The Si-tag for Immobilizing Proteins on a Silicon Device

Akio Kuroda, Yumehiro Hata, and Kenichi Noda
Department of Molecular Biotechnology, Hiroshima University, Hiroshima 739-8530, Japan
Tel: (+81)82-424-7758; Fax: (+81)82-424-7047
E-mail: akuroda@hiroshima-u.ac.jp

ABSTRACT

Targeting functional proteins to specific sites on a silicon device is essential for the development of new biosensors and supramolecular assemblies. Proteins have mainly been immobilized on silica surfaces by nonspecific adsorption or covalent bond formation between readily available functional groups on protein molecules (e.g., -NH2) and complimentary coupling groups on the solid surfaces. The nonspecific nature of these usual approaches inevitably contains the following difficulties; (i) it requires chemical modification of the solid surfaces, (ii) proteins may be denatured when the interaction with the surface is too strong, (iii) it is difficult to control protein orientation. Recently, we found ribosomal protein L2 as a silica-binding protein, which can be used to immobilize fused proteins on a silicon device. Indeed, a fusion of L2 and green fluorescence protein (GFP) adsorbed to the silicon/silica particles with a Kd of 0.7 nM at pH 7.5. This fusion protein was retained on the silica surface even after washing for 24 h with a buffer containing 1 M NaCl. This L2 silica-binding tag, which we call the "Si-tag", can be used for one-step targeting of functional proteins on silica surfaces. We also constructed a Si-tagged cell wall binding (CWB) protein and demonstrated that bacteria bound to a silicon wafer through the Si-tagged CWB.

Keywords: silicon device, protein targeting, Si-tag, bacteria, and biosensor

1 INTRODUCTION

The ability to target proteins to specific sites and anisotropically immobilize them on a silicon biodevice while preserving their function is necessary for the development of new biosensors such as nanowire sensor arrays [1,2] and silicon-based optical sensors [3]. One of the most exciting tools in proteomics is protein microarray technology, wherein a large number of proteins or peptides can be immobilized on a slide glass or a solid substance for high-throughput analysis of biochemical properties and biological activities [4].

Several efforts have been made to immobilize proteins with a controlled orientation. Immobilized protein A or G has been used to target immunoglobulin to the silica surface of a protein chip [5, 6]. Also, a poly-His tag has been used to immobilize proteins on silica surfaces via surface-chelated metal ions [7]. These methods, however, can require the modification of the silica surface. Addition of a sequence of nine arginine residues (poly-Arg) to a protein has been used for directed adsorption of a fusion protein onto a glass slide or silica resin without loss of enzymatic activity [8]. This method seems to be effective for targeting proteins to silica surfaces without chemical modification of the surface; however, poly-Arg tagged protein is slowly released from the silica surface, and a tag that binds more strongly than poly-Arg is needed for targeting of proteins to silica surfaces.

Recently, we tested the intracellular proteins from several bacterial strains as a silica-binding protein. We found that ribosomal protein L2 binds the most strongly to silica surfaces [9]. We further demonstrated that an L2-fusion protein binds to silica surfaces 20- to 100-fold more strongly than poly-Arg-tagged proteins. Here we demonstrated that the L2 silica-binding tag, which we call the "Si-tag", can be used for one-step targeting of functional proteins to a silica surface. This new technique can be applied to protein array and biosensor technologies (Fig. 1).

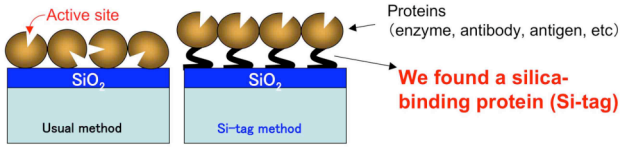

Figure 1: The principle of this technique to immobilize protein on a silicon device by using Si-tag. In usual method (left), proteins are randomly immobilized/adsorbed on a silicon device. Si-tag can be used to one-step immobilization of protein with a controlled orientation (right).

2 MATERIALS AND METHODS

2.1 Materials

Silicon particles were obtained from Junsei Chemical (Tokyo, Japan). Silica particles (diameter 0.8 μm) were obtained from Soekawa Chemicals (Tokyo, Japan). Plasmid pGFPuv was from Clontech (Palo Alto, CA, USA) and pET21-b was from Novagen (Madison, WI, USA). B-PER

lysing solution was purchased from PIERCE (Rockford, IL, USA). All other reagents were from Wako Chemical (Osaka, Japan) or Sigma Chemical (St. Louis, MO, USA) and of highest available quality.

2.2 Identification of silica-binding proteins

E. coli was grown on 2xYT medium at 37°C for 18 h. Cells were collected by centrifugation and disrupted in the presence of 0.25 mg/ml of lysozyme by ultrasonication (Branson, CT, USA). The lysate was subjected to centrifugation at 10,000 x *g* for 15 min. The protein concentration of the cleared supernatant was adjusted to 1 mg/ml with a buffer containing 25 mM Tris-HCl (pH 7.5), 0.5% Tween 20, and 1 M NaCl. Silicon particles (10 mg) were mixed with 1 ml of the diluted supernatant and incubated with rotary mixing for 30 min at 4°C. The silicon particles were precipitated by centrifugation at 4400 x *g*, washed three times with 1 ml of the buffer, and suspended in a SDS-sample buffer. After boiling for 5 min, proteins were separated by 12.5% SDS-PAGE. To perform the mass spectrometric analysis, the protein bands were excised from Coomassie blue-stained gels. In-gel digest of the proteins was performed using sequencing-grade trypsin (Promega, Madison, WI, USA). For matrix-assisted laser desorption ionization (MALDI)-time of flight (TOF) analysis, the peptide extracts were directly eluted from C18 ZipTip microcolumns (Millipore, Bedford, MA, USA) with 1.5 µl of saturated cyano-4-hydroxycinnamic acid directly onto the MALDI target and analyzed with a MALDI-TOF apparatus (Bruker, Bremen, Germany). The peptide fingerprints obtained by MALDI-TOF were used for protein searches using Mascot (Matrix science Ltd, London, UK).

2.2 Construction of GFP, L2-GFP and poly Arg-tagged GFP plasmids

A DNA fragment encoding GFP was amplified and then inserted into the pET21-b. The resulting plasmid was designated pETGFP. A DNA fragment encoding ribosomal protein L2 was amplified and then inserted into the pETGFP. The resulting plasmid was designated pETL2GFP. For the addition of poly-Arg to the N-terminus of GFP, synthetic oligonucleotides were annealed and inserted into pETGFP, and the resulting plasmid designated pETR9GFP.

2.3 Construction of CWB-L2 plasmid

A DNA fragment encoding the CWB of *atl* was amplified with PCR using *S. aureus* ATCC6538 chromosomal DNA as a template, then inserted into *Eco*RI and *Sac*I sites of pETL2GFP. The resulting plasmid was designated pET L2CWB.

2.4 Expression and purification of the recombinant proteins

The plasmids described above were introduced into *E. coli* Rosetta™ (DE3) pLysS (Novagen). GFP, L2-GFP, R9-GFP and CWB-L2 proteins with a His tag were expressed in 2xYT medium for 6 h in the presence of 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were collected by centrifugation and disrupted with lysozyme and ultrasonication. Polyhistidine-tagged proteins were purified by chromatography on a HiTrap Chelating column (Amersham Biosciences, Piscataway, NJ, USA). The fractions containing the recombinant proteins were obtained by elution with a buffer consisting of 20 mM phosphate buffer (pH 7.4), 0.5 M imidazole, and 15% glycerol. The fractions containing fusion proteins were then applied to an HS cation exchange column (Perspective Biosystems, Cambridge, MA, USA) equilibrated with 20 mM HEPES-NaOH (pH 7.5) containing 1 mM dithiothreitol, 1 mM EDTA, and 20% glycerol. The fusion proteins were eluted with a linear gradient of 0 to 1 M NaCl in the same buffer.

2.5 kinetic analysis of L2-GFP and R9-GFP to a silica particle

Proteins (GFP, L2-GFP, or R9-GFP) were diluted to the indicated concentrations in 1 ml of a buffer containing 0.5% Tween-20, 0.5 M NaCl, and 25 mM of phosphate (pH 6.0), Tris-HCl (pH 7.0, 7.5, or 8.0), or glycine-NaOH (pH 9.0). The diluted proteins were then mixed with 0.1 mg of silica particles. After 15-min incubation, the silica particles were precipitated by centrifugation and then washed with the same buffer. The amount of GFP that bound to the silica particle was determined by measuring the reduction in GFP fluorescence in solution. The dissociation constant (K_d) and maximum binding of L2-GFP and R9-GFP to silica particles were determined by Scatchard analysis.

2.6 Absorption of protein on a glass slide

An aliquot of protein solution was spotted onto a slide glass (Matsunami, Tokyo, Japan). The slide glass was shaken in a solution of 25 mM Tris-HCl (pH 8.0), 0.5% Tween 20, and 1 M NaCl at room temperature. At the indicated times, the slide was removed from the buffer, and GFP fluorescence remaining on the slide was visualized with an image analyzer (Typhoon; Amersham Biosciences).

2.7 Binding of bacteria to a silicon wafer through Si-tagged CWB

To test whether bacteria (*Bacillus subtilis*) bind to silicon wafer, *B. subtilis* was incubated for 12 h on a 2 x YT medium and collected by centrifugation. The pellet was washed by 1 ml of 20 mM Tris-HCl [pH 9] and suspended in the same buffer. The purified Si-tagged CWB was

adjusted to 0.2 mg/ml with the buffer and its 1 μl was plotted onto a silicon wafer. After incubation for 1 min at room temperature, the wafer was washed with 3 ml of the buffer. The bacterial suspension was plotted over the same region on the silicon wafer. After washing the bacterial suspension with the buffer, the wafer was dried and observed under scanning microscopy (JSM-5610LV).

3 RESULT AND DISCUSSION

3.1 Silica-binding protein in *Escherichia coli*

Cleared supernatant from *E. coli* intracellular lysate was mixed with silicon particles and incubated with rotary mixing for 30 min at 4°C. The silicon particles were precipitated by centrifugation and washed three times with a buffer containing 0.5% Tween 20 and 1 M NaCl. Proteins retained on the silicon particles were analyzed by SDS-PAGE (Fig. 2). Ribosomal protein L2 appeared to bind strongly to the silicon particle. This protein also bound to silica particles (data not shown). Because the silicon surface is oxidized to silica under the assay conditions, we concluded that these proteins bind to silica.

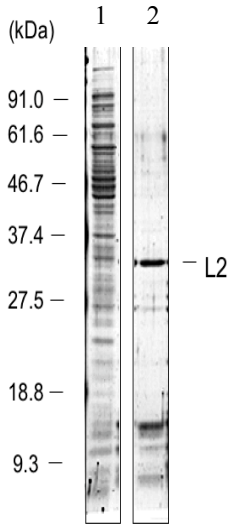

Figure 2: Silica-binding proteins in *E. coli*. Lane 1, cleared supernatant prepared from cell lysates. Lane 2, proteins that bound to silicon particles in the presence of 0.5% Tween and 1 M NaCl.

3.2 Characterization of a L2-Green Fluorescent Protein (GFP) fusion protein

Because GFP does not bind to silica particles in the presence of 0.5% Tween 20 and 0.5 M NaCl (data not shown), we generated a fusion of *E. coli* L2 and GFP (L2-GFP) to perform kinetic analyses of the interaction between L2 and a silica surface. We found that L2-GFP bound to the silica particles at pH values above 7.0. The dissociation constants (K_d's) of L2-GFP to the silica particle at pH 7.5, 8.0, and 9.0 were 0.7, 0.55, and 0.46 nM, respectively (Fig. 3). The maximum amounts of L2-GFP bound to the silica particle at pH 7.5, 8.0, and 9.0 were 25, 32, and 31 μg L2-GFP protein/mg silica particle, respectively (Fig.3).

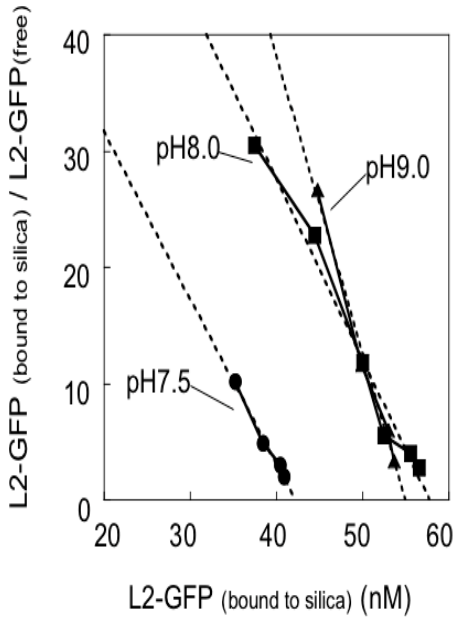

Figure 3: Scatchard analysis of L2-GFP binding to silica particles. The binding assay was performed as described in Fig. 2 at pH 7.5, 8.0, and 9.0.

Addition of a poly-Arg tag has been used for direct adsorption of protein on glass slides or silica particles without loss of enzymatic activity [8]. Poly-Arg tagged GFP (R9-GFP) bound to silicon particles with K_d's at pH 7.5, 8.0, and 9.0 of 123, 18, and 25 nM, respectively (data not shown). The maximum amounts of R9-GFP bound to silica particles at pH 7.5, 8.0, and 9.0 were 5.9, 14, and 16 μg GFP protein/mg silica particles, respectively. These results indicated that L2-GFP binds to the silica particles 30- to 200-fold more strongly than R9-GFP. Furthermore, we found that L2-GFP but not GFP or R9-GFP is retained on the slide glass after washing with a buffer containing 1 M NaCl for 24 h (Fig. 4).

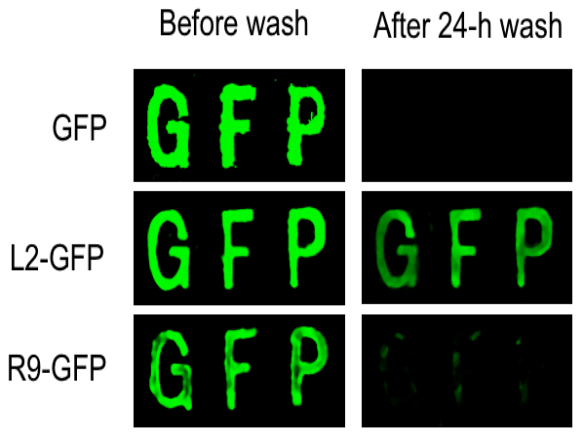

Figure 4: Immobilization of GFP, L2-GFP, and R9-GFP on a slide glass. Protein solution (GFP, L2-GFP, or R9-GFP) at a concentration of 10 nM was used to draw the letters "GFP" on a glass slide. The slide was dipped in a solution of 25 mM Tris-HCl (pH 8.0), 0.5% Tween 20, and 1 M NaCl solution and shaken at room temperature. After 24 h, the slide was removed from the buffer, and GFP fluorescence remaining on the slide glass was visualized with an image analyzer.

3.3 Bacteria bound to a silicon wafer through Si-tagged CWB

B. subtilis suspension was applied to a silicon wafer, on which the purified Si-tagged CWB was adsorbed. After washing the bacterial suspension with the buffer, the wafer was dried and observed under scanning microscopy (Fig. 5). *B. subtilis* was observed on the silicon wafer only where Si-tagged CWB was adsorbed. This result indicated that bacteria bound to a silicon wafer through the Si-tagged CWB.

As a brief summary, here the Si-tag can be applied to immobilization of functional proteins on a silicon wafer. We are now going to apply this technique to development of silicon-based biosensors.

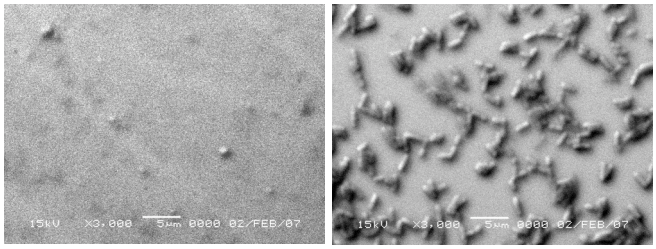

Figure 5: Binding of *B. subtilis* on a silicon wafer through Si-tagged CWB. *B. subtilis* was applied to a wafer (left) and washed. On the right wafer, Si-tagged CWB was applied in advance and then *B. subtilis* was applied to a wafer. *B. subtilis* was observed under scanning microscopy.

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