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# Two-Dimensional Crystal Film of Streptavidin: A Binding Base for Biotinylated Proteins for Protein Device Application

# Taiji Furuno

Summary—The two-dimensional crystal film of streptavidin (SA) has been prepared at the air/water interface and transferred onto hydrophobic silicon wafer. The transfer ratio has been improved from our previous studies, resulting in the entire covering of the surface of silicon wafer with 2D crystal domains of SA. Stable and controlled immobilization of biotinvlated proteins in their active form is to be achieved on this SA surface. Binding of biotinylated protein G to the SA surface and thereafter immunoglobulin G (IgG) to this immobilized protein G have been demonstrated by atomic force microscopy (AFM) under water. The biotin-streptavidin linkage is so stable that the bound protein G/IgG complex withstands the lateral pressure of the AFM cantilever tip during scanning. The number of bound molecules can be counted directly in the AFM images, suggesting the possibility to develop an AFM-based protein device in which the binding assay is ensured at high signal to noise rarios. The present study achieved the densest packing of SA on a solid support compared with those using self assembled film of thiol molecules on gold or biotinylated lipid at the air/water interface, which so far led to a random packing of streptavidin molecules or scattered assembly of 2D crystal patches of SA on solid supports. As demonstrated in this paper, uniformity of the 2D crystal film of SA is essentially important for the binding assay by means of AFM.

Keywords: Biotin, AFM, Protein G, IgG, Protein Array, Protein Chip

# Introduction

Application of protein arrays such as to sensors and bioassay devices requires technical establishment of immobilizing protein on solid support with controlled orientation. The two-dimensional (2D) crystal surface of protein might be promising for this aim, since its surface can be structurally well characterized and denaturation of immobilized proteins is thought to be diminished due to the hydrophilic nature of the

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surface. In the current nanotechnologies, the 2D crystals of protein have found applicability as nanometer templates on which proteins of interest can be immobilized and form an ordered array.<sup>1</sup>

The history of 2D crystallization of protein began with the work of ferritin assembled in a 2D form which adsorbed to a lipid monolayer spread at the air/water interface.<sup>2</sup> Antibody molecule,<sup>3</sup> cholera toxin B subunit<sup>4</sup> and tetanus toxin<sup>5</sup> were 2D crystallized by binding to the antigen or receptor molecules incorporated into lipid monolayers. Nonspecific electrostatic attraction between protein and lipid, which had positive or negative head group, was also utilized for 2D crystallization of RNA polymerase,<sup>6</sup>  $\alpha$ -toxin<sup>7</sup> and annexin V.<sup>8</sup>

The 2D crystallization of antibody molecule<sup>3</sup> was one of the earliest studies aimed at protein device application. The antibody molecules were, however, bound with their Fab portion orienting toward the substrate surface, indicating an inability to bind with antigen molecules. To my knowledge, the 2D crystals of antibody molecules, in which the Fab portion is exposed outside, have not been obtained till today. Further, there is no report that genetically modified protein introduced with surface cysteine was 2D crystallized on gold surface. This fact is different from that of alkanethiols that form self-assembled crystalline monolayers on gold surfaces.<sup>9</sup> In a recent nanobiotechnological application of protein arrays, His-tagged proteins were immobilized on a gold surface coated with self assembled alkyl thiols derivatized with nitrilotriacetic acid (NTA). Metal coordination bonds between His-tags and nitrilotriacetic acid produced uniform 2D protein arrays. Writing, reading and erasing the nanopattern on this 2D array has been demonstrated by lithographic application of AFM cantilever tip.<sup>10</sup>

Bacterial cell surface (S-layer) proteins, which exhibit a strong tendency to assemble into 2D crystal forms under a variety of crystallization conditions, hold promise for nanodevice applications.<sup>11</sup> Iron storage protein ferritin is also interesting for device applications. Ferritin is a protein of spherical shape storing maximally 4000 iron atoms inside its cavity as a nanoparticle of iron oxide. This core particle can be removed chemically and substituted with different kinds of metallic nanoparticles.<sup>12</sup> Ferromagnetic core of CoPt was prepared for the development of high density data storage.<sup>13</sup> Another application was toward an electronic device in which Listeria ferritin was used as an electron-confinement component for floating gate memory.<sup>14</sup>

The specific interaction between streptavidin (SA) and biotin is versatile and has long been used extensively for localization of molecules. The 2D crystals of SA which cover uniformly the surface of solid support is therefore supposed to have a wide range of application for bio-assay systems. The 2D crystallization of SA began with the study of binding to biotinylated lipid mixed in a lipid monolayer.<sup>15</sup> The 2D crystal domains of SA thus formed at the air/water interface tend to show a butterfly shape with the domain size extending to 100  $\mu$ m. For structural studies by transmission electron microscopy, the 2D crystals had to be transferred from air/water interface to a supporting grid. The coverage ratio of the grid approached 50% according to an improved transfer method.<sup>16</sup> The atomic force microscopy is another choice for structural analysis of SA 2D crystals. The hydrophobic surface of highly oriented pyrolytic graphite (HOPG) was shown to be useful for successful deposition of SA 2D crystals with the lipid film facing the HOPG surface.<sup>17</sup> However, the transferred crystal patches were discontinued and perturbed at the edges that appeared on the cleaved HOPG surface.

A versatile method was developed to 2D crystallize general proteins, where lipid heads derivatized with metal-chelating moieties of nitrilotriacetic acid (NTA) or iminodiacetic acid (IDA) were exploited to coordinate surface histidine residues or Histags to chelated nickel or copper ions and thus anchoring protein molecules at the surface of lipid monolayer spread at the air/water interface.<sup>18</sup> A number of proteins including SA have been 2D crystallized according to this method.<sup>19</sup>

We have used a nonspecific binding method, where soluble proteins such as ferritin, catalase and SA were densely bound to a polypeptide film of poly(1-benzyl-L-hisitidine) (PBLH) spread at the air/water interface.<sup>20</sup> The protein-bound PBLH film was then thermally annealed at the air/water interface to facilitate crystallization, and transferred onto hydrophobic silicon surface.

A high affinity constant of streptavidin for biotin ( $K_D = 10^{-15}$  M) is a merit for device application of 2D crystal film of SA. In the studies by electron microscopy or by AFM, the transferred SA crystal patches did not compose a homogeneous dense packing on the support<sup>16, 17, 20</sup>. However, for actual device application, the deposited SA film needs to cover the solid surface uniformly with little void space, since the protein molecules in a bulk solution may easily adhere to this region where the bare hydrophobic surface is exposed. Nonspecific protein binding to the hydrophobic area is unwanted since it acts probably as a noise source in assaying processes.

The SA film on a glass substrate appears to have profound potential also for single molecule biophysics and biochemistry, in which immobilization of the target molecule in active state is critically important and the use of transparent substrate is essential for fluorescence microscopy. However until now, the application of SA 2D crystal has rarely been pursued as a surface for binding biotinylated proteins.<sup>21</sup> The present study shows that the deposition of SA crystal film on glass or quartz plate is the next step.

# **Experimental Section**

# 1. Materials.

The synthetic polypeptide, poly(1-benzyl-L-histidine) (PBLH) with polymerization degree of 100 was obtained from Sigma Chem. Co. PBLH was dissolved in chloroform which contained dichloroacetic acid (DCA) with a ratio of PBLH : DCA = 1 : 1(mg/µl). The concentration of PBLH was ~0.6 mg/ml. Affinity purified streptavidin (SA) and human IgG were purchased from Sigma-Aldrich and biotinylated recombinant protein G ( $M_w$  ~22 kDa) removed of albumin binding site was purchased from Pierce. SA was further purified twice with a size exclusion chromatography (G3000XL column, Toso, Japan). After each elution, the SA solution was concentrated with a molecular filter with the cutoff molecular weight of 30,000.

#### 2. Two-Dimensional Crystallization of SA

Two-dimensional crystallization of SA was carried out basically according to the method reported previously,<sup>20, 22</sup> where SA molecules dissolved in a buffer were bound to the PBLH monolayer spread at the air/water interface. To facilitate crystallization, the densely packed arrays of SA molecules were then thermally treated in situ. A number of experimental parameters have been altered from the previous ones: the phosphate buffer was replaced by HEPES with EDTA and without salt, spreading of the PBLH film was very quickly done by pushing out the chloroform solution continuously with one stroke from a microsyringe with the top of the needle kept touching the subphase surface, and the incubation time at different stages was changed. Although we are not so confident how and which of these changes improved the 2D crystal quality, we can definitely say that both uniformity and reproducibility of the transferred film have been further improved than before. Among many crystallization parameters we have surveyed, the incubation time of a silicon wafer in contact with the interfacial SA/PBLH film seemed most concerned with the transferred film quality.

Recovering of the wafer from air/water interface immediately after deposition resulted in low coverage ratios so often. In the present protocols for 2D crystallization this incubation time is longer than 1 h. A kind of matching or commensuration between SAbound PBLH and HMDS-treated Si surface might be present. The preparation condition for 2D crystallization is depicted as the following.



# 3. Atomic Force Microscopy

An atomic force microscope (Explorer, Topometrix Co., USA) was used. A cantilever (OMCL-TR800, 100 µm in length, spring constant 0.6 nN/nm, Olympus Co., Japan) with the electron-beam deposited carbon contamination tip (supertip or EBD tip) at the apex of the pyramidal tip was used. The end radius of the sharpest supertip was estimated to be ~3 nm.<sup>23</sup> Most of the tips used in the present study are of similar end radius with some variations. In the fabrication process, each EBD tip was viewed from top in the SEM and the sharpness of the end was confirmed. This confirmation was very quickly terminated to avoid unwanted contamination deposition. The tapping mode was employed to image 2D arrays under water with a scan rate of  $5-15 \ \mu m/s$  and with a cantilever oscillation frequency of 46~52 kHz. The oscillation amplitude of the cantilever employed should have been around a few nanometers, and the setpoint, i.e., the damping ratio was initially set to be about 2-3 % of the amplitude of free oscillation of the cantilever. Therefore the actual damping would be 1 Å or less. To determine the real amplitude of the cantilever just before entering the feedback loop, go-and-stop cycle in the automatic approaching was repeated manually with short steps and the amplitude was re-registered at each time. The drift of the setpoint during imaging was compensated manually with 0.5 or 1.0 % step during scanning. The drive frequency for tapping was not always set at the peak of the resonance spectrum, instead a frequency in a flat region or shoulder which gave as random noise as possible in the sensor signal was used.

The supertip was robust and had a long lifetime unless damaged by an accidental approaching failure which occurred occasionally. The supertip was used repeatedly by UV cleaning just before AFM imaging. Furthermore and very strangely enough, the contamination of the supertip during protein imaging seemed quite less compared with pyramidal silicon nitride tip, probably coming both from its surface property and high aspect ratio of the tip shape.

The original AFM images were processed only for tilt.

# 4. Immobilization of Protein G/IgG Complex on SA Crystal Surface

To examine nonspecific binding of protein to SA surface, a drop of solution containing human IgG (10  $\mu$ g/ml in 10 mM HEPES pH 7.0) was applied to the SA film transferred onto Si wafer, incubated for 10min, and rinsed with pure water for AFM imaging.

Biotinylated protein G was immobilized by incubating the SA film in the buffer solution (10 mM HEPES, pH 7.0) containing 0.6  $\mu$ g/ml of protein G for 30 s to prepare low-density binding, and 5  $\mu$ g/ml for 10 min for high-density binding. A drop of HEPES buffer containing IgG was subsequently applied to the protein G-bound SA surface to achieve IgG immobilization via protein G-IgG interaction.

#### 5. Effect of Tween 20 on SA Crystal Structure

The effect of detergent on the SA crystal structure was studied using Tween 20 at a concentration range from 0.1 % (1 mg/ml) to 0.0002 % (2  $\mu$ g/ml). A drop of HEPES solution (pH 7.0) containing Tween 20 was applied to the SA film on the wafer, incubated for 1 min and rinsed with pure water for AFM imaging.

# **Results and Discussion**

#### 1. 2D Crystallization Conditions

The experimental parameters for 2D crystallization of SA at air/water interface are various: pH and ionic strength of the subphase, purity and concentration of protein in the subphase, spreading and surface density of PBLH, temperature and time for incubation, cooling rate after annealing, method for film transfer onto the support, and so forth. The SA films obtained in this study consisted of small crystal patches with the size less than 500 nm (Figure 1), containing void spaces between patches and single molecule vacancies in each patch. However, the lattice lines in the neighboring crystal patches were consecutive, suggesting that the larger crystal patches formed at the air/ water interface were fragmented into pieces on transfer onto substrate. For inspection with an AFM, more than ten areas over one sample surface (area ~3×4 mm<sup>2</sup>) were randomly chosen from center to edges around. Failure in the film transfer was sometimes accompanied by large void areas in which almost no crystal patches were

found. The dust particles and/or air bubbles that happened to be sandwiched between the wafer and the surface film are supposed to be the reasons.

The property of the force which allows SA molecules to bind to the PBLH film has not been well characterized. In our previous 2D crystallization studies of streptavidin and catalase,<sup>20, 22</sup> the subphase was adjusted to the pH at which PBLH was charged positively and proteins had net negative charge, ensuring electrostatic attractive force between them. A high salt concentration of the subphase buffer, e.g. ~100mM NaCl retarded the protein binding to PBLH film. In this context, the interaction between PBLH and protein in the buffer appeared to be electrostatic. However, the 2D crystals once formed and transferred onto the wafer were attached stably on the PBLH surface at high salt solutions, e.g. in KCl solutions of 1-2 M concentrations. Then, the non-electrostatic interactions such as of van der Waals interaction or hydrogen bonding between protein and imidazole moieties in PBLH is to be presumed.

Although only topographic images are presented in this paper, the error-signal images (equivalent to sensor-current or differential images) provided finer impression of the 2D crystal surface. We notice in Fig.1 that the resolution or impression of the AFM images appears better for the pair of Fig.1(a) and (b) than for Fig.1(c) and (d). We speculate that the main factor which caused this difference was the effective radius of the tip apex. The tip radius used for Fig.1(c), (d) should have been larger than for Fig.1(a), (b), resulting also in the smoother image of the crevice in Fig.1(c), (d) than in Fig.1(a), (b). We notice another point that the film surface is undulated, which resulted from the surface roughness of the silicon wafer on which the film is deposited.

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Figure 1. AFM images of 2D crystals of streptavidin deposited on Si wafer.

# 2. Nonspecific Binding and Stability of SA Film

The 2D crystal film of SA was incubated in a solution containing immunoglobulin G molecules. The brightly seen particles in Figures 2 a-d are presumably IgG molecules nonspecifically bound from the solution to crystal boundaries or to disordered areas. The 2D crystal surface appears to be fairly inactive for nonspecific binding of protein. This feature is significant for the application of the SA crystal film as the surface for immobilizing biotinylated proteins via biotin-streptavidin linkage.

Two-Dimensional Crystal Film of Streptavidin

(Taiji Furuno)



Figure 2. Nonspecific binding of immunoglobulin G on SA surface. The SA film was incubated in a solution of IgG with concentration of 10  $\mu$ g/ml in 10 mM HEPES pH 7.0 for 10 min, and rinsed in pure water for AFM imaging.

The addition of detergent to the buffer, a usually used method to reduce nonspecific adsorption of proteins, should be avoided. It is shown that the crystal structure of SA suffers easily from even a low concentration of surface active molecules contained in the solution. Figure 3 shows the effect of non-ionic detergent Tween 20 on the 2D crystal structure of SA in the concentration range from 2  $\mu$ g/ml to 200  $\mu$ g/ml. Figure 3 shows that the ordered array of SA was completely destroyed and large granules, which were probably the composite of protein, PBLH and detergent, were formed on the hydrophobic Si surface. At a lower concentration of Tween 20, we observed a disordered

and bumpy 2D assembly, in which we can barely identify each SA molecule (Figure 3c). This result suggests that, when we use the SA/PBLH system for application to proteinbinding assay, a care must be taken against the surface active impurities contained in the sample solution.



Figure 3. Effect of Tween 20 on SA crystal structure. The concentration of Tween 20 was (a) 200  $\mu$ g/ml, (b) 20  $\mu$ g/ml, and (c) 2  $\mu$ g/ml.

# 3. Immobilization of Protein G/IgG Complex onto SA Crystal Surface

We can demonstrate an application of the SA crystal film as a basis for immobilizing proteins. We achieved this first by binding biotinylated protein G onto the SA crystal surface via biotin-streptavidin interaction, then IgG to this immobilized protein G, which should have captured the Fc portion of IgG. Accordingly, protein G/IgG complex formed at the SA surface should be oriented with their Fab portion protruding from the SA crystal surface.

Two-Dimensional Crystal Film of Streptavidin



**Figure 4.** High and low density binding of biotinylated protein G on SA surface. (a) and (b), high-density binding prepared by incubating a transferred SA film in a protein G solution with concentration of 5  $\mu$ g/ml for 10 min; (c) and (d), low-density binding prepared by incubation in a 0.6  $\mu$ g/ml solution for 40 s.

A recombinant protein G ( $M_w$  ~22 kDa) removed of albumin binding site was applied to the SA surface. Figures 4a, b show that the biotinylated protein G molecules were bound at high densities to the SA 2D crystals. It is difficult to identify each protein G molecule. The crystal surface appears to be contaminated or smeared with many small dots instead. On the other hand, the bound protein G molecules are clearly seen in Figures 4c, d, in which the density of the bound protein G was lower and the apex of the AFM cantilever tip used was blunter probably than in Figures 4a, b. Then, the tip convolution effect enhanced the apparent molecular size of the surface-bound small protein. The lower resolution images of the underlying SA crystal lattice in Figures 4c, d than in Figures 4a, b also suggest the bluntness of the tip apex. It is confirmed inversely that an AFM tip with adequately blunt apex is helpful for counting the number of small molecules sparsely bound to a flat surface.



Figure 5. Binding of IgG to biotinylated protein G bound to SA crystal surface. (a) and (b), high density sample, prepared by incubation in 10 mM HEPES (pH 7.0) containing 10 μg/ml IgG for 10 min; (c) and (d), low density sample, 5 μg/ml IgG for 60 s.

Figure 5 shows the AFM images of higher (Figures 5a, b) and lower density (Figure 5c, d) binding of protein G/IgG complex on the SA film. The cantilever tip used to take

Figures 5a, b was sharper than for Figure 5c, d, representing higher-resolution lattice images, but disturbing heavily the surface bound protein G/IgG complex that resulted in streaky particle images. When the complex formation was suppressed by reduced incubation time, a part of protein G molecules seemed to be left solely on the SA crystal surface non-reacted with IgG. Figure 5c, d appear to contain both non-reacted form of protein G (low contrasted smaller dots) and protein G/IgG complex (larger and brighter streaky dots), although unambiguous discrimination between the two forms was not easy in the present study. However, from the comparison between Figure 4c, d and Figure 5c, we notice that the lower density sample contained non-reacted protein G, though the contrast of the image of protein G is higher in Figure 4c, d due to the narrower height range in grey scale than in Figure 5c.

It is difficult to perceive the typical shape of IgG molecule in Figure 5a, b. However, it seems assured that the binding of IgG to protein G is tight enough to withstand the lateral pressure from the scanning cantilever tip. If they are forced to be dissociated from the surface during scanning, the AFM should present a partially lost image of the particulate shape, e.g. like a semicircle with its bowstring placed down when the scanning direction is horizontal from top to bottom.<sup>24</sup> Most of the particles in Figures 5a, b are of whole shape, indicating that the IgG/protein G complex linked to the SA is not swept away during scanning. The lower binding samples, Figures 5c, d, also suggest that we can count the number of protein G/IgG complex directly by AFM imaging. This direct counting using AFM is considered to have high sensitivity if applied to a protein chip for immunoassay.

#### 4. Proposal for Higher Resolution Imaging to Discriminate the Shape of Immobilized Proteins

To achieve high resolution AFM imaging of protein molecules, immobilization is substantially important. It seems difficult, however, to obtain the shape of single protein molecules immobilized on the SA crystal surface. The usual-size ( $M_w < 100$  kDa) globular proteins derivatized with a number of biotinyl residues would form one or two biotin-streptavidin linkages on the crystal surface with the unit cell dimensions of ~9×9 nm<sup>2</sup> which contains two SA molecules, i.e., providing maximally four biotin binding sites exposed outside. Such immobilization is insufficient to keep the bound molecule unmoved during being scanned with the AFM tip. Further, the single protein molecules sitting solely on the SA surface may be intrinsically fluctuating by Brownian motion in water. Figure 6 schematically depicts these situations for the case of protein G/IgG complex immobilized on the SA crystal surface and scanned with a sharp AFM tip.



Figure 6. Cartoon depicting an inherent difficulty to obtain exact AFM images of immobilized but fluctuating single molecules on SA surface under water.

We showed a kind of trick using the PBLH system, in which the bound single protein molecules were stabilized further and provided higher-quality images. The samples were the mixtures of two protein species bound to the PBLH film.<sup>24</sup> In one sample, the single ferritin molecules were embedded in a 2D packing of catalase, and in another, the single catalase molecules were embedded in streptavidin arrays. Both of these samples presented well preserved images of single proteins, ferritin and catalase, respectively. We propose a similar method in which intermolecular space of IgG-antigen complexes is filled up with single species of smaller biotinylated proteins. Those smaller molecules will increase the lateral pressure if densely packed and assist stabilization of the solely bound larger molecules. This treatment will also change the topography of the sample surface, where the height of the larger molecules are effectively lowered, resulting also in a decrease in the lateral force applied from the scanning AFM tip and the reduction of the tip convolution effect. Moreover, if the two species, i.e., embedded minority molecules and embedding major components, are similar in height, we have a possibility to obtain higher resolution molecular image. Actually, we have shown that a dense 2D packing of actalase.

packing of catalase, not in crystalline form, can provide a high resolution AFM image resolving individual molecules showing a concave and twisted rectangular shape representative of its tetrameric subunit structure.<sup>25</sup> If AFM is capable of providing direct identification of the bound molecular species, it will become one of the powerful assaying tools in protein chip technology.

### 5. Preservation of SA Film.

Preservation of the transferred film is another issue when we plan a practical use of

the SA 2D crystal film. The transferred SA film was well preserved for a few days in pure water at refrigerator temperature, but the bound molecules detached gradually with time scale of days. We need to find out more adequate storing method or further treatment after deposition, if we desire higher-quality preservation which ensures a uniform dense packing of SA molecules for several days or longer.

# 6. Deposition of SA Film onto Glass or Quartz Plate

For applications of the SA film as a protein-binding base, the use of transparent solid support is desirable, when considering the use of fluorescence optical microscopy for characterization. Deposition of the SA film onto hydrophobic surface of glass cover slip, glass slide or optically polished quartz plate was surveyed tentatively. All of the experiments, however, failed in a very poor deposition of the SA film. Small patches of fragmented SA film resided sparsely on their surfaces. It was also evident from experiments that the PBLH/SA film could not be transferred onto hydrophilic Si surface. Although hydrophobicity of the Si surface was essential to ensure successful transfer, the deposition seemed to require other properties of the solid surface. Actually, the hydrophobicity of the surface checked by contact angle of water-droplet was very similar between Si wafer and quartz plate both treated with HMDS after UV-cleaning. It is therefore inferred that both of the PBLH/SA film.

# Conclusions

In conclusion, we have successfully prepared two-dimensional crystal film of streptavidin (SA) that entirely covers the hydrophobic surface of silicon wafer. This high coverage is significant for the SA film to be used for protein devices. We could discriminate by AFM between protein G/IgG complex and non-reacted protein G which were bound to the same SA surface simultaneously. Counting the number of bound protein molecules directly from AFM image is possible. However, identification of the bound protein species by their shape seems still improbable since the detailed molecular shape is smeared by the mechanical pressure of the scanning AFM tip against these loosely bound molecules and the intrinsic obscurity caused by the tip convolution effect. Nevertheless, the present study suggests that the uniform SA film is useful as one of protein-immobilizing surfaces for a variety of protein device applications.

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