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Abstract

A groundbreaking method for ordered molecular layer preparation on a solid surface employing the drop-stamp method has been developed by us taking advantage of the characteristics of the HFB molecule as a self-organizer/adsorption carrier. It is a smart method which can be used to prepare a self-organized protein layer on a solid surface without unspecific adsorption or defects. In our previous report, we clarified the self-organizing nature of HFB-tagged protein molecules on a surface of a solution droplet. In this report, a protein layer was prepared on a HOPG surface by using the drop-stamp method with a maltose binding protein (MBP)-tagged HFBII molecule. The structure of the stamped protein layer was investigated using Frequency Modulation Atomic Force Microscopy (FM-AFM) in a liquid condition. The FM-AFM images show that the drop-stamp

method can prepare an ordered protein layer on a solid surface smartly. The drop-stamp method using a HFB carrier is a practical method which can be used to prepare an ordered protein layer on a solid substrate surface without unspecific adsorption defects.

Keywords:

Self-organized protein layer, molecular interface, Hydrophobin, Drop-stamp

Introduction

Protein molecules, such as enzymes, have prominent functionalities, *i.e.* catalytic activity and molecular recognitions. By taking advantage of functional proteins, researchers have used proteins in the development of many different types of molecular device, such as biosensors[1, 2], protein-chips[3] bio (enzyme) fuel cells[4, 5]. However, there are difficulties in efficiently creating such molecular devices. In most cases, functional protein molecules are immobilized on the surface of a solid substrate (ex. electrodes, semi-conductors, carbon, glass etc) and they provide the substrate's functions. However, such protein molecules originally functioned in a homogeneous condition in solution. In the case of molecular devices, immobilized molecules have to provide functions in heterogeneous form and have to control a physical structure of molecular layer on solid surface, e.g. molecular density and molecular orientation, in order to provide both good stability and good function. Importance of optimized structure in molecular layer on solid has been noticed by

some careful researches. In case of immobilized enzyme activity, the apparent catalytic reaction ratio was drastically changed by a changing of immobilized molecular orientations on solid. Actually, immobilized enzyme can provide higher apparent catalytic activity in case of optimized molecular orientation on solid surface[6]. Affinity reaction of antibody is also very much depending on immobilized molecular orientations[7]. Cultured cell adhesion on solid material is also can be controlled with the structure of molecular layer on a solid surface. Cell adherent can be modulated using thermally responsive polymer-grafted layer on a solid substrate[8]. In that case, cell adherent/non-adherent is modulated through a changing by temperature. As described about some example, accurate molecular layer fabrication on a solid is an indispensible way to design functionalities of biomaterials. In the designing of interfacial function, it is important that the protein-solid interface (molecular-solid interface) is an ordered molecular layer on a solid surface in order to achieve an efficient molecular solid interface[9]. However, conventional methods to immobilize molecules on solid are unable to control this aspect of the molecular layer; control is clearly required in the fabrication of an ordered molecular layer on a solid surface. A controllable methodology for molecular layer preparation is strongly demanded by those in the fields of molecular interfaces and other protein molecular applications.

The authors previously proposed that the fabrication of a protein molecular layer could be achieved by using the “drop-stamp method”, which is based on the properties of the unique protein molecule hydrophobin[10-14]. Hydrophobin (HFB) is originally from the cell wall of fungi and is a small

amphiphilic protein molecule. HFB can form a very stable self-organized mono-layer at the air/liquid interface. Our research has found that the self-organized HFB monolayer at the air/liquid interface of a solution droplet can be transferred onto a solid surface through a stamping of the droplet onto the solid[15]. If the HFB is tagged with functional proteins through a genetic engineering technique, the fusion protein can form a stable self-organized monolayer at the air/liquid interface, in a similar way to that of the HFB molecule alone, as reported in our previous study[16]. This finding indicates that the tagged HFB can act as a molecular carrier to form a self-organized monolayer at the air/liquid interface. In our previous report, a fusion protein molecule of MBP and HFBII was genetically engineered. The HFBII-MBP fusion protein molecules could form a self-organized monolayer at the air/liquid interface, in a similar way to that of HFBII alone.

In this study, we investigate a stamped self-organized MBP-HFBII fusion protein layer on a HOPG surface in a solution using custom-built Frequency Modulation Atomic Force Microscope (FM-AFM) [17, 18]. The FM-AFM was developed to image soft surfaces in a liquid condition with a high degree of sensitivity. Nano-structures of a drop-stamped self-organized molecular layer with MBP-HFBII fusion protein and HFBII were investigated on a solid substrate surface. It will give a valuable information when the drop-stamp method is employed to prepare an biomolecular devise that needs an accurate ordered protein layer on a solid substrate.

Materials and Methods

Expression and Purification of HFBII and MBP-HFBII

HFBII was expressed in *Escherichia coli* BL21 (DE3), as previously described[16]. HFBII was purified from a soluble extract to obtain a high-purity level by a two-step process using Ni-NTA chromatography (HisTrap HP column, GE Healthcare) and reversed phase chromatography (Resource RPC Column, GE Healthcare). Maltose-binding protein (MBP) and HFBII fusion protein were designed and expressed in *E. coli* TB1, as previously described[16]. High-purity MBP-HFBII was purified from the *E.coli* cell extract by two-step chromatography: MBPTrap HP column (GE Healthcare) and HisTrap HP column (GE Healthcare). Both purification samples were stored at -20 ^{o}C after desalting by Slide-A-Lyzer (Pierce) and were lyophilized. The molecular weight and purity was determined, as previously described.

Analysis of the molecular properties at the air/liquid interface

The π-A isotherms were measured by using a KSV minitrough LB system (KSV Instruments) with 1 mM acetate buffer, pH 5.0, and 20 ^{o}C for the subphase. HFBII and MBP-HFBII fusion proteins were solved with 1 mM acetate-HCl buffer (pH=5.0). The protein solutions were spread on the subphase. After stabilization of the surface pressure, the protein molecules were compressed at a rate of 10 mm/min.

Fabrication of molecular layer on a solid surface by drop-stamp process

HFBII and MBP-HFBII fusion proteins were solved with 1 mM acetate-HCl buffer (pH=5.0). A 10

μ L droplet containing HFBII and/or MBP-HFBII fusion protein was positioned onto a polystyrene substrate. The droplet was incubated for 90 min in a humid condition to form a self-organized protein layer. Then, the self-organized protein layer was placed into contact with High Orientated Pyrolytic Graphite (HOPG: NT-MDT) in order to transfer it onto the solid substrate. The drop-stamped HOPG substrate was washed by acetate buffer immediately.

AFM imaging of the stamped protein layer

A custom-built liquid-environment FM-AFM with an ultra-low noise cantilever deflection detection system [17, 18] was used for the imaging of the stamped protein layers. A commercially available AFM controller (Asylum Research, Santa Barbara, CA, USA) was used to operate the FM-AFM. Silicon cantilevers (PPP-NCH: Nanosensors) with a spring constant in the range of 20-40 N/m were used in the experiments. FM-AFM imaging was performed in an acetate buffer solution at room temperature. All data were obtained through repetition experiments in each mixture ratio of MBP-HFBII and HFBII. Similar result of molecular layer structure could be observed in the repetition experiments.

Results and Discussion

HFBII is a unique amphiphilic protein molecule. It is a small protein molecule but has a planar hydrophobic domain in about 4 nm^2 of its surface[19]. The HFBII molecule also has self-organizing functionality at the air/liquid interface. HFBII molecules automatically self-organize into a

mono-layer membrane at the air/liquid interface. By taking advantage of the unique properties of HFBII, we employed HFBII as a molecular carrier to form an ordered molecular layer on a solid surface. Figure 1 shows the molecular design of HFBII and the genetically engineered HFBII-MBP fusion molecule. The size dimensions of HFBII and MBP are about 2.5 nm and 6 nm, respectively, as shown in Figure 1. The HFBII molecule was tagged to an MBP molecule through a 10-amino-acid linker (sequence; NNNNNNNNNN). The linker length is approx. 3nm. Therefore, it would appear that the MBP molecule exhibits fluctuation on HFBII molecular membrane. The fabrication of intermixed system is a tactic technique to perform controlled density of MBP-HFBII fused protein on a solid surface. In the system, density of tagged MBP on self-organized HFB layer can be changed through just change the mixed ratio of MBP-HFBII fused protein and HFBII protein. The π-A isotherms were analyzed to investigate the organized formation of the protein membrane at the air/liquid interface in various mixture ratio of MBP-HFBII protein and HFBII protein (Figure 2). As shown in the result, the surface pressure sharply increased in the case of HFBII molecules at the pure water surface. The high ratio of surface pressure increment suggested that HFBII self-organized on the solution surface smoothly and tightly. The π-A indicated that the occupation area of HFBII was 4 nm^2 . On the other hand, surface pressure increased gradually in the case of HFBII-MBP fusion molecules. The fusion molecules may have a larger occupation area of 30 nm^2, which was calculated from the result of the π-A isotherm. This suggested that self-organization of the HFBII-MBP molecule was probably inhibited through steric hindrances caused by the tagged

MBP (MW=55700) molecule because it is much bigger than the fused HFB molecule (MW=9300). Such molecular steric hindrance obstructs formation of a self-organized membrane on a solution surface. In order to avoid such obstructions, we developed a groundbreaking tactic. It is a very efficient tactic that employs HFBII molecules in the HFBII-MBP fusion molecule at the air/water interface of the solution droplet when the self-organized membrane is prepared[16]. HFBII plays a role in reducing the obstructions caused by the tagged bulky MBP molecule. In this study, in order to prepare an ordered molecular layer of HFBII-MBP fusion molecules on a HOPG surface with control of its density, HFBII molecules were intermixed with HFBII-MBP fusion molecules at a molar ratio of 4:1, 9:1 and 15:1. In the case of 4:1, 9:1 and 15:1 mixing ratios, the π-A isotherm shows that the slope of increment of surface pressure is sharper than that of the only-MBP fusion protein. This result suggests that these membranes of self-organized proteins consisted of mixed proteins (HFBII and HFBII-MBP) in the ratio of 4:1, 9:1 and 15:1, respectively. The data from our previous report showed, that the mixed HFBII and HFBII-MBP system forms a more stable self-organized membrane at the air/liquid interface, especially in the case of mixing ratios at 4:1 and 9:1. This suggests that the HFBII molecule mitigated steric hindrance between the tagged MBP molecules and acts as an alleviator in the cases where there is an appropriate mixing ratio. The membrane of the self-organized protein molecules is formed on the surface of a droplet with a HFBII protein, and it can be transferred to a solid surface by just stamping it onto the substrate[16]. As we described in our previous report, we were able to modulate the number of MBP molecules in

a HFBII self-organized membrane at air/liquid surface by changing the mixing ratio of HFBII and HFBII-MBP. This finding indicated that the number of MBP molecules on a stamped layer can be controlled both easily and accurately. A self-organized membrane with HFBII and HFBII-MBP fusion molecules (in a mixing ratio of 4:1, 9:1 and 15:1 respectively) were stamped on a HOPG substrate and their structures were investigated using FM-AFM in a liquid condition. The self-organized membrane of HFBII molecules stamped on the HOPG was observed to be uniform (Figure 3a). This result corresponded with the data that showed that HFBII was adsorptive when immobilized as a mono-layer in a stable condition[20]. In the intermixed system, bright points increased on the AFM images when the ratio of MBP-HFBII/HFBII was increased (Figure 3b-d; height of the bright spot was 3-6 nm. Diameter was 5 nm). These results indicated that the MBP molecule was tagged onto the HFBII molecules which formed a self-organized molecular membrane. Basically, the density of the MBP molecule can be controlled by changing the mixing ratio between HFBII and MBP-HFBII. The structure of the stamped protein layer of HFBII on a HOPG substrate is an important factor in the scaffold being able to fabricate a functional protein layer on it successfully. In order to demonstrate the scaffold structure, the stamped HFBII self-organized layer was investigated by FM-AFM in solution. The results showed that the drop-stamped HFBII layer maintained a self-organized ordered structure similar to that at the air/liquid interface (Figure 4a). The HFBII molecules were 2-5 nm in size. This result indicated that the self-organized layer of HFBII was not a crystallized structure, but that these molecules on a

HOPG surface were packed at certain intervals of operating molecular interaction. This image is similar to that in the previous report by another group that was observed on dry surface (not stamped on a solid substrate) [19]. In Figure 4b, the arrows show that the molecular layer is consisted of a structure of 2 nm molecules. The molecular size dimension corresponded to that of the HFBII molecule. There is a black part can be observed in the image. We are considering that the black point is a partly-penetrated hole based on z axis profile. As the current procedure of the drop-stamp method has been done manually, stamped solution droplet may stir on the HOPG when the solution droplet was stamped. The mechanical distortion causes some defects. However, most part of the stamped HFBII and HFBII fusion molecules formed ordered structure as seen in other view of AFM images. The structure of the MBP molecule on the HOPG substrate is an important factor to take into account when fabricating a functional protein layer on a solid surface. Our AFM investigations found that, the stamped self-organized fusion protein layer was well ordered due to the use of our mixed drop-stamp technique (HFBII/MBP-HFBII fusion=9:1). The stamped fusion protein layer was analyzed by FM-AFM in solution. White bright points were suspended (Figure 5a). These islands of white bright points are consistent with particles of about 5 nm (Figure 5b). The observed size dimension clearly corresponded to that of the MBP molecule (Figure 5c). However, in some parts of the HOPG, there are conditions where the MBP is directly adsorptive on a substrate directly and onto HFBII molecular layer (Figure 5d). Most of the MBP molecule in the 9:1 mixing ratio was measured as having a height between 3 and 6 nm. These results suggest that most of the

MBP molecules exhibit fluctuation caused by the 10-amino-acid linker connecting HFBII onto the HOPG substrate.

Conclusion

The MBP molecule tagged HFBII is adsorptive when it is stamped on a solid surface. The molecules are successfully self-organized as a monolayer membrane on a solution droplet surface. MBP was tagged to the self-organized HFBII layer on a solid. In the case, MBP and HFBII were linked through 10 amino acid peptide (sequence; NNNNNNNNNN) that provided hint of flexible structure. Then the MBP may fluctuate in solution. It is a very applicable process which can facilitate the design of efficient, functional molecular interfaces for molecular devices.

Acknowledgement

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Figure captions

Figure 1: Predicted schematic illustration of MBP-HFBII fusion molecule [PDB ID 2B97 and 1N3X]

Figure 2: Surface pressure-area (π-A) isotherms of HFBII (●), MBP-HFBII fusion protein (♦), HFBII:MBP-HFBII=4:1 (▼), HFBII:MBP-HFBII=9:1 (■) and HFBII:MBP-HFBII=15:1 (▲)

Figure 3: FM-AFM images of HFBII and HFBII intermixed with MBP-HFBII on HOPG in acetate buffer solution (400×400 nm^2). Height range of all images is standardized for analysis of difference from solid substrate (black to white: 10 nm). (a) HFBII, A=0.136 nm, Δf=+20 Hz, scan speed: 300nm/s. (b) HFBII:MBP-HFBII=15:1, A=0.136 nm, Δf=+79 Hz, scan speed: 976 nm/s. (c) HFBII:MBP-HFBII=9:1, A=0.136 nm, Δf=+68 Hz, scan speed: 1μm/s. (d) HFBII:MBP-HFBII=4:1, A=0.23 nm, Δf=-70 Hz, scan speed: 975 nm/s.

Figure 4: FM-AFM images of HFBII on HOPG in acetate buffer solution (a) 100×100 nm^2, A=0.271 nm, Δf=+67 Hz, scan speed: 500 nm/s, height range (black to white): 2.5 nm. (b) 30×30 nm^2, A=0.271 nm, Δf=+76 Hz, scan speed: 293 nm/s, height range (black to white): 1.2 nm.

Figure 5: FM-AFM images of HFBII intermixed with MBP-HFBII (9:1) on HOPG in acetate buffer solution. (a) 250×250 nm^2, A=0.136 nm, Δf=+66 Hz, scan speed: 1.25 μm/s, height range (black to white): 6 nm. (b) 100×100 nm^2, A=0.136 nm, Δf=+65 Hz, scan speed: 500 nm/s, height range (black to white): 6 nm. (c) Profile measured along the lines A-B in (a). (d) Schematic illustration

of molecular interface on HOPG substrate.

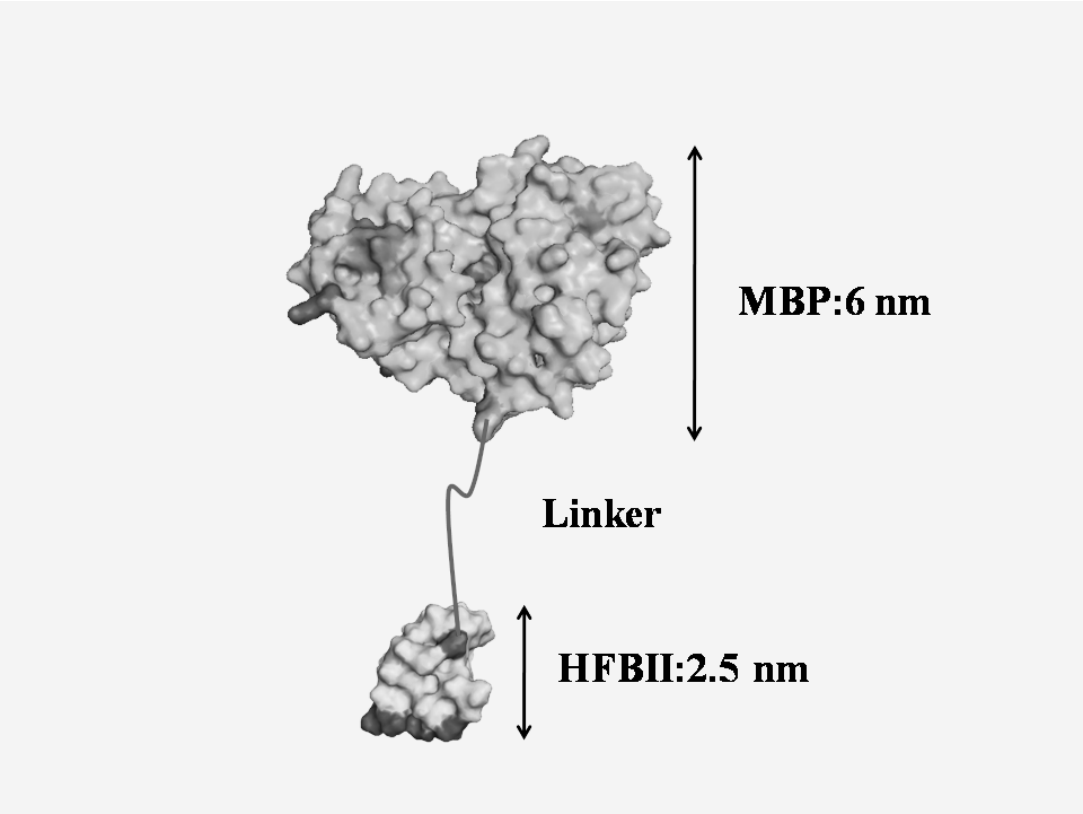

Figure 1

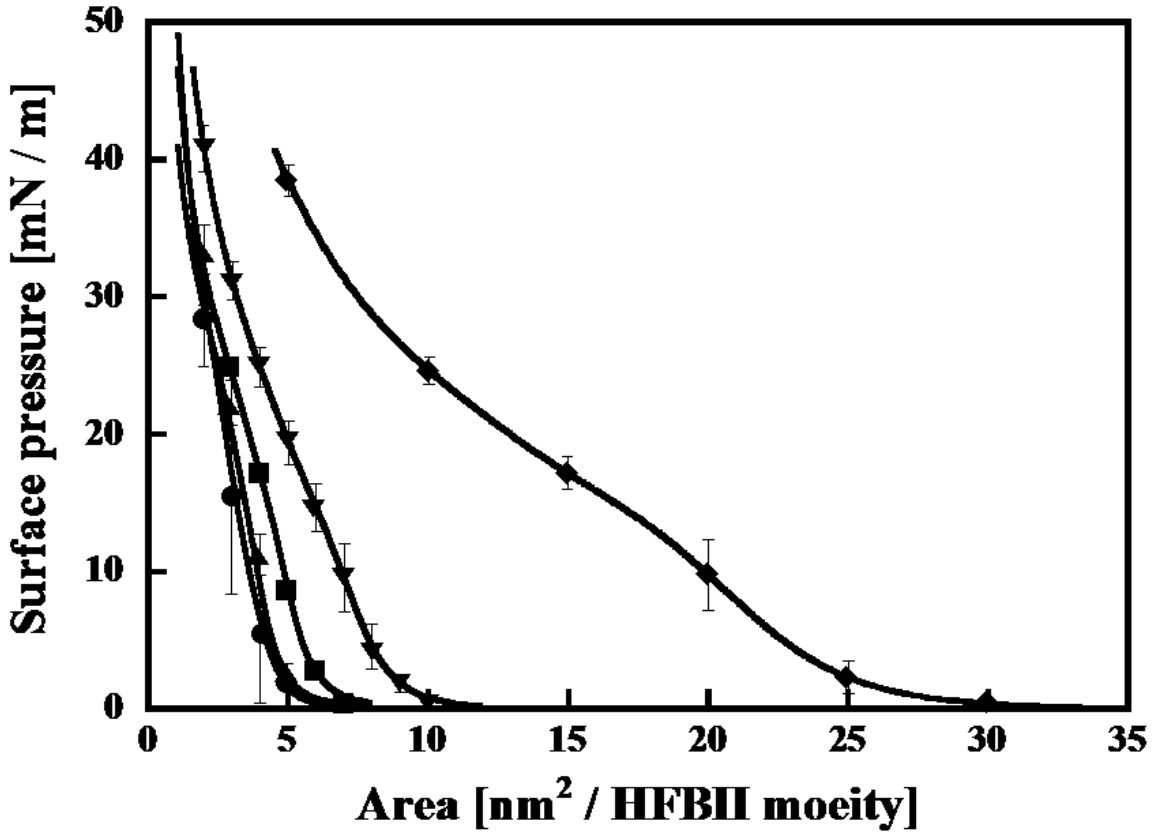
Surface pressure [mN / m]
50
40
30
20
10
0
0
5
10
15
20
25
30
35
Area [nm^2 / HFBII moeity]

Figure 2

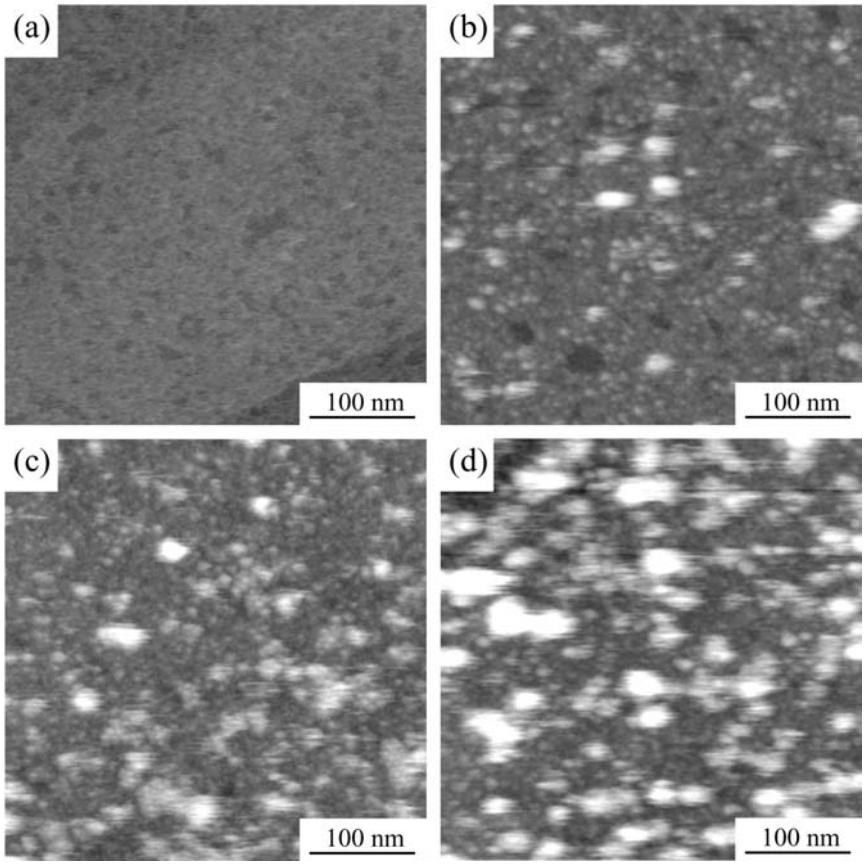

Figure 3

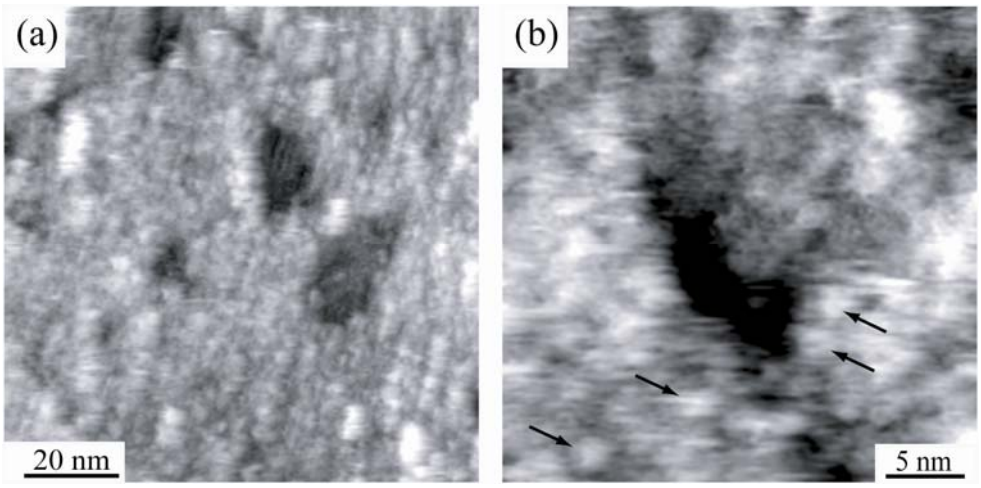

Figure 4

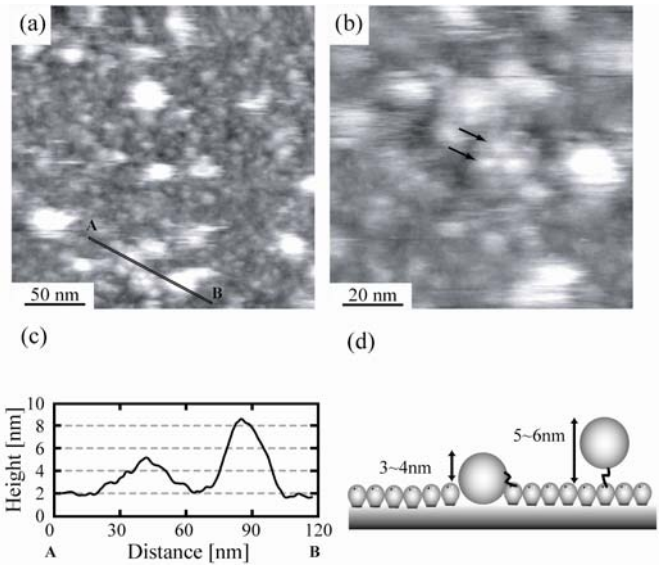
(a)
A
B
50 nm
(b)
20 nm
(c)
Height [nm]
10
8
6
4
2
0
0
30
60
90
120
A
Distance [nm]
B
(d)
5~6nm
3~4nm

Figure 5