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# Detection of Carbamate Pesticides based on MWNTs-chitosan Modified Carbon Nanotube Electrode Biosensor with Immobilized Acetylcholinesterase

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***Abstract***

A stable and highly sensitive enzyme biosensor based on MWNTs-chitosan modified glassy carbon electrode (GCE) with efficient immobilization of acetylcholinesterase (AChE) has been devised for rapid determination of carbamate pesticides especially methomyl, aldicarb, carbaryl and fenobucarb. AChE was immobilized via layer-by-layer self-assembly modification technique. The study indicated that the inhibition of carbamate pesticides on the AChE activity was proportional to concentration of carbamate pesticides in the $10^{-10}$ g·L$^{-1}$ to $10^{-3}$ g·L$^{-1}$ concentration range and the limitation of detecting methomyl is up to $10^{-12}$ g·L$^{-1}$ and others is $10^{-11}$ g·L$^{-1}$. So the enzyme biosensor may be an alternative new tool for analyzing pesticide remains in environment or food.

***Keywords: Enzyme Biosensor; AChE; LBL; CS; Carbamate Pesticides; Methomyl***

## 1 INTRODUCTION

Since the 1970s, because organochlorine pesticides have been restricted or prohibited, and insect species number of anti-organophosphorus pesticides are increasing, the use of carbamate pesticides in our country has become more and more common. Carbamate pesticides include carbaryl, carbofuran, fenobucarb, aldicarb, methomyl and so on, they not only can be used as pesticides, but also can stimulate crop growth significantly. However, most of them show great poison to animals and others show much more permanent than expected. They have brought a great harm to the environment [1]. Although environmentally degradable, they still pose a great hazard to the human health due to the high acute toxicity. Therefore, people are badly in need of a sensitive measurement of pesticide residues. At present, the carbamate pesticide residue analysis has drawn greater attention.

A great number of methods can be used for pesticide detection. For example, gas chromatography (GC) method is the most common one. But this method requires expensive instrumentation and highly trained personnel, it's time consuming, and is not easily adapted for in field analysis. Ellman-based spectrophotometry is a new method for carbamate pesticide detection, but it's time-consuming and troublesome. Electrochemical biosensor is an ideal analytical apparatus for in situ analysis due to its good selectivity, simple treatment, inexpensive instrument, and easy operation procedure. Therefore among many pesticide detection methods, the study of electrochemical biosensor has attracted increasing attention. Enzyme based electrochemical biosensors represents a promising alternative to the classical methods because of its smaller volume, higher sensitivity and stability, excellent selectivity and quick reaction. A variety of enzymes such as organophosphorous hydrolase, alkaline phsosphatase, ascorbate oxidase, tyrosinase and acid phosphatase have been employed. During pesticide analysis, amperometric AChE biosensors have shown satisfactory results for carbamate determination. AChE biosensor is made up of electrodes, electrochemical workstation and a computer. AChE was immobilized on the glassy carbon electrode

(GCE) to constitute the work electrode, and then use the work electrode to detect the actual sample. AChE will react with acetylthiocholine substrate and produce an oxidation peak which is irreversible, when it was fixed on the surface of working electrode. On the basis of the inhibition on the enzymatic activity, carbamate pesticides can be examined according to the measurement of the thiocholine oxidation current. So how to enhance the sensitivity and the stability of the work electrode is the central issue of this kind of biosensor.

Due to their nanometer-scale dimensions, nanomaterials have surface effect and quantum effect. Carbon nanotubes are a new kind of nanomaterials, besides common capacity of nanomaterials, they have high mechanical strength, and very high thermal and electrical conductivity. Since discovered in 1991[2], CNTs have attracted much attention due to their unique mechanical and electrical properties [3,4]. However, to our best knowledge, not many applications of CNTs with amperometric biosensors using acetylcholinesterase in carbamate pesticide detection have been reported. At home and abroad, the method of immobilizing enzyme for acetylcholinesterase electrode is generally cross-linking method at present. It is an immobilization method which employs bifunctional or multifunctional reagents to make enzyme molecules reticular structure with cross-linked between each other. Because chitosan [5] (CS) contains a large amout of $-NH_2$ functional groups and it is convenient for operation, non-toxic, inexpensive, higher mechanical strength and biologically compatible, it is widely used for preparing modified electrode as a modifying material. For the past few years, its advantages are that people use CS to immobilize biomoleculars. Espinosa, etc. [6] made carbon electrode as the working electrode utilizing printing technology to make a potentiometric biosensor, which was made by fixing cetylcholinesterase and cholesterol oxidase together on carbon electrode surface. Its detection limit of organophosphorus pesticides was less than 1nmol·$L^{-1}$. The entire detection process was not more than 20min. E.Suprun, etc.[7] fastened acetylcholinesterase on modified Prussian blue electrode through cross-linking of glutaraldehyde to get the enzyme electrode. Its detection limits for aldicarb, paraoxon, methyl parathion were 30, 10 and 5 ppb, respectively. Dan Du, etc.[8,9] mixed chitosan solution and glutaraldehyde solution to attach the free aldehyde to chitosan and MWNTs were added later. This mixture of liquid was applied to the pretreatment glassy carbon electrode and became AChE–CMC/GCE with dispensing method by adding AChE. To detect substrate thiocholine with the electrode, when the range of thiocholine concentration was 2.0-400 μmol·$L^{-1}$, the detection limit could reach 0.10 μmol·L-1 with a good linear relationship. And then the electrode was used for the detection of carbaryl. Its detection correlation coefficients were respectively 0.9985 and 0.9977 within the two concentration ranges of 0.01-0.5 μg·$mL^{-1}$ and 2-20 μg·$mL^{-1}$, and the detection limit was 0.004 μg·$mL^{-1}$. Zuxin Zhou, etc.[10] immobilized acetylcholinesterase with nylon mesh as carrier and then fixed the film and the glass electrode surface with an "O"-ring to get the bio-sensor to detect organophosphate and carbamate pesticides. Under the conditions of substrate concentration being $2\times10^{-3}$ mol·$L^{-1}$, 25℃, pH 7.0, the linear range was detected as $1.0\times10^{-8}$-$1.0\times10^{-4}$g·$L^{-1}$, the detection limit was $0.8\times10^{-8}$g·$L^{-1}$ and the samples recovery rate was 86.0%. As a result of functional groups of the enzyme protein such as amino, phenyl, thiol and imidazole were in the reaction of cross-linking of immobilized enzyme, the active center structure of enzyme may be affected and singularized enzyme inactivation.

In this paper, a unique method to assess the concentration of a pesticide has been developed. We adopted electrodeposit (ED) and layer-by-layer (LBL) self-assembly modification to modify the glassy carbon electrode (GCE) with multi-wall carbon nanotubes (MWNTs). The exist of the MWNTs increased the effective surface area of the electrode that laid a good foundation for the pesticides detections. Then the modified electrode was used to detect the thiocholine (TCh) and found that the detection potential was lower and the peak current was increased. The method involved immobilization of AChE on a modified electrode by chitosan (CS) and sodium alginate and this was achieved by self-assembly modification technique on the working electrode surface. The AChE was immobilized by the physical adsorption and embedding function. The electrical signal given by an enzymatic reaction was measured amperometrically to give an overall estimate of the pesticide residue. By the activity decreasing of immobilized AChE caused by pesticides, the composition of pesticide can be determined. The enzyme electrode was compared with the one that the AChE was immobilized only by the CS and the result showed that the enzyme electrode we made had good effect on the enzyme activity and the electrode life.

## 2 EXPERIMENTAL

According to our previous work practice, Oxidation of MWNTs and pretreatment of GCE were prepared. Afterwards, dipped the electrode which has been modified into sodium borate for about 15 min, its pH = 9.18, and negatively charged MWNTs surfaces during the process. After the modified electrode surface was rinsed with deionized water, then it was immersed into 0.5% concentration of CS acetic acid solution of pH = 5.0 and added 1 $mg \cdot mL^{-1}$ MWNTs into the sodium borate buffer solution for about 15 min. Then it's found that there would form $\{MWNTs/CS\}_5$ bilayer. Afterwards, the $\{MWNTs/CS\}_5$/ED/GCE was dipped into 0.5% CS acetic acid solution of pH=5.0 and AChE phosphate buffer solution of pH=7.4 for 15 min alternately. The modified electrode was carefully rinsed with deionized water after each dipping step. Thus a layer of the electrostatic self-assembly had been completed. FIG. 1 shows the relationship between the number of AChE/CS layers (n) and the current response of biosensor with $1.0\times10^{-3}$ $mol \cdot L^{-1}$ ATChCl in 0.1 $mol \cdot L^{-1}$ PBS (pH 7.40) at 0.30 V. We can conclude in FIG. 1 that when the number of {AChE/CS} layers is less than 4, the current response of biosensor will increase with the number of layers increase. When the number of {AChE/CS} layers is more than or equal to 4, the current response of biosensor will no longer increase but have a downward trend. Therefore, the number of this experiment's {AChE/CS} self-assembly layer was four.

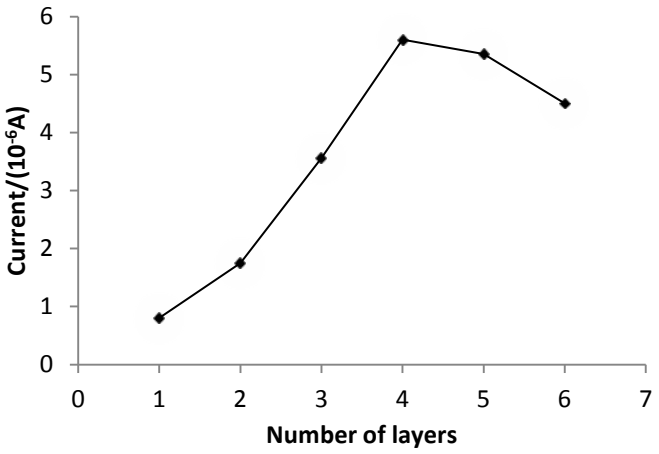


FIG. 1 THE RELATIONSHIP BETWEEN THE NUMBER OF ACHE/CS LAYERS (N) AND THE CURRENT RESPONSE OF BIOSENSOR.

A three-electrode system was used with a saturated calomel electrode as a reference electrode, a platinum wire as an auxiliary electrode, and a glassy-carbon electrode as a working electrode. Cyclic voltammetric and amperometric experiments were used to study the current response of biosensor. It was connected to a computer through an electrochemical analyzer CHI800. The amperometric curve was received at +0.3 V (versus SCE). Through adding 60 μL 0.1 $mol \cdot L^{-1}$ acetylthiocholine chloride (ATChCl) to 6 ml PBS with stirring, the oxidation current rapidly increased to a stable value. The biosensor achieved almost 95% of the stable condition current within 10 s. This was likely due to the fact that the MWNTs promoted electron transfer reaction. Four kinds of carbamate pesticides' 100ppm standards were serial diluted as concentrations ranged from $10^{-12}$ $g \cdot L^{-1}$ to $10^{-3}$ $g \cdot L^{-1}$. In order to detect the concentration of these pesticides, the prepared $\{AChE/CS\}_4$/$\{MWNTs/CS\}_5$/ED/GCE was dipped into these pesticides for about 10 min. FIG. 2 shows methomyl as an example. It is the relationship between immersing time in $1.0\times10^{-7}$ $g \cdot L^{-1}$ methomyl and the inhibition rate of the biosensor with $1.0\times10^{-3}$ $mol \cdot L^{-1}$ ATChCl in 0.1 $mol \cdot L^{-1}$ PBS of pH=7.40 at 0.30 V.

Afterwards, in order to study the current response of biosensor by amperometric curve, transformed the electrode to the electrochemical cell of $1.0\times10^{-3}$ $mol \cdot L^{-1}$ ATChCl in 0.1 $mol \cdot L^{-1}$ PBS of pH=7.40 at 0.30 V. The inhibition rate (IR) (%) was plotted against the carbamate pesticides concentrations to obtain a linear calibration graph. Pesticide inhibition was determined by the following equation: Pesticide inhibition (%)=$[(i_0-i_1)/i_0]\times100\%$, where $i_0$ is the peak ATChCl current on the prepared $\{AChE/CS\}_4$/$\{MWNTs/CS\}_5$/ED/GCE, $i_1$ is the peak ATChCl current on the prepared $\{AChE/CS\}_4$/$\{MWNTs/CS\}_5$/ED/GCE with methomyl inhibition. Recovery was determined by this enzyme biosensor using a given sample (seawater) of methomyl, aldicarb, carbaryl and fenobucarb.

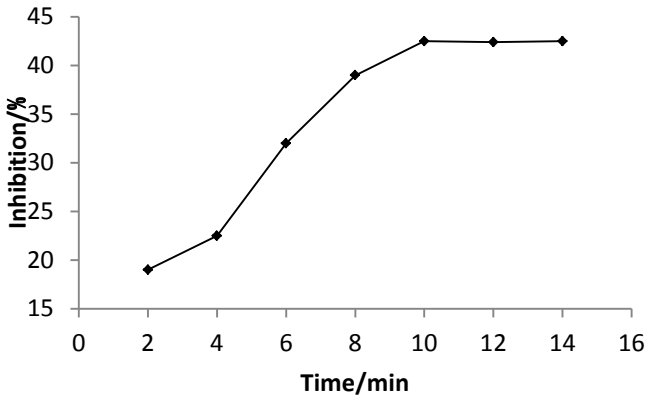


FIG. 2 THE RELATIONSHIP BETWEEN IMMERSING TIME AND THE INHIBITION OF THE BIOSENSOR.

## 3 RESULTS AND DISCUSSION

TCh is a product enzymatically generated from ATChCl. Under a certain potential, the oxidation process of TCh occurred on the GCE surface and it is determined by the following reaction formulas [11]:

$$2[H_3C]_3\text{-}N^+\text{-}CH_2\text{-}CH_2\text{-}SH \rightarrow [H_3C]_3\text{-}N^+\text{-}CH_2\text{-}CH_2\text{-}S\text{-}S\text{-}CH_2\text{-}CH_2\text{-}N^+\text{-}[CH_3]_3 + 2H^+.$$

FIG. 3 shows the TCh cyclic voltammetry curve which is used to record the current and potential, where curves A, B, and C represent the anodic oxidation peaks of TCh on GCE, ED/GCE, and {MWNTs/CS}$_5$/ED/GCE, respectively, in $5.0\times10^{-3}$mol·L$^{-1}$ TCh. In this cyclic voltammetry experiment, scanning potential range from -0.1 to 1.0 V, potential scanning speed is 100 mV·s$^{-1}$. The scanning result showed that the anodic peak current of TCh on the {MWNTs/CS}$_5$/ED/GCE ( Ipa= -61μA ) was increased greatly. Compared with the other two electrodes, its peak current was higher than that at ED/GCE ( Ipa= -57.5μA ) and exceeded twice that at GCE( Ipa= -29.5μA ).

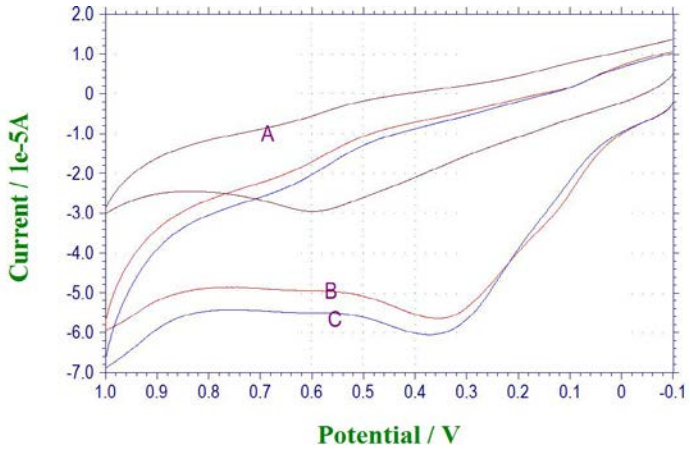


FIG. 3 THE TCH CYCLIC VOLTAMMETRY CURVE, WHERE CURVES A, B, AND C REPRESENT THE ANODIC OXIDATION PEAKS OF TCH ON GCE, ED/GCE, AND {MWNTS/CS}$_5$/ED/GCE, RESPECTIVELY, IN $5.0\times10^{-3}$MOL·L$^{-1}$ TCH.

In the meantime, the scanning result showed that the overvoltage of TCh on the GCE ( Epa=0.60V ) was higher than that at {MWNTs/CS}5/ED/GCE (Epa=0.35V). Because MWNTs which adsorbed by CS shows their better electrocatalytic activity, this can lead to low oxidation overvoltage and high anodic oxidation peak of TCh at the ED/GCE [12]. It is found that there are many amino groups (-NH2) on the surface of Chitosan. There are two processes in the whole reaction process: process of protonating and process of adsorbing. For example, at low pH acetic acid solution, amino groups (-NH2) on the surface of Chitosan are protonated and become -$NH_3^+$, that is the process of protonating. And then -$NH_3^+$ can adsorb –$COOH^-$ of MWNTs by electrostatic forces, that is the process of adsorbing. It can be determined by the following reaction formulas:

Process of protonating : $H^+ + CS\text{-}NH_2 \rightarrow CS\text{-}NH_3^+$;

Process of adsorbing: $COOH^- + CS\text{-}NH_3^+ \rightarrow CS\text{-}NH_3^+\ COOH^-$.

It is found that the Chitosan has strong adsorption ability and fast adsorption rate. It will be a good application foreground that Chitosan can adsorb the biomolecules stably. In the current study, control the pH of the enzyme solution at 7.40, which is higher than the isoelectric point of AChE (pH 4.50). Thus, the enzyme solution (pH 7.40) was present as anion [13], it can help to promote its adsorption to the CS.

FIG. 4-1 shows the relationship between inhibition rate of biosensor and concentration of pesticide. Inhibition rate can be detected from their inhibitory effect on AChE after the 10 min immersion of enzymatic electrode in different concentrations of methomyl solutions. The results indicate a good linearity between inhibition rate and concentration of methomyl in the $10^{-10}$ g·L$^{-1}$ to $10^{-3}$ g·L$^{-1}$ concentration range and the limitation of detecting methomyl is up to $10^{-12}$ g·L$^{-1}$. Meanwhile, FIG. 4-2, 4-3 and 4-4 show the datas of aldicarb, carbaryl and fenobucarb, respectively.

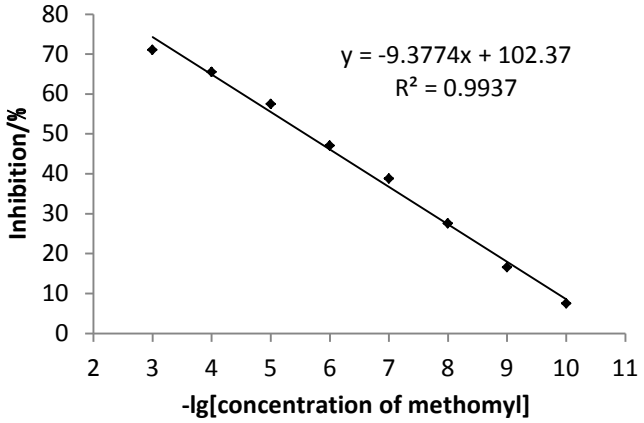


FIG. 4-1 THE STANDARD CURVE OF ACETYLCHOLINESTERASE ELECTRODE FOR THE DETERMINATION OF METHOMYL.

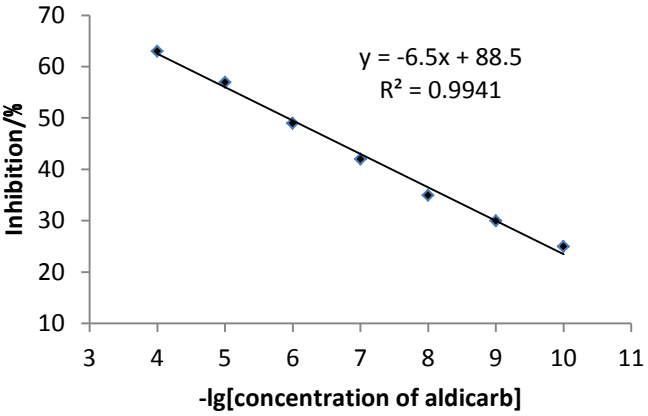


FIG. 4-2 THE STANDARD CURVE OF ACETYLCHOLINESTERASE ELECTRODE FOR THE DETERMINATION OF ALDICARB.

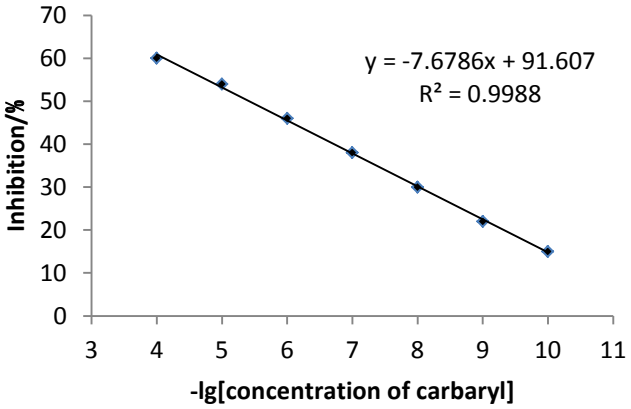


FIG. 4-3 THE STANDARD CURVE OF ACETYLCHOLINESTERASE ELECTRODE FOR THE DETERMINATION OF FENOBUCARB.

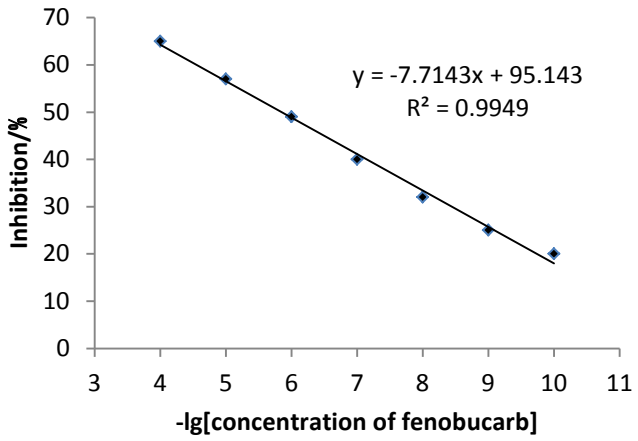


FIG. 4-4 THE STANDARD CURVE OF ACETYLCHOLINESTERASE ELECTRODE FOR THE DETERMINATION OF CARBARYL.

It can be seen in TABLE 1 that 3 times determination of recovery of methomyl is between 96.88%-152.68%. Considering the error of the electrochemical detection method itself and differences in the production of enzyme electrode, we can estimate that detection of $10^{-7}$g·L$^{-1}$ methomyl using this enzyme biosensor is accurate.

TABLE 1 RESULTS OF THE DETERMINATION OF RECOVERY OF METHOMYL

| Sample | Actual concentration (g·L$^{-1}$) | Detected concentration (g·L$^{-1}$) | Recovery (%) | Average Recovery （%） |
|---|---|---|---|---|
| 1 | $10^{-7}$ | $10^{-7.01}$ | 96.88 | 125.91 |
| 2 | $10^{-7}$ | $10^{-6.89}$ | 128.17 | |
| 3 | $10^{-7}$ | $10^{-6.82}$ | 152.68 | |

And here are the results of the other three carbamate pesticides.

TABLE 2 RESULTS OF THE DETERMINATION OF RECOVERY OF ALDICARB

| Sample | Actual concentration (g·L$^{-1}$) | Detected concentration (g·L$^{-1}$) | Recovery （%） | Average Recovery （%） |
|---|---|---|---|---|
| 1 | $10^{-6.00}$ | $10^{-5.98}$ | 104.71 | 103.19 |
| 2 | $10^{-6.00}$ | $10^{-6.01}$ | 97.72 | |
| 3 | $10^{-6.00}$ | $10^{-5.97}$ | 107.15 | |

TABLE 3 RESULTS OF THE DETERMINATION OF RECOVERY OF CARBARYL

| Sample | Actual concentration (g·L$^{-1}$) | Detected concentration (g·L$^{-1}$) | Recovery （%） | Average Recovery （%） |
|---|---|---|---|---|
| 1 | $10^{-6.00}$ | $10^{-6.01}$ | 97.72 | 99.31 |
| 2 | $10^{-6.00}$ | $10^{-6.02}$ | 95.50 | |
| 3 | $10^{-6.00}$ | $10^{-5.98}$ | 104.71 | |

TABLE 4 RESULTS OF THE DETERMINATION OF RECOVERY OF FENOBUCARB

| Sample | Actual concentration (g·L$^{-1}$) | Detected concentration (g·L$^{-1}$) | Recovery （% ） | Average Recovery （%） |
|---|---|---|---|---|
| 1 | $10^{-6.00}$ | $10^{-6.02}$ | 95.50 | 98.52 |
| 2 | $10^{-6.00}$ | $10^{-6.01}$ | 97.72 | |
| 3 | $10^{-6.00}$ | $10^{-5.99}$ | 102.33 | |

The storage stability and operational stability of the {AChE/CS}$_4$/{MWNTs/CS}$_5$/ED/GCE were examined to prove the practicability and precision of this study. From Fig 5, it can be concluded that the {AChE/CS}$_4$/{MWNTs/CS}$_5$/ED/GCE retained about 80 % of its original sensitivity even after 3 weeks when stored in 0.1 mol ·L$^{-1}$ PBS of pH=7.4 at 4 ℃ and measured every day. Through five repeated measurements, both the storage stability and operational stability of the {AChE/CS}$_4$/{MWNTs/CS}$_5$/ED/GCE were much better than the AChE/{MWNTs/CS}$_5$/ED/GCE. The results show that the stability of the electrode was very good, it wouldn't be influenced by high seawater salinity. The storage stability and operational stability should be owed to the following reasons: Chitosan contains a large amount of functional groups and it has good biocompatibility, it is widely used for preparing modified electrode as a modifying material. Furthermore, the manufacturing process is mild and has no effect on the enzyme molecules.

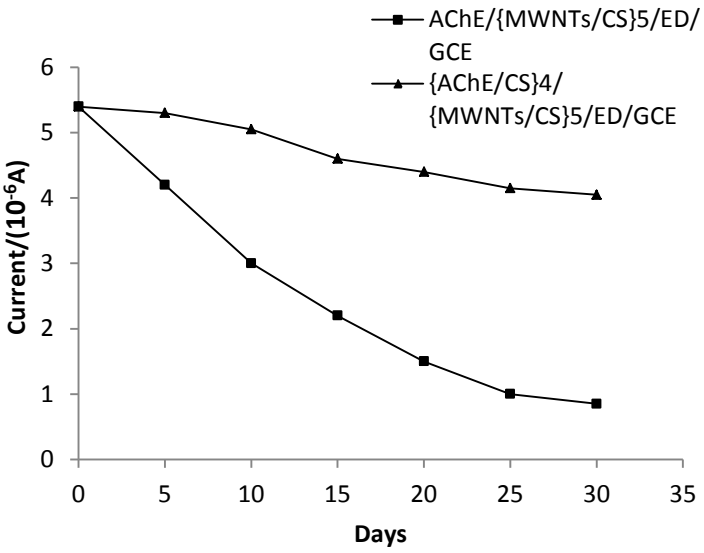


FIG. 5 THE STORAGE STABILITY CHECKED BY PERFORMING THE ELECTRODE IN 1.0×10$^{-3}$MOL ·L$^{-1}$ATCHCL EVERYDAY WITH THE BIOSENSOR BEING STORED IN 0.1 MOL ·L$^{-1}$ PBS OF PH=7.4 AT 4℃.

## 4 CONCLUSIONS

In conclusion, we gathered the good conductivity of MWNTs and the excellent biocompatibility of CS together by LBL self-assembly modification technique for the first time. The presence of MWNTs as a good conductor in the {AChE/CS}4/{MWNTs/CS}5/ED/GCE decreases the electron transfer impedance. CS can maintain the enzymatic activity of the immobilized AChE. Under the optimal conditions, the resulted biosensor exhibits many advantages, such as high sensitivity, low detection limit and good stability, etc. The proposed sensor shows good performance in seawater. We anticipate that this electro-biosensor will be useful for the development of other biosensors.

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