Fusion protein bilayer fabrication composed of recombinant azurin/cytochrome P450 by the sortase-mediated ligation method

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Recently, the fabrication of protein bilayer has been required for the development of protein or enzyme complex formation. In the present study, we fabricated a fusion protein bilayer composed of recombinant azurin–cytochrome P450, which was synthesized by a site-specific sortase-mediated ligation method. The \emph{Pseudomonas aeruginosa} azurin was modified by DNA recombinant technique, for enzymatic ligation and immobilization. The \emph{Pseudomonas putida} cytochrome P450 was also modified for enzymatic ligation. The recombinant metalloproteins were conjugated via the sortase A. The conjugation was confirmed by SDS-PAGE and UV–vis. Then, the prepared fusion protein was immobilized on Au substrate, by the self-assembly method. The Azu–P450 (recombinant azurin–cytochrome P450) fusion protein layer was confirmed by AFM (Atomic Force Microscopy) and SERS (Surface-enhanced Raman Spectroscopy), to confirm the fusion protein bilayer orientation. Moreover, the electrochemical property of Azu–P450 was observed by cyclic voltammetry (CV). As a result, the Azu–P450 fusion protein bilayer shows good orientation on the Au substrate. Also, the original redox property of this fusion protein bilayer has been well maintained. The proposed fusion protein bilayer can be used for bioelectronics devices. In particular, functional biomolecules, such as enzymes or antibodies, should be needed to control their orientation on the substrate [7]. Because these molecules react with external substrates, the signals are thereby transduced to the electronic part.

Since the 2000s, Choi’s group has researched the fabrication of biofilm, and its applications. Initially, they developed Langmuir–Blodgett film-based bioelectronics devices, such as a biophotodiode, which consisted of protein heterolayer [8–11]. After the development of this initial device, they suggested a protein layer-based biomemory device, using a self-assembly method [12]. More recently, they have focused on fabricating protein heterolayer-based biomemory devices, composed of cysteine-modified azurin, and cytochrome c. Various types of biomemory devices have been developed, using recombinant protein layers [13,14]. Controlling the biofilms is an important technique for making the future bioelectronic device that is composed of multiple proteins or enzymes.

Herein, we developed a novel protein bilayer fabrication method composed of recombinant azurin–cytochrome P450, which was conjugated by a site-specific sortase-mediated ligation method.

1. Introduction

The conventional goal of biotechnology is to understand the behavior of living organisms, with regard to agriculture, medicine, and engineering applications. However, in the last few decades, the field of biotechnology has broadened, and has become integrated with other scientific fields, including in particular, nanotechnology, information technology, and electrical engineering [1,2]. Consequently, various new areas of research have been created, which include nanobiotechnology, molecular electronics, bioelectronics, and nanomedical engineering [3,4]. Bioelectronics has led to the development of the biochip, as well as biosensors and bioelectronic devices [5]. Bioelectronics devices conventionally consist of a biological actuation part, and an electronic signal transduction part. The biological part is usually composed of biomolecules, such as protein, and DNA-based biofilm [6]. For that reason, controlling the biofilm is an essential technique in fabricating a bioelectronics device. In particular, functional biomolecules, such as enzymes or antibodies, should be needed to control their orientation on the substrate [7]. Because these molecules react with external substrates, the signals are thereby transduced to the electronic part.

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Herein, we developed a novel protein bilayer fabrication method composed of recombinant azurin–cytochrome P450, which was conjugated by a site-specific sortase-mediated ligation method.
For this, four experimental steps were conducted. Step 1: The cysteine group was introduced into wild-type azurin. Step 2: GGGSLPETGG peptide sequences were introduced at the C-terminal of recombinant azurin. Step 3: Recombinant azurin and GGG-modified cytochrome P450 were conjugated via sortase A. Step 4: Azu–P450 fusion protein was immobilized onto Au substrate.

Fig. 1(a) shows a schematic diagram of this process. The azurin molecule and cytochrome P450 molecule were designed and modified for ligation. The recombinant azurin was designed to introduce two cysteine residues, for direct immobilization on an Au substrate. In addition, the C-terminal of recombinant azurin was also modified, to insert GGGSLPETGG sequences for connecting cytochrome P450, using the site-specific ligation method with sortase A. The cytochrome P450 was also modified to contain triglycine (GGG) sequences, for linking directly with the azurin molecule. The sortase A that recognizes the LPETG sequence was cleaved, between the T and G residues. Subsequently, it was linked with the carboxyl group of T to the new amino group of G, by a native peptide bond [15]. Fig. 1(b) shows a schematic diagram of the sortase-mediated site-specific ligation strategy for azurin–cytochrome P450 fusion protein. The basic transpeptidation mechanism has previously been studied [16]. A specific ligation between the recombinant azurin and cytochrome P450 was obtained. Then, the prepared Azu–P450 fusion protein was immobilized onto Au substrate, to investigate the characteristics by self-assembly technique.

For this purpose, the recombinant azurin, cytochrome P450, and sortase A were expressed, and purified. Then, the Azu–P450 fusion protein was confirmed, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and UV–vis spectroscopy. The prepared Azu–P450 fusion protein was self-assembled onto an Au substrate. Atomic force microscopy (AFM) and surface-enhanced Raman spectroscopy (SERS) were used, to confirm the topography and composition of the fusion protein layer. The redox property of

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**Fig. 1.** (a) Schematic representation: fabrication of Azu–P450 fusion protein heterolayer to Au substrate by self-assembly method. (b) The sortase-mediated ligation of GGGSLPETGG-tagged cys1,2-modified azurin to triglycine modified cytochrome P450.
Azu–P450 fusion protein heterolayer was also monitored, by cyclic voltammetry (CV).

2. Materials and methods

2.1. Construction of recombinant azurin plasmids

The gene encoding cysteine-modified azurin was amplified by PCR, from the genomic DNA of *Pseudomonas aeruginosa*. The forward primer (5′-GCCATGCATATGCACTCTGCGG-3′) was designed to include the NdeI restriction enzyme site. The reverse primer, which contained a BamHI restriction enzyme site (5′-GCCATGCGGATCCCTGCAGCTGCCG-3′), was designed to include the GGGSPLPETGG sequence, for sortase-mediated ligation. The PCR product was obtained and purified, using a DNA purification kit (Qiagen, Valencia, CA, USA), and digested with NdeI and BamHI restriction enzymes (New England Biolabs, Ipswich, MA, USA). The digested DNA fragments were ligated with a pET-21b (+) vector (Novagen, Gibbstown, NJ, USA), which was predigested with NdeI and BamHI, using a ligation kit (TaKaRa, Shiga, Japan) [12].

2.2. Expression and purification of 6-histidine tagged-sortase A

The sortase A gene-containing the pET30b plasmid was transfected into *Escherichia coli* BL21 [DE3] phsex5. The cells were grown in TB medium to an OD (600 nm) value of 0.7, at which time the expression of the protein was induced, by adding 1 mM IPTG, at 25 °C. After growth for an additional 16 h at 37 °C, the cells were harvested by centrifugation. The cell pellets were resuspended in 0.05 M phosphate buffer and 0.3 mM NaCl, at pH 7.0, and lyzed by sonication. 6-Histidine-tagged sortase A was purified from the soluble fraction of the lysate, using TALON metal affinity resin (Clontech, Palo Alto, CA, USA), according to the manufacturer’s protocol, and dialyzed against 50 mm Tris–HCl and 150 mm NaCl, at pH 8.0. Figure 3(a) shows the SDS-PAGE result, without a His-Trap column. Figure 3(b) shows the SDS-PAGE, after His-Trap column treatment. This result shows that the purified sortase A was well obtained [17].

2.3. Preparation of recombinant cytochrome P450 and substrate

The recombinant cytochrome P450 was provided by the Nagamune Group from the University of Tokyo [18]. Distilled and deionized water was used to clean the substrates. To investigate the surface topography, Au (50 nm)/Cr (2 nm)/SiO2 substrate was fabricated and purchased from G-mek (Korea), for AFM analyses. The fabricated Au substrate was cleaned in a piranha solution, composed of 30 vol% H2O2 (Duksan Pure Chemical Co. Ltd., Seoul, Korea) and 70 vol% H2SO4 (Daejung Chemical Co. Ltd., Korea), at 60 °C for 5 min. The Au substrate was then rinsed with deionized water, and dried, using a stream of nitrogen.

For SERS measurement, ITO-coated glass substrates (G-mek, Korea) were cleaned by sonication for 30 min in 1% Triton X-100, DIW, and ethanol. After washing, cleaned ITO substrates were exposed to O2 plasma, to modify the surface to be hydrophilic; and then 100 μl of 5% APTES ([3-aminopropyl]triethoxysilane) in 95% ethanol was dropped onto the ITO substrate for 2 h, at room temperature. After cleaning with ethanol, the APTES-modified ITO substrates were heated at 99 °C for 20 min, to cure the organosilane molecules. Then, 20 nm of GNPs (gold nanoparticles, 100 μl, BBI, UK) was dropped onto the APTES-modified ITO surface, and incubated for 24 h, at 4 °C. Finally, the GNP-modified substrate was rinsed with deionized water, and then dried using a stream of nitrogen, to remove the unbound GNPs.

2.4. Conjugation and immobilization of the recombinant azurin/cytochrome P450 fusion protein on the gold substrates

The conjugation reaction between cys1,2,GGGSPLPETGG-modified azurin and GGG-modified cytochrome P450 was carried out in 50 mM Tris–HCl buffer at pH 8.0, containing 150 mM KCl and 10 mM CaCl2. The reaction mixture, containing 30 μM recombinant azurin and 30 μM recombinant cytochrome P450, was ligated with 10 μM sortase A at 25 °C, with shaking. The reaction product was purified by gel filtration.

To investigate the fusion protein film, the purified fusion protein solution was dropped onto purified Au substrate, for a direct immobilization process by self-assembly technique, for 6 h at 4 °C. A self-assembled recombinant azurin/cytochrome P450 fusion protein layer was formed on the Au surface by covalent bonding, due to the cysteine group of azurin on its surface.

2.5. Investigation of fusion protein immobilization by AFM, SERS

Atomic force microscopy (AFM) images were acquired with a Nanoscope (R) III (Digital Instruments, Hayward, CA, USA), to confirm the immobilization of the recombinant azurin/cytochrome P450 fusion protein. The AFM tips were phosphorous (n-type doped Si) (spring constant: 20–80 N/m, resonant frequency range: 230–305 kHz). Raman spectra were recorded using a 785 nm NIR laser, with an irradiation power of 3 mW, on the sample plane. Twenty five scans of 5 s from 400 to 1600 cm−1 (100 μm × 100 μm) were monitored, and the mean data were used. Prior to each step, a blank spectrum was recorded, which assigned the absorbance to be subsequently measured. Background spectra were subtracted from all of the Raman signals obtained from the proteins, and the SERS measurements of each sample were repeated 5 times [19].

2.6. Electrochemical property of recombinant azurin/cytochrome c heterolayer

The electrochemical properties of the prepared recombinant proteins were investigated by a potentiostat CHI660a electrochemical workstation (CH Instruments Inc., Houston, TX, USA). A three-electrode system was used for the CV experiments. The fabricated recombinant azurin/cytochrome P450 complex modified Au substrate (0.25 cm2) was used as the working electrode. Pt and Ag/AgCl electrodes were used as the counter electrode and reference electrode, respectively. CV was performed of the recombinant proteins immobilized on the Au surface in 10 mM HEPES buffer (pH 7.0), in the potential range of 0.6 to −0.1 V, vs Ag/AgCl electrode, at a scan rate 50 mV/s.

3. Results and discussion

3.1. Production of recombinant azurin

A cysteine-modified azurin gene was designed in our previous studies [12]. The recombinant azurin gene was re-designed to introduce the GGGSPLPETGG sequence at the end of the C-terminal. Figure 2(a) depicts a schematic representation of the azurin and plasmids for the expression in recombinant azurin, and the primer information for polymerase chain reaction (PCR). Figure 2(b) shows the vector containing the recombinant azurin. This prepared azurin-containing plasmid was amplified by PCR, and the product was confirmed by DNA electrophoresis and UV–vis (Figs. 2(c) and (d)). As shown in Figure 2(e), a cys1,2,GGGSPLPETGG-modified azurin gene was verified by DNA sequencing, and compared to that of the wild-type azurin gene. This observation suggests that a recombinant azurin gene was successfully expressed in *E. coli*. This was used to purify cys1,2,GGGSPLPETGG-modified azurin, which
was confirmed by 12% SDS–PAGE gel. Fig. 2(f) shows the azurin band size differences between the wild-type azurin and recombinant azurin. Protein bands corresponding to the predicted size of wild-type azurin and recombinant azurin are visible on the gel. The molecular weight of wild-type azurin is about 14.4 kDa. However, the molecular weight of cys1,2,GGGSLPETGG-modified azurin is about 15.2 kDa. Lane 1 indicates the size markers, lane 2 shows the wild-type azurin, and lane 3 shows the recombinant azurin. These results suggest that the recombinant azurin was successfully expressed and purified.

3.2. The sortase-mediated ligation between recombinant azurin and cytochrome P450

The sortase A was expressed and purified (Fig. 3(a) and (b)) by his-tag entrapment. The cys1,2,GGGSLPETGG-modified azurin and the GGG-modified recombinant cytochrome P450 were prepared for sortase-mediated site-specific ligation. The SDS–PAGE results show the Azu–P450 fusion protein (Fig. 3(c), left panel). Lane 1 shows the size markers; whereas, lanes 2 and 3 depict the recombinant azurin (15.2 kDa) and cytochrome P450 (47.1 kDa), respectively. Lane 4 shows sortase (25.0 kDa). The conjugation result is shown in lane 5, which shows a well-formed Azu–P450 fusion protein, yielding a retarded migration of the fusion protein on a SDS–PAGE gel (62.3 kDa). The conjugation mixture was collected and purified by gel ultrafiltration (Fig. 3(c), right panel), and purified fusion proteins were displayed.

However, as reference controls, (1) the fusion protein conjugation between wild-type azurin and GGG-modified cytochrome P450 was reacted by sortase A; and (2) the fusion protein conjugation between cys1,2-modified azurin and GGG-modified cytochrome P450 was also reacted by sortase A. Fig. S1 displays this SDS–PAGE result. In these cases, the fusion proteins did not show the band in the SDS–PAGE result. This means that the GGGSLPETGG peptide sequences of azurin in the C-terminal can play a critical role, in conjugating with GGG-cytochrome P450.

Additionally, the UV–vis absorption spectrum was analyzed, to confirm the copper uptake by the refolded recombinant azurin. P. aeruginosa azurin is a blue copper protein, with a Greek key topology. The recombinant azurin has a blue color, due to the ligand-to-metal charge transfer between the cysteine sulfur ligand (Cys112), and the oxidized copper ion. The copper coordinates five residues (Gly45, His46, Cys112, His117, and Met121) in a unique geometry that gives rise to intense absorption at 630 nm (Fig. 4(d), blue line) [14]. The observed adsorption at 630 nm confirmed the copper uptake by the folded recombinant apo-azurin. As a result, the copper uptake by folded cys1,2,GGGSLPETGG-modified apo-azurin was well investigated. The cytochrome P450, as a heme-thiolate protein, shares similar absorption spectral characteristics with a heme coordinated to the sulphhydril group from the cysteine residues [18]. The observed absorption Soret peaks at 417 nm and 447 nm indicate the heme iron in the cytochrome P450 molecule (Fig. 4(d), red line). These two shoulders with a broad band are observed for the reduced cytochrome P450. It seems that this may reflect the coordination of a nitrogen base as the sixth iron ligand [20]. In the case of Azu–P450 fusion protein, the absorption spectra show the combined spectra of recombinant azurin and cytochrome P450 (Fig. 4(d), green line). Thus, the conjugation of the Azu–P450 fusion protein was well confirmed. Based on this analysis, the Azu–P450 fusion protein was produced, and retained its unique structure.

3.3. The surface investigation of recombinant azurin–cytochrome P450 fusion protein

The purified Azu–P450 fusion protein was immobilized onto an Au substrate, using the self-assembly method. AFM and SERS were used to confirm the surface characteristics of the fabricated
fusion protein layer. The topographical features of the recombinant azurin, cytochrome P450, and Azu–P450 fusion protein were examined by AFM (Fig. 4(a)–(c)). As shown in Fig. 4(a), the cysteine-modified azurin was well immobilized on the Au substrate, and had small lumps of ~20 nm in diameter. In addition, Fig. 4(b) shows images of the cytochrome P450 self-assembled onto an 11-MUA-modified Au substrate. The thiol group of 11-MUA was anchored onto Au substrate, by covalent bonding. The carboxyl group of 11-MUA molecules provides the free amine group of cytochrome P450 by electrostatic bonding. The cluster size of the cytochrome P450 (~40 nm) was larger than that of the recombinant azurin. The Azu–P450 fusion protein immobilized on the Au substrate had various cluster sizes, corresponding to the Azu–P450 fusion protein (20–40 nm). However, in the case of Azu–P450 mixture as the reference control, the irregular morphology was investigated (Fig. S2). It is difficult to fabricate protein bilayer. This difference demonstrates that the immobilization of the Azu–P450 fusion protein was well retained.

Moreover, Fig. 4(d)–(f) shows the SERS analysis of recombinant azurin, cytochrome P450, and the Azu–P450 fusion protein self-assembled layer, respectively. In agreement with previous reports [21–23], the Raman spectra of the azurin from P. aeruginosa were recorded near the region of 750 cm\(^{-1}\), where the peaks are attributed to the C\textendash{}S (cys) stretching, and around the region of 923 cm\(^{-1}\), which corresponds to the C\textendash{}C stretching in the \(\alpha\)-helix (Fig. 4(d)). In this respect, the Raman spectrum of azurin indicates that the copper site maintains its structure, upon immobilization onto GNP-modified ITO. In the case of cytochrome P450 self-assembled on 11-MUA/GNP-modified ITO substrate, Fig. 4(e) shows the Raman spectra features of cytochrome p450, which are in agreement with previous reports [24,25]. The amide I peak at about 1430 cm\(^{-1}\) and the peak at 1557 cm\(^{-1}\) correspond to the amide II. The v3 mode in cytochrome P450 is at 1473 cm\(^{-1}\), which is consistent with the five-coordinate low-spin (5C/LS), so that the thiolate sulfurs are coordinated with the heme iron. These peaks indicate the successful immobilization of cytochrome p450/11-MUA, self-assembled onto GNP-coated ITO substrate.

In the case of Azu–P450 fusion protein, the Raman spectra show combination characteristics between azurin and cytochrome P450. Fig. 4(f) depicts the Raman spectra of Azu–P450 fusion protein. The peaks at 767 cm\(^{-1}\) and 927 cm\(^{-1}\) correspond to the C\textendash{}S (cys) stretching and C\textendash{}C stretching in the \(\alpha\)-helix, respectively. It is
likely that these peaks are attributed to the azurin molecule. Furthermore, the Raman spectra also show a peak at 1430 cm$^{-1}$ that corresponds to amide I, and a peak at 1473 cm$^{-1}$ that corresponds to the heme iron five-coordinate high-spin (5C/LS) configuration. The Raman spectrum of Azu–P450 fusion protein indicates that the fusion protein retained its unique structure and properties, on the Au substrate through the self-assembly process, resulting in the formation of an Azu–P450 fusion protein on the Au surface.

3.4. The electrochemical properties of recombinant azurin–cytochrome P450 fusion protein

Cyclic voltammetry (CV) was carried out, to determine the redox behavior of Azu–P450 fusion protein self-assembled onto Au substrate. The redox properties of GGGGLPETGG, cys1,2-modified azurin layer and GGG-modified cytochrome P450/11-MUA heterolayer were also investigated by CV. The cyclic voltammograms of each case (GGGGLPETGG, cys1,2-modified azurin, GGG-cytochrome P450/11-MUA, Azu–P450) are shown in Fig. 5(a)–(c), respectively. The CV measurements of each sample were repeated 5 times.
The reduction and oxidation potentials of the azurin are 159 ± 23 mV, and 433 ± 47 mV, respectively. These showed the redox process of recombinant azurin center Cu\(^{2+/+1}\). Also, the reduction and oxidation currents are monitored as 3.357 ± 0.174 µA, and −4.335 ± 0.319 µA, respectively. This result of redox property shows the recombinant azurin has contained its copper ion well, even though after recombination [12]. In the case of GGG-cytochrome P450/11-MUA heterolayer, the reduction potentials and oxidation potentials are 246 ± 41 mV, and 335 ± 37 mV, respectively. Also, the reduction and oxidation currents are monitored as 0.295 ± 0.079 µA, and −0.206 ± 0.092 µA, respectively. The redox currents values are relatively low, compared to recombinant azurin. In the case of Azu–P450 fusion protein, the reduction potentials and oxidation potentials are monitored as 356 ± 22 mV, and 422 ± 19 mV, respectively. The reduction and oxidation currents are monitored as 0.485 ± 0.058 µA, and −0.893 ± 0.070 µA, respectively. These redox potentials and values are listed in Fig. 5(d).

Presumably, this result shows the unique redox property between Cu\(^{1+/2+}\) ion from recombinant azurin, and Fe\(^{2+/3+}\) ion from recombinant cytochrome p450, because the reduction potential seemed to be combined between the recombinant azurin and recombinant cytochrome P450 molecules. In conclusion, the redox property of Azu–P450 was observed, and this can be a possible application for a biosensor.

4. Conclusion

In summary, we developed a protein bilayer fabrication method, composed of recombinant azurin and cytochrome P450, by the sortase-mediated ligation method. The cys1,2-modified azurin gene was re-designed, by the introduction of GGGSLPETG sequences at the C-terminal. The recombinant azurin gene was confirmed by DNA sequencing. The GGGSLPETG, cys1,2-modified azurin was expressed and purified, and the recombinant azurin was well conjugated with GGG–cytochrome P450, via sortase A. As a result, the fusion protein was obtained. The prepared fusion protein was self-assembled onto Au substrate, via cysteine-groups of azurin. The morphology of the azurin–cytochrome P450 fusion protein layer on the Au surface was investigated by AFM imaging. Also, the composition of the fusion protein layer was confirmed by SERS. Besides, the fabricated Azu–P450 fusion protein layer shows the redox property. As a result, the fabricated fusion protein layer was well organized onto the Au substrate. Therefore, it is possible for the proposed fusion protein bilayer to be applied, not only for the fusion protein bilayer, but also for protein complex multiple layer fabrication. Therefore, the proposed bilayer fabrication technique appears to be a good method for multiple protein layer construction, for applications such as the multiple detection biosensor.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.colsurfb.2014.03.034.

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