

Direct electron transfer of cytochrome c immobilized on a NaY zeolite matrix and its application in biosensing

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Abstract

The immobilization and electrochemical behaviors of cytochrome c on a NaY zeolite modified electrode were studied. The interaction between cytochrome c and NaY zeolite particles was examined by using UV-Vis spectroscopy and electrochemical methods. The direct electron transfer of the immobilized cytochrome c exhibited a pair of redox peaks with the $E_{1/2}$ of $-(44 \pm 3)$ mV (versus SCE) in 0.1 M pH 7.0 PBS. The electrode reaction showed a surface-controlled process with a single proton transfer at the scan rate range from 20 to 500 mV s⁻¹. Based on the immobilization of cytochrome c on NaY zeolite a high performance biosensor was constructed, which displayed an excellent response to the reduction of hydrogen peroxide (H₂O₂) without the aid of an electron mediator and could be used for H₂O₂ detection. NaY zeolite provided a good matrix for protein immobilization and biosensor preparation.

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1. Introduction

Cytochrome c is a very basic redox metalloprotein with an over charge of +7/+8 at neutral pH. Since the direct electron transfer of cytochrome c was first observed in 1977 [1], its direct electrochemistry and voltammetric measurements have been extensively described at various chemically modified electrodes [2–11]. These works have led to a good understanding of the electron transfer mechanism between cytochrome c and chemically modified electrodes. The modifiers for preparation of these chemically modified electrodes are all organic compounds. Compared with organic compounds, inorganic materials are intrinsically more stable catalysts because of their layered oxide structure.

Recently, a series of inorganic porous materials such as clay [12], montmorillonite [13–15], porous alumina [16] and sol-gel matrix [17] have been proven to be promising as the immobilization matrices. They have the advantages of high mechanical, thermal, and chemical stability, good adsorption and penetrability due to their regular structure and apprecia-

ble surface area. Besides them, the unique structural and catalytic properties of zeolites for structuring an electrochemical/electron transfer environment and resistance to biodegradation have also attracted considerable attention [18]. NaY zeolite possesses a microporous diameter of 0.81 nm [18]. The electrochemical behaviors of redox substances such as glucose oxidase [19,20] and horseradish peroxidase [21] incorporated in NaY modified matrixes have been studied. This work uses polyvinyl alcohol (PVA) as a supporting medium for NaY immobilization on electrode surface and reports on the direct electrochemistry of cytochrome c incorporated in NaY–PVA mixed media. An interaction between cytochrome c and NaY zeolite particles is observed with UV-Vis spectroscopy and cyclic voltammetry. NaY zeolite particles effectively retain the activity of the immobilized cytochrome c and facilitate the electron exchange between the cytochrome c and electrode.

The research on the direct electron exchange between redox proteins or enzymes and electrodes is largely driven by their potential applicability in fabricating biosensors and biomedical devices by removing the requirement of chemical mediators, which has a great significance in preparing the third generation biosensors [22–25]. The possibility of electrocatalytic sensing without addition of electron

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mediators based on the direct electron transfer of cytochrome c immobilized on electrode surfaces have been demonstrated [25–27]. In this paper, a new mediator-free H_2O_2 amperometric biosensor is constructed based on the direct transfer of cytochrome c immobilized on NaY zeolite modified glassy carbon electrode by a very simple process. The analytical performance of the biosensor with respect to response time, sensitivity, and stability are evaluated. The biosensor has a high sensitivity and a good stability, indicating NaY zeolite is a good matrix for protein immobilization and preparation of the third generation biosensor.

2. Experimental

Cytochrome c was purchased from Sigma (USA) and used as received. The response of immobilized cytochrome c indicated this production was enough for this work without need of further purification. NaY zeolite was purchased from Nanjing Inorganic Chemical Engineering Factory and used after mesh screening (>300 mesh). XRD results showed that the NaY zeolite was highly pure. Its lattice parameter a_0 was calculated to be 24.68 Å. The ratio of Si:Al atom was 2.7. Hydrogen peroxide (30%, w/v solution) was purchased from Shanghai Biochemical Regent (China). The concentrations of more diluted hydrogen peroxide solutions were determined by titration with cerium (IV) to a ferroin end point [28]. Polyvinyl alcohol (average degree of polymerization: 1800 ± 100) was purchased from Shanghai Laize Factory of Fine Chemicals. All other chemicals were of analytical grade and used without further purification. Phosphate buffer solutions (PBS, 0.1 M) with various pH values were prepared by mixing stock standard solutions of K_2HPO_4 and KH_2PO_4 and adjusting the pH with H_3PO_4 or NaOH. All the solutions were prepared with doubly distilled water.

Some NaY zeolite was calcinated at 500°C for 2 h, and dispersed in 10% NH_4Cl solution to perform an exchange process for 2.5 h at $90\text{--}95^\circ\text{C}$. The zeolite particles were filtrated under vacuum and washed until no chloride ion was found. After drying at 120°C for 4 h and further calcination at 500°C for 6 h, HNaY was obtained. After the exchange process, 66.0% of sodium atoms were substituted by hydrogen atoms, which was measured with ICP and defined as the exchanged degree of sodium. After two and three repeating exchange processes, 87.6 and 97.2% of sodium atoms were substituted by hydrogen atoms, respectively.

NaY or HNaY zeolite particles (30 mg) were dispersed into 10 ml water to obtain a suspension of 3.0 mg ml^{-1} NaY or HNaY zeolite. 100 μl of suspension was then mixed with 5 μl 3% PVA solution of ethanol/water (v/v, 1:1) to produce colloidal NaY or HNaY zeolite solution.

A glassy carbon electrode (GCE, 3 mm in diameter) was polished to a mirror-like finish with fine emery papers and 1.0, 0.3, and 0.05 μm alumina slurry (Beuhler) followed by rinsing thoroughly with doubly distilled water. The electrode was then successively sonicated in 1:1 nitric acid, ace-

tone and doubly distilled water, and allowed to dry at room temperature. Cytochrome c ($2\ \mu\text{l}$ 3.0 mg ml^{-1}) (in pH 7.0 PBS) and 2 μl colloidal NaY or HNaY zeolite solution were dropped on the pretreated GCE surface, respectively, and allowed to dry under ambient conditions for 3 h. The modified electrode was rinsed with doubly distilled water for twice or thrice to get rid of the non-firmly adsorbed cytochrome c. It was then immersed into 0.1 M pH 7.0 PBS to perform cyclic voltammetric sweep until a stable electrochemical response of cytochrome c was observed. The obtained cytochrome c/NaY or HNaY zeolite modified electrode was stored in 0.1 M pH 7.0 PBS at 4°C in a refrigerator when not in use.

Cyclic voltammetric and amperometric measurements were performed on 270 Electrochemistry System (EG&G, USA). A three-electrode system comprising a platinum wire as auxiliary, a saturated calomel electrode as reference and the cytochrome c/NaY or HNaY zeolite modified electrode as working electrode was used for all electrochemical experiments, which were carried out in a cell containing 2.0 ml 0.1 M PBS at room temperature ($25 \pm 2^\circ\text{C}$). All experimental solutions were deoxygenated by bubbling highly pure nitrogen for at least 10 min and maintained under nitrogen atmosphere during the measurements. In amperometric experiments, the current–time data were recorded by applying a potential of -200 mV on a stirred cell after a constant residual current had been established and successive additions of H_2O_2 solution into the buffer were done. The sensor response was measured as the difference between total and residual currents. UV-Vis absorbance spectroscopy was performed using a UV-2201 spectrophotometer (Shimadzu, Kyoto, Japan). ICP was performed with a PS-1 excitation light source (Leeman Co., USA).

3. Results and discussion

3.1. Spectroscopic analysis of cytochrome c/NaY zeolite system

The UV-Vis absorption spectrum of cytochrome c solution displayed a maximum absorption at 407 nm, while no absorption of colloidal NaY solution was observed (curves a and b in Fig. 1). Obviously, this absorption peak was attributed to the Soret band of cytochrome c [29]. Upon mixing of cytochrome c solution with colloidal NaY solution, a slight decrease in the absorbance of cytochrome c was observed and the absorption peak of cytochrome c shifted to 409 nm (curve c in Fig. 1). Previous studies have demonstrated that the absorption band would diminish after the full protein denaturation [29,30]. The red shift in the absorption peak and the decrease in its absorbance indicated an interaction between colloidal NaY zeolite particles and cytochrome c molecules due to the surface potential energy and adsorption properties of colloidal NaY particles. Such interaction did not destroy the structure and did not change the fundamental microenvironment of cytochrome c.

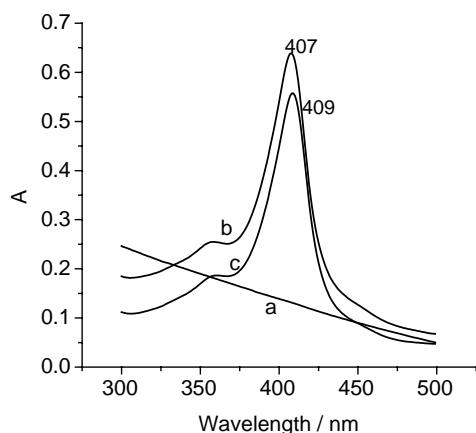


Fig. 1. UV-Vis absorption spectra of 3.0 mg ml^{-1} NaY colloidal solution (a); 3.0 mg ml^{-1} cytochrome c in 0.1 M pH 7.0 PBS (b); and 0.1 M pH 7.0 PBS containing 3.0 mg ml^{-1} NaY colloid and 3.0 mg ml^{-1} cytochrome c (c).

3.2. Direct electrochemistry of cytochrome c immobilized on NaY zeolite modified electrode

Fig. 2 shows the cyclic voltammograms of different electrodes in 0.1 M pH 7.0 PBS. No obvious electrochemical response was observed at both GCE and NaY/GCE, which indicated NaY was electroinactive in the potential window. At cytochrome c/NaY/GCE the cyclic voltammogram showed a couple of stable redox peaks at $+17$ and -104 mV at 100 mV s^{-1} . Obviously, these peaks were attributed to the redox reaction of the electroactive center of cytochrome c. Cytochrome c/GCE also showed the response of cytochrome c, but the response was much smaller than that of cytochrome c/NaY/GCE. Thus the adsorption of cytochrome c on NaY zeolite particles played an important role in facilitating the electron exchange between the electroactive center of cytochrome c and GCE. The $E_{1/2}$ of cytochrome c, estimated as the midpoint of reduction and oxidation potentials, was $-(44 \pm 3) \text{ mV}$. The interaction between cytochrome c and

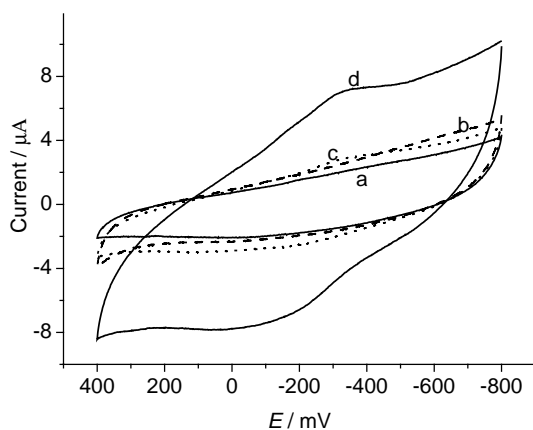


Fig. 2. Cyclic voltammograms of GCE (a); NaY/GCE (b); cytochrome c/GCE (c); and cytochrome c/NaY/GCE (d) in 0.1 M pH 7.0 PBS at 100 mV s^{-1} .

NaY zeolite particles made the $E_{1/2}$ shift negatively when compared to that of cytochrome c in solution ($+17 \text{ mV}$ versus SCE) [31].

With an increasing scan rate the redox peak currents of the immobilized cytochrome c on NaY zeolite particles increased linearly, indicating a typical of the surface-controlled electrode process. From the integration of the reduction peak of cytochrome c/NaY/GCE at different scan rates, an average surface coverage of cytochrome c was calculated to be $4.77 \times 10^{-10} \text{ mol cm}^{-2}$.

With the increase of scan rate the peak-to-peak separation increased. The electron transfer rate constant k_s could be estimated with the model of Laviron [32]. The peak-to-peak separation was 86, 121, 156, and 183 mV at 50, 100, 150 and 200 mV s^{-1} , respectively, producing an average k_s value of $0.69 \pm 0.04 \text{ s}^{-1}$. While the peak-to-peak separation was 210, 233, 276, and 312 mV at 250, 300, 400 and 500 mV s^{-1} , respectively, producing an average k_s value of $0.78 \pm 0.04 \text{ s}^{-1}$. These values were comparable with those of 0.4 s^{-1} for cytochrome c adsorbed on C_{13}COOH SAM on gold electrode [33], 10^{-4} to 10^{-1} s^{-1} for cytochrome c adsorbed on 11 different SAMs immobilized on gold electrode [34], 0.4 s^{-1} for cytochrome c immobilized on macro and micro sized carbon electrodes [35] and smaller than that of 45 s^{-1} for cytochrome c immobilized on mixed SAMs of carboxyl-methyl-terminated alkanethiolates on Au electrode [36]. The faster electron transfer rate resulted from the strong interaction between cytochrome c molecules and NaY zeolite particles. Thus, NaY zeolite provides a microenvironment for cytochrome c to undergo facile electron transfer reaction.

3.3. Effects of NaY zeolite component and solution pH on direct electron transfer of cytochrome c

Fig. 3 shows the cyclic voltammograms of cytochrome c immobilized on zeolites with different exchanged degrees

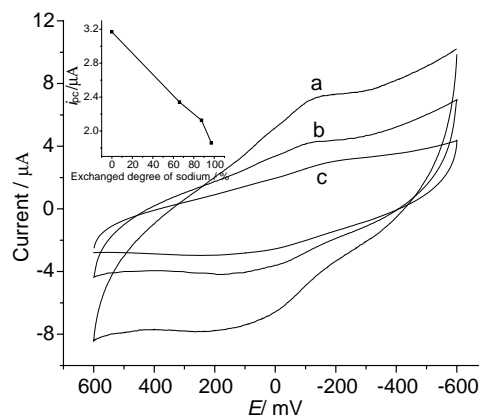


Fig. 3. Cyclic voltammograms of immobilized cytochrome c on zeolites with the sodium exchanged degrees of 0 (a); 66.0% (b); and 97.2% (c) in 0.1 M pH 7.0 PBS at 100 mV s^{-1} . Inset: plot of peak current vs. sodium exchanged degree.

of sodium. With the decreasing sodium content both redox peak currents and peak areas decreased. Thus the adsorption of cytochrome c on zeolite surface depended on the acidity of zeolite. The increasing acidity was not in favor of its adsorption. However, the change of exchanged degree of sodium did not influence the $E_{1/2}$ of cytochrome c.

The direct electrochemistry of immobilized cytochrome c also showed a strong dependence on solution pH. An increase in solution pH caused a negative shift in both cathodic and anodic peak potentials. Considering the fact that the $E_{1/2}$ was independent of the exchanged degree of sodium, the shift in $E_{1/2}$ upon pH increase was due to the thermodynamic change of the electrochemical reaction. The plot of $E_{1/2}$ versus pH showed a slope of -56 mV/pH in the pH range from 4.5 to 9.0, which was near the expected value of -58 mV/pH for a single proton transfer coupled to single electron transfer. Thus, one proton participated in the electron transfer process of the immobilized cytochrome c for neutralizing the excess charge that accumulated at the interface upon electrochemical reduction [37]. The surface-controlled electrode process indicated the diffusion of proton was very fast.

3.4. Electrocatalysis of cytochrome c/NaY/GCE to the reduction of H_2O_2

Upon addition of H_2O_2 to 0.1 M pH 7.0 PBS, the shape of cyclic voltammogram for the direct electron transfer of immobilized cytochrome c changed dramatically with an increase of reduction current and a decrease of oxidation current (Fig. 4), while the change of cyclic voltammogram of bare or NaY modified GCE (inset in Fig. 4) was very small, displaying an obvious electrocatalytic behavior of the cytochrome c to the reduction of H_2O_2 . Thus, cytochrome c was of good electrocatalytic activity.

The electrocatalytic response of the H_2O_2 sensor in presence of 10.0 mM H_2O_2 increased with an increasing scan rate (Fig. 5). The peak current was proportional to the square root of the scan rate, indicating a diffusion-controlled process. Thus, the electrocatalytic reaction was quite fast and

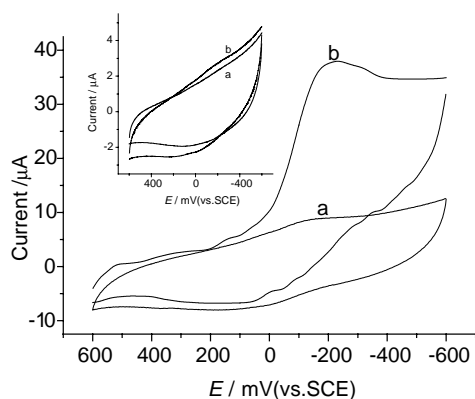


Fig. 4. Cyclic voltammograms of cytochrome c/NaY/GCE and NaY/GCE (inset) in 0.1 M pH 7.0 PBS containing 0 (a) and 10.0 mM H_2O_2 (b) at 100 mV s^{-1} .

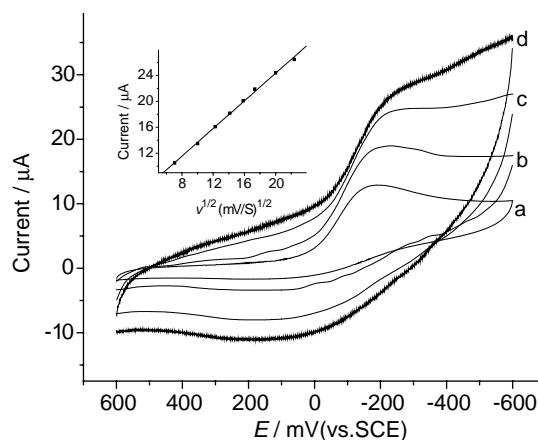


Fig. 5. Cyclic voltammograms of the H_2O_2 sensor in presence of 10.0 mM H_2O_2 at (a) 50, (b) 100, (c) 150, and (d) 200 mV s^{-1} .

the electrode process was controlled by the diffusion rate of H_2O_2 to the electrode surface.

The amperometric response of the cytochrome c/NaY/GCE with successive additions of H_2O_2 to 0.1 M pH 7.0 PBS at an applied potential of -200 mV was shown in Fig. 6. Upon addition of an aliquot of H_2O_2 to the buffer solution, the reduction current increased steeply to reach a stable value. The modified electrode achieved 95% of the maximum steady-state-current in less than 10 s. The results demonstrated clearly that the electrocatalytic response was very fast, which was even much faster than that reported for cytochrome c immobilized on 3-mercaptopropionoc acid monolayer modified Au electrode [38]. Although the current steps for the catalyzed signal displayed a decreasing current over time, we did not observe the difference among the signals determined for several times at the same concentration after H_2O_2 was added for 30 s. The catalytic current was stable and reproducible after 30 s. The decrease was due to the uneven concentration of H_2O_2 on the electrode surface that resulted from the addition of new H_2O_2 solution.

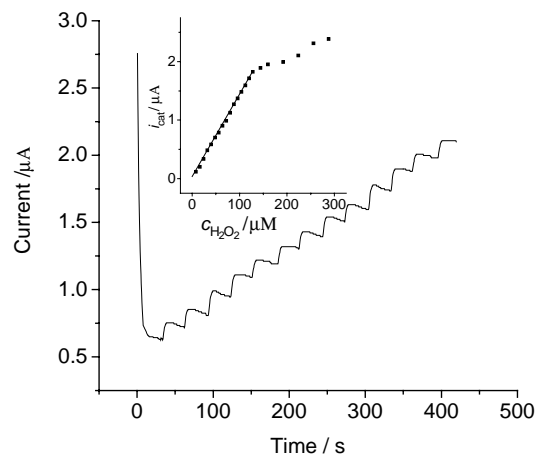


Fig. 6. Amperometric response of cytochrome c/NaY/GCE at -200 mV upon successive additions of 5 μ l 8.0 mM H_2O_2 to 5.0 ml 0.1 M pH 7.0 PBS. Inset: calibration curve of H_2O_2 sensor.

With an increasing solution pH from 5.1 to 8.3 the amperometric response increased and reached a maximum value at pH 7.0 and then decreased slightly. At the solution pHs less than 7.0 the increase in amperometric response was attributed to the increase in electrocatalytic activity of cytochrome c. The isoelectric points of NaY and cytochrome c are about 11 [39] and 10, respectively. Thus, both NaY zeolite particle and cytochrome c were positive-charged in the studied pH range. The slight decrease of amperometric response at solution pHs more than 7.0 was due to the denaturation or inaction of the immobilized cytochrome c, as some proteins that were reported to be inactive at pH 7.5 [40] and denaturant at pH 8.0 [41].

The calibration curve from $8.0\ \mu\text{M}$ to $1.0\ \text{mM}$ H_2O_2 showed a linear response range of the sensor to H_2O_2 concentration from 8.0 to $128\ \mu\text{M}$ with a correlation coefficient of 0.9995 ($n = 16$) (inset in Fig. 6). From the slope of $0.0143\ \mu\text{A}\ \mu\text{M}^{-1}$, a detection limit of $0.32\ \mu\text{M}$ was obtained at a signal-to-noise ratio of 3. The sensitivity of the sensor was $202\ \mu\text{A}\ \text{mM}^{-1}\ \text{cm}^{-2}$.

The influences of foreign species were investigated by analyzing a standard solution of $1 \times 10^{-5}\ \text{mol}\ \text{l}^{-1}$ hydrogen peroxide to which interfering species were added. One millimole K^+ , Na^+ , Al^{3+} , Cl^- , Br^- , NO_3^- , SO_4^{2-} , $0.3\ \text{mM}$ Ca^{2+} , Zn^{2+} , NH_4^+ and $0.1\ \text{mM}$ Fe^{3+} , Mg^{2+} produced the relative response of $<2\%$, respectively, indicating these ions coexisting in the sample matrix did not affect the determination of hydrogen peroxide.

3.5. Effect of temperature on the H_2O_2 sensor

Temperature is an important parameter affecting the electrocatalytic activity of enzyme or protein. Fig. 7 shows the effect of temperature on sensor response. With an increasing temperature from 15 to $40\ ^\circ\text{C}$ the amperometric response and the electrocatalytic activity of the immobilized cytochrome c increased. The immobilized cytochrome c had activity even at $60\ ^\circ\text{C}$. It was evident that the immobilized cytochrome c had good thermal stability because of the unchangeability

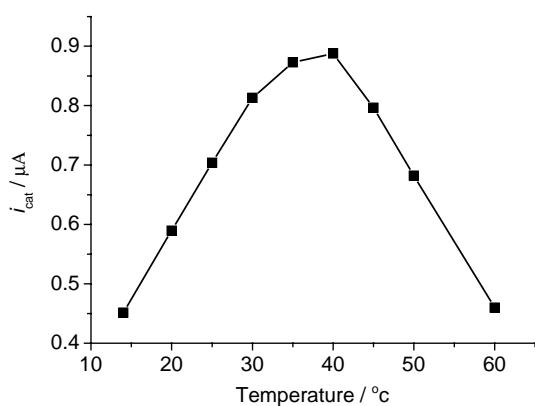


Fig. 7. Effect of temperature on amperometric response of $48\ \mu\text{M}$ H_2O_2 at pH 7.0.

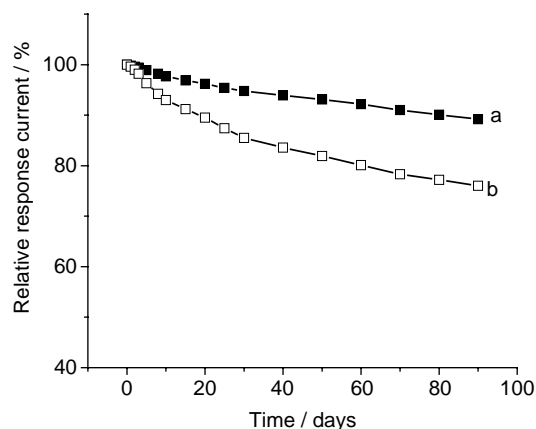


Fig. 8. Stability of the H_2O_2 biosensor stored in $0.1\ \text{M}$ pH 7.0 PBS (a) and in air (b) at $4\ ^\circ\text{C}$.

of microenvironment and its native structure upon temperature change. These results indicated that this sensor could handle in a wide range of temperature.

3.6. Stability and reproducibility of the H_2O_2 sensor

The direct electrochemistry of the cytochrome c/NaY/GCE could retain the constant current values upon the continuous cyclic sweep over the potential range from $-0.6\ \text{V}$ to $+0.6\ \text{V}$ at $100\ \text{mV}\ \text{s}^{-1}$. The immobilized cytochrome c/NaY/GCE only lost 7.7% of its initial activity after more than 300 successive measurements. Thus, NaY zeolite particles were very efficient for retaining the electrocatalytic activity of cytochrome c and preventing it from leaking out of the sensor.

The fabrication reproducibility of ten electrodes, made independently, showed an acceptable reproducibility with a relative standard deviation of 5.1% for the current determined at a H_2O_2 concentration of $48\ \mu\text{M}$. At one sensor the mean steady-state current was $0.70\ \mu\text{A}$ with a relative standard deviation of 3.2% for six determinations at a H_2O_2 concentration of $48\ \mu\text{M}$.

In addition to good reproducibility, NaY membrane imparted H_2O_2 biosensor a good long-term stability. The storage stability of H_2O_2 biosensor stored in $0.1\ \text{M}$ pH 7.0 PBS or air at $4\ ^\circ\text{C}$ was examined by checking periodically its relative response currents in PBS containing $48\ \mu\text{M}$ H_2O_2 (Fig. 8). After a storage period of 3 months in $0.1\ \text{M}$ pH 7.0 PBS the biosensor showed an 11% loss of activity.

4. Conclusions

Cytochrome c can be effectively immobilized on NaY zeolite particles to produce a fast direct electron transfer. At cytochrome c/NaY/GCE the cyclic voltammogram exhibits a pair of redox peaks corresponding to a surface-controlled electrode process with a single proton transfer. The adsorption of cytochrome c on zeolite surface depends on

the acidity of zeolite. NaY zeolite retains the activity of the immobilized cytochrome c and facilitates the electron exchange between cytochrome c and electrode. The immobilized cytochrome c displays a high affinity and sensitivity to H₂O₂. The sensor shows a good reproducibility and stability. NaY zeolite provides an efficient matrix for study of direct electron transfer of proteins and development of biosensors.

Acknowledgements

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