자리 지정 돌연변이화된 산화·환원 단백질을 이용한 바이오메모리의 개발

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Development of Biomemory Consist of Recombinant Redox Protein using Site-Directed Mutagenesis

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Introduction

Molecular electronic device has advanced to overcome the limit of current electronic device. Current trends of the molecular electronic device were the development of molecular-scale diode, organic thin-film transistor, organic wire like nanowire, and organic photovoltaic cell using the organic materials [1]. In our previous works, it has been reported that oxidoreductase film can be fabricated by using chemical linker material that have thiol-group to assemble it on gold (Au) substrate. However, the chemical linker can interfere with electron transfer because it is acted as an insulator of the system. Extraordinarily, In this study, we suggested novel immobilization technique of cupredoxin azurin on the Au surface. A recombinant azurin with cysteine residue using site-directed mutagenesis (SDM) was designed and then directly immobilized on Au surface without chemical linker. The immobilization of the functionalized protein could be confirmed by surface plasmon resonance (SPR) and its surface morphology could be analyzed by scanning tunneling microscopy (STM). The redox property was investigated by the electrochemical approach those are cyclic voltammetry (CV) and open circuit potential amperometry (OCPA).

Theory and experiments

Escherichia coli strain DH5α was used as the host for subcloning. Standard techniques were employed throughout this work [2]. The gene, encoding blue copper protein azurin was amplified by a polymerase chain reaction (PCR) from the genomic DNA of *Pseudomonas aeruginosa*. The forward primer was designed to contain a *Nde*I restriction enzyme site and the reverse primer was designed to contain a *BamH*I restriction enzyme site (Fig. 1a). The PCR product was purified by using a DNA purification kit (QIAZEN, USA) and digested with two restriction enzymes of *Nde*I and *BamH*I (New England Biolabs, UK). The digested DNA fragments were ligated with a pET-21a(+) vector (Novagen, Germany), which was predigested with *Nde*I and *BamH*I, by using a ligation kit (TaKaRa, Japan). Azu Cys F and Azu Cys R primers (Fig. 1b) were designed to contain mutant site for site-directed mutagenesis (SDM) and used to effect the mutations, changing the codon for Lys92Cys (K92C) from AAG to TGC. Mutations in the azu gene were introduced by site-directed mutagenesis [3,4]. Fig. 1c,d show schematic representations of plasmids for the expression of cysteine-modified azurin. The Plasmids, containing genes for azurins, were transformed into *E. coli* BL21 (DE3). The transformants were grown to 0.6 OD at 37 °C in shake flasks containing 1L of Luria-Bertani medium (0.5 % yeast extract, 1.0 % tryptophan, and 1.0 % NaCl) with 50 mg/mL ampicillin. The expression was induced by adding isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.839 mM. The transformed cells were grown for an additional 16 hr at 37 °C. The cells were harvested by centrifugation at 5000 g for 15 min at 4 °C. The cell paste was resuspended in sucrose buffer (20 % sucrose, 0.3 M Tris-HCl, pH 8.1, 1 mM EDTA) and subjected to osmotic shock (0.5 mM $MgCl_2$). Contaminating proteins were precipitated from the periplasmic preparation by decreasing the pH to 3.8 (50 mM sodium acetate), yielding azurin-containing supernatant. Apo-azurin and cysteine-modified apo-azurin fractions (Elution pH=4.6 and 4.8, respectively) were separated on an CM excellulose ion-exchange column with a pH gradient from 4.0 to 6.0 (50 mM sodium acetate) [5]. 0.5 M $CuSO_4$ was added to the protein solution and taken up by apo-azurin and cysteine-modified apo-azurin. Wild type azurin and cysteine-modified azurin were purified by MWCO 5k Amicon Ultra centrifugal filter (Millipore, USA). For the fabrication of gold (Au) substrate, cover glass composed of BK7 (18 mm X 18 mm, Superior, Germany) was used as a solid support. Chromium (Cr) was sputtered onto the glass substrate initially, as an adhesion material, to a thickness of 20 Å and followed by a gold (Au) sputtering to a thickness of 430 Å. The sputtered Au substrate was cleaned using piranha solution composed of 30 vol % H_2O_2 (Sigma-Aldrich, USA) and 70 vol % H_2SO_4 (Sigma-Aldrich, USA) at 70 ℃ for 5 min, and then the cleaned Au substrate was immersed into pure ethanol solution for 1 hr. The Au substrate was rinsed with deionized (DI) water. For

the preparation of azurin sample, azurin of 0.10 mg/ml is dissolved in 10 mM HEPES (pH 7.0). Drop 0.10 mg/ml cysteine-modified azurin solution on the substrate for 2 hr. After 2 hr, the substrates were slightly washed with DI water. It is important thing to wash the substrate sufficiently. And then the residual solution on Au surface was removed by N_2 gun. It is very simple step compared with reported previously method using alkanethiol. The schematic description of azurin immobilization is shown Fig. 1e,f. Modify the protein with cysteine residue is a novel technique that immobilized protein on Au surface directly.

Results and discussion

The fabricated protein film was investigated using surface plasmon resonance (SPR), because the angle shift of adsorbed surface is proportion to the quantity of adsorbed proteins. Fig. 2a shows the change of SPR angle with respect to the immobilized concentration of cysteine-modified azurin. Therefore, in this works, we defined the optimal concentration of cysteine-modified azurin layer. As the concentration of cysteine-modified azurin was increased, the amount of SPR angle shift was also increased and finally saturated. The results are presented in Fig. 2a. Moreover we determined the optimized cysteine-modified azurin concentration from the saturated SPR angle curve. After saturation concentration was determined, all experiment was carried out using optimized concentration. Optimal concentration was 0.10 mg/ml and optimal time for azurin immobilization was about 2 hr (Fig. 2b). Fig. 2c shows the SPR angle shift according to the deposition on gold. To confirm effective immobilization of cysteine-modified azurin, the angle shift is composed with wild type azurin. For confirming effectiveness of modified cysteine group, compare the angle shift of cysteine-modified azurin with wild type azurin. Therefore, it can be naturally immobilized on gold substrate without any chemical modification. When 0.10 mg/ml of cysteine-modified azurin was introduced on the cleaned Au surface, the SPR minimum angle shift was 0.78 degree were observed. But in the case of wild type azurin, the SPR minimum angle shift was only 0.19 degree at the same condition. The results were compared in Fig. 2d. It means that cysteine-modified azurin was well immobilized than wild type one. That is, cysteine-modified azurin could be successfully immobilized without any linker materials. The formation of the self assembled layer of recombinant proteins was investigated by SPR. Cysteine-modified azurin which are assembled on the gold surface directly was compared with wild type one. Surface morphology was also measured by scanning tunneling microscopy (STM). Fig. 3a shows the bare gold STM image. And Fig. 3b shows cysteine-modified azurin immobilized surface. In 50 nm scale, immobilized cysteine-modified azurin assumes the form of small lumps. Whereas adsorbed wild type azurin covered whole surface by forming the aggregates of 15 nm-20 nm in height in our previous work. Furthermore, it can be expected that cysteine-modified proteins are immobilized with good orientation probably due to the effective linking of thiol-group onto Au surface. The redox property of cysteine-modified azurin layer was investigated by cyclic voltammetry method. The redox reaction was reversible, suggesting that the self-assembled azurin maintain the reduction-oxidation properties. We take a optimal pH conditions by pH-control experiments from pH 5.0 to the pH 9.0 (Fig. 4a). From pH 5.0 to pH 7.0, there is little difference. But over the pH 8.0, The peaks was rapidly demolished since protein has been denatured. Therefore, we selected most reasonable pH condition as pH 7.0. As seen in Fig. 4b, the reduction potential was 265 mV and the oxidation potential was 146 mV. And the calculated SRP (Standard Redox Potential) of cysteine-modified azurin layer was 205.5 mV during the experiments in our system. That is matched with our previous results and references. It could be proved that protein still remain their structure and redox property in immobilized state. These facts are continuously reproduced in 200 cycles (Fig. 4c). There are little difference in first cycle and 200th cycle. In our previous works, cyclic voltammetry of wild type azurin without cysteine anchor did not maintain over 100 cycles. Because of their binding depends on non specific adsorption. Peak currents were rapidly decreased and peak interval was dramatically increased as cycle repeated. These results have a lot of noticeable meaning, since biomolecule was unstable and fragile. Therefore, to obtain the linear signal of the redox signal was very difficult. However, in our system using cysteine-modified azurin presented the oxidation potential and the reduction potential for 200 cycles. We have concluded that direct immobilized azurin layer have three distinct conducting states : Reduced states when reduction voltage (265mV) was applied to the azurin layers, oxidized states when oxidation voltage (146mV) was applied to the azurin layers, and equilibrium state under open circuit voltage (OCV) condition. Azurin has been reached the stable equilibrium state between assembled azurin and electrolytes when OCV applied. If we applied the OCV to the reduced azurin, it will be oxidized and discharged the electrons. We measured OCPA (open circuit potential amperometry) using three specific voltages (Fig. 5). Applying reduction voltage and measuring reduced current was defined as 'Write' step (electron inflow). And applying OCV and measuring conducted current was defined as 'Read' step (electron outflow). As similar to this logic, reduction voltage and OCV was applied to the assembled azurin layer one after the other, switching write and read function could be successfully repeated. 'Oxidation potential' plays a role of artificially "Erase step" by releasing the all trapped charges repeatedly.

Conclusion

Therefore, we can storage/read/release information by regulating cysteine-modified azurin with these three conducting states. The results show the biomemory concept proposed here is well validated. It is usually difficult for biomolecules, particularly proteins, to be introduced to electronic device because of their intrinsic problem such as

their instabilities.

Acknowledgment

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Figure Cations

Figure 1. Schematic representations of primers for genetic engineering and plasmids for expression.(a) Primers for Azu gene amplication. (b) Primers for site-directed mutagenesis. (c) Azu gene and Cysteine-modified Azu gene. (d) Vector. ; Schematic diagram of protein immobilization. (e) Wild type azurin. (f) Cysteine-modified azurin.

Figure 2. Characteristics of the protein immobilized layer under optimized condition by SPR. (a) Optimal immobilization concentration. (b) Optimal immobilization time. (c) SPR spectroscopy of a:bare gold surface, b:wild type azurin immobilized surface, and c:cysteine-modified azurin immobilized surface. (d) Compare angle shift of directly immobilized wild type azurin and cysteine-modified azurin.

Figure 3. Surface analysis of cysteine-modified azurin. (a) Annealed Au substrate. (b) Cysteine-modified azurin immobilized surface.

Figure 4. Various cyclic voltammograms (a) a : pH 5.0, b : pH 6.0, c : pH 7.0, d : pH 8.0, e : pH 9.0 (10mM HEPES, pH 7.0, azurin sample concentration = 0.10 mg/ml, electrode area = 0.25cm^2). (b) Cyclic voltammetry at pH 7.0. (c) 200 Cyclic voltammogram.

Figure 5. Open circuit potential amperometry (OCPA) of cysteine-modified azurin. (Write-Read-Erase : 3step)

Fig. 1 Kim, *et al.*

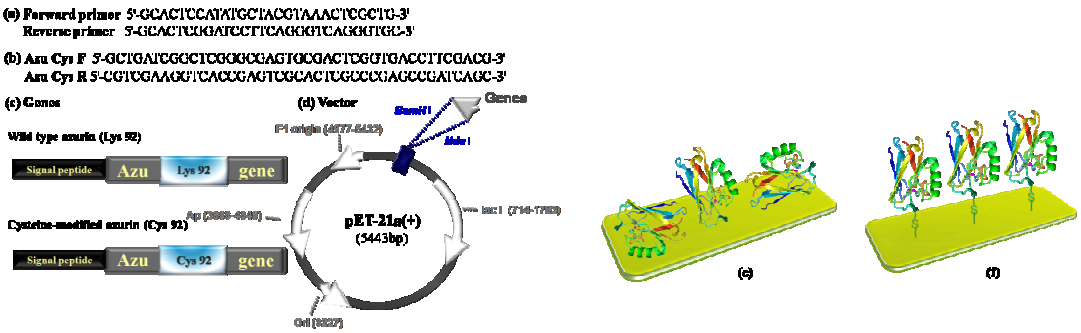
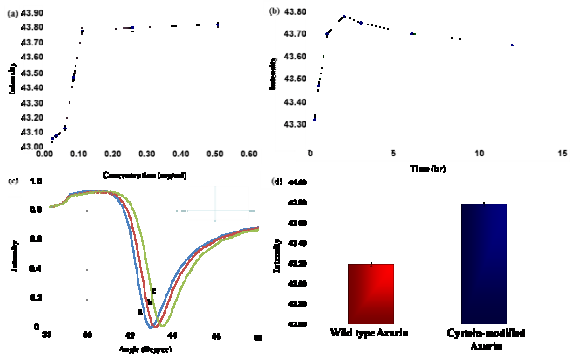


Fig. 2 Kim, *et al.*



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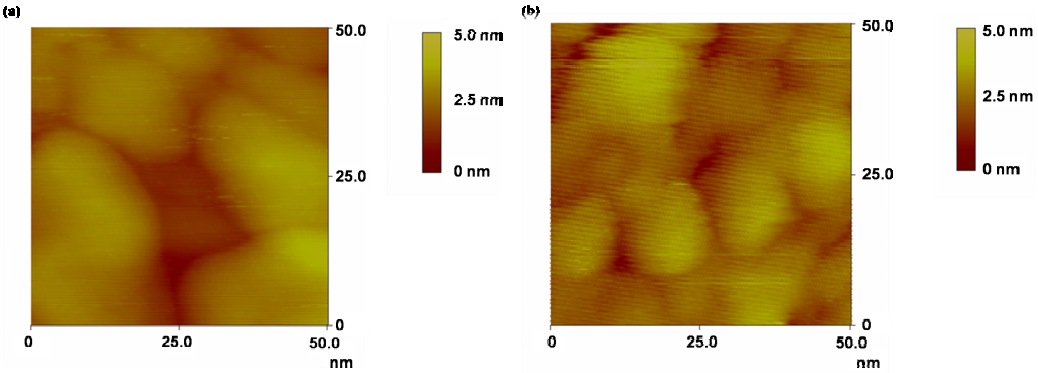

Fig. 4 Kim, *et al.*

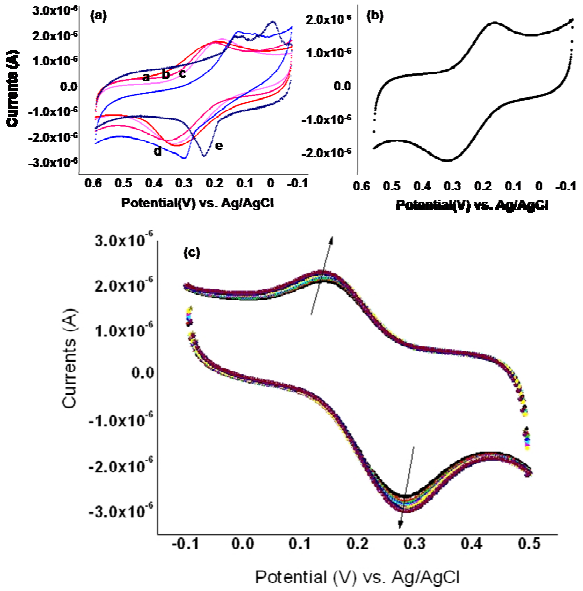

Fig. 5 Kim, *et al.*