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Self-Assembled Monolayer of DTSSP Modified Azurin for Biomolecular Electronic Device

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The electrochemical properties of immobilized Pseudomonas aeruginosa Azurin protein was investigated for its application to biomolecular electronic device. DTSSP (3,3-dithiobis(sulphosuccinimidyl propionate)) was used for the -thiol modification of Azurin Protein to immobilize a protein to gold electrode in order to enhance its orientation and immobilization capability. The redox characteristic influence of protein modification by DTSSP was investigated by UV spectroscopy. The effect of modification using DTSSP for the immobilization capability of protein was validated by surface plasmon resonance (SPR) spectroscopy and scanning tunneling microscopy (STM) investigation. The electrochemical results obtained by cyclic voltammetry (CV) showed that the well defined protein layer was formed on the gold electrode. This well ordered assembled Azurin on the target electrode can be used by the molecular size electronic device.

Keywords: 3,3-dithiobis(sulphosuccinimidyl propionate) (DTSSP); cyclic voltammetry (CV); *P. aeruginosa* azurin; scanning probe microscopy (SPM); self-assembly (SA); surface plasmon resonance (SPR)

1. INTRODUCTION

Molecular electronic device has advanced to overcome the limit of current electronic device. Current trends of the molecular electronic

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device were the development of molecular-scale diode, organic thin-film transistor, nanowire, and organic photovoltaic cell using the organic materials [1–3]. Various concepts for molecular information storage have been proposed [4–8]. Hopfield *et al.* proposed the concept for the shift register memory. The memory elements are based on a chain of electron-transfer molecules incorporated on a very large scale integrated substrate, and the information is shifted by photo-induced electron-transfer reactions [4]. Guisinger *et al.* proposed the probing charge transport at the single-molecule level on silicon. It means that single molecule can be applied to silicon-based molecular electronic devices [5].

In bio-molecular electronic device field, many researches proposed the biomolecular information devices [9–10]. Choi *et al.* investigated the shift register memory using the biomolecular hetero LB layer. Biomolecular hetero layers were functioned as the molecular diode and switching device with photocurrent generation and rectifying properties [11–15]. However, the biomolecules utilized were immobilized by the weak force such as electrostatic force or hydrophobic interaction although the biomolecular device concept was well proved. The immobilization properties of a protein layer also have not been well-investigated and improved in order to increase its electrochemical signal and stability.

We formed self assembled Azurin layer on the gold electrode because they most likely function as soluble electron carriers, transferring charge between redox partners in membrane or soluble condition although no physiological role has been established for Azurin [16–20].

In this study, we adopted 3,3-dithiobis(sulphosuccinimidyl propionate) (DTSSP) as a linker material. DTSSP contains thiol group (–SH) and amine group ($-NH_2$) at the both end sides. The DTSSP modification can give increased thiol group to the Azurin. The short length (12Å) of DTSSP can give profitable structure to electrochemical reaction. In previous experiments, we used SPDP (N-succinimidyl 3-(2-pyridyldithio)-propionate) as a linker material for azurin immobilization [21]. It is good methods for making azurin layer. However it also have limitation about two step process. In that case, protein is not assembled it just adsorbed on the linker assembled layer. DTSSP methods solve that problem through directly azurin assembly. From these investigation output shows fabricated thin protein film using DTSSP could be a useful protein film formation technique.

2. EXPERIMENTAL DETAILS

2.1. Material

P. aeruginosa azurin was purchased from Sigma-Aldrich Chemical Company (USA). 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

buffer (HEPES, Sigma-Aldrich, USA) was used as solvent to prepare Azurin sample and washing HEPES buffer solution. 3,3-dithiobis(sulphosuccinimidyl propionate) (DTSSP) was purchased from PIERCE chemical company. Distilled and deionized Milipore [(Milli-Q) water (DDW; $>18\,M\Omega$)] was used in this experiment. Benzyl benzoate (Merck, Germany) was purchased and used as index matching fluid for SPR measurement.

2.2. Fabrication of DTSSP Modified Azurin Film

0.5 mg/mL Azurin was dissolved by HEPES buffer (10 mM). DTSSP was dissolved into the HEPES buffer. DTSSP solution concentration was 5 mM. The mixture of DTSSP solution and Azurin dissolved solution was reacted into the ice for 3 hrs. And add stop solution Tris-HCl buffer.

For the fabrication of gold (Au) thin film, cover glass (BK7, 18 mm × 18 mm, Superior, Germany) was used. Chromium (Cr) was sputtered onto the glass substrate initially as an adhesion promoter material to a thickness of 20Å. Gold (Au) sputtering to a thickness of 430Å was followed on the sputtered Cr layer. The gold substrate was annealed by rapid thermal annealing system (ULTECH Co. Ltd., UTR-100 system) for 4 min at 400°C. It was consist of vacuum pump, halogen lamps. Therefore this system can be used by thermal annealing processes in an environment from ultra-high vacuum to ambient pressure with different atmospheres like oxygen and nitrogen.

The Au substrate was cleaned using piranha solution composed of 30 vol% H_2O_2 (Sigma-Aldrich Co., USA) and 70 vol% H_2SO_4 (Duksan Chemical Co. Ltd., Korea) at 70°C for 5 min, and then the cleaned substrate was immersed into pure ethanol solution for 1 hr.

2.3. Surface Plasmon Resonance (SPR) Spectroscopy and Scanning Tunneling Microscope (STM) Analysis

Bi-molecular interaction was monitored by surface plasmon resonance spectroscopy (Multiskop TM, Optrel GmbH, Germany) using He-Ne laser light source with a wavelength of 632.8 nm. The p-polarized light beam by the polarizer was used as a reference and the intensity of the reflected beam was measured by photo multiplier tube (PMT) sensor. A glass prism (BK 7, $n = 1.5168$) with 90° angle was used as a Kretschmann coupler. The plane face of the 90° glass prism was coupled to cover glass via index matching oil. The resolution of the angle reading of the goniometer was 0.01°. All samples were monitored in the condition of constant temperature of 20°C. The incidence angle was verified from 38° to 50°. SPR depends on a bound electromagnetic

wave that is proportion to the film thickness on the metal surface. The spatial change distribution creates an electric field which is localized at the metal-dielectric interface.

The surface morphology of the prepared metalloprotein layer was obtained by commercially available scanning probe microscopy (DI multimode, Veeco, USA). Image acquisition was carried out under the condition of I set = 1.0 nA, When the applied voltage was 0.1 V ~ 1.0 V. STM image can support with SPR data for confirming immobilization. STM analysis may be used to subsidiary method of SPR. The benefit of combining SPR and scanning probe microscopy (SPM) imaging allows the inter-relationships between surface topography and biological interaction with biomaterials to be efficiently analyzed.

2.4. Redox Characteristic Investigation by Electrochemical Method

Cyclic voltammogram were obtained using a 660 A system (CHI, USA). The electrochemical cell volume was a 5 mL, and fabricated by quartz. The electrochemical system was made up of three electrode system. The working electrode was fabricated by 43 nm gold deposition, and working electrode size was 0.5 cm × 0.5 cm. Three-electrode, counter electrode was platinum wire, reference electrode was Ag/AgCl electrode. HEPES buffer (pH = 5.4) was used by electrolyte. All experiments were conducted at room temperature.

3. RESULTS AND DISCUSSION

3.1. UV Spectroscopy Investigation

Figure 1 is a schematic diagram of DTSSP modified Azurin immobilization system. Azurin was reacted by DTSSP. DTSSP modified Azurin was assembled directly to the gold substrate. Figure 2 shows the UV spectra of both wild type Azurin and the DTSSP modified Azurin. The UV-vis characterization was carried out on a Nano drop system (ND-1000, USA). In living systems, the azurin coordinates one copper ion. In addition to being essential for function (electron-transfer processes), the copper ion also stabilizes the protein structure. Like these Azurins have an intrinsic property to absorb visible light at a wavelength near 627 nm. This UV spectrum wave length peak can show the capability of the Azurin redox property [22]. DTSSP made a function with amine group of the Azurin. DTSSP reaction might have given an influence to the Azurin original structure and the redox capability. So in the initial state, we had experience many try and

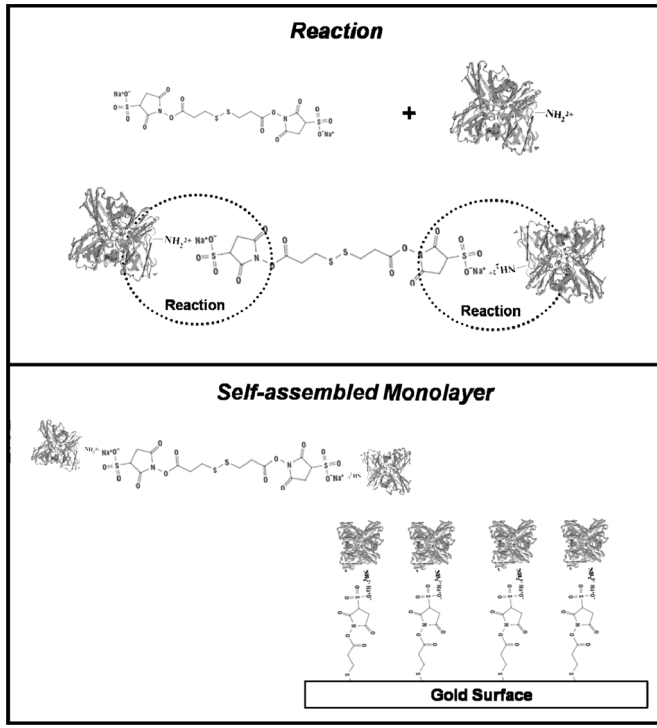


FIGURE 1 Schematic diagram of DTSSP modified Azurin immobilization by molecular assembly onto the gold surface.

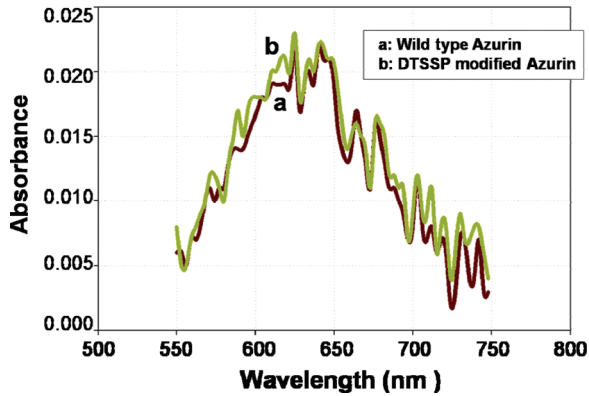


FIGURE 2 Investigation of redox property by the UV-visible absorption spectra of DTSSP modified Azurin.

errors to optimize the reaction time and proper concentration. The UV spectrum experiment result showed, the redox capability of DTSSP modified Azurin was maintained after DTSSP modification.

3.2. Confirm the Increased Immobilization Capability by SPR

In order to confirm the molecular self assembly capability, SPR spectroscopy was carried on the same concentration and assembly time. The changes of SPR curves by adsorbing, could be changed by binding of wild type Azurin and DTSSP modified Azurin on Au substrate. Figure 3 was the result that resonance angle comparison

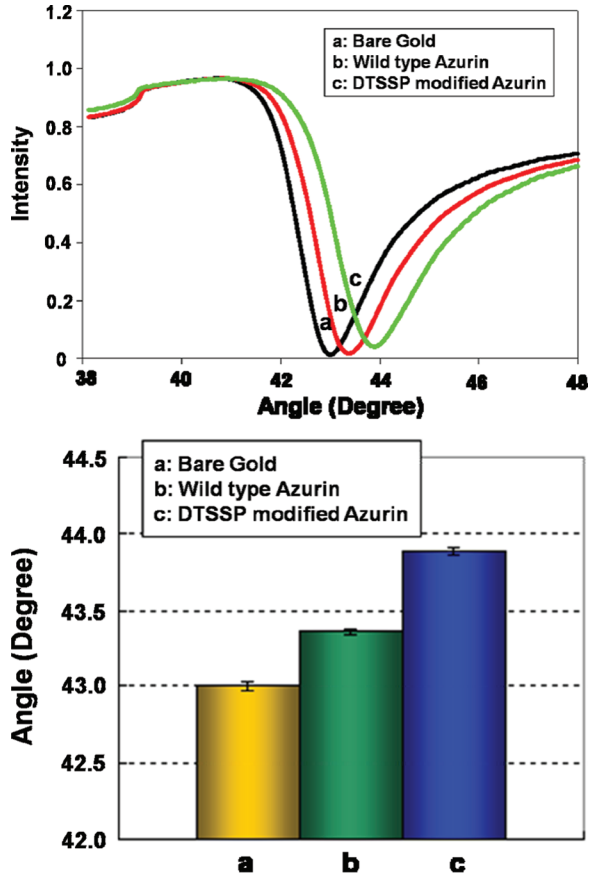


FIGURE 3 Confirming the immobilization of DTSSP modified Azurin by SPR.

between wild type Azurin assembly on the bare gold electrode and the DTSSP modified Azurin assembly layer. From the SPR spectroscopy results, resonance angle of each substrates were like these; bare gold is 43.0 degree, wild type Azurin assembly layer is 43.4 degree, DTSSP modified Azurin layer is 43.9 degree. The angle shift of surface plasmon resonance was proportion to the material amount reaction with metal substrates [23–25]. Therefore immobilization capability of DTSSP modified Azurin was increased by the SPR spectroscopy resonance angle shift investigation.

3.3. Syrface Analysis Using STM

STM, operating at constant current, has allowed us to obtain single molecule images for Azurin monolayer on gold in air. Figure 4 shows topography variation by the change of the surface modification. The sputtered deposited gold substrate had about 30–40 nm size morphology. When this substrate was annealed by rapid thermal process, nanoscale gold morphology was disappeared, and more platen surface was obtained. Generally, SA layer is monolayer, thus the surface morphology is influenced on the surface morphology of SA layer. An analysis of the molecular lateral size gives an average value of

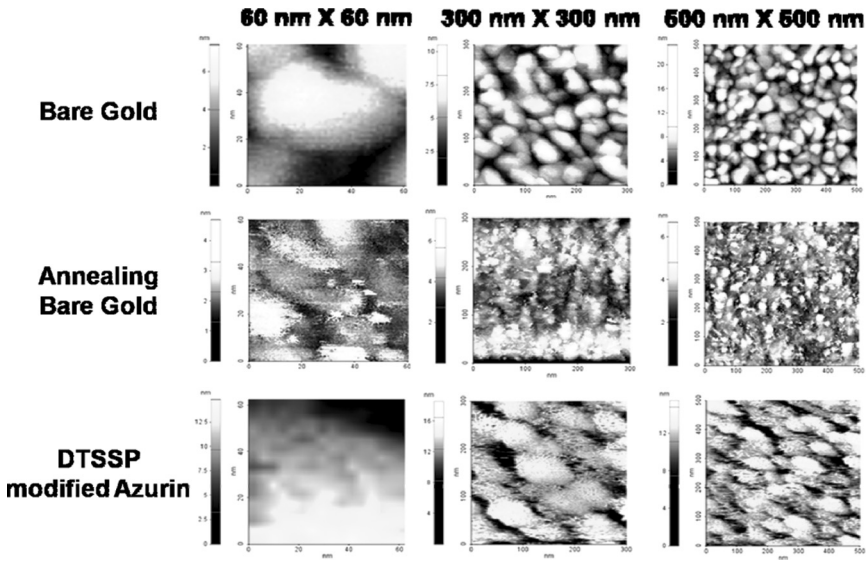
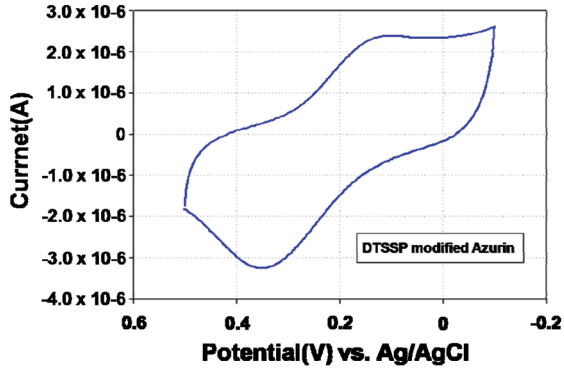


FIGURE 4 Surface analysis of DTSSP modified Azurin by STM.

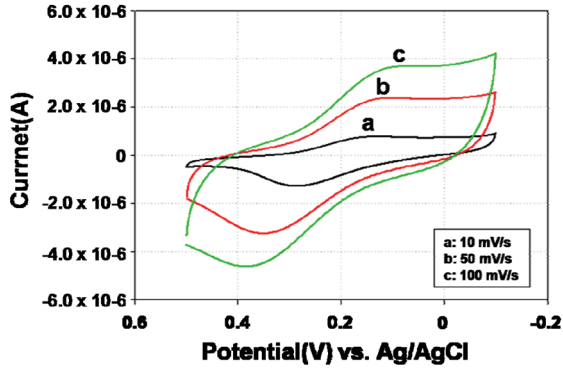
$4 \sim 5$ nm. It was well matched with a data by the crystallographic dimensions [26]. From thses results, The Azurin molecular film was fabricated on the annealed gold surface by the well-ordered structure.

3.4. Electrochemical Analysis Using CV

Cyclic voltammetry (CV) experiment was carried out in Azurin modified gold working electrode, 10 mM HEPES buffer was used by electrolyte. Figure 5 shows the redox property of the DTSSP modified Azurin. It was difficult to define the original Azurin redox property. So no redox inactivity potential was initially confirmed by potential



(a)



(b)

FIGURE 5 Confirm the redox property DTSSP modified Azurin by the CV. (a) Confirm the DTSSP modified Azurin redox property by cyclic voltammetry. (b) Confirm the redox peak separation by the change of scan rate variation.

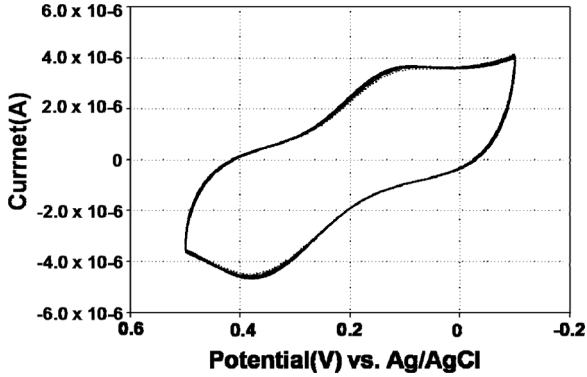


FIGURE 6 Cyclic voltammogram at 100 mV/s in 100 cycles.

sweep at the target voltage region. Not reacted DTSSP on the DTSSP modified Azurin was blocked by Tris-HCl buffer. This blocking process was optimized before DTSSP modified Azurin redox characteristic investigation experiment. From the cyclic voltammetry investigation, the reduction potential was a 100 mV, the oxidation potential was a 360 mV. Therefore SRP (Standard Redox Potential) is calculated about 230 mV by the equation of $(Ep + Ec)/2$. Redox properties such as the standard redox potential were sustained under the repeat of over 100 cycles in air (Fig. 6).

4. CONCLUSION

Azurin was modified by the organic compound that gave an additional -thiol group to the Azurin surface. The redox capability maintenance was confirmed by UV-vis spectrum, and the increased immobilization capability was confirmed by SPR spectroscopy. The more amounts of Azurin was immobilized on the gold substrate than wild type Azurin immobilization. The fabricated Azurin film was confirmed by STM investigation. On the annealed gold substrate, DTSSP modified Azurin made a well-packed protein film. The redox capability investigation was confirmed by electrochemical investigation. The reduction and oxidation peak of Azurin was verified at the 100 mV and 360 mV. From the uniform 100 cycles reduction and oxidation variation, the uniformity of the fabricated Azurin film was suitable to make a molecular electronic device. The proposed immobilization method of DTSSP modified Azurin can be used for making high quality protein film, and applied to the fabrication of nano-scale bioelectronics.

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