Design of Functional Protein Materials Self-Assembled on Solid-Phase Surface

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Abstract

For the construction of intelligent materials using protein molecules, it is essential to assemble protein molecules stably on a solid-phase surface in a highly oriented manner. We have designed novel proteins consist of hydrophobic peptide sequence as a self-assembled structural unit and molecular recognition peptide as a functional unit. These designed proteins were expressed in *E. coli* and purified with affinity chromatography. The resulting proteins retained their functions on a solid-phase surface and they were applied to biosensing system and cellular engineering.

Introduction

In order to construct intelligent materials consisting of functional proteins, the development of techniques for immobilizing proteins with retaining their functions even on a solid surface is important. However, when proteins are immobilized on solid surfaces, their activities are usually less than those of soluble proteins. So far, several methods have been developed to immobilize proteins. Protein molecules may be stably immobilized by covalent bonding to the solid surface. However, it often results in a loss of their activities since this method is generally utilized randomly existing reactive groups such as amino-, carboxy-, and thiol-groups in proteins. Self-assembling technique, which is based on the formation of stable gold-sulfide (Au-S) bonds and van der Waals interactions between the side chains of the molecules, has been utilized for the controlled molecular assembling on a solid surface of sulfer-bearing organic structure (1, 2). This technique is also applied to immobilize protein molecules on a solid surface (3). We introduced cystein residue at the terminus of proteins and the

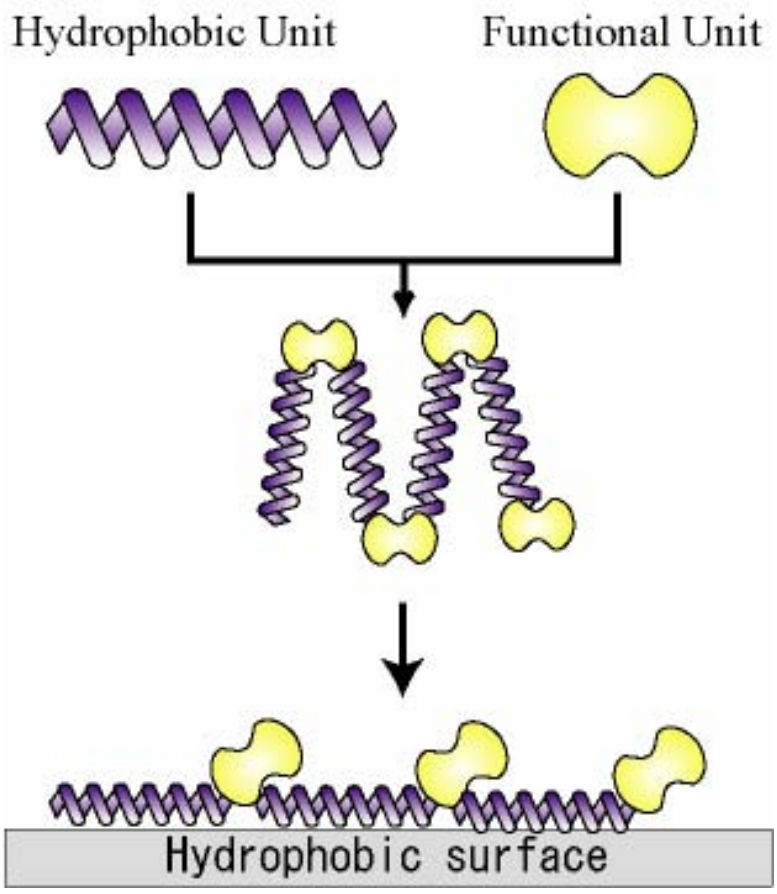

Fig.1 Design concept of functional protein assembled on solid-phase surface

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resulting recombinant proteins could be assembled on gold surfaces (4, 5). However, this method is limited to assemble proteins on gold surfaces. The easiest way for immobilization of proteins is simple adsorption, however, it is difficult to control the orientation of protein on a solid-surface.

To overcome these disadvantages, we have proposed a novel technique for assembling functional proteins. It is based on a simple hydrophobic interaction between protein and solid-surface. In this method, a target protein is fused with a hydrophobic peptide unit, which has an ability to assemble on a hydrophobic solid-surface (Fig. 1). In this condition, a relatively hydrophilic target protein part in the fusion protein should be oriented to the opposite side of solid-surface. Therefore, it is expected to realize a simple and efficient method for assembling functional proteins.

A common hydrophobic unit was utilized for the construction of these proteins. We have paid attention to the motif in the elastin-based polyhexapeptide repeat (Ala-Pro-Gly-Val-Gly-Val) as the hydrophobic unit. Synthetic polypeptides based on the repeating sequences of elastin have been studied extensively by Urry *et al* (6, 7). They have suggested that the polyhexapeptide [(APGVGV)n] consisting of hydrophobic amino acids forms a rigid β-spiral structure. This peptide unit is expected to provide the protein with hydrophobicity strong enough to be assembled on a hydrophobic solid surface. Another noteworthy characteristic of the elastin peptides is coacervation (8). The peptides form clear solutions at room temperature or below, but the solution becomes turbid on heating. In this reversible process, called coacervation, a hexapeptide can retain its β-spiral structure. We expected the functional small peptide incorporated into the hexapeptide-based protein retained its function even after heating, because the conformation of the functional peptide was protected by the thermostable rigid structure.

As functional units, we have selected some kinds of molecular recognition peptides. One is an antibody-binding peptide, B-domain of staphyrococcal protein A (SpA) (9). The structural gene for 2-repeat of the B-domain (B2) was fused to the gene for 12-repeat of the APGVGV sequence in frame. The 2-repeat of the B-domain has been confirmed to exhibit enough binding affinity to stably immobilize the Fc region of IgG. Immobilization of antibody molecules on a solid surface is a widely used technique in various fields such as immunoassays, immunosensors, and affinity chromatography. In these systems, it is essential to assemble antibody molecules stably on a solid surface in a highly oriented manner with retaining their antigen-binding affinity. A hydrophobic unit is expected to assemble on a hydrophobic surface preferentially hence a relatively hydrophilic molecular recognition unit should retain its function. As the result, one can immobilize antibody molecules efficiently on the surface. The other model for a functional peptide unit is a cell-adhesive peptide unit. The peptide sequence of only three amino acids, Arg-Gly-Asp (RGD), shows the minimal cell-recognition function through integrin on the cell surface (10). This cell-adhesive unit should be immobilized on a cell-culture plate through the strong hydrophobic interaction between a hydrophobic peptide unit and hydrophobic plate surface. It is also expected to have a thermostable structure, and it will be a useful biomaterial for cellular engineering and tissue engineering.

Materials and Methods

Materials

E. coli JM109 (Takara Shuzo, Shiga, Japan) was used for propagation and construction of plasmids, and *E. coli* DE3 (Novagen, Madison, WI) was used for production of proteins. Mouse fibroblast 3T3-L1 was obtained from Riken Cell Bank (Tsukuba, Japan). Synthesized DNA fragments were purchased from Gliner Japan (Tokyo, Japan). Restriction enzymes and ligase were purchased from Toyobo (Shiga, Japan) and Takara Shuzo. Plasmids, pGEX-3X and pET32c were obtained from Amersham Phrmacia (Uppsala, Sweden) and Novagen, respectively. All other chemicals were of analytical grade.

Plasmid Construction

pGEX-E12: A synthetic DNA fragment encoding APGVGV sequence was inserted into pGEX-3X under the structural gene of glutathione S-transferase (GST) in frame at *Bam*HI-*Eco*RI for the construction of pGEX-E. The inserting fragment included non-palindromic *Ban*I sits at both terminals of the repeating sequence for tandem insertion. pGEX-E and the synthetic DNA fragment were digested with *Ban*I and ligated. Twelve fragments of the gene encoding APGVGV sequence were connected in head-to-tail ligation and oriented in transcription direction of the vector. The resulting plasmid was designated pGEX-E12.

pET-EB4: The plasmid pKK-B2 was constructed by inserting a 2-repeat of the gene encoding the B-domain of protein A. This inserted fragment (B2) was amplified with polymerase chain reaction (PCR) by using the reverse primer containing *Bam*HI and *Eco*RI sites.

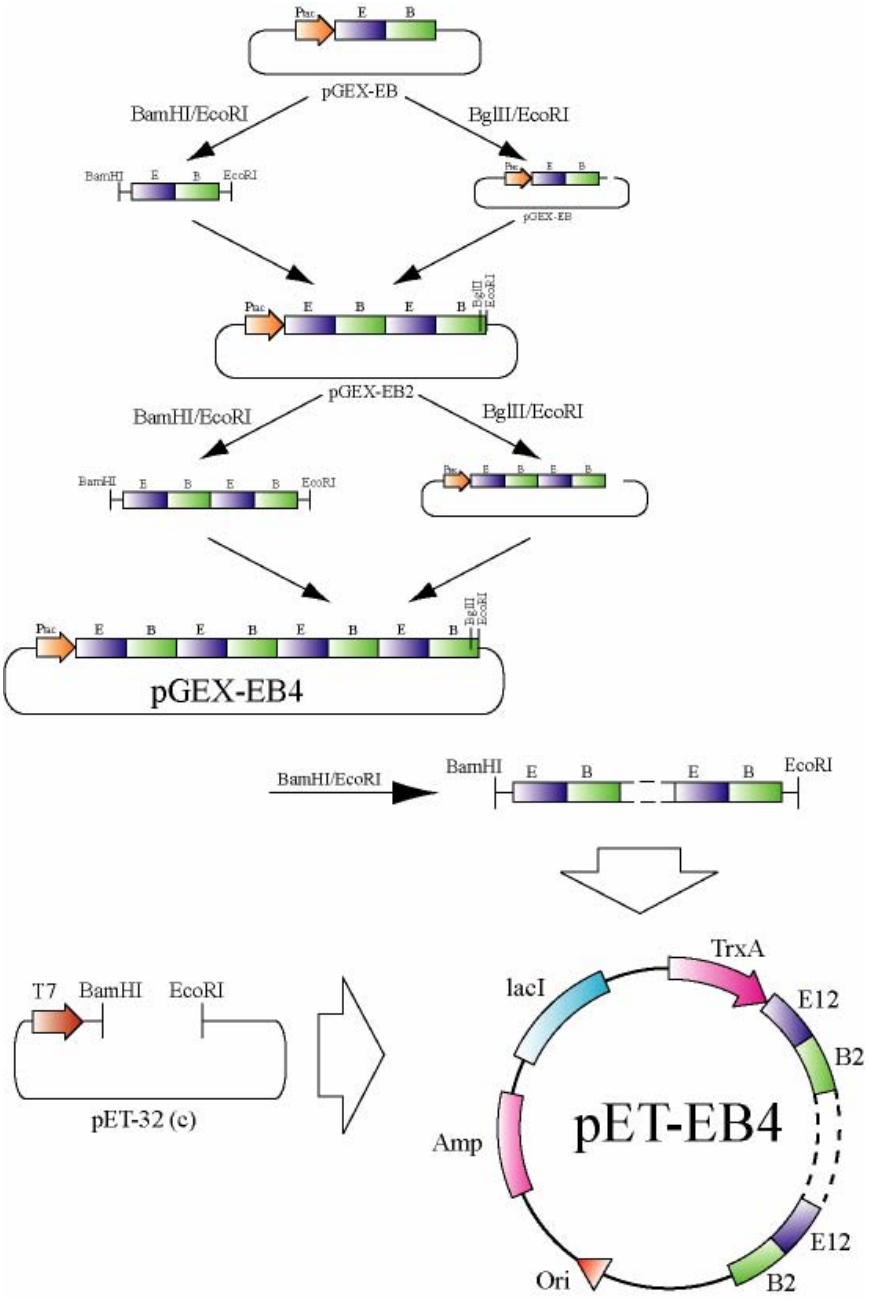

Fig.2 Construction of plasmid pET-EB4

This amplified fragment was digested with *Bam*HI and *Eco*RI, and inserted into the *Bgl*II-*Eco*RI site of pGEX-E12. The resulting plasmid was designated pGEX-EB. A *Bam*HI-*Eco*RI fragment of the pGEX-EB was inserted at *Bgl*II-*Eco*RI site of the same plasmid, and pGEX-EB2 was constructed. The same procedure was repeated for pGEX-EB2, and pGEX-EB4 encoding a 4-repeat of $(APGVGV)_{12}$-$(B\text{-domain})_2$ sequence was constructed. Finally, the fragment of *Bam*HI-*Eco*RI from pGEX-EB4 was inserted into the pET-32c at the same site as a fusion with the gene of thioredoxin in frame. The resulting plasmid was designated pET-EB4. The construction of pET-EB4 is schematically shown in Fig.2.

pET-ER4: The plasmid pGEX-E12 was digested with *Bgl*II and *Eco*RI and another DNA fragment encoding RGD sequence was inserted in frame (pGEX-ER). A *Bam*HI-*Eco*RI fragment from the plasmid was inserted at *Bgl*II-*Eco*RI sites of the same plasmid, and pGEX-ER2 was constructed. This procedure was repeated again for pGEX-ER2, and pGEX-ER4 encoding 4 repeats of $(APGVGV)_{12}$-RGD sequence was constructed. Finally, a fragment of *Bam*HI-*Eco*RI from pGEX-ER4 was inserted into the pET-32c (Novagen, Inc., WI, USA) at the same sites as a fusion with the gene of thioredoxin in frame. The resulting plasmid was designated pET-ER4.

Expression and Purification of Proteins

E.coli BL21(DE3) cells transformed with pET-EB4 or pET-ER4 were grown in LB medium supplemented with 50 μg/ml ampicillin to OD_{660} = 0.5 at 37 . After induction with isoprpylthio-β-D-galactoside (IPTG), cells were cultured for another 4 h at 30 , then were washed and resuspended in a binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl; pH 7.9), and disrupted by sonication. After centrifugation, the supernatant was applied to a Ni^{2+}-charged-column to purify the fusion protein with thioredoxin by using histidine-tag at the N-terminus. The protein was eluted with a buffer containing 100 mM imidazole. The purified fusion protein was digested with thrombin for 8 h at room temperature and removed thioredoxin moiety by applying Ni^{2+}-charged-column again. The solution containing desired protein was dialyzed against phosphate-buffered saline (PBS). The purified sample was analyzed by SDS-PAGE and Western blotting. Purified proteins from cells transformed with pET-EB4 and pET-ER4 were designated EB4 and ER4, respectively.

AFM Imaging of EB4

Purified protein samples were dissolved in PBS. Twenty microliters of the solution was incubated on a freshly cleaved highly oriented polygraphite (HOPG) or mica for 1 ht at 37 , which was washed with 1 mL of distilled water and dried shortly in a stream of compressed and filtered air. All samples were further dried in 2 L glass desicator in the presence of P_2O_5 for 10 min.

The samples were imaged with an AFM of NanoScopeIII multimode system in the tapping mode in air. The tip of the cantilevers is modulated sinusoidally to touch briefly the surface of the sample. Standard silicon cantilever of 125 μm in length was used. Cantilever oscillation frequency was turned to the resonance frequency of the cantilever (280-350 kHz). The 512 x 512 pixel images were captured with a scan size between 1.0 and 2.0 μm at a scan rate of 1-2 scan lines/s.

Cell Adhesion Assay for ER4

Mouse fibroblasts 3T3-L1 cells were used for the experiments of cell adhesion assays. Cell adhesion assays on ER4 or fibronectin were performed by a modification of the method of Yamada and Kennedy (11). ER4 and fibronectin were diluted into sterilized water to 500 nM and 100 nM, respectively, then 500 μl aliquots were added to a 24-well tissue culture plate (Sumitomo Bakelite Co., Tokyo, Japan). After incubation at room temperature for 12 h, all remaining protein adsorption sites were saturated with 0.1% BSA for 2 h. Cultured cells were washed with PBS, detached with 0.25% trypsin, 0.02% EDTA, then washed and resuspended to approximately 1.0 x 10^6 cells /ml in DMEM. One milliliter of cell suspensions were added to wells coated with the adhesive proteins coated wells. After incubation for 90 min at 37 , attached cells were fixed with 3.8% formaldehyde in PBS. Attached cells in four randomly chosen fields (1.02 mm^2) of a well were counted using a microscope and those of three wells were averaged.

Results and Discussion

Expression of EB4

Designed antibody-binding protein, EB4 (Fig. 3) was produced in *E.coli*. by using pET-system as the fusion with thioredoxin. Through the use of this system, EB4 protein was produced in large amounts in the soluble fraction. SDS-PAGE analysis of these purified samples revealed the single band in the predicted molecular mass, 84 kDa (data not shown). Western blotting analysis revealed the same band as SDS-PAGE analysis, which indicated the protein possessed the antibody-binding ability.

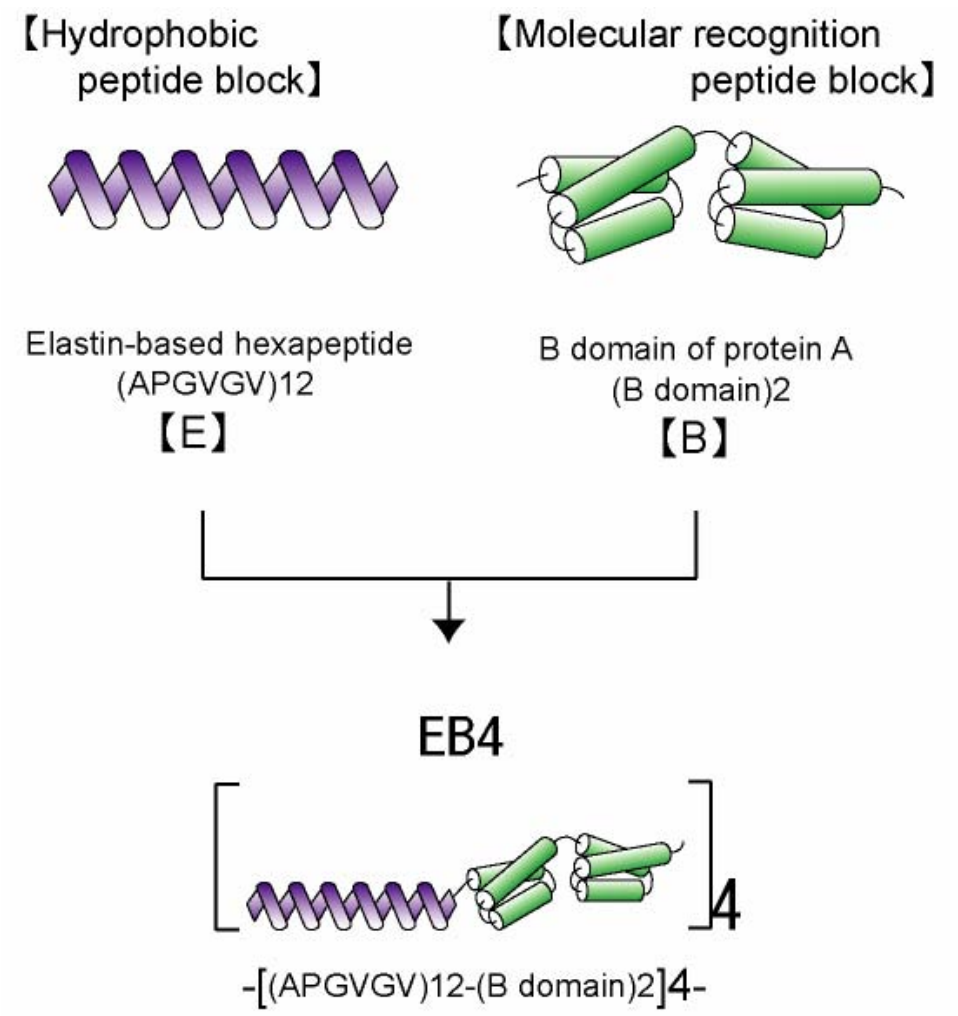

Fig.3 Design of EB4

AFM Imaging of EB4 on HOPG or Mica Surface

Simple adsorption of protein molecules on solid surface is mostly dependent on the hydrophobic interaction between

protein and solid surface. EB4 is expected to assemble efficiently on the hydrophobic solid surface by hydrophobic interaction. To investigate the assembling efficiency of EB4 onto hydrophobic surfaces, the protein was assembled on HOPG and mica plates as the hydrophobic and hydrophilic surfaces, respectively, and they were observed by AFM.

On the HOPG surface (Fig. 4), EB4 was observed at very low concentration (0.1 nM). Assembling amounts of protein on the surface increased with an increase of concentrations. Over 100 nM of concentration, almost all the surface area was covered with EB4. Furthermore, the structure of assembled EB4 showed the plane surface. They seemed to be assembled with intermolecular hydrophobic interactions. In contrast, the protein was scarcely assembled on the mica surface even at high concentrations (100 nM) of proteins (Fig. 5). At 1000 nM of concentration, they were observed as cohered structure. The hydrophobic peptide blocks of EB4 may be repelled with the hydrophilic mica surface, and they should be formed a cohered structure by inter-molecular interaction.

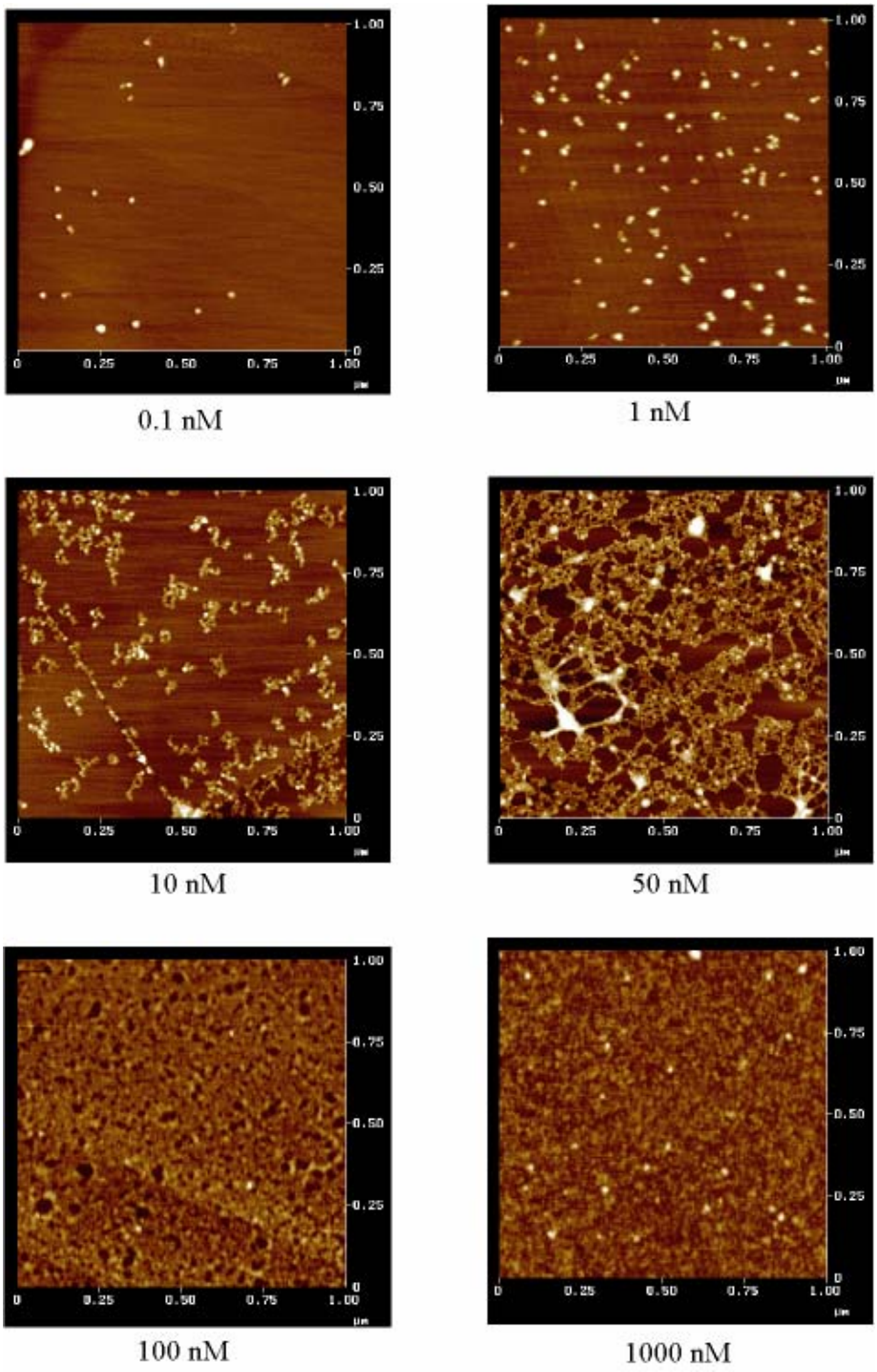

Fig.4 AFM image of EB4 assembled on HOPG surface

Antibody Binding Ability of EB4 Assembled on Solid Phase Surface

Firstly, the antibody-binding ability of EB4 in liquid phase was investigated by comparing with SpA. The various concentrations of EB4 or SpA solution including SpA-POD were reacted to rabbit IgG-immobilized microplate. The amount of reacted SpA-POD to immobilized IgG was estimated by peroxidase activity. As the

result, EB4 gave an excellent antibody binding ability, which was about 10 times higher than that of SpA.

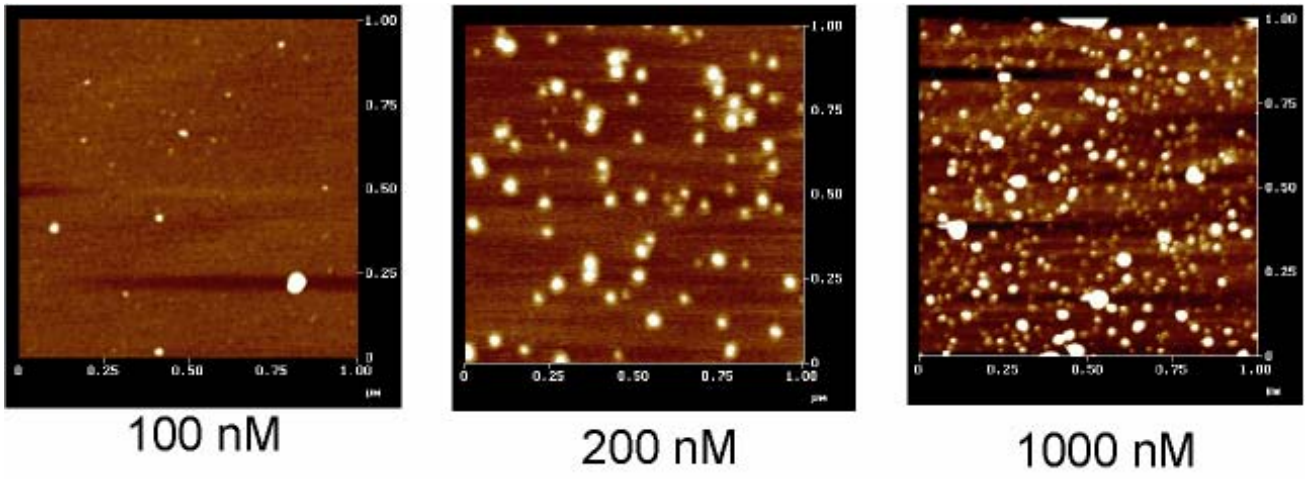

Fig.5 AFM Image of EB4 assembled on mica

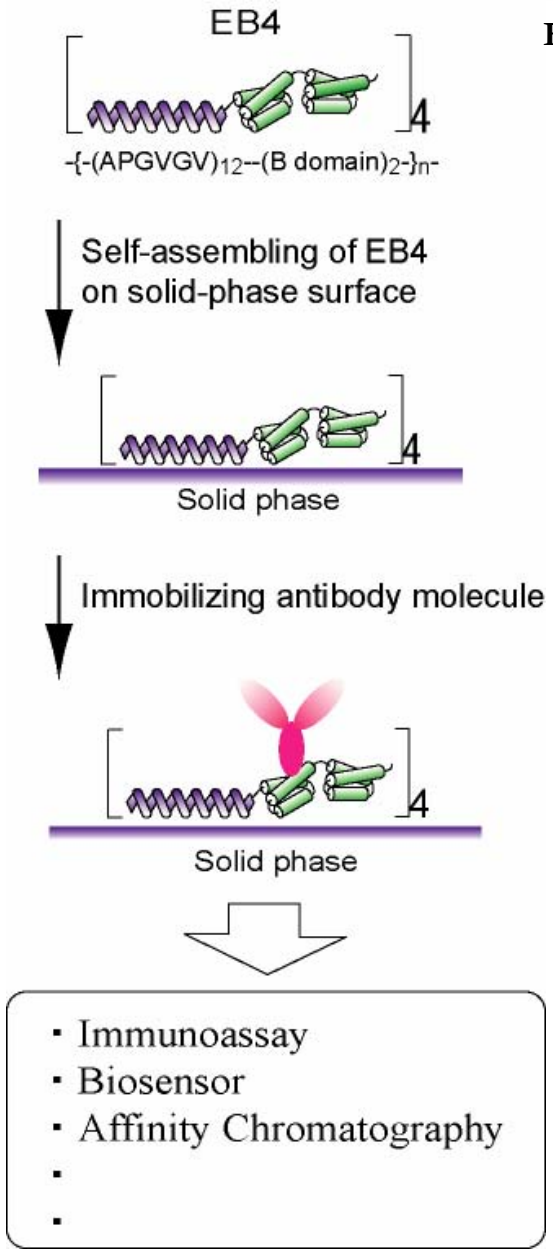

Fig.6 Assembled EB4 for imobilization of antibody

Next, the antibody-binding ability of EB4 on a solid phase was investigated by enzyme immunoassay. At first, EB4 was coated on the microplate surface in various concentrations. A constant amount of rabbit anti-peroxidase antibody was reacted to each protein-coated microplate and the peroxidase was added. Amount of immobilized peroxidase was estimated by peroxidase activity, which reflected the amount of assembled EB4 retained antibody binding ability on the microplate surface. As the result, it was clarified that EB4 had the sufficient antibody-binding ability even on a solid surface.

Finally, the antibody assembling ability of the EB4-coated plate was investigated by comparing with that of the noncoated plate. In general immunoassay methods, antibody was assembled on a microplate surface by simple adsorption. Therefore, the antibody-assembling ability of the EB4-coated plates was investigated by comparing with that of the noncoated plate. Rabbit anti-peroxidase antibody was reacted to EB4-coated and noncoated microplate in various concentrations, then was estimated by peroxidase activity, which reflected the amount of immobilized antibody molecule on the microplate. As the result, antibody could be assembled on microplate surface by physical adsorption. The antibody assembling ability on EB4-coated microplate was more efficiently by than noncoated one. In the model case of immunoassay, we tried the sandwich ELISA for targeting mouse IgM

on EB4-coated or noncoated microplate. The required amount of primary antibody for assembling on microplate surface is reduced with EB4-coating. These results suggest that EB4 is a good material for assembling antibody with high orientation on a hydrophobic matrix (Fig.6).

Expression and Purification of ER4

The design concept of ER4 is shown in Fig. 7. As the ER4 has the extreme hydrophobic structure, thioredoxin was chosen as the fusion partner for the expression in *E. coli*. Because LaVallie *et al*. (12) reported that the linkage to thioredoxin dramatically increased the solubility of heterologous proteins synthesized in the *E. coli* cytoplasm, and that thioredoxin fusion proteins usually accumulated to high levels. Furthermore, pET/DE3 system was used for protein production. Since the host *E. coli* cells strain DE3 have a gene for T7 RNA polymerase under the control of tac promoter in their chromosomal DNA, production of T7 RNA polymerase is induced with IPTG. As the result, large amounts of expression of the gene under T7 promoter can be expected by adding IPTG. Through the use of this system, the fusion protein between ER4 and thioredoxin was produced in larger amounts in soluble fraction than insoluble fraction. The protein could be purified in one step with Ni^{2+}-column from the soluble fraction. ER4 of approximately 33 kDa could be observed by SDS-PAGE after cleaving with thrombin and removing thioredoxin. Approximately 4 mg of purified ER4 was obtained from 1 l culture.

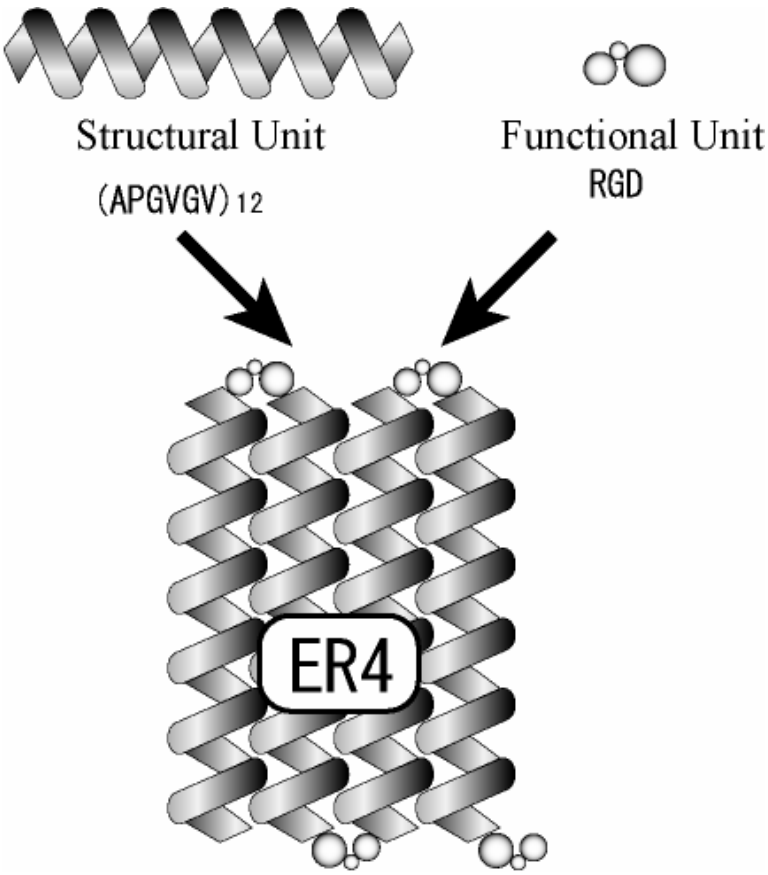

Fig.7 Design concept of ER4

Cell Adhesion Activity of ER4

The 3T3-L1 cells were seeded on an ER-4-coated cell culture plate. Number of cells were counted in each plate, and shown in Fig. 8. As the same as fibronectin-coated plate, cells were attached well and spread on the ER4-coated plate, while the cell attachment was scarcely observed on a non-coating plate. ER4 possesses quite high cell adhesion activity, which is nearly 80% of fibronectin activity.

To confirm the cell attachment on ER4-coated plate was attributed to the RGD sequence in ER4, 100 mg ml^{-1} of soluble RGD peptide was added in cell suspension before seeding the cells. As shown in Fig. 8, the cell adhesion on the ER4-coated plate was completely inhibited by the presence of soluble RGD peptide (GRGDSP) in

the cell suspension. Contrary to this, when RGE peptide (GRGESP) was added, no inhibition effect was observed. The substitution of glutamic acid (E) for aspartic acid (D) has been demonstrated to abolish the adhesive-promoting activity of the peptide (13). Furthermore, E13, which is 13-repeating unit of APGVGV without RGD sequence, -coated plate had no cell adhesion activity. These results indicate that cells attached to the ER4-coated plate by interaction between cell receptor and RGD sequences in ER4.

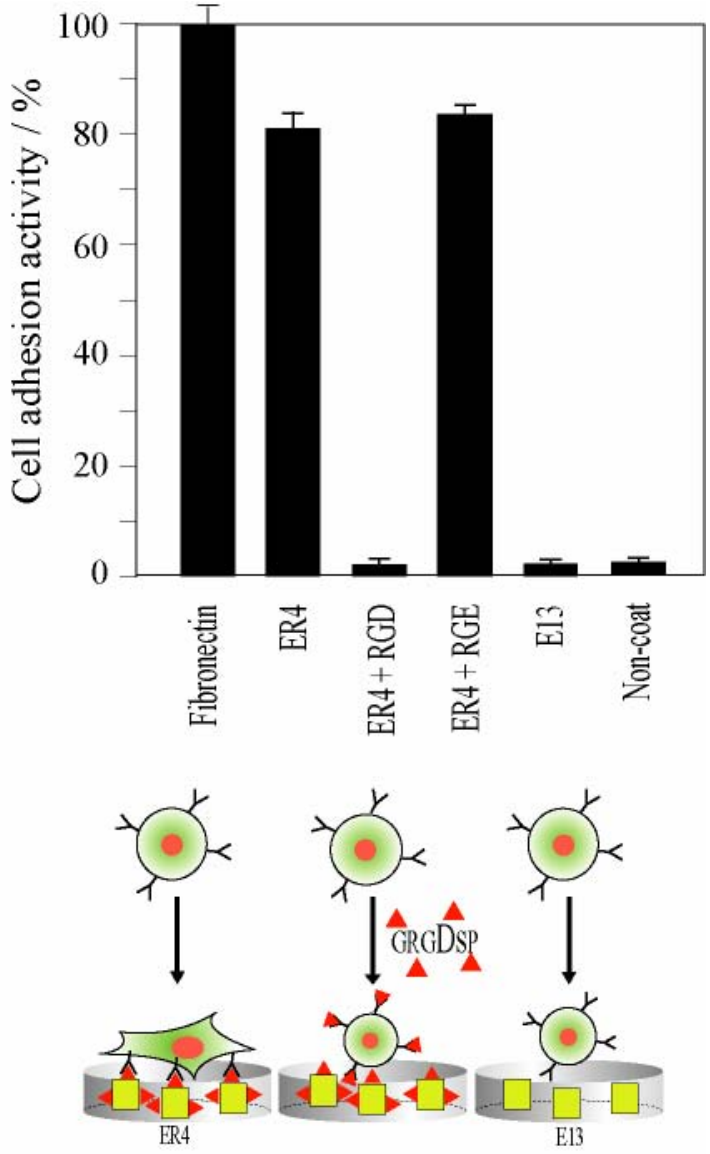

Fig.8 Cell adhesin activity of proteins

Thermal Stability of ER4

As also shown in Fig. 8, ER4 retained approximately 80% of cell adhesion activity of fibronectin. To investigate the thermostability, the solution of fibronectin and ER4 were heated at 120 for 20 min in an autoclave, then they were coated on the plastic surfaces. The cell adhesion activities were estimated by counting the number of cells attached on each plate. The cell adhesion activity of each autoclaved protein was compared with that of respective native protein. In the case of autoclaved fibronectin, cell adhesion activity decreased to approximately 50% of that of native fibronectin. The RGD sequence in fibronectin has been predicted to assume β-turn conformation (10). Based on NMR study, Main *et al.* (14) have reported RGD sequence in fibronectin is solvent exposed and lies on a conformationally mobile loop between two β-strands. Therefore this decrease of cell adhesive activity may be caused by denaturation of higher-ordered structure of fibronectin, and RGD sequence on the surface of fibronectin should be buried in the inside of the structure. Contrary to this, over 90% of cell adhesion activity remained in the autoclaved ER4. This result suggests that higher-ordered structure of the protein was protected by rigid β-spiral structure of polyhexapeptide even after autoclaving. The hydrophilic RGD sequences in ER4 should be exposed on the surface of the substrate while ER4 adsorbed on the substrate by hydrophobic interaction with APGVGV sequences. The cell adhesion activity of autoclaved ER4 was approximately 20% higher than that of fibronectin.

Conclusion

In the present study, a novel principle has been proposed to design easily assembled functional proteins on solid-surface. A hydrophobic peptide sequence as a self-assembled structural unit and molecular recognition peptide as a functional unit were fused by genetic engineering. Based on this concept, some kinds of proteins were constructed. EB4 is an antibody-binding protein with hydrophobic unit. Antibody molecules could be assembled with high orientation on hydrophobic solid surface. EB4 will find a wide application in immunoassays, immunosensors, and affinity chromatography. ER4 is a thermostable cell adhesion protein. This protein is useful as an artificial extra cellular matrix for cellular engineering and tissue engineering.

References

(1) Whitesides, G. M., Laibinis P. E. (1990) *Langmuir* **6**: 87-96.

(2) Whitesides, G. M., Mathias, J. P., Seto C. T. (1991) *Science* **254:** 1312-1319.

(3) Sasaki, Y. C., Yasuda, K., Suzuki, Y., Ishibashi, T., Satoh, I., Fujiki, Y., Ishiwata S. (1997) *Biophysical J.* **72**: 1842-1848.

(4) Kanno, S., Yanagida, Y., Haruyama, T., Kobatake, E., Aizawa, M. (2000) *J. Biotechnol.* **77**: 207-214.

(5) Kobatake, E., Suzuki, S., Yanagida, Y., Haruyama, T., Aizawa, M. (1999) *J. Intell. Mater. Sys. Struc.* **10**: 446-450.

(6) Urry, D.W., Cunningham, W.D., Ohnishi, T. (1974) *Biochemistry* **13**: 609-616.

(7) Urry, D.W., Ohnishi, T., Song, M.M., Mitchell, L.W. (1975) *Int. J. Pept. Protein Res.* **7**: 367-378.

(8) Urry, D.W. (1978) *Perspective Biol. Med.* **21**: 265-295.

(9) Uhlen, M., Guss, B., Nilsson, B., Gatenbeck, S., Philipson, L., Lindberg, M. (1984) *J. Biol.Chem.* **259**(3): 1695-1702.

(10) Pierschbacher, M. D., Ruoslati, E. (1984) *Nature* **309**: 30.

(11) Yamada, K. M., Kennedy, D. W. (1984) *J. Cell. Biol.* **99**: 29.

(12) La Vallie, E. R., DiBlasio, E. A., Kovacic, S., Grant, K. L., Schendel, P. F., MaCoy, J. M. (1992) *Bio/Technology* **11**: 187.

(13) Gray, W. R., Sandberg, L. B., Foster J. A. (1973) *Nature* **13**: 609.

(14) Main, A. L., Harvey, T. S., Baron, M., Boyd, J., Campbell, I. D. (1992) *Cell* **71**: 671.