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Sensitive Acetylcholinesterase Biosensor Based on Screen-Printed Carbon Electrode Modified with Cerium Oxide-Chitosan/Mesoporous Carbon-Chitosan for Organophosphorus Pesticide Residue Detection

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A novel electrochemical acetylcholinesterase (AChE) biosensor modified with cerium oxide-chitosan (CeO_2-CS) and ordered mesoporous carbon-chitosan (OMC-CS) using a screen-printed carbon electrode (SPCE) was fabricated to detect organophosphorus pesticides (OPs). Compared with a common electrode, SPCEs do not need to be polished, can be conveniently carried, and are suitable for on-site detection. CeO_2 has good redox properties and a larger electron transfer rate. In addition, OMC has good electrical conductivity and a larger specific surface area, and chitosan (CS) has a considerable number of hydroxyl and amine groups in polymer chains, which have strong complexation ability. Herein, CeO_2-CS and OMC-CS were modified in the SPCE layer by layer to improve the electrochemical response and increase the fixed amount of AChE. The results obtained with this biosensor revealed that its sensitivity was significantly improved. Taking into account the possible interaction between the test conditions, the experimental parameters were optimized using a quadratic orthogonal rotation combination design to achieve a high electrochemical signal. The properties of AChE/OMC-CS/CeO_2-CS/SPCE were characterized by differential pulse voltammetry and cyclic voltammetry techniques. Under optimum conditions, the fabricated acetylcholinesterase biosensor showed great reproducibility and high stability. In addition, the as-prepared AChE biosensor was successfully utilized for analysis of OPs in vegetables.

Keywords: Acetylcholinesterase biosensor; Cerium oxide-chitosan/mesoporous carbon-chitosan; Screen-printed carbon electrode; Organophosphorus pesticides residues

1. INTRODUCTION

Organophosphorus pesticides (OPs) are widely used in agricultural production because of their high efficiency; however, they cause significant risks to the environment and can contaminate food and water, resulting in risks to human health [1]. One of the most significant exposure pathways to these pesticides for humans is the daily consumption of fresh vegetables [2]. Thus, it is important to be able to detect pesticide residues in fresh vegetables. Many approaches have been successfully used to detect OPs, such as high-performance liquid chromatography (HPLC) [3-5], gas chromatography mass spectrometry (GC/MS) [6-8], and gas chromatography (GC) [9]. However, most of these methods are time-consuming and complicated to operate and are not suitable for rapid on-site analysis of vegetables.

An acetylcholinesterase (AChE) biosensor method is a rapid method to detect OPs that has been studied in recent years [10-13]. The detection principle is as follows: AChE interacts with substrates of acetylthiocholine chloride (ATCl) and produces an electro-active product, resulting in an obvious oxidation peak. When pesticides are present, the AChE can form stable complexes with them, resulting in a decrease in the production of thiocholine. Therefore, pesticide concentrations can be detected by the changes in the current signals. So far, a growing number of simple methods using an AChE biosensor for determination of pesticides have been established [14-16].

Multifarious nanometre materials have been widely introduced to improve the performance of AChE sensors, such as gold nanoparticles [17-19], Prussian blue [10, 20, 21], multiwall carbon nanotubes [22, 23], nano cerium oxide (CeO_2) [24] and ordered mesoporous carbon (OMC) [25]. Among these methods, CeO_2 and OMC have drawn growing interest in the manufacture of electrochemical sensors. CeO_2 is a kind of rare earth semiconductor material with a low price and wide application range. It has good redox properties between the two valence states of Ce^{3+} / Ce^{4+}, and an "electronic ladder" is formed on its surface after its nanocrystallization, which greatly accelerates the speed of electron transfer [26-28]. Saha et al. have used nanoporous CeO_2 to fabricate a glucose biosensor [29]. Nesakumar et al. have developed a lactic acid biosensor based upon CeO_2 and lactate dehydrogenase CeO_2 nanoparticles [30]. OMC has a large superficial area and a large pore volume, which can increase the fixed amount of biological molecules and therefore improve the sensitivity of the biosensor. In addition, OMC has good conductivity, biocompatibility and adsorption compared with other nano materials [31, 32]. Jiang et al. modified electrodes with a platinum-OMC composite to prepare glucose biosensors [33]. Zhang et al. reported an electrochemiluminescent biosensor based on OMC as the sensing material for the detection of glyphosate [34]. Chitosan (CS) has characteristics of antibacterial properties and biodegradability, and it can be used to immobilize biomolecules to maintain satisfactory bioactivity [35]. More importantly, chitosan solutions can disperse CeO_2 and OMC uniformly. Compared with common electrodes, screen-printed carbon electrodes do not need to be polished, can be conveniently carried, and are suitable for on-site detection, so, SPCEs have been used for many biosensors [36, 37].

Thus, utilizing the strong synergistic effect of a CeO2-CS and OMC-CS nanocomposite, we designed a novel AChE biosensor for OP detection based on SPCEs modified with CeO_2-CS/OMC-CS. Chlorpyrifos and methamidophos as templates were analysed with this AChE biosensor to verify its sensitivity and accuracy.

2. EXPERIMENTAL

2.1 Reagents, materials and apparatus

Acetylcholinesterase (AChE), acetylthiocholine chloride (ATCl) and chlorpyrifos were purchased from Sigma (USA). Methamidophos was obtained from Lifeholder (USA). CS was purchased from Sangon Biotech Co., Ltd. (Shanghai, China). OMC was purchased from Nanjing Jicang Nano Co., Ltd. (China). CeO_2 was supplied by Shanghai Aladdin Biological Technology Co., Ltd. (China). The phosphate buffer solutions (PBSs) with different pH values were prepared by mixing 0.01 M NaH_2PO_4 and 0.01 M Na_2HPO_4 solutions in different ratios. All other reagents were of analytical grade. Electrochemical measurements were performed using a CHI660D electrochemical workstation (Shanghai Chenhua Co., China). The commercially screen-printed carbon electrode (TE100) was purchased from Zensor R&D (Taiwan), which consists of a working electrode (carbon, diameter of 3 mm), an Ag/AgCl reference electrode and a graphite counter electrode. All ultra-purified water was prepared by water purification systems (PALL, USA, 18.2 MΩ•cm at 25°C).

2.2 Preparation of CeO_2-CS and OMC-CS

First, 4.0 mg CeO_2 was dissolved in 4 mL 0.2% CS solution, and it was dispersed for 3 h with an ultrasonic cleaning machine, producing an ivory CeO_2-CS solution. And a highly dispersed black solution of OMC-CS was obtained by using similar method.

2.3 Fabrication of the AChE biosensor

Before each experiment, the SPCE was first carefully cleaned with ultra-purified water, and then CV measurements were performed at 0–1 V in pH 7.5 PBS until a stationary current-voltage curve was achieved. The resulting SPCE was used for the following procedure. CeO_2-CS (8.0 μL) was dropped on the surface of the SPCE (CeO_2-CS/SPCE). After air drying, the electrode was modified with 8.0 μL OMC-CS (OMC-CS/CeO_2-CS/SPCE). Finally, 5.0 μL of 0.02 U AChE was immobilized onto the SPCE to obtain an AChE/OMC-CS/CeO_2-CS/SPCE and was used in the following study.

2.4 Electrochemical detection of pesticides

In the pesticide assay, a prepared AChE electrode was immersed in an ATCl solution (1.0 mM, pH 8.0), and then, using DPV technology to measure the peak current, and it was recorded as $I_{P,control}$. Next, after being gently washed with PBS (pH 8.0), the electrode was incubated for 12 minutes in a solution with a known pesticide concentration. Finally, after it was washed with PBS, it was measured in the ATCl solution again, and the peak current was recorded as $I_{P, exp}$. The inhibition rate of pesticides was calculated as follows [20]:

$$\text{Inhibition}(\%)=(I_{P, control}-I_{P, exp})/I_{P, control}\times 100\%$$

2.5 Pretreatment of real samples

Some fresh vegetables (Oilseed rape, Lettuce, Chinese cabbage and Agaricus bisporus) were purchased at the local supermarket and proven to be free of pesticide residues by gas chromatography. Then, a 2 mL chlorpyrifos solution (0 μg/L, 100 μg/L) was sprayed on 2.0 g samples of the vegetables, and they were stored at 4°C for 24 h. Then, the adsorbed chlorpyrifos was extracted by ultrasonic treatment for 5 min in a 10 mL solution containing acetone and PBS (1/9, v/v), and the filtered suspensions were examined using the fabricated AChE biosensor.

3. RESULTS AND DISCUSSION

3.1 SEM characterizations of CeO_2-CS/SPCE and OMC-CS/CeO_2-CS/SPCE

The morphology of the nanocomposites was characterized by using a scanning electron microscope (SEM). As shown in Figure 1 (A), CeO_2-CS presented a more compact and uniform structure than the only CeO_2 nanomaterials [24]. Figure 1 (B) shows that OMC-CS can form a rather regular and smooth film on the SPCE surface. In addition, compared with many nanomaterials, the combination of CeO_2-CS and OMC-CS not only promotes electron-transfer, but they can also form a favourable microenvironment to immobilize AChE [38, 39].

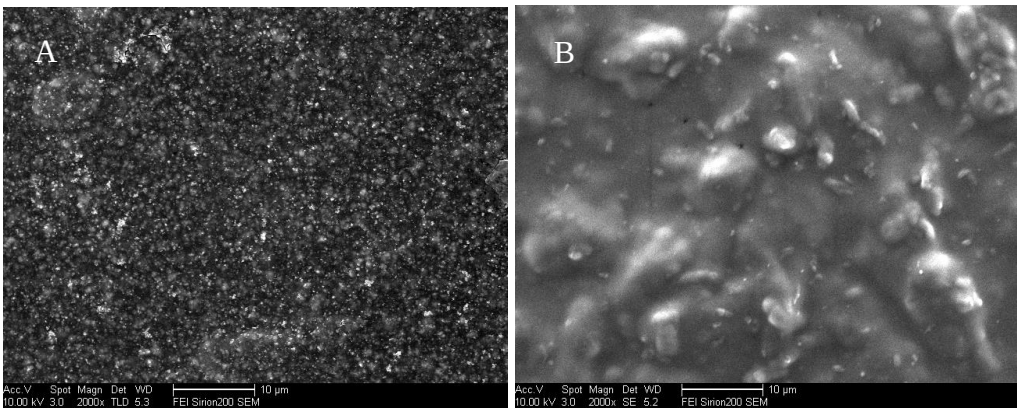


Figure 1. SEM characterizations (A) CeO_2-CS/SPCE; (B) OMC-CS/CeO_2-CS/SPCE

3.2 Current characterization of the AChE biosensor

CV measurements were carried out in $[Fe(CN)_6]^{3-/4-}$ solution, and the results of each immobilization step are recorded in Figure 2. Here, you can see that the redox peak of bare SPCE is the smallest peak (Figure 2a). After the CeO_2-CS (Figure 2b) was immobilized on the surface of SPCE, its current response increased because of its good redox properties and electron transfer rate of CeO_2-CS. When OMC-CS was added to SPCE (Figure 2c), a larger peak was obtained because of the good electroconductivity. The OMC-CS/CeO_2-CS/SPCE had good electrochemical activity with the highest redox peak current (Figure 2e) due to the synergistic effects of the CeO_2-CS and OMC-CS, which improved the electrochemical response of the biosensor. Because large protein molecules (e.g.,

enzymes, etc.) can hinder electron transfer, when 5 μL AChE added to the above electrode, the current significantly decreased (Figure 2d). The decline in its peak value also indirectly illustrated that AChE was successfully fixed.

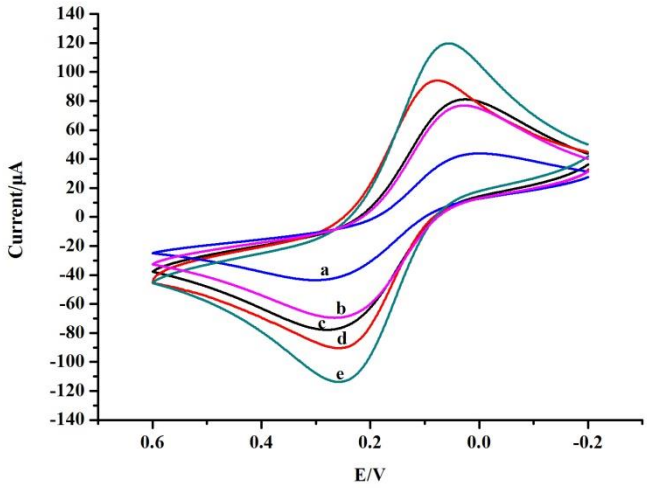


Figure 2. CVs of modified SPCEs (in 0.1 M PBS (pH 7.5) containing 5.0 mmol/L $[Fe(CN)_6]^{3-/4-}$ and 0.1 mol/L KCl): (a) bare SPCE; (b) CeO_2-CS/SPCE; (c) OMC-CS/SPCE; (d) AChE/OMC-CS/CeO_2-CS/SPCE; (e) OMC-CS/CeO_2-CS/SPCE

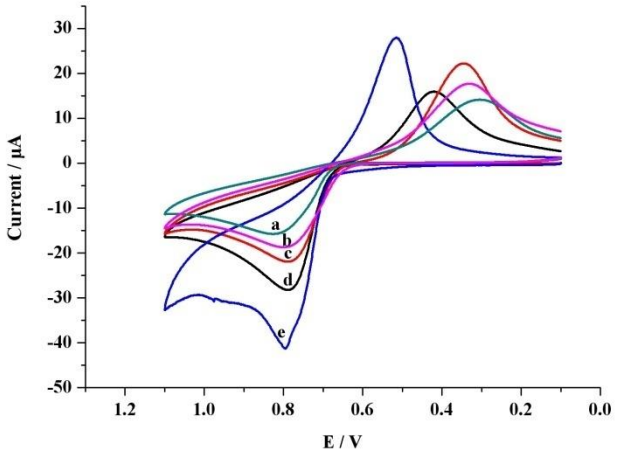


Figure 3. CVs of modified SPCEs(in pH 7.5 PBS solution containing 1.0 mM ATCl): (a) bare SPCE; (b) CeO_2-CS/SPCE; (c) OMC-CS/SPCE; (d) AChE/OMC-CS/CeO_2-CS/SPCE after inhibition with chlorpyrifos for 10 min; (e) AChE/OMC-CS/CeO_2-CS/SPCE

ATCl was used as the substrate to record the biosensor assembly process, as shown in Figure 3. In comparison with the bare SPCE (Figure 3a), the current had a small increase when the electrode surface was modified with CeO_2-CS (Figure 3b) and OMC-CS (Figure 3c), respectively. Figure 3e shows that the current was significantly enhanced after 5 μL AChE was added onto the OMC-CS/CeO_2-CS/SPCE, which is because a current was generated through the catalytic action of AChE. After AChE/OMC-CS/CeO_2-CS/SPCE was inhibited with 100 μg/L chlorpyrifos for 10 min, the CV response reduced significantly (Figure 3d) because chlorpyrifos inhibited the activity of AChE.

3.3. Optimization of the biosensor for pesticides determination

The experimental parameters were optimized through the method of quadratic orthogonal rotation combination design test with three factors and five levels. The levels of independent variables are displayed in Table 1. The arrangement of the test and response are shown in Table 2.

The optimization results are shown in Figure 4. The inhibition rate increased first and then decreased with the increase in pH, and the maximum value was at pH 8.0. The inhibition rate increased obviously as the AChE amount increased in the range from 0.06 to 0.1. However, the inhibition rate decreased slightly as the amount of AChE increased further, which may be due to the excessive thickness of the enzyme layer hindering electronic transmission. When the inhibition time was higher than 12 min, the inhibition rate decreased slightly, but it tended to be stable. Therefore, the optimum test conditions include: a pH of 8.0, an enzyme load of 0.1 U, and an inhibition time of 12 min. These values are in accordance with data reported in our previous studies [40, 41].

Table 1. Levels of independent variables in quadratic orthogonal rotation combination design

Independent variables	Levels of variation				
	-1.682	-1	0	1	1.682
X_1 = pH of the bottom liquid	7	7.4	8	8.6	9
X_2 = fixed amount of enzyme/U	0.06	0.076	0.1	0.124	0.14
X_3 = inhibition time/min	8	9.6	12	14.4	16

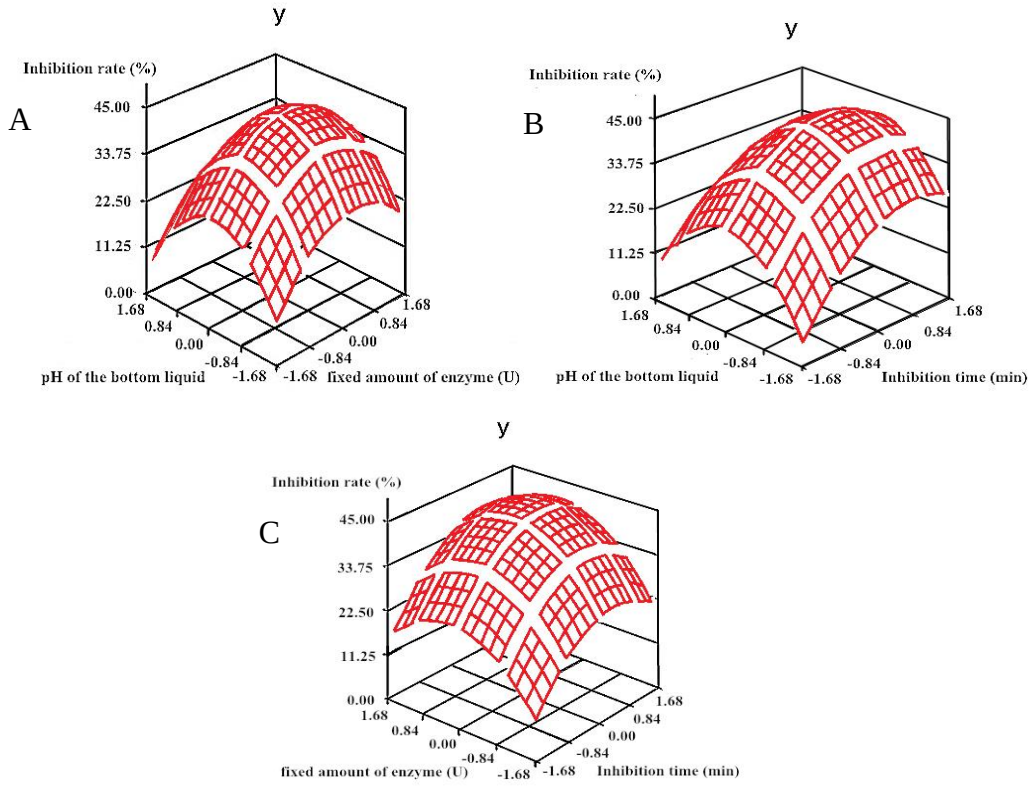


Figure 4. Response surface of the inhibition effect by test parameters (A) pH of the bottom liquid and fixed amount of enzyme; (B) fixed amount of enzyme and inhibition time; (C) pH of the bottom liquid and inhibition time

Table 2. Quadratic orthogonal rotation combination design and response

Assay	Independent variable			Response
	X_1	X_2	X_3	Inhibition rate /%
1	1	1	1	29.386
2	1	1	-1	22.692
3	1	-1	1	23.143
4	1	-1	-1	18.96
5	-1	1	1	36.406
6	-1	1	-1	25.609
7	-1	-1	1	30.372
8	-1	-1	-1	21.768
9	1.682	0	0	27.282
10	-1.682	0	0	26.998
11	0	1.682	0	38.477
12	0	-1.682	0	28.821
13	0	0	1.682	43.054
14	0	0	-1.682	27.03
15	0	0	0	40.669
16	0	0	0	39.841
17	0	0	0	44.475
18	0	0	0	43.923
19	0	0	0	42.856
20	0	0	0	41.341
21	0	0	0	44.625
22	0	0	0	44.604
23	0	0	0	42.397

3.4 Determination of pesticides

Under the optimum test conditions, we found a linear relationship between the inhibition rate and the logarithm of the target concentrations. As shown in Figure 5, the DPV peak value decreased gradually (curves a–k) as the concentration of chlorpyrifos increased. The linear equations were calculated as $y=8.1536x+30.649$($0.01-10^5$ μg/L, $R^2=0.99155$) for chlorpyrifos and $y=18.188x+9.3262$ (1−600 μg/L, $R^2=0.9937$) for methamidophos within the detection limits of 0.01 μg/L and 1 μg/L, respectively (Figure 6).

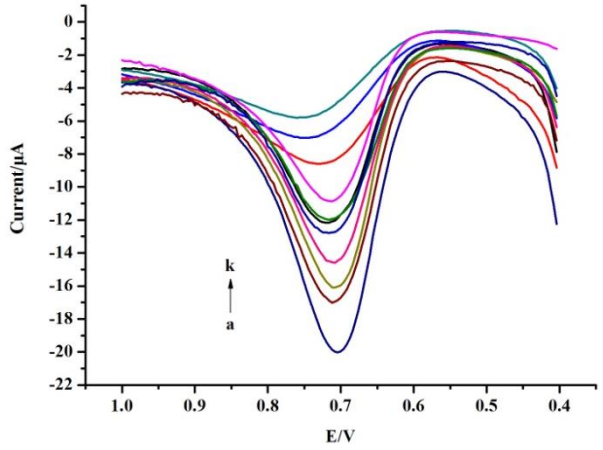
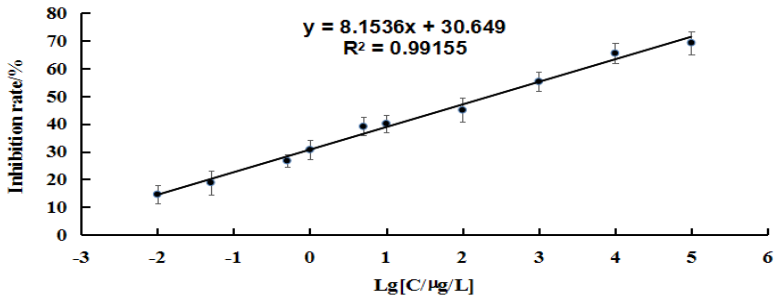
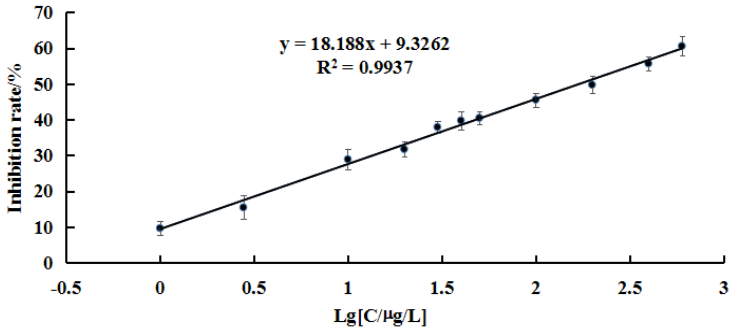


Figure 5. DPV of AChE/OMC-CS/CeO_2-CS/SPCE exposed to different concentrations of chlorpyrifos. chlorpyrifos concentration: a–k: 0 μg/L; 0.01 μg/L; 0.05 μg/L; 0.5 μg/L; 1 μg/L; 5 μg/L; 10 μg/L; 100 μg/L; 1000 μg/L; 10000 μg/L; 100000 μg/L



(A)



(B)

Figure 6. (A) Standard curve of the inhibition rate and chlorpyrifos concentrations; (B) Standard curve of the inhibition rate and methamidophos concentrations

The comparison between the AChE/OMC-CS/CeO_2-CS/SPCE biosensor with other AChE biosensors is summarized in Table 3. The results demonstrated that the OMC-CS/CeO_2-CS composite could adsorb more AChE, maintain the activity of AChE, and promote electron transfer. Therefore, the biosensor developed in this study could be used to detect pesticides with lower detection limits.

Table 3. Comparison of the fabricated biosensor with other AChE biosensors for detection of chlorpyrifos and methamidophos

Analytical methods	Linear range	Detection limit	Analyte	Reference
NA/Ag@rGO-NH_2/AChE/GCE	0.021-0.122 μg/mL	14 ng/mL	chlorpyrifos	42
NF/AChE-CS/AgNPs-CGR-NF/GCE	1.0×10^{-13}-1×10^{-8} M	5.3×10^{-14} M	chlorpyrifos	38
CLDH-AChE/GN-AuNPs/GCE	0.05-150 μg/L.	0.05 μg/L	chlorpyrifos	18
AChE/MWCNTs/DCHP/SPE	0.05-1.0×10^{5} μg/L	0.05 μg/L.	chlorpyrifos	43
AChE/OMC-CS/CeO_2-CS/SPCE	0.01-10^{5} μg/L	0.01 μg/L	chlorpyrifos	This work
AChE/AuNPs/GCE	28×10^{-3}-170×10^{-3} μM	70×10^{-3} μM	methamidophos	39
AChE/Pt-CAs/BDD	10^{-11}-10^{-6} M	3.1×10^{-13} M	methamidophos	44
AChE/OMC-CS/CeO_2-CS/SPCE	1-600 μg/L	1 μg/L	methamidophos	This work

3.5 Repeatability and storage stability

The repeatability and long-term stability were also studied. A series of six repetitive measurements of chlorpyrifos (100 μg/L) was performed on AChE biosensors prepared by the same method, and the relative standard deviation (RSD) was 4.5%, which indicated that the biosensor had good repeatability. Six individual measured results demonstrated that the response current could reach 98% and 91.2% of its initial activity after 7 and 30 days of storage in a refrigerator at 4°C, respectively.

3.6 Analysis of pesticide in real samples

For researching the practicability of the fabricated biosensor, spiked samples (Oilseed rape, Lettuce, Chinese cabbage and Agaricus bisporus) with a profenofos concentration of 100 μg/L were detected. The recoveries are summarized in Table 4, and they were from 95.0% to 102.0%. These satisfactory results showed that the biosensors proposed in this paper can directly analyse actual samples.

Table 4. Recovery of the proposed AChE/OMC-CS/CeO_2-CS/SPCE biosensor in real samples

Samples	Added (μg/L)	Found (μg/L)	RSD(%)(n=3)	Recovery (%)
Oilseed rape	0	0	0	—
	100	97	3.5	97
Lettuce	0	0	0	—
	100	95	3.6	95
Chinese cabbage	0	0	0	—
	100	102	2.5	102
Agaricus bisporus	0	0	0	—
	100	96	4.2	96

4. CONCLUSIONS

In this work, a novel biosensor (AChE/OMC-CS/CeO_2-CS/SPCE) with excellent stability, reproducibility and a short response time was successfully fabricated and shown to be sensitive to OPs. The presence of CeO_2, OMC and CS can significantly improve the detection sensitivity by capturing massive biomolecules and accelerating electron transfer because of its good conductivity and biocompatibility. The AChE biosensor had low limits of detection, a wide linear range and high sensitivity. Spiked samples were also tested, and the results agreed well with the known concentrations. Hence this is a viable strategy for the detection of OPs residues in practical applications.

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