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Application of a C6-OH of Chitosan Immobilized Cyclodextrin Derivates on an Electrochemical H_2O_2 Biosensor

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ABSTRACT: Biosensor detecting techniques have attracted much attention in the content determination of H_2O_2, which has been used illegally as a food additive. An electrochemical biosensing membrane for the detection of H_2O_2 was developed with C6-OH of chitosan immobilized cyclodextrin derivates (6-CD–CTS), which possessed a high cyclodextrin loading capacity (2.12×10^{-4} mol/g), as the carrier. The biosensor was prepared through the inclusion of ferrocene as the electron mediator in a hydrophobic cavity of cyclodextrin and crosslinking catalase (CAT) to 2-NH_2 of 6-CD–CTS. The ferrocene-included complex was evaluated by ultraviolet–visible spectrophotometry and thermogravimetric analysis. Its electrochemical behavior was also studied. The impact of the reaction conditions on the CAT immobilization capacity was evaluated. When previous membrane was used to detect the concentration of H_2O_2 (C_{H2O2}), we found that the catalysis of CAT and the signal amplification of ferrocene had a major impact on the cyclic voltammograms. The optimal working pH of the modified electrode was 7.0. The peak current (I) had a linear relationship with the H_2O_2 concentration (CH_2O_2) in the range 1.0×10^{-4} to 1.0×10^{-3} mol/L. The linear regression equation was $I = 0.00475C_{H2O2} - 0.03025$. The detection limit was 10^{-6} mol/L. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 41499.

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INTRODUCTION

In recent years, H_2O_2 has been used illegally as an additive in foods and health care products. H_2O_2 is easily decomposed to produce free radicals; this may bring great harm to human health. So, methods for detecting the content of H_2O_2 are of great importance in quality supervision and inspection.[1] Biosensor detecting techniques have attracted much attention in the content determination of H_2O_2.

Chitosan, a kind of important natural polymer, has been widely used in many applications, including medical materials, sanitarian materials, and matrices for enzyme immobilization.[2–4] Chitosan has excellent membrane formation and functionalization properties because of its active groups, such as —NH_2 and —OH, and it can be readily fixed on the electrode surface. Thus, in recent years, chitosan has received extensive attention in research in electrochemical biosensing membranes.[5–7] Chitosan is also a popular carrier for immobilizing enzymes and improving enzyme stability through 2-NH_2 crosslinking or the self-assembly of chitosan.[8] The immobilization of the enzyme on chitosan provided a favorable microenvironment for the enzymatic reaction, and the reactivity is well protected against acid, base, heat, and metal-ion interference.[9,10]

In practical applications, to improve the sensitivity of the membrane to electrical current, ferrocene, quinone, organic dyes, and other derivatives have often been used as mediators, and one of them was doped in the chitosan membrane to facilitate the electron transfer between the electrode and the enzyme.[11] However, because the electron mediator often has poor solubility in water, the homogeneity of doping is difficult to control; this significantly impacts the stability and service life of the membrane. Therefore, improving the binding stability and homogeneity of electron mediators in the chitosan membrane through the chemical modification of chitosan is a key issue in improving the sensor performance.[12,13]

Including the electron mediator inside the hydrophobic cavity of cyclodextrin promotes electron transfer from the enzyme reaction center to the electrode surface; this prevents the loss of the electron mediator and ensures the sufficient mobility of

mediator and improves the sensitivity and stability of the sensor.[14,15]

A major issue for the use of cyclodextrin in biosensing membranes involves its poor membrane formation properties. To ensure homogeneous dispersion on the surface of the sensor electrode, cyclodextrin is usually blended with polymers to form a membrane.[16,17] However, cyclodextrin has strong intermolecular hydrogen bonding; this makes it difficult to introduce it into a membrane or disperse it homogeneously. Consequently, this results in the instability and poor performance of membranes containing cyclodextrin and its derivatives. One can expect that stable membranes containing cyclodextrin with controlled orientation on the surface will definitely demonstrate superior performance in sensing applications.[18]

The chemical immobilization of cyclodextrin on the chitosan skeleton can be a feasible means for generating a supramolecular host material as a sensing membrane. The approach combines the excellent supramolecular inclusion properties of cyclodextrin with the remarkable advantages of chitosan in membrane formation, immobilization, functionalization, and biochemical properties; this allows the preparation of biosensing membranes with outstanding performance. The immobilization of cyclodextrin on chitosan has been well studied. Prabaharan and Mano[19] and Sashiwa and Aiba[20] provided literature reviews in this field. However, in most cases, the immobilization was made on the 2-NH_2 of chitosan.[21–24] As the amino group of chitosan brings it great physiological activity and at the same time gives a good modification handle, the previous derivates are not conducive for performing further amino group modification and manipulating its unique properties.

Several researchers have reported different ways of preparing C6-OH of chitosan immobilized cyclodextrin derivates.[25–28] Because the 6-OH group has a lower reactivity than 2-NH_2, the preparation of the previous derivates were more difficult and yielded a loading capacity that was relatively low ($<7 \times 10^{-5}$ mol/g). In comparison, the derivatives of chitosan immobilized with cyclodextrin at the 2-NH_2 position reported in the literature attain a value of 2.4×10^{-4} mol/g.[29] Then, the uses of the 6-OH immobilized cyclodextrin derivate of chitosan were limited for its lower immobilized loading. In our previous study, the preparation routes for 6-OH cyclodextrin-immobilized chitosan (6-CD–CTS) derivatives were explored in depth.[30–32] The reaction routes via nucleophilic substitution or click reactions were established. The loading capacity of the prepared derivates were greater than 2×10^{-4} mol/g. Applications of such chitosan derivates in the sensitive films of sensors or in other fields become possible with significant improvements in the immobilized loading.

In this study, the prepared 6-CD–CTS was used as a sensitive electrochemical biosensor membrane via the inclusion of ferrocene as an electron mediator in the cyclodextrin hydrophobic cavity and with the immobilization of catalase (CAT) by crosslinking to the 2-amino groups of 6-CD–CTS. The resulting membrane was fixed on the surface of the glassy carbon electrode, and the response of the modified electrode to H_2O_2 was determined by cyclic voltammetry to explore the application of this methodology in electrochemical biosensing.

EXPERIMENTAL

Materials

6-CD–CTS, with a loading capacity of 2.12×10^{-4} mol/g, was synthesized according to a method in the literature.[31] Ferrocene was chemical grade and was supplied by Beijing Chemical Reagents Co. (China). CAT was purchased from Shanghai Shengke Bio-Tech Co., Ltd. (China). Ethanol, tetrahydrofuran, dimethyl sulfoxide, HCl, $FeCl_3$, Na_2HPO_4, and NaH_2PO_4 were all analytical grade and were used as received.

Inclusion of Ferrocene in 6-CD–CTS

To the solution of 6-CD–CTS (0.05 mol/L) in ethylenediamine–ethanol (1:1 v/v) was added a ferrocene solution in anhydrous ethanol, and the mixture was heated in an 80°C oil bath and refluxed for 6 h. The stoichiometric ratio of 6-CD–CTS to ferrocene was varied with values of 1:1, 1:2, 1:3, 1:4, and 1:5 in different runs. After the reaction was complete, the precipitate was washed with doubly deionized water, then washed with tetrahydrofuran to remove unreacted ferrocene, and finally dried to give the inclusion product of ferrocene in 6-CD-CTS (6-CD-in-FE–CTS).

Immobilization of Catalase

6-CD-in-FE–CTS or 6-CD–CTS was dissolved in 1% aqueous acetic acid, and the concentration was kept at 0.02 mol/L. Then, the solution was adjusted to neutral by the addition of the aqueous sodium hydroxide. Ultimately, the solution of the CAT (pH 7), which was dissolved in 0.1 mol/L phosphate-buffered saline (PBS), was added to the previous solution with a concentration of 30.0 mg/mL. The immobilizing reaction was processed at a certain temperature for a certain time. Afterward, a glutaraldehyde solution was added to the reaction system, and the crosslinking reaction was carried out at a certain temperature for a certain time. Then, the mixture was centrifuged, filtered, and washed with deionized water to remove unreacted glutaraldehyde and with 0.1 mol/L PBS buffer to remove unreacted CAT, respectively. After this, the immobilization product catalase immobilized 6-CD-in-FE–CTS–CAT (6-CD-in-FE–CTS–CAT) or catalase immobilized 6-CD–CTS (6-CD–CTS–CAT) was gained. The details of the reacting conditions are listed in Table I.

Determination of the Activity of the Immobilized Catalase in 6-CD-in-FE–CTS–CAT

The activity of the immobilized CAT in 6-CD-in-FE–CTS–CAT was determined by spectrophotometry.

The standard solution of H_2O_2 in the PBS buffer was prepared by the dilution of 120 μL of 30% aqueous H_2O_2 with 0.05 mol/L PBS buffer solution (pH 7.0) and the final volume was set to 100 mL. The PBS buffer solution (0.05 mol/L, pH 7.0) was used as the blank. The absorbance of H_2O_2 at 240 nm was measured.

The solution of 6-CD-in-FE–CTS–CAT (0.005 mol/L in 1% aqueous acetic acid) was adjusted to neutral with aqueous sodium hydroxide, and 1.0 mL of the resulting solution was mixed with 3.0 mL of a standard H_2O_2 solution in PBS buffer. The mixture was reacted at 25°C for 5 min, and the absorbance at 240 nm was then measured.

The activity of the immobilized CAT on 6-CD-in-FE–CTS–CAT could be calculated as follows:

Table I. Effects of the Immobilization Conditions on the Activities of the Immobilized CAT

$m_{glutaraldehyde}/m_{6\text{-CD-in-FE-CTS}}$	Temperature (°C)	pH	Time (h)	$m_{CAT}/m_{6\text{-CD-in-FE-CTS}}$	P (U/g)
1.25					43.1
0.57					98.7
0.50					317.5
0.25	25	7.0	2	3.50×10^{-2}	367.4
1.25×10^{-1}					420.3
6.25×10^{-2}					289.6
3.13×10^{-2}					168.4
1.57×10^{-2}					93.7
	4				375.5
	15				405.7
0.25	25	7.0	2	3.50×10^{-2}	420.3
	40				411.6
	60				372.1
		6.0			178.9
		6.0			349.0
0.25	25	7.0	2	4.00×10^{-2}	420.3
		7.5			362.9
		8.0			248.6
			0.5		324.7
			1		420.3
			2		294.0
0.25	25	7.0	3	3.50×10^{-2}	280.1
			4		257.0
0.25	25		5		244.9
			6		227.6
				1.00×10^{-2}	59.6
				1.50×10^{-2}	121.0
				2.00×10^{-2}	193.2
				2.50×10^{-2}	279.7
0.25	25	7.0	1	3.00×10^{-2}	311.2
				3.50×10^{-2}	420.3
				4.00×10^{-2}	408.2
				4.50×10^{-2}	405.3
				5.00×10^{-2}	392.3

$$P = \frac{AV}{\varepsilon l t m} \quad (1)$$

where P is the specific activity of the immobilized CAT in 6-CD-in-FE–CTS–CAT, A is the difference in the absorbance between the test solution and the blank solution, V is the volume of the test solution, ε is the molar absorptivity of H_2O_2 at 240 nm (36 L $mol^{-1} \cdot cm^{-1}$), l is the cuvette thickness, t is the reaction time, and m is the mass of the added immobilized CAT.

Electrochemical Properties of 6-CD-in-FE–CTS–CAT

The glassy carbon electrode was mirror-polished with 0.3 μm of Al_2O_3 and ultrasonicated sequentially in deionized water, absolute ethanol, and deionized water for 1 min in each step. The electrode was then hung to dry at 25°C.

The solution of 6-CD-in-FE–CTS, 6-CD–CTS–CAT, or 6-CD-in-FE–CTS–CAT (0.005 mol/L in 1% aqueous acetic acid) was oscillated for 30 min, and 6 μL of the previous solution was added dropwise onto the glassy carbon electrode. The electrode was hung to dry at 25°C to furnish the modified electrode. The cyclic voltammograms of the modified electrode in the test solutions were recorded on an electrochemical workstation (Shanghai Chenghua Instrument Co., Ltd., China) with a three-electrode system, that is, with the modified electrode as the working electrode, a saturated calomel electrode as the reference electrode, and a platinum electrode as the counter electrode.

Characterization

Thermogravimetric analysis of the samples was done on a TG-Pyris 1 thermogravimetric analyzer (Shimadzu Co., Japan) at a

heating rate of 20°C/min within the temperature range from 50 to 600°C and nitrogen used as the purge gas. Ultraviolet–visible (UV–vis) spectra were recorded with a Pgeneral TU-1810 UV–vis spectrophotometer (Beijing Purkinje General Instrument Co., Ltd., China).

RESULTS AND DISCUSSION

Inclusion Ratio of Ferrocene in 6-CD–CTS (*Ir*)

Under acidic conditions (hydrochloric acid), ferrocene in aqueous ethanol was quantitatively oxidized by Fe^{3+} to the ferrocene ion:[33]

$$Fe^{3+} + Fe(C_5H_5)_2 = Fe^{2+} + [Fe(C_5H_5)_2]^+$$

This reaction is a single-electron stoichiometric process and can be used to quantify ferrocene. In the following discussion, $[Fe(C_5H_5)_2]^+$ is abbreviated as FE^+.

Ferrocene was dissolved in acidic aqueous ethanol (1:1 v/v containing 2.4 mol/L hydrochloric acid) and an equal volume of 1.000 g/L Fe^{3+} solution was added. After 30 min of the reaction, the absorption of the resulting ferrocene ion was measured by UV–vis spectrophotometry. The maximum absorption was observed at 619 nm (Figure 1). As shown in Figure 1, ferrocene or Fe^{3+} had no absorption at 619 nm. The inclusion product 6-CD-in-FE–CTS was also dispersed in acidic aqueous ethanol (1:1 v/v containing 2.4 mol/L hydrochloric acid) and hydrolyzed by the addition of an equal volume of concentrated sulfuric acid. To the hydrolyzed mixture, we then added an equal volume of 1.000 g/L Fe^{3+} solution. After 30 min of the reaction, UV–vis spectrophotometry also showed an absorption peak at 619 nm. In contrast, under identical conditions, the hydrolysis product of 6-CD–CTS showed no absorption peak at 619 nm after the addition of Fe^{3+}. The observations proved the inclusion of ferrocene within the cyclodextrin cavity of 6-CD–CTS and demonstrated that the inclusion could be quantified by UV–vis spectrophotometry.

Ferrocene was dissolved in acidic aqueous ethanol (1:1 v/v containing 2.4 mol/L hydrochloric acid) at different concentrations and mixed with an equal volume of 1.000 g/L Fe^{3+} standard solution. After 30 min of the reaction, the absorbance at 619 nm was measured and fitted into the standard working curve as follows:

$$A = 1.682C - 0.031 \quad (R = 0.9992) \qquad (2)$$

where A is the absorbance of the mixture solution at 619 nm, R is the correlation coefficient of the curve, and C is the concentration of the ferrocene solution (g/L).

6-CD-in-FE–CTS was dispersed in acidic aqueous ethanol (1:1 v/v containing 2.4 mol/L hydrochloric acid) at different concentrations and hydrolyzed by the addition of an equal volume of concentrated sulfuric acid. To the hydrolyzed mixture was then added an equal volume of a 1.000 g/L Fe^{3+} standard solution. After 30 min of the reaction, the absorbance at 619 nm was measured, and the amount of ferrocene included in 6-CD–CTS (C_{FE}; mol/g) could be calculated from eq. (2). *Ir* was calculated as follows:

$$Ir = C_{FE}/C_{CD} \qquad (3)$$

where *Ir* represents the inclusion ratio of ferrocene by the cyclodextrin hydrophobic cavity in 6-CD–CTS and C_{CD} is the amount of the immobilized cyclodextrin in 6-CD–CTS (mol/g).

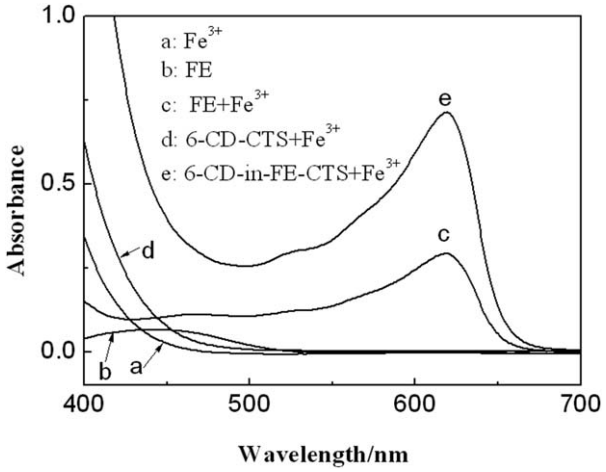

Figure 1. UV–vis spectra for the reacting products of different states of ferrocene with Fe^{3+}.

We found that in the synthesis of 6-CD-in-FE–CTS, when the molar ratios of 6-CD–CTS to ferrocene were 1:1, 1:2, 1:3, 1:4, and 1:5, *Ir* reached values of 23.51, 31.69, 38.76, 40.71, and 42.20%, respectively. We observed that as the amount of the reactant ferrocene increased, *Ir* improved but with a gradually slowing trend. In the subsequent enzyme immobilization studies, 6-CD-in-FE–CTS with an *Ir* of 42.20% was used.

Thermogravimetric Analysis

The thermal stabilities of the ferrocene, 6-CD–CTS, ferrocene/6-CD–CTS mixture, and 6-CD-in-FE–CTS inclusion compound were studied (Figure 2). Ferrocene had a low sublimation temperature, and weight loss occurred rapidly at low temperatures. The weight loss of 6-CD–CTS over 242.1–414.1°C corresponded to the decomposition of the pyran ring, with the maximum weight loss rate occurring at 315.5°C, and the weight loss amounted to 55.35% over this range. The mixture of ferrocene and 6-CD–CTS exhibited weight losses at 40.7–117.8 and 275.2–396.5°C; these temperatures corresponded to the sublimation of ferrocene and the decomposition of the pyran ring in 6-CD–CTS, respectively. The thermogravimetric curve of the inclusion compound 6-CD-in-FE–CTS was remarkably different from that of the ferrocene/6-CD–CTS mixture. The weight loss of 6-CD-in-FE–CTS occurred over 227.6–414.8°C, with the maximum weight loss rate occurring at 301.9°C, and the weight loss amounted to 59.15% over this range. Compared with 6-CD–CTS, the onset and maximum weight loss temperatures were both lower, and the weight loss was greater, but the ending weight loss temperature was similar. These results indicate that the inclusion of ferrocene in the cyclodextrin cavity affected the stability of the pyran ring in cyclodextrin and promoted the decomposition of cyclodextrin. In addition, the decomposition of cyclodextrin was also accompanied by ferrocene sublimation; this resulted in the higher weight loss. No ferrocene sublimation weight loss was observed in the low-temperature range for 6-CD-in-FE–CTS. The observations proved the inclusion of ferrocene in 6-CD–CTS and also proved that the inclusion product was free of ferrocene residue after washing.

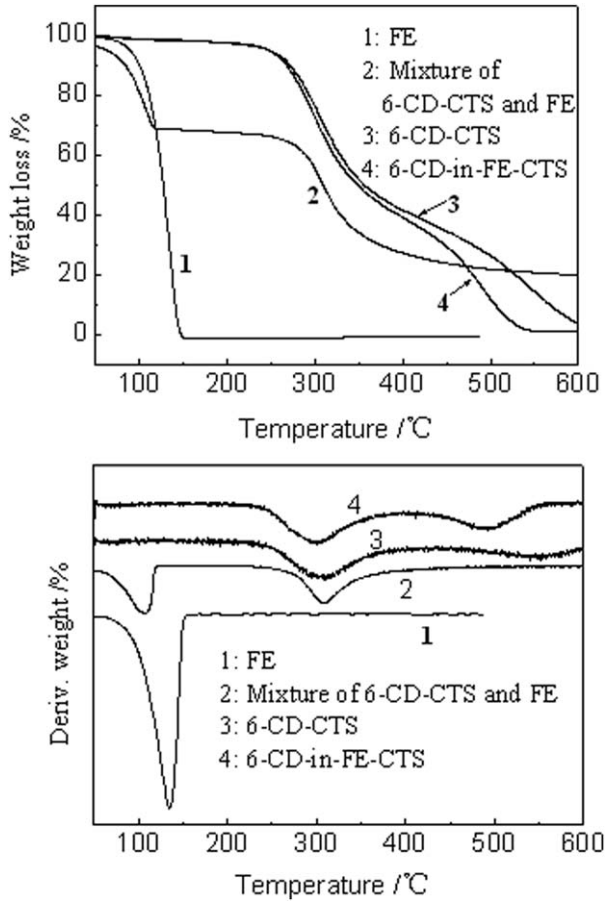

Figure 2. Thermogravimetry and differential thermogravimetry curves of the ferrocene, 6-CD–CTS, ferrocene/6-CD–CTS mixture, and inclusion compound (6-CD-in-FE–CTS).

Electrochemical Properties of 6-CD-in-FE–CTS

With a CHI660 electrochemical workstation and three-electrode test system composed of a glassy carbon working electrode, a platinum counter electrode, and a calomel reference electrode, the electrochemical properties of 6-CD-in-FE–CTS were studied.

The solution of 6-CD-in-FE–CTS (0.001 mol/L in 1% aqueous acetic acid) was adjusted to neutral by the addition of aqueous sodium hydroxide. An amount of 50 μL of the previous solution was mixed with 5 mL of PBS buffer solution (0.1 mol/L, pH 7). Then, the cyclic voltammograms of the previous solution in the range 0–0.60 V at different scan rates were recorded and are shown in Figure 3.

The figure shows a pair of redox peaks that could be attributed to the FE/FE$^+$ pair of ferrocene. The peak potential was almost independent of the scan rate. The potential difference between redox peaks (ΔE_p) was about 60 mV. The ratio of the oxidation peak current to the reduction peak current (i_{pa}/i_{pc}) is largely a constant close to 1:1. According to the ΔE_p value and the i_{pa}/i_{pc} ratio, over the scan rate range of 20–500 mV/s, the FE/FE$^+$ redox process is reversible or quasi-reversible. Furthermore, the inset in Figure 3 shows that the peak current had a linear relationship with the square root of the scan rate; this indicated that the FE/FE$^+$ redox process in the inclusion product was under diffusion control.

Study of the Optimum Reaction Conditions of the Catalase Immobilized onto 6-CD-in-FE–CTS

Catalase was immobilized onto 6-CD-in-FE–CTS via a crosslinking reaction. The immobilization and the activity of the immobilized enzyme were obviously affected by the reaction conditions, including the concentration of the crosslinking agent, immobilization temperature, pH value, *t*, and concentration of CAT. The effects of the immobilization conditions on the activity of the immobilized CAT were studied to determine the optimum enzyme immobilization conditions.

The impact of the glutaraldehyde concentration on CAT immobilization was examined. We found that the activity of the immobilized CAT reached a maximum when $m_{\text{glutaraldehyde}}/m_{\text{6-CD-in-FE–CTS}}$ was 0.25% (where $m_{\text{glutaraldehyde}}$ means the mass of glutaraldehyde, and $m_{\text{6-CD-in-FE–CTS}}$ means the mass of 6-$_{\text{CD-in-FE–CTS}}$). Ideally, the bifunctional glutaraldehyde molecule should have been crosslinked with the amino group in chitosan at one end and crosslinked with the amino, phenol, and thiol groups in CAT at the other end. When the glutaraldehyde concentration was too low, the crosslinking between the 2-NH_2 group of chitosan and glutaraldehyde was inadequate and left few active sites for immobilizng CAT; this resulted in poor enzyme activity. However, an excessively high glutaraldehyde concentration led to intramolecular or intermolecular crosslinking; this also reduced the active sites (aldehyde group) for CAT immobilization and increased the steric hindrance of the carrier; this gave a low CAT loading. In addition, glutaraldehyde was also an enzyme inactivating agent.[34] A high glutaraldehyde content on the carrier may have triggered enzyme denaturation and reduced enzyme activity. Therefore, in the CAT immobilization reaction, the most suitable $m_{\text{glutaraldehyde}}/m_{\text{6-CD-in-FE–CTS}}$ ratio was 0.25.

The optimal crosslinking temperature for the activity of the immobilized CAT was found to be 25°C, although the variation

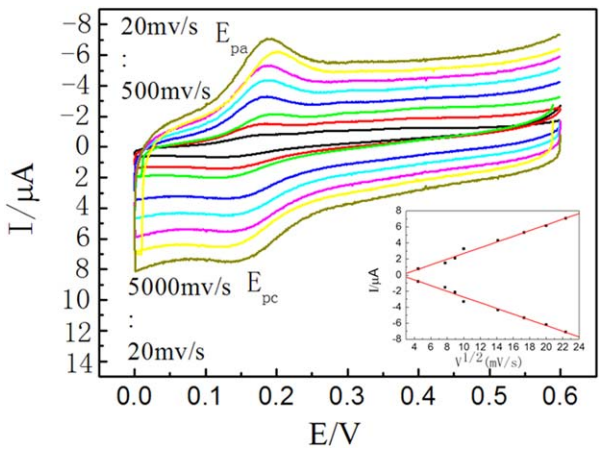

Figure 3. Cyclic voltammograms of 6-CD-in-FE–CTS at different scan rates (20, 60, 80, 100, 200, 300, 400, and 500 mV/s) and the relationship curve of the peak current, the square root of the scan rate (the inset figure), where E means the electrode potential, E_{pa} means the peak potential of the anode, E_{pc} means the peak potential of the cathode, and $V^{1/2}$ means the square root of the scan rate. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

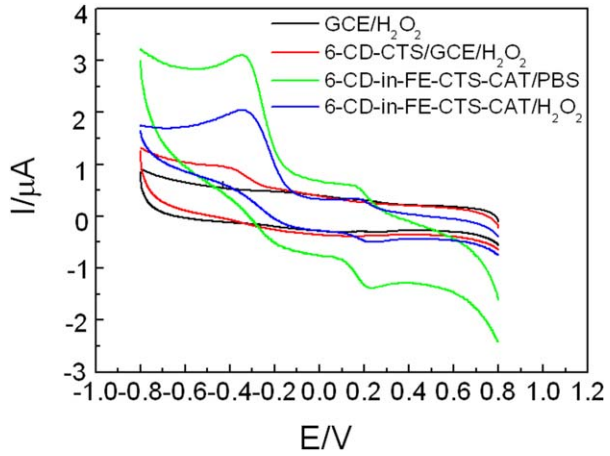

Figure 4. Cyclic voltammetry curves of the bare electrode, 6-CD–CTS modified electrode, and 6-CD-in-FE–CTS–CAT modified electrode in H_2O_2 solution (scan rate = 100 mV/s), where *E* means the electrode potential, and GCE means the glassy carbon electrode. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

in the activity of the immobilized CAT was small over the temperature range of 4–60°C. The most suitable crosslinking temperature was thus selected as 25°C, which gave the highest activity of the immobilized CAT.

The optimal pH value of the immobilization reaction system for the activity of the immobilized CAT was studied. When the pH value was less than 7.0, the activity of the immobilized CAT was gradually enhanced with increasing pH value. When the pH value increased further, the activity of the immobilized CAT tended to drop off. The activity of the immobilized CAT reached its maximum value around a pH value of 7.0. Because the immobilization reaction took a relatively long time to complete, the prolonged exposure of CAT to the environment with a suboptimal pH may have induced enzyme denaturation to some extent.

By changing *t* from 0.5 to 6 h and keeping the other reaction conditions constant, we also evaluated the effect of *t* on activity of the immobilized CAT. The crosslinking reaction was relatively rapid. The activity of the immobilized CAT increased with the extension of the crosslinking *t* up to a maximum at about 1 h. However, a prolonged *t* led to intramolecular or intermolecular crosslinking; this reduced the CAT activity of the final product. Thus, the appropriate crosslinking *t* was taken to be 1 h.

The amount of added CAT on the activity of the immobilized CAT was also evaluated. As shown in Table I, when $m_{CAT}/m_{6\text{-CD-in-FE–CTS}}$ was less than 4.00×10^{-2}, the activity of the immobilized CAT increased with increasing mass ratio of the added enzyme. When $m_{CAT}/m_{6\text{-CD-in-FE–CTS}}$ was around 4.00×10^{-2}, the activity of the immobilized CAT reached its highest value. When the mass ratio was increased further, the activity of the immobilized CAT was set for a small drop. As the $m_{CAT}/m_{6\text{-CD-in-FE–CTS}}$ ratio increased, the CAT loading increased, and the activity improved. Nevertheless, because there were only limited active sites (aldehyde) on the carrier, the carrier became saturated after the $m_{CAT}/m_{6\text{-CD-in-FE–CTS}}$ ratio reached a certain level, and the CAT loading and activity no longer improved. The suitable $m_{CAT}/m_{6\text{-CD-in-FE–CTS}}$ ratio in the CAT immobilization process should have been 4.00×10^{-2}.

With studies under different conditions, we found that the optimum immobilization conditions for 6-CD-in-FE–CTS–CAT with the highest activity of the immobilized CAT were as follows: $m_{glutaraldehyde}/m_{6\text{-CD-in-FE–CTS}}$ was around 0.25, the immobilization reaction temperature was 25°C, the pH value of the reaction system was around 7, *t* was 1 h, and $m_{CAT}/m_{6\text{-CD-in-FE–CTS}}$ was around 4.00×10^{-2}. The activity of the immobilized CAT for the 6-CD-in-FE–CTS–CAT prepared under these conditions was able to reach 420.3U/g.

Electrochemical Properties of 6-CD-in-FE–CTS–CAT

The cyclic voltammetry curves of the bare electrode, 6-CD–CTS modified electrode, and 6-CD-in-FE–CTS–CAT modified electrode in PBS buffer solution (5.00 mL, 0.1 mol/L, pH = 7.0) containing 5 μL of 0.1 mol/L H_2O_2 solution were measured from −0.80 to 0.80 V (Figure 4). We saw that the bare electrode and the 6-CD–CTS modified electrode gave no response to the PBS solution of H_2O_2. The 6-CD-in-FE–CTS–CAT modified electrode gave two pairs of peaks in the 0.1 mol/L blank PBS buffer; these were the FE/FE^+ redox peaks and the CAT redox peaks, respectively. Upon the addition of H_2O_2 to the PBS buffer, the reduction current decreased, and the oxidation current increased in the CAT redox peaks. This proved proves the occurrence of the enzymatic reaction.

Effect of the Included Ferrocene on the Electrocatalysis of 6-CD-in-FE–CTS–CAT

The working electrode was modified with 6-CD–CTS–CAT and 6-CD-in-FE–CTS–CAT, respectively and the cyclic voltammograms were scanned in 0.01 mol/L H_2O_2 solution (in Figure 5). Compared with the 6-CD–CTS–CAT modified electrode, the 6-CD-in-FE–CTS–CAT modified electrode gave a significantly higher oxidation current and reduction current in the CAT redox peaks. This observation proved that ferrocene amplified the electrical signal during detection, and this improved the sensitivity and limit of detection for H_2O_2.

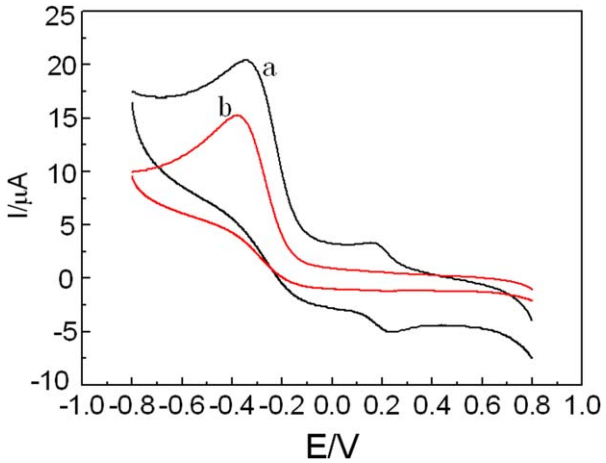

Figure 5. Cyclic voltammograms of the (a) 6-CD-in-FE–CTS–CAT modified electrode and (b) 6-CD–CTS–CAT modified electrode in H_2O_2 solution, respectively (scan rate = 100 mV/s), where *E* means the electrode potential. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Effect of the pH Value of the Detecting Solution on the Peak Potential of 6-CD-in-the FE–CTS–CAT Modified Electrode

The detecting solution was prepared with 5 μL of a 0.01 mol/L H_2O_2 solution mixed with 5 mL of a 0.1 mol/L PBS buffer solution (at a certain pH value). Then, the cyclic voltammograms of the 6-CD-in-FE–CTS–CAT modified electrode from −0.80 to 0.80 V were measured in the previous detecting solution. The relationship between the peak potential and the pH value of the detecting solution is shown in Figure 6. When the pH value was less than 7.0, the peak potential was raised when the pH of the detecting solution was increased. When the pH value was around 7.0, the peak potential reached its highest value. If the pH value of the detecting solution increased further, the peak potential was decreased with increasing pH value. So, the most suitable pH value of the detecting solution for the 6-CD-in-FE–CTS–CAT modified electrode was around 7.0.

Limit of Detection

The chronoamperometric curves of the 6-CD-in-FE–CTS–CAT modified electrode was measured at −0.30 V in PBS buffer (5.00 mL, 0.1 mol/L, pH 7.0). A certain amount of H_2O_2 solution was added at regular intervals (Figure 7), and the measured peak current was plotted against the H_2O_2 concentration to determine the limit of detection (the inset in Figure 7).

As shown in Figure 7, the 6-CD-in-FE–CTS–CAT modified electrode showed a fast and sensitive response to the addition of the H_2O_2 solution. The time it took for the reaction current to reach the saturation condition was less than 5 s. The previous results indicate that the CAT immobilized onto the 6-CD-in-FE–CTS–CAT modified electrode had efficient catalytic activity to the reduction reaction of H_2O_2.

The peak current (I) had a linear relationship with the H_2O_2 concentration (C_{H2O2}) in the range 1.0×10^{-4} to 1.0×10^{-3} mol/L. The linear regression equation was as follows:

$$I = 0.00475 C_{H2O2} - 0.03025 \quad (4)$$

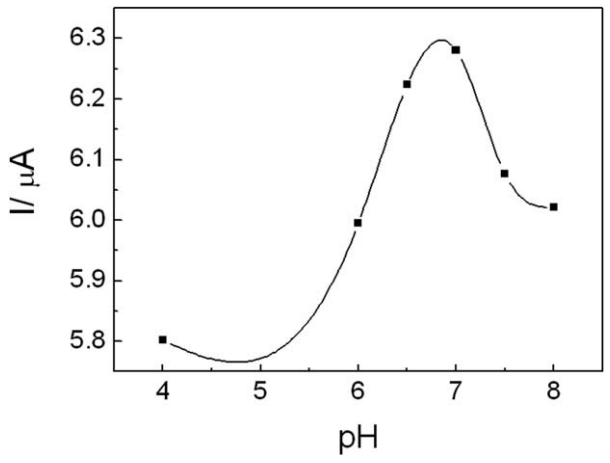

Figure 6. Effects of the pH value of the detecting solution on the peak potential of the 6-CD-in-FE–CTS–CAT modified electrode in H_2O_2 solution.

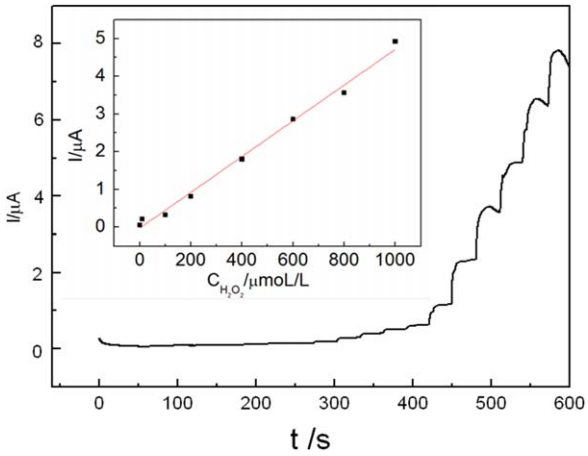

Figure 7. Chronoamperometric curve of the 6-CD-in-FE–CTS–CAT modified electrode in H_2O_2 solution and the relationship between the peak current with H_2O_2 concentration (inset). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

where I is the peak current and C_{H2O2} is the concentration of H_2O_2. The linear correlation coefficient of the curve was 0.99173. The limit of detection was 10^{-6} mol/L.

CONCLUSIONS

A high-loading-capacity (2.12×10^{-4} mol/g) 6-CD–CTS was used as an electrochemical biosensing membrane for the quantitative detection of H_2O_2 by the inclusion of ferrocene as the electron mediator in the cyclodextrin hydrophobic cavity and the crosslinking of CAT to the 2-amino groups. The electrochemical behavior of the 6-CD-in-FE–CTS solution was studied. The results show that at scan rates of 20–500 mV/s, the FE/FE$^+$ redox process of the included mediator in 6-CD-in-FE–CTS was reversible or quasi-reversible and under diffusion control. The impact of the reaction conditions on the CAT immobilization capacity in 6-CD-in-FE–CTS–CAT was evaluated. We found that the optimum immobilizing conditions for 6-CD-in-FE–CTS–CAT with the highest the activity of the immobilized CAT were as follows: $m_{glutaraldehyde}/m_{6\text{-CD-in-FE–CTS}}$ was around 0.25, the immobilizing reaction temperature was 25°C, the pH value of the reaction system was around 7, t was 1 h, and $m_{CAT}/m_{6\text{-CD-in-FE–CTS}}$ was around 4.00×10^{-2}. The activity of the immobilized CAT for 6-CD-in-FE–CTS–CAT prepared under these conditions reached 420.3 U/g. A glassy carbon electrode was modified by 6-CD-in-FE–CTS–CAT and was then used to detect C_{H2O2}. We found that the sensitivity and limit of detection to H_2O_2 were clearly improved because of the catalysis of CAT and the signal amplification of ferrocene. The optimal working pH of the 6-CD-in-FE–CTS–CAT modified electrode was 7.0. The peak current had a linear relationship with the H_2O_2 concentration in the range 1.0×10^{-4} to 1.0×10^{-3} mol/L. The linear regression equation was $I = 0.00475 C_{H2O2} - 0.03025$. The limit of detection was 10^{-6} mol/L. Via the previous study, the new application pattern of 6-CD–CTS derivatives in the electrochemical biosensor was established, and the results show great innovative meaning for the application of the previous derivatives.

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