



Development of glucose amperometric biosensor based on a novel attractive enzyme immobilization matrix: Amino derivative of thiacalix[4]arene

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ABSTRACT

Calixarenes and their derivatives may be a promising material for enzyme immobilization owing to their particular configuration, unique molecule recognition function and aggregation properties. In this paper, p-tert-butylthiacalix[4]arene tetra-amine (TC4TA) was first used as enzyme immobilization material. This attractive material was exploited for the mild immobilization of glucose oxidase (GOD) to develop glucose amperometric biosensor. GOD was strongly adsorbed on the TC4TA modified electrode to form TC4TA/GOD composite membrane. The adsorption mechanism was driven from the covalent bond between amino-group of TC4TA and carboxyl group of GOD and molecule recognition function of TC4TA. Amperometric detection of glucose was evaluated by holding the modified electrode at 0.60 V (versus SCE) to oxidize the hydrogen peroxide generated by the enzymatic reaction. The sensor (TC4TA/GOD) showed a relative fast response (response time was about 5 s), low detection limit (20 μM , $S/N=3$), and high sensitivity (ca. $10.2 \text{ mA M}^{-1} \text{ cm}^{-2}$) with a linear range of 0.08–10 mM of glucose, as well as a good operational and storage stability. In addition, optimization of the biosensor construction, the effects of the applied potential as well as common interfering compounds on the amperometric response of the sensor were investigated and discussed herein.

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1. Introduction

Enzyme biosensors are valuable devices for chemical and biomedical analysis, biotechnology, contamination monitoring, food and agriculture product processing [1–5]. In the near future, this promising and growing technology could substitute a great number of analytical methods currently employed at laboratory and industrial levels, due to its simplicity and low cost.

In order to fabricate excellent enzyme biosensors to improve their functionality and reusability, various materials have been used for the immobilization of enzymes in the preparation of biosensors. New materials such as synthetic polymer [6–8], natural polymers [9–12], nanostructure materials [13–19] and composite matrix [20–26] with nice biocompatibility, good stability and large surface area for immobilization of enzymes are the focus of studies.

Among these immobilization materials, superamolecular compounds – cyclodextrins, a class of torpidly shaped cyclic oligosaccharide with a hydrophilic outer surface and a hydropho-

bic inner cavity, are used as immobilization materials usually [27–29]. However, calix[n]arenes, as the third host molecules, are not reported as immobilization materials. Calix[n]arenes are cyclic oligomers synthesized by condensation of a p-alkylated phenol and formaldehyde. Calixarenes have a particular configuration because of their cavity. They have the flexibility to adjust the cavity dimension and the ability to form inclusion compounds with a great variety of guests, from charged molecules such as anions, metallic cations to apolar compounds. This versatility makes the calixarene family the third major class of macrocyclic binding agents after the crown-ethers and the cyclodextrins.

In this paper, we synthesized a new kind of calixarenes' derivative of p-tert-butylthiacalix[4]arene tetra-amine (TC4TA, the structures of host molecule was shown in Fig. 1A). We investigated a simple method for fabricating a calixarene film. Then a model enzyme, glucose oxidase (GOD), was adsorbed on the TC4TA modified electrode based on the EDC/NHS coupling reaction. Characteristics and performance of both the film and the resulting biosensor were studied in detail. The enzyme biosensors constructed with composite membrane showed excellent efficiency of electron transfer between the GOD and the electrode for the electrocatalysis of glucose.

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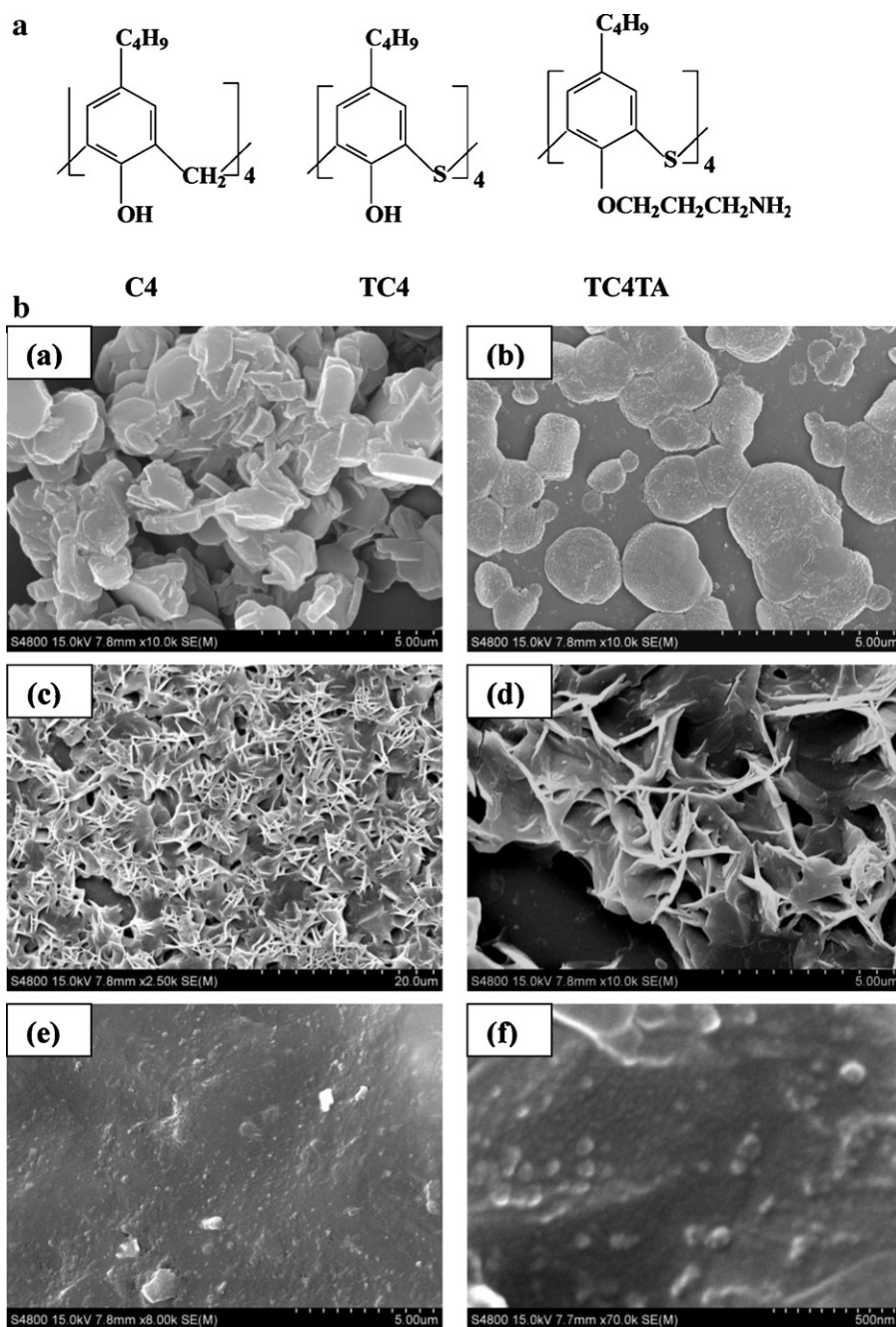


Fig. 1. (A) Chemical structure of p-tert-butylcalix[4]arene (C4), p-tert-butylthiacalix[4]arene (TC4), p-tert-butylthiacalix[4]arene tetra-amine (TC4TA). (B) SEM images of films (a) C4; (b) TC4; (c) and (d) TC4TA with different magnifications; (e) and (f) TC4TA film was immersed in GOD solution with different magnifications.

2. Experimental

2.1. Reagents

Glucose oxidase (GOD) (EC1.1.3.4, Type VII-S, 108 U mg⁻¹) from *Aspergillus niger* was purchased from Amresco. p-Tert-butylthiacalix[4]arene tetra-amine was synthesized according to the literature [30–32]. ¹H NMR (CDCl₃, 600 MHz) δ7.30 (s, 4H, ArHAr), δ7.40 (s, 4H, ArHAr), δ3.89 (t, 8H, OCH₂), δ2.41 (t, 8H, NH₂), δ1.27 (s, 36H, C(CH₃)₃), δ1.07 (s, 16H, CH₂). Elemental analysis for C₅₂H₇₆O₄S₄N₄: C, 66.2, H, 8.2, N, 5.6. N-Ethyl-N'-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide sodium salt (NHS) were purchased from Sigma–Aldrich Co. Other reagents used in this paper were of analytical grade and used as commercially available. All the chemicals

were used as received. Double distilled and sterilized water was used to prepare all solutions.

2.2. Instrumentations

¹H NMR spectrum was recorded on a Bruker AV 600-MHz instrument. Elemental analysis for TC4TA was measured by using a SERIES II2400 (PerkinElmer) elemental analyzer. The film of TC4TA/GOD was characterized by scanning electron microscope (SEM). SEM was taken by Philips XL-30ESEM operating at an accelerating voltage of 20 kV. ATR-FTIR spectra of TC4TA/GOD films on the platinum disk electrode were recorded on a Bruker TENSOR 27 FTIR spectrometer operated at a resolution of 4 cm⁻¹. Contact angle of water on the product was measured by using a Dataphysics OCA40 contact

angle analyzer. On TC4TA/GOD films of the platinum disk electrode, the contact angle can be measured by the sessile drop technique. All electrochemical experiments were carried out on a CHI660C electrochemical workstation (Chenghua, Shanghai) with a three-electrode cell. A platinum disk electrode (diameter: 1 mm) was used as the working electrode. The working electrode was polished to a mirror finish with 0.05 μm alumina aqueous slurry and rinsed with water in an ultrasonic cleaning bath before. The reference electrode was a saturated calomel electrode (SCE). All potentials reported in this paper were against SCE. A platinum wire was used as the counter electrode. All measurements were carried out in a thermostated cell at $25 \pm 0.1^\circ\text{C}$. The aqueous electrolyte solutions were prepared in 0.1 M PBS buffers in which the pH was measured as 7.0 by using a PHS-25 pH-meter (Leizi Instrumental Factory, Shanghai).

2.3. Construction of the TC4TA/GOD modified electrode

First, the working electrode (diameter: 3 mm) was polished to a mirror with 0.05 μm alumina aqueous slurry and rinsed with water in an ultrasonic cleaning bath before use. TC4TA was also dissolved in chloroform with concentration of 10 mg mL^{-1} . A defined amount of TC4TA in chloroform was spread on the surface of the platinum disk electrode. The coating was dried in air at room temperature. Second, the combination of GOD and TC4TA was based on the EDC/NHS coupling reaction [33–35]. In detail, the above TC4TA modified electrode was reacted with 2 mg mL^{-1} GOD solution containing a mixture of freshly prepared 0.02 M EDC and 0.05 M NHS of PBS solution (pH 7.0) for at least 10 h to fabricate covalently immobilized electrode. Finally, the modified electrode was rinsed with the same PBS solution and equilibrated under stirring for about 20 min in a phosphate buffer solution in order to remove unadsorbed enzyme. All electrodes were stored in PBS at 4°C .

3. Results and discussion

3.1. Characterization of the TC4TA and TC4TA/GOD films

SEM experiments were performed to characterize the structure of the films with different host molecule and the self-assembly process of enzyme on the TC4TA film. Fig. 1B(a)–(d) shows SEM images of *p*-tert-butylcalix[4]arene (C4), *p*-tert-butylthiacalix[4]arene (TC4), and TC4TA films (the structures of host molecule were shown in Fig. 1A). From Fig. 1B(a), it can be seen that the film of C4

displayed irregular crystal shape. Three-dimensional island structure of the film of TC4 was shown in Fig. 1B(b). The two kinds of films cannot form continuous membrane structure on the surface of the platinum disk electrode. But, from Fig. 1B(c), the film of TC4TA spread on the surface of the electrode and formed continuous membrane structure spontaneously. There were many folds on the self-assembly membrane (as shown in Fig. 1B(d)), which may increase the surface area of film effectively and be beneficial to adsorb much more enzyme on the surface of the electrode. When the film of TC4TA was immersed in GOD solution, enzyme was absorbed on the film of TC4TA to self-assemble TC4TA/GOD composite film (Fig. 1B(e) and (f)). This random adsorption mechanism was driven by the covalent bond between amino-group of TC4TA and carboxyl group of GOD and molecule recognition function of TC4TA through hydrophobic inner cavity. From Fig. 1B(e), the continuous membrane structure of composite film was still much clear, but the folds on the self-assembly membrane were disappeared. However, the distribution of the enzyme on the composite film was observed clearly. It was most likely that GOD was immobilized as a spherical aggregation with the diameter of 30–100 nm (Fig. 1B(f)) on the surface of TC4TA film. The significant difference in surface morphologies of the two electrodes demonstrated the presence of GOD on the TC4TA film.

Fig. 2A shows the ATR-FTIR spectra of TC4TA and TC4TA/GOD films on the platinum disk electrode. From Fig. 2A(a), the ATR-FTIR spectrum of TC4TA film, the main characteristic peaks were assigned as follows: CH_3 asymmetric and symmetric stretching vibrations appeared at 2954 cm^{-1} , 2867 cm^{-1} , respectively. The bands observed at 1574 cm^{-1} and 1441 cm^{-1} were assigned to the phenyl plane bending vibrations. The ATR-FTIR spectrum of TC4TA/GOD was shown in Fig. 2A(b). As compared with the spectrum of TC4TA, the $\text{C}=\text{O}$ stretching vibrations on the TC4TA/GOD film was clearly shown in 1643 cm^{-1} , which demonstrated that $-\text{COOH}$ of GOD could react with amino group of TC4TA through a dehydration reaction and covalent assembly on the electrode surface [32–35]. Furthermore, the appearance of new peaks in the $1200\text{--}600\text{ cm}^{-1}$ region were attributed to the characteristic peaks of GOD, which indicated that GOD was adsorbed on the surface of the TC4TA film intensively through covalent bond to construct TC4TA/GOD composite film.

The self-assembly process of enzyme on the TC4TA film can be confirmed by measurements of relative contact angle by the sessile drop method. It showed that when the water droplet dropped onto the thin chip of the TC4TA film on the platinum disk electrode

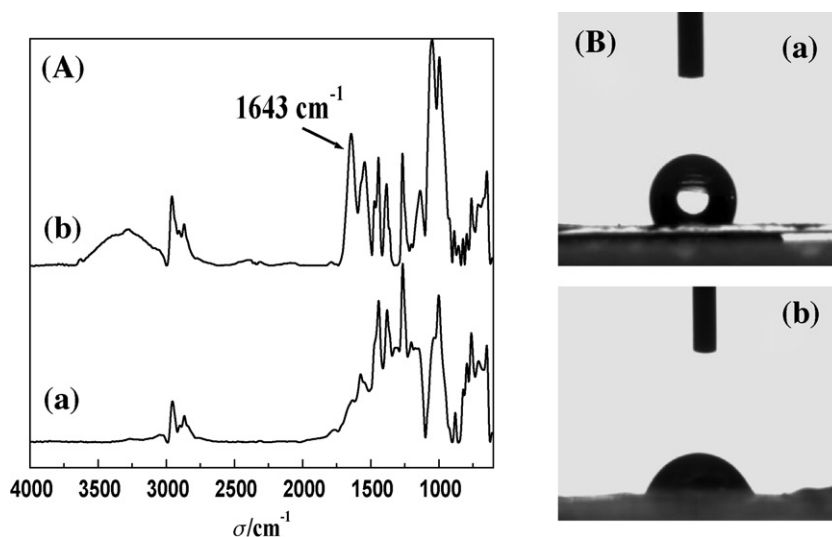


Fig. 2. (A) ATR-FTIR spectra; (B) image of water droplet behavior on (a) TC4TA modified electrode; (b) TC4TA/GOD modified electrode.

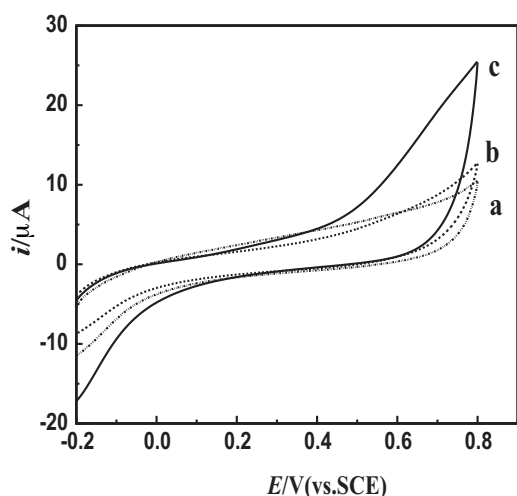


Fig. 3. Cyclic voltammograms obtained at (a) bare Pt; (b) TC4TA modified platinum electrode; (c) TC4TA/GOD modified platinum electrode in 0.1 M PBS buffer solution containing 1.0 mM glucose.

(Fig. 2B(a)), stable drop shape formed, and water cannot be imbibed in the chip. The contact angle of water on the TC4TA film was 118° , which showed that the surface of the TC4TA film was hydrophobic and very difficult to be wetted by water. Such high water contact angle indicated the surface polarity was weaker, and the surface energy was lower consequently. It was due to TC4TA molecules with predominant phenyl and tert-butyl exposed to the air–water interface. After the TC4TA film was immersed in GOD solution, the water contact angle, as shown in Fig. 2B(b), was decreased to 56° and the wettability was increased obviously. The experiment results of contact angle confirmed that enzyme was strongly adsorbed on the surface of TC4TA film, which resulted in a change of the surface of TC4TA film from hydrophobic to hydrophilic. Obviously, the driving force of adsorption of TC4TA film came from the covalent bond between carboxyl group of GOD and amino-group of TC4TA, and non-covalent bond between enzyme and host molecule, such as hydrogen bonding, hydrophobic interaction. At the same time, the control tests were carried out and C4 or TC4 was used to take the place of TC4TA. The water contact angle of C4 film was changed from 93° to 81° , and the value of contact angle of TC4 film decreased from 94° to 78° , which indicated that there was the weaker interaction between GOD and C4 or TC4. The difference of the structure of these host molecules led to the different results of contact angle experiment. Compared with C4 and TC4 (shown in Fig. 1A), amino-group in TC4TA represented important action with $-\text{COOH}$ in GOD. In other words, the adsorption of GOD on the TC4TA film was mainly driven by the covalent bond and non-covalent bond played the secondary role during the self-assembly process of the composite film.

3.2. Cyclic voltammograms of the glucose biosensor

To study the electrocatalytic effect of the TC4TA/GOD film to glucose, cyclic voltammetry was performed. Fig. 3 shows the cyclic voltammograms obtained at the TC4TA/GOD film modified electrode (curve a) in 0.1 M PBS buffer solution containing 1.0 mM glucose in comparison with a bare Pt electrode (curve a) and TC4TA film modified electrode (curve b). The electrochemical responses of all these electrodes were negligible in the solution in the absence of glucose (data not shown). As shown in Fig. 3, the oxidation currents at the bare Pt electrode and TC4TA film modified electrode were quite small, and the oxidation of substrate at the bare Pt electrode or TC4TA film modified electrode started at

~ 0.6 V, which indicated that the oxidation current came from the direct oxidation of glucose. However, the electrochemical response obtained at the TC4TA/GOD film modified electrode was much larger than that obtained at the bare Pt electrode or TC4TA film modified electrode. Moreover, it can be seen that the oxidation of substrate started at ~ 0.4 V, and the oxidation current increased as the potential increased at this modified electrodes, which implied that the GOD catalyzed glucose to produce H_2O_2 and the current was attributed to the oxidation of H_2O_2 [36]. Furthermore, it can be seen that the magnitude of the electrochemical response at these different electrodes increased in the following order: TC4TA/GOD modified electrode > TC4TA modified electrode $\approx$ bare Pt. Therefore, the TC4TA/GOD film modified electrode exhibited the highest electrocatalytic activity toward glucose and was used to detect glucose.

3.3. Optimization of experimental variables

In order to optimize the performance of the glucose biosensor based on TC4TA/GOD modified electrode, various experimental variables affecting the amperometric determination of glucose, such as the TC4TA loading, the immersion time of TC4TA modified electrode in GOD solution, pH of solution and applied potential, were investigated.

3.3.1. Influence of TC4TA loading

The effect of membrane loading of TC4TA was studied. The effect of TC4TA loading on the response characteristics of TC4TA/GOD modified electrode was examined in the presence of 1.0 mM glucose. Fig. 4(A) displays the relationship between peak current and TC4TA loading. The current increased as the TC4TA loading increased and the response currents reached the maximum when the TC4TA loading was $15 \mu\text{L}$ ($190 \mu\text{g mm}^{-2}$). However, with the TC4TA loading increased continuously, the current decreased, which implied that the overmuch TC4TA would make the resistance of the biomembrane increase and biosensor response slightly loss. Considering the response stability of the TC4TA/GOD biosensor, TC4TA loading for each test strip was set at $190 \mu\text{g mm}^{-2}$ in this study.

3.3.2. Influence of immersion time

The immersion time of the TC4TA modified electrode was researched and the experimental result was shown in Fig. 4(B). With the increasing of the immersion time of the TC4TA modified electrode in GOD solution, the current increased and the response currents reached the maximum when the immersion time was 10 h. If the immersion time was too short, the interaction between TC4TA and GOD was too weak to adsorb GOD effectively. However, with the immersion time increased continuously, the current was almost unchanged, which revealed that the enzyme immobilization was saturated, the current did not decrease after the optimal point. Considering the response stability and obtaining relative large response current of the TC4TA/GOD biosensor, the immersion time of TC4TA modified electrode in GOD solution was 10 h in this study.

3.3.3. Influence of pH

Solution pH is known to be one of the most important factors affecting the enzyme activity and stability, and hence the performance of enzyme electrodes. Effect of pH on the current of the biosensor was determined in the range of pH 5–9 in the presence of glucose (1.0 mM). Fig. 4(C) shows that the highest response current can be obtained at pH 6.8. The pH value of the highest activity for GOD was 5.5 as reported by the supplier. In this case, the optimal pH value was larger than that for GOD highest activity (ca. pH 5.5), which was attributed to that the presence of TC4TA may change the microenvironment of the enzyme and the resulting

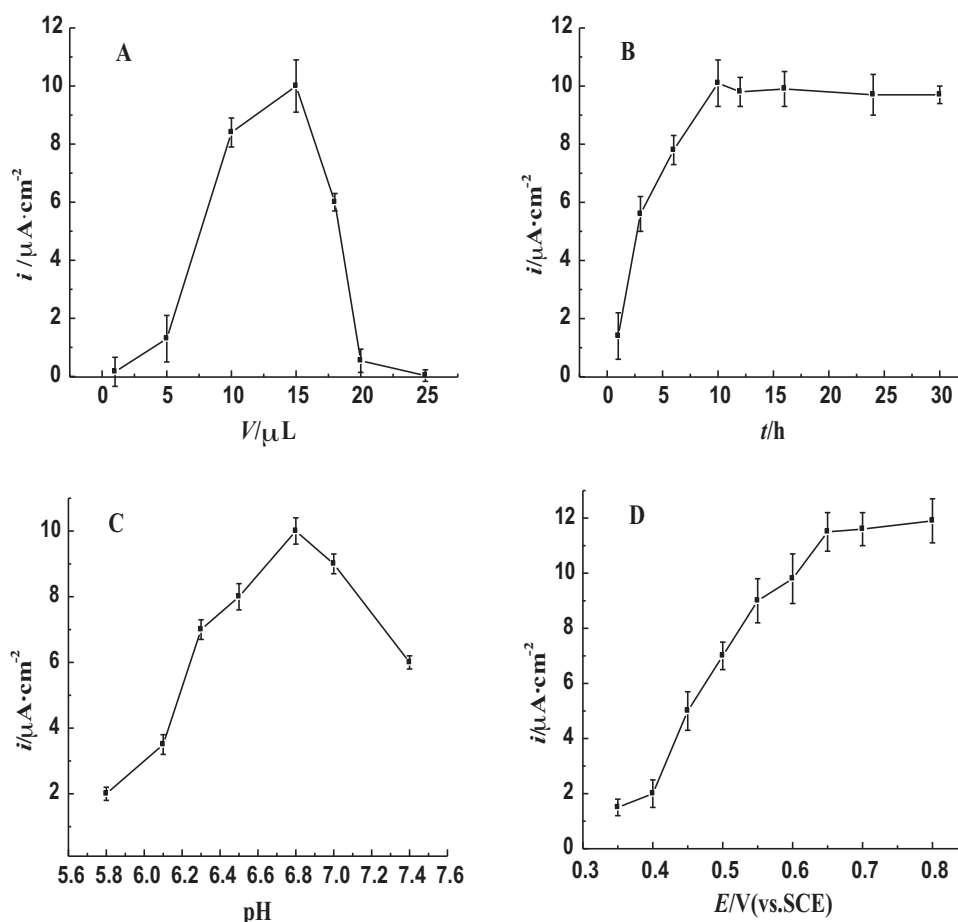


Fig. 4. (A) Influence of volume of TC4TA on the biosensor sensitivity ($E_{\text{app}} = 0.6\text{ V}$, in 0.1 M PBS with pH 7.0, at 25°C); (B) the influence of immersion time on the biosensor sensitivity ($E_{\text{app}} = 0.6\text{ V}$, in 0.1 M PBS with pH 7.0, at 25°C); (C) the influence of pH on the biosensor sensitivity ($E_{\text{app}} = 0.6\text{ V}$, in 0.1 M PBS, at 25°C); (D) the effect of operation potential on the biosensor response (in 0.1 M PBS with pH 7.0, at 25°C).

charge distribution of the enzyme surface was changed. Moreover, the physiological pH value of blood is known to be near 7. Herewith, pH 7 was selected for evaluating the performance of the glucose biosensor based on the TC4TA/GOD modified electrode.

3.3.4. Influence of applied potential

Fig. 4(D) shows the effect of the applied potential on the response current of the TC4TA/GOD modified electrode in 0.1 M PBS (pH 7.0) containing 1 mM glucose. It was noted that the response current increased with the increase of the applied potential. This meant that the response of the enzyme electrode in this potential range may be controlled by the electrochemical oxidation of hydrogen peroxide [37]. However, it was well known that the response current to the electroactive interferences also increased with the increase of the applied potential. In order to obtain relative large response current, short response time and good anti-interference ability, +0.60 V was selected as the applied potential for the amperometric determination of glucose [38].

3.4. Performance of the chronoamperometric glucose biosensors

The electrocatalytic oxidation of glucose at TC4TA/GOD modified platinum electrode was also studied by amperometry. The potential of 0.6 V (versus SCE) was chosen as the applied potential for the amperometric determination of glucose. Fig. 5(A) shows the typical response curves for the TC4TA/GOD modified platinum electrode on successive injections of glucose to the stirring PBS at pH 7.0. Fig. 5(B) shows the corresponding linear range of the

calibration curve obtained via the TC4TA/GOD-modified platinum electrode. A clearly defined oxidation current proportional to the concentration of glucose was observed. The biosensor achieved 95% of the steady-state current within 5 s, with a linear range of 8×10^{-5} to 1×10^{-2} M. This kind of biosensor had a low detection limit of 2×10^{-5} M based on $S/N = 3$ and a high sensitivity of $10.2\text{ mM}^{-1}\text{ cm}^{-2}$. The high sensitivity of the enzyme electrode can be attributed to the excellent adsorption ability, high electrocatalytic activity and good biocompatibility of the superamolecular membrane of TC4TA. The apparent Michaelis–Menten constant (K_M) can be obtained by the analysis of the slope and the intercept of the plot of the reciprocals of the steady-state current versus glucose concentration. K_M value for TC4TA/GOD-modified platinum electrode was found to be 7.4 mM.

3.5. Effects of the interferences on the response of mediated enzyme biosensors

Generally, the influence of the presence of easily oxidizable endogenous and exogenous interferences was examined at their physiological normal levels on the biosensor response to glucose as reported in literature [39,40]. However, the normal concentration of these interferences were rather low compared the content of glucose (5 mM), 0.1 mM for ascorbic acid, 0.5 mM for uric acid, 0.2 mM for dopamine, 0.05 mM for *p*-acetaminophenol, 0.02 mM for L-cysteine and 2 mM for glutathione reduced. The results were illustrated in Fig. 6. Except uric acid, the influences of ascorbic acid, dopamine, *p*-acetamidophenol, L-cysteine and glutathione reduced

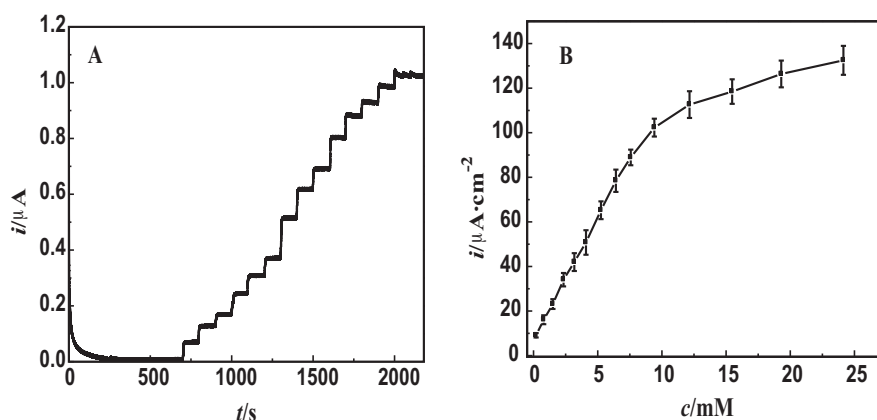


Fig. 5. (A) The *i-t* plot of TC4TA/GOD electrode response for glucose, under the optimum conditions; (B) calibration curves of TC4TA/GOD for glucose, under the optimum conditions (0.1 M phosphate buffer solution with pH 7.0, at 25 °C, E_{app} = 0.6 V).

Table 1
Glucose content determination in serum sample.

Samples	Content determined by local hospital (mM)	Content determined by current method ^a	Relative error (%)	Added (mmol L ⁻¹)	Found ^a (mmol L ⁻¹)	Recovery (%)
1	4.88	5.23	+6.7	2.00	7.11	94
2	4.26	4.13	-3.1	2.00	6.32	110
3	4.17	4.35	+4.1	2.00	6.28	97
4	5.83	5.69	-2.5	2.00	7.81	106
5	6.19	6.03	-2.7	2.00	8.13	105

^a Average of 5 times of determination.

were quite weak. This was attributed to the excellent adsorption ability and high electrocatalytic activity of the supermolecular membrane of TC4TA.

3.6. Reproducibility and stability of TC4TA/GOD biosensors

The reproducibility of biosensor was estimated by five TC4TA/GOD electrodes, which were prepared under the same condition and used to detect the current response of 1 mM glucose. The results revealed that the biosensors owned satisfying reproducibility with a relative standard deviation (RSD) of 5.32% for 50 successive measurements (each sensor was performed 50 mea-

surements). The storage stability of enzymatic biosensor in storage conditions (phosphate buffer of pH 7.0 at 4 °C) was investigated in the same phosphate buffer solution containing 1.0 mM glucose. In the storage stability test, 5 measurements of each biosensor were performed every day during the period of the 30 days. The corresponding result showed that 80% response current was still retained after 30 days.

3.7. Real sample analysis

The proposed method was applied to evaluate glucose content in serum samples. Fresh plasma samples were first analyzed in the local hospital with Olympus AU2700 biochemistry analyzer (Japan). The samples were reanalyzed with the TC4TA/GOD electrode. Before determination, the samples were diluted by buffer solution to the appropriate concentration. Under the optimum condition, the samples were tested by the TC4TA/GOD biosensors and the contents of glucose in blood can be calculated from the calibration curve. The results, which were shown in Table 1, were satisfactory and agreed closely with those measured by the biochemical analyzer done by local hospital and the recovery values ranged from 94% to 106%, which indicated that the fabricated glucose biosensor had practical potential.

4. Conclusions

The feasibility of an amperometric glucose biosensor based on immobilization of GOD in organic matrix TC4TA had been investigated. Owing to particular configuration, unique molecule recognition function and high adsorption ability, as well as its special aggregation, TC4TA was an available alternative substrate for the immobilization of enzyme molecules, inducing the covalent bond between carboxyl group of GOD and amino-group of TC4TA, and non-covalent bond between enzyme and host molecule, such as hydrogen bonding, hydrophobic interaction. The TC4TA/GOD

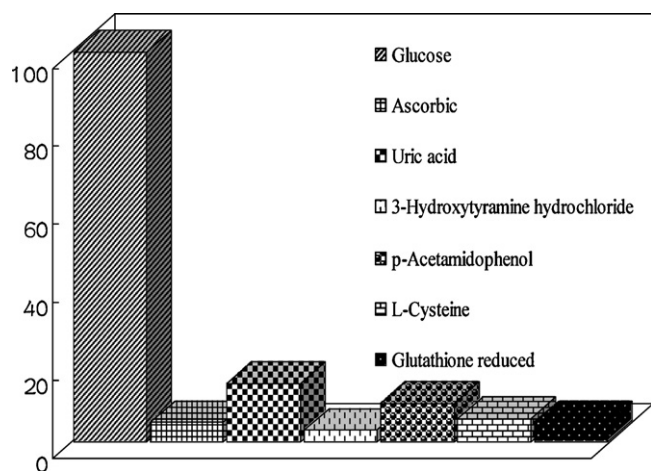


Fig. 6. The sensing selectivities of the glucose biosensor, TC4TA/GOD applied at 0.6 V vs SCE. The absolute responses were defined as the percentage of the reaction current of ascorbic acid, uric acid, 3-hydroxytyramine hydrochloride, *p*-acetamidophenol, L-cysteine, glutathione reduced and comparing to that of glucose in 0.1 M PBS (pH 7.0).

biosensors exhibited good analytical performance. We believed that amino derivative of thiocalixarene would have potential application in the field of bioelectrochemistry and biosensor.

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