Title	Deposition onto Glass Surface of Two-dimensional Crystal Film of Streptavidin
Sub Title	ストレプトアビジン2次元結晶膜のガラス表面への転写固定
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Publisher	慶應義塾大学日吉紀要刊行委員会
Publication year	2009
Jtitle	慶應義塾大学日吉紀要. 自然科学 (The Hiyoshi review of the natural science). No.46 (2009.) ,p.1- 11
JaLC DOI	
Abstract	The two-dimensional (2D) crystal film of streptavidin (SA) prepared at the air/water interface had been transferred onto a hydrophobic surface of silicon wafer and studied by atomic force microscopy1). In the present study the deposition of this film onto glass coverslip has been examined. Annealing the glass coverslip at 525 ºC rendered the surface smoother and stabilized adhesion of the transferred film. The use of glass as substrate for protein immobilization has the advantage that an optical microscope can be used in transmission mode, and the fluorescence quenching which generally occurs for dyes locating near metal or semiconductor surfaces can be avoided2) . Therefore, the 2D crystal film of SA deposited on glass would find wider applications than on silicon as the protein binding base using biotinylated proteins.
Notes	原著論文
Genre	Departmental Bulletin Paper
URL	https://koara.lib.keio.ac.jp/xoonips/modules/xoonips/detail.php?koara_id=AN10079809-20090930-0001

Hiyoshi Review of Natural Science
Keio University No. 46, 1-11 (2009)

Deposition onto Glass Surface of Two-dimensional Crystal Film of Streptavidin

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Summary——The two-dimensional (2D) crystal film of streptavidin (SA) prepared at the air/water interface had been transferred onto a hydrophobic surface of silicon wafer and studied by atomic force microscopy[1]. In the present study the deposition of this film onto glass coverslip has been examined. Annealing the glass coverslip at 525 ºC rendered the surface smoother and stabilized adhesion of the transferred film. The use of glass as substrate for protein immobilization has the advantage that an optical microscope can be used in transmission mode, and the fluorescence quenching which generally occurs for dyes locating near metal or semiconductor surfaces can be avoided[2]. Therefore, the 2D crystal film of SA deposited on glass would find wider applications than on silicon as the protein binding base using biotinylated proteins.

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

1. Introduction

In biological device applications specificity of one molecule to another is exploited and their reactions are shown on the surface of solid support to be assessed by most analytical means at high sensitivity. The immobilization of immunoglobulin molecules on solid supports have been studied for the development of protein microarrays which could speed up expression profiling[3-4]. From vast number of studies for immobilized proteins, it is believed that immobilization procedure has some danger leading to the denaturation of immobilized protein on interacting with the surface itself to be bound on[5]. Orientation control for the immobilized proteins can increase the analyte capturing efficiency[4]. The stability and density of the immobilized protein with feasible orientations are key factors which must be taken to be significant in the fabrication of protein arrays.

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One promising method to obtain immunoglobulin arrays with high antigen binding activity is to use high density packing of two-dimensional (2D) array of streptavidin (SA) immobilized on the surface of solid support[3-4]. The SA array binds immunoglobulin molecules with biotinylated Fc portions. Biotinylated Fab' part prepared by enzymatic digestion of IgG and subsequent biotinylation on the cysteinyl thiol exposed by reducing the $F(ab')_2$ also forms densely packed molecular array. The IgG or Fab' arrays have been reported to have high antigen capturing efficiency under the conditions of weak denaturation, high density packing and feasible orientation of antigen-binding site.

The 2D crystallization technique for soluble proteins is interesting from both the basic aspect of structural studies[6] and the application to fabricate protein devices. The 2D crystals of protein have so far been prepared by exploiting binding from subphase to various amphiphilic layers preformed at air/water interface, and the surface films thus prepared are transferred either on carbon films for electron microscopy, graphite for atomic force microscopy or hydrophobic surface of silicon wafer for scanning electron microscopy and/or atomic force microscopy.

We have so far deposited 2D crystals of proteins on the surface of silicon wafer. The study had been started for establishing the basic technique of 2D crystallization of soluble proteins, also aiming at the future development towards protein-based devices. Scanning electron microscopy had been routinely used there to examine the packing of proteins in two dimensions, where both the electroconductivity and surface smoothness were required for the substrate. The silicon wafer satisfied these requirements to considerable extent, although the surface of mechanochemically polished crystal of silicon is not as smooth as mica surface due to the formation of natural oxide layer. The specimen deposited on the silicon, which was negatively stained to enhance contrast, was conductive enough to escape from the problem of surface charging-up during scanning electron microscopy (SEM) using a focused electron beam. In the later work we have evidenced that the 2D assembly of protein could be characterized routinely under physiological conditions by means of atomic force microscopy (AFM), in which also the surface smoothness of the silicon wafer was realized to be important to obtain high quality AFM images with reduced lateral force exerted by the scanning AFM tip.

Although the silicon wafer is thus moderately suitable as substrate for characterization both by SEM and AFM and appears to be not bad as substrate for protein device application, it is inadequate without doubt for studies where transparency of the substrate is required. When application studies, e.g. patterning of the deposited film for immunoassay or protein-protein interaction analyses are presumed, the glass or silica plate is thought to be more advantageous than silicon for characterization by optical

microscopy. We have studied from these reasons the deposition of the 2D crystal film of protein especially streptavidin (SA) onto glass coverslip. As far as we know the 2D crystals of protein have never been deposited onto glass surface for structural assessment by any microscopic means.

We had reported the optimization for the deposition of 2D crystal films of SA onto silicon wafer[1]. In the present study we applied the same procedure for fused silica and glass coverslip with slight modifications of the deposition conditions. However, those attempts mostly failed. We have so far implicitly assumed that the complex film of SA crystals bound to a film of poly-benzyl-L-histidine (PBLH), a synthetic polypeptide, is robust enough to be bridged over a surface with nanometer-scale roughness. This assumption seemed to be valid for the surface of silicon wafer which had a sub-nanometer roughness due to natural oxide formation. The deposition onto glass and silica plates which had similar surface roughness was poor or very poor. The HMDS-treated surface of silica plate showed almost the same hydrophobicity as silicon when assessed by water droplet test. However, the SA crystals were rarely found on its surface, indicating that the deposition is not determined only by the hydrophobicity of the substrate surface. The surface of HMDS-treated glass showed a weaker hydrophobicity than HMDS-treated silica. However, the glass proved to have a more preferable surface for deposition of PBLH/SA than silica: the film could be transferred as assemblies of fragmented patches,

Annealing the glass at ~500 ºC seemed to render the surface smoother or at least changed the surface roughness as imaged by AFM, and the coverage ratio of the SA film and its uniformity were highly improved. The thermal treatment was examined very preliminarily for the silica plate, but no improvements were found in the temperature range we tested.

2. Experimental

2.1 Materials

Biological materials used in this study have already been described in the previous paper[1]. They are synthetic polypeptide, poly(1-benzyl-L-histidine) (PBLH), affinity purified streptavidin (SA), human IgG and biotinylated recombinant protein G. Only the SA was further purified with a size exclusion chromatography (G3000XL column, Toso, Japan). The glass coverslip (Matsunami Glass Ind. Ltd, Japan), fused silica plate with polished optically flat surface (Sigma Koki Co. Ltd., Japan) and mechanochemically polished N-type silicon wafer with orientation (111) (Sumitomo Sitix, Corp. Japan) were

used as solid supports..

2.2 Preparation and transfer of 2D crystal film of streptavidin

Preparation procedures for two-dimensional crystal film of streptavidin at air/water interface and the subsequent transfer onto substrates were essentially the same as previously reported for silicon substrate[1].

2.3 Thermal treatment of the glass coverslip

The pieces of coverslip cut to size of ~4×5mm^2 were ultrasonicated for 1 min in a neutral detergent solution, rinsed in water and subsequently in Milli-Q water. They were then put into eppendorf tubes one by one to be spun in a micro centrifuge blowing off the excess water on the surface for quick drying. Next, they were UV-O_3 cleaned with a low pressure mercury lamp (dominant wavelength ~185 nm) and then heated in an electric furnace (TMF-1000, Tokyo Rikakikai Co., Ltd., Japan) to a desired temperature and cooled down taking several hours. After the thermal treatment they were UV-O_3 cleaned again and finally treated in glass vials filled with HMDS gas at 60 ºC for 1 h.

2.4 Atomic force microscopy

The atomic force microscopy (AFM) is the main tool for the present study to examine the assembly of the transferred SA film on the glass surface. The scan parameters of AFM have already been described in detail[1].

3. Results and Discussion

3.1 Surface roughness of glass coverslip, fused silica plate and silicon wafer

The surface of glass, fused silica and silicon are compared in Fig.1 (a)-(c). These error-signal images are adjusted for brightness and contrast, so that the impression about roughness is not necessarily proportional to their actual surface roughness. The topography images taken simultaneously with these error-signal ones were analyzed for roughness profiles using the built-in AFM software. The same cantilever tip (supertip) was used throughout the measurements for the three kinds of substrate by changing scan areas. The roughness parameter R_a, the arithmetic average of the deviation from the mean lines, for three scan areas of 200×200 nm^2, 500×500 nm^2 and 1000×1000 nm^2 was 0.10-0.14 nm for glass (Fig.1 (a)), 0.23-0.27 nm for silica (Fig.1 (b)) and 0.13-0.15 nm for silicon wafer (Fig.1 (c)). Both glass and silicon presented granular surfaces with slightly finer one for the glass (compare Fig.1 (a) with (c)), which well corresponded to

the numerical difference in R_a parameters. The surface roughness R_a of the polished silica plate was about twice of the others.

The chemical properties of the surface should also be different among them. The hydrophobicity of each surface obtained by treatment with HMDS gas was compared by water droplet test, i.e. placing the same amount of water on each surface and measuring the droplet diameter. The droplets on silicon and fused silica had almost the same diameter, indicating the same degree of hydrophobicity. While, on the surface of the glass the droplet showed a slightly larger size and gradually widened with time, which indicates a weaker hydrophobicity of the surface than of silicon and silica, or may suggest some chemical sensitivity or reactivity of its surface. Hydrophobicity of the silicon surface was prerequisite for the success of transfer of PBLH/SA film. However, the degree of hydrophobicity of the surface is not the sufficient condition determining the degree of deposition. The attempts of depositing PBLH/SA film onto hydrophobic surface of fused silica eventually failed and only small fragments were found scattered on the surface (data not shown). This means that the PBLH/SA crystal film cannot reside on the silica plate bridging over such a rough surface as shown in Fig.1 (b). On the other hand, the film can fully cover the surface of silicon which is flat at sub-nanometer level but not atomically (Fig.1(c) and (d)). Both the mechanical strength of the PBLH/SA film and the adhesion force between the film and silica plate might be insufficient under the pulling force exerted upon touching and withdrawing the plate from the water surface by horizontal transfer method. The adhesion force per unit area might be weak or the number density of the contact point between PBLH/SA and fused silica surface might be insufficient due to its deep surface profile.

3.2 Deposition of PBLH/SA 2D crystal film onto thermally treated glass coverslip

It would be rational to assume that the transfer of PBLH/SA film is improved if we use a glass with smoother surface. We have previously shown that a prolonged incubation time of silicon in touch with the film at air/water interface improved the uniformity of the transferred PBLH/SA film[1)], suggesting the importance of matching or commensuration between the PBLH/SA film and the substrate surface. We have therefore pursued making the glass surface smoother by thermal treatment. In the production line, a thin glass plate like coverslip is produced by the drawdown method. The glass coverslip thus produced has fairly smooth surface as shown in Fig.1 (a).

We mentioned that the surface of as-purchased glass presented a morphology resembling that of silicon. This surface accepted deposition of PBLH/SA film, but it was not uniformly covered with the film when we applied the same procedure used to obtain

a uniform coverage of silicon. A large fragment of PBLH/SA film was deposited in Fig.2 (a), (c) and sometimes folded and overlapped (Fig.2 (c)). We notice in Fig.2 (b) and (d) that many 2D crystal fragments have the size of ~10×10 SA molecules. The isolated SA molecules sit in the narrow spaces between fragments.

The annealing of the coverslip was examined in the temperature range of 500~700°C. The finally chosen annealing procedure was simple as heating the coverslip to 525°C taking 15 min, keeping it still for 1 h, and cooling it slowly in air to the room temperature by turning off the power of the furnace. Fig.3 (a) suggests that the glass surface got smoother than in Fig.1 (a), and at the same time a number of small craters appeared on the surface. The diameter of the crater is ~20 nm, height of the outer ring is ~1 nm, and the depth of the pit which locates inside is ~2 nm. The surface granularity seems to have disappeared due to a kind of melting of the surface (compare between Fig.1 (a) and Fig.3 (a)). By carefully watching the Fig.3 (a) and (b), we notice the outbreak of small holes or pits, i.e. small dark spots in Fig.3 (b) overall. We can therefore conclude at least that the morphology of the glass surface has changed. Although AFM images gave an impression of change from rough to smooth, the R_a value increased on the contrary from 0.10-0.14 nm to ~0.18 nm. Further, annealing at a higher temperature made the surface to have bigger and deeper dimples with depth of ≥ 4 nm (Fig.3 (c)).

The deposition of the PBLH/SA film onto a 525 °C-treated surface was satisfactorily uniform. A single crystal domain, if the cracks neglected, occupies the left half area in Fig.4 (a) and also nearly single domain occupies in Fig.4 (b). We notice that the PBLH/SA film is highly perturbed or almost missing over the craters (encircled). Taking into account that the height of the crater ring is ~1 nm and bottom of a crater is fairly flat with a smaller pit, we conclude that the PBLH/SA film is neither stiff nor flexible enough to bridge over 1 nm roughness.

3.3 Protein G/IgG assembly fabricated

We have previously shown that the IgG molecules can be immobilized on the silicon surface via binding to adapter molecules of protein G[1)] which had been biotinylated and bound to SA layer (Fig.1 (d)). Similar binding experiment was preliminarily done for the PBLH/SA layer on a glass annealed at 525 ºC. A dense packing or a kind of molecular forest comprising of IgG was formed on glass (Fig.4 (c), (d)). We assume that the antigen binding site of IgG is oriented toward the far side of the substrate as depicted in Fig.4 (e). We would like to postulate that we can directly discriminate by AFM imaging the bound molecular species if they have large difference in the molecular size or shape.

However, as is seen from Fig.4 (c) and (d), the packing of IgG molecules are loose and they are waving with the scanning AFM tip like the forest under a strong wind, which severely degrades the AFM image resolution. If we really desire molecularly resolving image quality, the packing of the IgG molecules, i.e. the antigen binding surface must be much stiffer due to higher packing density of IgG.

In this configuration, denaturation of IgG molecules on interacting with the surface would be faint if probable, so that the stabilization of the PBLH/SA layer on glass becomes next a key property for the immunosensor application of the present system. It had been confirmed for three kinds of PBLH/protein systems, PBLH/SA, PBLH/catalase and PBLH/ferritin, that their binding is stable on silicon wafer, and those bound protein molecules were not quickly detached when agitated in pure water using an ultrasonicator (data not shown). The PBLH/SA on silicon was also stable at high salt concentrations at neutral pH. However, they were very sensitive to detergent molecules and the PBLH/SA structure was shown to be highly perturbed in the presence of low concentration of Tween 20[1)]. Thus, the increased binding stability would be the most important feature for the immunoassay application of the PBLH/SA/Protein G/IgG multilayered system.

Conclusion

We have clearly shown by AFM imaging that annealing of the glass made the surface smoother, resulting in an improvement of the deposition ratios of PBLH/SA film. Like on silicon surface we have prepared on a glass surface a dense packing of IgG molecule, which were bound to the biotinylated protein G immobilized on the SA array via biotin-streptavidin linkage. The patterning of PBLH/SA film with high stability is one of our next subjects of the present system towards protein device applications.

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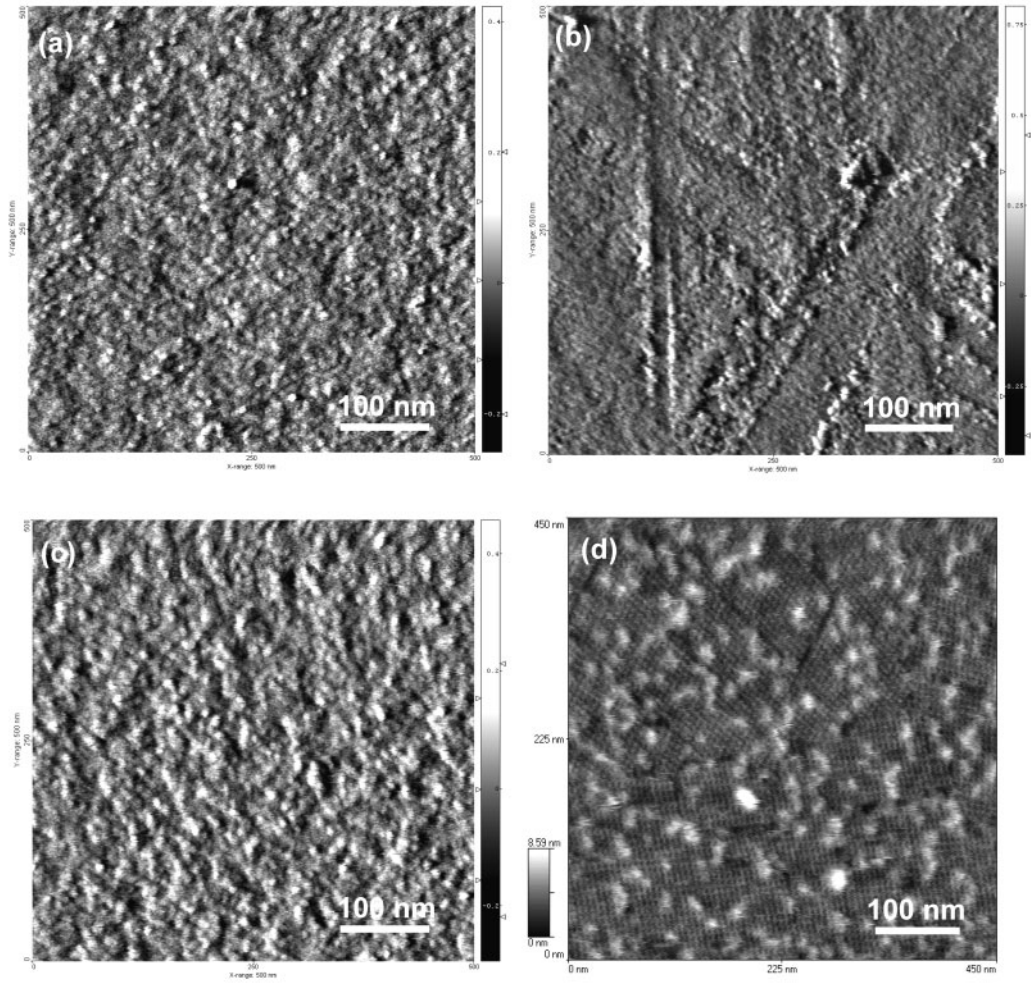

Fig.1 AFM images of the surface of (a) glass coverslip, (b) polished silica plate, (c) silicon wafer, taken by error-signal mode. (d) AFM image of IgG molecules bound to biotinylated protein G immobilized on the SA layer via biotin-SA linkage[1]. The transferred PBLH/SA 2D crystalline film has cracks along lattice lines, but is well packed to uniformly cover the underlying silicon surface.

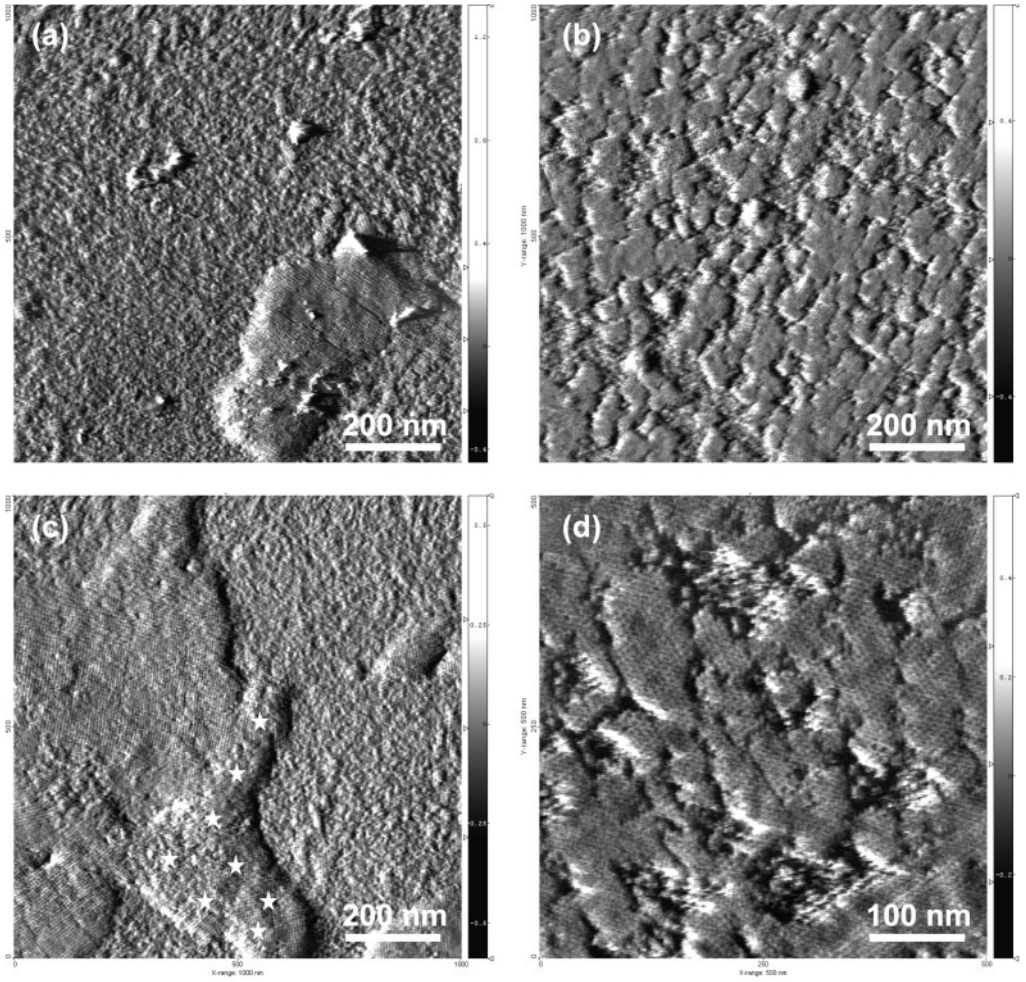

Fig.2 Error signal AFM images of 2D crystalline PBLH/SA film transferred onto as-purchased coverslips with hydrophobic surface. The PBLH/SA fragment in (c) was probably flipped and folded in the asterisked region, which showed about twice the height of the single-thick fragment in the topographic image taken simultaneously (data not shown).

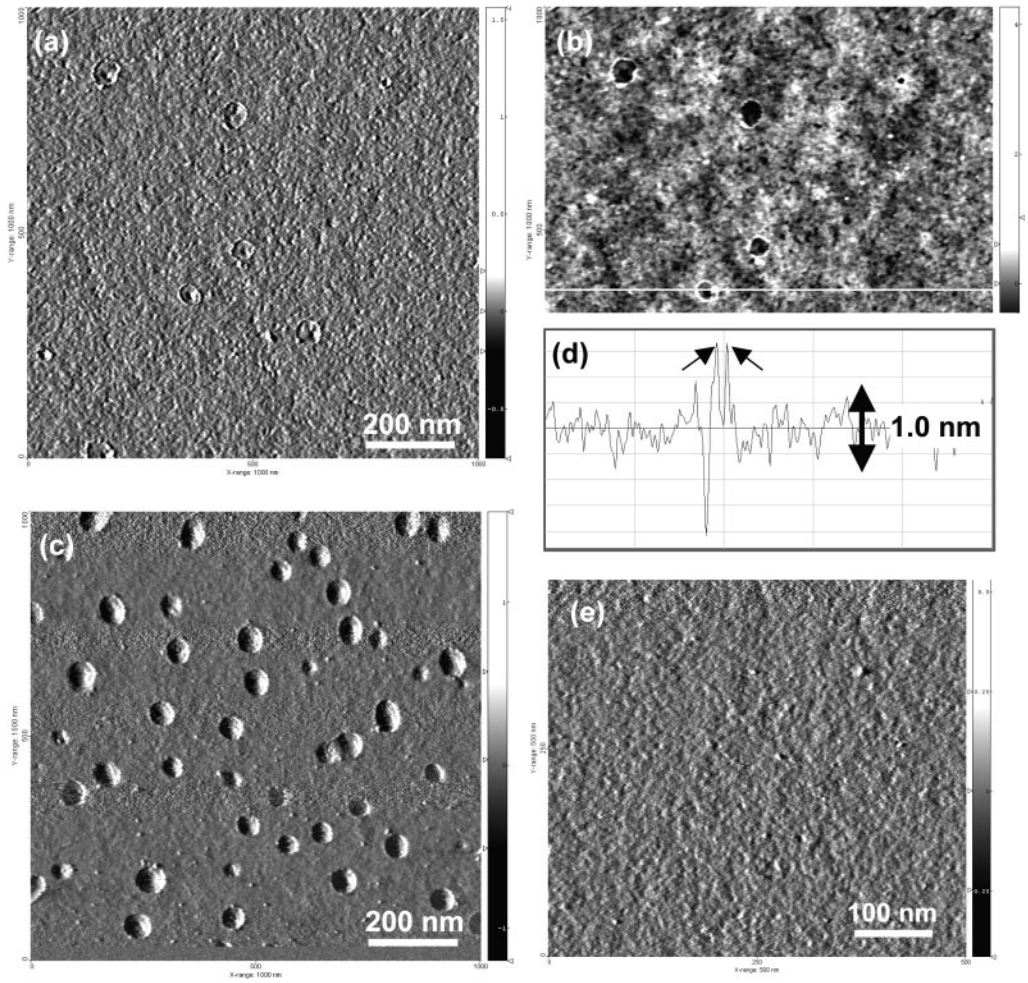

Fig.3 AFM images of the surface of glass. (a), (b) the same area of the coverslip treated at 525 °C, imaged by (a) error signal mode, (b) topography mode. (c) treated at 600 °C (error signal). (d) height profile of the treated coverslip along the white line in (b). The arrow (left) indicates a deposition of a nano-sized granule atop the crater ring and the right arrow indicates another particle sitting aside. (e) another area without crater, treated at 525 °C.

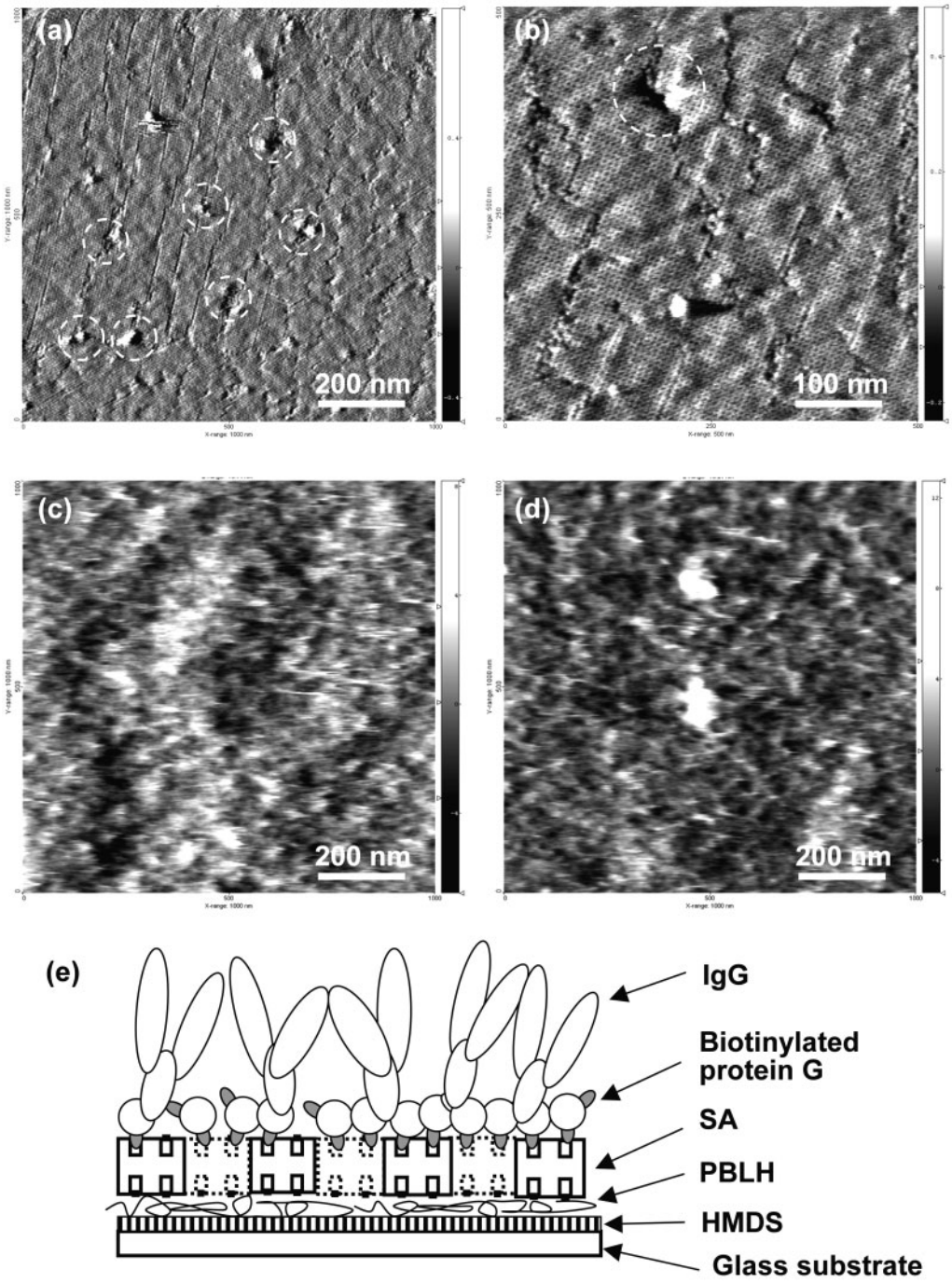

Fig.4 (a), (b) uniform covering with the PBLH/SA film of the surface of glass annealed at 525 °C. Dotted circles in (a) and (b) mark positions of underlying craters. Brightly seen blobs inside the circles are aggregates of PBLH/SA film peeled off at the crater positions. (c), (d) densely packed IgG molecules bound to biotinylated protein G immobilized on the uniform SA film as shown in (a) and (b). (e) cartoon summarizing this multilayer assembly.