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Escherichia coli with autodisplayed Z-domain of protein A for signal amplification of SPR biosensor

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ABSTRACT

Generally, an immunoaffinity SPR biosensor detects a target analyte in a sample through highly selective adsorption by using the antigen–antibody interaction. For improving the sensitivity, various kinds of particles have been added to the already bound analytes on the SPR biosensor (sandwich assay). In this work, signal amplification was demonstrated by the expression of the IgG-binding Z-domain of protein A on the outer membrane of *Escherichia coli* via "Autodisplay". The amount of Z-domain of protein A expressed on the outer membrane was calculated to be 280,000 molecules per cell. In addition, the IgG-binding ability of the expressed protein was characterized using FACS analysis. The signal amplification of the SPR biosensor was performed in the sandwich assay format using a model of horseradish peroxidase (HRP); the limit of detection was determined to be significantly improved from 1 μg/ml to 1 ng/ml. Finally, myoglobin analysis was demonstrated for the medical diagnosis of cardiac diseases. The detection limit was estimated to be improved from 10 ng/ml to <1 ng/ml. These results show that Z-domain-displaying *E. coli* can be successfully used for the signal amplification of immunoaffinity biosensors, thereby improving the sensitivity and the limit of detection.

1. Introduction

Immunoaffinity (IA) biosensors have been developed by using the highly specific interaction between antigens and antibodies. The IA layer for the molecular recognition of a specific target analyte has usually been produced by immobilizing the antibodies on the surface of a transducer (Luppa et al., 2001). It is well known that the antibodies (IgG's) have a 'Y'-shaped structure with two binding sites (called F_{ab}s) at the variable regions. These binding sites are localized at the two edges of three-dimensional structure of the antibody. Therefore, effective detection of a target analyte by the antibodies at the IA layer is possible when the F_{ab} regions of the antibodies are exposed toward the analyte by orientation control methods (Liddell, 2001). Density of antibodies on the IA layer should also be controlled to improve the sensitivity of the immunosensors. This is done by binding maximum amount of target analyte in a unit sample volume.

In order to improve sensitivity of immunosensors, both density and orientation of the antibodies should be controlled. Various methods aiming to achieve this have been tried. It has been reported that IA layers prepared by physical adsorption or chemical coupling result in randomly oriented antibodies, where only 20% of antibodies in the IA layer have proper orientation for the binding of analytes (Lu et al., 1996; Muramatsu et al., 1989). Especially for controlling the orientation of antibodies at the IA layer, protein A has been most frequently used for the immobilization of antibodies. Protein A is well known to bind polysaccharides that are located at the constant region (called F_c region) of antibodies. Due to this characteristic, protein A has been applied to immunosensors, as well as conventional immunoassays, for the control of antibody orientation (Chung et al., 2006; Anderson et al., 1997; Bae et al., 2005; Kanno et al., 2000; Nakanishi et al., 1996; Owaku and Goto, 1995). In this work, the antibody-binding domain of protein A from *Staphylococcus aureus*, that is the Z-domain of protein A, was produced at the outer membrane of *Escherichia coli* by "Autodisplay", exhibiting a controlled orientation and density of the expressed protein.

The name "Autodisplay" was initially introduced for the use of the autotransporter domain of a protein called adhesin involved in diffuse adherence from *E. coli* (AIDA-I) (Benz and Schmidt, 1992)

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for the outer membrane translocation of a recombinant protein in combination with the signal peptide of the cholera toxin-subunit (CTB) and an artificial promoter (Maurer et al., 1997). More recently, "Autodisplay" was established generally as a recombinant surface display of proteins or peptides by means of any autotransporter in any gram-negative bacterium (Jose and Meyer, 2007). In comparison to other cellular surface display systems, the "Autodisplay" system has several unique features as follows: (1) more than 10^5 recombinant molecules can be expressed on the outer membrane *E. coli* (Jose et al., 2001), (2) stable multi-meric proteins can be expressed from mono-meric genes (Jose et al., 2002; Jose and von Schwichow, 2004).

The recombinant passenger protein is transported by simply introducing its coding sequence in-frame between the signal peptide and the translocating domain of transformation vector as shown in Fig. 1 (Maurer et al., 1999). The introduction of a multiple cloning site instead of the native passenger allows in-frame insertion of various passenger domains. The C-terminal part of autotransporter protein forms a porin-like structure (β-barrel) within the outer membrane of gram-negative bacteria, such as *E. coli*. Through this pore, the recombinant passenger domain is self-translocated to the surface (Jose et al., 1995; Jose, 2005, 2006; Jose and Meyer, 2007). This autodisplay system was successfully used for the functional surface expression of a wide variety of recombinant proteins (Mauer et al., 1997; Jose et al., 2001; Jose and von Schwichow, 2004; Lattemann et al., 2000; Schulthesis et al., 2002; Rizos et al., 2003; Fischer et al., 2001; Krause et al., 2004; Veiga et al., 2003).

In the present study, we used antibody-binding domain of protein A (called the Z-domain) as a recombinant passenger protein in "Autodisplay". This work aimed to express Z-domain of protein A in a controlled orientation on the *E. coli* outer membrane. To characterize the autodisplayed Z-domain, the amount of Z-domain was estimated by comparison with a co-expressed reference protein called OmpA. Subsequently, the antibody-binding activity of the autodisplayed Z-domain of protein A was confirmed by using fluorescence-labeled antibody and flow cytometric analysis (FACS).

The *E. coli* cells with autodisplayed Z-domain of protein A on the outer membrane was used for the signal amplification of an SPR biosensor. Usually, SPR biosensors are reported to have a detection range between μM and nM for a single step assay for analytes with molecular weight of more than 1000 Da (Lundstroem, 1994; Kim et al., 2007; Pyo et al., 2007). In comparison with the sensitivity of the conventional methods for medical diagnosis, an SPR biosensor should have improved sensitivity (Chung et al., 2005). In order to improve the sensitivity of immunosensors, including SPR biosensors, various labeled proteins have been added to the already bound analyte on the sensor surface (Mullett et al., 2000). For this 'signal amplification', several methods, using various kinds of labeled proteins, have been reported. For instance, secondary antibodies (Goh

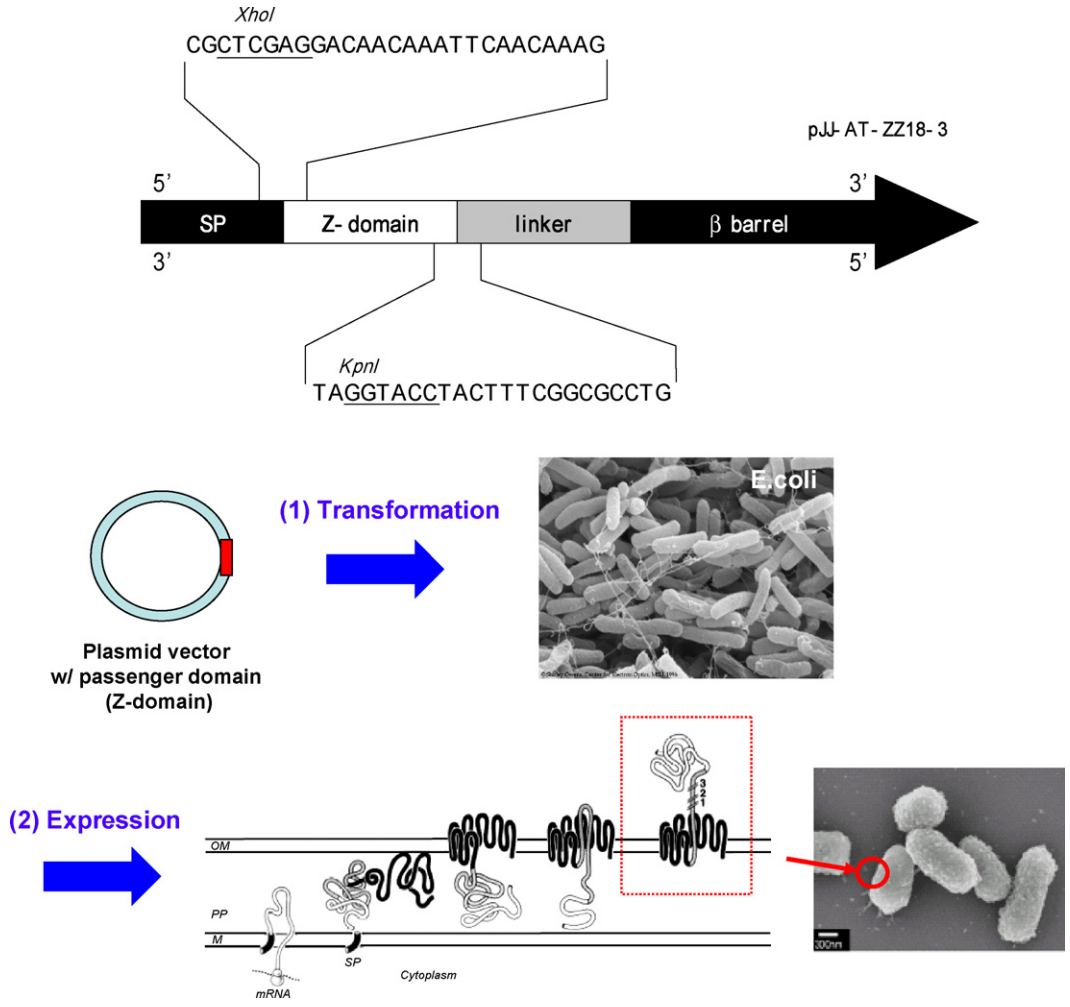

Fig. 1. Autodisplay of proteins at the *E. coli* outer membrane. The autodisplaying plasmid with a passenger domain (Z-domain of protein A) is transformed to *E. coli*, and then the passenger domain is expressed on the outer membrane of *E. coli*. Structure of the passenger domain which encodes the Z-domain–autotransporter fusion protein for Autodisplay as being a part of plasmid pJJ-AT-ZZ18-3. 'SP' represents signal peptide for the translocation of the passenger across the inner membrane.

et al., 2003), latex particles (Severs and Schasfoort, 1993), secondary antibodies conjugated with colloidal gold (Leung et al., 1994) and avidin-biotinylated liposomes (Wink et al., 1998), have all been used. In this work, the *E. coli* cells with the autodisplayed Z-domain was bound to the specific antibodies. The resulting antibody-bound *E. coli* cells were injected into the already bound target analytes on the SPR biosensor for signal amplification. In this work, horseradish peroxidase (HRP) and its antibody were used as a model system for testing the feasibility of the method, and the medical diagnosis of cardiac disorder was demonstrated by using protein A-displaying *E. coli* and a marker protein called myoglobin (Matveeva et al., 2005).

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA), protein A, and avidin were purchased from Calbiochem-Novabiochem GmbH (Schwalbach, Germany). Anti-*E. coli* antibodies (polyclonal), myoglobin, anti-myoglobin antibodies (polyclonal), and anti-HRP antibodies (polyclonal) were bought from AbCam (Cambridge, UK). Horseradish peroxidase and all of the other chemicals (of analytical grade) were purchased from Sigma–Aldrich Korea (Seoul, Korea). DyLight 647-coupled goat anti-mouse antibodies (polyclonal) were purchased from Pierce (Rockford, USA).

2.2. Construction of autodisplay vector, pJJ-AT-ZZ18-3

The antibody-binding Z-domain of protein A (from *S. aureus*) was first cloned by PCR, resulting in plasmid pEZZ18 (Löwenadler et al., 1987). The Z-domain for cloning in an autodisplay vector was obtained by PCR amplification using following primers with restriction enzyme sites for XhoI and KpnI, respectively: 5′-CGCTCGAGGACAACAAATTCAACAAAG-3′ and 5′-TAGGTACCTACTTTCGGCGCCTG-3′. The resulting PCR product (with 186 bp) was first inserted into pBluescipt KS(-), and the PCR product was confirmed by nucleic acid sequencing. The PCR product was then excised by XhoI and KpnI, and then it was ligated to autotransporter plasmid pET-Adx04 (Jose et al., 2002). The resulting plasmid was referred to be pJJ-AT-ZZ18-3 as shown in Fig. 1.

2.3. Surface display of the Z-domain of protein A

The autodisplaying vector pJJ-AT-ZZ18-3 was transformed into *E. coli* UT5600 (F– *ara*14 *leuB*6 *azi*-6 *lacY*1 *proC*14 *tsx*-67 *entA*403 *trpE*38 *rfbD*1 *rpsL*109 *xyl*-5 *mtl*-1 *thi*1, Δ*ompT*-*fepC*266) (Grodberg and Dunn, 1988), which is not able to form the outer membrane protease T (OmpT). The transformed *E. coli* cells expressed the recombinant passenger–autotransporter fusion gene of pJJ-AT-ZZ18-3 and subsequently translocated the Z-domain of protein A onto the cell surface of *E. coli* UT5600 through the autotransporter pathway (Maurer et al., 1997). *E. coli* cells were routinely cultured at 37 °C in a Luria–Bertani (LB) broth of 10 μM EDTA, 10 mM 2-mercaptoethanol, and ampicillin at a concentration of 100 mg/l.

2.4. Analysis of outer membrane proteins

2.4.1. Outer membrane preparation

The *E. coli* cells were grown overnight and this culture (1 ml) was used to inoculate fresh LB medium (20 ml). The *E. coli* cells were incubated at 37 °C with vigorous shaking (200 rpm) for about 5 h until the optical density at a wavelength of 578 nm reached a value of 0.7. Then, the *E. coli* cells were harvested and the outer membranes were prepared according to the rapid isolation method of Hantke (Hantke, 1981), using modifications by Schulthesis et al. (2002).

2.4.2. SDS-PAGE and densitometric analysis

The outer membrane preparations were diluted (1:2) with a sample buffer (100 mM Tris–HCl, pH 6.8, containing 4% SDS, 0.2% bromophenol blue, and 20% glycerol). The prepared outer membrane samples were boiled for 20 min and then analyzed using 12.5% SDS-PAGE. After electrophoresis, proteins were visualized by staining with Coomassie brilliant blue. The molecular weight of the autodisplayed Z-domain of protein A was confirmed by comparison with the pre-stained molecular weight markers (Bio-Rad Laboratories, Munich, Germany). The density of each band was estimated using a documentation system (ChemiDoc XRS^{TM}) and an analysis program (Quantity-One^{TM}) from Bio-Rad Laboratories.

2.4.3. Flow cytometer analysis

E. coli cells transformed with plasmid pJJ-AT-ZZ18-3 were grown overnight and diluted (1:20) in a freshly prepared medium. The *E. coli* cells were grown at 37 °C with vigorous shaking until the optical density reached a value of 3.0 at a wavelength of 578 nm (OD^{578}). The *E. coli* cells were harvested, washed three times with PBS, and then resuspended in PBS/3% fetal calf serum to a final OD^{578} of 1.0. After incubation at room temperature for 10 min, 20 μl of DyLight 647-labeled IgG was added to 1 ml of the cell suspension, resulting in a final concentration of 20 μg IgG/ml. After incubation for 30 min at room temperature, the *E. coli* cells were washed three times with 1 ml of PBS and then resuspended in filter-sterilized PBS to have OD^{578} of 0.5. For each experiment, at least 10,000 cells were analyzed with a FACSAria flow cytometer (Becton-Dickinson, Heidelberg, Germany), using an excitation wavelength of 488 nm and filter-sterilized PBS as a sheath fluid. The threshold trigger was set on side scatter to eliminate background noise and to analyze only intact cells.

2.5. SPR biosensor measurements

A Spreeta^{TM} chip from Texas Instruments Co. (Dallas, TX) was used for the SPR measurements. This SPR chip is equipped with a flow cell with a capacity of 5 μl and the measurements were performed using a home-made control circuit board, designed according to the technical specification of Spreeta^{TM} chip from Texas Instruments Co. In each case, the sample and the washing solution were injected into the flow cell using an integrated injection valve and a peristaltic pump. The pumping rate was set at 1.0 ml/min and the flow of the solution was programmed to stop during the incubation step. The whole instrument was kept in an incubator where the temperature was set at 37 °C. Each measurement step consists of sample injection (100 μl), incubation (30 min), and three repeated washing steps with 0.5% (v/v) Tween 20 (3 ml). In order to avoid any effect of buffer change, the signal was calculated from the difference between SPR response prior to sample injection and that after the washing steps, and the flow of buffer was stopped during the measurement and the incubation steps.

3. Results and discussion

3.1. Quantification of the surface-displayed protein A (Z-domain)

Recently, we reported that autodisplay method enables surface to display numbers up to 1.8×10^5 passenger molecules per cell without interfering with viability and growth of the cell (Jose et al., 2001; Jose and von Schwichow, 2004; Jose and Meyer, 2007). In this work, expression of 2.8×10^5 molecules per cell of Z-domain

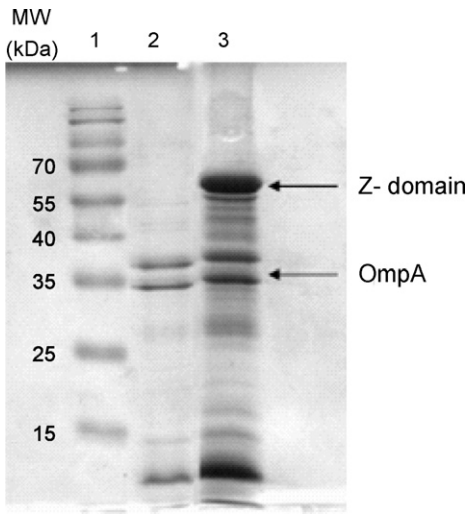

Fig. 2. Quantification of the autodisplayed Z-domain of protein A on SDS-PAGE gel: lane 1, protein molecular weight marker; lane2, the outer membrane sample of control cells (UT5600 (DE3)) without expressing Z-domain; lane 3, outer membrane with autodisplayed Z-domain of protein A.

of protein A was possible using the same autodisplay method. This number of molecules autodisplayed on a single *E. coli* cell was estimated by comparing the expression of the Z-domain with the expression of the natural outer membrane protein OmpA. The number of OmpA molecules on the intact outer membrane of *E. coli* UT5600 is known to be approximately 10^5 copies per cell (Koebnik et al., 2000). Based on this value, the amount of expressed Z-domain molecules were estimated by normalizing the intensity of protein bands on the SDS-PAGE gel, as described in Section 2.4. As expected from the specification of the *E. coli* strain of UT5600 (F– *ara*14 *leuB*6 *azi*-6 *lacY*1 *proC*14 *tsx*-67 *entA*403 *trpE*38 *rfbD*1 *rpsL*109 *xyl*-5 *mtl*-1 *thi*1, Δ*ompT*-*fepC*266), the protein bands of the Z-domain of protein A did not appear on the outer membrane sample of control cells (UT5600 (DE3)) without expressing Z-domain (lane 2) as shown in Fig. 2.

The protein band of the Z-domain was observed at the outer membrane sample of *E. coli*, transformed by autodisplaying plasmid of pJJ-AT-ZZ18-3 (lane 3), and the molecular weight was estimated to be 58.5 kDa, as expected from the fusion protein sequence of the Z-domain. Band intensities of the OmpA and Z-domain were estimated by densitometric analysis (Section 2.4), and the number of autodisplayed Z-domain molecules were estimated to be 2.8×10^5 molecules per cell. From these results, the expression of the Z-domain of protein A was confirmed and the amount of the expressed Z-domain was estimated from these densitometric analyses of the outer membrane samples.

3.2. Functional analysis of the autodisplayed Z-domain of protein A using flow cytometry (FACS)

The antibody-binding activity of the Z-domain, which was confirmed to be autodisplayed at the outer membrane (Fig. 2), was estimated by using fluorescence-labeled antibodies and FACS analysis. As shown in Fig. 3, DyLight 647 (a fluorescent dye)-labeled antibodies (IgG) were incubated with the intact *E. coli* UT5600 and the *E. coli* UT5600 autodisplaying Z-domains on their outer membranes. After washing steps described in Section 2, the *E. coli* samples were studied by FACS analysis. In the case of native *E. coli* UT5600, the labeled antibodies were not expected to be specifically bound to the outer membrane. As shown in Fig. 3(a), the labeled *E. coli* was estimated to be 9.6% of the 10,000 *E. coli* cells. In the case of the *E. coli* cells autodisplaying the Z-domain of protein A, the portion of fluorescence-labeled *E. coli* was estimated to be 90.8% and the number of *E. coli* without antibody-binding activity was evaluated less than 10% ($n = 10^4$) as shown in Fig. 3(b). These FACS analysis results indicated that the Z-domain of protein A (which was displayed by transformation of UT5600 with the Autodisplay plasmid pJJ-AT-ZZ18-3) had an antibody-binding activity, and the portion of the Z-domain active *E. coli* cells was more than 90% ($n = 10^4$).

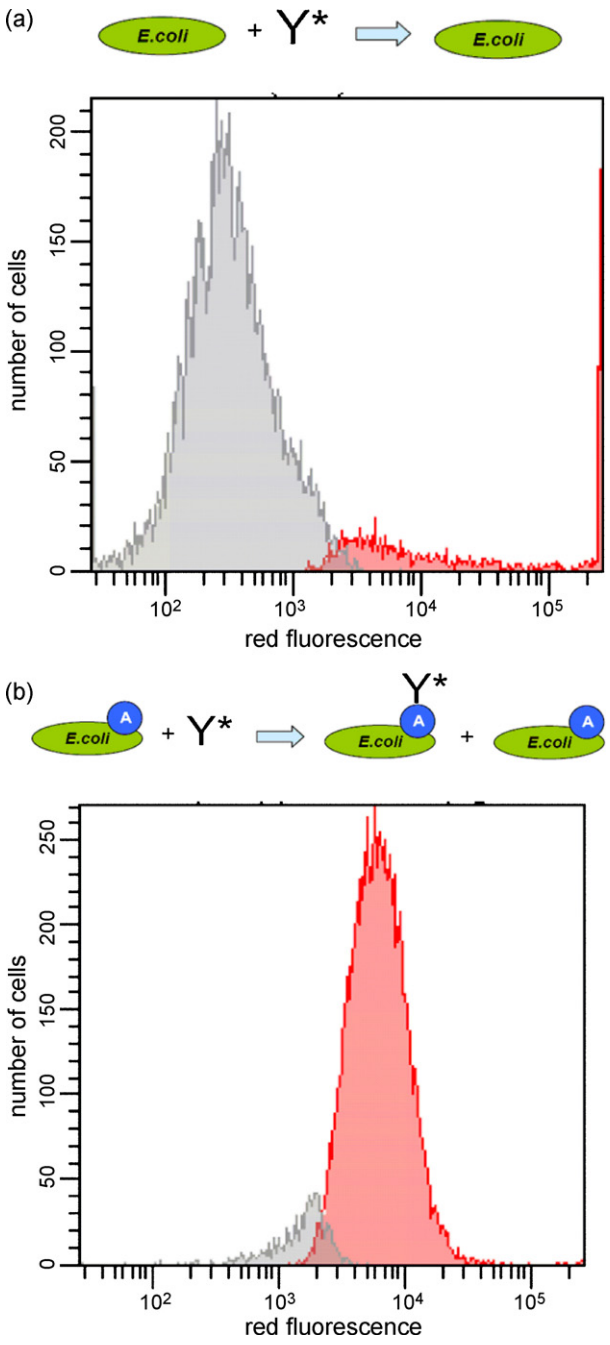

Fig. 3. FACS analysis for the antibody-binding activity of the autodisplayed Z-domain of protein A on the outer membrane of *E. coli* cells. (a) FACS analysis data of the native *E. coli* cells and (b) FACS analysis data of Z-domain autodisplaying *E. coli* cells.

3.3. SPR response by the specific binding of antibody-coupled E. coli

Usually, *E. coli* cells have a cylindrical shape with a length of 2.0 μm and a width of 0.5–1.0 μm (Muramatsu et al., 1989). As

the thickness of the sensitive layer of a SPR biosensor is known to be less than 200 nm (Caide and Sui, 2000), the binding of *E. coli* can saturate the SPR response. In order to show the feasibility of signal amplification, response of the SPR biosensor was measured when the Z-domain-displayed *E. coli* was specifically bound to the IA layer. For this measurement, the BSA was coated onto the SPR biosensor through physical adsorption. This was done by incubating BSA solution (at a concentration of 10 mg/ml) for 1 h. Subsequently, the anti-BSA antibody-coupled *E. coli* and antibody-unbound *E. coli* were sequentially injected onto the SPR biosensor. For the binding of anti-*E. coli* antibody to the autodisplayed Z-domain, the *E. coli* solution (at a concentration of 10^8 cell/ml) was mixed with the same volume of the anti-*E. coli* antibody solution (at a concentration of 100 μg/ml) for 1 h. The anti-BSA antibody-coupled *E. coli* and antibody-uncoupled *E. coli* (100 μl) were then sequentially injected onto the SPR biosensor. The anti-BSA antibody-bound *E. coli* indicated significant SPR response in comparison to that of antibody-unbound *E. coli*. The amount of non-specific binding of the antibody-uncoupled *E. coli* to the BSA surface (15 AU) was estimated to be approximately 10% in comparison to the specific binding (150 AU). This result shows that the antibody-bound *E. coli* with Z-domain can make a significant SPR response by the specific binding to the IA layer of the SPR biosensor.

3.4. Application of Z-domain autodisplayed E. coli for sandwich assay on the SPR biosensor

Sandwich assays have been widely used to increase sensitivity of immunosensors, including the SPR biosensor. In such an assay format, a target analyte is bound to the IA layer, and then additional antibodies or antibody-coupled particles were injected to make a sandwich complex with the already bound target analyte. To demonstrate feasibility of the sandwich assay using the Z-domain autodisplayed *E. coli*, a protein called horseradish peroxidase was used as a model target analyte. The anti-HRP antibodies were immobilized to produce an IA layer on the SPR biosensor and the HRP solutions (at different concentrations) were injected as samples.

For the HRP measurement, the IA layer was prepared through physical adsorption. This was done by incubating the anti-HRP antibody solution (at a concentration of 100 μg/ml) for 1 h. The binding of anti-HRP antibodies were bound to the *E. coli* cells with the autodisplayed Z-domain by mixing the *E. coli* (at a concentration of 10^8 cell/ml) with the same volume of the anti-HRP antibody solution (at a concentration of 100 μg/ml) for 1 h. After the HRP sample solution was injected onto the SPR biosensor, the anti-HRP antibody-bound *E. coli* (100 μl) was sequentially injected onto the SPR biosensor.

The typical procedure used for the signal amplification, by the sandwich assay, is shown in Fig. 4. At first, SPR response was measured after the HRP sample injection, and then the *E. coli* bound to the anti-HRP antibodies were injected sequentially. In the case of HRP detection, limit of detection was determined to be approximately 1 μg/ml. When the signal amplification is applied to the HRP detection by sandwich assay, limit of detection was improved more than 1000-fold, to be less than 1 ng/ml, and the concentration range from 1 ng/ml to 1 μg/ml could be measured with a significantly high sensor response. This result clearly shows applicability of the *E. coli* cell with Z-domain to signal amplification through the sandwich assay format. The signal amplification effect by the antibody-bound *E. coli* was compared with the effect by the antibody binding to the analyte. For this experiment, anti-HRP antibody (100 μg/ml) was sequentially injected onto the SPR biosensor after the HRP treatment. The LOD was improved to be less than 10 ng/ml and the signal amplification by the antibody-bound *E. coli* was estimated to be more than 10-fold higher than only-antibody treatment.

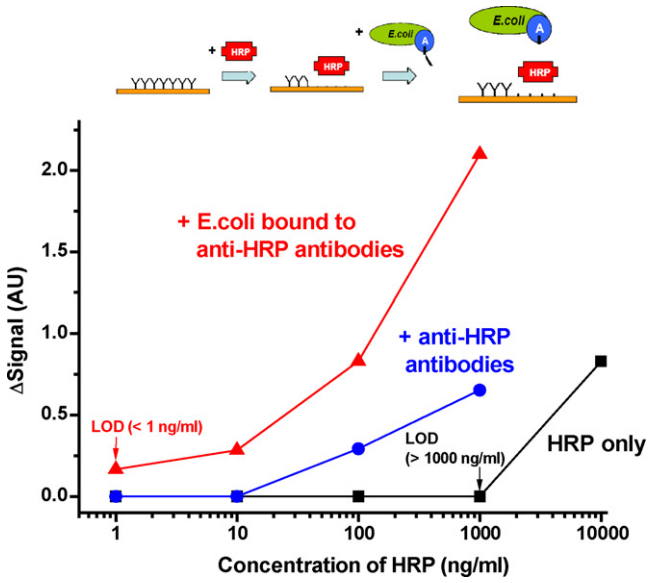

Fig. 4. SPR biosensor responses before and after the sandwich assay of a model protein (HRP) by using anti-HRP antibody-bound *E. coli* cells.

3.5. Detection of myoglobin using antibody-coupled E. coli

Cardiac disorder is usually known as 'silent killer' because the symptoms are trivial or vague right up until a dangerous heart attack. Myoglobin is known to be a specific biomarker for cardiac disorder, along with troponins and chreatine kinases (Matveeva et al., 2005). If heart infarction occurs by the clogging of a blood vessel, the tissue of the heart muscle is partially damaged and these proteins are released into the blood stream. If this condition is detected within 24 h after the infarction happens, life of a patient can be saved by the thrombolytic treatment. The normal concentration of myoglobin, 100 ng/ml, has been used as the cut-off value for diagnosis of cardiac disorder. In this work, medical diagnosis of cardiac disorder by the detection of myoglobin was demonstrated by using a SPR biosensor. The sandwich assay using *E. coli* cells with Z-domain was applied for the improvement of sensitivity.

For the myoglobin measurement, the IA layer was prepared through physical adsorption by incubating the anti-myoglobin antibody solution (at a concentration of 100 μg/ml) for 1 h. The anti-myoglobin antibody coupling to the *E. coli* with autodisplayed Z-domain was carried out by mixing the *E. coli* (at the concentration of 10^8 cell/ml) with the same volume of the anti-myoglobin antibody solution (at a concentration of 100 μg/ml) for 1 h. After the sample solution was injected onto the SPR biosensor, the anti-myoglobin antibody-bound *E. coli* solution (100 μl) was sequentially injected onto the SPR biosensor.

As shown in Fig. 5, the myoglobin sample was injected onto the SPR biosensor with the IA layer of anti-myoglobin antibodies. The *E. coli* cells bound with anti-myoglobin antibodies were then sequentially injected for signal amplification. Before and after amplification, the detection limit was estimated to be improved from 10 ng/ml to less than 1 ng/ml as shown in Fig. 5.

This result shows that *E. coli* displaying the Z-domain at its surface can be effectively used for signal amplification using the sandwich assay. Based on the result of a model protein (HRP) and myoglobin, limit of detection is expected to be more than 10-fold improved by the amplification method.

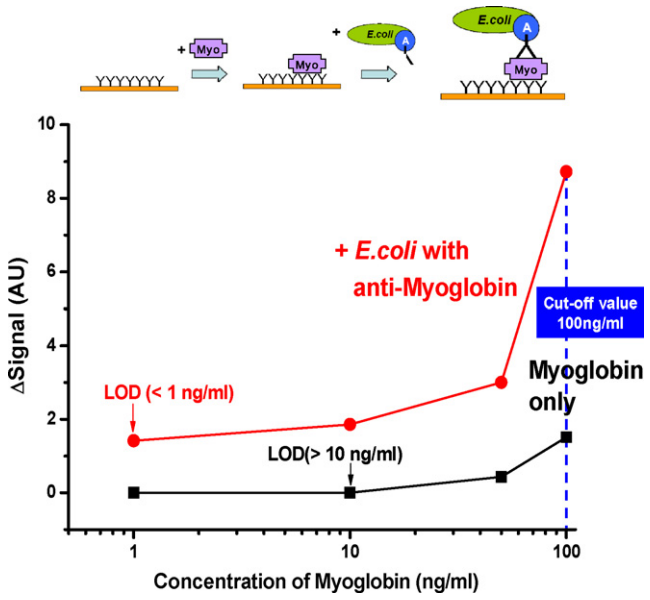

Fig. 5. Comparison of SPR biosensor response with and without the sandwich assay of myoglobin by using anti-myoglobin antibody-coupled *E. coli*.

4. Conclusions

In this work, signal amplification was demonstrated by using the *E. coli* cells autodisplaying the antibody-binding Z-domain of protein A at the outer membrane. The amount of Z-domain of protein, autodisplayed at the outer membrane, was calculated to be 280,000 molecules per cell. In order to confirm the antibody-binding ability of the autodisplayed Z-domain, the DyLight 647-labeled antibodies were added to the *E. coli* cells autodisplaying Z-domain and to the native *E. coli* (negative sample). The two samples were analyzed by flow cytometry and over 99% of *E. coli* cells autodisplaying Z-domain bound the DyLight 647-labeled antibodies. In the negative sample, no fluorescence was detected. This result showed that the autodisplayed Z-domain at the outer membrane has the expected antibody-binding activity. The signal amplification of the SPR biosensor was performed in the sandwich assay format by using *E. coli* cells displaying Z-domain. Antibodies against horseradish peroxidase were immobilized and then HRP was injected onto the HRP-bound SPR biosensor. The anti-HRP antibody was immobilized on the Z-domain autodisplayed at *E. coli* by incubation and then added to the HRP-bound SPR biosensor. By the signal amplification method, the limit of detection was significantly improved from 1 μg/ml to 1 ng/ml. To demonstrate the feasibility of using *E. coli* as a signal amplification tool, *E. coli* cells displaying Z-domain were applied to the myoglobin, and analyzed for the medical diagnosis of cardiac diseases. The detection limit was estimated to be improved from 10 ng/ml to <1 ng/ml. These results show that *E. coli* cells autodisplaying Z-domain can be successfully used for the signal amplification of immunoaffinity biosensors, thereby improving sensitivity and the limit of detection.

Acknowledgments

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