Biosensors and Bioelectronics 24 (2009) 3049–3054

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Direct electrochemistry of hemoglobin entrapped in cyanoethyl cellulose film and its electrocatalysis to nitric oxide

Shanshan Jia[a,c], Junjie Fei[a,c,*], Jinping Zhou[b,**], Xiaoming Chen[a,c], Jianqiang Meng[c]

[a] Key Laboratory of Environmentally Friendly Chemistry and Applications of Ministry of Education, Xiangtan University, Xiangtan 411105, China
[b] Department of Chemistry, Wuhan University, Wuhan 430072, China
[c] College of Chemistry, Xiangtan University, Xiangtan 411105, China

ARTICLE INFO

Article history:
Received 19 December 2008
Received in revised form 13 March 2009
Accepted 16 March 2009
Available online 25 March 2009

Keywords:
Cyanoethyl cellulose
Hemoglobin
Direct electrochemistry
Nitric oxide
Electrocatalysis
Biosensors

ABSTRACT

A water-soluble cyanoethyl cellulose (CEC), homogeneously synthesized in NaOH/urea aqueous solution, was used as an immobilization matrix to entrap proteins and enzymes. Then hemoglobin (Hb) was used as a template to fabricate CEC–Hb biomimetic membranes in which the Hb showed direct electrochemistry on a glass carbon electrode (GCE). The characterizations of CEC–Hb film were demonstrated by ultraviolet–visible (UV–vis) spectra, scanning electron microscopy (SEM), and electrochemical impedance spectroscopy (EIS). The electrochemical behaviors of Hb in CEC film have been investigated and a pair of well-defined and quasi-reversible cyclic voltammetric peaks for the protein heme Fe(III)/Fe(II) redox couples was observed at about −0.369 V (vs. SCE). The CEC–Hb film exhibited a good electrocatalytic activity for the reduction of nitric oxide (NO). The amperometric response of the biosensor varied linearly with the NO concentration ranging from 1.1×10^{-6} to 1.3×10^{-4} mol L^{-1}. Moreover, the studied biosensor exhibited high sensibility, good reproducibility, and long-term stability. Finally, this method has applied to monitoring the NO release from biologic samples.

1. Introduction

During the past few years, the direct electron transfer (DET) of protein or enzyme and underlying electrodes had attracted increasing attention (Katz and Willner, 2004; Elliott et al., 2002; Aguey-Zinsou et al., 2003). These studies provided an important foundation for mediator-free biosensors (Wang et al., 2005), bioreactors (Willner and Katz, 2000) and biomedical devices (Armstrong et al., 1988). To date, many research works were performed on the heme proteins (Wu and Hu, 2007). As one of heme proteins, hemoglobin (Hb) is well known for its function in the vascular system of animals, transporting oxygen from the lungs or gills to peripheral tissues. It also aids, both directly and indirectly, the transport of carbon dioxide and regulates the pH of blood. However, the direct electrochemistry of Hb on bare electrode is fairly difficult. Recently, various composite films containing Hb were modified on electrode surfaces to research its direct electrochemistry, these films may provide a favorable microenvironment for Hb and thus enhance the electron-transfer rates (Lu et al., 2005, 2006; Willner and Katz, 2000; Armstrong et al., 1988; Wu and Hu, 2007; Zhao et al., 2006; Pang et al., 2003). Entrapment or encapsulation of Hb within a biocompatible materials by using a simple procedure, especially physical entrapment of biomolecules without the need of complicated covalently attachment, is certainly desirable (Lu et al., 2006).

Nitric oxide (NO) is an intra- and extra-cellular messenger that mediates diverse signaling pathways in target cells and is known to play an important role in many physiological processes including neural signaling, immune response, inflammatory response, modulation of ion channels, phagocytic defence mechanism, penile erection and cardiovascular homeostasis along with its decompensation in atherogenesis (Moncada et al., 1988; Ignarro, 2002; Gong et al., 2004; Koshland, 1992). It is a highly diffusible inorganic radical gas and has been known for many years to be a toxic pollutant in car exhaust fumes, fossil fumes and cigarette smoke. The discovery of the biological functions of NO in the 1980s came as a complete surprise and caused quite a stir. Hence NO was named "Molecule of the Year" in 1992 by the journal *Science* (Koshland, 1992). First electrochemical electrode for detecting NO in biological samples was developed by Shibuki (1989). In 1992, a NO sensor based on a chemically modified carbon fiber electrode was reported (Malinski and Taha, 1992). Since then, the NO sensors based on chemically

* Corresponding author at: College of Chemistry, Xiangtan University, Xiangtan 411105, China. Tel.: +86 732 8292205; fax: +86 732 8292251.
** Corresponding author at: Department of Chemistry, Wuhan University, Wuhan 430072, China Tel.: +86 27 87219274; fax: +86 27 68754067.
E-mail addresses: fei_junjie@xtu.edu.cn, fei_junjie@hotmail.com (J. Fei), zhoujp325@whu.edu.cn (J. Zhou).

0956-5663/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.bios.2009.03.022

modified electrode have been widely researched by using various kinds of modifier such as NiTmPyP (Nitetra(4-*N*-methyl pyridyl) porphyrin) (Mitchell and Micheaelis, 1998), myoglobin-clay (Kroning et al., 2004), indium hexacyanoferrate (Casero et al., 2003), ionic polymers (Kitajima et al., 2001).

In the body, the synthesis of NO is involved in the reaction of various nitric oxide synthase (NOS) with L-arginine (L-Arg) in the presence of trace O_2. Production of NO plays a role in liver injury as an inhibitor or agonist of cell signaling events (Chen et al., 2003). So monitoring the NO concentration in liver is of great importance. Seldom report related to this kind of experiment based on the protein molecules modified electrode in electroanalytical chemistry field was published because of the serious interferences resulted by co-existing biological molecules in real biological samples (Wang et al., 2007).

During the past few decades, there has been an increasing interest in using natural polymers as an immobilization matrix for cell carriers, living organisms, proteins, and enzymes (Li et al., 2004; Lu et al., 2006). Cellulose is an organic compound with the formula $(C_6H_{10}O_5)_n$, a polysaccharide consisting of a linear chain of several hundred to over 10,000 β $(1 \rightarrow 4)$ linked D-glucose units. The hydroxyl groups of cellulose can be reacted with various reagents to afford derivatives with useful properties (Klemm et al., 2005). As a natural polymer, cellulose and its derivatives seem to be ideal supported materials for enzyme or protein systems, but their applications in protein immobilization are very limited (Li et al., 2004). Cyanoethyl cellulose (CEC, structure was shown in Scheme S1 of supplementary data), one of the important cellulose derivatives, is in great demand as binding polymers in inorganic ceramic electroluminescence cells, capacitors and electric components because of its high dielectric constant (Nakayama and Azuma, 1998). However, our literature search revealed that no work has been made to apply CEC as the materials for electrochemical sensors or immobilization of proteins. This may due to its shortcoming of low solubility in water or general organic solvents. Water-soluble CEC with low degree of substitution, synthesized by a simple and environmental-friendly method, has been developed in our previous work (Zhou and Zhang, 2006). When the aqueous solution of CEC was casted on electrode, a uniform film was formed and once the film formed at the glassy carbon electrode surface, it is unable to liquefy. This may be due to the excellent affinity between CEC and the glassy carbon. Therefore, the CEC film may be readily used as an immobilization matrix to entrap proteins and enzymes. We chose Hb as a model protein to investigate this system. The direct electrochemistry of Hb and the dramatically enhanced bioelectrocatalytic activity toward nitric oxide at the CEC–Hb film modified GCE were achieved. Our results showed that CEC may be ideal support material for many protein or enzyme systems, being both biosourced and biologically compatible.

2. Experimental

2.1. Reagents

Water-soluble cyanoethyl cellulose was prepared in NaOH/urea aqueous solution according the previous method (Zhou and Zhang, 2006). FTIR spectrum of the prepared CEC displayed a characteristic absorption band at around 2255 cm^{-1} (see Fig. S1 in the supplementary data), indicating that the cellulose has been cyanoethylated. Nitrogen content of CEC was determined to be 2.86%. The weight-average molecular weight (M_w) and number-average molecular weight (M_n) of CEC were determined to be 1.06×10^5 and 8.75×10^4 g/mol, respectively, by using size exclusion chromatography combined with laser light scattering (DAWN DSP, Wyatt Technology Co., United States).

Bovine hemoglobin (M_w 66,000) was purchased from the Sigma Chemical Company and used without further purification, and 5 mg/mL Hb stock solution was prepared with 0.1 M pH 7.0 phosphate buffer.

CEC was dispersed into the cold water to obtain a 2.0 mg/mL aqueous solution. Nitric oxide standard solution was freshly prepared by dropping H_2SO_4 (2 mol/L) into deoxygenated $NaNO_2$ saturated solution slowly (Butler and Williams, 1993). The concentration of NO in saturated stock solution is 1.8 mmol/L at 20 °C.

Rat (about 500 mg) was used for preparation of rat liver samples. The final biological sample was clear solution in yellow color, and the concentration of protein in the sample was 8 mg/mL which was measured by UV–vis spectroscopy. All other chemicals were of analytical grade and were used without further purification. All solutions were prepared by using double-distilled water.

2.2. Preparation of modification electrode

The glassy carbon electrode (GCE), before use, was first polished to a mirror-like with 1.0, 0.3 and 0.05 μm alumina slurry on a polish cloth, and then washed with 1:1 nitric acid/alcohol, sonicated in a deionized water bath to remove any residual alumina, and dried in the air before use. 5 mg/mL hemoglobin solution and 2 mg/mL CEC solution were mixed together according to the ratio of 1:1 (v/v). The modified working electrode was prepared by applying an 8 μL drop of a mixed CEC–Hb solution. The drop was allowed to dry under vacuum, resulting in the Hb-entrapped CEC modified GCE (CEC–Hb/GCE). The excellent affinity between CEC and the glassy carbon made this modified film was stable in solution. The surface concentration of electroactive Hb (Γ^*) could be estimated from integration of the reduction or oxidation peak in the CVs according to the following equation:

$$Q = nFA\Gamma^* \quad (1)$$

where Q is the charge involved the electrode reaction, n is the number of electron transferred, F is the Faraday's constant, and A is the effective area of the GCE. For CEC–Hb/GCE, A is estimated to be 0.082 cm^2 using $K_3Fe(CN)_6$ as a electrochemical probe.

2.3. Apparatus and measurements

The electrochemical experiments were carried out with a CHI 630C electrochemical workstation with a conventional three-electrode cell. The cell was sealed with Teflon cap with three-electrode holes and two small holes for nitrogen purging and NO solution spiking. A CEC–Hb film modified GCE was used as a working electrode. A saturated calomel electrode (SCE) was employed as the reference electrode and a platinum wire was connected as the counter electrode. For steady-state amperometric experiments were carried out in a well-stirred solution and the working potential was set at −0.8 V. The supporting electrolyte was phosphate buffer (pH 7.0, 0.1 M). For the deoxygenated experiments, all solutions were purged with high purity nitrogen for at least 30 min. During the experiments, nitrogen atmosphere was maintained in order to keep a pressure over the solution. Hence, NO is not going away from the solution. All experiments were carried out at room temperature.

UV–vis absorption spectra were performed on a Lambda 25 UV–vis spectrometer (PerkinElmer instrument, USA). All the relative measurements were carried out at ambient temperature under experimental conditions identical to those of the electrochemical studies. The Hb and Hb-CEC films for UV–vis were prepared by dropping Hb and Hb–CEL solution on a glass slide and air dried, respectively. Scanning electron microscopy (SEM) was done with a scanning electron microscopy (LEO 1530 VP, Germany). EIS measurements were carried out in a solution of 5 mmol/L

$K_3Fe(CN)_6 + K_4Fe(CN)_6$ phosphate buffer (pH 7.0) at a formal potential. The alternating voltage was 10 mV and the frequency range was 1.0×10^{-1} to 1.0×10^5 Hz.

3. Results and discussions

3.1. Characterization of CEC–Hb film

Structure variation of Hb molecules should be considered after their immobilization in CEC film. The shape and position of the Soret absorption band of heme iron can provide information on the possible denaturation of heme protein, especially the conformational change about the heme pocket (George and Hanania, 1953). UV–vis absorption spectra of Hb under different conditions are recorded. Fig. 1 illustrates the UV–vis spectra of Hb solution, Hb film and CEC–Hb film on glass slides. Film cast from Hb alone gives a Soret band at 415 nm (curve b), which is the same as that for native Hb in buffer (curve a). The Soret band of CEC–Hb film (curve c) at 415 nm was also the same as that of Hb film alone and there is no Soret band of CEC film (curve d), which indicates that no significant denaturation of Hb occurs in the presence of CEC at temperatures higher than the denaturizing temperature for the native protein. These findings confirm that CEC stabilizes the immobilized protein, and the Hb molecule retains its native conformation and thus its biological activity. Consistently, the CEC–Hb film prepared in this study shows the potential of dramatically enhancing the thermal stability of the resulting biosensor.

Scanning electron microscopy was used to compare the morphology of CEC and CEC–Hb films (see supplementary data Fig. S2). The top view of a CEC film on a GC block reveals approximately flat and featureless. While the top view of CEC–Hb film appears as dendrite. Moreover the crystal structure of CEC–Hb film shows an orderly arrangement, suggesting that the interaction between CEC and Hb indeed occurs and may even influence the morphology of the dry films.

As a powerful tool for probing the features of surface-modified electrodes, the EIS was used to study the surface-modified electrodes. The Nyquist plot of the EIS includes a semicircular portion and a linear portion. The semicircular portion at higher frequencies corresponds to the electron-transfer kinetics of the redox probe at the electrode interface. Meanwhile, the linear part at lower frequencies corresponds to the diffusion process. The typical Nyquist plots ($-Z''$ vs. Z') for the bare GCE, CEC/GCE, and CEC–Hb/GCE was obtained (see supplementary data Fig. S3). The EIS diagrams revealed that the Nyquist diameter of the CEC/GCE was much larger

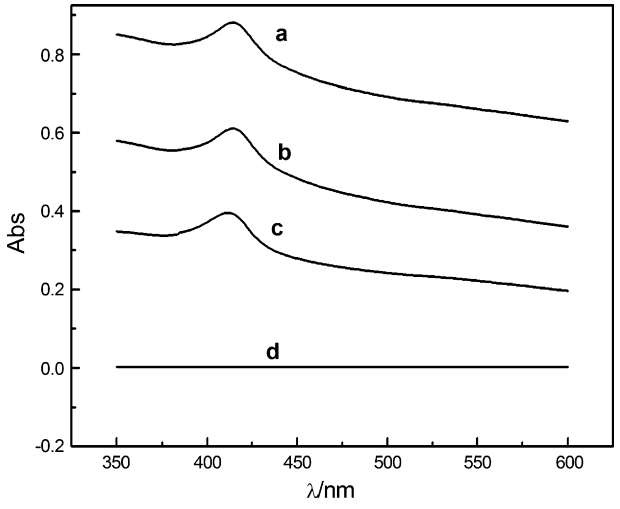

Fig. 1. UV–vis spectra of (a) Hb solution, (b) Hb film, (c) CEC–Hb film, (d) CEC film.

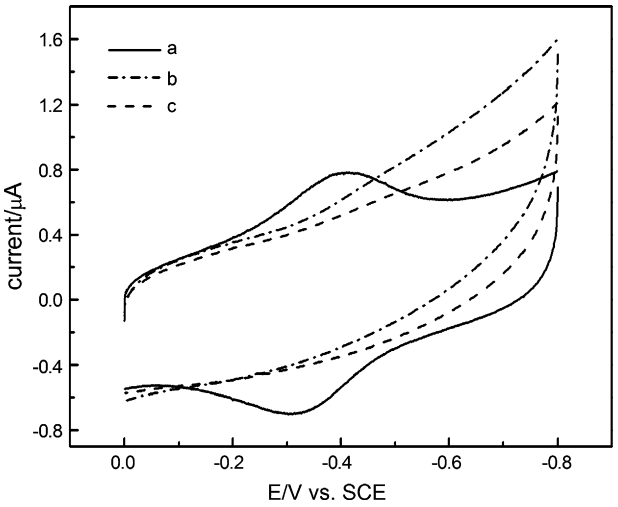

Fig. 2. Cyclic voltammograms of (a) CEC–Hb/GCE, (b) CEC/GCE, (c) bare GCE, in 0.1 M pH 7.0 phosphate buffer at a scan rate of 0.1 V s^{-1}.

than that of the bare GCE, which suggests that the CEC coated on the electrode could obstruct the electron transfer of the redox probe. Comparing with the CEC/GCE, an obvious increase in the interfacial resistance was observed at the CEC–Hb/GCE, which indicates that Hb was immobilized successfully onto the electrode.

3.2. Electrochemical characteristics of Hb immobilized in CEC films

The electrochemical behaviors of CEC–Hb/GCE, CEC/GCE, and bare GCE in pH 7.0 phosphate buffer were studied by cyclic voltammetry. As shown in Fig. 2, The CEC–Hb/GCE presents a pair of large redox peaks (curve a). However, there is no current peak in the scanned potential windows for the CEC/GCE (curve b) or bare GCE (curve c) under the same conditions. These results obviously indicate that the pair of peaks is characteristic of the Fe(III)/Fe(II) redox couple for the heme protein. The anodic peak potential (E_{pa}) and the cathodic peak potential (E_{pc}) are located at −0.336 and −0.402 V, respectively, with a formal potential $E^{\theta'}$ (defined as $(E_{pa} + E_{pc})/2$) at −0.369 V, which is similar to that previously reported for Hb entrapping into other materials, such as methyl cellulose (Li et al., 2004), Ag sol (Xu et al., 2008), pluronic films (Shan et al., 2005), zirconium dioxide nanoparticles (Liu et al., 2004) and carbon nanotube (Cai and Chen, 2004). The peak-to-peak separation (ΔE_p) is 0.066 V and the anodic and cathodic peak currents are almost equal, implying that the Hb undergoes a quasi-reversible electrochemical reaction. The obviously observed direct electron transfer from Hb in CEC film could be due to the fact that the three-dimensional structure of CEC film would provide a biocompatible microenvironment for Hb molecules, retaining the bioactivity of the Hb. Thus, it is possible to achieve fast direct electron transfer between the heme site of the immobilized Hb and electrode surface.

The cyclic voltammetric behaviors of the CEC–Hb/GC electrode in phosphate buffer (pH 7.0, 0.1 M) at different scan rates from 0.02 to 0.5 V/s were carried out (see supplementary data Fig. S4). Each voltammogram is symmetric and has a pair of well-defined redox peaks. The reduction and oxidation peak currents exhibited a linear relationship with the scan rate. Furthermore, plots of $\lg i_{pc}$–$\lg \nu$ and $\lg i_{pa}$–$\lg \nu$ have a ratio of the slopes (Si_{pc}/Si_{pa}) of about 1. All these results suggesting that the reaction was a surface-confined electrochemical process (Dai et al., 2004), which all electroactive ferric Hb (Hb–Fe(III)) produced by the oxidation of ferrous Hb (Hb–Fe(II)) on the anodic scan could be reduced to Hb–Fe(II) on the cathodic scan. In the range of scan rate from 0.02 to 0.5 V/s, the average Γ^*

value was 2.19×10^{-11} mol/cm^2. The value obtained in this paper is about 1.2 times higher than that of the theoretical monolayer coverage (1.89×10^{-11} mol/cm^2) (Wang et al., 2005). This shows that several layers of Hb entrapped in CEC film participated in the electron-transfer process.

When $n\Delta E_p > 0.2$ V, the kinetics of the heterogeneous electron transfer for the CEC–Hb films could be analyzed using the model of Laviron (1979). Laviron derived a simple relationship (Eq. (2)) between the logarithm of electron-transfer rate constant (K_s) at 0 overpotential and the scan rate

$$\log K_s = \alpha \log(1-\alpha) + (1-\alpha)\log\alpha - \log\frac{RT}{nF\nu} - \alpha(1-\alpha)\log\frac{nF\Delta E_p}{2.3RT} \quad (2)$$

where α is the charge transfer coefficient, n is the number of electrons transferred, F is the Faraday's constant, ν is the scan rate, R is the gas constant, and T is the absolute temperature. α was estimated to be 0.45 ± 0.02 ($n = 4$) from slope of E_p versus $\log \nu$ plot according to Laviron study (Laviron, 1979). Hence, the direct electron-transfer rate constant K_s of Hb in the CEC–Hb/GCE is calculated to be 1.10 ± 0.05 s^{-1}, suggests reasonably fast electron transfer.

The CVs of CEC–Hb/GCE exhibited a strong dependence on the pH of phosphate buffer and both the anodic and cathodic peak potentials shift negatively with the increase of pH (see supplementary data Fig. S5). An asymmetric redox CV was obtained at pH 4.0. Subsequently, the same CV obtained at pH 7.0 could reproduce when the same Hb modified electrode was put back to the pH 7.0 buffer. This was likely due to a reversible pH-induced conformational change of Hb at low pH. Moreover, E_{pa}, E_{pc} and $E^{\theta'}$ dependent on the solution pH linearly with the slope of −52.3, −43.2 and −51.0 mV/pH, respectively. These slope values are close to the theoretical value of −59 mV/pH for a reversible one-electron transfer coupled by single-proton transportation (Bond, 1980). Thus, the reaction scheme for the electrochemical reduction and oxidation of Hb can be represented in general terms by:

$$\text{HbhemeFe(III)} + \text{H}^+ + \text{e}^- \leftrightarrow \text{HbhemeFe(II)} \quad (3)$$

Those slope values were smaller than the theoretical value of −59 mV/pH for a single-proton coupled, reversible one-electron transfer, because of the influence of the protonation of trans and residue ligands around the heme and the protonation of the water molecules coordinated with the central iron (Yamazaki et al., 1978).

3.3. Electrocatalytic behaviors of CEC–Hb/GCE to the reduction of NO

In order to study the electrocatalytic activity of Hb immobilized in CEC–Hb film, its response to the reduction of nitric oxide was explored. The CVs of CEC–Hb film modified electrode in the absence and presence of NO are shown in Fig. 3. In the absence of NO, only the redox peaks of Hb are observed (curve a). Upon the addition of 2.25 μM NO into pH 7.0 phosphate buffer, the voltammetric behavior of the CEC–Hb/GCE changes dramatically, with a obvious catalytic reduction peak located at −0.83 V (curve c). For comparison, no evident reduction peak of NO is observed on the CEC film modified electrode (curve d). These results show that the immobilized Hb retains its electrocatalytic activity to reduce NO diffusing from solution onto CEC–Hb film.

The potential dependence of NO response (dynamic potential voltammogram) at a CEC–Hb/GCE was performed. The results showed that the reduction currents increase with the increasing potentials, and it finally reach a steady response at about 0.80 V. Hence the amperometric response of the biosensor to successive additions of 2.0 μM NO in 0.1 M phosphate buffer (pH 7.0) was carried out at an applied potential of −0.80 V (vs. SCE) and the results

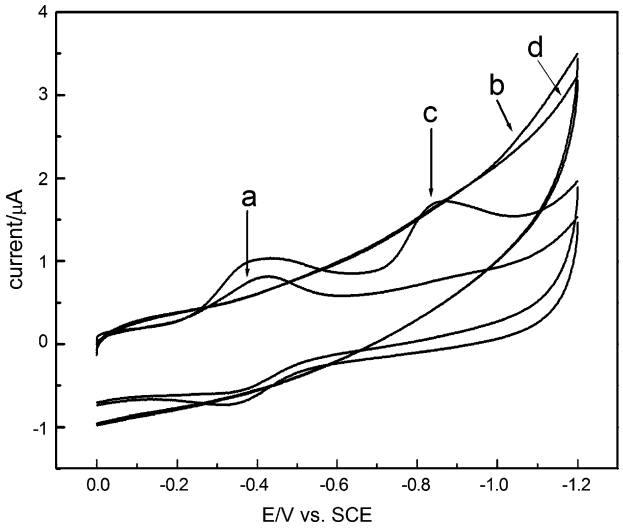

Fig. 3. Cyclic voltammograms of different modified electrodes in 0.1 M pH 7.0 phosphate buffer at scan rate of 0.1 V s^{-1} CEC–Hb (a, c) and CEC films (b, d) in the absence of NO (a, b) and in the presence of 2.25 μM NO (c, d).

are presented in Fig. 4. Consistent with the voltammetric measurement result, the biosensor shows a rapid and sensitive response to the addition of NO. Upon the addition of NO, the reduction current reached a stable value in few seconds indicating of the fast response of the biosensor. The reduction current response is proportional to the concentration of NO as shown in inset of Fig. 4 in the concentration ranges from 1.12×10^{-6} to 4.72×10^{-5} mol L^{-1} and from 4.72×10^{-5} to 1.26×10^{-4} mol L^{-1} with linear regression equations as following, respectively:

$$i(\mu\text{A}) = 0.011 + 0.025C\,(\mu\text{mol L}^{-1}) \quad (R = 0.999)(n = 9)$$

and

$$i(\mu\text{A}) = 0.47 + 0.016C\,(\mu\text{mol L}^{-1}) \quad (R = 0.996)(n = 9)$$

Different slopes of two curves suggested that there was some progressive enzyme inactivation in the presence of higher concentration of NO. In addition, the detection limit of the biosensor is 2.0×10^{-8} mol/L which is based on five times measurement for the standard deviation of the blank noise (95% confidence level, $k = 3$,

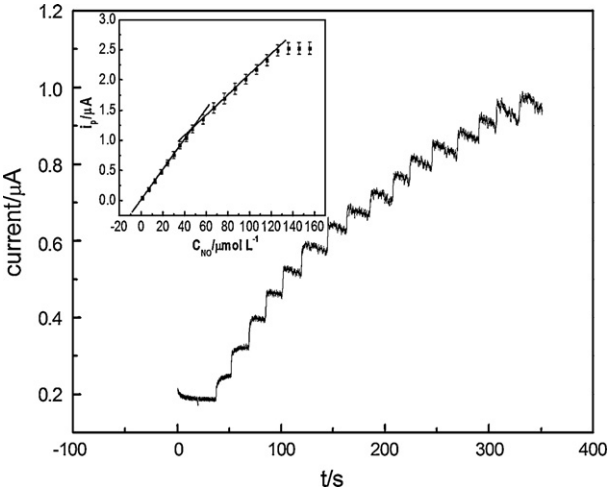

Fig. 4. Amperometric responses of CEC–Hb/GCE in 0.1 M pH 7.0 phosphate buffer to successively injecting of 2.0 μM NO, applied potential: −0.8 V. The inset is a plot of the current of NO against its concentrations (the error bars presented in this plot were calculated from five calibration curves).

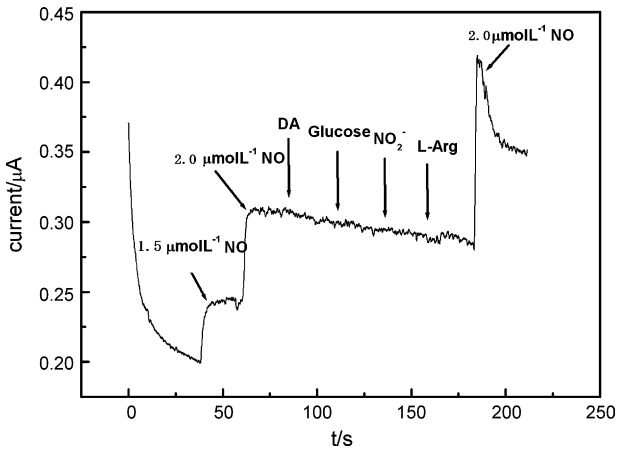

Fig. 5. Amperometric response of the CEC–Hb/GCE to NO in the presence of some coexisting compounds (6.0×10^{-6} mol/L DA, 1.5×10^{-5} mol/L glucose, 5.0×10^{-5} mol/L NO_2^-, 3.0×10^{-4} mol/L L-Arg) in 0.1 M phosphate buffer (pH 7.0), applied potential: −0.80 V.

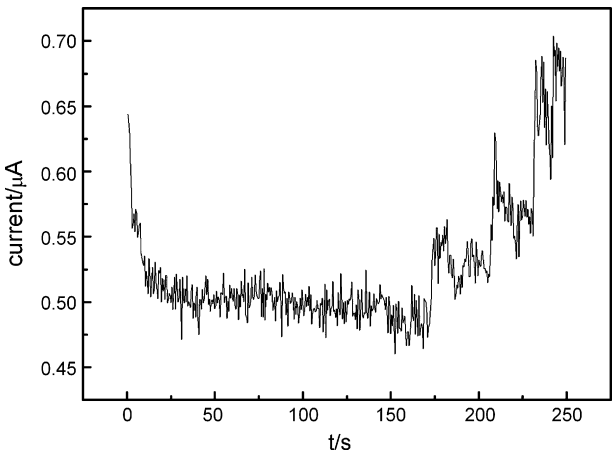

Fig. 6. Amperometric response of the CEC–Hb/GCE to NO released from rat liver mitochondria with adding 0.8 mM L-Arg, applied potential: −0.80 V.

$n = 5$). It was clear that there was a current plateau when the concentration of NO was high enough to be 1.26×10^{-4} mol/L, exhibiting a characteristic of the Michaelis–Menten kinetic mechanism. According to the Lineweaver–Burk equation:

$$i_{ss}^{-1} = i_{max}^{-1} + K_s^{app} c^{-1} i_{max}^{-1} \quad (4)$$

where i_{ss} is the steady-state current after the addition of NO, c is the bulk concentration of the substrate, and i_{max} is the maximum current measured under saturated NO conditions, the apparent Michaelis–Menten constant, K_m^{app}, representing dissociation constant (affinity for substrate) of the enzyme–substrate complex for enzyme reactions exhibiting simple Michaelis–Menten kinetics from the point of view of biology, just is an indicator of the affinity that Hb has for a NO, and the stability of the Hb–NO complex in present work. Here, it could be derived from the plot of steady-state current versus NO concentration with a result of 75.39 μmol/L, which is similar to those on hemoglobin/montmorillonite/polyvinyl alcohol multi-assembly at a PG electrode surface (Pang et al., 2003) and on Hb/cationic gemini surfactant film composite film modified glassy carbon electrode (Wang et al., 2007). The results clearly indicated that Hb molecules immobilized in CEC films retained their bioactivity and exhibit high affinity for NO.

3.4. Selectivity, stability, reproducibility and real samples assay

The selectivity of this NO biosensor was evaluated by NO measurements in the presence of some small biological molecules. Fig. 5 clearly indicates that these interference compounds at corresponding concentrations did not influence the detection of NO. The experimental results also showed that at least 1.0×10^{-5} mol/L dopamine (DA), 4.0×10^{-4} mol/L glucose, 1.0×10^{-4} mol/L nitrite (NO_2^-), 5.0×10^{-4} mol/L L-arginine (L-Arg), do not interfere with the determination of 2.0×10^{-6} mol L^{-1} NO within an error of 5%. We propose that Hb in the CEC–Hb film can exhibit a fine enzymatic characterization and a high catalytic activity to NO, which makes Hb-based NO biosensor to have a good selectivity and real biological samples assay.

As an essential parameter, the stability of this NO biosensor has been examined. After the modified electrode was exposed to air for 10 days at 4 °C the current response decreases by less than 12% of its initial current response to the reduction of NO (see supplementary data Fig. S6), which showed that the sensitivity of this modified electrode was decreasing over time. Thus, the biosensors were stored at 4 °C when not in use. The reproducibility of the CEC–Hb film modified GCE was estimated in phosphate buffer (pH 7.0) containing 4.5×10^{-6} mol/L NO and 1.2×10^{-6} mol/L NO. The relative standard deviation (RSD) for 10 repeatedly detections of NO with the same Hb modified electrode was calculated to be 2.1% and 3.6%, respectively. At the same time, RSD for seven repeatedly detections of NO with different Hb modified electrodes was calculated to be 6.8%. These results indicate that the NO biosensor possesses good stability and reproducibility, which originates from the essence of the preparing method.

Considering the good selectivity and stability of this biosensor, we tried to monitor the NO release from rat liver and just passable results were obtained as shown in Fig. 6. The addition of 0.80 mmol/L L-Arg in 2.0 mL biological samples resulted in the apparent increase of current responses under stirring condition. As it can be seen from Fig. 5, this biosensor showed no current response to L-Arg. So the change of current signal was corresponded to the reduction of NO released from the rat liver sample. NOS was activated since the addition of L-Arg and then activated NOS was converted into NO in the presence of trace O_2. A maximum value of the current signal change could be monitored within 20 s and after the addition of L-Arg, and then the current signal decayed slowly. This response time was satisfying as expected. L-Arg was successively injected for three times but only two similar current responses could be observed while the last time was in lost because of the obvious noise. The concentration of NO released from the rat liver sample was about from 1.3 to 2.5 μmol/L. This experiment provided a chance to construct perfect NO biosensor, which could be applied for the NO detection in vivo.

4. Conclusion

In summary, water-soluble CEC, synthesized by a simple and environmental-friendly method, was firstly used to immobilize Hb. The CEC film provides a good microenvironment for the immobilization of proteins, while retaining biological activity of Hb for the direct electron transfer between protein and electrode surface. Based on this, a novel biosensor has been developed without any sort of electron mediator. These biosensors showed excellent catalytic activity toward the reduction of NO and high affinity for NO. In view of the simplicity of fabrication, high stability and sensitivity, this configuration may provide a new aspect toward understanding the kinetics and thermodynamics of biological redox processes and a promising platform for the study of direct electron transfer in proteins and the development of NO biosensors in the future.

Acknowledgements

This work was supported by the Key Project of Chinese Ministry of Education (No. 206104), Hunan Provincial Natural Science Foundation of China, and the Open Project Program of Key Laboratory of Environmentally Friendly Chemistry and Applications of Ministry of Education.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.03.022.

References

Aguey-Zinsou, K.F., Bernhardt, P.V., De Voss, J.J., Slessor, K.E., 2003. Chem. Commun. 3, 418–419.
Armstrong, F.A., Hill, H.A.O., Walton, N.J., 1988. Acc. Chem. Res. 21, 407–413.
Bond, A.M., 1980. Modern Polarographic Methods in Analytical Chemistry. Marcel Dekker, New York, pp. 29–30.
Butler, A.R., Williams, D.L., 1993. Chem. Soc. Rev. 4, 233–241.
Cai, C.X., Chen, J., 2004. Anal. Biochem. 325, 285–292.
Casero, E., Pariente, F., Lorenzo, E., 2003. Anal. Bioanal. Chem. 375, 294–299.
Chen, T., Zamora, R., Zuckerbraun, B., Billiar, T.R., 2003. Curr. Mol. Med. 3, 519–526.
Dai, Z.H., Liu, S.Q., Ju, H.X., Chen, H.Y., 2004. Biosens. Bioelectron. 19, 861–867.
Elliott, S.J., McElhaney, A.E., Feng, C., Enemark, J.H., Armstrong, F.A., 2002. J. Am. Chem. Soc. 124, 11612–11613.
George, P., Hanania, G., 1953. Biochem. J. 55, 236–243.
Gong, L., Pitari, G.M., chulz, S.S., Waldman, S.A., 2004. Curr. Opin. Hematol. 11, 7–14.
Ignarro, L.J., 2002. J. Physiol. Pharmacol. 53, 503–514.
Katz, E., Willner, I., 2004. Angew. Chem. Int. Ed. 43, 6042–6108.
Kitajima, A., Teranishi, T., Miyake, M., 2001. Electrochemstry 69, 16–20.
Klemm, D., Heublein, B., Fink, H.P., Bohn, A., 2005. Angew. Chem. Int. Ed. 44, 3358–3393.
Koshland, D.E., 1992. Science 258, 1861–1865.
Kroning, S., Scheller, F.W., Wollenberger, U., Lisdat, F., 2004. Electroanalysis 16, 253–259.
Laviron, E., 1979. J. Electroanal. Chem. 101, 19–28.
Li, Y.M., Liu, H.H., Pang, D.W., 2004. J. Electroanal. Chem. 574, 23–31.
Liu, S.Q., Dai, Z.H., Chen, H.Y., Ju, H.X., 2004. Biosens. Bioelectron. 19, 963–969.
Lu, Q., Hu, S.S., Pang, D.W., He, Z.K., 2005. Chem. Commun. 20, 2584–2585.
Lu, X.B., Hu, J.Q., Yao, X., Wang, Z.P., Li, J.H., 2006. Biomacromolecules 7, 975–980.
Malinski, T., Taha, Z., 1992. Nature 358, 676–678.
Mitchell, K.M., Micheaelis, E.K., 1998. Electroanalysis 10, 81–88.
Moncada, S., Radomski, M.W., Palmer, R.M., 1988. Biochem. Pharmacol. 37, 2495–2501.
Nakayama, E., Azuma, J.I., 1998. Cellulose 5, 175–185.
Pang, J.T., Fan, C.H., Liu, X.J., Chen, T., Li, G.X., 2003. Biosens. Bioelectron. 19, 441–445.
Shan, W.J., He, P.L., Hu, N.F., 2005. Electrochim. Acta 51, 432–440.
Shibuki, K., 1989. Brain Res. 487, 96–104.
Wang, F., Chen, X.X., Xu, Y.X., Hu, S.S., Gao, Z.N., 2007. Biosens. Bioelectron. 23, 176–182.
Wang, S.F., Chen, T., Zhang, Z.L., Shen, X.C., Lu, Z.X., Pang, D.W., Wong, K.Y., 2005. Langmuir 21, 9260–9266.
Willner, I., Katz, E., 2000. Angew. Chem. Int. Ed. 39, 1180–1218.
Wu, Y.H., Hu, S.S., 2007. Microchim. Acta 159, 1–17.
Xu, Y.X., Hu, C.G., Hu, S.S., 2008. Sens. Actuators B 130, 816–822.
Yamazaki, I., Araiso, T., Hayashi, Y., Yamada, H., Makino, R., 1978. Adv. Biophys. 11, 249–281.
Zhou, J.P., Zhang, L.N., 2006. Preparation of cyanoethyl celluloses. Chinese Patent Appl. No.: CN 200610124958.7.
Zhao, G., Xu, J.J., Chen, H.Y., 2006. Electrochem. Commun. 8, 148–154.