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Fabrication of functional biomolecular layer using recombinant technique for the bioelectronic device

Sang-Uk Kim*, Young Jun Kim*, Cheol-Heon Yea, Junhong Min***, and Jeong-Woo Choi*,**,†**

*Interdisciplinary Program of Integrated Biotechnology, Sogang University, Seoul 121-742, Korea
**Department of Chemical and Biomolecular Engineering, Sogang University, Seoul 121-742, Korea
***Department of BioNano technology, Kyungwon University, Seongnam 461-701, Korea
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Abstract−A novel immobilization method of blue copper protein azurin on a gold surface was developed without a chemical linker using recombinant technique. Azurin was recombined with a cysteine anchors by site-directed mutagenesis (SDM) and used to effect the mutations, changing the codon for Leu39Cys (L39C) from CTG to TGC. The immobilization of the functionalized protein is confirmed by surface plasmon resonance (SPR) and its surface morphology is analyzed by scanning tunneling microscopy (STM). The immobilization efficiency has been increased about 76.3%, as compared to that of wild type azurin. The electrochemical property of the fabricated thin film was investigated by cyclic voltammetry (CV). As a result, cysteine-modified azurin can be used for making high quality protein film, and applied to the fabrication of nano-scale bioelectronics.

Key words: Bioelectronic, Recombinant Azurin, Cyclic Voltammetry, Site-directed Mutagenesis

INTRODUCTION

In recent years, there has been considerable research to overcome the limits of conventional electronic devices with molecular scale electronics. The current trends of molecular electronics is to develop devices like diodes and transistors by using organic as well as bio-organic materials [1-3]. Various concepts for molecular information storage have been proposed. 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 photoinduced electron-transfer reactions [4]. Hersam et al. proposed the probing charge transport at the single-molecule level on silicon. It shows that single molecules can be applied to silicon-based molecular electronic devices [5]. Bocian et al. investigated the redox kinetics of the redox active molecule attached to Si (100) surface. They demonstrated that porphyrin-based molecules bound to Si (100), which exhibit redox behavior useful for information storage [6,7].

In the field of bio-molecular electronics, many researchers have investigated the development of biomolecular information devices. Willner et al. proposed the information storage logic that was composed of DNA or enzyme. The possibility of encoding information in the base sequences of DNA was shown by manipulating DNA by enzymes [8,9]. Choi et al. investigated the shift register memory using the biomolecular hetero Langmuir Blodgett (LB) layer. Biomolecular hetero LB layers functioned as a molecular diode and switching device with photocurrent generation and rectifying property [10-12].

According to *in vitro* studies, azurin is very useful biomaterial in the field of bioelectronics. This metalloprotein with its redox property can function as an electron donor or acceptor. However, generally, the protein is unstable and fragile in the solid state. Therefore, efficient film fabrication technology is strongly required in order to use the protein as a component of bioelectronic devices. Given that artificially controlling the arrangement of a biomolecule in the solid state, a nano-scale bioelectronic device can be developed with high performance.

In the recent decade, the self-assembly (SA) technique has been studied, which offers a useful method for making a thin layer onto the metal substrate for various applications, such as biosensor, single-electron transistor (SET), field-effect transistor (FET), and so on [13,14]. The most general system of self-assembly is to use alkanethiols as chemical linker. In the long hydrocarbon chains structure, one side is reacted with solid substrate and another side is reacted with target protein. It has been found that sulfur compounds coordinate very strongly to various metal surfaces, such as Au, Ag, Cu, and Pt. In most work to date, the Au surface has been used for the self-assembly monolayer (SAM) formation of alkanethiols, because gold cannot oxidize easily. Therefore, it can be handled in ambient conditions. However, the alkanethiols based self-assembly method has a drawback for the application to bioelectronics, because it can function as an insulator [15].

In this study, we suggested a novel immobilization technique of cupredoxin azurin on the Au surface. A recombinant azurin with cysteine residue by using site-directed mutagenesis was designed and then directly immobilized on Au surface without chemical linker. The immobilization of the functionalized protein was confirmed by surface plasmon resonance (SPR), and its surface morphology was analyzed by scanning tunneling microscopy (STM). The electrochemical property of the fabricated thin film was investigated by cyclic voltammetry (CV). The direct immobilization of the recombinant azurin modified by site-directed mutagenesis for the development of efficient bioelectronic device has not been reported.

†To whom correspondence should be addressed.
E-mail: jwchoi@sogang.ac.kr
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MATERIALS AND METHODS

1. Design of Recombinant Azurin

Escherichia coli strain DH5α was used as the host for subcloning. Standard techniques were employed throughout this work [16-18]. 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

5'-GCACTCCATATGCTACGTAAACTCGCTG-3'

was designed to contain *Nde*I restriction enzyme site and the reverse primer

5'-GCACTCGGATCCTTCAGGGTCAGGGTGC-3'

was designed to contain a *BamH*I restriction enzyme site. 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

Azu Cys F 5'-CCTGTCCCACCCCGGCAACTGCCCGAAG AACGTCATGGGCC-3'
Azu Cys R 5'-GGCCCATGACGTTCTTCGGGCAGTTGCC GGGGTGGGACAGG-3'

were designed to contain mutant sites for site-directed mutagenesis (SDM) and used to effect the mutations, changing the codon for Leu39Cys (L39C) from CTG to TGC. Mutations in the azu gene were introduced by site-directed mutagenesis [19,20]. Fig. 1(a), 1(b) show schematic representations of plasmids for the expression of cysteine-modified azurin.

2. Expression and Purification of Recombinant Protein

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 1 L 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 5,000 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 a CM excellulose ion-exchange column with a pH gradient from 4.0 to 6.0 (50 mM sodium acetate) [21]. 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).

3. Fabrication of Protein Immobilized Biofilm

For the fabrication of gold (Au) substrate, a cover glass composed of BK7 (18 mm×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 with piranha solution composed of 30 vol% H_2O_2 (Sigma-Aldrich, USA) and 70 vol% H_2SO_4 (Sigma-Aldrich, USA) at 70 °C 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 was 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 to wash the substrate sufficiently. And then the residual solution on Au surface was removed by N_2 gun. It is a very simple step compared with reported previously method using alkanethiol [22-24]. The schematic description of azurin immobilization is shown Fig. 2(a), (b). Modifying the protein with cysteine residue is a novel technique

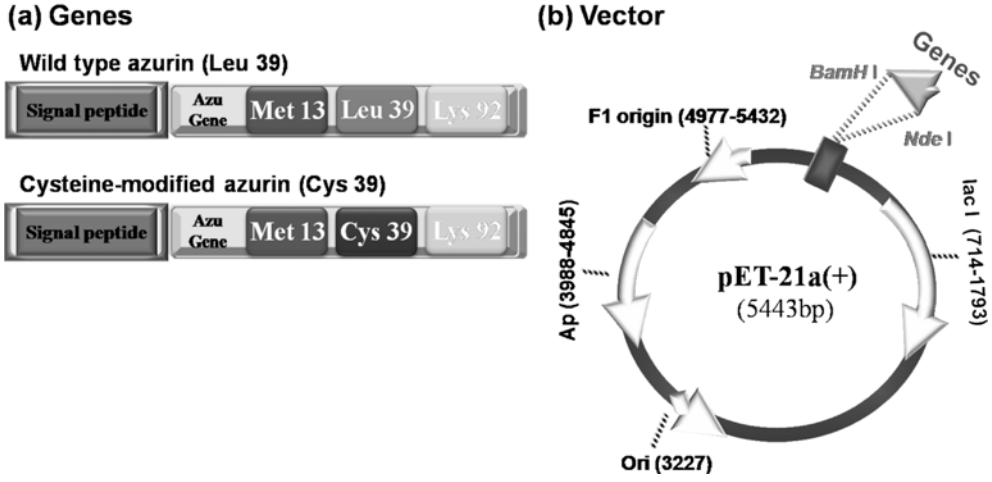

Fig. 1. Schematic representations of genes and plasmids for expression. (a) Azu gene and Cysteine-modified Azu gene. (b) Vector.

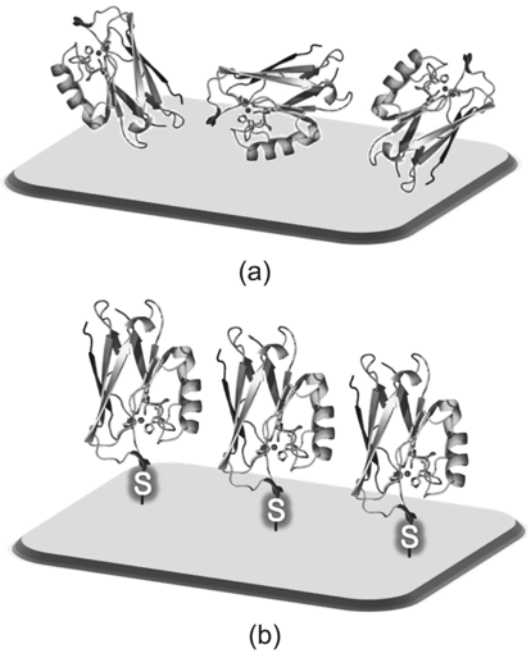

Fig. 2. Schematic diagram of protein immobilization. (a) Wild type azurin. (b) Cysteine-modified azurin.

that immobilizes protein on Au surface directly.

4. Confirming Immobilization using Surface Plasmon Resonance (SPR)

SPR depends on a bound electromagnetic wave that is proportional to the film thickness on the metal surface. Kretchmann's attenuated total reflection (ATR) configuration is well known as the design for the 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 TM, Optrel GmbH, Germany) by 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 Kretchmann coupler. The plane face of the 90° glass prism was coupled to the 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°.

5. Topography Analysis using Scanning Tunneling Microscopy (STM)

Surface topology images of the adsorbed azurin and bare gold surface were acquired by using STM (Multimode, Veeco, USA). STM has been used for the investigation of some solid surfaces at the nanometer scale. Since STM can provide a direct insight into the molecular topography under physiological conditions, the calculation of the coverage of thin films and surface roughness and the detection of protein-protein binding are possible. Image acquisition was carried out under the condition of I set=0.5 nA, When the applied voltage was 0.1-1.0 V the STM image could support SPR data for confirming immobilization. STM analysis could be utilized to complement the method of SPR. The benefit of combining SPR and scanning probe microscope (SPM) imaging allows the interrelationships between surface morphology and biological interaction with biomaterials to be efficiently analyzed.

6. Redox Characteristic Investigation by Electrochemical Method

Cyclic voltammograms (CV) were obtained with a 660A system (CHI, USA). The electrochemical cell volume was 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 area was 0.25 cm^2. Three-electrode, counter electrode was platinum wire, reference electrode was Ag/AgCl electrode. HEPES buffer (pH=7.0) was used by electrolyte. All experiments were conducted at room temperature.

RESULTS AND DISCUSSION

The fabricated protein film was investigated by using surface plasmon resonance (SPR), because the angle shift of the adsorbed surface is proportional to the quantity of adsorbed proteins. Fig. 3(a) shows the change of SPR angle with respect to the immobilized concentration of cysteine-modified azurin. Therefore, in this work, we defined the optimal concentration of cysteine-modified azurin layer. As the concentration of cysteine-modified azurin was increased,

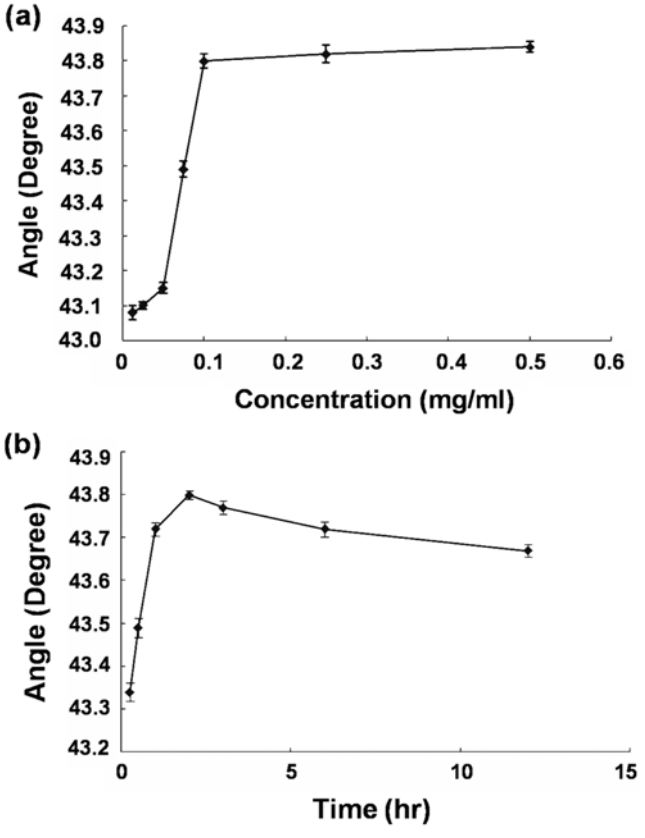

Fig. 3. Characteristics of the protein immobilized layer under optimized condition by SPR. (a) Optimal immobilization concentration. (b) Optimal immobilization time.

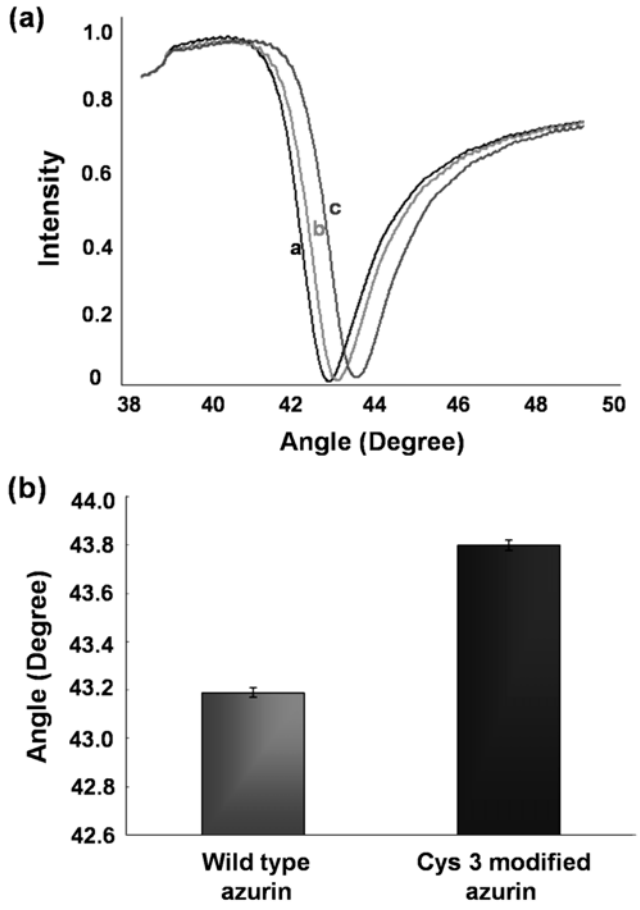

Fig. 4. Confirming the immobilization of azurin layer by SPR. (a) SPR spectroscopy of a: bare gold surface, b: wild type azurin immobilized surface, and c: cysteine-modified azurin immobilized surface. (b) Compare angle shift of directly immobilized wild type azurin and cysteine-modified azurin.

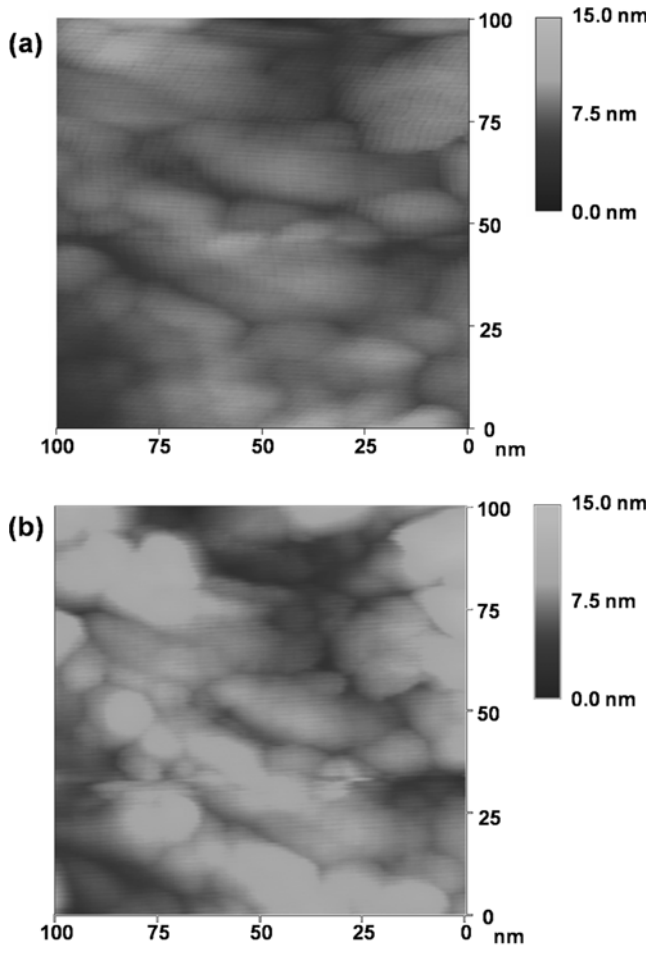

Fig. 5. STM surface analysis of cysteine-modified azurin. (a) Annealed Au substrate. (b) Cysteine-modified azurin immobilized surface.

the amount of SPR angle shift was also increased and finally saturated. The results are presented in Fig. 3(a). Moreover, we determined the optimized cysteine-modified azurin concentration from the saturated SPR angle curve. After saturation concentration was determined, all experiments were performed by using optimized concentration. Optimal concentration was 0.1 mg/ml and optimal time for azurin immobilization was about 2 hr (Fig. 3(b)). Fig. 4(a) shows the SPR angle shift according to the deposition on gold. For confirming the effectiveness of the modified cysteine group, one can 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 0.1 mg/ml of cysteine-modified azurin was introduced on the cleaned Au surface, an SPR angle of 0.80 degree was observed. But in the case of wild type azurin, the SPR angle was only 0.19 degree at the same condition. The results are compared in Fig. 4(b). It means that cysteine-modified azurin was more well immobilized than the 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 surface plasmon resonance (SPR). Cysteine-modified azurin which is assembled on the gold surface directly was compared with wild type one. Surface morphology was also measured by scanning tunneling microscopy (STM). Fig. 5(a) shows the bare gold STM image, and Fig. 5(b) shows the cysteine-modified azurin immobilized surface. At 100 nm scale, immobilized cysteine-modified azurin assumes the form of small lumps. Whereas, the adsorbed wild type azurin covered the whole surface by forming aggregates of 15-20 nm in height in our previous work [25]. 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 maintains the reduction-oxidation properties. The scan range was from 500 mV to −100 mV with 50 mV/s scan rate (positive to negative voltage direction). Azurin sample concentration was 0.1 mg/ml for immobilization based on SPR results. 10 mM HEPES(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) was used as a electrolyte. As seen in Fig. 6, the reduction potential was 123 mV and the oxidation potential was 247 mV. During the experiments the calculated standard redox potential of cysteine-modified azurin layer was 185 mV. That matches our previous results and reference [26]. These results have

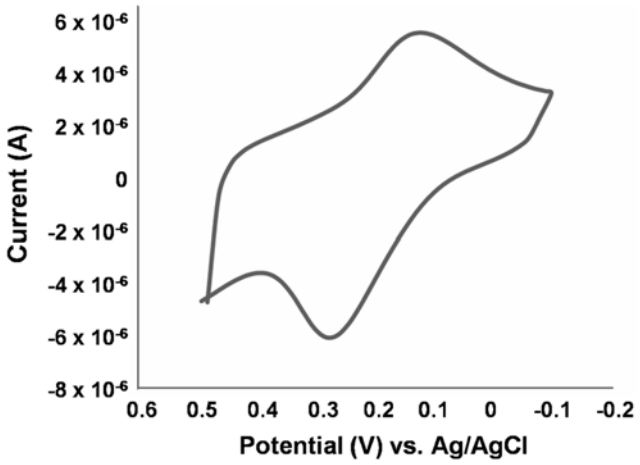

Fig. 6. Cyclic voltammetry at pH 7.0. (10 mM HEPES, pH 7.0, azurin sample concentration=0.10 mg/ml, electrode area=0.25 cm^2).

noticeable points. These stable electrical properties of azurin layer by the direct immobilization of cysteine-modified azurin imply that the direct immobilization technology can offer very stable and well oriented protein layer. In case of the use of a linker in the process of protein immobilization, not-completed immobilized protein existed in the protein layer and this protein seems to be detached under a harsh condition [27].

CONCLUSIONS

Recently, many studies have pioneered genetic engineering techniques to insert cysteine residues to various biomolecules with the purpose of improving their immobilization efficiency to develop better bioelectronic devices. In this work, we have suggested a novel method for fabricating azurin thin film based on genetic engineering technique without any chemical linker material. We designed cysteine-modified azurin using site-directed mutagenesis. The cysteine-modified azurin layer was self-assembled on the Au surface. Optimal concentration and time for the formation of azurin thin layer were determined as 0.1 mg/ml, 2 hr. As a result, the immobilization efficiency has been increased about 76.3%, as compared to that of wild type azurin. The proposed immobilization method of cysteine-modified azurin can be used for making high quality protein film, and applied to the fabrication of nano-scale bioelectronics.

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