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# Multi-step reduction of nitric oxide by cytochrome *c* entrapped in phosphatidylcholine films

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**Abstract**

By entrapping cytochrome *c* (Cyt *c*) in phosphatidylcholine (PC) film, we have obtained the direct electrochemistry of the protein. Meanwhile, the catalytic reduction of nitric oxide (NO) has been investigated. Besides the pair of peaks corresponding to the redox reactions of Cyt *c*, two new cathodic peaks can be observed after the addition of NO into the test solution. One, located at −0.510 V, is proposed to come from the formation of hydroxylamine. The other, at −0.690 V, is assigned to the electrochemical reduction of NO. These observations are very different from the previous reports, because only one peak can be obtained for the other studies. The difference is related to the electron transfer rate and the escape rate of the products in the NO reduction process. This study might bring clearer insight into the reduction mechanism of NO.


*Keywords:* Cytochrome *c*; Electrochemistry; Nitric oxide; Phosphatidylcholine; Catalytic reduction

## 1. Introduction

Nitric oxide (NO) is a molecule that has drawn a lot of attention in the last decade. It is an endogenously free radical synthesized from arginine by nitric oxide synthase. It plays an important role in many physiological processes [1]. Since it was proved to be the endothelium-derived relaxing factor (EDRF) in the cardiovascular system [2,3], the study on NO has been the focus of many scientists' interest. And lots of studies are based on the catalytic reduction by proteins or enzymes [4–10]. However, the mechanism of the NO reduction process is still unclear till now.

Electrochemical method, such as protein film voltammetry [11–15], is an efficient method for the characterization of electron-transfer process. This method can be sensitive to the amperometric response of NO reduction [16–26]. Some researchers have reported the different products for the NO reduction process [16,23]. In the meantime, Mimica et al. [27] have reported two NO reduction peaks (located at −0.6 and −1.0 V) in the electrocatalytic process by hemoglobin entrapped in surfactant films. But in this report the second peak is contributed to the reduction of Fe in the nitroxyl adducts.

Phosphatidylcholine (PC) has been a good membrane material for embedding proteins [5,28]. It is a component of a biological membrane, while can provide a mimic environment for the functioning of proteins and enzymes [11,29,30]. So, we have used PC in this work to embed cytochrome *c* (Cyt *c*) and to study the electrocatalytic activity of the protein towards NO. Interestingly, two reduction peaks rather than one can be observed. The reduction process has been discussed accordingly. This study might provide a new and clearer insight into the mechanism of NO reduction process.

## 2. Experimental

### 2.1. Materials

Cyt *c* was obtained from Sigma and used as received. PC was obtained from the Chemical Plant of Huadong Normal University in Shanghai (China). Other reagents were of ana-

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lytical grade. Water was purified with a Milli-Q purification system and was used to prepare all solutions. The saturated solution of 2.0 mmol/L NO was prepared according to the previous report [31].

### 2.2. Electrochemical experiments

Electrochemical experiments were carried out with a PAR 283 Potentiostat/Galvanostat (EG&G, USA). A three-electrode system was employed with a modified pyrolytic graphite (PG) working electrode, a saturated calomel reference electrode (SCE) and a platinum wire auxiliary electrode. All the potentials reported in this paper are versus SCE. The PC vesicle dispersion was prepared by ultrasonicating a 1.0 mmol/L PC suspension in water for at least 2 h until it became clear. The Cyt *c*/PC modified PG electrode was made as following procedures. The substrate PG electrode was first polished using rough and fine aluminum oxide papers. Then it was polished to mirror smoothness with an aluminum oxide (particle size of about 0.05 μm)/water slurry on silk. Finally, the electrode was thoroughly washed with doubly distilled water and treated in an ultrasonic water bath for five minutes. A mixture of 10 μL 0.1 mmol/L Cyt *c* and 10 μL 1.0 mmol/L PC was spread on the PG electrode surface. The film was then dried overnight at room temperature. The modified electrode was thoroughly rinsed with nanopure water and was then ready for use. Electrochemical measurements were carried out under an anaerobic condition. The test solution was first bubbled thoroughly with high purity nitrogen for at least 10 min. Then a stream of nitrogen was blown gently across the surface of the solution in order to maintain the anaerobic condition throughout the experiments.

## 3. Results and discussion

As is well known, Cyt *c*, which acts as an electron shuttle in the respiratory chain, displays a slow electron transfer rate at an electrode surface. Therefore, numerous efforts have been made to enhance the electron transfer reactivity of the protein [32–38]. In this work, we have used protein film voltammetry technique to facilitate the electron transfer between Cyt *c* and the electrode. Fig. 1 (solid line) is the cyclic voltammograms (CVs) of Cyt *c* incorporated in a PC membrane. A pair of redox peaks can be observed attributing to the redox reaction of Cyt *c*. Alternatively, if an electrode is coated with PC alone, no peak occurs in the potential range of interest (Fig. 1 dot line). No corresponding peak can be observed either with the bare PG electrode (Fig. 1 dash line). These results clearly demonstrate that Cyt *c* can take redox reaction after being entrapped in PC membrane. The anodic and cathodic peaks are located at −0.170 and −0.305 V, respectively. And the peak currents are proportional to scan rate in the range from 50 to 1000 mV/s (Fig. 2), which implicates a thin-layer electrochemical behavior.

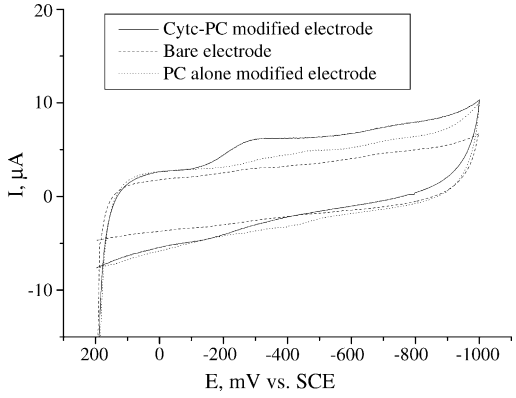



Fig. 1. Cyclic voltammograms obtained at (i) Cyt *c*/PC co-modified PG electrode; (ii) bare PG electrode; (iii) PC alone modified PG electrode in a pH 4.0 buffer solution.

Compared with the cathodic peak, the anodic peak of Cyt *c* is not obvious (Figs. 1 and 2), which suggests that the ferrous Cyt *c* (reduced form of Cyt *c*) on the electrode surface is only partially converted to ferric Cyt *c* (oxidized form of Cyt *c*). So, in the second cycle, the cathodic peak decreases remarkably (Fig. 3). These results clearly demonstrate that the electron transfer between Cyt *c* and electrode is not quick enough.

When NO is added to the test solution, besides the redox peaks of Cyt *c*, two new cathodic peaks appear. These two peaks are located at about −0.510 and −0.690 V, respectively (Fig. 4). It should be mentioned that the catalytic peak of NO can also be observed in the previous reports, but there is only one cathodic peak when NO is catalytically reduced by Cyt *c* [4,29]. So, the reductive process of NO in this system should be different from the previous models. We propose that these two peaks are related to two different products, which are formed via the catalytic reduction of NO by Cyt *c*.

Metalloporphyrins, phthalocyanines, schiff bases and related complexes have been reported in previous researches as electro-catalysts for NO reduction [16–27]. It has been proposed that NO first binds to the ferrous heme to form

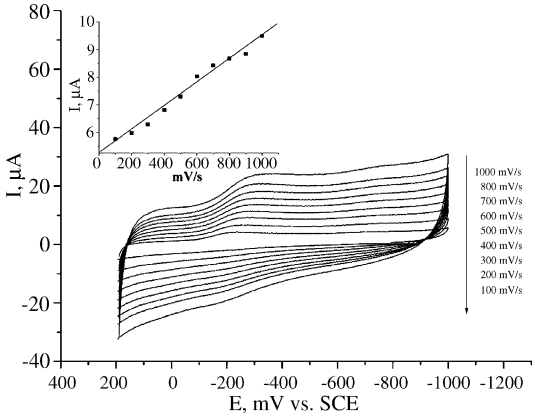



Fig. 2. Cyclic voltammograms obtained on the Cyt *c*/PC co-modified PG electrode in the pH 4.0 buffer solution with different scan rate. Inset is the relation of cathodic peak current and scan rate of Cyt *c*/PC modified electrode.

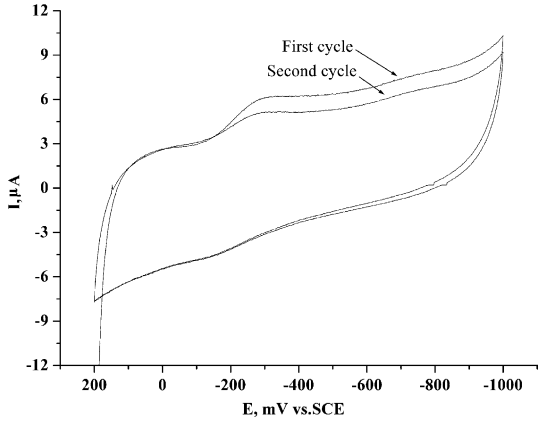



Fig. 3. Repetitive cyclic voltammograms. Other conditions as Fig. 2.

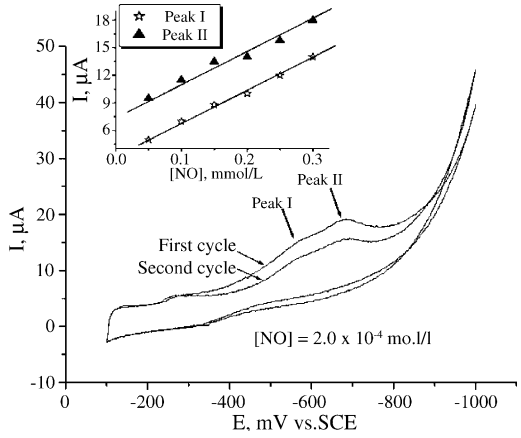
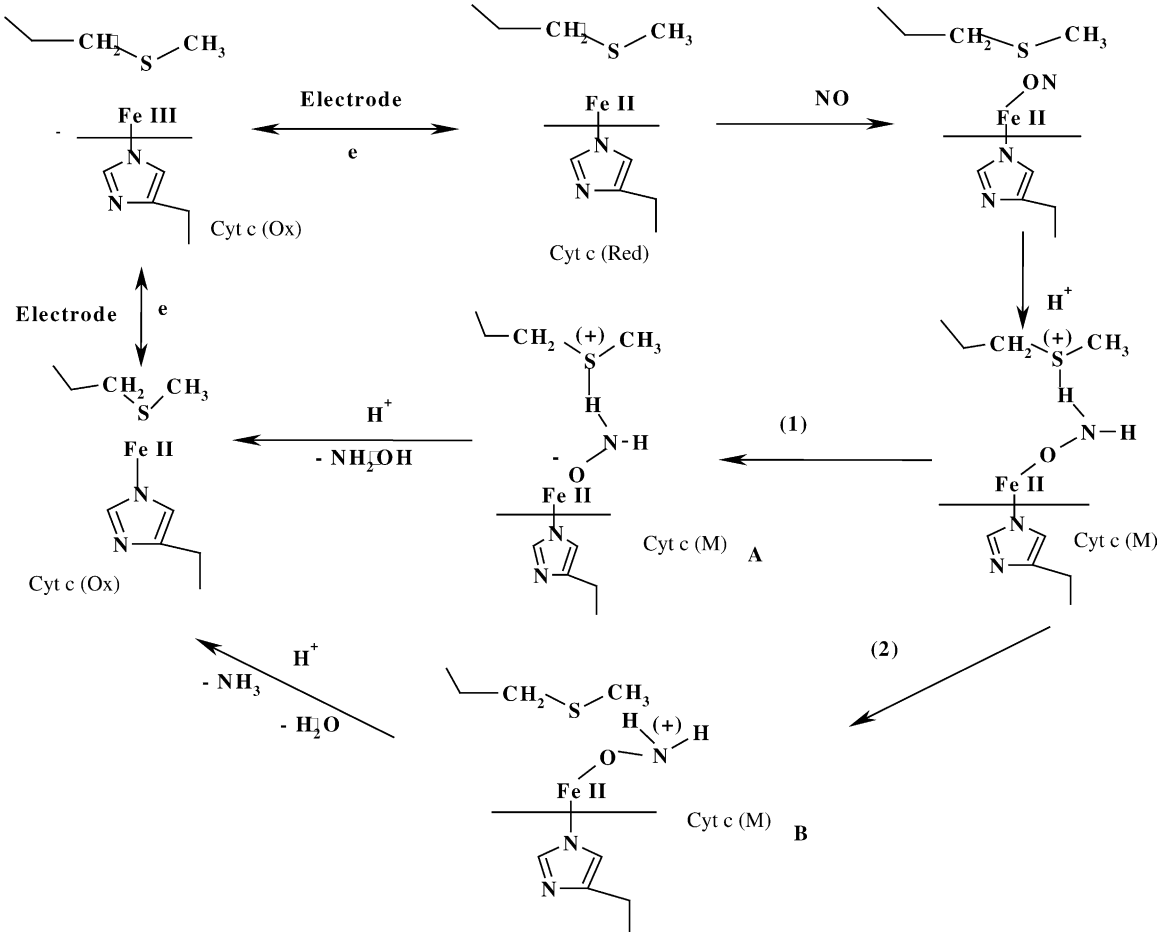



Fig. 4. Cyclic voltammograms obtained at a Cyt *c*/PC co-modified PG electrode in a pH 4.0 buffer solution containing $2 \times 10^{-4}$ mol/l NO. Scan rate: 100 mV/s. Inset is the relation between the peaks currents and NO concentrations.

ferrous-nitroxyl intermediate. The major decomposition process of reducing ferrous-nitroxyl adducts gives the product $N_2O$. And the oxygen of NO dominantly binds to the positive charge ferrous heme because, compared with nitrogen, oxygen tends to display a negative charge in the NO molecule. In some cases, it is suspicious that the negative charged $NO^-$. will escape from the positive charge of ferrous heme because of the electrostatic effect. Accordingly, we propose the mechanism shown in Scheme 1. Cyt *c*(Ox) and Cyt *c*(Red) are the oxidized and reduced forms of Cyt *c*, respectively. Cyt *c*(M) refers to the intermediate of a kinetically stable conformation of Cyt *c* binding to NO. Subscripts A and B refer to the kinetically stable conformations which tend to be decomposed and




Scheme 1. The process of the catalytic reduction of NO by Cyt *c*.

reduced, respectively. Cyt $c(M)_A$ is thus the intermediate state of a ferrous-nitroxyl adduct with the preferred decomposed state conformation; and Cyt $c(Ox)_B$ is the intermediate state of the protein with the preferred reduced state conformation. After NO is added into the test solution, it will bind to the ferrous heme to form ferrous-nitroxyl intermediate because NO has a high affinity to Cyt *c*. Under the negative potential, electron can transfer from Cyt *c* to NO and be handed over to electrode. Under acid condition, this unstable intermediate state will be protonized quickly to form Cyt *c*(M).

It is well known that the active site of Cyt *c* contains a heme that is bound to the protein through axial coordination by the alkyl sulfide side chain of a proximal methionine residue. In the NO reduction process, the methionine residue participates in the protonized process. The catalytic process of Cyt *c* involves the stepwise multi-electron oxidation of the heme by NO, generating an oxo ferryl protophyrin IX π-cation radical, Cyt *c*(M). At a slow electron transfer rate between electrode and Cyt *c*, the conformational change of Cyt *c*(M) will have adequate time to decompose and release hydroxylamine before it can be reduced. So, the observed rate of the whole reaction will be controlled exclusively by the electron transfer rate.

In general, the oxidized conformation can be subsequently reduced in response to a decrease in the oxidized heme potential. Unfortunately, as the aforementioned results, PC cannot effectively facilitate electron transfer of Cyt *c* with the electrode, which results in the different products of NO. As a result, when the electron transfer rate is slow for the –NHO– group reduction process, the $NH_2OH$ product has adequate time to release. If the electron-transfer rate is very fast, the –NHO– group will be reduced immediately. Generally, the second reduction process takes place prior to the releasing process, and single reduction wave is often obtained. But According to the aforementioned results, in this system, peak I should be attributed to the reduction of NO to hydroxylamine:

$$NO + 3H^+ + 3e^- \rightarrow NH_2OH \quad (1)$$

and then the hydroxylamine reduce to ammonia, thus peak II appears.

$$NH_2OH + 2H^+ + 2e^- \rightarrow NH_3 + H_2O \quad (2)$$

The complete reaction can be described as the following equation:

$$NO + 5H^+ + 5e^- \rightarrow NH_3 + H_2O \quad (3)$$

In order to confirm this proposal, a comparison experiment has been performed, i.e. $NH_2OH$ has been added to the buffer. In pH 5.0 buffer solution, hydroxylamine is protonized. It is conceivable that there is no response of protonated hydroxylamine, as is shown in Fig. 5, due to the electrostatic repulsion of PC films. However, under alkali conditions, there is an electrochemical response resulting from hydroxylamine. What is more, the reduction potential is located at −700 mV, nearly located at the same potential as the peak II in Fig. 4. Alternatively, if an electrode is coated with PC alone in the same buffer solution, no peak can be seen in the potential range of interest. No corresponding peak can be observed either from the bare PG electrode. We also find that the reduction peak of hydroxylamine increases with the congregation time, indicating that the hydroxylamine is not a strong ligand for ferrous Cyt *c* to form Cyt *c*(M).

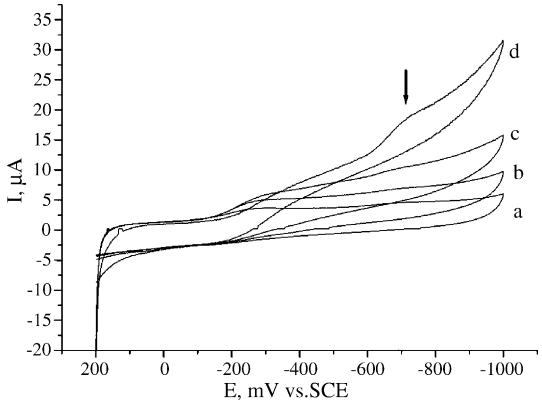



Fig. 5. Cyclic voltammograms obtained at a Cyt *c*/PC co-modified PG electrode for (a) pH 4.0 buffer solution in the absence of $NH_3OH{\cdot}Cl$; (b) pH 4.0 buffer solution containing $2 \times 10^{-3}$ mol/l $NH_3OH{\cdot}Cl$; (c) pH 8.0 buffer solution containing $2 \times 10^{-3}$ mol/l $NH_3OH{\cdot}Cl$; (d) pH 12.0 buffer solution containing $2 \times 10^{-3}$ mol/l $NH_3OH{\cdot}Cl$. Congregation time: 1 min.

## 4. Conclusions

Electrochemical method was employed in this work to study the NO catalytic reduction process. Two catalytic peaks of NO by Cyt *c* were first observed, which revealed the multi-reduction of NO by the protein entrapped in PC membrane. Mechanism has been proposed to explain this phenomenon. These interesting results would also offer an alternative method to investigate enzyme biomimetic towards NO.

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