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Nanoscale Fabrication of *P. aeruginosa Azurin* on Self-Assembled Monolayer

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Pseudomonas aeruginosa Azurin is a metalloprotein of 14 kDa with copper ion in its active site. Protein film formation onto the substrate was performed by the self-assembly technique. In order to make molecule assembled layer onto the gold substrate, Azurin was cross-linked with N-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP). SPDP have both a -thiol group to assembly with gold substrate and a linking part to assembly with protein. We detected optimized condition to make a protein film using Surface Plasmon Resonance (SPR). The surface structure of adsorbed Azurin film onto the gold surface was analyzed by scanning tunneling microscope (STM).

Keywords: metalloprotein; *P. aeruginosa Azurin*; self assembly; SPR (Surface Plasmon Resonance); STM (Scanning Probe Microscope)

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1. INTRODUCTION

In a biological electron transfer system, long-range electron transfer takes place very efficiently with one direction through the biomolecules [1]. If we could control these molecules, we can make a nano-scale electronic bio-device [2]. According to the previous studies, *Azurin* may have a redox property to transfer electron such as cytochrome c_{551}. It has been shown that *Azurin* can act as an electron donor or acceptor *in vivo* condition [3]. However, it is difficult to use redox property in nano device because protein is very fragile in vitro circumstance. Therefore, we need an efficient technology to control the fabrication of protein film on target substrate.

In recent decade, Self-Assembly (SA) technique has been studied, because self-assembly offers an useful method to make a thin layer onto the metal substrate for various applications [4–6]. Modified substrate with linker material can control the protein immobilization onto solid substrate. The most famous system of self- assembly is a system that is composed of alkanethiols. In the long hydrocarbon chain structure, there are two reaction parts. One part reacts with solid substrate and another with target protein. Many studies on self assembly formation of alkanethiol on gold substrate have been carried out in respect of structure, kinetics, effect of chain length, and solvent effect. It has been found that sulfur compounds coordinate strongly to various metal surfaces, such as gold, silver, copper and platinum. But gold surface usually has been used for the Self-Assembly Monolayer (SAM) formation of alkanethiols. When protein film is formed on the gold substrate by alkanethiol linker, the length of alkanthiol is related to the rate of electron transfer from the protein to the substrate. When we fabricate bioelectronic device or biosensor using biomolecules, the shorter alkanthiol linker material was more effective linker material than the longer one.

In this study, we adopted N-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP) as a linker material. SPDP contains sulfhydryl group ($-SH$) and amine- reaction group ($-NH_2$) and the length of SPDP is 6.8 Å. It is very short compared with the size of target protein. If we fabricate thin protein film using SPDP, it would be a useful linker material for bioelectronic device or biosensor.

The thin film formation using SA technique permits reliable control of the packing density and the immobilization environment on the substrate. In the previous reports, we reported the immobilization of the protein A using SPDP [6]. But the formation of *Azurin* self assembled layer using SPDP linker material and detection by STM and SPR have not been reported yet.

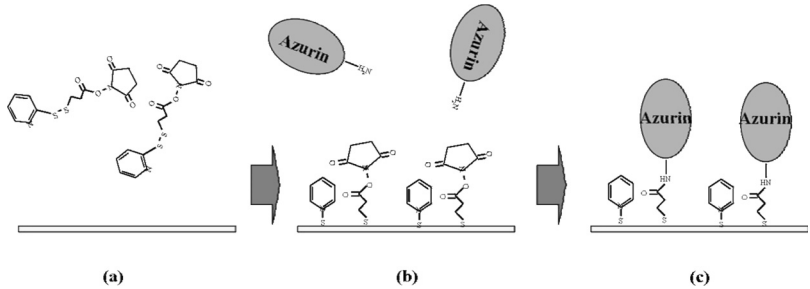

FIGURE 1 Schematic description of *Azurin* immobilization using SPDP by layer-by-layer molecular assembly onto the gold substrate. (a) SPDP immobilization on cleaned gold substrate, (b) *Azurin* SA on fabricated SPDP layer and (c) The formation of fabricated SA *Azurin* thin film.

In this study, the conformation of well-ordered *Azurin* layer is fabricated by the optimized SPDP concentration layer that was reported [7]. The Optimization of the assembled *Azurin* concentration is detected by SPR. And the morphology of immobilized *Azurin* is observed by STM. Figure 1 is a schematic diagram of this immobilization system. The surface of self assembled SPDP layer would be able to react with primary amine ($-NH_2$) in protein. When we make a SPDP layer onto the gold substrate, we can immobilize *Azurin* onto the gold substrate.

2. EXPERIMENTAL DETAILS

Materials

P. aeruginosa azurin was purchased from Sigma-Aldrich Chemical Company (USA). SPDP (N-Succinimidyl-3-(2-pyridyldithio)-propionate) was purchased from PIERCE Company (USA). To make a solution for self assembly of SPDP, pure acetone was purchased from Junsei Chemical Corporation (Japan). Phosphate buffered saline (PBS, Sigma-Aldrich, USA) and Phosphate buffer saline tween20 (PBS-T Sigma-Aldrich, USA) were used as solvent to prepare *Azurin* sample and washing buffer solution. Distilled and deionized Milipore [(Milli-Q) water (DDW; $>18\,M\Omega$)] is used in this experiment. Benzyl benzoate (Merck, Germany) was purchased and used as index matching fluid for SPR measurement.

Thin Film Fabrication

For the fabrication of gold (Au) thin film, cover glass (BK 7, $18\,mm \times 18\,mm$, Superior, Germany) was used. Chromium (Cr) was

sputtered onto the glass substrate initially as an adhesion promoter material to a thickness of 20 Å. Gold (Au) sputtering to a thickness of 430 Å was followed on the sputtered Cr layer. The sputtered Au substrate was cleaned using phirana solution composed of 30 vol% H_2O_2 (Sigma-Aldrich MO 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.

For the preparation of *Azurin* sample, *Azurin* of 0.1 mg/ml is dissolved in 10 mM phosphate buffer saline (pH 7.4). First cleaned bare gold plate is immersed in 1 mg/ml SPDP solution for 24 h. The Au substrate was rinsed with acetone and deionized water. It is important thing to wash the substrate sufficiently. And then the residual solution on Au surface was removed by N_2 gun. After the SPDP modified Au substrate was prepared, Au substrate was immersed on 0.1 mg/ml *Azurin* solution for 12 h. *Azurin* adsorbed onto the Au substrate that was rinsed with 10 mM phosphate buffer saline solution, and immersed into 0.05% PBS-T buffer solution for 30 min. After these, the Au substrate was rinsed with deionized water and dried in desiccator which is filled with N_2 gas.

Surface Plasmon Resonance Detection and STM Analysis

SPR depends on a bound electromagnetic wave that is proportional to the film thickness on the metal surface. The Kretchmann's attenuated total reflection (ATR) configuration is well known as the design for SPR instrumental method. The external laser field drives the free electron of metal in a distinct mode. The spatial change distribution creates an electric field which is localized at the metal-dielectric interface.

Bi-molecular interaction was monitored by surface plasmon resonance spectroscopy (Multiskop™, 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 Kreschmann 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°.

The surface topography of the prepared molecular film was obtained by commercially available scanning probe microscopy (XE-100, PSIA, Korea). Image acquisition was carried out under the condition of $I_{set} = 0.5$ nA. The applied voltage was 0.1 V ~ 1.0 V.

3. RESULTS AND DISCUSSION

Detection of Optimized *Azurin* Concentration using by SPR

The plasmon resonance characteristics of fabricated protein surface were investigated to optimized assembled *Azurin* concentration on SPDP layer, which verify the optimized condition since the angle shift of adsorbed surface is proportion to the quantity of adsorbed materials. Optimized SPDP condition to assemble on gold substrate was defined in the previous research [7]. Figure 2 shows the change of SPR angle with respect to the change of concentration of *Azurin*. The saturation concentration of SPDP was defined in the previous works [7]. So in this study, the optimal *Azurin* concentration was defined on the optimized SPDP layer. As the concentration of *Azurin* increased, the amount of SPR angle shift also increased and finally saturated. In Figure 2, we determine the optimized *Azurin* concentration from the saturated SPR angle curve. From the saturated SPR resonance angle, we can assume the optimized *Azurin* concentration is a 0.1 mg/ml in *Azurin* self-assembly system onto the gold substrate.

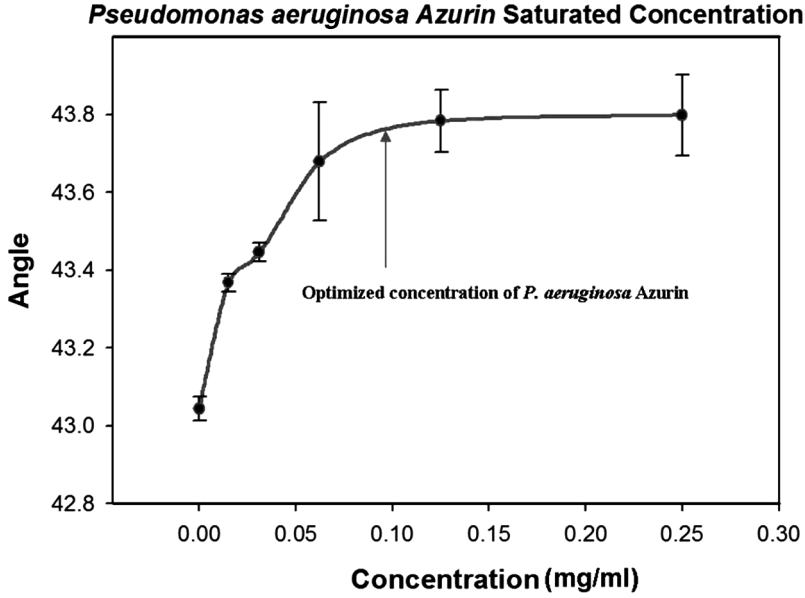

FIGURE 2 Change of SPR angle shift with respect to the *Azurin* cooncentration.

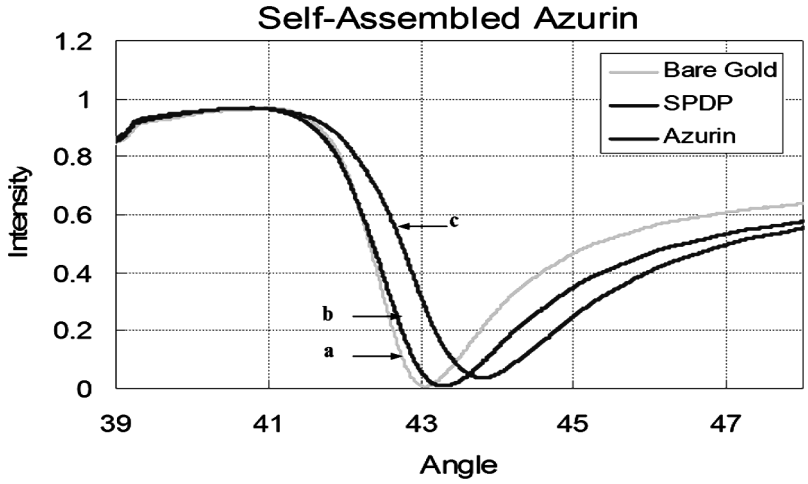

FIGURE 3 SPR spectra of (a) Bare gold surface, (b) Modified surface using SPDP and (c) Immobilized *Azurin* on SPDP surface on gold substrate.

Resonance Angle Shift by the Increased Layer Thickness

Figure 3 shows the SPR angle shift. Resonance angle is proportional to a new layer formation. When SPDP was introduced on the cleaned Au surface, the resonance angle shift was 0.18°. The following immobilization of 0.1 mg/ml of *Azurin* made the SPR resonance angle shifted by 0.26°. In this SPR spectroscopy system, we changed the position more than ten point. And we averaged these results. So this result means the average film formation on the gold substrate. From this result, we can assume the uniform *Azurin* film formation.

Natural Self-Assembly and Artificial Self Assembly

Self assembly technique onto the gold substrate generally uses -thiol group (−SH). But *Azurin* has a -thiol group in it's own outside structure. Therefore it can be naturally immobilized onto the gold substrate. In Figure 4, we compared SPDP modified self assembly and natural self assembly that immobilized directly onto the gold surface using *Azurin* thiol- group. The SPR angle shift of the natural self assembly is shifted 0.07°. But the SPR angle shift of the artificial self-assembly is 0.324°. The larger SPR angle shift means the more amount proteins was immobilized on gold substrate than the natural *Azurin* assembly. Several previous research used a natural self-assembly technique [10,11]. However, through this experiment, we can explain it is more efficient to use a linker material for self assembly.

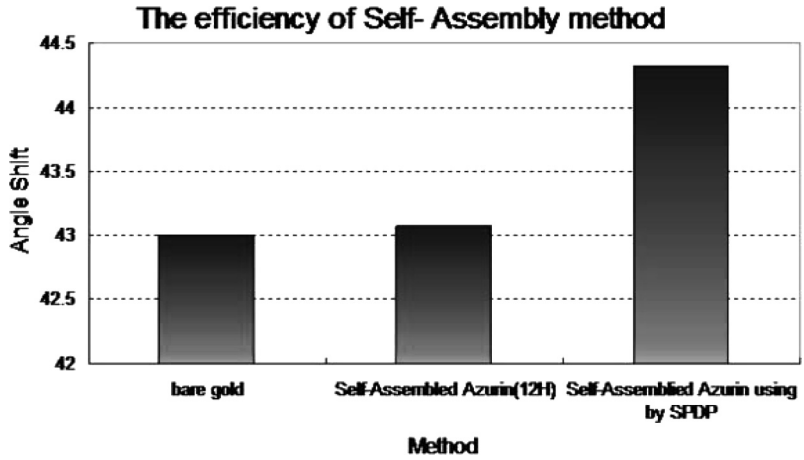

FIGURE 4 Natural self assembly and SPDP modified self assembly on gold surface.

Surface Analysis using STM (Scanning Tunneling Microscope)

For the visualization of the fabricated *Azurin* thin film, Scanning Tunneling Microscope (STM) study was carried out. STM analysis may be used to compliment method of SPR. The benefit of combining SPR and Scanning Probe Microscope (SPM) imaging allows the inter-relationships between surface morphology and biological interaction with biomaterials to be efficiently analyzed [9]. Figure 5 shows the morphologies of the clean bare gold surface and the *Azurin* immobilized gold substrate.

In Figure 5, the RMS (Root-Mean-Square) value of the bare gold substrate is 6.24 Å, while the RMS of the self assembled *Azurin* layer is 13.46 Å. The surface of the self assembled *Azurin* layer is rougher than the surface of the bare gold substrate. In Figure 5, the height bar of *Azurin* immobilized substrate is longer than that of the bare gold substrate. It can explain the formation of *Azurin* film onto the gold substrate.

4. CONCLUSIONS

The *Azurin* layer was self-assembled on the gold surface using SPDP. Optimal concentration for *Azurin* is determined as 0.1 mg/ml using SPR saturation curve. When we make an *Azurin* film on the gold surface, the method of using linker material like SPDP is more efficient than natural self assembly. STM topography showed that uniform

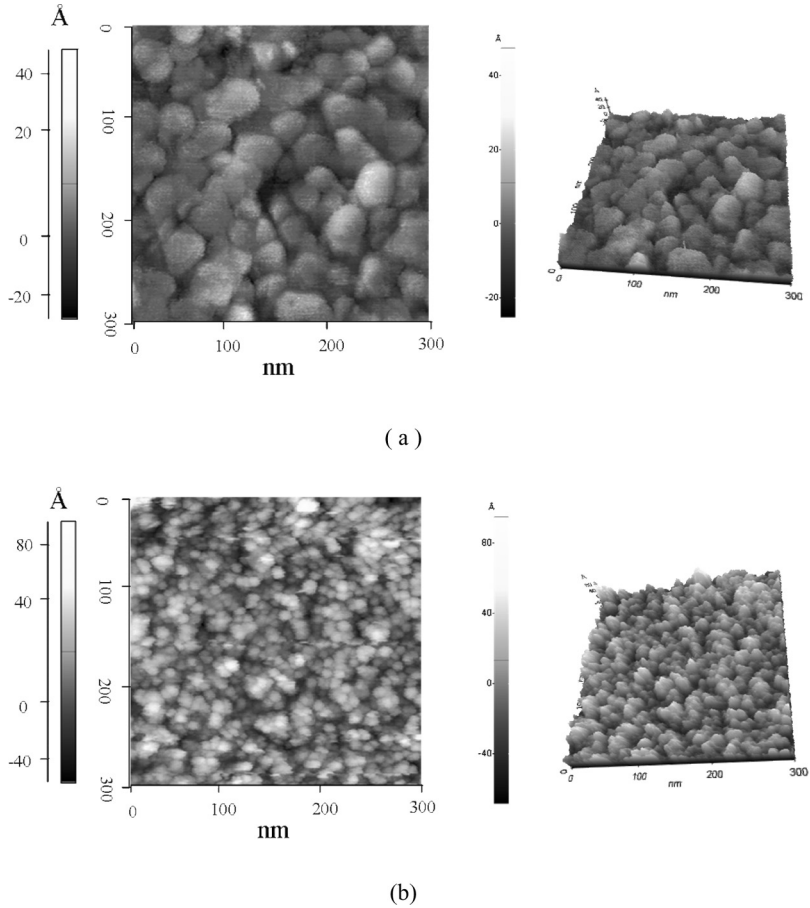

FIGURE 5 STM topography analysis: (a) Au substrate image (300 nm) (b) *P. aeruginosa Azurin* immobilized layer.

hetero layer was fabricated on the gold substrate. It can be explained that the formation of the functional proteins is well fabricated on gold substrate. From this work, we can develop the fabrication of nanobio device using *Azurin* protein in the next step.

REFERENCES

[1] Chi, Q., Zang, J. D., & Nielsen, J. U. (2000). *J. Am. Chem. Soc.*, *122*, 4047.
[2] Choi, J. W. & Fujihira, M. (2004). *Applied Physics Letters*, *84*, 2187.
[3] Messerschmidt, A., Huber, R., Poulos, T., & Wieghardt, K. (2001). *Hand Book of METALLOPROTEIN*, Wiley: Chichester, UK.

[4] Nuzzo, R. G. & Allara, D. L. (1983). *J. Am. Chem. Soc.*, *105*, 4481.
[5] Devaraj, N. K., Dinolfo, P. H., Chidsey, C. E. D., & Collman, J. P. (2006). *J. Am. Chem. Soc.*, *128*, 1794.
[6] Strong, L. & Whitesides, G. M. (1988). *Langmuir*, *4*, 546.
[7] Lee, W. C., Lee, D. B., Oh, B. K., Lee, W. H., & Choi, J. W. (2004). *Enzyme and Microbial Technology*, *35*, 678.
[8] Choi, J. W., Park, S. J., Nam, Y. S., Lee, W. H., & Fujihira, M. (2002). *Colloids and Surfaces B: Biointerfaces*, *23*, 295.
[9] Friis, E. P., Andersen, J. E. T., Madsen, L. L., Moiler, P., & Ulstrup, J. (1907). *Journal of Electroanalytical Chemistry*, *431*, 35.
[10] Chen, X., Davies, M. C., Roberts, C. J., Shakesheff, K. M., Tendler, S. J. B., & Williams, P. M. (1996). *Anal. Chem. 68*, 1451.
[11] Choi, J. W., Nam, Y. S., & Fujihira, M. (2004). *Biotechnol. Bioprocess Eng.*, *9*, 76.