Characterization of Protein-Attached Conducting Polymer Monolayer

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Cytocrome c (cyt c)-immobilized monolayers and multiple monolayers of a conducting polymer [poly(terthiophene-3-carboxylic acid) polymer (poly-TTCA)] were prepared, where the monolayer of monomer precursor was fabricated with the Langmuir—Blodgett technique. Covalent immobilization of cyt c was achieved by the formation of an amide bond between the carboxylic groups of the conducting polymer and amino groups of lysine in cyt c. The monolayer of poly-TTCA and poly-TTCA/cyt c was characterized by cyclic voltammetry, XPS, EQCM, Auger electron spectra (AES), and atomic force microscopy (AFM). The immobilization of cyt c on the polymer layer reveals the direct electron-transfer processes of cyt c. Cyclic voltammetry of the poly-TTCA/cyt c-modified electrode showed a pair of reversible peaks at +0.212/0.212 mV (Epa/Epc) versus Ag/AgCl in a 0.2 M phosphate buffer solution (pH 7.0). The peak separation and the redox peak current of the poly-TTCA/cyt c-modified electrodes were gradually increased by increasing the number of poly-TTCA/cyt c layers on the electrode. The heterogeneous electron-transfer rate constant (kct) of cyt c at the poly-TTCA/cyt c-monolayer-modified electrode was estimated to be 0.874 s−1. The method provides a novel route for the fabrication of protein (cyt c)-immobilized and/or lipid (palmitoyloleoylphosphatic acid)—immobilized monolayers and multiple monolayers of a conducting polymer. Cyt c bonded on the conductive polymer layers was applied for bioelectronic devices with unique functionality.

Conducting polymers are well-known as functional materials for applications in batteries, electrodes, fuel cells, sensors, etc., due to their unique properties.1 They have attracted much attention in recent years as an immobilization matrix of biomaterials, such as proteins and enzymes. Also, they act as an electron communication linker for redox active organics and proteins.1–3 Particularly, poly-5,2,5′-terthiophene-3′-carboxylic acid (poly-TTCA) is a promising candidate for a matrix to immobilize biomolecules for biosensor systems, because it contains carboxyl acid groups in the polymer structure that are easily grafted onto its molecule skeleton and exhibits environment stability and good electrical properties.4 The polymer acts as a redox stabilizer (redox buffer) for redox enzymes and proteins that resists the oxidation of these compounds in air.3

Electrical contacting of redox proteins and electrode surfaces is one of the important issues in the field of bioelectronic devices (biosensors, biofuel cells, and bioelectronics), a rapidly progressing research field that aims to integrate biomolecules and electronic elements into functional groups.4 Immobilization of redox proteins to the electrode surface without denaturalization is essentially required to develop biosensing devices based on electroactive properties of biomaterials.5 One popular strategy utilizing the functional-group-terminated layer to get biomaterial immobilization on the electrode is the self-assembled monolayer (SAM). Many studies have been performed for redox protein, such as cytochromes c (cyt c) and c with the SAM method.6 However, the preparation of conducting or electroactive multiple monolayers, which make it possible to increase the well-oriented coverage of cyt c complex in a limited electrode area, is difficult. Additionally, controlling the orientation of functional groups and the polymerizing direction during the electropolymerization of monomers in a solution phase is difficult.7 Thus, there is a need of a much more convenient method to overcome these difficulties.

In the present study, the fabrication and characterization of protein (cyt c)-immobilized monolayers and multiple monolayers of the conducting polymer were carried out with Langmuir—Blodgett (LB)7 and electrochemical techniques. The poly-TTCA and poly-TTCA-attached cyt c (poly-TTCA/cyt c) monolayers were characterized by cyclic voltammetry (CV), XPS, EQCM, Auger electron spectra (AES), and atomic force microscope (AFM) experiments. The monolayer of poly-TTCA/cyt c was used for bridge-like electronic communication in bioelectronics device applications. Finally, a lipid (palmitoyloleoylphosphatic acid, POPA) monolayer, an insulating material, which could control the current flow depending on the geometry of the lipid

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monolayer onto poly-TTCA and poly-TTCA/cyt c monolayers, was used to construct passive bioelectronics device elements.

**Experimental Section**

**Reagents.** Cytochrome c from a horse heart (type VI, Sigma Co.) was used after purification by the previously described procedure. First, cyt c was converted to the fully oxidized form by addition of excess K$_3$Fe(CN)$_6$. Next, it was purified by ion-exchange chromatography on Whatman CM-32 and eluted with 0.5 M NaCl + 10 mM PBS (phosphate buffer solution, pH 7.0). Eluent containing the purified protein was concentrated by ultrafiltration using Amicon YM-3 membranes, and then diazylated extensively to remove phosphate.

A terthiophene monomer bearing a carboxylic acid group, 5,2′:5′′,2″-terthiophene-3′-carboxylic acid (TTCA), was synthesized following our previous report; 1-Ethyl-3-(3-dimethylaminopropyl)-carboxamide (EDC) and dichloromethane (98.8%, anhydrous, sealed under nitrogen) were received from Sigma Co. Tetraethylammonium perchlorate (TBAP, electrochemical grade) was obtained from Fluka Co., and dried under vacuum. Using Na$_2$HPO$_4$/NaH$_2$PO$_4$ and sodium acetate/acetic acid mixtures, respectively. All other chemicals were of extrapure analytical grade and used without further purification. All aqueous solutions were prepared in doubly distilled water, which was obtained from a Milli-Q pure water system.

**Apparatus.** The glassy carbon (GC) and gold (Au) electrodes were polished with 0.5-μm alumina/water slurry and a polishing cloth to a mirror finish, followed by sonication and rinsing with distilled water. A Kosentech PT-2 potentiostat and galvanostat was used for all electrochemical experiments. The counter electrode was a Pt wire and the reference electrode was an Ag/AgCl electrode. The highly oriented pyrolytic graphite (HOPG) was freshly cleaved before the AFM experiment and was purchased from Advanced Ceramics Co. AFM experiments were carried out on a multimode AFM system from Digital Instrument Inc. Commercial Si$_3$N$_4$ tips were attached to a triangular cantilever made of the same material of 125-μm length, 300-kHz resonance frequency, and 5-10-nm radii. The force (spring) constant was 20-100 N/m. A Laumguir—Blodgett trough (KSV-5000) equipped with two symmetrical compartments, each 71 × 12 cm$^2$, was used to record surface pressure—area isotherms. The QCM experiment was performed using a SEIKO EG & G model QCA 917 and the EG & G PAR software package (M270/250 Electrochemical Analysis Software). Au-coated working electrodes (area, 0.196 cm$^2$, 9 MHz: AT-cut quartz crystal) were used. AES was performed using a VG Scientific Microlab 350 (at KBIS, Busan). A primary electron beam energy of 5.0 keV and primary beam current of ~200 nA were used in all AES analysis. XPS experiments were performed using a VG Scientific Escalab 250 XPS spectrometer with monochromatic Al Kα source with charge compensation at KBIS (Busan, Korea).

**Preparation of TTCA Monolayers.** A two-step method was followed to fabricate the monolayers and multilayers of protein-attached conducting polymers. First, the preparation of the poly-TTCA monolayer bearing selectively oriented carboxyl groups of TTCA molecules on the electrode surface was followed by casting a thin TTCA monolayer by the LB method. Second, the formation of monolayers and multilayers of protein-immobilized conducting polymer (poly-TTCA/cyt c) and (poly-TTCA/cyt c)$_n$, where $n = 1, 2, 3, ...$ was undertaken by immobilizing cyt c onto the poly-TTCA layer. The LB trough was treated before use by rinsing with distilled water and chloroform. It was then filled with a 0.1 M NaOH solution. The TTCA monomer solution (1.0 mg mL$^{-1}$) was prepared in CH$_2$Cl$_2$ and used without further purification. All aqueous solutions were prepared in doubly distilled water, which was obtained from a Milli-Q pure water system.

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**Fabrication of the Monolayers and Multiple Monolayers of Poly-TTCA/cyt c.** The TTCA monolayer transferred from the LB trough was immediately electropolymerized by cycling the potential between 0.0 and +1.5 V (1 cycle) in a 0.2 M acetic acid buffer solution (pH 5.0) at a scan rate of 50 mV/s to obtain the poly-TTCA monolayer (Scheme 1). The poly-TTCA-monolayer-modified electrode was then immersed in a 10 mM phosphate buffer solution (pH 7.0) containing 5.0 mM EDC at 25 °C for 12 h. Thereafter, it was incubated in a phosphate buffer solution (pH 7.0) containing cyt c (4.0 mg mL$^{-1}$) at 4 °C for 48 h to form a covalent bond via the carboxylic groups in poly-TTCA and amines of lysine in cyt c (Scheme 1a). The electrode was then washed three times with PBS. To obtain a poly-TTCA/cyt c-multilayer-modified electrode, the fabrication procedure for the poly-TTCA/cyt c monolayer was repeated in a successive fashion. On the other hand, the lipid, such as the POPA monolayer, was prepared using the LB technique. This followed the same procedure described previously for the fabrication of a TTCA monolayer, with the exception of using chloroform as a solvent instead of CH$_2$Cl$_2$ for making POPA solutions (1.0 mg mL$^{-1}$).

**Fabrication of the Bioelectronics Device.** Au electrodes for the device were fabricated on a clean polyethylene film by vacuum deposition followed by treating with (3-mercaptopropyl)trimethoxysilane (MPS) and washing with ethanol and vacuum drying. A thin plastic mask was used to define the electrode pattern having a gap width of ~5.4 μm between the two Au disks sized 0.5 × 1.0 cm, and contact pads were each 0.3 cm in diameter. The gold was vapor-deposited between 800 and 1000 A with a Hummer X sputter coater (Anatech Co.).

At the first stage of the electronic device fabrication, the poly-TTCA film was electrochemically formed on the two separated Au electrodes together in a 0.1 mM TTCA/CH$_2$Cl$_2$ solution. The 430 polar groups (COO$^-$) of TTCA will form attachments with the subphase layer, with the thioephene backbone jutting outward. The resultant LB monolayer of TTCA was transferred onto the electrode surface at different surface pressures at a speed of 5 mm/min, where the electrode was only being inserted into the subphase (the monolayer transfers upon the downstream only), or it was only being removed (the monolayer transfers upon the upstream only). These depositions of the TTCA monolayer are assigned as tail-first [because the sulfur (S) groups of TTCA come first (in the former case)] or head-first [because the COO$^-$ groups of TTCA come first (in the latter case)]. The transfer ratio, defined as the ratio of the surface area of the monolayer on water to that of the electrode onto which the monolayer was transferred, was measured with the software control. The transfer ratio was close to 1 in each case, indicating the quantitative transfer of the LB monolayer of TTCA onto the electrode surface. TTCA-monolayer-coated electrodes were dried for 24 h under nitrogen before use.

**Scheme 1. Schematic View for the Preparation of the Poly-TTCA/cyt c-Modified Electrode**

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potential cycling at the scan rate of 1 V/s produced very thin polymer layer growth that made a narrow gap width between the two separated electrodes. The continuous potential cycling at the very fast sweep rate made controlling the gap width between the two separated electrodes easy through growth of the poly-TTCA film, which gave a 40–195 nm gap width between two separated electrodes. After that, the two separated poly-TTCA-coated electrodes were connected immobilizing cyt c, which shows the bridge-like connection by cyt c. The next TTCA monolayer was coated by the LB technique and polymerized in an aqueous solution for the multilayer fabrication.

Results and Discussion

Characterization of Transfer Conditions of the TTCA Monolayer. In the early stage of the experiment, to get a better alignment of TTCA molecules on an air–water interface, the composition of subphase was examined under neutral, acidic, and basic conditions. When distilled water was used as a neutral subphase, the formation of TTCA monolayers was impossible due to the complete aggregation of TTCA molecules upon spraying. This phenomenon could be due to the poor solubility of the TTCA monomer in water. Under acidic media (in the 1.0, 100, 500 mM HCl solutions), the larger aggregation of TTCA molecules was observed and possibly caused by the overlapping of thiophene rings of TTCA molecules by the strong interaction between -COOH groups of TTCA.12 On the other hand, the basic medium displays a well-oriented monolayer. Thus, a basic medium was used to make the TTCA monolayer.

Figure 1A shows the π–A isotherms obtained for a TTCA monolayer at 20 ± 0.1 °C, where the compression rate was 5 mm/min. When a 0.01 M NaOH solution was used as a subphase, the surface pressure increased rapidly to 62 mN/m and the limited molecular area (LMA) was found to be 1.1 Å. Over 62 mN/m, the floating monolayer collapsed. When the 0.1 M NaOH solution was used, the surface pressure rose gradually to 47 mN/m, and the LMA value was found to be 2.1 Å. With a further increase in the surface pressure above 47 mN/m, the floating monolayer collapsed. Using a 0.5 M NaOH solution as a subphase, the LMA value was 2.7 Å. Under this condition, the monolayer collapsed above 48 mN/m. No change in the isotherm of TTCA was observed at >0.5 M NaOH concentrations. Such differences in isotherms could be due to the different ionic repulsion of polar groups (COO-) of TTCA under basic conditions. In a 0.01 M NaOH solution, the surface pressure for collapsing of a TTCA monolayer was high due to the higher repulsion between COO- groups. At >0.5 M NaOH solutions, the monolayer was formed at lower surface pressure, mainly due to the lower ionic repulsion between COO- groups of TTCA and/or OH- ion in the subphase.

The TTCA monolayer formed at different NaOH conditions was transferred to the electrode surfaces; next, the electropolymerization was carried out (one cycle, Scheme 1) to get the polymer monolayer. The poly-TTCA/cyt c-modified electrode, after immobilization of cyt c on the poly-TTCA layer fabricated in a 0.1 M NaOH subphase followed by the EDC activation step, gave the largest redox peaks of cyt c in a 0.2 M PBS, pH 7.0. Thus, all experiments for the TTCA monolayer transfer were performed using a 0.1 M NaOH subphase at 46 mN/m (20 ± 0.1 °C).

Immobilization of Cyt c on the Oppositely Oriented TTCA Monolayer. The formation and electropolymerization of the TTCA monolayer were performed, leading to the two different orientations, tail-first (Scheme 1a) and head-first (Scheme 1b),

Figure 1. (A) π–A isotherm curves for floating monolayer of TTCA at the air–water interface. (B) Electropolymerization of the TTCA monolayer as the spatial orientation in a 0.2 M acetate buffer solution (pH 5.0) at a scan rate of 50 mV/s. (C) CV’s recorded with the poly-TTCA/cyt c-monolayer-modified electrode according to orientation of TTCA monolayer in a 0.2 M phosphate buffer solution (pH 7.0). Scan rate, 50 mV/s. (D) Frequency changes during the immobilization of cyt c onto the poly-TTCA monolayer on a gold electrode.

Figure 2. (A) Auger electron spectra for poly-TTCA and poly-TTCA/cyt c-monolayer-modified surfaces. XPS spectra of (B) C1s, (C) O1s, and (D) N1s for a poly-TTCA-monolayer-modified electrode (black) and poly-TTCA/cyt c-monolayer-modified surfaces (red).

Conductivity Measurements of TTCA Film. After formation of the TTCA monolayer, we measured the surface conductivity of the poly-TTCA film followed by our previous report. The resistance was measured across the 20 µm gap in a dichloromethane solution containing 0.1 M TBAP while the potential was varied from −0.5 to 1.6 V. The calculated resistance and conductivities are plotted as a function of applied potential. The conductivity increases monotonically as the potential becomes more positive. This result indicated that the oxidized quinoid form of this polymer was also quite conductive.

QCM Analysis. The immobilization of cyt c depending on the orientation of the TTCA monolayer was monitored by EQCM experiments (Figure 1D). The TTCA-monolayer-coated gold electrode was electropolymerized as depicted in Scheme 1a. It was then mounted in the EQCM cell, followed by measuring frequency changes in a 5.0 mM phosphate buffer solution (pH 7.0) containing 4.0 mg mL⁻¹ cyt c. The poly-TTCA-monolayer-modified electrode having a tail-first orientation showed immediate frequency changes upon adding the cyt c solution, whereas no change was observed for the bare gold electrode, due to the absence of a polymer layer. During immobilization of cyt c onto the poly-TTCA film having a tail-first orientation, the frequency decreased rapidly in the first 25 min and attained a steady state after 137 min. This indicates that immobilization of cyt c onto the poly-TTCA film was completed within 137 min at room temperature. After 137 min, the frequency change (ΔF) was about 42.7 Hz. The mass change (Δm) was determined using the equation reported previously. The amount of cyt c immobilized onto the poly-TTCA film having a tail-first orientation after 137 min was 46.2 ng for a 0.196 cm² electrode, which corresponded to a surface coverage of 3.78 × 10⁻¹² mol. On the basis of the molecular mass of 12 230 Da and the hydrodynamic diameter of 3.4 nm of cyt c globular from bovine heart, the quantity of cyt c in the monomolecular layer was calculated to be 3.59 × 10⁻¹² mol. The value is almost the same as the quantity of cyt c adsorbed on the first poly-TTCA monolayer, indicating the formation of a monomolecular layer of cyt c in each layer. The amount of cyt c increased with increasing the poly-TTCA/cyt c layer from the first to the third layer and decreased thereafter. The immobilized cyt c after 137 min was about 75.4, 134.5, 91.2 ng for the second, third, and fourth layers of poly-TTCA/cyt c, respectively. The increased amounts of cyt c loading in QCM experiments with increasing of the poly-TTCA/cyt c layer clearly indicated the formation of the poly-TTCA/cyt c multilayer. On the other hand, for the poly-TTCA film having the head-first orientation (Scheme 1b), ΔF was only about 2.3 Hz after 137 min of immobilization. This resulted in a very little amount of cyt c (2.53 ng) being immobilized onto the poly-TTCA film having a head-first orientation.

AES and XPS Analysis. Figure 2A shows the AES spectra for the poly-TTCA monolayer and cyt c-immobilized poly-TTCA
monolayer. For the poly-TTCA monolayer, Auger peaks were observed at 91, 265, and 510 eV for S\textsubscript{LMM}, C\textsubscript{KLL}, and O\textsubscript{KLL}, respectively, which indicated the successful formation of the poly-TTCA monolayer on the electrode. The peak for S\textsubscript{LMM} in the case of the cyt c-bonded poly-TTCA surface shifted to a lower energy, while the peaks for C\textsubscript{KLL} and O\textsubscript{KLL} were found in the same position as for the poly-TTCA monolayer. An additional N\textsubscript{KLL} peak at 510 eV was observed for the cyt c-bonded poly-TTCA surface, which provided a guide for the immobilization of cyt c onto the poly-TTCA layer, due to the fact that TTCA has no nitrogen atoms.

Figure 2B–D shows the XPS spectra of C1s, O1s, and N1s for the poly-TTCA and poly-TTCA/cyt c-monolayer-modified electrodes. The C1s peak of the poly-TTCA monolayer was observed at 284.6 eV for C–C, C–S, and C–H bonds, while the peak for C–O and C=O bonds was found at 287.1 eV. At the poly-TTCA/cyt c-monolayer-modified electrode, a new peak corresponding to a C–N bond was observed at 286.0 eV. In addition, the C–O bond was shifted to a higher energy, confirming the immobilization of cyt c onto the poly-TTCA monolayer by the amide bond formation between the carboxylic acid group of poly-TTCA and amines of cyt c.14 The O1s peak of poly-TTCA was found at 531.4 eV, which assigned the C–O–H bond of the -COOH group.13 At the poly-TTCA/cyt c-monolayer-modified electrode, a peak corresponding to a C–O bond was observed at 530.8 eV, which indicated the removal of O–H groups upon the amide bond formation between the carboxylic acid and amine groups. No N1s peaks in the spectra were observed for poly-TTCA only. However, the N1s peaks for the poly-TTCA/cyt c-monolayer-modified electrode were confirmed at 398.8 eV (–NH\textsubscript{2}) and 400.9 eV (–NH).15 Here, the latter peak indicated the formation of an amide bond and the former peak came from the nitrogen-containing side chains like lysine of cyt c.

AFM Images. The TTCA monolayer prepared by the LB technique showed a uniform distribution of the monolayer in ATM images (Figures 3a, d). After electropolymerization of the TTCA monolayer, the small bright domains, ~3.5 nm in height and ~35 nm in size, were randomly distributed into the poly-TTCA monolayer. The rough surface was observed in 3D images (Figure 3b, c). We assumed that small domains were ascribed to the formation of salts by counterionic species onto the cationic poly-TTCA backbone, because the rough surface and the domains disappeared after immobilization of cyt c (Figure 3c, f). The diameter of immobilized cyt c was ~12.5 nm, which was 3 times larger than the previously reported value of 3.7 nm,16 indicating that cyt c was adsorbed as a big cluster form.

Although we have studied the poly-TTCA/cyt c thin film with QCM, AES, XPS, AFM, and conductivity measurements techniques, some other techniques such as IR and CD may also possibly determine the structure of the protein-attached conducting polymer.5

The Electrochemical Behavior of Immobilized Cyt c. The electron-transfer (ET) reaction of cyt c at bare electrodes such as platinum, gold, nickel, mercury, and p-type silicon has been shown to be slow due to adsorption-induced denaturation.17 In addition, it is not possible to observe the direct electron-transfer process of cyt c at bare electrodes, such as glassy carbon. However, modification of the electrode surfaces with electron promoters have made it possible to observe direct ET reaction of cyt c. We have previously studied the electrochemical behavior of cyt c for a cardiolipectin and a biomembrane of POPA/cardiolipectin-bonded conducting polymer layers. Herein, the direct electron transfer of cyt c at the monolayer and multiple monolayers of poly-TTCA/cyt c-modified electrodes was studied, as shown in Figure 4A, B.

As shown in Figure 4A, the peak currents of cyt c were directly proportional to the scan rates, indicating that the redox reaction was controlled by adsorbing cyt c18 at the poly-TTCA/cyt c-monolayer-modified electrode. The peak separations were also found to have increased upon increasing the scan rates, indicating a quasireversible system. The formal potential determined for cyt c was little positive compared with the previous results obtained with various negatively charged surfaces. This was possibly due to the interaction of cyt c with the interior of the underlined polymer, or the protein-attached polymer monolayer exposes the heme to an environment of low dielectric constant, which might shift the heme redox potential to the higher positive values.56 The redox peak current of the poly-TTCA/cyt c-multilayer-modified electrode (n = 1–4) was also examined (Figure 4B). The redox peak currents of cyt c gradually increased up to the triple layer and then decreased at the quadruple layer. It was certain that the increase of the peak current was due to the increment of the total coverage of cyt c on to the electrode; however, the decrease occurring at the quadruple layer might have arisen from the following possibilities. These include ET limitations caused either by the semiconducting effect19 (hindered charge hopping mechanism) and/or by the elongated ET distance within the quadruple layer, which increases the resistance of the polymer layer. Moreover, this suggests the existence of an optimum ET length (~12–17 nm) for bioelectronics device applications of a poly-TTCA/cyt c multilayer system. The ratio of the oxidation to the reduction peak currents (I\textsubscript{pox}/I\textsubscript{pox}) and the peak separation increased with an increase in the number of layers. This demonstrates that the delayed ET rate was generated by the electrochemical irreversibility caused by the elongated ET distance, because I\textsubscript{pox}/I\textsubscript{pox} was equal to 1 for an ideally reversible ET reaction.20 The quasireversible ET rate constants (k\textsubscript{e}) of the poly-TTCA/cyt c-multilayer-modified electrode (n = 1–4) were calculated by using Laviron’s equation.21 The effect of the poly-


Figure 3. AFM images of (a) TTCA, (b) poly-TTCA, and (c) poly-TTCA/cyt c-monolayer. (d–f) 3-D AFM images of parts a–c.
conducting polymer-modified electrode\textsuperscript{23} and a lipid membrane-modified indium oxide electrode.\textsuperscript{21} However, the formal potential of the direct ET reaction of cyt c at the poly-TTCA/cyt c-modified electrode was shifted to a more positive value than the value obtained in a previous report employing different mixed self-assembled monolayers.\textsuperscript{6} At the S(CH\textsubscript{2})\textsubscript{2}-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 V 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 a control experiment, we compared the electrochemical properties of a poly-TTCA/cyt c-monolayer-modified electrode prepared using the present method with a conventional poly-TTCA electrode immobilized with cyt c. The conventional modified electrode was prepared by the electropolymerization of a TTCA monomer in TBAP/CH\textsubscript{2}Cl\textsubscript{2} solution,\textsuperscript{2,3a} followed by the EDC activation and subsequent cyt c immobilization steps. The redox peak separation was found to be 50 mV \((E\textsubscript{pc}/E\textsubscript{pa} = 0.221/0.171 \text{ V}, \Delta E\textsubscript{p} = 50 \text{ mV})\) with the conventional poly-TTCA/cyt c-modified electrode in 0.2 M PBS. The peak separation of 50 mV was ~40 mV more positive than the values obtained (~10 mV) with the poly-TTCA/cyt c-monolayer-modified electrode. The results indicated that the poly-TTCA/cyt c-monolayer-modified electrode has a much closer interaction between cyt c and the modifier monolayer, which led to more efficient electronic communication between the electrode surface and cyt c. A series of ten successive measurements using poly-TTCA/cyt c-monolayer-modified electrodes in 0.2 M PBS yielded a good reproducible signal with a relative standard deviation of 3.2%.

### Application to Bioelectronics Device

Two split poly-TTCA-covered electrodes, separated with a narrow gap that was controlled by growth of the conductive polymer, were used to fabricate a device. The images obtained by the scanning electron microscopy (SEM) confirmed the distance between the bare Au electrodes as 5.4 ± 0.5 \(\mu\text{m}\) (not shown). After polymerization in a 0.1 mM TTCA/CH\textsubscript{2}Cl\textsubscript{2} solution at the scan rate of 1 V/s, the SEM images of the Au/poly-TTCA//poly-TTCA/Au structure confirmed that the distance between the electrodes decreased to ~40 and 190 nm when covering the electrodes with a poly-TTCA layer (not shown). To form a bridge-like connection by a protein between the polymer-modified electrodes on a Au/\{poly-TTCA/cyt c/poly-TTCA/Au device, cyt c was immobilized onto it. The connection between the two separated electrodes by cyt c was confirmed by the SEM images. The electropolymerization of the TTCA monolayer was performed to form the poly-TTCA monolayer, followed by the TTCA monolayer casting by the LB method (tail-first deposition) onto the cyt c-immobilized poly-TTCA-coated Au electrodes. The monolayer casting, electropolymerization, and cyt c immobilization processes followed by the EDC activation steps were repeated in successive fashion to construct a Au/\{poly-TTCA/cyt c/poly-TTCA\}_n/Au device \((n = 1 \sim 3)\). The current–voltage \((I–V)\) curve was measured at a linear sweep (the scan rate of 50 mV/s) using the Au/cyt c/Au and Au/\{poly-TTCA//cyt c/poly-TTCA\}_n/Au devices (not shown). For the Au/\{poly-TTCA//cyt c/poly-TTCA\}_n/Au device, the measured currents at −4 V were 96 \((n = 1)\) and 297 nA \((n = 3)\), whereas it was 0.21 nA for the Au/cyt c/Au device. At the three layers electrode, a sharp

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The redox peaks of cyt c were observed at ~0.210/0.200 V \((E\textsubscript{pc}/E\textsubscript{pa}, \Delta E\textsubscript{p} = ~10 \text{ mV})\) when the CV was recorded with poly-TTCA/cyt c-modified electrodes in 0.2 M PBS. The potential is very close to the values obtained in previous reports using a lipid-bonded

Table 1. Electrochemical Properties of Poly-TTCA/cyt c-Monolayer- and Multiple-Monolayer-Modified Electrodes

<table>
<thead>
<tr>
<th>No.</th>
<th>(I_{pa}/I_{pc}) (nA) (±SD) (\Delta E\textsubscript{p} (mV)) (±SD)</th>
<th>(k_0 (s^{-1})) (±SD)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>29.6 ± 0.8/29.6 ± 0.7 210 ± 1/200 ± 1.3 10 ± 1.1 0.874 ± 0.02</td>
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<tr>
<td>2</td>
<td>48.3 ± 0.9/47.0 ± 0.6 228 ± 1.4/196 ± 1.2 32 ± 1.3 0.712 ± 0.02</td>
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<tr>
<td>3</td>
<td>87.9 ± 1.3/81.0 ± 1.2 235 ± 1.5/198 ± 1.7 37 ± 1.5 0.679 ± 0.01</td>
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<tr>
<td>4</td>
<td>59.2 ± 0.9/59.5 ± 0.8 261 ± 1.3/161 ± 1.5 100 ± 1.4 0.367 ± 0.01</td>
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\(\Delta E\textsubscript{p}\) is the peak separation, \(k_0\) is the rate constant of the direct ET reaction of cyt c.

\* Number of poly-TTCA/cyt c monolayers. \(n = 3.\)
polarization $I-V$ curve was observed, indicating the typical rectifier property of the device fabricated with the poly-TTCA/cyt c multilayer (Au{(poly-TTCA//cyt c)//poly-TTCA}$_3$/Au). The device exhibits amplified currents at a voltage range of −4 to 4 V compared to the direct-coupled cyt c device (Au/cyt c//Au). This is so because poly-TTCA coating can minimize the side effects, such as the adsorption-induced denaturation or the nonoptimal orientation of cyt c, which will appear when cyt c molecules are directly adsorbed onto the bare Au electrode. The control of the layer thickness of Au{(poly-TTCA//cyt c)//poly-TTCA}$_n$/Au reveals the potential of passive bioelectronics elements.

In the fabrication of bioelectronic elements with a protein, according to the variable geometries of the lipid monolayer deposited using the LB method, we provided a physiological environment similar to a cell. Here, the $I-V$ curve was obtained to investigate the control of the ET direction (Figure 5). The device structure, as shown in Figure 5A, was obtained by the following: (a) electropolymerization of the poly-TTCA film onto the two separated Au electrodes, (b) immobilization of cyt c followed by the EDC activation step, (c) deposition of the POPA monolayer onto the poly-TTCA//cyt c layer by the LB technique, and (d) the connector formation of the poly-TTCA layer onto the poly-TTCA/cyt c/POPA layer. This device displayed no current response due to the insulation by lipid (POPA) molecules (Figure 5C). Similar to the device shown in Figure 5A, the device structure shown in Figure 5B was also obtained by utilizing four steps with the exception of the following two steps: (i) immobilization of cyt c was carried out only on one electrode (left side as shown), while POPA was deposited onto some part of the second (right side) electrode, and (ii) the poly-TTCA monolayer was formed onto both electrodes with the remaining poly-TTCA-coated parts of the right electrode providing a communication bridge between the poly-TTCA/cyt c of the left and poly-TTCA of the right electrodes. This device exhibited a good current flow through the contact between poly-TTCA layers through the cyt c molecular bridge (Figure 5D). This result demonstrated that the control of protein and lipid geometry in a device can adapt the fabrication of bioelectronics passive elements, which can be used to adjust the desired current flow direction in a specific electronic circuit dimension.

Conclusions

We have demonstrated the immobilization of well-oriented and stable monolayers and multilayers of a protein, cyt c, onto the monolayered conducting polymer layers through the formation of covalent bond between the carboxylic acid groups in a conducting polymer of poly-TTCA and amines of cyt c. The poly-TTCA was grown electrochemically as the spatial orientation of the TTCA monolayer onto the electrode followed by the LB method. The well-oriented and stable cyt c layer on the poly-TTCA monolayers and multilayers offers the redox peaks of cyt c with an excellent reproducibility. This solves the problems of long-term instability and poor adhesion that commonly arise in conventional LB films. The electrochemical behavior of well-ordered cyt c at poly-TTCA/cyt c-monolayer-modified electrodes was studied using cyclic voltammetry. It was found that the redox reaction of cyt c is a quasi-reversible process. The favorable ET properties of monolayers and multilayers of poly-TTCA/cyt c-modified electrodes indicated that the method might be generally useful for protein devices. The monolayers and multilayers of poly-TTCA not only provide an adequate immobilization environment but also facilitate a possible applicability for developing bioelectronics devices such as biosensors and biofuel cells.

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