Lipid-bonded Conducting Polymer Layers for a Model Biomembrane: Application to Superoxide Biosensors

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Model biomembranes composed of poly-DATT/DGS/POPA and poly-DATT/DGS/CL were separately prepared on gold electrodes. A monolayer of 1,2-dioleoyl-sn-glycero-3-succinate (DGS) was covalently bonded onto electrochemically grown poly-(3,4-diamino-2,2:5,2-terthiophene) (DATT) layers (thickness of ~300 nm; particle size of ~50 to 70 nm). The numbers of unit molecules of the poly-DATT layer and of the DGS immobilized onto the poly-DATT layers were $1.53 \times 10^{-7}$ and $1.56 \times 10^{-9}$ mol cm$^{-2}$, respectively, using a quartz crystal microbalance technique. The lipid bilayers of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate (POPA) and cardiolipin (CL) were formed onto the poly-DATT/DGS layer using the Langmuir–Blodgett technique. The surface characterizations of each step were investigated by SEM, AFM, and XPS analyses. Cytochrome c (cyt c) was immobilized onto these model biomembranes through the charge interaction between the positive charges of cyt c and the negative charges of phosphate groups in CL or POPA lipids. At the POPA- and CL-modified biomembranes, the formal potentials of the redox couple of the immobilized cyt c were 0.22 and 0.23 V (vs Ag/AgCl), respectively. The redox reaction of the immobilized cyt c at the POPA- and CL-modified biomembranes was quasi-reversible, and the electron-transfer rate constants were 0.121 s$^{-1}$ and 0.133 s$^{-1}$, respectively. The applicability of these cyt c immobilized biomimetic membranes as the biosensors was tested for the determination of superoxide.

The development of new model biomembrane systems shows promise for biochemical applications and has aroused the interest of more and more researchers because these model biomembrane systems can supply a biological environment to the detecting surface of a biosensor. Scientifically, phospholipids, membrane receptors, or proteins of an extracellular matrix immobilized onto conductor and semiconductor devices can provide physical models of cell and tissue surfaces, which allow the investigation of the basic principles of their complex functions in nature.1

Various biomembrane model systems based on lipid bilayers, unilamellar/bilamellar lipids vesicles, detergent micelles, and other biologically important molecules and structures which possess appropriate amphipathic properties have been being used as biomembranes.2 Lipids are amphiphilic, one part of the molecule being hydrophilic and other part being lipophilic. The lipid layer on a solid substrate can be formed by casting the lipid molecules. Evaporation of the solvent after casting, using the Langmuir–Blodgett (LB) technique, renders a self-assembled layer to an ordered stack of lipid layers. This technique is one of the most useful methods to prepare mono-, bi-, and multilayer films.3–5 Especially the Y-type6 lipid bilayer on solid surfaces behaves in a manner that is quite similar to a biomembrane in biological cells.7 For example, a number of bio-compounds (e.g., protein, enzyme, cofactor) can be incorporated into the lipid layers for specific chemical applications. Furthermore, lipid layers can greatly reduce interference by effectively excluding hydrophilic electroactive materials from the detecting surface. However, formation of the lipid layers on solid surfaces by mere physical adsorption with a casting method can eventually lead to a detachment or replacement of the surface-active components, thus rendering the lifetime of the layers too short for practical purposes. Thus, the physical adsorption of lipid molecules is unattractive due to its low stability. To mimic model biomembranes, functional coupling of lipid constituents with solid surfaces has been studied and has become popular.8 To enhance the stability of lipid layers, solid surfaces have been modified with the self-assembled monolayers (SAMs) or conducting polymers. SAMs are suitable for surface modification and for making very thin insulating layers that prevent leak currents, nonspecific adsorption, and surface decomposition in aqueous electrolytes. To date, SAMs of alkanethiols on gold electrodes have been most intensively studied to enhance the stability of modified surfaces.9

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To fabricate a more stable biomimetic membrane, we modified the solid surface with a conducting polymer layer, which serves as a base substrate for the biomembrane. A conducting polymer of terthiophene is known to be very stable over a wide pH range and to be resistant to chemicals. In the present study, gold electrodes were modified by coating them with a conducting polymer, poly-(3, 4-diaminoo-2,2,5,2-terthiophene) (poly-DATT), that has two reactive amine functional groups for covalent attachment with the carboxylic acid groups of lipid, 1,2-dioleoyl-sn-glycero-3-succinate (DGS). The modification of the polymer layer was accomplished by the electropolymerization of the DATT monomer in a 0.1 M tetrabutylammonium perchlorate (TBAP)/CH2Cl2 solution using the potential cycling method. This method is very attractive because the polymer layer thickness and the particle size of the polymer can be controlled by changing the cycle number and the scan rate, respectively, in the electropolymerization process. The polymer-coated electrodes allow the lipid constituents to attach in a chemically stable way by establishing covalent bonds between the reactive groups of lipid and the modified surface. Thus, a stable covalently bound DGS monolayer on the poly-DATT-coated gold electrode can be obtained. The formation of a Y-type phospholipid bilayer on the DGS-modified poly-DATT was further studied using 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate (POPA) and cardiolipin (CL) according to the LB technique. A schematic presentation of these biomimic membranes is shown in Figure 2. The electrochemical behavior of a redox protein, cytochrome c (cytc), was studied for these biomimetic membranes. For possible applications in biosensing devices, superoxide detection was carried out by immobilizing cytc onto these biomimetic membranes. We choose the superoxide radical detection for application because it is a primary component of so-called reactive oxygen species and is a potentially damaging agent for the living system. The superoxide radical released during the respiratory burst of neutrophils and macrophages has been implicated in the pathology of a variety of pathological conditions, including rheumatoid arthritis and neurodegenerative disorders. It is also closely related to the aging problem, inflammatory diseases, ischemic-reperfusion injury, atherosclerosis, and cancer. There is a strong demand for a sensitive analytical method for real-time monitoring of the superoxide radical in living cells and tissues. Thus, we examined a lipid-modified electrode containing cytc, which has good physical adherence and communication with living cells.

**EXPERIMENTAL SECTION**

**Materials.** DGS, POPA, and CL were purchased from Sigma Co. and Avanti Polar Lipids Inc., Alabaster, AL, and were used without further purification. The structures of the lipids are displayed in Figure 1. Dichloromethane (99.8%, anhydrous, sealed under N2 gas) and 1-ethyl-3-(3-dimethylamino)-propyl carbodiimide (EDC) were acquired from Sigma and were used as received. Cytochrome c from horse heart (type VI, Sigma Co.) was used after purification. The purification process of cyt c has been described in our previous paper. Briefly, cyt c from horse heart was converted to the fully oxidized form by addition of excess K3Fe(CN)6 and then purified by ion-exchange chromatography on Whatman CM32, eluted with 0.5 M NaCl + 10 mM phosphate buffer solution at pH 7.0. Tetraphenylammonium perchlorate (electrochemical grade) was obtained from Fluka (USA) and purified according to the general method, followed by drying under vacuum at 10⁻³ Torr. A terthiophene monomer bearing two amine groups (DATT), was synthesized according to a previous report. A phosphate buffer solution was prepared by using 0.1 M disodium hydrogen phosphate (Aldrich) with 0.1 M sodium dihydrogen phosphate (Aldrich). All other chemicals were of extra pure analytical grade and were used without further purification. All aqueous solutions were prepared in doubly distilled water, which was obtained from a Milli-Q water purifying system (18 MΩ·cm).

**Instrumentation.** Lipid bilayer-modified DGS/poly-DATT/Au (area = 0.07 cm²), Ag/AgCl (in saturated KCl), and a Pt wire were used as working, reference, and counter electrodes, respectively. Cyclic voltammograms were recorded using a potentiotstat/galvanostat, Kosentech model KST-P2 (South Korea). A quartz crystal microbalance (QCM) experiment was performed using a SEIKO EG&G model QCA 917 and a PAR model 263A potentiostat/galvanostat (U.S.A.). A Au working electrode (area, 0.196 cm²; 9 MHz; AT-cut quartz crystal) was used for the QCM experiment. Electron spectroscopy for chemical analysis (ESCA) was performed using a VG Scientific ESCALAB 250 XPS spectrometer with a monochromated Al Kα source with charge compensation at KBSI (Busan). Scanning electron microscopy (SEM) images were obtained using a Cambridge Stereoscan 240. Atomic force microscopy (AFM) experiments were carried out

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on a multimode AFM system of Digital Instrument Inc. (U.S.A.). Commercial Si$_3$N$_4$ tips were attached to a triangular cantilever made of the same material and of 125-$\mu$m length, 300-kHz resonance frequency, and 5–10-μm radius. The force (spring) constant was 20–100 N/m. A Laugmuir–Blodgett KSV-5000 (Finland) trough equipped with two symmetrical compartments, each of 71 × 12 cm$^2$, was used to record surface pressure–area isotherms. Each isotherm represents the average of four experimental π–A isotherms.

RESULTS AND DISCUSSION

Preparation and Electrochemical Characterization of the Poly-DATT Layer on a Au Electrode. The poly-DATT layer on a Au electrode was obtained through the electropolimerization of a DATT monomer in 0.1 M TBAP/CH$_2$Cl$_2$ solution. After electropolimerization, the poly-DATT film was washed with CH$_2$Cl$_2$ to remove the excess monomers from the electrode surface. Figure 3A shows a series of cyclic voltammograms (CVs) recorded for a 1.0 mM DATT monomer on a Au electrode in a 0.1 M TBAP/CH$_2$Cl$_2$ solution while the potential was cycled between 0.0 and +1.6 V versus Ag/AgCl. The CVs exhibited two oxidation peaks at +0.75 and +1.35 V versus Ag/AgCl during the first anodic scan, which were due to the oxidation of the amine group to the imine group and the oxidation of the monomer to form the polymer, respectively. The currents of these peaks decreased as the cycle numbers increased. This clearly demonstrates that the polymer film immediately forms after the oxidation of the DATT monomer at +1.35 V. The thickness of the polymer film increased as the cycle number increased. A small and broad reduction peak of the polymer was observed at +0.48 V versus Ag/AgCl of the cathodic scan.

To obtain a nanoscale thickness of the poly-DATT layer with a maximum surface coverage, the effect of the scan rate during the electropolimerization experiment was studied. Figure 3B shows SEM images of the nanostructured poly-DATT grown on the Au electrode at various scan rates. It can be seen from the SEM images that as the scan rate increased in the electropolimerization step, the particle size of the conducting polymer tended to be smaller. The thickness of the poly-DATT layer after seven cycles also decreased with the increasing of the scan rate, indicating the formation of smaller particles at higher scan rates. However, the maximum surface coverage of poly-DATT was obtained at 100 mV/s, and the thickness of the poly-DATT layer formed after seven cycles was estimated from the SEM image to be ~300 nm. Thus, a scan rate of 100 mV/s was used in all subsequent experiments.

Figure 2. Schematics of the formations of the biomimic membranes.

Figure 3. (A) CVs recorded for the electropolimerization of a 1.0 mM DATT in a 0.1 M TBAP/CH$_2$Cl$_2$ for seven consecutive potential cycles between 0 and 1.6 V on a Au electrode. The scan rate was 100 mV/s. (B) SEM images of the poly-DATT layers according to the scan rate in the electropolimerization using CV (scan rate: b ~ h = 5, 10, 25, 50, 100, 200, and 350 mV/s).
Figure 4. (a) Frequency shifts obtained from the EQCM experiment concurrently recorded with the CV for the formation of the poly-DATT layer and (b) frequency and mass changes during the immobilizations concurrently recorded with the CV for the formation of the poly-DATT layer.

QCM Analysis. EQCM studies were carried out with concurrently recording CVs at the scan rate of 100 mV/s to determine the amount of poly-DATT formed on a Au electrode surface, as shown in Figure 4a. The frequency shift increased linearly with the cycle number, indicating that the amount of the formed poly-DATT increased as the cycle number increased. The amount of poly-DATT formed after seven cycles was 8800 ± 200 Hz. The amount of DGS immobilized onto the poly-DATT layer was determined using the QCM technique. Figure 4b shows that the frequencies changed during the DGS attachments onto the poly-DATT film on the Au electrode. During the immobilization of the DGS onto the poly-DATT film, the frequency decreased and attained a steady state after 2.5 h. This indicates that the immobilization of the DGS onto the poly-DATT film was completed within 2.5 h at room temperature. After 2.5 h, the frequency change (Δf) was ~200 Hz. The mass change (Δm) during the DGS immobilization onto the poly-DATT film was determined using an equation reported previously.

ESCA Analysis. Figure 5 shows C1s and N1s spectra for poly-DATT and lipid-bonded poly-DATT surfaces obtained from the ESCA analysis. The C1s spectrum for the poly-DATT surface exhibited two peaks at 284.4 and 286.0 eV. The peak at 284.4 eV corresponded to C−H, C−S, or C−C bonds, whereas the peak at 286.0 eV corresponded to C−N bonds. In the case of the lipid-bonded poly-DATT, the C1s spectrum exhibited four peaks at 290.2, 288.2, 286.5, and 284.6 eV, C−H, C−S, or C−C bonds and C−N bonds were observed at 284.6 and 286.5 eV, respectively. The peaks for C−N in the case of the lipid-bonded poly-DATT shifted to a higher energy, indicating an interaction between the −NH2 groups of the poly-DATT and the −COOH groups of the lipid (DGS). The peaks at 290.2 and 288.2 eV corresponded to the O−C−N and C−O bonds, respectively. The poly-DATT film exhibited two N1s peaks at about 399.4 and 401.0 eV due to the presence of the −NH2 and =NH groups. This indicates that some of amine groups in the monomer were oxidized to imine groups during electropolymerization. A −NH2 peak at 399.4 eV disappeared in the spectrum of the lipid-bonded poly-DATT. This indicates that the formation of the amide bond results in the disappearance of the −NH2 group. The presence of C=O and O−C−N peaks at about 288.2 and 290.2 eV in the C1s spectrum and the disappearance of the −NH2 groups in the N1s spectrum obtained for the lipid-bonded poly-DATT confirmed that the lipid molecules covalently bonded to the poly-DATT layer.

The Formations of the Lipid Bilayers. The formations of the lipid bilayers of POPA and CL on the DGS-bonded poly-DATT were studied using the LB technique. At first, the σ−A isotherms for the lipid films of POPA and CL spread on pure water were studied, and then the monolayers of these lipids were transferred to the DGS-bonded poly-DATT layer on Au electrodes using an LB transfer process. Figure 6 shows the σ−A isotherm curves of POPA and CL monolayers on water. The isotherm for POPA shows a lift-off area of 0.9 nm2 molecule−1, and then the surface pressure rose gradually to 44 mN m−1. Over this point, the floating monolayer of POPA collapsed. The limited molecule area (LMA) with a highly condensed monolayer of POPA at 22°C was 0.55 nm2. The isotherm for CL shows a lift-off area of 1.75 nm2 molecule−1, and then the surface pressure rose gradually to 40 mN m−1. Over this point, the floating monolayer of CL also collapsed. The limited molecule area (LMA) with a highly condensed monolayer of CL at 22°C was 0.9 nm2. The isotherm for POPA deviated from the isotherm for CL by a shift of 0.85 nm2 molecule−1 to smaller surface areas. This shift suggests that the packing orientations of the two lipids were different, and in fact, tighter packing for POPA was observed at a given pressure. The resultant LB monolayers of the POPA and CL lipids were transferred onto the DGS-bonded poly-DATT at different surface pressures by immersing the electrodes at a speed of 4 mm min−1. The transfer ratio, defined as the ratio of the decrease in the surface area of the monolayer on water to the surface areas of the electrode onto which the monolayer is transferred, was measured with software control. The transfer ratio was close to 1 in each case, indicating the quantitative transfer of the LB monolayers of POPA and CL onto the DGS-bonded poly-DATT.

Surface Characterization using AFM. The different steps involved in the fabrication of the biomimic membranes were separately confirmed by taking AFM images of the surface for...
each step. Figure 7 shows the 3D AFM images obtained for (a) poly-DATT and (b) poly-DATT/DGS surfaces. The surface of the poly-DATT layer exhibited a plane and uniform conducting polymer particles 50 to 90-nm diameter. The DGS constituents immobilized onto the poly-DATT layer through covalent bonding can be clearly seen in the AFM image obtained for poly-DATT/DGS. The particle size of the lipid was found to be ~2 nm. The AFM images for the poly-DATT/DGS/POPA and poly-DATT/DGS/CL were also obtained (not shown), which exhibited rather cloudy images with some roughness.

The Electrochemical Behavior of Cytochrome c at the Biomimic Membranes. Cytochrome c is a basic redox metalloprotein with an overall positive charge of +8 at neutral pH. Basic lysine residues are clustered around its active site, which is a heme iron porphyrin. Cyt c consists of a single polypeptide chain having 104 amino acid residues arranged in a globular tertiary structure. It plays an important role as an electron carrier in the mitochondrial intermembrane space between two membrane-bound protein complexes, cyt c reductase and cyt c oxidase. The direct electron-
transfer reaction of cyt c has been the most widely investigated
among the redox proteins. The direct electron-transfer reaction of cytochrome c at electrodes such as platinum, gold, nickel, mercury, and p-type silicon has been shown to be slow due to adsorption-induced denaturation. It is not always possible to observe the direct electron-transfer process of cyt c at a bare electrode such as glassy carbon, etc., due to inadequate orientation. However, modification of the electrode surfaces with electron promoters has made it possible to observe direct electron-transfer reactions of cyt c. There have been reports of the observation of direct electron-transfer reactions of cyt c using promoters. The biological lipid film can also act as a suitable medium for the immobilization of the proteins. Previously, we have also studied the electrochemical behavior of cyt c at a cardiolipin layer. In the present study, the electrochemical behavior of cyt c at the bilayer-bonded poly-DATT, as a biomimetic membrane, was studied using cyclic voltammetry. Figure 8A shows the CVs recorded for the poly-DATT- (dotted line), poly-DATT/DGS- (dashed line), and poly-DATT/DGS/POPA-modified electrodes (solid line), and Figure 8B shows the CVs recorded for poly-DATT/DS (dashed line) and poly-DATT/DGS/CL-modified electrodes (solid line) in a 0.1 M phosphate buffer solution containing 0.1 mM cyt c.

where \( I_p \) is the peak current; \( n \), the number of electrons; \( F \), the Faraday constant; \( R \), the gas constant; \( T \), temperature; \( \nu \), the scan rate; \( A \), the area of the electrode; and \( \bar{\Gamma} \), the surface coverage of cyt c. Assuming one electron transferred for cyt c, the surface coverage of cyt c was calculated as 1.5 \( \times 10^{-12} \) mol cm\(^{-2}\) and 2.8 \( \times 10^{-12} \) mol cm\(^{-2}\) at the POPA- and CL-based biomembranes, respectively. The observed higher surface coverage of cyt c at the CL-based biomembrane might have been due to the stronger interaction of cyt c with CL than that with POPA. These \( \bar{\Gamma} \) values of cyt c obtained for POPA- and CL-based membrane are comparable to that obtained for the imidazole-terminated (3.3 \( \pm 0.2 \) \( \times 10^{-12} \) mol cm\(^{-2}\)) and pyridine-terminated (2.5 \( \pm 0.3 \) \( \times 10^{-12} \) mol cm\(^{-2}\)) thiol monolayers. The peak separations between the anodic and cathodic peaks might have been due to the redox reaction of cyt c. For the Au/poly-DATT/DS/POPA and Au/poly-DATT/DGS/CL electrodes, the formal potentials of the redox reaction were 0.22 and 0.23 V vs Ag/AgCl, respectively, which are close to the values obtained in a previous report in a solution. The formal potentials of the electron-transfer reaction of cyt c at the Au/poly-DATT/DGS/CL and Au/poly-DATT/DGS/POPA electrodes were shifted to a more positive value than that obtained at the different mixed self-assembled monolayers. For example, at the S(CH2)\(_{12}\)-Py and hydroxyl-terminated monolayers, the reported formal potential is 0.005 and 0.044 V, respectively, versus Ag/AgCl. The apparent formal potential is 0.012 mV for carboxylic acid-terminated monolayers. For the mixed monolayer films, which are composed of pyridine, imidazole, and nitrile functionalities that can interact with the heme site of the cyt c, a significant negative shift in redox potential was observed, ranging from \(-0.172 \) V for the pyridine system to \(-0.415 \) V for the nitrile system. In the present case, penetration of cyt c into the interior of the bilayer lipid membrane exposes the heme to an environment of low dielectric constant, which should shift the heme redox potential to the higher positive values. These formal potentials of cyt c are comparable to one obtained at an indium oxide working electrode modified with a lipid membrane from a mixture of egg phosphatidylcholine and 20 mol % CL.

The peak currents were proportional to the scan rates, indicating that the redox reaction was associated with a surface adsorption of cyt c at the thicker lipids layer rather than the Nernstian diffuse layer. The surface coverage of adsorbed cyt c was calculated by using the following equation:

\[
I_p = n^2F^2\nu\bar{\Gamma}/ART
\]

cathodic peaks were 0.11 and 0.09 V. This means that the redox reactions of cyt c at the Au/poly-DATT/DGS/POPA and Au/poly-DATT/DGS/CL electrodes were controlled by the kinetic effect. The peak separations were found to be increased with an increase in the scan rates, indicating that the redox reaction was quasi-reversible. The electron-transfer rate constants (k°) of the adsorbed cyt c at the Au/poly-DATT/DGS/POPA and Au/poly-DATT/DGS/CL biomimic membranes were 0.121 and 0.133 s⁻¹, respectively, using a method for a surface-bound electrochemical system. Although the electron transfer (ET) distance between the polymer substrate and the heme site of cyt c was not experimentally determined, it is expected that the ET distance should be ~3 nm because the thickness of a lipid monolayer is ~2–3 nm, and cyt c penetrated into the interior of the lipid bilayer to a significant extent due to the electrostatic interaction between the positive charges of cyt c and the negative charges of the phosphate groups. The tunneling rate is expected to depend approximately exponentially on distance. Thus, in the present study, the long-range electron-transfer reaction of cyt c was expected to occur in a fashion similar to other well-defined systems. The values of electron-transfer rate constants are comparable to the reported values obtained at the cyt c immobilized onto a 16-carboxylic acid-terminated SAM-modified gold electrode (0.09 s⁻¹). Applications of Cyt c-Immobilized Biomimic Membranes to Superoxide Detection. The possible applications of these biomimetic membranes to the construction of the superoxide anion radical sensor were preliminarily tested by immobilizing cyt c onto these membranes. The superoxide anion radical (O₂⁻) is a short-lived species resulting from the 1 e⁻ reduction of oxygen and is a primary component of so-called reactive oxygen species. However, it is stabilized in the lipid layers due to the interaction between the superoxide radical and a hydrophobic protonated site of lipids. Moreover, lipid layers can improve the physical adherence and communication between cyt c and biological samples, such as cells in the in vitro and in vivo experiments. Thus, a conventional voltammetric method can be employed for the detection of O₂⁻ with the modified electrode. In addition, the present modified electrodes have very stable binding of lipid layers because lipid layers were covalently bonded with a conducting polymer.

The O₂⁻ was generated by the injection of K₂O₂ in dimethyl sulfoxide (DMSO) into the phosphate buffer solution. This method was followed because the half-life of O₂⁻ is very short in aqueous solutions and the O₂⁻ undergoes natural disproportionation reaction in water according to the following equation:

\[
2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2
\]

Figure 9 shows the CVs recorded for (A) Au/poly-DATT/DGS/POPA/cyt c and (B) Au/poly-DATT/DGS/CL/cyt c-modified electrodes in phosphate buffer solution with (solid line) or without O₂⁻ (dashed line) after purging the nitrogen gas for 20 min. In the absence of O₂⁻, the CVs exhibited only the cyt c redox peak for both electrodes. When the DMSO solution of K₂O₂ was injected into the phosphate buffer solution with stirring, a new cathodic peak was observed at about −0.29 V and −0.26 V for Au/poly-DATT/DGS/POPA/cyt c and Au/poly-DATT/DGS/CL/cyt c, respectively. These cathodic peaks might be related to the reduction of O₂⁻ by the adsorbed cyt c according to the following equations: The currents of these cathodic peaks increased with increasing concentrations of O₂⁻, indicating that the cathodic peaks solely arose from the reduction of O₂⁻. The CVs recorded for Au/poly-DATT/DGS/POPA (dotted line) and Au/poly-DATT/DGS/CL (dotted line) in the presence of O₂⁻ did not show any peak for the O₂⁻ reduction. The O₂ reduction was observed at the more negative potential (at about −0.5 V versus Ag/AgCl) with the modified electrodes as shown in Figure 9C. However, in the present study, O₂ was completely removed from the solution by purging a pure N₂ gas for 20 min before each experiment.

The O$_2^-$ detection at these low potentials is very effective for the elimination of other interfering compounds. The interferences from uric acid, dopamine, and glutamate were not observed for the O$_2^-$ detection. However, ascorbic acid, a potential interfering compound, reduced the O$_2^-$ sensor response significantly. The 50% reduction of current response was observed as a result of the addition of 0.1 mM ascorbic acid to the O$_2^-$ system. This result indicated that ascorbic acid is a scavenger of the O$_2^-$ anion.

The amperometric detection of O$_2^-$ was performed at −0.29 and −0.26 V with Au/polyp-DATT/DGS/POPA/cyt c- and Au/polyp-DATT/DGS/CL/cyt c-modified electrodes, respectively. Figure 10 shows the typical current–time plots for the addition of varying amounts of KO$_2$ (in DMSO) in a 0.1 M phosphate buffer solution (Figure 10A). The catalytic reduction currents rose steeply to a stable value as soon as the KO$_2$ was added. The superoxide reduction response was confirmed using a superoxide scavenger, superoxide dismutase (SOD), and is shown in Figure 10B. During the amperometric experiment, addition of SOD greatly decreased the reduction current, whereas the addition of catalase did not change the reduction current. This indicates that the reduction current was due to the reduction of the superoxide radical. Figure 10C shows the calibration plots for the O$_2^-$ detection obtained for Au/polyp-DATT/DGS/POPA/cyt c- and Au/polyp-DATT/DGS/CL/cyt c-modified electrodes. The sensitivity of the Au/polyp-DATT/DGS/CL/cyt c-modified electrode was about 2 times higher than that of the Au/polyp-DATT/DGS/POPA/cyt c electrode. The relative standard deviations (RSD) at 1.0 nM of O$_2^-$ were 6.3 and 6.1% with the Au/polyp-DATT/DGS/POPA/cyt c and Au/polyp-DATT/DGS/CL/cyt c-modified electrodes, respectively. These linear dependences of the O$_2^-$ concentration yielded the regression equations of $i_0$ (nA) = (53.1 ± 2.8) + (51.6 ± 1.71) [C] (μM) and $i_p$ (nA) = (17.91 ± 1.6) + (33.2 ± 0.94) [C] (μM) for the Au/polyp-DATT/DGS/CL/cyt c- and Au/polyp-DATT/DGS/POPA/cyt c-modified electrodes, respectively, with correlation coefficients of 0.998 and 0.997. The detection limits were 40 ± 9 and 80 ± 6 nM, respectively, based on triplicate measurements for the standard deviation of the blank noise (95% confidence level, $k = 3, n = 5$). The lower detection limit and higher sensitivity obtained for the Au/polyp-DATT/DGS/CL/cyt c-modified electrode were related to the larger amount of immobilized cyt c in the Au/polyp-DATT/DGS/CL due to the presence of two phosphate groups in CL, which compared with one phosphate group in POPA.

The dynamic ranges and the detection limits were found to be wider and lower, respectively, than those obtained recently from a superoxide sensor based on a monolayer and multilayer cytochrome c electrodes. The stabilities and the detection limits of the present superoxide sensors are also comparable to a superoxide dismutase-immobilized cysteine-modified gold electrode.

The stability of the O$_2^-$ sensor was tested by measuring the response three times each day for 7 days. No drastic change in the O$_2^-$ response was observed when the sensor was kept at 4 °C after every measurement. After 7 days, the response decreased significantly. The protein, cyt c, was immobilized by only electrostatic interactions; thus, a higher number of O$_2^-$ measurements certainly resulted in a loss of protein from the electrode surface.

**CONCLUSIONS**

Two types of biomimic membranes were prepared by immobilizing DGS lipid onto a nanoparticle-composed conducting polymer through covalent bonding. The POPA and CL lipid bilayers were formed onto the DGS-bonded poly-DATT using the LB technique. The surface characterizations of these biomimic membranes were investigated using EQCM, SEM, AFM, and XPS analyses. The electrochemical behavior of cyt c at these biomimic membranes was studied using cyclic voltammetry, and it was found that the redox reaction of cyt c is a quasi-reversible process. Two types of O$_2^-$ biosensors were constructed by immobilizing cyt c on the biomimic membranes through the charge interactions between the positive charges of cyt c and the negative charges of the phosphate groups in the lipids. The linear range for the O$_2^-$

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detection was between 0.2 and 3.2 μM for both biosensors. The detection limits were ~40 and 80 nM for the Au/poly-DATT/DGS/CL/cyt c- and Au/poly-DATT/DGS/POPA/cyt c-modified electrodes, respectively. The long-term usability of the modified electrodes might be extended for a long time by repeating adsorbing of cyt c. Miniaturization of the electrodes is also possible, which is another advantage of the present electrodes. In fact, microelectrode fabrication with the present methods can be a suitable biosensor for the in vivo detection of the superoxide radical in cells and tissues, a goal in our current study.

ACKNOWLEDGMENT

Financial support for this work from the Ministry of Health and Welfare (Grant No. 02-PJ3-PG6-EV05-0001) and from the Korean Science and Engineering Foundation (KOSEF) through the Center for Integrated Molecular Systems is gratefully acknowledged.

Received for review June 8, 2005. Accepted October 24, 2005.

AC0510080