Fabrication of a biomimetic membrane with biomaterials-attached conducting polymer: Application to a NADH sensor

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Abstract—A electrochemical sensor for β-nicotinamide adenine dinucleotide (NADH) has been developed. To fabricate a stable sensor probe, we modified the probe surface with a conjugated polymer (5,2′:5′,2″-terthiophene-3′-carboxylic acid, TTCA), which serves as a base substrate to construct the artificial biomembrane. The AuNPs were electrodeposited onto a carbon electrode (CE) to enhance its electrocatalytic activity, sensitivity, and stability. Cytochrome c (cyt c) and lipid (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, DOPE) were simultaneously immobilized on the poly-TTCA/AuNPs/CE. DOPE and cyt c immobilized onto the poly-TTCA layers were 2.8 X 10⁻⁹ and 1.8 X 10⁻¹⁰ mole/cm², respectively. To improve the specific biomimetic properties, ubiquinone (UQ₁₀) was immobilized onto the modified electrode together. The characteristics of modified electrode were investigated by cyclic voltammetry, impedence spectrometry, differential pulse voltammetry, and QCM.

I. INTRODUCTION

Electron transfer reactions are essential processes in respiration, photosynthesis, and many other biochemical reactions. The study for the factors that control such electron transfer reactions is key point to understanding these processes and being able to reproduce them in synthetic systems [1]. Phospholipids, membrane receptors, or proteins of an extracellular matrix immobilized onto the 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 [2]. 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 used as biomembranes [3]. The development of a model of biomembrane systems is interest of more researchers because these model biomembrane systems can supply a biological environment to the surface of a biosensor [4].

Reduced β-nicotinamide adenine dinucleotide (NADH) and its oxidized form, NAD⁺, are very important coenzymes, which play an essential role in the energy production/consumption of all living systems. NADH participates in a variety of enzymatic reactions by more than 300 dehydrogenases [5]. Electrochemical reactions of NADH has been an important issue of amperometric biosensors [6] and biofuel cells [7]. However, the direct electrochemical conversion of both NAD⁺ and NADH takes place at a high overvoltage with less sensitivity of the electrode surface [8]. Thus, there have been lots of effort in designing another electrochemical system to investigate NADH reactions.

Conjugated polymers have attracted wide attention owing to their applicability to biosensors as well as optical devices and energy conversion devices, etc. [9]. Of these, conjugated polymers, having functional groups such as -COOH or -NH₂, are attractive for the fabrication of biosensors because biomolecules can be covalently immobilized onto conjugated polymers [4]. Stable immobilization of biomolecules is crucial to obtain a sensitive biosensor. Previously, -COOH group-functionalized terthiophene conjugated polymer was used to fabricate a DNA sensor [10] and immunosensor [11]. To fabricate a more stable biomimetic membrane, we modified the solid surface with a conjugated polymer layer, which serves as a base substrate for the biomembrane. A conjugated polymer of terthiophene is known to be very stable over a wide pH range and resistant to chemicals [10]. To enhance the stability, selectivity, and sensitivity, nanomaterials such as gold nanoparticles (AuNPs), carbon nanotubes and quantum dots can be used for sensor. AuNPs have been widely used in bioanalysis due to their size-dependent electrical properties, high surface area-to-volume ratios, high electrocatalytic activities, and ease of chemical modification [12]. Thus, AuNPs were electrodeposited onto a carbon electrode to enhance sensor performance of the present system.

In the present study, the use of artificial biomaterials with comparable recognition properties has been proposed for designing biomimetic sensors. Firstly, AuNPs were electrodeposited onto the carbon electrode (CE) to enhance the conductivity of the electrodes. The CE was modified by coating with a conjugated polymer, 5,2′:5′,2″-terthiophene-3’-carboxylic acid (TTCA), that has reactive carboxylic acid group for covalent attachment with the amine groups of lipid, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and cytochrome c (cyt c). To improve the specific biomimetic characteristics, ubiquinone (UQ₁₀) was simultaneously immobilized onto the modified electrode. The polymer-coated electrodes allow the lipid constituents to attach in a
chemically stable way through the covalent bond formation between the reactive groups of biomaterials and the modified surface. A schematic presentation of the biomimetic membrane is shown in Scheme 1. The electrochemical behavior of a cyt c with UQ_{10} was investigated and these biomimetic membranes applied for NADH detection.

II. METHODS

A. Materials

Cytochrome c (cyt c, from equine heart, molecular mass: 12,384 Da, Sigma Co.) was used after purification as follows: cyt c was converted to the fully oxidized form by addition of excess K_2Fe(CN)_6 and then purified by ion-exchange chromatography on Whatman CM-32, eluted with 0.5 M NaCl in 10 mM phosphate buffer at pH 7.0. Eluent containing the purified protein was concentrated by ultra-filtration using Amicon YM-3 membranes, and then dialyzed extensively to remove phosphate [13]. Decylubiquinone (UQ_{10}, Sigma Co.), β-nicotinamide adenine dinucleotide (NADH, Sigma Co.), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE, C18:1 (Δ 9-cis), Avanti Polar Lipid Inc.) were used without further purification. A terthiophene monomer having a carboxylic acid group, 5,2′:5′,2″-terthiophene-3′-carboxylic acid (TTCA), was synthesized according to a previous method [10]. 1-Ethyl-3-(3-(dimethylamino)-propyl) carbodiimide (EDC), N-hydroxy succinimide (NHS), di(propylene glycol) methyl ether, and tri(propylene glycol) methyl ether were received from Aldrich Co. (USA). All aqueous solutions were prepared in doubly distilled water, obtained from a Milli-Q water purifying system (18 MΩ/cm). The buffer solutions were prepared using NaH_2PO_4 + Na_2HPO_4 mixtures (phosphate buffer solution, PBS). All other reagents were of the best commercial quality available.

B. Apparatus and instruments

All electrochemical experiments were performed in a three-electrode cell. Modified CEs (area = 0.07 cm^2) were used as working electrodes, with Ag/AgCl (in saturated KCl) as a reference electrode and Pt wire as the counter electrode. Cyclic, linear sweep, and differential pulse voltammograms were recorded using a potentiostat/galvanostat (Kosentech model KST-P2, S. Korea). The impedance spectra were measured with the EG&G Princeton Applied Research PARSTAT 2263 at an open circuit voltage from 100 kHz down to 100 mHz, at a sampling rate of five points per decade (AC amplitude: 10 mV). A quartz crystal microbalance (QCM) experiment was performed using a SEIKO EG&G model QCA 917 and a PAR model 263A potentiostat/galvanostat (USA). An Au-coated working electrode (area: 0.196 cm^2; 9 MHz; AT-cut quartz crystal) was used for the QCM experiment.

C. Preparation of the modified electrode

For preparation of the modified electrode, AuNPs were electrodeposited onto the CE from a 0.5 M H_2SO_4 solution containing 0.001% HAuCl_4, using linear sweep voltammetry from +1.4 to +0.5 V. The electrodeposition conditions were as follows: deposition time, 60 s; deposition potential, -1.0 V; scan rate, 0.1 V/s; potential cycling, three times [12]. The poly-TTCA layer was formed on the AuNP/CE according to a previous method [11]. The CE was dipped in 1.0 mM TTCA monomer containing solution prepared in 1:1 of di(propylene glycol) methyl ether and tri(propylene glycol) methyl ether and polymer growth was obtained by potential cycling two times from 0.0 to 1.4 V (vs. Ag/AgCl) at 50 mV/s in a 10.0 mM PBS (pH 7.0). After polymerization, the poly-TTCA/AuNP electrode was immersed for 12 h in PBS (pH 7.0) containing 10.0 mM EDC and 10.0 mM NHS to activate the carboxylic acid groups of the poly-TTCA layer. Then, the electrode was washed with a buffer solution and subsequently 1.5 μL of 0.5 mM DOPE + 0.5 mM UQ_{10} mixtures in chloroform was dropped onto the electrode surface. The electrode was simultaneously incubated in 10.0 mM PBS containing 5.0 mg/mL of cyt c at pH 7.0 for 48 h at 4 °C and washed again with the same buffer.

III. RESULTS AND DISCUSSION

Fig. 1 shows the linear sweep voltammograms recorded during AuNPs electrodeposition on to the carbon electrode (CE) in a 0.5 M H_2SO_4 solution containing 0.001% HAuCl_4 from +1.5 to +0.4 V. The peak currents increased as the number of potential cycling increased. The size of deposited AuNPs onto the electrode was estimated in the range of 25-40 nm. To evaluate the surface characteristics, impedance spectroscopic measurements were performed for each modified surface in a 4.0 mM ferricyanide solution (not shown). AuNP deposition on the bare CE resulted in lower impedance values compared to those without AuNP, indicating that the conductivity of electrode surface largely increased due to AuNP treatment.
Fig. 1. (A) Linear sweep voltammograms for electrochemical deposition of AuNPs. The electrodeposition conditions were as follows: deposition time, 60 s; deposition potential, -1.0 V; potential range, from +1.4 to +0.5 V scan rate, 0.1 Vs⁻¹; potential cycling, three times.

Fig. 2. Cyclic voltammograms of polymer growing on AuNPs/CE in 0.1 M PBS (pH 7.0). Potential cycling was between 0.0 and +1.4 V; scan rate, 0.1Vs⁻¹; two times.

The CV recorded during anodic electropolymerization of the carboxyl terthiophene monomer coated AuNPs/CE in a 0.1 M PBS of pH 7.0 is shown in Fig. 2. During the first anodic scan from 0.0 to +1.4 V on the TTCA monomer coated electrode in a buffer solution at the scan rate of 0.01V/s, the oxidation peak of the monomer appeared at around +1.15 V and the reverse scan from +1.4 V showed a very small cathodic peak. These results correlate to those previously reported [10], and the large reduction peak current at around +0.5 V, corresponds to effect of AuNPs. EDC and NHS catalyze the formation of amide bonds between carboxylic acids and amines by activating the carboxylic groups. The EDC reaction proceeds at room temperature and pH 7.0. Covalent bonds were formed between the activated carboxylic terminal groups of the conjugated polymer coated on the CE and amide groups of DOPE and cyt c.

The amount of DOPE and cyt c covalently bonded onto the poly-TTCA layer was assessed by using QCM (quartz crystal microbalance) studies. The frequency shift has been monitored during the immobilization of DOPE and cyt c onto the surface of poly-TTCA film which was electrochemically deposited onto the substrate electrode. The mass changes (Δm) were calculated using the following equation [10]:

\[ Δm = Δf X 5.608 \text{ (ng/cm}^2\text{/Hz}) X 0.196 \text{ cm}^2 \]

Where, Δf was the change in frequency, 5.608 (ng/cm²)/Hz was sensitivity factor calculated from the physical constant for quartz, and 0.196 cm² was the electrode area. The frequency changes (Δf) obtained were (A) ~370 and (B) ~393Hz. The amount of DOPE and cyt c immobilized onto the poly-TTCA layer was calculated to be 406.8 ng (2.8 x 10⁻⁹ mole/cm²) and 432.1 ng (1.8 x 10⁻¹⁰ mole/cm²), respectively.

A pair of redox peaks was observed at +0.09/+0.06 V resulting from the redox process of the immobilized cyt c onto the modified electrode at a scan rate of 0.05 V/s in 0.1 M PBS (not shown). The peak separation was measured to be approximately 60 mV and observed to be large and well defined.

Fig. 3 displays the decrease in the current as the concentration of NADH (0.0 to 15.0 mM) in the solution increased and the calibration plots of the voltammetric response according to the concentration of NADH in the presence of UQ₁₀. This linear dependency of the NADH concentration yielded the equation: \( I_{pc} (\mu A) = (0.63833) + (-0.04988) [C] \) (mM), with a correlation coefficient of -0.994. There was, however, no significant change in the cyt c redox peak amplitudes due to varying NADH concentrations in the absence of UQ₁₀. This shows that UQ₁₀ affects NADH detection to a certain extent.

VI. CONCLUSION

The stable biomimetic membrane was successfully developed. DOPE (lipid), UQ₁₀ (coenzyme), and cyt c (redox protein) were covalently bonded to a conjugated polymer layer coated on the AuNPs-deposited CE. The quantities of immobilized molecules and the reaction time were determined with QCM experiments. The electrochemical behavior of cyt c at these biomimetic membranes was studied using cyclic voltammetry, and it was found that the redox reaction of cyt c is a quasireversible process. The modified electrode, CE/AuNPs/poly-TTCA/DOPE+UQ₁₀:Cyt c, was used to determine the NADH concentration with a sensitivity -0.04988 μA/mM. This new biomimetic system can be applied for bioelectronic devices and biosensors.

REFERENCES


