Fabrication of avidin single molecular layer on silicon oxide surfaces and formation of tethered lipid bilayer membranes

R. Tero†
Institute for Molecular Science, Myodaiji-cho, Okazaki, 444-8585, Japan,

N. Misawa
Graduate University of Advanced Studies, Myodaiji-cho, Okazaki 444-8585, Japan

H. Watanabe
Computer and Communication Center, Kyushu university, Maidashi 3-1-1, Higashi-ku, Fukuoka, 812-8582, Japan

S. Yamamura
Graduate University of Advanced Studies, Myodaiji-cho, Okazaki 444-8585, Japan,

S. Nambu
Computer and Communication Center, Kyushu university, Maidashi 3-1-1, Higashi-ku, Fukuoka, 812-8582, Japan,

Y. Nonogaki and T. Urisu‡
Institute for Molecular Science, Myodaiji-cho, Okazaki, 444-8585, Japan, and Graduate University of Advanced Studies, Myodaiji-cho, Okazaki 444-8585, Japan
(Received 23 June 2005; Accepted 26 July 2005; Published 30 August 2005)

Single molecular layer of avidin is fabricated on an atomically flat SiO$_2$ surface and characterized by atomic force microscopy (AFM) and infrared reflection absorption spectroscopy. Immobilization of avidin is performed as follows; i) ester-modification of the surface by silane-coupling agent, ii) carboxylation by hydrolysis in HCl and iii) amide bonding between the surface -COOH and -NH$_2$ in avidin molecules. Large dome structures (∼60 nm height) are formed after the ester-modification, but an atomically flat surface is obtained after the hydrolysis reaction. AFM topographs and function-recognizing images show that the each of avidin molecules adsorbs as a single molecule and retains the biotin-binding activity. Formation of a tethered bilayer membrane of a biotinylated phospholipid on the avidin layer is also described. [DOI: 10.1380/ejssnt.2005.237]

Keywords: Atomic force microscopy; Ab initio quantum chemical methods and calculations; Surface chemical reaction; Surface structure, morphology, roughness, and topography; Silicon oxides; Protein immobilization; Molecular recognition; Lipid bilayers

I. INTRODUCTION

Modification of solid surfaces by biomaterials is widely used in the analyzing method in biological and medical fields [1]. Biofunctionalization of device materials, such as silicon and silicon oxides, is an attractive research theme and important to the development of new biosensors and screening methods in which biological reactions are directly detected on electronic circuits. Silicon substrates have several other advantages as the supports of biomaterials; biochemically inactive and does not inhibit biological reactions; high accumulation and condensation on a chip are anticipated by applying the surface fine processing techniques. In order to utilize these advantages, it is necessary to construct and characterize so-called 'well-defined' biofunctionalized surfaces, on which the biological molecules are deposited in a single molecular level retaining their functions.

We have fabricated an avidin single molecular layer covalently immobilized on a SiO$_2$ surface. Avidin-biotin bonding is a well known protein-ligand interaction for the exceptionally high affinity ($K_b > 10^{15}$ M$^{-1}$) [2], and widely used in surface modification in biochemical and bioanalytical fields [1, 2]. A chemically oxidized SiO$_2$ surface was modified with ester group followed by hydrolysis in HCl to obtain a carboxyl-terminated surface. The surface morphological change was observed by atomic force microscopy (AFM), and the transformation of the terminal group was measured by infrared reflection absorption spectra using a buried metal layer (BML-IRRAS) [3]. Avidin molecules are immobilized through the amide bond between the surface COOH and NH$_2$ in the avidin. It is clarified that each of the avidin molecules adsorbs as a single molecule, not in an aggregated state. Function-recognizing AFM observation [4, 5] using a biotinylated cantilever shows that the avidin molecules retain the biotin-binding activity even after the covalent immobilization. Tethered bilayer membranes of biotinylated phospholipid are successively formed on this avidin single molecular layer.

*This paper was presented at International Symposium on Molecule-Based Information Transmission and Reception - Application of Membrane Protein Biofunction- (MB-ITR2005), Okazaki, Japan, 3-7 March, 2005.
†Corresponding author: tero@ims.ac.jp(R.Tero)
‡Corresponding author: urisu@ims.ac.jp(T.Urisu)
AFM images in air were obtained by SPA3800 (Seiko Instruments Inc.) in dynamic force mode. AFM observations in the buffer solutions were performed using PicocPlus (Molecular Imaging ) equipped with recognition imaging system (PicotREC) [4, 5] in magnetic AC mode. The modification of the cantilever by biotin with polyethylene glycol linker is followed to the reference [8].

IRRAS was measured by FT/IR620 (JASCO) in vacuum using the BML substrate which was made by wafer-bonding method [3]. After the spectrum of a CMETS- or COOH-modified surface was obtained, the surface was exposed to UV light for 30 min in air in order to remove the CMETS or COOH layer. Then the spectrum of the UV-exposed sample was measured and subtracted as the background. Calculations of the structural optimization and vibrational frequency were carried out with a Gaussian 98 system of programs using the Hartree-Fock (HF) level with the 6-31+G* basis set. Scaling factors (SF) for the vibrational modes are determined to calibrate the calculated frequency values to the previously reported vibrational frequencies [9–12]. The SF for the CH stretching, CH bending and CO stretching modes are 0.8901, 0.8962 and 0.8820, respectively.

III. RESULTS AND DISCUSSION

Figure 2a shows the SiO2 surface after CMETS deposition (Fig. 1, step a). Large domes of ~60 nm height were observed. The SiO2 surface before the CMETS deposition was atomically flat (rms: 0.18 nm). The roughness of the flat regions between the domes was 0.24 nm (rms). These large domes are probably polymerized CMETS molecules, since the aggregation of polymerized siloxane is sometimes observed in the surface modification using silane coupling agent, for example octadecyltrichlorosilane/SiO2 [7, 13]. Covalent immobilization of CMETS on a SiO2 surface proceeds as follows [14]:

$$\text{R-SiCl}_3 + 3\text{H}_2\text{O} \rightarrow \text{R-Si(OH)}_3 + 3\text{HCl}$$ (1)
$$\text{R-Si(OH)}_3 + 3\text{R}^-\text{OH} \rightarrow \text{R-SiR}_3 + 3\text{H}_2\text{O}$$ (2)

where R is CH$_3$O(CO)C$_2$H$_4$ of CMETS and R$^-$OH is a surface hydroxyl group or another hydrated CMETS molecule. If the hydrated CMETS molecule formed by Eq. (1) reacts with a surface hydroxyl group, the molecule is bound to the surface. The domes shown in Fig. 2a are formed through the reaction (2) between hydrated CMETS molecules. The CMETS domes were resistive to organic solvents, such as acetone and chloroform. The domes stably remained after the sonication in these solvents (data not shown). After sonicated in pure water, some of the domes were broken as shown in Fig. 2b. The image shows that the inside of the dome is empty. It was difficult to remove these domes by washing in water or organic solvents, but these domes can physically be wiped out rather easily. They were almost completely removed by rubbing gently using a cotton swab (Fig 2c).

Figure 3 shows the morphological changes of the COOCH$_3$-modified SiO$_2$ surfaces during the hydrolyzation (Fig. 1, step b). The polymerized-CMETS domes

II. EXPERIMENTAL

As-delivered Si(111) wafers were RCA-cleaned by successive treatment with following solutions; conc. H$_2$SO$_4$+H$_2$O$_2$ (30 %) (4:1 in the volume ratio), HF (5%) and conc. HCl+H$_2$O$_2$ (30%)+H$_2$O (1:1:4). A quite flat (rms roughness of 0.18 nm) chemically oxidized SiO$_2$ layer formed after these processes. Figure 1 shows the reaction scheme of the avidin immobilization on the SiO$_2$ surface. The SiO$_2$ is modified with COOCH$_3$ groups in a buffer solution (150 mM NaCl, 1.0 mM CaCl$_2$) (Avanti) (99:1). The mixture of DMPC+b-DOPE was vacuum-dried in a glass tube and agitated and conc. HCl+H$_2$O$_2$ (30%)+H$_2$O (1:1:4). A quite flat (rms roughness of 0.18 nm) chemically oxidized SiO$_2$ layer formed after these processes. Figure 1 shows the reaction scheme of the avidin immobilization on the SiO$_2$ surface. The SiO$_2$ is modified with COOCH$_3$ groups in a 0.5 mM 2-(carboxmethoxy)ethyltrichlorosilane (CMETS) (Gelest)/toluene solution at -15°C for 1h (Fig. 1, step a). The COOH/SiO$_2$ reagent contains a structural isomer (1-CMETS) less than 15%. The COOCH$_3$/SiO$_2$ was hydrolyzed in 35% HCl aq. at room temperature (RT) for 24 h to obtain COOH-modified SiO$_2$ surface (Fig. 1, b). The COOH/SiO$_2$ was activated in 3.0 mM N-(hydroxysuccinimide (Aldrich) and 1.0 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (Dojindo) of a buffer solution (0.1 M HEPES/NaOH (pH 7.4)) (Fig. 1, c) and was covalently bonded with egg-white avidin (Sigma) in 5-160 mM of avidin/ buffer (0.1 M HEPES/NaOH (pH 7.4)) solution (Fig. 1, d). The excess COOH was deactivated by 0.5 M ethanolamine (Fig. 1, e). Unilamellar vesicles of dimyristoylphosphatidylcholine (DMPC) (Avanti) and biotinylated dioleoylphosphatidylethanolamine (b-DOPE) (Avanti) (99:1). The mixture of DMPC+b-DOPE was vacuum-dried in a glass tube and agitated in a buffer solution (150 mM NaCl, 1.0 mM CaCl$_2$, 10 mM HEPES/NaOH (pH 7.4)), and followed by extraction through a 100 nm polycarbonate filter (Liposo-Fast, Avestin, Inc.). The details about the sample preparation and the experimental conditions were described elsewhere [6, 7].

FIG. 1: Reaction scheme of the surface carboxylation and the avidin immobilization on the SiO$_2$ surface. Details of the steps a–e are described in the experimental section.
FIG. 2: (a) AFM topographs (10 × 10 µm²) and the line profiles of the CMETS-modified SiO2 surfaces. (b) The CMETS/SiO2 surface sonicated in water for 10 min. (c) The CMETS/SiO2 surface wiped by a cotton swab. All images are obtained in air.

FIG. 3: AFM topographs (10 × 10 µm²) and the line profiles of the CMETS-modified SiO2 surfaces (a) before and (b-d) after the hydrolyzation in HCl. The hydrolysis times are (b) 1 h, (c) 4 h and (d) 24 h. All images are obtained in air.

(Fig. 3a) became smaller after 1h hydrolysis in HCl (Fig. 3b). The surface of the domes seemed to be rough and to have many holes. Some of these may be artificial structures caused by AFM cantilever, but Fig. 3b shows that the domes become thin and fragile after the HCl treatment. The domes were hardly recognized after 4 h hydrolyzation (Fig. 3d). The transformation from COOCH3 to COOH was strongly indicated from the change in the surface hydrophilicity. The CMETS/SiO2 surface shown in Fig. 3a was quite hydrophobic, but the hydrolyzed surface shown in Fig. 3d turned hydrophilic. The roughness of the COOH/SiO2 surface (Fig. 3d) was 0.12 nm (rms), as atomically flat as the original SiO2 surface (rms: 0.18 nm).

Figure 4 shows the BML-IRRAS spectra measured for the CMETS-modified SiO2 surfaces before and after hydrolyzation. The IRRAS spectrum of the CMETS/SiO2 (Fig. 4a) surface is obtained from the wiped sample, without the polymerized domes, like Fig. 2c. Vibrational modes at CH stretching, CH bending and CO stretching regions were detected in 2840-2970 cm⁻¹, 1430-1480 cm⁻¹ and 1700-1740 cm⁻¹, respectively. Absorption bands of the CMETS were identified by the theoretical calculation using the clusters models shown in Fig. 5. They are molecular models of two CMETS isomers. The CMETS molecules immobilized on a SiO2 surface through the reactions (1) and (2) are represented by exchanging the three chlorine atoms of the CMETS to OSiT3. Calculated frequencies and assignment of the absorption are summarized in Table 1. The absorbance at 2960 cm⁻¹ is assigned to νa(CH3). The νs(CH2) and νa(CH2) modes of the two carbon atoms in the methylene chain are coupled and appeared at 2918, 2905 cm⁻¹ and 2854, 2845 cm⁻¹, respectively [11]. δ(CH2) modes are 1420 cm⁻¹ (C1) and 1437 cm⁻¹ (C2), and CH2 wagging modes are 1295 cm⁻¹ (C1) and 1364 cm⁻¹ (C2). The strong absorbances at 1738 cm⁻¹ and 1719 cm⁻¹ are assigned to the ν(C=O) of 2-CMETS and 1-CMETS, respectively. The ν(C=O) red-shifted and their intensity drastically decreased after the hydrolysis in HCl (Fig. 4b). These changes will be because of COOH formation; ν(C=O) red-shift is caused by hydrogen bond

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TABLE I: Wave numbers of CMETS vibrational modes measured by BML-IRRAS (Fig. 4a) and theoretically calculated using the cluster models in Fig. 5.

| Vibrational mode | Experiment (cm\(^{-1}\)) | Calculation (cm\(^{-1}\)) |
|------------------|--------------------------|-----------------------------|
| \(\nu_a(CH_3)\)  | 2960                     | 2976                        |
| \(\nu_a(CH_2)\) (C1, C2) | 2918  | 2903                        |
|                  | 2905                     | 2891                        |
| \(\nu_c(CH_2)\) (C1, C2) | 2854  | 2854                        |
|                  | 2845                     | 2840                        |
| \(\delta(CH_2)\) (C2) | 1437  | 1432                        |
|                  | 1420                     | 1409                        |
| \(CH_2\) wagging (C2) | 1364  | 1375                        |
|                  | 1295                     | 1293                        |
| \(\nu(C=O)\)     | 1738                     | 1761                        |
|                  | 1719                     | 1732                        |
| \(\nu(C-O)\)     | 1224                     | 1225                        |
|                  | 1212                     |                             |

FIG. 4: BML-IRRAS spectra of CMETS-modified SiO\(_2\) surfaces (a) before and (b) after the hydrolyzation in HCl for 24 h.

formation between the neighboring COOH groups; the decrease in intensity is due to the orientational change of the molecule caused by the hydrolyzation, since the vibrational modes perpendicular to the surface is enhanced in IRRAS measurements. The \(\nu(CH_3)\) mode is still observed at 2966 cm\(^{-1}\) even after the hydrolyzation. It is partially due to the CH\(_3\) group of the 1-CMETS, but a part of the 2-CMETS molecules may not be hydrolyzed. The results shown in Fig. 3 and Fig. 4 reveal that the SiO\(_2\) surface is modified by COOH keeping the atomic flatness.

Avidin molecules are covalently immobilized on the COOH-modified SiO\(_2\) surface following the reaction scheme in Fig. 1, steps c and d. The deposited amount of the avidin is controlled by the concentration of the avidin solution (5-160 nM). Figures 6a and 6b show the AFM images of the COOH/SiO\(_2\) surface after the deposition of avidin molecules in 5 nM and 160 nM solutions, respectively. Protrusions with uniform size are observed on the surface, and increase with the avidin concentration. The averaged height and radius of the protrusions is 1.7 nm and 12.7 nm (n=270), respectively. We have evaluated the volume of the protrusion at 150 nm\(^3\), assuming the curvature radius of the cantilever 20 nm [6]. The value is well corresponding to the volume of a single avidin molecule, 135 nm\(^3\), which has been estimated from the molecular weight of avidin (\(\sim\)70 kDa) [15]. Therefore, each of the protrusions observed in Fig. 6a is a single avidin molecule, not an aggregation of the avidin. When we immersed mica substrates in an aqueous solution of streptavidin, the streptavidin physisorbed on the surface in aggregated states (data not shown). The single molecular adsorption of avidin is achieved by the covalent immobilization using the protocol in Fig. 1 on the atomically flat SiO\(_2\) surface. After the surface is soaked in the 160 nM avidin solution, the surface is almost fully covered with the avidin molecules, and the single molecular layer of avidin is successively fabricated (Fig. 6b).
Functions of proteins are closely related to their structures, which is strongly affected by the surrounding environment. In the deposition of proteins onto the solid surfaces, it is necessary to pay attention to the denaturing and the deactivation of the proteins and their functions. Hence we have investigated whether each of the immobilized avidin molecules retains the biotin-binding function, by means of function-recognizing-AFM imaging [4, 5]. In this imaging mode, the attractive force mapping between the surface and the cantilever is obtained simultaneously with the topograph. An additional attractive force due to the avidin-biotin interaction is detected when the biotin-modified cantilever [8] scans above the avidin molecules on the surface, if they retain the biotin-binding activity. Figures 7a and 7b show the topograph and the recognition image obtained by a biotin-modified cantilever.
tion of the avidin molecules in the topograph is observed as a depression in the recognition image. This means that an attractive force is applied to the biotin-modified cantilever on the avidin molecules. We have performed the blocking experiment [4] in order to exclude the possibilities of artificial attractions, such as a hydrophobic affinity of contaminations. Figures 7c and 7d are the topograph and the recognition image, respectively, continuously observed after the observation of Figs. 7a and 7b. Avidin solution is injected into the liquid phase at the blue arrow in Fig. 7d, and the avidin reached to the cantilever at the red arrow. After the avidin in the solution adsorbed on the biotin on the cantilever, recognition signal of the avidin disappeared (Fig. 7d) even though the avidin is observed in the topograph (Fig. 7c). This result shows that the avidin molecules covalently immobilized on the SiO$_2$ surface retains their biotin-binding function.

We have performed deposition of a tethered bilayer membrane of a biotinylated phospholipid on the avidin layer, as an example of the deposition of biomaterials on solid surfaces bridged by avidin-biotin bond. The ‘tethered membrane’ is a supported lipid bilayer membrane, which is linked to substrates through for example poly(ethylene glycol) (PEG) [16–18]. The linker holds the fluid membrane stably, and works as a separator between the substrate and the membrane or membrane proteins, since these delicate biomaterials may be denatured by the direct interaction with inorganic substrates. Figure 8a shows the full-coverage avidin layer which is prepared in the same condition as that in Fig. 6b after the incubation in the 100-nm-filtered DMPC+b-DOPE (99:1) vesicle suspension. The adsorbed vesicles remained on the surface and planar lipid bilayer does not form. It was reported recently that the adsorbed vesicles on the avidin-modified aluminum oxide surface transformed to planar membrane by the addition of PEG [19], which was a generally used cell-fusion inducer [20]. We have used smaller unilamellar vesicles to fabricate the planar bilayer without using the fusion agent. The 100-nm-filtered suspension was sonicated for 10 min and deposited on the avidin/SiO$_2$ surface. Not only adsorbed vesicles, but also a planar membrane of 5.5 nm thickness is observed (Fig. 8b). The thickness is corresponding to that of the single bilayer membrane [7, 21]. We suppose that the sonicated suspension is a mixture of various size of vesicles (but less than 100 nm) and hence crushed smaller vesicles by sonication transform to planar membrane and rather larger ones remained without rupturing, because smaller vesicles become planar membrane more easily due to the large surface tension [21]. In a low membrane coverage region, a layer of 3.3 nm thickness is observed under the lipid bilayer (5.7 nm) (Fig. 8c). The results shows that single tethered membrane is formed on the avidin layer as illustrated in Fig. 8d.

The atomically flat COOH-modified SiO$_2$ surface (Fig. 3d) and the formation of the densely packed, but not aggregated, single molecular layer of avidin in sufficiently wide area (Fig. 6c) make it possible to observe a single lipid bilayer membrane of ~5 nm thickness. The surface roughness is an important factor in the experimental methods of surface science. We believe that the single molecular layer of avidin in the present study can be used as a suitable substrate to investigate the function and structure of biomaterials, such as antigen, antibody and DNA, using surface science techniques, since the biotinylation of these biomolecules is already established and widely used technique.

IV. CONCLUSION

Covalently immobilized single molecular layer of avidin is fabricated on a chemically oxidized SiO$_2$ surface. It is confirmed from the AFM observation and BML-IRRAS measurement that each of avidin molecules adsorbs as a single molecule, not in an aggregated state. Function recognizing AFM observation using biotinylated cantilever shows that the biotin-binding activity is retained even after the covalent immobilization. Tethered bilayer membranes of biotinylated phospholipid are formed on the full-coverage avidin layer by incubation in a sonicated vesicle suspension.

Acknowledgments

The authors appreciate the valuable advises from Dr. A. Hirano and Prof. M. Sugawara in Nihon University on avidin immobilization reactions. We are also grateful to Dr. T. Jing and Dr. W.T. Johnson in Molecular Imaging for the instruction in the recognition AFM imaging. This work was partially supported by the Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports Science and Technology, (2001-2006, 13GS0016).

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