

Electron transfer reactivity and the catalytic activity of horseradish peroxidase incorporated in dipalmitoylphosphatidic acid films

Xinjian Liu, Yinxi Huang, Libin Shang, Xiaoyue Wang, Han Xiao, Genxi Li*

Department of Biochemistry and National Key Laboratory of Pharmaceutical Biotechnology, Nanjing University, Nanjing 210093, PR China

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Abstract

Horseradish peroxidase (HRP) was incorporated in dipalmitoylphosphatidic acid (DPPA) to form a film and the film was modified on pyrolytic graphite electrode. UV–Vis spectra suggested that HRP in the film could keep its secondary structure similar to the native state. A pair of stable, well-defined, and quasi-reversible cyclic voltammetric peaks was observed with the formal potential at -276.2 mV (vs. saturated calomel electrode), characteristic of heme $\text{Fe}^{\text{III}}/\text{Fe}^{\text{II}}$ redox couple of HRP. The apparent heterogeneous electron transfer rate constant and other electrochemical parameters were presented. The catalytic activity of HRP in DPPA film toward oxygen, hydrogen peroxide and nitric oxide were also examined.

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Keywords: Horseradish peroxidase; Dipalmitoylphosphatidic acid; Hydrogen peroxide; Nitric oxide; Cyclic voltammetry

1. Introduction

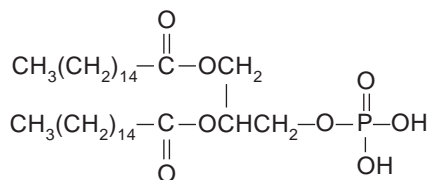
Direct electron transfer between proteins (enzymes) and electrode is of great importance because of not only theoretical but also practical interest. Research on direct electrochemistry of proteins may provide a model for the investigation of electron transfer reactivity in biological systems [1,2]. Also, an enzyme immobilized on electrode without mediator(s) would permit the electrochemical measurement of the substrate with more feasibility, such as low interference, high sensitivity, good stability and reproducibility, etc. [3,4].

By means of immobilizing proteins into some kinds of films, direct electrochemistry of the macromolecules can be achieved more easily and efficiently [5–13]. As is well known, the electron transfer activities of many proteins (enzymes) are closely related to the membrane environment. In order to get their direct electrochemistry, it is very necessary to construct a biomembrane-like microenviron-

ment which is beneficial to the electron transfer of redox proteins (enzymes). Based on this thought, there has been an increasing interest recently in using biomembrane-mimic materials as matrix for immobilizing enzymes [14–19]. A long-term goal of this kind of research is to make stable films on electrodes with good enzyme activity, which is helpful for a variety of electrochemical, spectroscopic, and other studies. Others and we have fabricated lots of films in which incorporated redox proteins (enzymes) demonstrate direct and quasi-reversible voltammetry [19–25]. All these films have effectively enhanced the direct electron transfer between the proteins and electrodes.

Here we will report a new film of lipid, based on the fact that many proteins (enzymes) acting as electron carriers have known to be embedded in biomembranes which are primarily composed of lipids. Recent research indicates that besides being used as matrix for proteins, the lipid bilayer structure have further biological functions. For instance, cytochrome *c* gets *N*-demethylase activity after incorporated in lipid bilayer [26]. Also, clear evidence has shown that proteins in lipid membranes can remain the structure similar to their native states.

* Corresponding author. Tel.: +86 25 83593596; fax: +86 25 83592510.
E-mail address: genxili@nju.edu.cn (G. Li).



Scheme 1.

Dipalmitoylphosphatidic acid (DPPA), an anionic-type lipid, is able to form a bilayer-like arrangement as in membranes structure (Scheme 1). Its merit such as the biodegradability, non-toxicity, low cost, readily availability, and especially its well-defined, three-dimensional network has made it a significant material for various purposes [27–29]. We then expect that DPPA would be a good film-forming material for the immobilization of redox proteins (enzymes), and thus, for the study of their electrochemical and catalytic properties.

Horseradish peroxidase (HRP) is a heme-containing glycoprotein. It has been intensively studied with electrochemical method, although its direct electrochemistry is relatively difficult [18,25,30–33]. In this paper, we report that the direct electron transfer reactivity of HRP can be obtained after it is incorporated in DPPA films. Furthermore, oxygen (O_2), hydrogen peroxide (H_2O_2), and nitric oxide (NO) can be electro-catalytically reduced at HRP–DPPA-modified electrodes, showing the potential applicability as biosensors. The possible catalytic mechanism for the reduction of H_2O_2 has also been presented.

2. Experimental

Lyophilized HRP (MW 42,000) was from Shanghai Chemical Reagent Company. Dipalmitoylphosphatidic acid (DPPA) was from Sigma. They were all used without further purification. Pyrolytic graphite (PG) was purchased from Shanghai Xinxing Carbon Co. Ltd (Shanghai, China). All other chemicals were of reagent grade. Their stock solutions were stored in a refrigerator at a temperature of 4 °C. H_2O_2 was freshly prepared before being used. Water was purified with a Milli-Q purification system to a specific resistance $>16 \text{ M}\Omega \text{ cm}^{-1}$ and was used to prepare all the solutions.

The supporting electrolyte was 0.1 M HAc–NaAc (pH 4.0–5.0) buffer solution containing 0.1 M KBr. Other buffers were 0.05 M potassium dihydrogen phosphate (pH 5.0–8.0), 0.1 M boric acid (pH 8.0–10.0), and 0.1 M citric acid (pH 3.0–4.0), all containing 0.1 M KBr. The pH values of buffers were regulated with HCl or NaOH solutions.

Cyclic voltammetry was performed with a PARC 263 Potentiostat/Galvanostat (EG&G, USA). A three-electrode configuration was employed. The cell was a 10-mL, single-electrolyte compartment. The working electrode was a PG disk electrode ($A=6.25 \text{ mm}^2$). The PG electrode was made as follows: Press a PG rod (geometric area: 6.25 mm^2) into a glass tube (with a diameter of 5 mm) and put epoxy resin at

the glass/rod interface to fix it. Electrical contact was made by adhering a copper wire to the rod with the help of Wood alloy. A saturated calomel electrode (SCE) was used as the reference electrode and all potentials reported here were referred to this electrode. A platinum wire electrode served as the counter electrode.

Because DPPA could not be dissolved or suspended in water, we prepared DPPA suspension (1.0 mg mL^{-1}) by dispersing DPPA in ethanol solvent with ultrasonication for about 5 min. Before preparing the HRP–DPPA film, the dispersion was ultrasonicated for another 2 min.

The substrate PG electrode was first polished using rough and fine sandpapers. It was then polished to mirror smoothness with an alumina (particle size of about 0.05 mm^2)/water slurry on silk. After that, it was ultrasonicated in both water and ethanol for about 2 min. A mixture of HRP and DPPA was spread evenly onto the surface of the substrate PG disk electrode to prepare the HRP–DPPA film-modified electrode. The best amount of the mixture and the best proportion is $10 \mu\text{L}$ of 0.1 mM HRP combined with $10 \mu\text{L}$ of 1.0 mg mL^{-1} DPPA. Alternatively, only DPPA or only HRP was cast onto the PG electrode.

An Eppendorf tube was fitted over the electrode for 2 h to ensure that water evaporated slowly and a more uniform membrane structure could be formed. This electrode was then dried overnight at room temperature. Finally, the electrode was thoroughly rinsed with pure water and was ready for use. The modified electrode was kept in a phosphate buffer solution with pH 7.0 at 4 °C when not in use.

UV–Vis absorption spectroscopy was performed using a UV-2201 spectrophotometer (Shimadzu; Kyoto Japan). The spectra were recorded with 0.10 mg mL^{-1} HRP in various solutions.

3. Results and discussion

The location of the Soret band of iron heme can provide information about the conformation of heme proteins. A shift or disappearance of the Soret band may indicate some structural changes or possible denaturation of proteins [34,35]. Thus, UV–Vis spectroscopy has been used to inspect the conformation alterations of HRP incorporated in DPPA films. The spectrum of 0.10 mg mL^{-1} HRP solution shows a Soret band at 405.8 nm, while it locates at 407.4 nm after HRP is dissolved in DPPA solution (Fig. 1). A red shift of only 1.6 nm is observed, indicating that although some changes in the microenvironment of the protein around the heme take place, HRP basically maintains its native secondary structure after being entrapped in DPPA. When merely mixed with ethanol in proportion of 1:1, the Soret band of HRP appears at 406.6 nm, shifting 0.8 nm, which indicates a slighter change compared to that of HRP–DPPA solution. In contrast, the Soret band will shift largely and decrease greatly if HRP is denatured by 8 mol L^{-1} urea

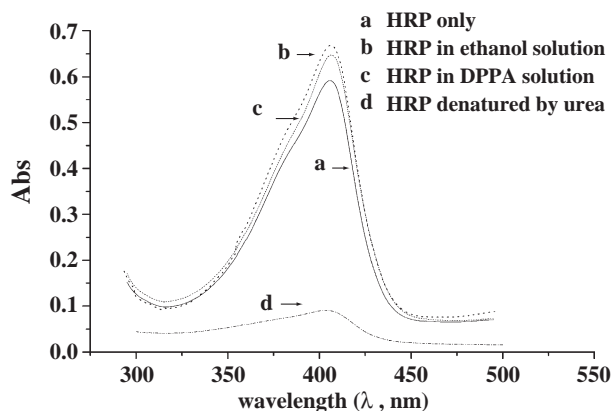


Fig. 1. UV–Vis absorption spectra of (a) 0.10 mg mL^{-1} HRP in HAc–NaAc buffer solution, (b) 0.10 mg mL^{-1} HRP in ethanol–water (v/v 1:1) solution, (c) 0.10 mg mL^{-1} HRP in ethanol–water (v/v 1:1) solution containing 0.10 mg mL^{-1} DPPA, and (d) 0.10 mg mL^{-1} HRP denatured by 8 M urea.

(Fig. 1d). Therefore, ethanol can be used to suspend DPPA for the preparation of HRP–DPPA film-modified electrode.

As is shown in Fig. 2a and b, no voltammetric peak can be observed either at a bare PG electrode or at a DPPA-alone-modified PG electrode. Fig. 2c shows the cyclic voltammogram (CV) of the PG electrode coated with HRP–ethanol in a pH 4.0 HAc–NaAc buffer solution in the potential scan range of -900 and 200 mV at a scan rate of 200 mV s^{-1} . It displays a small pair of peaks at -359.4 mV for E_{pc} and -286.1 mV for E_{pa} , which is caused by the redox reaction of HRP. This result is consistent with the UV–Vis absorption spectroscopic studies, which suggests some small effects of ethanol on HRP structure. A pair of well-defined, quasi-reversible CV peaks can be observed for HRP–DPPA films (Fig. 2d). The peaks are much larger than those of HRP treated only with ethanol, indicating a significant facilitating effect of DPPA. These results indicate that DPPA membrane provides a mimic environment for the

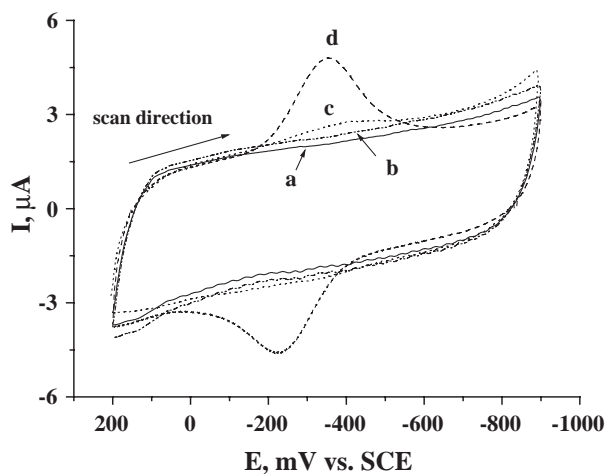


Fig. 2. CVs of (a) bare PG electrode, (b) coated with DPPA, (c) coated with HRP in ethanol solution, and (d) coated with HRP in DPPA solution. Scan rate: 200 mV s^{-1} ; the pH value of buffer solution: 4.0.

functioning of proteins, in which the native conformations of the proteins are retained and the electron-transfer rates are greatly enhanced compared with those involving proteins at bare electrode. The peaks are characteristics of heme $\text{Fe}^{\text{III}}/\text{Fe}^{\text{II}}$ redox couple [11,21,23]. The formal potential, estimated from its half wave peak potential, is at about -276.2 mV (vs. SCE), similar to our previous report [11].

For quasi-reversible electrochemistry, according to the Laviron equation [36], there is a linear relationship between the reduction peak potential and the natural logarithm of scan rate (Fig. 3). From the slope of the straight line, we get $\alpha \times n$ to be 0.5335, in which α stands for transfer coefficient and is normally between 0.3 and 1. So n equals 1, which is the number of electrons transferred during the redox procedure.

According to the integrals of the reduction peaks and Faraday laws, the surface concentration of electroactive HRP in the films (Γ^*) is estimated according to the following formula:

$$I_p = \frac{n^2 F^2 A \Gamma v}{4RT}$$

I_p is the reduction peak current; n is the electron transfer number; A is the electrode area; Γ is the surface concentration of electroactive HRP; F , v , R , and T are defined as forenamed. In this study, $I_p = 1.8 \times 10^{-6} \text{ A}$; $n = 1$; $A = 6.25 \times 10^{-2} \text{ cm}^2$; $F = 96,500 \text{ C mol}^{-1}$; $v = 0.2 \text{ V s}^{-1}$; $R = 8.314 \text{ J K}^{-1} \text{ mol}^{-1}$; $T = 298.15 \text{ K}$. So the value of Γ can be calculated to be $1.5 \times 10^{-10} \text{ mol cm}^{-2}$. The amount of electroactive HRP is higher than $5.0 \times 10^{-11} \text{ mol cm}^{-2}$ which is the saturated concentration of HRP in one layer [30]. This suggests multiple but not single layers of HRP coated on the electrode. Compared with $5.1 \times 10^{-11} \text{ mol cm}^{-2}$ of HRP concentration in DNA films [37], DPPA films are more efficient, i.e., more proportion of electroactive HRP are present in HRP–DPPA films.

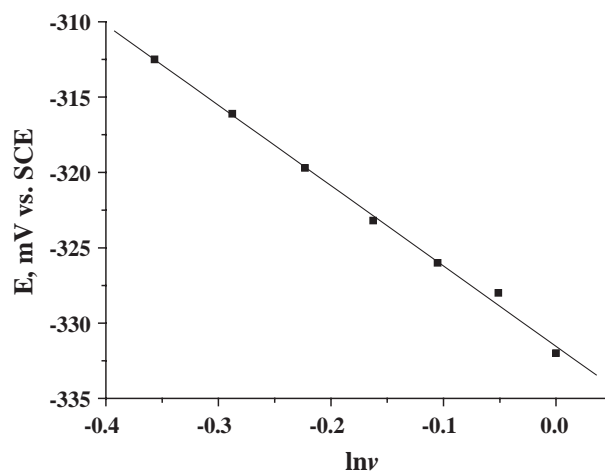


Fig. 3. Relationship between the reduction peak potential of HRP–DPPA films and the natural logarithm of scan rate. The pH value of buffer solution: 4.0.

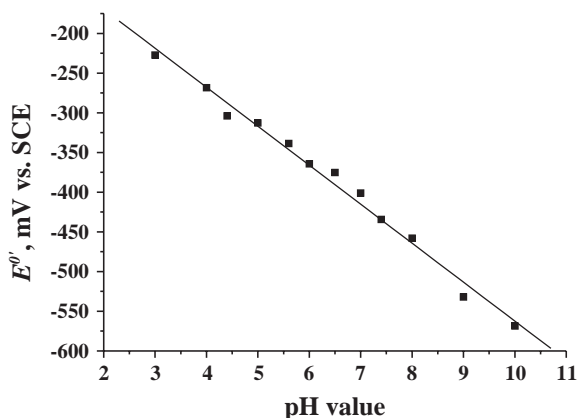


Fig. 4. Relationship between the formal potentials of HRP–DPPA films and the pH value of buffer solution. Scan rate: 200 mV s^{−1}.

At the scan rate ranging from 0.01 to 1 V s^{−1}, ΔE is less than 200 mV without exception. When $n \times \Delta E < 200$ mV, the apparent heterogeneous electron transfer rate constant (k_s) can be estimated by using the formula [36]:

$$k_s = \alpha n F v / RT$$

in which F stands for the Faraday constant, v stands for scan rate, R stands for molar gas constant, and T stands for thermodynamic temperature. When scan rate is 0.2 V s^{−1}, k_s equals to 4.07 s^{−1}, and the average value of k_s , while scan rate is ranged from 0.01 to 1 V s^{−1}, is 11.2 s^{−1}, is much higher than that of HRP–DNA films which is 1.13 s^{−1}, indicating a faster electron transfer. This result suggests that compared with DNA, DPPA can much more feasibly facilitate the electron transfer of HRP with the sPG electrode.

Fig. 2 also shows that CVs of HRP embedded in DPPA films have basically symmetric peaks shapes, and nearly equal heights of the reduction and oxidation peaks can be obtained. On the other hand, the peak separation (ΔE) is less than 100 mV at a scan rate of 200 mV s^{−1}, and the reduction and oxidation peaks currents are found to increase linearly with the potential scan rate from 10 to 900 mV s^{−1}. All these results are characteristic of quasi-reversible, diffusion-less, thin-layer electrochemistry [38].

Electrochemical behaviors of HRP–DPPA films at different pH values have been examined. HRP in DPPA films can give well-defined reversible CVs in a wide range of pH, and the effect of pH on CVs is also reversible, namely, we can get almost the same figures when the modified electrode is returned to the former buffer solution, after having been put into another buffer solution with different pH values. On the other hand, the peaks currents of the HRP–DPPA-modified electrode will increase by decreasing the pH value of the solution. We have also checked the stability of the modified electrode in the pH range from 3.0 to 10.0 and have found that the modified electrode is more stable at pH 4.0–7.0 than at the other pH values. So we select pH 4.0 in the following experiments.

A negative shift in potentials is found for both the reduction and oxidation peaks potentials with the increase of pH value. Fig. 4 shows that the formal potentials ($E^{0'}$), estimated as the midpoint of CV reduction and oxidation peak potentials of heme Fe^{III}/Fe^{II} redox couple, have a linear relationship with pH with a slope of -49.6 mV pH^{−1} for HRP–DPPA films (pH 3.0–10.0). This slope value is close to the theoretical value of -59 mV pH^{−1} for a reversible proton-coupled single-electron transfer [23]. Though the mechanism is still unclear, the linear relationship between $E^{0'}$ and pH at least suggests that the electron transfer between the electrode surface and the protein is accompanied by proton transfer.

O₂, H₂O₂, and NO can all be catalytically reduced by HRP in DPPA films. The catalytic reduction of O₂ is first examined. It has been known that a pair of peaks characteristic of the heme Fe^{III}/Fe^{II} redox couple can be obtained at -450 mV and -355 mV for a pH 4.0 HAc–NaAc buffer. After being injected a certain volume of air into the buffer solution, a new reduction peak appears at about -310 mV (Fig. 5). Meanwhile, the peaks of the heme Fe^{III}/Fe^{II} redox couple diminish gradually with the addition of O₂, implying a fast electrocatalysis process. Furthermore, the height of this peak increases with the addition of air and reaches a plateau at a high concentration. The oxygen concentration is determined according to the previous studies [39]. Fig. 5 inset shows the linear dependence of the reduction peaks currents on the O₂ concentration in the range of 1.9×10^{-5} to 8.8×10^{-4} mol L^{−1}. For the electrodes modified merely with DPPA, one peak for the reduction of O₂ at about -700 mV can be noticed, far more negative than that of the catalytic peak (figure not shown). Therefore, it is HRP entrapped in DPPA films that significantly decreases the activation energy for the reduction of O₂.

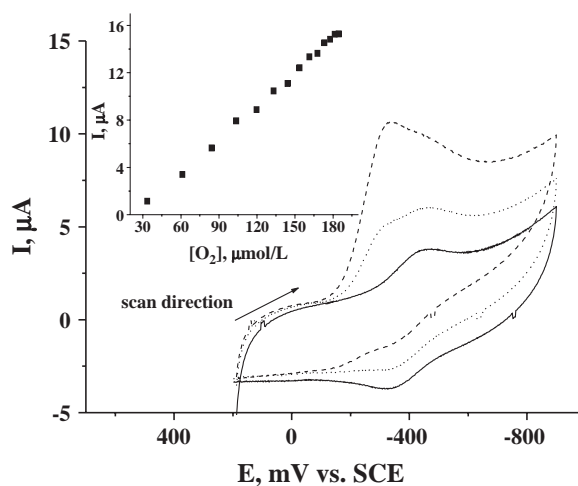
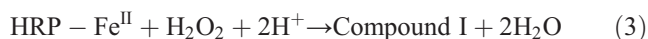


Fig. 5. CVs obtained at an HRP–DPPA-modified PG electrode containing a series of concentrations of O₂. Inset is the plot of the catalytic peak current vs. the concentration of O₂. Others are the same as in Fig. 2.

We have then investigated the electrocatalytic activity of HRP toward H_2O_2 . Apparently, the cathodic wave increases after the addition of H_2O_2 into the buffer solution, accompanied by a decrease in the anodic peak, which is characteristic of an electrochemically catalytic reaction (Fig. 6). Further studies reveal that the reduction peak current increases with the concentration of H_2O_2 . Since no similar results can be observed at DPPA-alone-modified electrodes, we consider the catalytic reduction as the result of the effect HRP on H_2O_2 . The catalytic curves for HRP–DPPA films indicate a linear range of H_2O_2 analysis from 2.0×10^{-5} to $1.0 \times 10^{-3} \text{ mol L}^{-1}$ (Fig. 6 inset). When the H_2O_2 concentration is larger than 1.0 mmol L^{-1} , the calibration curve inflects, and the curve tends to maximum at 1.8 mmol L^{-1} of H_2O_2 , indicating a saturation of enzyme–substrate reaction.

It is noticeable that the electrochemical catalysis toward the reduction of O_2 and H_2O_2 may have some intrinsic relation. For instance, CV of HRP–DPPA films in the buffer solution containing $2.0 \times 10^{-5} \text{ mol L}^{-1}$ H_2O_2 coincides perfectly with that after being added 10 mL air (Fig. 7). Thus, a possible mechanism of the reaction of H_2O_2 catalyzed by HRP in DPPA films is postulated as follows [40]:



Reaction (5) is the slow step and it is the limiting step in the whole catalytic reaction [41].

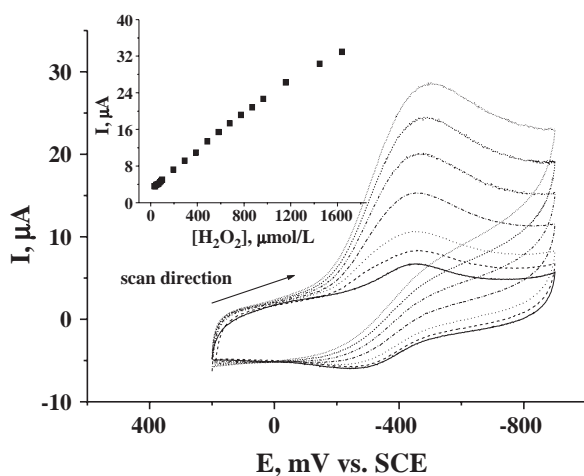


Fig. 6. CVs obtained at an HRP–DPPA-modified PG electrode containing a series of concentrations of H_2O_2 . Inset is the plot of the catalytic peak current vs. the concentration of H_2O_2 . Others are the same as in Fig. 2.

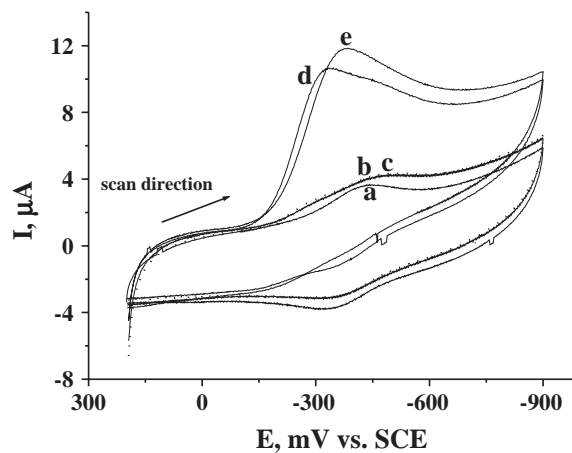


Fig. 7. A series of CVs of HRP–DPPA films achieved under the following circumstances: The buffer solution (a) containing neither O_2 nor H_2O_2 ; (b) being injected 10 mL O_2 (dotted curve); (c) containing $2.0 \times 10^{-5} \text{ mol L}^{-1}$ H_2O_2 ; (d) being injected 50 mL O_2 ; (e) containing $4.0 \times 10^{-4} \text{ mol L}^{-1}$ H_2O_2 . Others are the same as in Fig. 2.

Electrochemical catalysis of HRP toward the reduction of NO has also been studied. Aliquots of NO ($2.0 \times 10^{-3} \text{ mol L}^{-1}$) are added to the bulk solution to generate a series of NO concentrations. In this case, a new reduction wave, aside from the redox waves of HRP, is observed at -700 mV (Fig. 8). The height of this new wave increases linearly with the concentration of NO. A linear dependence of the peak current on the NO concentration is obtained ranging from 2.5×10^{-6} to $2.0 \times 10^{-3} \text{ mol L}^{-1}$ (Fig. 8 inset). Meanwhile, the reduction peak current of HRP slightly decreases compared with that obtained in the blank solution, which indicates an interaction between HRP and NO. According to our previous studies, this new wave can be ascribed to the facilitated catalytic reduction of NO in the presence of HRP [25].

Further studies reveal that pH can have some obvious effect on these catalytic reactions. The catalytic peaks

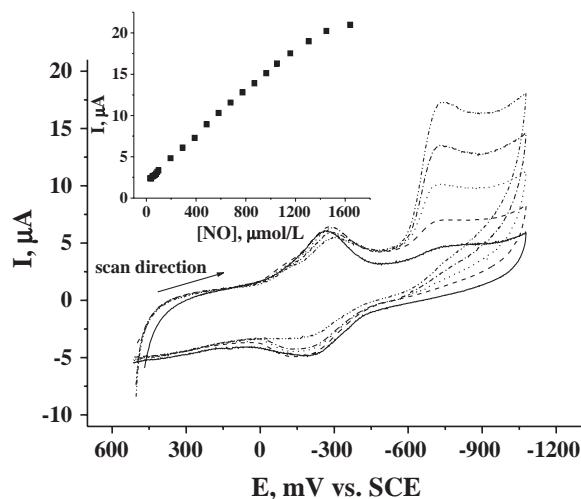


Fig. 8. CVs obtained at an HRP–DPPA-modified PG electrode containing a series of concentrations of NO. Inset is the plot of the catalytic peak current vs. the concentration of NO. Others are the same as in Fig. 2.

currents of O_2 , H_2O_2 , and NO will all increase by decreasing the pH value of the test solutions. So a lower pH value should be selected for the determinations of these species. The reproducibility and stability of the HRP–DPPA film-modified electrode has also been examined and satisfactorily obtained. A successive measurement of O_2 , H_2O_2 , or NO can all give consistent data, and the modified electrode can be stored for 2 weeks with about 5% peak current decrease.

4. Conclusion

HRP can retain its native state in DPPA films. After being entrapped in DPPA films, which are modified on PG electrodes, HRP displays direct, stable and nearly reversible CV responses. Experimental results reveal that the apparent heterogeneous electron transfer of HRP has been greatly accelerated between HRP and the electrode. Furthermore, HRP can exhibit catalytic activity toward the reduction of O_2 , H_2O_2 and NO.

Acknowledgments

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