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ScienceDirect
Talanta 70 (2006) 852–858
Talanta
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Amperometric choline biosensors prepared by layer-by-layer deposition of choline oxidase on the Prussian blue-modified platinum electrode

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Received 8 September 2005; received in revised form 4 February 2006; accepted 7 February 2006
Available online 23 March 2006

Abstract

An amperometric choline biosensor was developed by immobilizing choline oxidase (ChOx) in a layer-by-layer (LBL) multilayer film on a platinum (Pt) electrode modified with Prussian blue (PB). 6-*O*-Ethoxytrimethylammoniochitosan chloride (EACC) was used to prepare the ChOx LBL films. The choline biosensor was used at 0.0 V versus Ag/AgCl to detect choline and exhibited good characteristics such as relative low detection limit (5×10^{-7} M), short response time (within 10 s), high sensitivity (88.6 $\mu A\, mM^{-1}\, cm^{-2}$) and a good selectivity. The results were explained based on the ultrathin nature of the LBL films and the low operating potential that could be due to the efficient catalytic reduction of H_2O_2 by PB. In addition, the effects of pH, temperature and applied potential on the amperometric response of choline biosensor were evaluated. The apparent Michaelis–Menten constant was found to be $(0.083 \pm 0.001) \times 10^{-3}$ M. The biosensor showed excellent long-term storage stability, which originates from a strong adsorption of ChOx in the EACC multilayer film. When the present choline biosensor was applied to the analysis of phosphatidylcholine in serum samples, the measurement values agreed satisfactorily with those by a hospital method.
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Keywords: Choline oxidase; Amperometric biosensor; Layer-by-layer; Prussian blue; 6-*O*-Ethoxytrimethylammoniochitosan chloride

1. Introduction

The quantitative determination of choline in biological samples such as human bile, serum, amniotic fluid, brain extracts and pharmaceutical products is important in clinical analysis [1–4]. Thus, the development of amperometric choline biosensors utilizing choline oxidase (ChOx) is an active research area because biosensors offer simple, inexpensive, rapid and reliable means for the analysis [5–16].

ChOx has been immobilized onto the electrode surfaces by a variety of techniques, including casting and electropolymerzation, cross-linking and self-assembly techniques [6–15]. Recently, ordered protein multilayers can also be produced with a layer-by-layer (LBL) technique based on physical adsorption driven by an ionic force of attraction [17]. The LBL technique has recently been extended to a variety of materials, including synthetic polymers, nanoparticles, nucleic acids and proteins [5,16,18–25]. As far as proteins are concerned, the major advantage of the LBL technique lies on the possibility of minimizing denaturing because the adsorption process is carried out in aqueous solutions under mild conditions. The LBL film-modified enzyme sensors have been shown to exhibit a good reproducibility, stability and high sensitivity [5,16,18,19,23–25].

Most of amperometric choline biosensors have been used based on the direct oxidation of enzymatically liberated hydrogen peroxide (H_2O_2) or mediator-based determination [5–16]. The direct detection of H_2O_2 is usually accomplished by the oxidation at anodic potentials (greater than +0.6 V versus Ag/AgCl) [5,6,10–13,15–16]. However, electrochemically active interfering species which are usually present in biological fluids, such as ascorbic acid, uric acid and acetaminophen, are easily oxidized at the same potentials and produce an interfering current

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doi:10.1016/j.talanta.2006.02.012

[18,26]. This results in a difficulty in the quantitative analysis of H_2O_2 and the enzyme substrate.

This drawback can be circumvented by operating the sensor at lower potential range (from −0.2 V to 0.0 V versus Ag/AgCl) where interfering reactions cause only a minor influence on the biosensor response [7–9,12,14]. Up to now, there are only two low-potential transducers for H_2O_2 reduction: peroxidase [9,12] and metal hexacyanoferrate [7,8,14]. Prussian blue (PB) with particular characteristic of catalyzing H_2O_2 reduction at a potential range between −0.2 V and 0.0 V (versus Ag/AgCl), is defined as "artificial peroxidase" and the "range offering the most sensitive and interference-free detection" for amperometric biosensors [7,27–30]. Thus, the effects of the most common interfering species can be avoided or greatly reduced. Additionally, its long-term stability makes it more suitable than horseradish peroxidase (HRP) in assembling modified electrode [7,30]. Recently, papers have appeared on constructing oxidase enzyme-based biosensors for the detection of choline [7,8,14], glucose [24,30–32], glutamate [32–34], lactate [32,35,36], cholesterol [37,38], oxalate [39] and sucrose [40].

We report here an efficient and simple way to prepare choline biosensors using ChOx and 6-*O*-ethoxytrimethylammoniochitosan chloride (EACC) multilayer films deposited on a PB-modified platinum (Pt). According to our knowledge, LBL technique has never been used for the immobilization of ChOx on PB-modified electrode. In addition, no work has concerned the immobilization of enzymes with EACC for biosensor applications. In this paper, EACC is first used instead of chiotsan to fabricate layer-by-layer film to improve the characteristics of choline biosensor. EACC films have an excellent film-forming ability, high permeability towards water and small molecules, good adhesion, non-toxicity, high mechanical strength and good biocompatibility [41–43]. All these virtues make it a promising matrix for enzyme immobilization for the practical use of biosensors [42,43]. The experimental conditions related to the preparation and characteristics of the deposition behavior of EACC and ChOx to fabricate the choline biosensor have also been studied in details and discussed. The choline biosensor thus fabricated exhibits excellent performances, such as relative low detection limit, fast response time, high sensitivity and good stability.

2. Experimental

2.1. Reagents

Choline oxidase (ChOx, EC 1.1.3.17, from Alcaligenes species, 17 units mg^{-1}) and phospholipase D (PLD, EC 3.1.4.4, from Streptomyces species, 150 units mg^{-1}) were obtained from Sigma (USA). 6-*O*-Ethoxytrimethylammoniochitosan chloride (EACC, degree of deacetylation was >85%, MW: 80,000) was gifted from Tianjin University (China). Choline chloride was obtained from Sigma. Serum samples were obtained from the Hospital at Nankai University. All other reagents were of analytical grade and used without further purification. All aqueous solutions were prepared using doubly distilled water.

2.2. Preparation of PB film on the Pt electrode

The Pt electrode (diameter 3 mm) was polished with alumina powder, washed with double distilled water, and finally sonicated thoroughly in double distilled water before use. Then, the Pt electrodes were modified with a PB film according to the reported procedure [24]. The Pt electrode was potentiostatically deposited at a potential of +0.4 V during 60 s from an aqueous 2×10^{-3} M $K_3[Fe(CN)_6] + 2 \times 10^{-3}$ M $FeCl_3$ solution in 0.1 M KCl + 0.01 M HCl. After deposition, the modified electrodes were rinsed with double distilled water and immersed into a solution containing 0.1 M KCl + 0.01 M HCl, where the electrode potential was cycled between 0.0 V and 1.0 V at a scan rate of 50 mV s^{-1}, until a stable voltammetric response was obtained [24].

2.3. Preparation of $(EACC/ChOx)_n$ multilayer film-modified choline sensor

ChOx can be used as polyanionic material for preparing LBL film around pH 7.4, because the isoelectric point (p*I*) of ChOx lies around pH 4.5 [16]. The pH of ChOx solution has been set apart from p*I* so that the protein is sufficiently charged. The solution for enzyme immobilization thus used was consisted of 0.2 mg mL^{-1} of ChOx in 0.1 M phosphate buffer solution at pH 7.4.

The multilayer films containing EACC and ChOx were prepared on the PB-modified Pt electrode according to the reported procedure [18]. The PB-modified Pt electrode was immersed in an EACC solution (2 mg mL^{-1} in Dulbecco's buffer solution [18], pH 7.4) for 20 min to deposit the first polycationic layer. After being rinsed in phosphate buffer solution (pH 7.4) for 5 min to remove weakly adsorbed polycations, the Pt electrode was immersed in a ChOx solution for 20 min to immobilize enzyme on the polycation-modified electrode through electrostatic force. Then the Pt electrode was washed with phosphate buffer solution (pH 7.4) for 5 min to remove weakly adsorbed enzymes. Such deposition and rinsing processes were carried out repeatedly to obtain the desired number of layers. The resulting enzyme electrodes were thoroughly washed with phosphate buffer solution (pH 7.4) and stored in phosphate buffer solution (pH 7.4) below 4 °C for future use. Fig. 1 shows the chemical structure of EACC and a possible structure of the EACC/ChOx multilayer film.

2.4. Electrochemical measurements

The electrochemical instrument consisted of a Potentiostat–Galvanostat (EG&G PARC Model 283 with a software M270). The electrochemical measurements of the choline biosensor were carried out with a conventional three-electrode system. A PB/$(EACC/ChOx)_n$ film-modified electrode (3 mm diameter) was used as a working electrode, a Pt wire (1 mm diameter) as an auxiliary electrode and an Ag/AgCl as a reference electrode. All the electrochemical measurements were carried out in 20 mL of 0.1 M phosphate buffer solution with a gently stirring. Prior to the experiment, the phosphate buffer solution was deoxygenated by bubbling thoroughly with

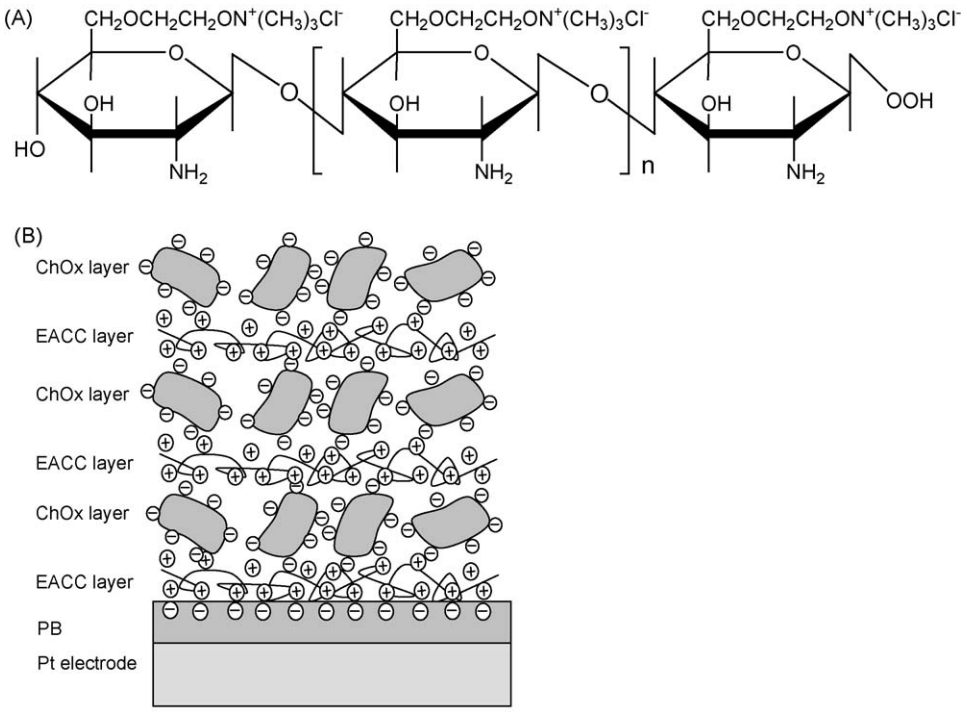

Fig. 1. (A) Chemical structure of 6-*O*-ethoxytrimethylammoniochitosan chloride. (B) Possible structure of Pt/PB/(EACC/ChOx)$_n$ LBL films.

high-purity nitrogen at least 15 min. Then a stream of nitrogen was blown gently across the surface of the phosphate buffer solution to maintain the solution anaerobic in the experiment. The background current was allowed to decay to a steady value before aliquots of stock choline solution were added.

2.5. Gravimetric measurements

A quartz crystal microbalance (QCM) (QCA 917 Model, EG & G) with an AT-cut quartz resonator (9 MHz) whose surface (effective surface area: 0.2 cm^2) is covered with a Pt thin layer was used to monitor the deposition of EACC and ChOx. The deposition process of PB/(EACC/ChOx)$_n$ is the same as above. In the QCM device used, the adsorption of 1 ng mass induces a −0.91 Hz change in the resonance frequency as discussed in the next section [5].

3. Results and discussion

3.1. QCM Study

Prior to fabricating choline biosensors, a set of QCM measurement was performed to clarify the deposition behavior of the EACC and ChOx. The relationship between the change in resonant frequency (ΔF) resulting from a change in mass (m), is given by Eq. (1) [44]:

$$\Delta F = -2F_0^2(\rho_q\mu_q)^{-1/2} m A^{-1} \quad (1)$$

where ΔF is the measured frequency shift due to the adsorbed mass in Hz, F_0 the fundamental oscillation frequency of the dry crystal (9×10^6 Hz), ρ_q the density of quartz (2.65 g cm^{-3}), μ_q the shear modulus (2.95×10^{11} dyn cm^{-2}), A the quartz area (0.2 cm^2) and m is the deposited mass. For the 9 MHz quartz crystal used in this work, Eq. (1) predicts that an adsorbed mass of 1 ng corresponds to a frequency reduction of 0.91 Hz [5].

The insets of Fig. 2 show the typical adsorption kinetics for two consecutive steps of the EACC/ChOx bilayer assembly. The inset A of Fig. 2 shows frequency changes induced by the adsorption of EACC (curve a) and that during the subsequent washing process (curve b). In curve a, one can see a time course of the frequency decrease due to the adsorption of EACC, where the adsorption of EACC required ca. 20 min to reach equilibrium. Curve b shows the desorption of the small excess parts of the adsorbed EACC by washing with phosphate buffer solution probably due to a weak binding. After washing, the final loading of EACC was ca. 154 ± 10 ng (ca. 140 ± 10 Hz). Frequency changes due to the adsorption of ChOx onto the resulting EACC-covered film are shown in the inset B of Fig. 2, where a similar adsorption and washing behavior was observed. The loading of ChOx was ca. 143 ± 10 ng (ca. 130 ± 10 Hz). The thickness of the each EACC and ChOx layers can be estimated to be 6.4 nm and 5.6 nm, respectively, assuming the density of 1.2 ± 0.1 g cm^{-3} for EACC layer and 1.3 ± 0.1 g cm^{-3} for ChOx layer according to the reported procedure [45]. These results suggest that EACC and ChOx can be assembled successfully into a thin film (ca. 12 nm) by the layer-by-layer deposition technique. Furthermore, EACC is easily soluble in both acidic and basic solutions and is a better candidate for a polycationic material in fabricating biosensors. In fact, the results shows that the assemble effect of water-soluble EACC (6.4 nm) is better than chitosan (4.9 nm) [19]. The reason is that EACC is a stronger polycationic than chitosan, because it has the cationic group of

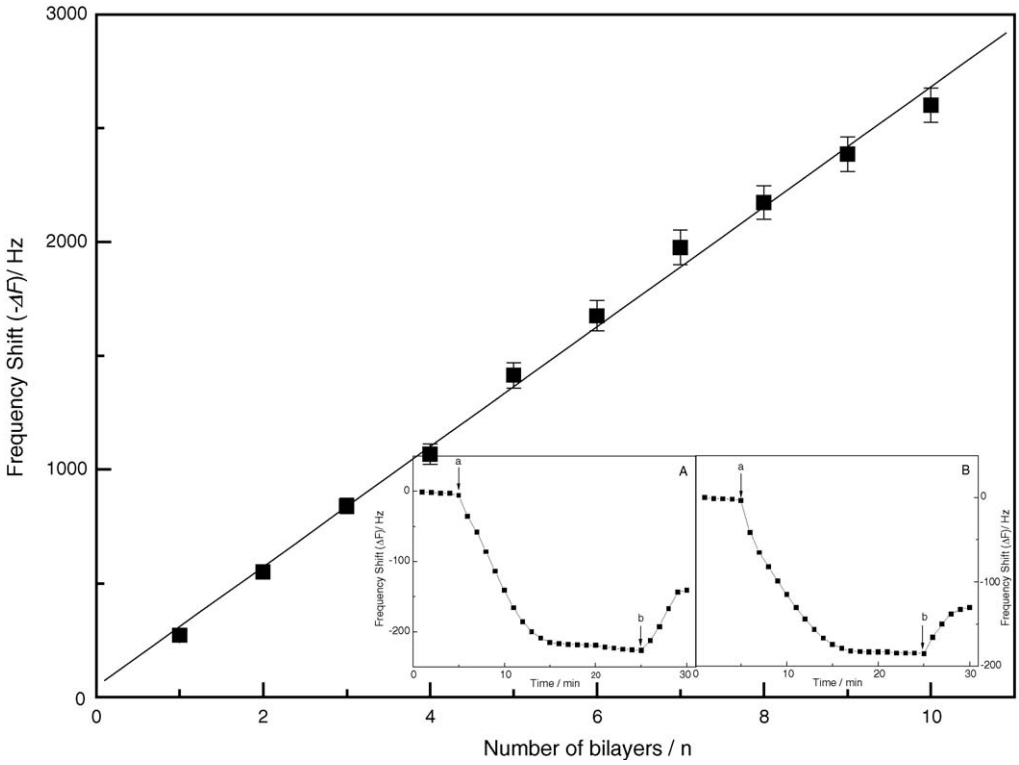

Fig. 2. Relationship between the frequency change and the number of EACC/ChOx bilayers deposited on the quartz resonator. The error bars show the deviations within five measurements. The inserts display the frequency changes of QCM induced by the deposition of EACC (A) and ChOx (B). The quartz resonator was immersed in EACC or ChOx solution at (a) and in phosphate buffer solution at (b).

quaternary ammonium salt in its "*O*-"site and improves protonation of the amino groups. Therefore EACC is easier to be protonated and stronger base than chitosan, and provides the better properties for fabricating multilayer film with negative enzyme through electrostatic force than chitosan itself.

The multilayer films containing EACC and ChOx were prepared on the quartz resonator to monitor the deposition behavior. Fig. 2 shows the frequency shifts of the resonator during the deposition of the EACC/ChOx bilayer. The resonance frequency changed linearly, confirming a linear growth of the EACC/ChOx multilayer film as a function of the number of bilayers. This results mean that the EACC/ChOx multilayer film is successfully formed through electrostatic force of attraction between the protonated EACC and negatively charged ChOx (p*I*, 4.5) [16] and that, in each deposition step, the amount of enzyme immobilized is approximately the same.

3.2. Effect of the number of bilayers on the sensitivity of choline sensors

Electrochemical measurements were carried out to evaluate the effects of the number of EACC/ChOx bilayer on the sensitivity of the choline biosensor. The wide concentration range (5×10^{-7} M to 1×10^{-4} M) of choline solutions were used in the present assays. Table 1 shows the sensitivity for choline increases almost linearly with the number of EACC/ChOx bilayers stepwise from 14.7 μA mM^{-1} cm^{-2} for 2 bilayers to 88.6 μA mM^{-1} cm^{-2} for 10 bilayers. The sensitivity of the biosensors obtained from the slope of the initial linear part of the calibration, with different limits of detection respectively (three times the noise, see Table 1). Such an increase in the sensitivity is attributed to the fact that the larger amount of enzyme was immobilized in the multilayer films when the number of bilayers is increased. These results are promising because the sensitivity could be controlled by changing the number of bilayers. Moreover, the LBL method allows fine control of thickness of the enzyme layer to avoid mass transport problems. As already discussed, the fact that the sensitivity increased well proportionally with the increased EACC/ChOx bilayer means the choline molecular can pass smoothly through the thin EACC/ChOx films and the enzymatic reaction is mainly preceded in diffusion control process. The sensitivity further increased when the number of bilayers was more than 10. However, the response time

Table 1
Effect of the number of bilayers on the sensitivity and limit of detection (LOD) of choline

The number of EACC/ChOx bilayers	Sensitivity (μA mM^{-1} cm^{-2})	LOD (M)
2	14.7	1×10^{-4}
3	24.3	1×10^{-4}
4	31.7	5×10^{-5}
5	40.9	5×10^{-5}
6	49.3	5×10^{-5}
7	58.4	5×10^{-6}
8	69.4	5×10^{-6}
9	80.2	5×10^{-7}
10	88.6	5×10^{-7}

became slower for the thicker films probably due to the diffusion problem. Based on the above results, we chose 10 bilayers of PB/(EACC/ChOx)$_{10}$ film-modified electrodes to construct choline biosensors.

3.3. Optimization of experimental parameters

The effects of pH, temperature and applied potential on the behavior of the PB/(EACC/ChOx)$_{10}$ multilayer film-modified electrode have been investigated.

The effect of the pH value of the sample solution on the current response of the PB/(EACC/ChOx)$_{10}$ film modified electrodes to 5×10^{-5} M choline was studied at 0.0 V over the pH range 6.0–10.0 in a 0.1 M phosphate buffer solution (data not shown). The currents increased from pH 6.0 to 7.4 and then decreased over pH 7.4. This decrease might be due to the poor enzyme activity. Hence, the optimum pH for the choline biosensor to obtain the maximum response was determined to be 7.4. The result was similar to the literatures data with a maximum of the response current at around pH 7.4 reported at PB modified electrodes [8,14]. The result indicated that the present choline biosensor has not altered the optimum pH of ChOx (pH 7.0–8.0) [46].

The effect of temperature on the current response of PB/(EACC/ChOx)$_{10}$ film modified electrodes was also studied in 5.0×10^{-5} M choline solutions (pH 7.4) at 0.0 V (data not shown). The current response increased with the increasing temperature and reached the maximum response at ca. 30 °C. Then the current began to decrease as the temperature increased over 30 °C. The current decreased above 30 °C may be due to thermal inactivation of ChOx. Thus, the optimum temperature was ca. 30 °C for the choline biosensor.

The effect of the applied potential on the current response of sensor was also studied. The current response increased from +0.2 V and reached the maximum at 0.0 V (versus Ag/AgCl). Application of the more negative potential did not improve the current response (data not shown). Thus, the choline biosensor was polarized at 0.0 V (versus Ag/AgCl). Based on the results, we used the choline sensors at 0.0 V (versus Ag/AgCl) in this study.

3.4. Effect of interfering materials

The selectivity of the biosensor for choline detection was evaluated in the presence of possible interfering compounds such as ascorbic acid, uric acid and acetaminophen. The effect was tested by adding these substances to the choline solution. As shown in Fig. 3, the electrode was first kept in a stirred phosphate buffer solution (0.1 M, pH 7.4) at 0.0 V and a 0.05 mM choline solution was added. Then, 0.1 mM of ascorbic acid, uric acid and acetaminophen were added sequentially, which were indicated by arrows in Fig. 3. Little changes in current due to the interferences were observed. Following the addition of the interferences, further choline (0.05 mM) was added into the solution. The results confirmed the high selectivity of the choline biosensor, which may be attributed to the low potential (0.0 V versus Ag/AgCl).

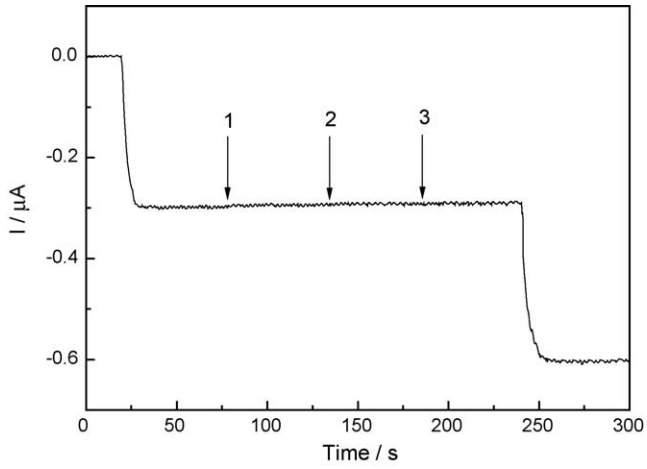

Fig. 3. Effect from the possible interferents in choline sensors. Amperometric response was obtained with 10 bilayers Pt/PB/(EACC/ChOx) biosensor in a 0.1 M phosphate buffer solution (pH 7.4) at 0.0 V vs. Ag/AgCl. The arrows show the each moment at the injection of the interfering compounds of ascorbic acid (1), uric acid (2), acetaminophen and (3) (each injection was controlled to 0.1 mM of interfering compounds). The current plateaus correspond to the injection of choline (the attained concentration is 0.05 mM).

3.5. The properties of choline biosensors

Fig. 4 shows the typical current–time plots for a choline biosensor built with PB/(EACC/ChOx)$_{10}$ multilayer film-modified Pt electrode upon the addition of successive choline solution in a 0.1 M phosphate buffer solution (pH 7.4) at 0.0 V. Each addition corresponds to a 0.01 mM solution increase of choline concentration. The response time achieved in the 95% value of the steady states current is short (approximately 10 s), probably because the film is ultrathin and the diffusion barrier at the electrode is very low. It is similar to that of choline biosensor prepared with [poly(ethyleneimine)/ChOx]$_{10}$ film (10 s), and shorter than that of choline biosensor prepared with

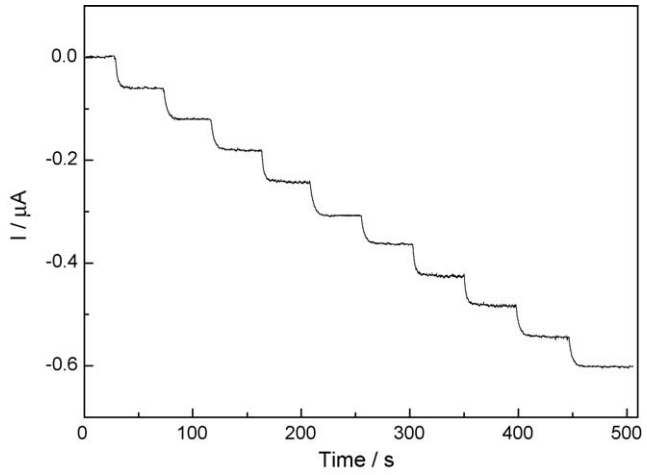

Fig. 4. Amperometric responses obtained upon the successive addition of choline solution at the applied potential 0.0 V in phosphate buffer solution at pH 7.4 with a 10-bilayer LBL film of (EACC/ChOx) deposited on to Pt/PB. Each current step corresponds to an increase of 0.01 mM in choline concentration.

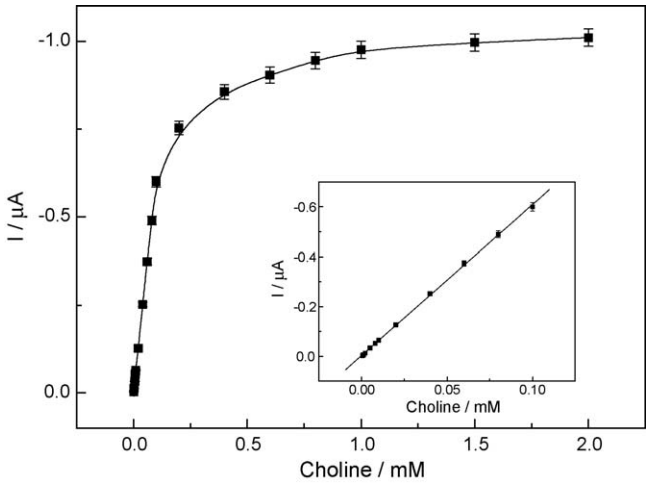

Fig. 5. Calibration curve of the response current of the sensor to choline concentration in phosphate buffer solution at pH 7.4 at the applied potential 0.0 V vs. Ag/AgCl. The error bars show the deviations within five measurements.

[poly(diallyldimethylammomium choride)/ChOx]$_8$ film (15 s) [16].

A typical calibration curve of the choline biosensor was obtained toward a function of choline concentration at the applied potential of 0.0 V as shown in Fig. 5. According to the Michaelis–Menten equation, the apparent Michaelis–Menten constant K_m is calculated to $(0.083 \pm 0.001) \times 10^{-3}$ M from Fig. 5. As shown in the insert of Fig. 5, the PB/(EACC/ChOx)$_{10}$ film-coated biosensor indicated a linear calibration graph in the concentration range of 5×10^{-7} M to 1.0×10^{-4} M, with a linear regression equation for I (μA) = $(-0.00316 \pm 0.00177) + (-6.06002 \pm 0.04116) \times c$ (mM) and relative standard deviation $R = -0.9977$. The detection limit of choline biosensor was 5×10^{-7} M, based on three times measurements with the standard deviation of the blank (95% confidence level, $k = 3$, $n = 5$). This value is same to that of PB-modified enzyme electrode [8] and lower than some of other PB-modified ChOx biosensors [7,14] (see Table 2). The sensitivity of choline biosensor obtained from the slope of the initial linear part of the calibration is 88.6 μA mM^{-1} cm^{-2}. This value is also similar to the reported PB-modified enzyme electrode [8] and larger than some of other PB-modified ChOx biosensors [7,14] (see Table 2). These results show that this kind of choline biosensor based on ChOx immobilized in LBL film at PB-modified electrode is actually valuable and sensitive.

Table 2
Prussian blue-based choline biosensors - sensitivity and limit of detection (LOD) in comparison with the literatures

Electrode	Sensitivity (μA mM^{-1} cm^{-2})	LOD (M)	Reference
LBL Pt/PB/(EACC/ChOx)$_{10}$	88.6	5×10^{-7}	This paper
Carbon paste electrode	3.5	2×10^{-5}	[7]
Screen printed electrode	110	5×10^{-7}	[8]
Pt electrode	67	5×10^{-5}	[14]

Table 3
Analysis of choline in serum samples[a]

Sample number	Determined by spectrophotometric method (1.0×10^{-4} M)	Determined by choline biosensor (1.0×10^{-4} M)	Recovery (%)
1	0.248 ± 0.005	0.259 ± 0.006	104.4 ± 1.2
2	0.418 ± 0.006	0.442 ± 0.011	105.7 ± 1.8
3	0.674 ± 0.008	0.632 ± 0.028	93.8 ± 3.5
4	0.846 ± 0.010	0.817 ± 0.024	96.6 ± 2.4
5	1.032 ± 0.007	1.096 ± 0.043	106.2 ± 6.1

[a] The values are averages of five measurements by the choline biosensor and spectrophotometric method.

We checked a long-term stability of the choline sensor by measuring the current response to 0.05 mM choline once in a day and stored in 0.1 M phosphate buffer solution (pH 7.4) at 4 °C. The choline biosensor retained 85.7% of its initial current response to choline after ca. 2 month. Thereafter, the response deteriorated gradually. The good stability implies that the existence of EACC film is actually helpful for improving the stability of the enzyme electrode. Good stability may be also attributed to the enzyme entrapped strongly in the good biocompatible EACC film which is stable in the detection solutions.

3.6. Analysis of serum samples

Phosphatidylcholine makes up a considerable proportion of the lipid such as brain lipids and the determination of serum phosphatidylcholine is important for the clinical diagnosis of liver diseases. The phosphatidylcholine can be hydrolyzed by phospholipase D (PLD) to phosphatidic acid and free choline, which can become the substrate for ChOx oxidation [47]. In the present work, for the determination of phosphatidylcholine in a serum sample, 1 mL of the sample was first incubated with a stock PLD solution (100 μL, 3 mg mL^{-1}) at 37 °C for 20 min to ensure the complete hydrolysis of phosphatidylcholine and then diluted with phosphate buffer solution (pH 7.4). To verify the reliability of the responses of the choline biosensor, the sample was analyzed by the choline biosensor comparing with a standard hospital method (spectrophotometric measurement). The analytical values of choline by the present choline biosensor agreed satisfactorily with those by spectrophotometric method and the recoveries values were in the range of 93.8 ± 3.5–106.2 ± 6.1% (see Table 3).

4. Conclusions

The layer-by-layer (LBL) deposition technique has been exploited to produce the sensitive and stable choline biosensors. The high sensitivity and fast response time may be due not only to the efficacy in the enzyme immobilization but also to the ultrathin nature of the LBL films, in which mass transport problems are minimized. Use of PB layer is useful for electrocatalytic reduction of H_2O_2 at a low potential (0.0 V versus Ag/AgCl), which makes it possible to avoid interference arising

from ascorbic, uric acids and acetaminophen. Another merit is that the choline sensors are stable for approximately 2 months. When the present choline biosensor was applied to the analysis of phosphatidylcholine in serum samples, the obtained values agreed well with those by a hospital method.

Acknowledgements

This work was supported by the National Natural Science Foundation (No. 90209054), the Project 863 (No. 2002AA326060) and the Grant-in-Aid for the High-Tech Cooperation Project of Nankai University and Tianjin University from the Chinese Ministry of Education.

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