An Amperometric Biosensor Based on Ascorbate Oxidase Immobilized in Poly(3,4-ethylenedioxythiophene)/Multi-Walled Carbon Nanotubes Composite Films for the Determination of L-Ascorbic Acid

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An amperometric L-ascorbic acid (AA) biosensor fabricated by immobilizing ascorbate oxidase (AO) in poly(3,4-ethylenedioxythiophene) (PEDOT) and multi-walled carbon nanotubes (MWCNTs) composite films was reported for the first time. The entrapment of AO in PEDOT/MWCNTs composite films was performed during an electrochemical polymerization process. The influence of various experimental conditions was examined for determining the optimum analytical performance. The response of the biosensor towards AA under the optimized conditions is linear from 0.05 to 20 mM with a detection limit of 15 μM ($S/N = 3$). The biosensor shows a response time of 20 s and a sensitivity of 23.95 $\text{mA M}^{-1} \text{cm}^{-2}$. The apparent Michaelis-Menten constant (K_m) and apparent activation energy (E_a) are 19.5 mM and 21 kJ mol^{-1} , respectively. Moreover, the biosensor exhibits good anti-interferent ability, good reproducibility and remarkable storage stability.

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L-Ascorbic acid (vitamin C, AA), a natural water-soluble vitamin, is a potent reducing and antioxidant agent that functions in fighting bacterial infections, in detoxifying reactions, and in the formation of collagen in fibrous tissue, teeth, bones, connective tissue, skin, and capillaries. Found in citrus and other fruits, and in vegetables, AA cannot be produced or stored by humans, and must be obtained in the diet. It is thus very important to develop quick and effective methods for the quantitative detection of AA in fruits, vegetables, grain crops, agro-food products and beverages. Some methods have been available for AA detection, such as chromatographic methods^{1,2} and spectrophotometric methods.^{3,4} Nevertheless, complex sample pretreatment procedures, time-consuming manipulations and low sensitivities limit their practical applications. Electrochemical methods⁵⁻⁹ have long been used to detect AA either directly or combined with other techniques. Since development of the first amperometric enzyme-based AA biosensor, tremendous attention has been focused on improving the response performances of enzyme electrodes for biosensor research, because this class of technique is characterized by high sensitivity and selectivity, simplicity, and easy miniaturization.¹⁰

The immobilization of an enzyme on the sensing bioelement, which specifically recognizes the analyte (substrate), onto a

transducing surface, is the keystone in constructing biosensing devices. Different configurations usually exploited for the immobilization of enzyme are adsorption, covalent binding, entrapment in gels and membranes or polymer matrices, and the cross-linking of a specific enzyme.¹¹⁻¹⁶ Electropolymerization, a recent focus among immobilization strategies, is an electrochemical route to form polymeric coatings by the entrapment of biomolecules, and involves the application of an appropriate potential to a working electrode immersed in an aqueous solution containing the electropolymerizable monomer and the immobilizable biomolecule, which is homogeneously incorporated in the growing polymer. The mechanism of incorporation of biomolecules into the growing polymers, however, is not yet fully understood.¹⁷ The entrapment of enzymes in electropolymerized conducting polymeric films easily ensures proximity between the active site of the enzyme and the conducting surface.¹⁸⁻²⁰ This method provides a facile and controllable way for depositing biologically active molecules of any size and geometry to defined areas on electrodes without a chemical reaction that could affect the activity of the entrapped biomolecules.

Intrinsically conducting polymers (ICPs), in particular, are especially amenable for electrochemical biosensor development for providing biomolecule immobilization. Their advantages for biosensing applications include biocompatibility, an ability to entrap and controllably release biological molecules (reversible doping), efficient charge-transfer from a biochemical reaction

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and an ability for tuning electrical, physical, chemical and optical properties in view of a particular application.²¹ Among ICPs, poly(3,4-ethylenedioxythiophene) (PEDOT) is a very popular material by virtue of its high conductivity, good redox reversibility and stability, as well as having an environment-friendly feature.^{22,23} These excellent properties of ICPs, along with the possibility to entrap enzymes during electrochemical polymerization, have been exploited for fabricating amperometric enzyme-based biosensors. Carbon nanotubes (CNTs) are certainly one of the most popular nanosupports for biomolecule immobilization, since their peculiar structures, including high porosity and reactivity, assure a large active surface area and suitability for the production of miniaturized biosensors.²⁴⁻²⁶ Recently, ICPs incorporated with CNTs have been largely prepared for the fabrication of biosensing devices.²⁷⁻³⁰ The resulting ICPs/CNTs composites have lower charge transfer resistance and mass-transfer impedance than CNTs alone, thus yielding a nanomaterial with synergic and improved properties. It is expected that the combination of ICPs with CNTs may generate composites with new and interesting properties, providing higher sensitivity and stability of the immobilized molecules, thus constituting the basis for new and improved analytical devices for biosensing and other applications.

Previously, Wen *et al.* in our group fabricated an amperometric AA biosensor based on ascorbate oxidase (AO) immobilized in a PEDOT matrix by a one-step electropolymerization method. The proposed biosensor could detect AA successfully, and had a good linear range. However, the biosensor suffered from bad storage stability due to easy leakage of AO from the PEDOT matrix, which limits its application as biosensor materials.³¹ Inspired by preceding studies, we reasoned that multi-walled carbon nanotubes (MWCNTs) could facilitate the fabrication of an amperometric biosensor with better storage stability. The primary objective of this study was to prove this hypothesis. Subsequently, an amperometric AA biosensor with better storage stability was successfully fabricated by the one-step electrochemical immobilization of AO on a PEDOT/MWCNTs composite modified platinum (Pt) disc electrode. Besides, our work represents the first attempt to immobilize AO in PEDOT/MWCNTs composite films to develop an amperometric biosensor for the determination of AA.

Experimental

Chemicals

AO (1800 U mg⁻¹, EC 1.10.3.3) from cucumber (*Cucurbita* sp.) and MWCNTs suspension were purchased from Chengdu Institute of Organic Chemistry, Chinese Academy of Sciences (MWCNTs content, 4.39 wt%; outer diameter, 50–100 nm; length, 5–15 µm). EDOT (99%; Aldrich), sodium *N*-lauroylsarcosinate (SLS, 95%; Aldrich). Phosphate buffer solutions (PBS; 0.05 M) with various pH values were prepared with Na₂HPO₄ and NaH₂PO₄ (obtained from Sinopharm Chemical Reagent Co., Ltd.). AA was purchased from Bio Basic Inc. Lithium perchlorate trihydrate (LiClO₄; Sinopharm Chemical Reagent Co., Ltd.) of analytical reagent grade was used as received. Double-distilled deionized water was used throughout this study.

Apparatus

All electrochemical experiments were performed using a potentiostat-galvanostat (Model 263A, EG&G Princeton Applied Research), with a conventional three-electrode cell

Scheme 1 Illustration of the fabrication process and the electrochemical oxidation of AA.

system, at room temperature. A 3-mm diameter Pt disc electrode was used as the working electrode, a 1-mm diameter stainless-steel wire was the auxiliary electrode and a saturated calomel electrode (SCE) was the reference electrode. The addition of the sample was carried out with a micropipettor. The pH value was measured with a Delta 320 pH meter. The temperature was controlled with a type-HHS thermostat.

Preparation of the PEDOT/MWCNTs/AO-modified electrode

Before modification, a bare Pt disc electrode was carefully polished with a 0.05-µm alumina slurry, then thoroughly rinsed with double-distilled deionized water, followed by ultrasonically for 5 min, respectively.

The PEDOT/MWCNTs/AO-modified electrode was prepared by one-step potentiostatical polymerization in PBS containing 0.02 M EDOT, 0.075 mg mL⁻¹ AO, 1 mg mL⁻¹ MWCNTs, 0.02 M SLS, and 0.02 M LiClO₄ at a polymerization potential of 1.1 V vs. SCE for 60 s on the Pt disc electrode surface. Also, the thickness of PEDOT/MWCNTs/AO composite films was controlled by the total charge passing through the cell, which was read directly from the current-time curves by computer. The enzyme electrode was washed repeatedly with double-distilled deionized water to remove any loosely bound enzyme, the electrolyte and monomer from the electrode surface, and then stored in 0.05 M PBS (pH 6.5) in a refrigerator (4°C) when not in use. The fabrication procedure of the biosensor is schematically illustrated in Scheme 1.

Amperometric biosensor measurements

The amperometric detection of AA was carried out by applying a potential of 0.4 V vs. SCE for 100 s. The supporting electrolyte was 0.05 M air-saturated PBS (pH 6.5). Each experiment was repeated three times at three sample cells with a constant concentration of AA, respectively, and each data point represented the average of data collected three times.

Results and Discussion

The mechanism of AA determination

In the present work, the amperometry technique was used to measure AA. AO was used to catalyze the oxidation of AA to L-dehydroascorbic acid (DHA) and water with the oxygen present in solution (illustrated in Scheme 1). The biocatalytical process for the oxidation of AA can be summarized as the following reaction:

Fig. 1 Cyclic voltammograms of the PEDOT/MWCNTs/AO-modified electrode in 0.05 M PBS (pH 6.5) containing 0.02 M LiClO₄ at scan rates of 25, 50, 100, 150, 200, 250, and 300 mV s⁻¹. The inset shows a calibration plot of the peak currents vs. the square of root of scan rate.

This reaction was denoted as a double-displacement reaction mechanism,^{32,33} which is a two-substrate reaction mechanism, two compound reactants AA and O₂ resulting in products of DHA and H₂O. Substrates appear to bounce on and off the enzyme just like a ping-pong ball bouncing up and down on a table; thus, the mechanism is also called the “Ping-Pong” reaction mechanism. This is caused by a nucleophilic attack of one group onto another, followed by the release of another group to form a different compound. The double-displacement reaction does not occur sequentially; AA first binds to the free enzyme AO_{ox} (oxidized AO) and forms AA·AO_{ox}, then transforms into AO_{red} (reduced AO)·DHA, next releases the product DHA and forms a modifying enzyme, AO_{red}. The second substrate, O₂ also binds to modifying the enzyme AO_{red} to form O₂·AO_{red}, which transforms into AO_{ox}·H₂O, then releases another product H₂O and forms the free enzyme AO_{ox} again. In the AA and O₂ reaction catalyzed by AO, the concentration of O₂ was held constant, whereas AA varied in the as-obtained electrochemical biosensor. Therefore, freshly prepared sample solutions with different concentrations of AA were used prior to each measurement. Thusly, the concentration of O₂ was kept constant in air-saturated PBS in all experiments.

Cyclic voltammograms of the PEDOT/MWCNTs/AO-modified electrode

Cyclic voltammetry was used to characterize the electrochemical behavior of the PEDOT/MWCNTs/AO-modified electrode. Figure 1 depicts cyclic voltammograms of the modified electrode in 0.05 M PBS containing 0.02 M LiClO₄ with the scan rate from 25 to 300 mV s⁻¹. The PEDOT/MWCNTs/AO composite films could be cycled repeatedly without any obvious decomposition, which indicated that the composite films had good redox activity and stability. In addition, the peak currents vs. the square of root of scan rate exhibited a linear relationship, which suggested a diffusion-controlled process.

As is well known, the active center of AO is deeply embedded in a protective protein shell, which makes direct electron transfer between the enzymes and the electrodes extremely difficult. An important feature for the immobilized enzyme electrode lies in that the active immobilization matrix can improve the electron-transfer ability between the enzyme and the electrode surface. Therefore, the electrochemical performance of the immobilized enzyme electrode is largely dependent on the electrochemical properties of the active immobilization matrix on the electrode. In our previous work, we proved that the

Fig. 2 Effect of the electropolymerization time on the biosensor response in 0.5 M PBS (pH 6.5) containing 0.5 mM AA at 25°C. Applied potential, 0.4 V.

PEDOT/MWCNTs composite films showed better electrochemical activity compared with pure PEDOT films.³⁴ MWCNTs can form a network and project outward from the electrode, acting like bundled ultramicroelectrodes that permit access to the active sites of enzymes, facilitating direct electron transfer, which may improve electrochemical performance of the PEDOT/MWCNTs-based biosensor.^{35,36}

Optimization of experimental conditions

To improve the performance of the biosensor, various factors influencing the response of the biosensor, such as the film thickness, pH of the detection solution, applied potential used in the determination and temperature were investigated in detail.

Normally, the thickness of the PEDOT/MWCNTs/AO composite films defines the response of an enzyme-based biosensor. The electrodeposition time plays an important role in the thickness of the deposited PEDOT/MWCNTs/AO composite films. In this study, the composite films were electrodeposited on the electrode by the potentiostatic method. The effect of the amount of deposited films on the response current of the biosensor was investigated (Fig. 2). The response current of the PEDOT/MWCNTs/AO-modified electrode to 0.5 mM AA increased with an increase of the electrodeposition time from 30 to 60 s. However, when the electrodeposition time was more than 60 s, the response current gradually decreased, which indicated the thickness increase of the polymer, the obstacle of electron-transfer. Besides, a longer deposition time would result in a longer response time. Therefore, the deposition time of 60 s was selected in a following investigation.

The response of the AA biosensor depends on the activity of immobilized AO, which is associated with the pH of the ambient environment. The effect of the pH on the performance of the biosensor was studied in PBS with different pH values (5–8) when the biosensor was subject to 0.5 mM AA standards, as shown in Fig. 3. The current gradually increased from pH 5.0 to 6.2, and after achieving the maximum current at around pH 6.2, it decreased, indicating that the optimum activity occurred at around pH 6.2.

The effect of varying the temperature on the biosensor response was also examined between 20 and 50°C. As illustrated in Fig. 4, the current response increased progressively with an increase in temperature, reaching a maximum response current at about 40°C, and then decreased sharply. This could be caused by the denaturation of AO at higher temperatures. Although the biosensor showed a maximum response at about 40°C, room temperature (25°C) was still chosen for this work in

Fig. 3 Effect of the pH on the biosensor response in PBS containing 0.5 mM AA at 25°C. Applied potential, 0.4 V.

Fig. 4 Effect of the temperature on the biosensor response in PBS (pH 6.5) containing 0.5 mM AA. Applied potential, 0.4 V; inset, plot of the $\ln I$ vs. T^{-1} .

order to keep the stability of the biosensor and to prevent possible solution evaporation at higher temperature.

The dependence of the response current on the temperature in an initial region can be expressed as an Arrhenius relationship,^{37,38}

$$I(T) = I_0 \exp\left(\frac{-E_a}{RT}\right). \quad (2)$$

Here, I represents the steady-state current response, I_0 represents a collection of currents, R is the universal gas constant, T is the absolute temperature in Kelvin and E_a is the apparent activation energy. The activation energy for an enzymatic reaction could be calculated according to the slope of the plots of $\ln I - 1/T$ in the range of 20–35°C (inset of Fig. 4). Thus, an estimated value for E_a of 21 kJ mol⁻¹ was obtained for the AO-catalyzed reaction. This E_a value was smaller than that (43.496 kJ mol⁻¹) reported for AO immobilized in PEDOT film.³¹ This smaller E_a value means that the AO immobilized in PEDOT/MWCNTs composite films possesses higher enzymatic activity.

The effect of the applied potential on the amperometric response of the PEDOT/MWCNTs biosensor was studied, as shown in Fig. 5. The amperometric current response for AA increased as the applied potential increased from 0 to 0.4 V. The maximum response was attained at 0.4 V, and the application of a more positive potential up to 0.5 V did not improve the current response. Thus, 0.4 V was chosen as the optimal potential for the amperometric detection of AA.

Fig. 5 Effect of the applied potential on the biosensor response in PBS (pH 6.5) containing 0.5 mM AA at 25°C.

Fig. 6 Relationship between the response current and the concentration of AA in 0.05 M PBS (pH 6.5) at 25°C. Applied potential, 0.4 V. Inset (a) shows the determination of the apparent Michaelis-Menten constant; inset (b) is the calibration curve for the biosensor response vs. different concentrations of AA.

Amperometric response of the biosensor and calibration curve

Amperometric AA measurements were performed with the prepared biosensor at a fixed working potential (0.4 V) in PBS containing different AA concentrations (0 to 100 mM), represented in Fig. 6. The concentration of O₂ was kept constant in air-saturated PBS, because O₂ was a co-substrate of the AO reaction. The steady-state currents gradually increased with increasing concentration of AA, and exhibited a linear relationship with the concentration of AA in the range from 0.05 to 20 mM with a detection limit of 15 μM (estimated at $S/N = 3$) with a correlation coefficient of 0.99. The sensitivity (determined from the slope of the initial linear part of the calibration) is 23.95 mA M⁻¹ cm⁻². It was observed that the biosensor response gradually deviated from the linear feature as the AA concentration up to 20 mM. This is the characteristic of a typical Michaelis-Menten kinetics. The apparent Michaelis-Menten constant, K_m , which depicts the enzyme-substrate kinetics of the biosensor, can be calculated from the Lineweaver-Burk equation,³⁹

$$\frac{1}{I_{ss}} = \left(\frac{K_m}{I_{max}}\right) \left(\frac{1}{C}\right) + \frac{1}{I_{max}}, \quad (3)$$

where I_{ss} is the steady-state current, C the concentration of AA,

Table 1 Performances of the fabricated AA biosensor compared to the reported biosensors

Property	Present	Wen <i>et al.</i> ³¹	Chauhan <i>et al.</i> ⁴⁰	Akyilmaz <i>et al.</i> ⁴¹	Wang <i>et al.</i> ⁴²
Support for immobilization	PEDOT/MWCNTs	PEDOT	Egg shell membrane	Teflon membrane	Polystyrene
Method of immobilization	Entrapment	Entrapment	Covalent coupling	Adsorption	Adsorption
Mode of measurement	Current	Current	Current	Current	Dissolved O ₂
Response time	20 s	30 s	10 s	45 s	Within 1 min
Detection limit	15 μ M	33 μ M	10 μ M	5.0 $\times 10^{-5}$ and 1.2 $\times 10^{-3}$ M	2 – 4 μ M
Range/M	0.5 $\times 10^{-4}$ – 2 $\times 10^{-2}$	1 $\times 10^{-4}$ – 1 $\times 10^{-2}$	1 $\times 10^{-5}$ – 4 $\times 10^{-4}$	4.0 $\times 10^{-4}$ – 1 $\times 10^{-3}$	1 $\times 10^{-6}$ – 4 $\times 10^{-4}$
Storage stability	Retained 99% of its initial activity after 10 days and retained 60% after 30 days	Retained 88% of its initial activity after 6 days and retained 58% after 12 days	Maintained 50% of its initial activity after 120 days	Retained 85% of its initial activity after 60 days	Retained 60% of its initial activity after 14 days

Fig. 7 Reproducibility of the PEDOT/MWCNTs/AO-modified electrode.

Fig. 8 Storage stability of (a) PEDOT/MWCNTs/AO-modified electrode, (b) PEDOT/AO-modified electrode.

K_m the apparent Michaelis-Menten constant and I_{max} the maximum current. From the curve of I_{ss}^{-1} vs. C^{-1} , based on the experimental data from Fig. 6, the apparent Michaelis-Menten constant, K_m , was estimated to be 19.5 mM.

The analytical performance of the constructed biosensor was compared with those of other AA biosensors (Table 1). From the data presented in Table 1, it could be observed that the AA biosensor in our work was characterized by a wider linear range, lower detection limit and better storage stability compared with the pure PEDOT-based biosensor prepared with the similar method, which may benefit from the synergistic activity between PEDOT and MWCNTs.

Reproducibility and storage stability studies of AA biosensor

The operational stability of the biosensor for AA was determined with 24 repetitive experiments by using the same enzyme electrode. At the end of the 24th experiment for AA, the biosensor retained 97% of its original activity. The reproducibility of the enzyme electrode is given in Fig. 7. The satisfying result demonstrated that a small amount of enzyme was leaked out, which indicates the good reproducibility of the PEDOT/MWCNTs/AO-modified electrode.

The storage stability is an extremely important parameter to be considered for a biosensor. The storage stability of the AA biosensor was examined by intermittently measuring the current response to an AA standard solution every 3 days over a period of one month (Fig. 8a). The amperometric response of the biosensor remained at 99% of its initial activity for the first 10 days, and 65% activity was retained after one month. The good storage stability of the biosensor could be attributed to the biocompatible environment of PEDOT and the strong sorption

capability of MWCNTs. For a comparison, the storage stability of the PEDOT/AO-modified electrode was also investigated (Fig. 8b), by intermittently measuring the current response to the AA standard solution every 2 days over a period of two weeks. During the first 2 days, the current response had about 5% decreasing, and in the next 2 weeks the current response decreased sharply by about 65% of its initial activity. It can be seen that the PEDOT/MWCNTs/AO-modified electrode in our work had a better storage stability than the PEDOT/AO-modified electrode. There are two reasons for this good storage stability. First, the PEDOT/MWCNTs composite films on the electrode surface were very stable. Second, the three-dimensional network structure of the PEDOT/MWCNTs composite could provide a biocompatible microenvironment for maintaining the native activity of the enzyme, and preventing its possible leakage from the electrode surface.

Interference test

The effects of the interference of a variety of compounds (fructose, glucose, sucrose, citric acid, L-alanine, ethanol, sodium chloride, oxalic acid, inositol and nicotinic acid) in monitoring AA was also investigated. Only L-alanine decreased the sensor response by 15%, while others had no obvious effect on the amperometric response (Table 2). Thus, the biosensor exhibits good anti-interferent ability.

Conclusions

In this paper, an amperometric AA biosensor was firstly fabricated by immobilizing AO in PEDOT/MWCNTs composite

Table 2 Influence of the electroactive interferents of the AA response

Interferent	Relative activity, %	
	Interferent concentration, 0.5 mM (AA 0.5 mM)	Interferent concentration, 1 mM (AA 0.5 mM)
Fructose	100	100
Glucose	100	100
Sucrose	100	100
Citric acid	100	100
L-Alanine	85	85
Ethanol	100	100
Sodium chloride	100	100
Oxalic acid	100	100
Inositol	100	100
Nicotinic acid	100	100

films using the one-step electrochemical polymerization method. Subsequently, the optimal experimental conditions of the AA biosensor were studied in detail, such as the film thickness, pH value, detection potential and temperature. The AA biosensor could determine the concentration of AA successfully, and showed superior performance, such as wide linear range, fast response time, low detection limit and good reproducibility by combining the merits of PEDOT and MWCNTs. Moreover, the biosensor displays a good anti-interferent ability and excellent storage stability. Hence, our work proves that the PEDOT/MWCNTs composite material is a promising enzyme immobilization platform for fabricating enzyme-based biosensors. All of these advantages are also suitable for practical applications in the agro-industry.

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