

RESEARCH ARTICLE

Protein nanoarray on ProlinkerTM surface constructed by atomic force microscopy dip-pen nanolithography for analysis of protein interaction

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Protein nanoarrays are addressable ensembles of nano-scale protein domain on solid surfaces. This method can serve as a useful platform for ultraminiaturized bioanalysis. In this study, we investigated single molecular nanopatterning and molecular interaction of proteins that were immobilized on ProlinkerTM surface of gold-coated silicon wafer by using dip-pen nanolithography (DPN) method. Contact force and humidity were optimized at 0.01 nN and 80%, respectively. The domain features of protein nanoarrays were developed at the contact time of 5 s. The optimized conditions for the nanoarray process were applied to create protein nanoarray using integrin $\alpha_v\beta_3$ and angiogenin. Constructed protein nanoarrays using integrin $\alpha_v\beta_3$ have single molecular monolayer with regular domain shape (height 15 ± 5 nm). The changed height value due to the single molecular interaction between integrin $\alpha_v\beta_3$ and vitronectin was approximately 30 ± 5 nm on ProlinkerTM surface as measured with atomic force microscopy tip. Taken together, these results suggest that protein nanoarray on ProlinkerTM surface fabricated by well-controlled DPN process can be used to analyze single molecular interaction of protein.

Received: May 31, 2005
Revised: October 25, 2005
Accepted: November 7, 2005

Keywords:

Atomic force microscopy / Dip-pen nanolithography / ProlinkerTM / Protein nanoarray / Single molecular interaction of protein

1 Introduction

The solid-phase microarrays have emerged as the method for high throughput and high-parallel hybridization testing for DNA and RNA samples. The microarray methods have

proven useful as high-throughput (HT) analytical tools in proteomics and genomics as well as future HT screening of new drugs [1–6]. For example, DNA microarrays can be used to probe gene expression and in panel assays for research and clinical-based diagnostics [7–13]. In addition, protein microarrays are very useful for screening of drug candidates and studying of protein expression profiling. Proteins are not only the final product of the genome but also the functional unit of diverse biological processes. Therefore, proteins are considered as important content material for microarray chip. For the fabrication of protein microarrays, molecular linker system that holds protein molecule tightly is essential. ProlinkerTM was developed as a novel molecular linker for

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Abbreviations: AFM, atomic force microscopy; DPN, Dip-Pen nanolithography; MUDA, mercaptoundecanoic acid

stable immobilization of proteins such as antibodies and integrin receptors on solid substrate. ProteoChip base plate, a new protein microarray chip, was coated with Prolinker™ to make use of wide-spread biological fields including molecular diagnostics, new drug screening, and functional proteomics [14, 15].

Assays on protein microarrays are commonly read using optical properties such as fluorescence, which requires a fluorescently labeled molecule to detect protein-protein interactions [16]. However, chemical attaching by labeling reagent on protein surface can make a deformational change of protein molecule and, unfortunately, structural change can affect the function of that protein directly. For this reason, many researchers focused on the label-free detection method and try to develop new detection method for protein-protein interaction. Recently, dip-pen nanolithography (DPN) method using atomic force microscope has been developed by Chad A. Mirkin group and applied to the fabrication of protein nanoarrays with antibody and enzyme. Protein nanoarray is generated by DPN in which a cantilever tip of atomic force microscopy (AFM) is used to deliver protein molecules to a surface via a solvent meniscus. For the evaluation of arrayed nanostructure and the detection of molecular interaction of proteins, the heights of protein nanoarrays are measured by AFM [17–20]. Nanoarray technology based on DPN has several advantages compared to microarrays. Using nanoarray technology, very small quantities of individual proteins can be used to study functional proteomics [21]. In addition, protein nanoarrays using AFM directly examine the molecular interactions of individual proteins and thus the requirement for ‘reporter’ elements is completely eliminated [20]. With Mirkin’s technology, the height measurement of protein double layer that was constructed by DPN was performed by AFM cantilever to deter-

mine individual molecular interaction of proteins. In this study, we investigated fabrication of protein nanostructures constructed on Prolinker™ surface and protein molecular interaction by using AFM.

2 Materials and methods

2.1 Chemicals and reagents

For the fabrication of protein nanoarray, angiogenin and integrin $\alpha_v\beta_3$ were purchased from Chemicon (CA, Temecula, USA) and used as source materials of protein nanoarray. Sample solutions of angiogenin and integrin were prepared and used at concentration of 500 $\mu\text{g}/\text{mL}$ in PBS buffer solution. For studying single molecular interaction on Prolinker™ surface, vitronectin was used as a target protein against integrin and BSA was used as a control. Protein solution contained 1 mM MgCl_2 and 0.1 mM CaCl_2 . Mercapto-undecanoic acid (MUDA), PBS solution (10X), and other reagents were obtained from Sigma Chemicals (St. Louis, MO). Milli-Q grade ($>18.2 \text{ m}\Omega/\text{cm}$) water was used for the preparation of the sample and buffer solutions. Prolinker™, which is one of calix[4]arene derivatives with crown-ether moiety, was purchased from Proteogen (Seoul, Korea) and used as a linker system for the immobilization of protein.

2.2 Construction of protein nanoarray

Figure 1 shows schematic representation of protein nanoarray using DPN. To increase the hydrophilicity of cantilever surface, gold-coated cantilever tips were immersed into 1 mM MUDA in ethanol for 30 min, rinsed with ethanol, and then dried under the N_2 gas stream at room tempera-

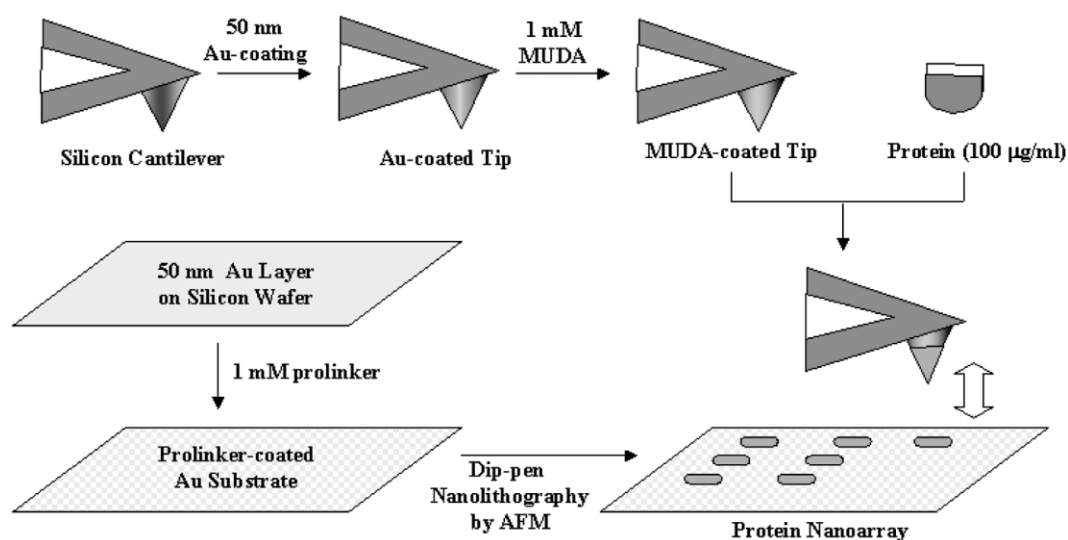


Figure 1. Schematic representation of protein nanoarray on Prolinker surface of gold-coated silicon wafer using AFM dip-pen nanolithography.

ture. The hydrophilic tips with the carboxylic acid-terminated self-assembled monolayers facilitate protein adsorption on the tip surface. Surface-modified tip was immersed into the solutions of capture proteins (angiogenin or integrin) for 1 h, and then used immediately for making protein nanopatterning. On the other hand, the 50-nm gold-coated silicon wafer was used as a substrate for protein nanopatterning. For the stable immobilization of protein on the silicon wafer, we formed a Prolinker™ layer on the surface of gold-coated silicon wafer as a linker system. The surface modification using Prolinker™ was performed through immersing the gold-coated substrate into the 1 mM Prolinker™ solution in chloroform for 1 h. After that, it was rinsed with acetone followed by methanol, and then dried under the N₂ gas stream at room temperature. To construct protein nanoarray, the AFM cantilever adsorbed with capture protein solution was spotted on the Prolinker™ surface of gold-coated wafer using AFM XE-100 (PSIA, Sungnam, Korea) with contact mode and the wafer was incubated for 3 h. After the incubation, capture protein nanoarray was incubated with interacting protein through immersing method. After 2-h incubation, the wafer was washed three times with distilled water and topography images and height profiles of protein nanoarrays were obtained using non-contact mode (NSC 14, PSIA) for protein-protein interactions.

2.3 Optimization of nanoarray fabrication

To optimize the patterning process of protein nanoarray, some essential factors such as contact force, humidity and contact time were controlled and estimated. Protein nanoarray patterning was performed with different contact forces (100, 10 and 0.01 nN) and different contact time (5, 7 and 9 s) between AFM cantilever tip adsorbed with protein solution and Prolinker™ surface of gold-coated silicon wafer with different humidity conditions (30 and 80%). Topography images and height profiles of protein nanoarray were obtained using AFM non-contact mode.

2.4 Formation of single molecular nano-dots of integrin receptor

After construction of integrin $\alpha_v\beta_3$ (500 $\mu\text{g/mL}$) nanoarray, it was immersed into PBS solution and shaking on the orbital shaker for 1 h. After rigorous rinsing process, image and line profile of the integrin nanoarrays were investigated by AFM tip in non-contact mode.

2.5 Height measurement of integrin nano-dots using AFM cantilever tip

Integrin $\alpha_v\beta_3$ nanoarrays (500 $\mu\text{g/mL}$) were constructed on the Prolinker™-coated gold surface by AFM DPN as described in Section 2.2 and blocked with 10% PEG for 1 h to avoid nonspecific binding of vitronectin. The integrin nanoarray was washed three times with distilled water and

then incubated with vitronectin (10 $\mu\text{g/mL}$), major extracellular matrix ligand of integrin $\alpha_v\beta_3$, for 1 h in incubation chamber with 80% humidity. After rinsing and drying, the height of the integrin nano-dots was measured by scanning of the protein surface using AFM cantilever tip in non-contact mode.

3 Results

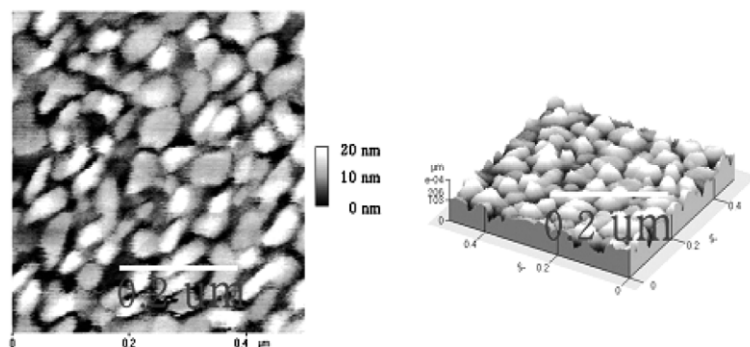
3.1 Surface modification by Prolinker™

Prolinker™ has crown-ether moiety that act as a host cavity for the amine group of protein surface. Our previous reports demonstrated that Prolinker™ held protein molecules tightly and its self-assembled monolayer is a very efficient molecular linker system for immobilization of protein onto substrate surface without any loss of activity [14, 15]. To construct protein array in nano-scale on solid substrate, we prepared Prolinker™ surface onto gold-coated silicon wafer and carried out DPN using AFM. The AFM images of gold-coated surface and Prolinker™-modified surface are shown in Fig. 2. Bare gold surface showed relatively large size domain (50–100 nm), whereas Prolinker™ surface (Fig. 2B) had a remarkably smooth and densely uniform surface characteristics compared with bare gold surface (Fig. 2A). These increases of smoothness and uniformity on Prolinker™ modified surface were caused by the formation of self-assembled monolayer of Prolinker™ molecules, calix[4]arene-based small organic molecule. These results suggest that the self-assembled Prolinker™ monolayer can provide favorable condition for promoting detection resolution of protein molecular interaction through reducing signal-to-noise ratio via height measurement of proteins using AFM tip.

3.2 The effect of contact force on protein nanopatterning

To examine the effect of contact force on the process of nanopatterning by DPN, we fabricated protein nanoarray using angiogenin. Angiogenin spots were arrayed onto the Prolinker™-coated surface with different contact forces (100, 10 and 0.01 nN). Topographic images and height profiles of angiogenin nanoarray that spotted in different contact forces are shown in Fig. 3. Under the condition of strong contact forces (100 and 10 nN), gold substrate coated with Prolinker™ layer was removed and only few angiogenin was remained at the edge of the surface. On the other hands, the other condition with weak contact force (0.01 nN) was evaluated as the best applicable method in the patterning process of protein nanoarray Fig. 3C. These data suggest that protein pattern was well regulated by contact force of protein-adsorbed AFM cantilever on Prolinker™ surface of gold substrate.

A



B

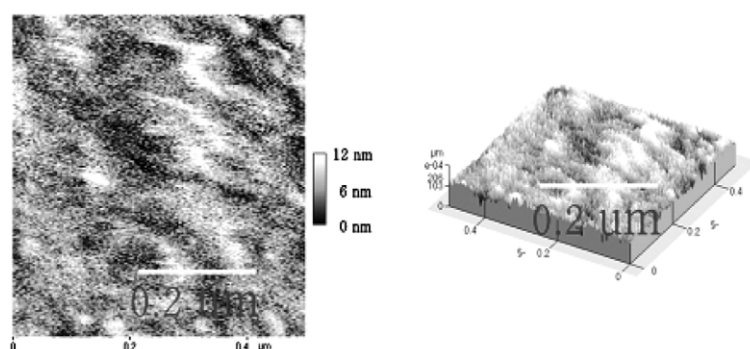


Figure 2. AFM topographic images of Prolinker™ surface on gold-coated silicon wafer. Topographic and 3-D images of gold surface on silicon wafer (A) and Prolinker™ surface onto gold-coated silicon wafer (B) were scanned by AFM cantilever tip in contact mode. A tip-substrate contact force of 0.3 nN was used to avoid damaging the surfaces. Spring content of silicon cantilever was ~40 N/m.

3.3 The effect of humidity on protein nanopatterning

In general, it is known that humidity is a critical factor in the nano-process of biomaterials such as protein and DNA. In particular, hydration is essential for maintaining the functional activity of protein [22]. Many steps of nano-scale processes including protein adsorption on the tip surface and protein transfer onto the substrate surface were significantly influenced by humidity condition. Therefore, humidity is important factor to control biomolecules delivering process in nano-scale using AFM cantilever. In this study, we were able to determine an optimal humidity condition for fabrication of protein nanoarray on Prolinker™ surface. Protein nanoarrays spotting with two different humidity conditions (30 and 80%) are compared in Fig. 4 and Table 1. With 30% humidity, arrayed spot features and spot heights were irregular (Fig. 4A). The spot irregularity under low humidity condition was demonstrated by showing dimensional data with SD in Table 1. In the condition of 30% humidity, the average and SD value of spot height are 44.3 and 33.0 nm, respectively (Table 1). These results indicate that protein transfer from tip to surface of substrate is uncertain and uncontrolled under the low humidity condition. On the other hand, in the condition of 80% humidity, arrayed spot features are relatively regular and the height values of protein spots are similar to each other. In the high humidity condi-

Table 1. Height and width value of protein nanoarrays arrayed with different humidity^{a)}

Humidity	Height (nm)		FWHM (nm)	
	AVG	SD	AVG	SD
30%	44.3	33.0	163	57.7
80%	80.2	18.6	150	25.4

a) FWHM: full width at half max; AVG: average.

tion, average and SD value of spot heights are 80.2 and 18.6 nm, respectively (Table 1). In particular, SD value of spot width in low humidity condition (30%) is remarkably higher than that of high humidity condition (80%). These findings strongly suggest that 80% humidity condition guarantee protein molecules to keep the hydration state during DPN process to make regular and active protein spots without dehydration of proteins.

3.4 The effect of contact time on protein nanopatterning

To assess the influence of contact time in the protein nanopatterning process, the protein nanoarrays were constructed on Prolinker™ surface with different contact time (5, 7 and

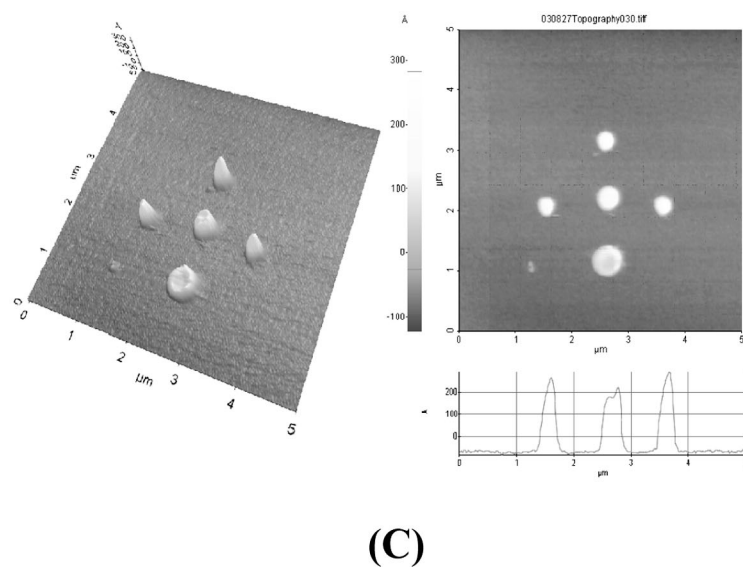
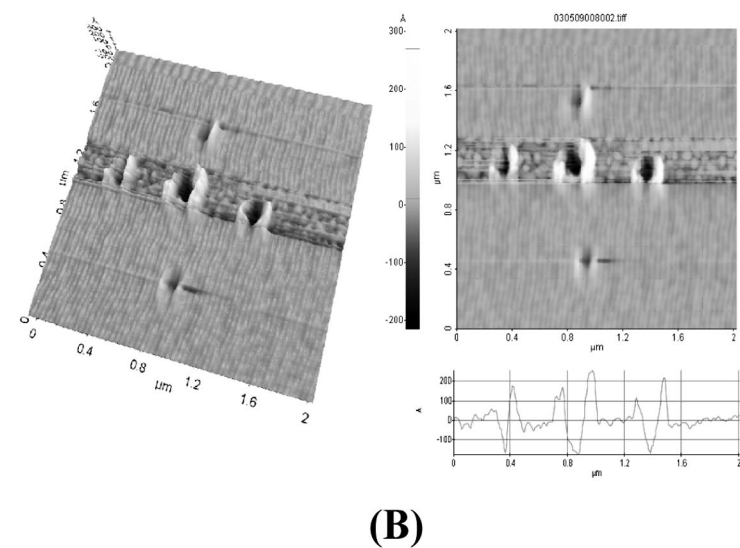
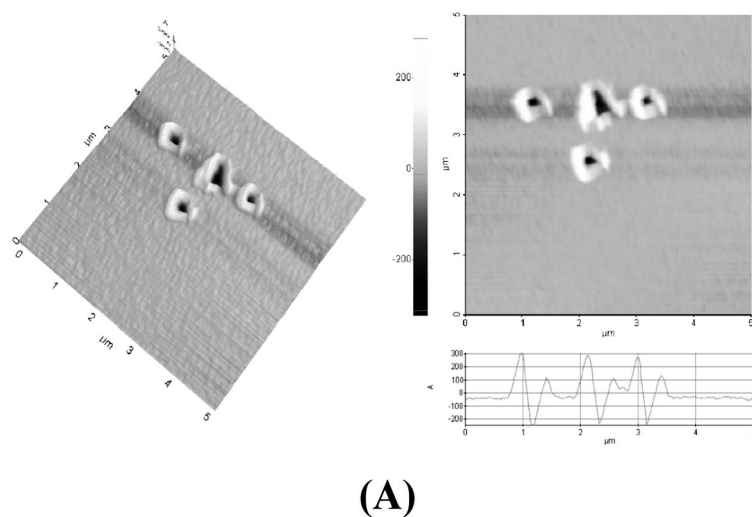


Figure 3. The effect of contact force on the construction of protein nanoarray. Angiogenin spots were arrayed onto a Prolinker™-coated gold wafer with different contact forces, *i.e.* 100 nN (A), 10 nN (B) and 0.01 nN (C), respectively. Topographic images and height profiles of arrayed angiogenin spots were obtained by AFM tip in non-contact mode. For the high-resolution of AFM image, scan rate was maintained at 0.5 Hz.

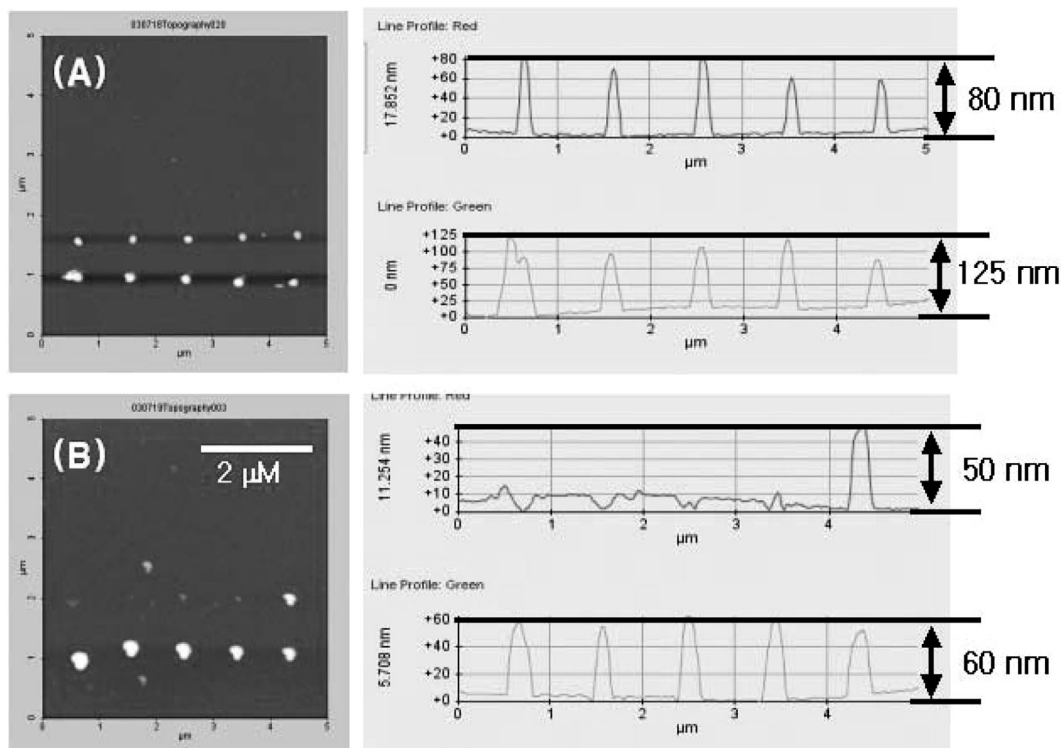


Figure 4. The effect of humidity on the construction of protein nanoarray. Angiogenin spots were arrayed onto a Prolinker™-coated gold wafer with 30% (A) and 80% (B) of humidity. Topographic images and height profiles of arrayed angiogenin spots were measured by AFM in non-contact mode. The image was taken at a scan rate of 0.5 Hz to obtain a high-resolution image.

9 s). The numerical value of protein nanoarray according to contact time is summarized in Table 2. As shown in Table 2 and Fig. 5, the diameter of protein nanoarray increased proportionally in contact time-dependent manner. These findings indicate that long contact time leads to large spot size, while the feature height decreased with the increase of contact time (Table 2). Therefore, it is essential for the optimization of protein nanopatterning process, to control the contact time of AFM tip on solid surface. On the other hands, it was previously reported that the rate of transport is highly dependent on protein composition in the nano-patterning process [22]. These results suggest that the structural characteristics of nanoarray are determined by diverse elements.

3.5 Analysis of integrin $\alpha_v\beta_3$ -vitronectin interaction on single molecular monolayer by AFM

For the fabrication of well-controlled protein nanoarray, we optimized several factors such as humidity, contact force and contact time. Angiogenin and integrin $\alpha_v\beta_3$ were used as model proteins for construction of protein nanoarray. The nanoarrays of two proteins were constructed using optimized

Table 2. Height and width value of protein nanoarrays arrayed with different contact time

Contact time (sec)	Height (nm)	Width at base (nm)	FWHM (nm)
5	54	403	140
7	36	460	226
9	20	685	480

condition. Optimal contact force (0.01 nN) and humidity (80%) were used for adsorption of proteins on Prolinker™ surface. Contact time was taken 5 s for each spot. To get the image of nanoarray, scanning of nano-patterned protein heights was taken at a scan rate of 0.5 Hz. Integrin nanoarray was constructed as a lattice structure with 12 spots (3×4) within $12 \mu\text{m}^2$ area. As shown in Fig. 6A, the width and height of integrin nanoarrays were 120 and 60 nm, respectively. The angiogenin nanoarrays including 12 protein spots (4×4) within $9 \mu\text{m}^2$ area are shown in Fig. 6B. The width and height value of angiogenin nanoarray were observed at the level of 60 and 120 nm, respectively. These results suggest that one nanospot may contain multiple layers of protein molecules on the surface.

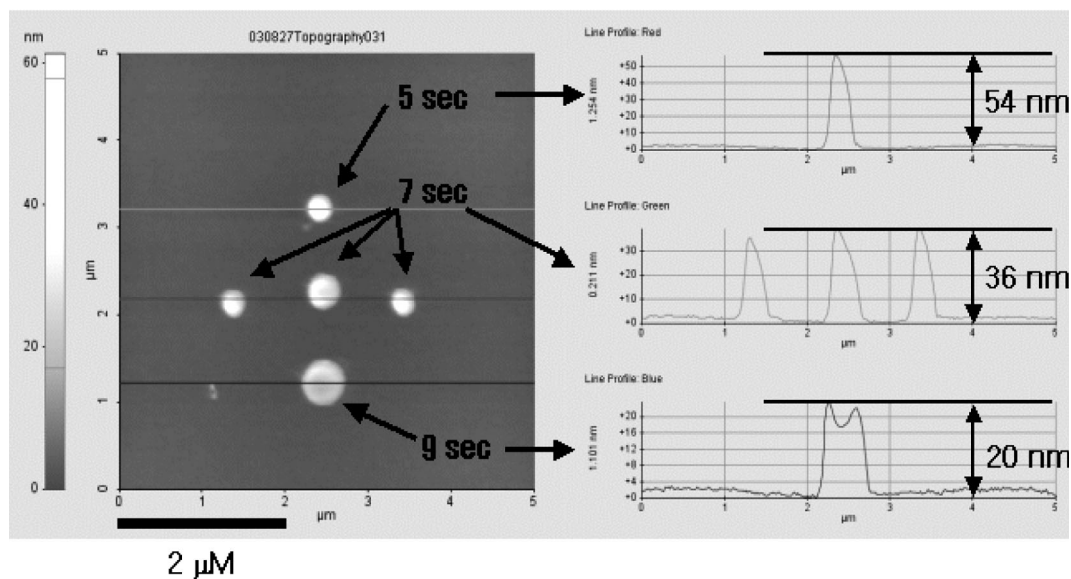


Figure 5. The effect of contact time on the construction of protein nanoarray. Milk bovine angiogenin were arrayed onto Prolinker™-coated gold wafer with different contact time (5, 7 and 9 s). Topographic images and height profiles of arrayed angiogenin spot were measured by AFM tip in non-contact mode. The image was taken at a scan rate of 0.5 Hz to obtain a high-resolution image.

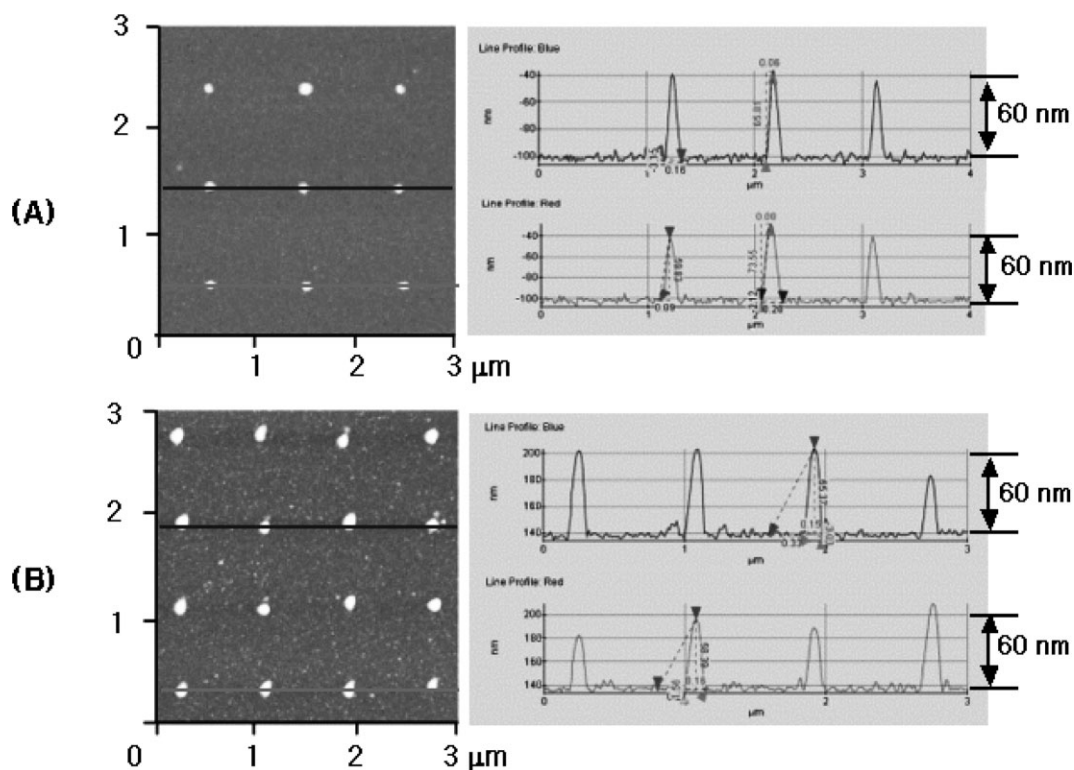


Figure 6. AFM images of angiogenin and integrin $\alpha_v\beta_3$ nanoarrays. Topographic images and height profiles of protein nanoarrays constructed by optimal conditions of AFM dip-pen nanolithography are shown. Optimal contact force (0.01 nN) and humidity (80%) were used for adsorption of proteins on Prolinker™ surface. Contact time was 5 s for each spot. The image was taken at a scan rate of 0.5 Hz. Width and height of arrayed angiogenin (A) and integrin $\alpha_v\beta_3$ (B) spots were 120 nm and 60 nm, respectively.

As shown in Fig. 7, the single molecular integrin nano-dots were constructed by AFM DPN method. Integrin $\alpha_v\beta_3$ was arrayed within $20\ \mu\text{m}^2$ as 24 spots (4×6). After rigorous rinsing, some nano-scale small dots remained in the same region of arrayed integrin nanopots (Fig. 7B and D). We used some of circle trace, which was occurred by the whirling of inorganic particle during shaking incubation, as a positioning marker. As shown in Fig. 7C, each nano-dot has regular height values of $15 \pm 5\ \text{nm}$. In the previous works on the structure of integrin, it was reported that integrin $\alpha_v\beta_3$ formed a shape of a prolate spheroid and the single molecular dimension of integrin was either about 10 nm (inactivated form) or 20 nm (activated form) along major axes [23, 24]. Based on these reports, the height value of integrin nano-dot in Fig. 7C means that the integrin $\alpha_v\beta_3$ was immobilized as a single molecular monolayer on the Prolinker™ surface.

Single molecular interaction between integrin $\alpha_v\beta_3$ and its cellular ligand, vitronectin, was found on Prolinker™ surface as a double layer of two proteins that could be detected by height measurement using AFM cantilever tip (Fig. 8B). The integrin and BSA nanoarray constructed by AFM DPN was incubated with vitronectin through immersing method (Fig. 8A). BSA was used as a control without any interaction with vitronectin. After incubation with vitronectin ($10\ \mu\text{g}/\text{mL}$) for 1 h, the height of integrin nanoarray increased from $15 \pm 5\ \text{nm}$ to $30 \pm 5\ \text{nm}$ (Fig. 8B). The height of protein double layer formed by binding of vitronectin onto integrin $\alpha_v\beta_3$ increased as much as the height of vitronectin compared with that of the integrin spot alone. In contrast, the height value of BSA spots did not change or decreased slightly. It is evident that the height change in integrin spots is due to the integrin-vitronectin interaction in a single mol-

ecule level, suggesting that height measurement by AFM makes possible the detection of molecular interaction of proteins. These data strongly suggest that AFM is a breakthrough tool for functional proteomics and high-throughput screening of protein-protein interaction in nano-scale. Taken together, these data suggest that the ability to make protein nanoarrays on Prolinker™ surface with well-defined feature size, shape, and spacing, should increase the capabilities of studying the fundamental interactions between biological structures (cell, complementary proteins, and DNA *etc.*) and solid surfaces. Although we have explored only protein nanopatterning, we expect that it can be extended, with straightforward modification of the aforementioned protocols, to a wide range of biomolecular structures and that the resolution of the technique optimized as described should improve conventional DPN technology.

4 Discussion

We have shown that protein nanoarray formed on Prolinker™ surface by DPN method is applicable to basic studies of functional proteomics for analysis of protein functions, in particular, molecular interactions of proteins. Since the DPN method using AFM has been developed by Chad A. Mirkin group, this technique opened a wide range of novel applications for biologists [17–20, 24]. Our study using integrin and angiogenin presents an additional challenge to expand the application field of DPN method. In particular, we demonstrated that Prolinker™ can serve as a stable linker system not only for protein microarray but also for protein nanoarray because of the fabrication process of protein nanoarray on the Prolinker™ surface by DPN method [14, 15].

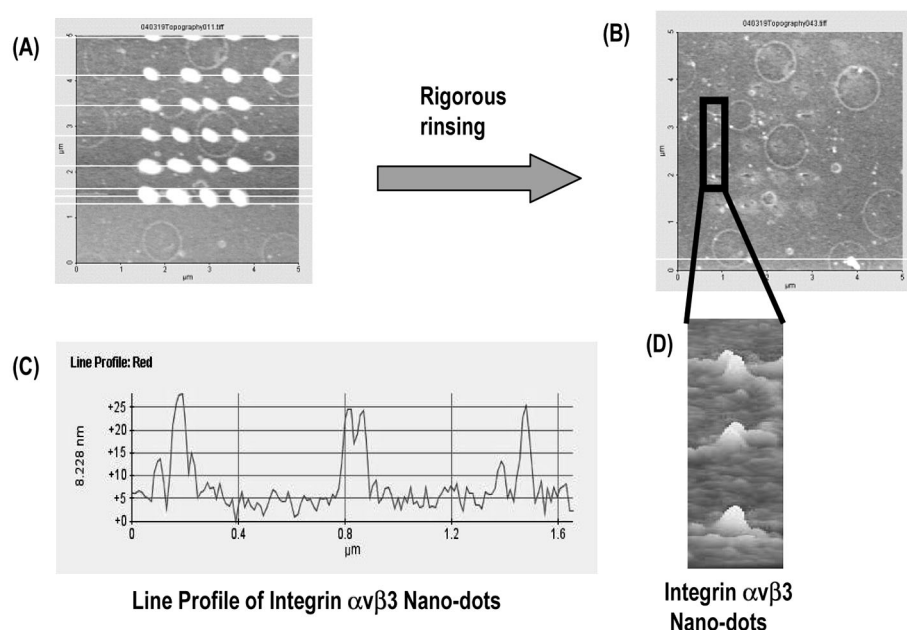


Figure 7. AFM image of single molecular integrin $\alpha_v\beta_3$ nano-dots and its line profiles. Topographic image of integrin $\alpha_v\beta_3$ nanoarray (A) and integrin $\alpha_v\beta_3$ nano-dots after rigorous rinsing (B) are shown. (C) and (D) reveal the line profile of integrin $\alpha_v\beta_3$ nano-dots and 3-D topographic single molecular image of integrin $\alpha_v\beta_3$ nano-dots, respectively.

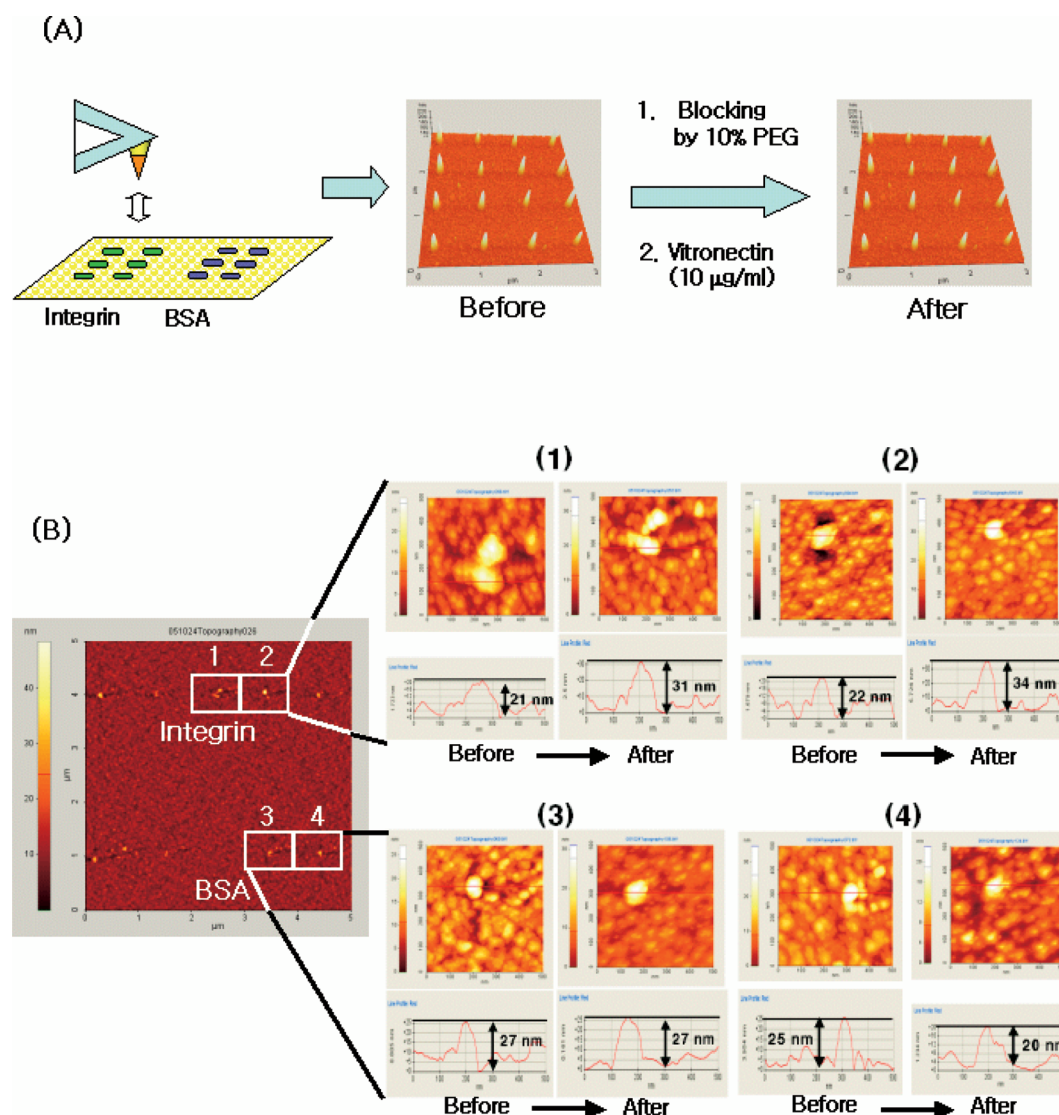


Figure 8. Single molecular interaction of integrin $\alpha_v\beta_3$ -vitronectin on Prolinker surface. Integrin and BSA nanoarray constructed by DPN was incubated with vitronectin through immersing method. The process for interaction between integrin and vitronectin on Prolinker™ surface is shown (A). AFM images of protein nanospots (including integrin and BSA) before and after interaction with vitronectin show the height change (B). After interaction with vitronectin (10 µg/mL), the height value of integrin nanospots increased [(1) and (2) in (B)], but the height value of BSA nanospots did not increase or decreased slightly [(3) and (4) in (B)]. Line profile shows the height change of integrin nano-dot depending upon the molecular interaction with vitronectin.

It has been shown in the evaluation of humidity condition that the protein nanospots arrayed with low humidity condition showed larger width value than those with high humidity condition. The reason may lie in the hydrophobic interaction between Prolinker™ surface and protein solution. Prolinker™ surface has hydrophobic nature that provides favorable environment for making compact protein spots on protein microarray chip without spreading out [14]. In DPN process with low humidity condition, the protein layer on the cantilever surface changes to the dry and high viscosity state because of the fast vaporization of water molecules. For this

reason, the hydrophobic interaction between Prolinker™ surface and PEG in protein solution may increase and, this increased hydrophobic interaction may allow the spots to spread out laterally.

In addition, through the estimation of DPN condition, we showed that nano-scale processes of DPN including protein adsorption on the tip surface and protein transfer onto the substrate surface were significantly influenced by various technical conditions such as humidity, contact force, and contact time. Especially, our data showed the importance of humidity in the DPN process not only for the maintenance of

protein activity but also for the well-controlled construction of protein nano-dots. Through the evaluation of some important factors in DPN condition, we established the optimized array condition in nano-scale and applied it to the construction of protein nanoarray with integrin and angiogenin.

In general, the AFM instrument directly observes the formation of a molecular complex by reporting a height change of the topography [22, 25]. In this study, the height of protein nanoarray was measured and was evaluated by AFM, for the investigation of protein-protein interactions in nano-scale. This detection process is label free without any requirement for fluorescent, radioactive, enzymatic or other molecular probes. Thus, this approach is applicable to a wide range of molecular species, many of which are difficult to label with conventional tags, including proteins and drug candidate molecules [26]. In our experiments, we were able to construct protein nanoarrays of angiogenin and integrin $\alpha_v\beta_3$ on the Prolinker™ surface and the detection of protein interaction was performed by measuring the height of protein molecular complex using AFM tip. Interestingly, we obtained a single molecular image of integrin $\alpha_v\beta_3$ monolayer with height of 15 ± 5 nm (Fig. 8) in agreement with a previous report showing X-ray crystallographic data of integrin $\alpha_v\beta_3$ [23]. Mirkin *et al.* [22] showed that single molecular interaction of two different proteins such as antibody and antigen could be detected by scanning over the surface of the protein complex. Using the integrin nanoarray, we also demonstrated that analysis of integrin $\alpha_v\beta_3$ -vitronectin interaction was observed at a height level of approximately 30 nm through height-detection method of molecular complex of integrin $\alpha_v\beta_3$ and vitronectin scanned by AFM probe, indicating that these two proteins bind each other in single-molecule level (Figs. 7 and 8). It is evident that height-detection method using AFM is an enabling technology for analysis of single protein interaction. In addition, the integrin $\alpha_v\beta_3$ immobilized on Prolinker™ surface was capable of binding to vitronectin in nano-scale without any loss of activity. We have previously demonstrated the function of Prolinker™ for stable immobilization of protein containing intact activity on protein microarray chip, ProteoChip [14]. Our result suggests that surface chemistry for protein immobilization may play an important role in the successful development of protein nanoarray.

In conclusion, we have successfully developed a novel protein nanoarray on Prolinker™ surface via AFM DPN to detect protein-protein interaction in nano-scale. We optimized DPN conditions for the construction of protein nanoarray. In addition, we found single molecular interaction between integrin $\alpha_v\beta_3$ and vitronectin using integrin $\alpha_v\beta_3$ nanoarray via detecting the height of the protein complex by scanning method of AFM tip. Taken together, these data suggest that the protein nanoarray can be a breakthrough technology applicable to biomarker assay in an ultrasensitive manner and basic research of functional proteomics. Further studies will be needed to demonstrate and confirm the possibility for biological applications of protein

nanoarray and develop other sensitive detection technology for high-throughput analysis of single protein molecular interaction using protein nanoarray.

This research was supported in part by a grant from National R&D Project for Nano Science and Technology (grant no. M1-0214-00-0194), the Molecular and Cellular BioDiscovery Research Program (grant no. M1-0311-00-0114) and the Protein Chip Technology Program of the Ministry of Science and Technology, Korea.

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