Direct Electrochemistry of Cytochrome c Embedded in Membrane Films of Anionic-Type Lipids Prepared *via* Ion-Exchange

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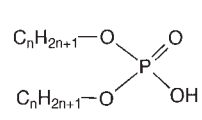
Cytochrome c was embedded in a membrane film composed of anionic-type lipids *via* ion-exchange. The cytochrome c embedded in lipid films coated on a graphite electrode showed well-defined redox waves. Redox potentials and specular reflectance spectra indicated that the native structure of cytochrome c was retained in the lipid films.

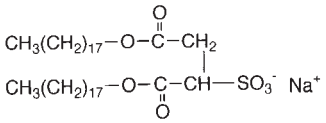
The redox reactions of proteins incorporated into lipid films on an electrode have been extensively investigated. The electrochemical behavior and spectroscopic characterization of ferredoxin,[1–3] myoglobin[4,5] and hemoglobin[6,7] incorporated into lipid films has been previously studied. Some approaches to development of biosensors have also been reported, which use membrane film-entrapped peroxidase[8,9] and catalase-peroxidase.[10] In almost all studies previously reported, protein-lipid film-modified electrodes were prepared using a mixed-solution of lipid and protein cast onto the electrode.

In the present study, we developed a system for incorporation of the cationic protein, cytochrome c (*ca.* 12 k daltons[11]), by ion-exchange, using a membrane film composed of anionic-type lipids. This ion-exchange incorporation system permits satisfactory protein electrochemistry without denaturing of the protein conformation.

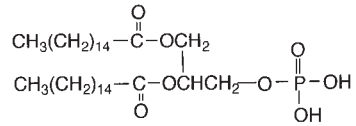
Bovine heart cytochrome c was purchased from Sigma and was used as received. Five different lipids were used for modification of the electrode. $2C_{16}N^+Br^-$, $2C_nPO_4H$ (n = 16, 18) and $2C_{18}SucSO_3^-Na^+$, and DPPA were purchased from Tokyo Kasei Kogyo, Sogo Pharmaceutical, and Sigma, respectively. Purchased lipids were used as received.



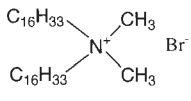
$2C_nPO_4H$ (n = 16, 18)



$2C_{18}SucSO_3^-Na^+$



DPPA



$2C_{16}N^+Br^-$

The lipid film-modified electrodes were prepared according to methods described in a previous study.[1,2] Briefly, a basal plane of pyrolytic graphite (Union Carbide Co.; geometric area, 0.26 cm^2), BPG, was used as the working electrode. Prior to casting of the lipid solution, the fresh basal plane of a BPG electrode was exposed by abrasion with No. 1500-grid SiC paper. Ten microliters of a chloroform solution consisting of 0.1 mol dm^{-3} $2C_{16}N^+Br^-$ or $2C_nPO_4H$ (n = 16, 18), or a benzene solution consisting of 0.1 mol dm^{-3} DPPA, respectively, was placed on a BPG electrode. Sixty microliters of 5 mmol dm^{-3} $2C_{18}SucSO_3^-Na^+$ in an ethanol solution was placed on a BPG electrode. The electrodes were then modified with lipids and allowed to air-dry for 12 h at ambient temperature.

Cyclic voltammetric measurements were carried out using an electrochemical analyzer (Bioanalytical Systems, BAS 100 B/W), in 50 mmol dm^{-3} tris-HCl buffer solution (pH 7.2) at 23 °C under nitrogen gas. An Ag|AgCl (saturated KCl) electrode and a platinum electrode were used as reference and counter electrodes, respectively. All potentials are reported with respect to the Ag|AgCl (saturated KCl) electrode.

Specular reflectance spectra were obtained with a Shimadzu UV-3100 spectrophotometer. Twenty microliters of 0.1 mol dm^{-3} $2C_{16}N^+Br^-$, $2C_nPO_4H$ (n = 16, 18) or DPPA, or sixty microliters of 5 mmol dm^{-3} $2C_{18}SucSO_3^-Na^+$ in the organic solution was placed on a propyltrichlorosilane-modified quartz glass plate and allowed to air-dry for 12 h. The modified quartz glass plate was immersed in 50 mmol dm^{-3} tris-HCl buffer solution (pH 7.2) containing 0.1 mmol dm^{-3} cytochrome c at 23 °C for 30 min, and then rinsed with a tris-HCl buffer solution. Specular reflectance spectra were subsequently measured. The oxidized-form of cytochrome c embedded in the lipid was reduced by immersion into the buffer solution containing sodium dithionate.

To investigate the incorporation of cytochrome c into the lipid membrane film, the lipid film-modified electrodes were immersed in 50 mmol dm^{-3} tris-HCl buffer solution (pH 7.2) containing 0.1 mmol dm^{-3} cytochrome c at 23 °C. The peak currents of well-defined redox waves attributable to cytochrome c were found to increase at the electrodes modified with $2C_nPO_4H$ (n = 16, 18), $2C_{18}SucSO_3^-Na^+$ and DPPA as anionic-type lipids. Eventually, the peak current reached a steady state. The elapsed time to achieve a steady state was strongly dependent on the lipid used: the elapsed times were approximately 3, 6, 1 and 1 h for $2C_{16}PO_4H$, $2C_{18}PO_4H$, $2C_{18}SucSO_3^-Na^+$ and DPPA, respectively. These differences in incorporation behavior could be attributable to the microstructure of the lipid membrane film.

After the peak current reached a steady state, these lipid film-modified electrodes were gently rinsed with the buffer solution, and placed in the buffer solution in the absence of cytochrome c. The redox waves representing cytochrome c were still retained, as shown in Figure 1. No significant change in the voltammograms was observed while keeping the modified electrodes in the buffer solution at 23 °C for at least 6 h. The results indicated that cytochrome c was incorporated into the cast film of lipid membrane,

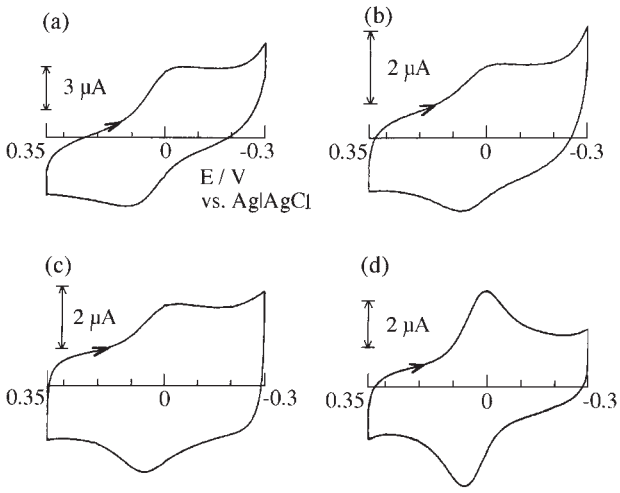


Figure 1. Typical cyclic voltammograms of cytochorome c embedded in $2C_{16}PO_4H$ (a), $2C_{18}PO_4H$ (b), $2C_{18}SucSO_3^-Na^+$ (c) and DPPA (d) - modified BPG electrodes in a tirs-HCl buffer solution (pH 7.2). Potential sweep rate: 100 mV s^{-1}. Temperature: 23 °C.

and that direct electron transfer of incorporated cytochrome c occurred at the electrode surface.

The redox potentials evaluated from the midpoint of the anodic and cathodic peak potentials of the redox waves representing cytochrome c embedded in $2C_{16}PO_4H$, $2C_{18}PO_4H$, $2C_{18}SucSO_3^-Na^+$ and DPPA were 35, 40, 30 and 40 mV, respectively. The redox potential of cytochrome c at oxide semiconductors and promoter modified electrodes in a buffer solution was reported to be 50–70 mV.[11] There was no significant difference in the redox potentials between cytochrome c embedded in the lipid films and cytochrome c in the buffer solution, indicating that the native cytochrome c structure was maintained in the lipid films. Figure 2 shows the specular reflectance spectra of oxidized- and reduced-forms of cytochrome c embedded in DPPA film. The peak maxima in the spectra were seen at 528 and 410 nm for the oxidized-form, and 550, 520 and 416 nm for the reduced-form, which were in good agreement with the spectra of the oxidized- and reduced-forms of cytochrome c in the buffer solution. The same results were observed when $2C_{16}PO_4H$, $2C_{18}PO_4H$ or $2C_{18}SucSO_3^-Na^+$ was used. These results also confirm that the native cytochrome c structure is maintained in the lipid films.

The peak currents in the voltammograms of cytochrome c embedded in $2C_{16}PO_4H$, $2C_{18}PO_4H$, $2C_{18}SucSO_3^-Na^+$ and DPPA were proportional to the potential sweep rate, v, over a sweep range of 2–10 mV s^{-1}, as expected for thin-layer electrochemical behaviour, and were proportional to the square root of v over a sweep range of 200–1000 mV s^{-1} as expected for a diffusion-controlled process.[2]

Compared to anionic-type lipids, the incorporation of cytochrome c into $2C_{16}N^+Br^-$, a cationic-type lipid film, was spectroscopically and electrochemically undetectable. This result together with the fact that cytochrome c was incorporated into the anionic-type lipid films suggests that the incorporation of cytochrome c into the lipid film is attributed to ion-exchange between the cation produced as a counter ion for negatively charged-lipid by ionization in the buffer solution and the positively charged cytochrome c (pI 10[12]). This ion-exchange mechanism was also supported by the results of an electrolyte concentration dependence experiment. For example, the incorporation of cytochrome c into DPPA film on the

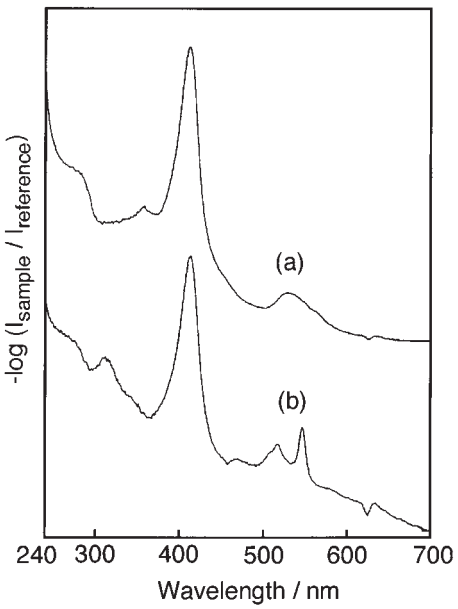


Figure 2. Specular reflectance spectra of oxidized- (a) and reduced- (b) forms of cytochrome c embedded in DPPA film of a quartz plate.

electrode was gradually inhibited with increasing electrolyte (NaBr) concentration in the buffer solution. The values of peak currents observed were approximately 30, 18 and 15% at 0.25, 0.5 and 1.0 NaBr mol dm^{-3}, respectively, against the value obtained in a solution containing no electrolyte.

In conclusion, cytochrome c was embedded, without denaturation, into membrane films composed of an anionic-type lipid *via* ion-exchange. The cytochrome c embedded in the lipid films on a graphite electrode showed well-defined redox waves. The results obtained in this study indicate that the lipid films are useful not only as a matrix for incorporation of proteins but also as an electron transfer interface between metallo-proteins and electronic devices.

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References and Notes

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