levels (Table S1, in Supplemental Information). Consequently, it was possibly concluded that the AChE-Cs/Pd-Cu NWs/GCE biosensor would be served as a promising monitoring method for OPs residues analysis.

Table 2. Correlation of the concentrations of pesticides content in vegetables and fruits determined directly by the proposed biosensor and LC-MS/MS.

Sample	Malathion biosensors				LC-MS/MS method		
	Measured value (1 %)	Averaged value	Recovery (%)	RSD (%) n=3	Averaged value	Recovery (%)	RSD (%) n=3
Malathion ^a		9.87 ppb	98.7	3.4	10.39 ppb	103.9	1.6

		24.33 ppb	97.3	2.1	24.73 ppb	98.9	2.3
Courgettes	8.34	N/A ^b		5.1	N/A ^c		
Carrots	3.24	N/A ^b		3.4	N/A ^c		
Lettuces	9.06	N/A ^b		6.7	N/A ^c		
Orange	5.17	N/A ^b		2.3	N/A ^c		
Added (0.2 ppb)							
Courgettes	35.24	0.227 ppb	113.5	4.3	N/A ^c		
Carrots	21.87	0.197 ppb	98.5	7.9	N/A ^c		
Lettuces	27.93	0.218 ppb	109	3.7	N/A ^c		
Orange	31.54	0.223 ppb	111.5	5.4	N/A ^c		
a. Since the limit of detection (LOD) of the proposed AChE biosensor is much lower than the LC-MS/MS method, the standard samples for LC-MS/MS were diluted before measurement with AChE biosensor.							
b. The inhibition rate is lower than 10%, so we assume that pesticides are not detected.							
c. Concentration of malathion lower than 1.4 ppb cannot be detected out by LC-MS/MS method.							

Conclusion

In summary, a simple and reliable AChE-Cs/Pd-Cu NWs/GCE biosensor was constructed for monitoring OPs. The Pd-Cu NWs showed obvious advantages, such as a large active area (0.268 cm²), remarkable catalytic activity and conductivity, relatively low cost, unique synergy properties, and favorable chemical stability. The biosensor was characterized by CV, DPV, EIS and amperometry, exhibiting unprecedented performance, such as a favorable affinity to ATCl ($K_M = 50.56 \mu\text{M}$), acceptable precision and reproducibility, long-time stability, wide linear detection range and a low detection limit for malathion. The determination of spiked malathion in vegetable and fruit samples (courgettes, carrots, lettuces, orange) were tested with excellent recoveries by the proposed biosensor.

Acknowledgments

The authors acknowledge the financial support from the National Natural Science Foundation of China (Grant No. 21371149) and the Natural Science Foundation of Hebei (Grant No.

B2016203498, 11965152D) and Research Fund for the Doctoral Program of Higher Education of China (Grant 20131333110010).

References

- [1] D. Du, S.Z. Chen, D.D. Song, H.B. Li, X. Chen, Development of acetylcholinesterase biosensor based on CdTe quantum dots/gold nanoparticles modified chitosan microspheres interface, *Biosens. Bioelectron.* 24 (2008) 475–479.
- [2] J. Kirsch, C. Siltanen, Q. Zhou, A. Revzin, A. Simonian, Biosensor technology: recent advances in threat agent detection and medicine, *Chem. Soc. Rev.* 42 (2013) 8733–68.
- [3] Q.L. Lang, L. Han, C.T. Hou, F. Wang, A.H. Liu, A sensitive acetylcholinesterase biosensor based on gold nanorods modified electrode for detection of organophosphate pesticide, *Talanta*. 156–157 (2016) 34–41.
- [4] N.J. Ronkainen, H.B. Halsall, W.R. Heineman, Electrochemical biosensors, *Chem. Soc. Rev.* 39 (2010) 1747–1763.
- [5] W.Y. Zhang, A.M. Asiri, D. Liu, D. Du, Y.H. Lin, Nanomaterial-based biosensors for environmental and biological monitoring of organophosphorus pesticides and nerve agents, *Trends Anal. Chem.* 54 (2014) 1–10.
- [6] D.G. Rackus, M.H. Shamsi, A.R. Wheeler, Electrochemistry, biosensors and microfluidics: a convergence of fields, *Chem. Soc. Rev.* 44 (2015) 5320–5340.
- [7] C. Zhai, X. Sun, W.P. Zhao, X.Y. Wang, Acetylcholinesterase biosensor based on chitosan/prussian blue/ multiwall carbon nanotubes/hollow gold nanoparticles nanocomposite film by one-step electrodeposition, *Biosens. Bioelectron.* 42 (2013) 124–130.
- [8] C.Z. Zhu, G.H. Yang, H. Li, D. Du, Y.H. Lin, Electrochemical sensors and biosensors based on nanomaterials and nanostructures, *Anal. Chem.* 87 (2014) 230–249.

- [9] A. Aminea, F. Arduinib, D. Moscone, G. Palleschi, Recent advances in biosensors based on enzyme inhibition, *Biosens. Bioelectron.* 76 (2016) 180–194.
- [10] N. Nesakumar, S. Sethuraman, U.M. Krishnan, J.B.B. Rayappan, Electrochemical acetylcholinesterase biosensor based on ZnO nanocuboids modified platinum electrode for the detection of carbosulfan in rice, *Biosens. Bioelectron.* 77 (2016) 1070–1077.
- [11] P. Scrimin, L.J. Prins, Sensing through signal amplification, *Chem. Soc. Rev.* 40 (2011) 4488–4505.
- [12] J.P. Lei, H.X. Ju, Signal amplification using functional nanomaterials for biosensing, *Chem. Soc. Rev.* 41 (2012) 2122–2134.
- [13] Y.T. Zhao, W.Y. Zhang, Y.H. Lin, D. Du, The vital function of Fe₃O₄@Au nanocomposites for hydrolase biosensor design and its application in detection of methyl parathion, *Nanoscale.* 5 (2013) 1121–1126.
- [14] J.L. Huang, L.Q. Lin, D.H. Sun, H.M. Chen, D.P. Yang, Q.B. Li, Bio-inspired synthesis of metal nanomaterials and applications, *Chem. Soc. Rev.* 44 (2015) 6330–6374.
- [15] S. Upadhyay, G.R. Rao, M.K. Sharma, B.K. Bhattacharya, V.K. Rao, R. Vijayaraghavan, Immobilization of acetylcholineesterase–choline oxidase on a gold–platinum bimetallic nanoparticles modified glassy carbon electrode for the sensitive detection of organophosphate pesticides, carbamates and nerve agents, *Biosens. Bioelectron.* 25 (2009) 832–838.
- [16] C.X. Xu, A.H. Liu, H.J. Qiu, Y.Q. Liu, Nanoporous PdCu alloy with enhanced electrocatalytic performance, *Electrochem. Commun.* 13 (2011) 766–769.
- [17] C.X. Xu, Y.Q. Liu, F. Su, A.H. Liu, H.J. Qiu, Nanoporous PtAg and PtCu alloys with hollow ligaments for enhanced electrocatalysis and glucose biosensing, *Biosens. Bioelectron.* 27 (2011)

160–166.

[18] X. Cao, N. Wang, S. Jia, Y.H. Shao, Detection of glucose based on bimetallic PtCu nanochains modified electrodes, *Anal. Chem.* 85 (2013) 5040–5046.

[19] C.Z. Zhu, D. Du, A. Eychmüller, Y.H. Lin, Engineering ordered and nonordered porous noble metal nanostructures: synthesis, assembly, and their applications in electrochemistry, *Chem. Rev.* 115 (2015) 8896–8943.

[20] L. Dai, S.Z. Zou, Enhanced formic acid oxidation on Cu–Pd nanoparticles, *J Power Sources*. 196 (2011) 9369–9372.

[21] D.C. Yang, J. Carpena-Núñez, L.F. Fonseca, A. Biaggi-Labiosa & G.W. Hunter, Shape-controlled synthesis of palladium and copper superlattice nanowires for high-stability hydrogen sensors, *Sci. Rep.* 4 (2014) 3773.

[22] X.L. Qin, H.J. Yin, W.J. Guo, M.S. Pei, A novel signal amplification strategy of an electrochemical aptasensor for kanamycin, based on thionine functionalized graphene and hierarchical nanoporous PtCu, *Biosens. Bioelectron.* 77 (2016) 752–758.

[23] C. Ye, M.Q. Wang, X. Zhong, S.H. Chen, Y.Q. Chai, R. Yuan, Highly sensitive electrochemiluminescence assay of acetylcholinesterase activity based on dual biomarkers using Pd–Au nanowires as immobilization platform, *Biosens. Bioelectron.* 79 (2016) 34–40.

[24] T.D. Thanh, J. Balamurugan, S.H. Lee, N.H. Kim, J.H. Lee, Novel porous gold-palladium nanoalloy network-supported graphene as an advanced catalyst for non-enzymatic hydrogen peroxide sensing, *Biosens. Bioelectron.* 85 (2016) 669–678.

[25] A.H. Liu, H.R. Geng, C.X. Xu, H.J. Qiu, A three-dimensional hierarchical nanoporous PdCu alloy for enhanced electrocatalysis and biosensing, *Anal. Chim. Acta* 703 (2011) 172–178.

- [26] M. Janyasupab, C.W. Liu, Y. Zhang, K.W. Wang, C.C. Liu, Bimetallic Pt-M (M=Cu, Ni, Pd, and Rh) nanoporous for H₂O₂ based amperometric biosensors, *Sens. Actuators B: Chem.* 179 (2013) 209–214.
- [27] C.X. Xu, Y.Q. Liu, J.P. Wang, H.R. Geng, H.J. Qiu, Nanoporous PdCu alloy for formic acid electro-oxidation, *J Power Sources*. 199 (2012) 124–131.
- [28] L. Shang, F.Q. Zhao, B.Z. Zeng, 3D porous graphene-porous PdCu alloy nanoparticles-molecularly imprinted poly(para-aminobenzoic acid) composite for the electrocatalytic assay of melamine, *ACS Appl. Mater. Interfaces*. 6 (2014) 18721–18727.
- [29] M. Yuan, A.P. Liu, M. Zhao, W.J. Dong, T.Y. Zhao, J.J. Wang, W.H. Tang, Bimetallic PdCu nanoparticle decorated three-dimensional graphene hydrogel for non-enzymatic amperometric glucose sensor, *Sens. Actuators, B* 190 (2014) 707–714.
- [30] Y.Y. Cai, H. Li, Y.Y. Li, Y.F. Zhao, H.M. Ma, B.C. Zhu, C.X. Xu, Q. Wei, D. Wu, B. Du, Electrochemical immunoassay for carcinoembryonic antigen based on signal amplification strategy of nanotubular mesoporous PdCu alloy, *Biosens. Bioelectron.* 36 (2012) 6–11.
- [31] C.Y. Du, M. Chen, W.G. Wang, G.P. Yin, Nanoporous PdNi alloy nanowires as highly active catalysts for the electro-oxidation of formic acid, *ACS Appl. Mater. Interfaces*. 3 (2011) 105–109.
- [32] X. Cao, N. Wang, Y. Han, C.Z. Gao, Y. Xu, M.X. Li, Y.H. Shao, PtAg bimetallic nanowires: Facile synthesis and their use as excellent electrocatalysts toward low-cost fuel cells, *Nano. Energy*. 12 (2015) 105–114.
- [33] J. Warner, S. Andreescu, An acetylcholinesterase (AChE) biosensor with enhanced solvent resistance based on chitosan for the detection of pesticides, *Talanta* 146 (2016) 279–284.
- [34] D. Du, M.H. Wang, J. Cai, Y.H. Qin, A.D. Zhang, One-step synthesis of multiwalled carbon

nanotubes-gold nanocomposites for fabricating amperometric acetylcholinesterase biosensor, Sens. Actuators, B 143 (2010) 337–341.

[35] H.Y. Zhao, X.P. Ji, B.B. Wang, N. Wang, X.R. Li, R.X. Ni, An ultra-sensitive acetylcholinesterase biosensor based on reduced graphene oxide-Au nanoparticles- β -cyclodextrin/Prussian blue-chitosan nanocomposites for organophosphorus pesticides detection Biosens. Bioelectron. 65 (2015) 23–30.

[36] G.X. Yu, W.X. Wu, Q. Zhao, X.Y. Wei, Q. Lu, Efficient immobilization of acetylcholinesterase onto amino functionalized carbon nanotubes for the fabrication of high sensitive organophosphorus pesticides biosensors, Biosens. Bioelectron. 68 (2015) 288–294.

[37] Q. Zhou, L. Yang, G.C. Wang, Y. Yang, Acetylcholinesterase biosensor based on SnO₂ nanoparticles-carboxylic graphene-nafion modified electrode for detection of pesticides, Biosens. Bioelectron. 49 (2013) 25–31.

[38] D. Du, X.X. Ye, J. Cai, J. Liu, A.D. Zhang, Acetylcholinesterase biosensor design based on carbon nanotube-encapsulated polypyrrole and polyaniline copolymer for amperometric detection of organophosphates, Biosens. Bioelectron. 25 (2010) 2503–2508.

[39] N.H. Chauhan, N. Narang, C.S. Pundir, Immobilization of rat brain acetylcholinesterase on porous gold-nanoparticle-CaCO₃ hybrid material modified Au electrode for detection of organophosphorous insecticides, Int. J. Biol. Macromol. 49 (2011) 923–929.

[40] T. Liu, H.C. Su, X.J. Qu, P. Ju, L. Cui, S.Y. A, Acetylcholinesterase biosensor based on 3-carboxyphenylboronic acid/reduced graphene oxide-gold nanocomposites modified electrode for amperometric detection of organophosphorus and carbamate pesticides, Sens. Actuators, B 160 (2011) 1255–1261.

- [41] X. Zhao, J. Zhang, L.G. Wang, Z.L. Liu, W. Chen, Pd_xCu_{100-x} networks: an active and durable electrocatalyst for ethanol oxidation in alkaline medium, *J. Mater. Chem. A*, 2 (2014) 20933–20938.
- [42] EU. Method Validation And Quality Control Procedures For Pesticide Residues Analysis In Food And Feed[S]. EU, Document No. SANCO/12495/2011.
- [43] Determination of 450 pesticides and related chemicals residues in fruits and vegetables, GB/T 20769-2008, Standardization Administration of the People's Republic of China, 2008, vol. GB/T 20769-2008.
- [44] N.B. Oujji, I. Bakas, G. Istamboulie, I. Ait-Ichou, E. Ait-Addi, R. Rouillon, T. Noguer, Solgel immobilization of acetylcholinesterase for the determination of organophosphate pesticides in olive oil with biosensors, *Food Control* 30 (2013) 657–661.
- [45] N. Chauhan, C.S. Pundir, An amperometric biosensor based on acetylcholinesterase immobilized onto iron oxide nanoparticles/multi-walled carbon nanotubes modified gold electrode for measurement of organophosphorus insecticides, *Anal. Chim. Acta* 701 (2011) 66–74.
- [46] J. Ding, H. Zhang, F. Jia, W. Qin, D. Du, Assembly of carbon nanotubes on a nanoporous gold electrode for acetylcholinesterase biosensor design, *Sens. Actuators, B* 199 (2014) 284–290.
- [47] N. Chauhan, C.S. Pundir, An amperometric acetylcholinesterase sensor based on Fe₃O₄ nanoparticle/multi-walled carbon nanotube-modified ITO-coated glass plate for the detection of pesticides, *Electrochimica Acta* 67 (2012) 79–86.

Table 1 Comparisons of this proposed AChE biosensor with other biosensors for the detection of malathion.

AChE biosensors	Linear range	K_M (mM)	Detection limit	References
AChE/Chit-PB-MWNTs-HGNs/Au	0.05-75 nM	0.21	0.05 nM	[7]
AChE-MWCNTs-Au-CHIT/GCE	3-3000 nM and 6-45 μ M	0.268	1.8 nM	[34]
CS/AChE/PB-CS/ERGO-AuNPs- β -CD/GCE	24-6000 pM	0.106	12.4 pM	[35]
AChE/CNT-NH ₂ /GCE	0.2-1 and 1-30 nM	0.067	0.08 nM	[36]
AChE/PAn-PPy-MWCNTs/GCE	0.03-1.5 and 3-75 μ M	-----	3 nM	[38]
AChE-AuNPs-CaCO ₃ /silica sol-gel/AuE	0.1-100 nM	-----	0.1 nM	[39]
AChE/CPBA/AuNPs/RGO-CS/GCE	1.5-30 and 60-300 nM	0.16	1.5 nM	[40] [44] [45] [46] [47]
AChE/sol-gel/SPE	10-500 nM	-----	3.6 nM	
Fe ₃ O ₄ NP/c-MWNT/Au	0.1-40 nM	-----	0.1 nM	
AChE/MWCNT/CA/NPG	3-1500 nM	-----	1.5 nM	
AChE-Fe ₃ O ₄ NPs/c-MWCNTs/ITO	0.1-70 nM	-----	0.1 nM	
AChE-Cs/Pb-Cu NWs/GCE	5-1000 ppt and 500-3000 ppb (15-3000 pM and 1500-9000 nM)	0.05	1.5 ppt (4.5 pM)	This issue

Table 2. Correlation of the concentrations of pesticides content in vegetables and fruits determined directly by the proposed biosensor and LC-MS/MS.

Sample	Malathion biosensors				LC-MS/MS method			
	Measured value (I %)	Averaged value	Recovery (%)	RSD (%)	Averaged value		Recovery (%)	RSD (%)
				n=3				n=3
Malathion a		9.87 ppb	98.7	3. 4	10. 39 ppb		103. 9	1. 6
		24.33 ppb	97.3	2. 1	24. 73 ppb		98. 9	2. 3
Courgettes	8.34	N/A b		5. 1	N/Ac			
Carrots	3.24	N/A b		3. 4	N/Ac			
Lettuces	9.06	N/A b		6. 7	N/Ac			
Orange	5.17	N/A b		2. 3	N/Ac			
Added (0.2 ppb)								
Courgettes	35.24	0.227 ppb	113.5	4. 3	N/Ac			
Carrots	21.87	0.197 ppb	98.5	7. 9	N/Ac			
Lettuces	27.93	0.218 ppb	109	3. 7	N/Ac			
Orange	31.54	0.223 ppb	111.5	5. 4	N/Ac			
a. Since the limit of detection (LOD) of the proposed AChE biosensor is much lower than the LC-MS/MS method, the standard samples for LC-MS/MS were diluted before measurement with AChE biosensor.								
b. The inhibition rate is lower than 10%, so we assume that pesticides are not detected.								
c. Concentration of malathion lower than 1.4 ppb cannot be detected out by LC-MS/MS method.								

