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Direct Observation of Adsorption-Induced Inactivation of Antibody Fragments Surrounded by Mixed-PEG Layer on a Gold Surface

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Abstract: To examine the adsorption behavior of antibody fragments (Fab′) directly immobilized on a gold surface through S−Au linkage, analyses by surface plasmon resonance (SPR), fluorometry, and atomic force microscopy (AFM) with an excellent blocking technique by the consecutive treatments of longer-poly(ethylene glycol) (PEG) (MW = 5k) and shorter-PEG (MW = 2k), abbreviated as mixed-PEG layer formation, were performed. The results of the SPR analysis suggest that the adsorption-induced inactivation of the antigen-binding activity of Fab′ took place gradually on the gold surface, where the activity disappeared almost completely at 60 min after Fab′ immobilization. In contrast, in the case of Fab′ coimmobilized by the mixed-PEG layer, 70% of the initial antigen-binding activity of the Fab′ was retained even 60 min after the construction of the hybrid surface. Using fluorescein-labeled Fab′ (FL-Fab′), fluorescence measurement of the constructed surface was carried out. The fluorescence of the FL-Fab′ without any blocking agent on the gold surface was gradually quenched and finally decreased to 40% of the initial intensity 60 min after Fab′ immobilization. The decrease in the fluorescence intensity is considered to be caused by the change in the distance between the fluorophores labeled on the Fab′ and the gold surface, due to the energy transfer from the fluorophores to the gold surface. In contrast, 75% of the initial intensity was observed on the Fab′/mixed-PEG coimmobilized surface. The results obtained from the SPR and fluorometric analyses correlated well with each other; thus, the surface-induced inactivation of the antigen-binding functionality was presumably due to the conformational and/or orientation change of Fab′ on the gold surface. AFM studies provided direct information on the time-dependent decrease in the height of the immobilized Fab′ on the gold surface. In contrast, the coimmobilization of densely packed mixed-PEG tethered chains around the Fab′ on the gold surface suppressed the decrease in the height of Fab′, presumably indicating that the conformational and/or orientation change of Fab′ was suppressed by the coimmobilized mixed-PEG layer. The new findings obtained in this study are expected to be useful for the improvement of the antibody fragment method and, thus, for the construction of high-performance immuno-surfaces.

Introduction

Protein-immobilized substrates have been widely used in a number of applications for biosensing and bioseparation systems.[1,2] For most of these systems, a critical consideration regarding protein immobilization is the effective expression of the protein's function on a solid surface. Protein adsorption onto the surface is a complex process induced by van der Waals, hydrophobic and electrostatic interactions, as well as hydrogen bonding. The quantity, density, conformation, and orientation of the adsorbed protein strongly depend on the immobilization technique and the surface characteristics of the protein and the substrate.[3−7] Although surface−protein interactions are not well

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understood, immobilization chemistry has played a fundamental role to determine protein function on a solid surface. Early immobilization techniques were based on the physical adsorption of antibodies onto a solid surface without any linkers and layers such as plastic, glass, or inorganic substrates. To construct protein-immobilized substrates through covalent linkage, numerous linkage strategies have been developed using reactive group-terminated surfaces.[8,9] A common method for protein immobilization is based on the reaction between amino residues in the protein and amino-reactive groups located on substrates such as *N*-hydroxysuccinimide, periodate, gluteraldehyde, isothiocyanate, and active ester.[8,10,12] Although these techniques are simple and protein-immobilized surfaces are easy to construct using this method, it often induces a subsequent decrease in protein activity due to the immobilization with random orientation[11−14] and the adsorption-induced conformational change[15−20] of the protein.

Several linkage chemistries have been suggested to improve the protein orientation on a solid surface. When the structure of a protein is known, amino- or carboxy-terminal tags can be introduced into the protein by the recombinant technique. For instance, a histidine tag can be used to immobilize the protein in an oriented fashion using a Ni-NTA-terminated surface.[21,22] In the case of antibodies, some methods for their immobilization on a substrate in a highly oriented manner have been suggested. Since an antibody contains a conserved glycosylation site on the Fc domain, it can be oxidized and then biotinylated easily using biotin hydrazide. The biotinylated antibody thus prepared can be immobilized onto a streptavidin-immobilized substrate. Alternatively, antibodies can also be oriented on a surface through binding to immobilized protein A or G,[23−25] which specifically targets the Fc region of an antibody, but a proper methodology for protein A or G immobilization in a highly oriented manner is required.[25] In recent years, there has emerged another oriented immobilization methodology for antibodies which uses the native sulfanyl[26] (SH) groups on the antibody liberated after the splitting of the intact immunoglobulin (IgG) into two antibody fragments (Fab′). Since the SH group of Fab′ is structurally opposite to the antigen-binding site, Fab′ immobilization in a highly oriented manner can be accomplished on an SH reactive linker-terminated substrate through covalent attachment.[14,27−29] For example, Lu et al. have reported that a Fab′ oriented immuno-surface constructed on an SH-terminated silica surface resulted in enhanced antigen-binding activity when compared to surfaces prepared via random immobilization.[28] Since the chemisorption of the SH group to Au is very selective and strong, direct immobilization of antibody fragments onto a gold surface has been proposed.[30−34] O'Brien and co-workers have revealed that directly immobilized Fab′ fragments on a gold surface are useful for preparing antigenic surfaces with enhanced epitope densities relative to surfaces modified with nonspecifically adsorbed whole-molecule IgG.[31] Brogen et al.[33] and Harada et al.[34] have also reported that atomic force microscopy (AFM) probes functionalized with directly immobilized Fab′ more specifically detected surface-bound antigens than those modified with randomly immobilized Fab′ using several spacers.

As described above, direct immobilization of Fab′ onto a gold surface through S−Au linkage is one of the efficient strategies for constructing an immune-surface in a highly oriented manner. Several researchers have investigated the binding activity of immobilized Fab′ from the perspective of surface coverage[33,33] and linker effect;[29,35−37] however, its conformational change on a bare gold surface has not been investigated sufficiently to date.

Here, we focus on the time-dependent changes in the binding activity and structure of Fab′ directly immobilized on a gold surface though Au−S linkage. To evaluate the antigen-binding

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properties accurately by means of a surface plasmon resonance (SPR) sensor while avoiding possible nonspecific binding, we employed our original blocking technique in this study[10,38,39] viz., mixed poly(ethylene glycol) (PEG) tethered chains composed of PEGs with different molecular weights, PEG(5k) and PEG(2k), abbreviated as mixed-PEG layer formation, were constructed around the Fab′ immobilized on the gold surface. The mixed-PEG layer consisting of a combination of PEG(5k) and PEG(2k) showed an extremely high nonfouling characteristic as compared to a packing of the PEG chains.

During the course of this study, we found that the reactivity of the Fab′ immobilized on the gold surface gradually decreased and finally disappeared completely without any blocking treatment. Interestingly, SPR analysis revealed that the consecutive mixed-PEG layer formation just after Fab′ immobilization prevented the inactivation of its antigen-binding function. Energy transfer analysis by fluorescence spectrometry indicated the conformational and/or orientation change of the immobilized Fab′. In addition, the direct surface analysis by AFM suggested that the conformational change of the immobilized Fab′ took place on the gold surface, while the densely packed PEG tethered chains prepared by mixed-PEG layer formation with PEG(5k) and PEG(2k) just after Fab′ immobilization effectively prevented a large conformational change. Although the conformational change of proteins adsorbed on surfaces has been addressed in previous issues,[15–20] to our knowledge, this is the first report to reveal the time-dependent inactivation and conformational change of Fab′ immobilized on a gold surface. Furthermore, the effective prevention of the inactivation and conformational change of Fab′ immobilized on the gold surface by the coimmobilized mixed-PEG layer surrounding the pre-immobilized Fab′ was demonstrated in this study.

Materials and Methods

Materials. SH-terminated semitelechelic poly(ethylene glycol)s [PEG-SH (M_n = 5000 and 2000); NOF Corp., Tokyo, Japan], dithiothreitol (DTT; Wako Pure Chemical Industries, Ltd., Osaka, Japan), fluorescein isothiocyanate (FITC; Sigma-Aldrich, Inc., St. Louis, MO), bovine serum albumin (BSA; Sigma-Aldrich, Inc., St. Louis, MO), c-reactive protein (CRP; Mitsubishi Kagaku Iatron, Inc., Chiba, Japan), polyclonal anti-CRP antibody Fab′$_2$ (Mitsubishi Kagaku Iatron, Inc., Chiba, Japan), disodium hydrogenphosphate 12-hydrate (Wako Pure Chemical Industries, Ltd., Osaka, Japan), sodium dihydrogenphosphate dihydrate (Wako Pure Chemical Industries, Ltd., Osaka, Japan), sodium chloride (Nakalai Tesque, Inc., Tokyo, Japan), Na^{125}I (PerkinElmer Co., Ltd., Japan) and sodium *N*-chloro-*p*-toluenesulfonamide (chloramine T; Wako Pure Chemical Industries, Ltd., Osaka, Japan) were used as received. The water used in this study was purified using the Milli-Q system (Nihon Millipore Co., Tokyo, Japan).

Preparation of Fab′, ^{125}I-Labeled Fab′, and Fluorescein-Labeled Fab′. Fab′ of CRP was prepared by the following procedure: 153 μL of Fab′$_2$ (23 μM) was mixed with 12 μL of DTT (10 mM) to reduce the disulfide bond in the hinge region, and the mixture was diluted with 835 μL of 50 mM phosphate buffer solution containing 150 mM NaCl and 5 mM EDTA (pH 7.4) (PBS−EDTA buffer). After 90 min of reaction at room temperature, the mixture was poured into a centrifugal filter unit (Microcon YM-3, MWCO = 3000, Millipore Corp., Billerica, MA) to remove the excess DTT. Further purification based on fast protein liquid chromatography (FLPC) was performed using an AKTA purifier with an HR column (GE Healthcare Europe) at a constant flow rate of 0.5 mL/min; PBS−EDTA buffer was used as the elution buffer.

^{125}I-labeled Fab′ of CRP was prepared by a procedure based on the chloramine T method, as follows: 200 μL of Fab′$_2$ solution (1 mg/mL) was mixed with 200 μL of Na^{125}I and 90 μL of chloramine T solution (3 mg/mL). After stirring for 1 min at room temperature, the reaction was stopped by adding 100 μL of sodium persulfate solution (10 mg/mL). To remove the unreacted ^{125}I, gel filtration was carried out on a PD-10 column (Amersham Biosciences, Uppsala, Sweden) using PBS−EDTA buffer. The purified ^{125}I-labeled Fab′$_2$ was then reduced by the procedure described above, and ^{125}I-labeled Fab′ was obtained after purification in a centrifugal filter unit (Microcon YM-10, MWCO = 10 000).

Fluorescein (FL) labeled- Fab′ of CRP was prepared by the following procedure: FL labeled-Fab′$_2$ was prepared by reacting excess FITC with Fab′$_2$ (overnight at 4 °C and pH 7.4), followed by purification in Microcon YM-10. The Fab′ fragmentation of FL-labeled Fab′$_2$ was carried out by the procedure described above, followed by purification in Microcon YM-10, MWCO = 10 000.

The obtained Fab′, ^{125}I-labeled Fab′, and FL-labeled Fab′ were checked using 10% SDS−polyacrylamide gel electrophoresis (SDS−PAGE) and Coomassie Blue staining or autoradiography techniques. The concentrations of these Fab′s were determined by measuring their absorbance, ε = 1.48 L cm^{-1} g^{-1} at 280 nm.

SPR Analysis. SPR analysis was performed on a Biacore 3000 device (Biacore AB, Uppsala, Sweden), and the adsorption behavior of Fab′ and PEG-SH on the bare gold surface was monitored by the SPR angle shift on the sensor surface. Gold sensor chips (SIA kit Au, Biacore AB, Uppsala, Sweden) for SPR analysis were purchased from Biacore AB. The bare gold SPR sensor chips were cleaned with piranha solution (3:1 volume of concentrated sulfuric acid and 30% hydrogen peroxide) at room temperature and then rinsed with a copious amount of water prior to use. After the bare gold sensor chip was docked into the SPR instrument, the Fab′/mixed-PEG coimmobilized surface was constructed as follows. The immobilization of Fab′ onto the bare gold surface was first carried out by the injection of 1 μM Fab′ in PBS-EDTA buffer for 4 min. Then, the surface was PEGylated by the injection of 200 μM of the longer PEG-SH(5k) for 30 min, followed by the injection of 200 μM of the shorter PEG-SH(2k) for 30 min twice. As control surfaces, monolithic Fab′/PEG(5k) and Fab′/PEG(2k) coimmobilized surfaces were constructed by the immobilization of Fab′ followed by PEGylation with PEG-SH(5k) and PEG-SH(2k), respectively. All sample injections were carried out at a constant flow rate of 5 μL/min at 25 °C, using PBS-EDTA buffer as a running buffer solution.

To evaluate the antigen-binding activity of Fab′ on the Fab′/PEG coimmobilized gold surfaces, the detection of CRP on the constructed surfaces was carried out by the injection of 1 μM CRP in a PBS-EDTA buffer for 30 min. As a control, the SPR response caused by the injection of 1 μM BSA was also measured on the constructed surfaces. All the SPR measurements were carried out at a constant flow rate of 5 μL/min at 25 °C. The obtained SPR angle shifts were averaged from four individual experiments (n = 4).

Radiometric Assay of ^{125}I-Fab′ on the Gold Surface. To determine the surface density of Fab′ immobilized on the gold surface before and after PEGylation, a radiometric assay using ^{125}I-Fab′ was carried out. In this experiment, in order to avoid the adsorption of ^{125}I-Fab′ on the opposite side of the gold chip surface (glass surface), the glass surface was covered with adhesive tape and the tape was peeled off prior to the radiometric measurement. The ^{125}I-Fab′ immobilized and ^{125}I-Fab′/mixed-PEG coimmobilized gold surfaces were prepared in batch experiments, as follows: the immersion of a bare gold chip for SPR into 1 μM Fab′ in PBS-EDTA buffer was carried out for 4 min. After washing with the buffer solution, the chip was immersed in the buffer solution for

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different time (0, 10, 20, 30, and 60 min). After peeling off the adhesion tape, the surface density of Fab′ on Fab′ immobilized gold surfaces was determined as 0.054 ± 0.002 molecules/nm^2 by measuring the radioactivity using γ-counter (Cobra 5005/5010, Auto-Gamma Counting Systems, Packard, Meriden, CT). The adhesion tape prevented the adsorption of ^{125}I-Fab′ to the back side of surface effectively. The counts per minute (cpm) of radioactivity of the constructed surfaces exceeded 12 000 cpm, while the that of reference surface prepared by immersion into buffer solution only was extremely low value (about 100−200 cpm).

To construct Fab′/mixed-PEG coimmobilized surface, the ^{125}I-Fab′-modified gold surface was immersed in 200 μM PEG-SH (5k) solution with the buffer for 30 min, followed by 200 μM PEG-SH(2k) solution with the buffer for 30 min twice. The constructed ^{125}I-Fab′/mixed-PEG coimmobilized chip was washed and immersed in the buffer solution for different times (0, 10, 20, 30, and 60 min). The radioactivity on the ^{125}I-Fab′/mixed-PEG coimmobilized surface thus prepared was measured by a γ-counter. As shown in Figure S1, the surface density of Fab′ on Fab′/mixed-PEG coimmobilized gold surface was determined as about 0.05 molecules/nm^2, indicating that the desorption of Fab′ by PEG coimmobilization did not take place during this experimental time (60 min). The radioactivities of the ^{125}I-Fab′ immobilized on the gold surface from three individual experiments (n = 3) were averaged.

Fluorescence Measurement of the FL-Fab′-Immobilized Gold Surface. A FL-Fab′-immobilized gold surface was constructed and monitored by a fluorescence micro plate reader (ARVO MX, PerkinElmer Japan Co., Ltd., Yokohama, Japan). In this experiment, all the incubation steps were carried out by the formation of 15-μL droplets of the solutions on the surface. FL-Fab′-immobilized and FL-Fab′/mixed-PEG coimmobilized gold surfaces were prepared in batch experiments, as follows: the incubation of a bare gold chip for SPR (SIA kit Au, Biacore AB, Uppsala, Sweden) by 1 μM Fab′ in PBS-EDTA buffer was carried out for 4 min. After washing with the buffer solution, the chip was incubated in the buffer solution for different times (0, 10, 20, 30, and 60 min). In the case of the FL-Fab′/mixed-PEG coimmobilized gold surface, consecutive PEGylation just after Fab′ immobilization was performed with 200 μM PEG-SH(5k) solution and the buffer for 30 min, followed by 200 μM PEG-SH(2k) solution and the buffer for 30 min twice. The constructed FL-Fab′/mixed-PEG coimmobilized chip was washed and incubated with the buffer solution for different times (0, 10, 20, 30, and 60 min). As a control, a physically adsorbed Fab′-modified glass surface was prepared by the incubation of a glass surface by 1 μM Fab′ in PBS-EDTA buffer. After washing with the buffer solution, the chips were incubated in the buffer solution for different times (0, 10, 20, 30, and 60 min). The obtained fluorescence data were averaged from three individual experiments (n = 3).

Atomic Force Microscopy. A commercial AFM (MFP-3D-Bio AFM, Asylum Research, Santa Barbara, CA) was used. The AFM has a closed loop on the *x*-, *y*-, and *z*-axes. AFM imaging was obtained in the tapping mode (AC mode) using commercial Si_3N_4 microfabricated cantilevers (BL-AC40TS-C2, Olympus). The spring constant of the cantilevers and the scan rate were 0.1 N/m and 0.7−1 Hz, respectively. Before each experiment, the cantilevers were cleaned with ozone, and their spring constant was determined in air using the thermal power spectrum of the cantilevers. For the AFM analysis, Fab′-immobilized and Fab′/mixed-PEG coimmobilized gold surfaces were constructed on a gold-coated mica substrate. The Fab′/mixed-PEG coimmobilized gold surface was prepared as follows: the immersion of a bare gold chip into 1 μM Fab′ in PBS-EDTA buffer was carried out for 4 min. After washing with the buffer solution, the Fab′-modified gold chip was immersed in 200 μM PEG-SH(5k) in the buffer solution for 30 min, followed by 200 μM PEG-SH(2k) in the buffer solution for 30 min twice. All the measurements were carried out in PBS-EDTA buffer solution.

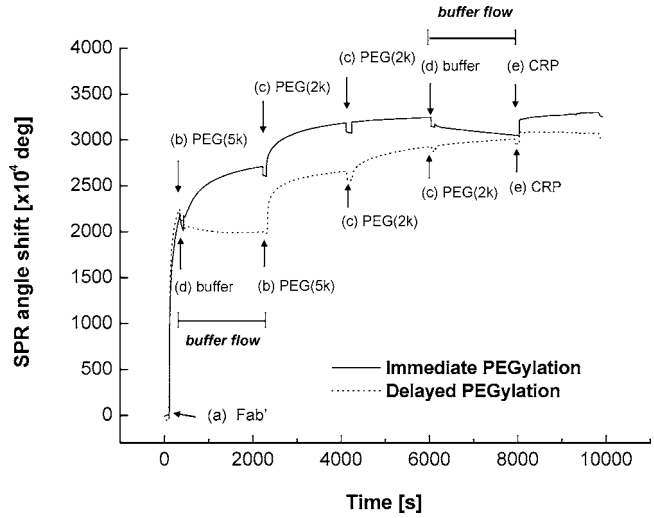

Figure 1. Typical SPR sensograms for the construction of Fab′/mixed-PEG coimmobilized gold surfaces. The changes in the SPR angle shift were caused by the injection of (a) 1 μM Fab′, (b) 200 μM PEG-SH(5k), (c) 200 μM PEG-SH(2k), (d) 50 mM sodium phosphate buffer containing 150 mM NaCl (pH 7.4), and (e) 1 μM CRP. The solid line is the sensogram for immediate PEGylation, where modification with PEG-SH(5k) was carried out immediately after the end of Fab′ immobilization. The dotted line indicates that, 30 min after the end of the injection of the Fab′ solution, PEGylation with PEG-SH(5k) was carried out.

Results and Discussion

SPR Studies of the Antigen-Binding Activity of Fab′/PEG Coimmobilized Gold Surfaces. The CRP-binding activity of Fab′ of anti-CRP antibody immobilized on the gold surface was evaluated by means of an SPR sensor. In this study, we performed PEG immobilization to prevent nonspecific adsorption on the surface. The gold surface for the analysis was prepared by Fab′ immobilization, followed by modification with a mixed-PEG layer by consecutive treatments with the longer PEG-SH(5k) and the shorter PEG-SH(2k). The utility of the mixed-PEG layer on the gold surface has been reported in our previous papers;[9,38,39] the gold surface modified with the mixed-PEG layer showed a higher nonfouling characteristic than the monolithic PEG(5k)- or PEG(2k)-modified gold surface.[39] Table S1 in the Supporting Information shows the SPR angle shift caused by BSA adsorption on the Fab′/mixed-PEG surface, Fab′/PEG(2k) surface, and Fab′/PEG(5k) surface, where the Fab′/mixed-PEG surface showed an almost completely nonfouling characteristic; this indicates that the formation of a mixed-PEG layer is applicable to the Fab′-immobilized gold surface under these experimental conditions.

The CRP-binding activity of the Fab′ immobilized on the gold surface was evaluated on a Fab′/mixed-PEG surface. During the experimental trials for the construction of the Fab′/mixed-PEG surface, we found that the CRP-binding activity of Fab′ on the constructed Fab′/mixed-PEG surface was strongly affected by the length of time from the end of the Fab′ injection to the start of the PEG-SH(5k) injection. Figure 1 shows the typical sensograms for the construction of the Fab′/mixed-PEG coimmobilized gold surface and the detection of CRP. Two kinds of sensograms for the construction of the Fab′/mixed-PEG coimmobilized gold surface are shown in this figure. One

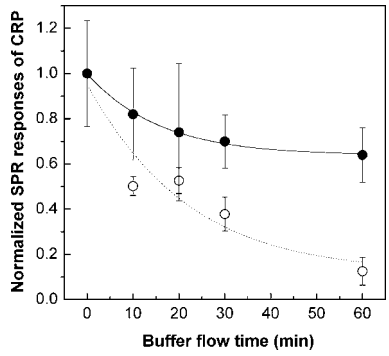

Figure 2. Normalized SPR response of the binding of CRP on the Fab′/mixed-PEG coimmobilized gold surfaces prepared by immediate PEGylation (closed circles) and delayed PEGylation (open circles). PEGylations were carried out after the end of Fab′ immobilization. The buffer flow times for the surfaces prepared by immediate and delayed PEGylations were taken after Fab′ immobilization and PEGylation, respectively. All the SPR experiments were carried out at a constant flow rate of 5 μL/min at 25 °C ($n = 4$, ± SEM).

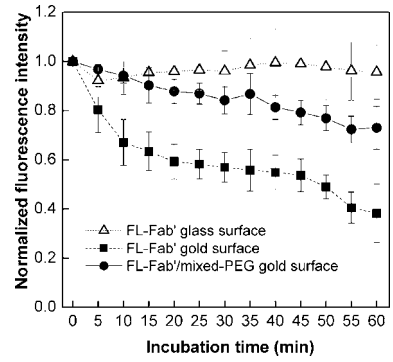

Figure 3. Changes in the fluorescence intensity of three FL-Fab′-modified surfaces, a glass surface (open triangles), a gold surface (closed squares), and a gold surface with immediate PEGylation (closed circles). The incubation in buffer solution was demonstrated after preparation of these surfaces.

represents consecutive PEGylation with PEG(5k) just after Fab′ immobilization [immediate PEGylation (solid line in Figure 1): (a) Fab′ → (b) PEG-SH(5k) → (c) PEG-SH(2k) → (c) PEG-SH(2k) → (d) buffer flow for 30 min, each PEG immobilization being performed for 30 min]. The other is delayed PEGylation with PEG(5k) at 30 min after Fab′ immobilization [delayed PEGylation (dotted line in Figure 1): (a) Fab′ → (d) buffer flow for 30 min → (b) PEG-SH(5k) → (c) PEG-SH(2k) → (c) PEG-SH(2k), each PEG immobilization being performed for 30 min]. Interestingly, a definite SPR angle shift (ca. 200°) was observed when 1 μM CPR solution was injected onto the Fab′/mixed-PEG surface that was prepared by immediate PEGylation [solid line (e)], while a slight SPR angle shift (ca. 50°) was observed when the surface Fab′ was left for 30 min without PEGylation (delayed PEGylation) [dotted line (e)]. These results suggested that the antigen-binding activity on the Fab′/mixed-PEG surface, which had been left exposed for 30 min prior to PEG(5k) injection, had drastically decreased [dotted line (d)−(b)]. Figure 2 shows the changes in the SPR responses of CRP binding onto the Fab′/mixed-PEG gold surfaces as a function of delayed time length from the end of Fab′ injection to the start of PEG-SH(5k) injection. On the Fab′/mixed-PEG surface prepared by delayed PEGylation, the normalized SPR response caused by CRP binding, which was normalized to the initial response (at 0 min-delayed time), decreased as the length of delayed time increased, and about 90% of the binding activity was lost on the constructed surface at 60 min. In contrast, on the Fab′/mixed-PEG surface prepared by immediate PEGylation, the CRP binding activity did not decrease significantly as the delayed time prior to CRP injection [solid line (d)−(e) in Figure 1] increased, and about 70% of the initial CRP binding activity was observed at 60 min. To confirm if the observed decrease in binding activity was derived from the desorption of Fab′ from the gold surface, quantitative analysis was carried out, and the results are shown in Figure S1 in the Supporting Information. From the radiometric assay using ^{125}I-labeled Fab′, no desorption was observed during this experimental time period (60 min). These results clearly indicate that the binding activity of the Fab′ immobilized on the gold surface without any PEGylation strongly decreased and finally disappeared almost completely after 60 min. In contrast, the immobilization of densely packed mixed PEGs around the Fab′ immobilized on the surface effectively prevented its inactivation.

Fluorescence Studies of Fab′ Immobilized on the Gold Surface. To find any correlation between the adsorption-induced inactivation and structural change of Fab′, the changes in the fluorescence intensity of fluorophore-labeled Fab′ on the gold surface were measured. Gold surfaces in proximity to a fluorophore are well-known to strongly quench its fluorescence, primarily through the nonradiative energy transfer from the dye to the metal.[40−42] The quenching efficiency decreases as the distance from the gold to the fluorophore increases. Figure 3 shows the changes in the fluorescence intensity of the three constructed surfaces as a function of time before mixed-PEG immobilization on the FL-Fab′-immobilized gold surface. In the case of the FL-Fab′-modified gold surface, the fluorescence intensity of the FL-Fab′ immobilized on the gold surface drastically decreased with the increase in the length of time before PEGylation, and about 60% of the initial intensity was lost when the immobilized Fab′ was kept for 60 min before PEGylation. In contrast, the fluorescence intensity of the FL-Fab′-modified glass surface did not decrease at all for the present experimental time period (60 min). These findings, along with the results of the radio-labeling experiments shown in Figure S1, suggest that the reduction in fluorescence intensity of FL-Fab′ on the gold surface did not derive from the desorption of the immobilized FL-Fab′ and the self-quenching of the dyes, but from other mechanisms such as nonradiative energy transfer (NRET) from the dye to the gold surface. If the observed fluorescence decrease was derived from NRET, it is strongly suggested that a time-dependent conformational and/or orientation change of Fab′ took place on the gold surface. Interestingly, when the densely packed mixed PEGs were immobilized just after the Fab′ immobilization, fluorescence quenching was effectively prevented, and about 75% of the initial fluorescence intensity was observed even after 60 min. The obtained data strongly indicate that the coimmobilized PEG layer effectively prevented the conformational and/or orientation change of Fab′ on the gold surface. Taking into account the data obtained from the SPR and the fluorescence-based analyses, which correlated well with each other, that is, the remarkable inactivation of the antigen-binding function of the immobilized Fab′ caused by the

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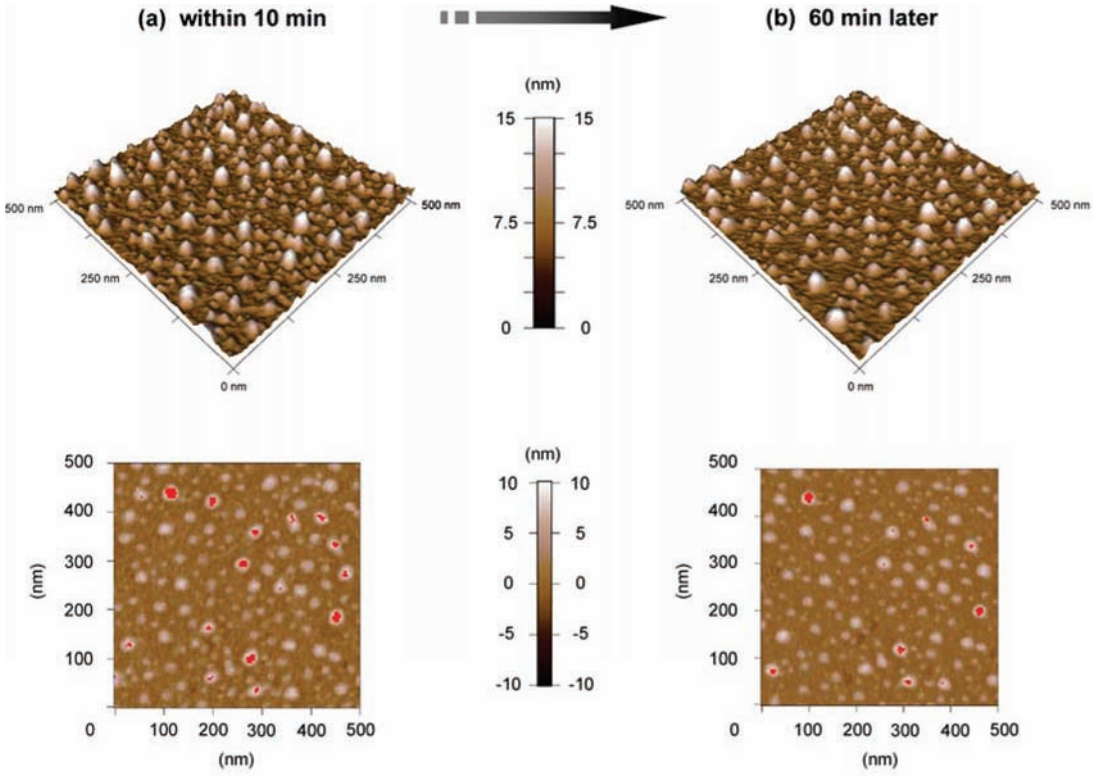

Figure 4. Changes in height of Fab′-immobilized gold surfaces as observed in AFM images in 3D (above) and 2D (bottom), as a function of time after Fab′ immobilization. AFM measurements were carried out (a) within 10 min and (b) at 60 min after the end of Fab′ immobilization. In the 2D images, the regions with a height of more than 8 nm are distinguished by red coloring. Tapping-mode (AC-mode) AFM in phosphate buffer solution at pH 7.0 was employed. The scan was performed at 1000 × 1000 nm^2, and the images were extracted at 500 × 500 nm^2.

adsorption-induced conformational and/or orientation change on the gold surface, it can be concluded that the prevention of Fab′ inactivation by the formation of the mixed-PEG layer resulted from the structure stabilization of Fab′ on the gold surface by the densely packed tethered PEG chain layer around the Fab′ on the surface. Recently, several groups have pointed out conformational changes in proteins upon adsorption to a gold surface which are due to the high free energy of the gold surface, a phenomenon which has been studied by indirect methods.[15,17,43] A similar conformational change in Fab′ might be induced by its adsorption onto the gold surface. Although the mechanism of conformational change is not yet sufficiently clear, the mixed-PEG layer effectively improved the stability of the Fab' structure on the surface and prevented contact between the gold surface and Fab'.

AFM Analysis of Fab′-Immobilized and Fab′/Mixed-PEG Coimmobilized Gold Surfaces. To obtain direct information on the conformational change in the immobilized Fab′ and the prevention effect of the PEG layer on the denaturation of Fab′ on the surface, AFM studies were performed using Fab′-modified surfaces with and without densely packed mixed-PEG coimmobilization in phosphate buffer solution at pH 7.4. The gold surface used in this study had an rms roughness of 0.36 ± 0.06 nm in a 1000 × 1000 nm^2 scan. The AMF images in Figure 4 show the Fab′-immobilized gold surfaces as a function of time after complete Fab′ immobilization. The size and molecular weight of Fab′ are known as 7 nm ×5 nm ×4 nm and 46 kDa, respectively.[44] The height plots of the Fab′ immobilized surface was shown in Figure S2a in Supporting Information and the observed heights correlate with the size of Fab′. The surface within 10 min after Fab′ immobilization, shown in Figure 4a, was covered with Fab′ and had an rms roughness of 2.06 ± 0.15 nm in a 1000 × 1000 nm^2 scan. On the other hand, the surface at 60 min after Fab′ immobilization, shown in Figure 4b, had an rms roughness of 1.83 ± 0.16 nm in a 1000 × 1000 nm^2 scan. The decrease in the rms roughness from 2.06 to 1.83 might indicate a conformational change in the Fab′ on the bare gold surface. The Figure S2a also clearly showed the time-dependent decrease in the height of immobilized Fab′. Furthermore, the 2D images in Figure 4a,b clearly indicate changes in the height of the Fab′ immobilized on the gold surface. In the 2D images of Figure 4, the regions that show a height of more than 8 nm are distinguished by red coloring. As compared with Figure 4a, the number of surface deposits with a height of more than 8 nm is lower in Figure 4b. Thus, it is directly confirmed that the adsorption-induced conformational change in Fab′ on the gold surface was observed with time, which strongly supports the idea that the immobilized Fab′ undergoes surface-induced denaturation, as indicated by the SPR and fluorescence analyses.

AFM studies of the Fab′/mixed-PEG coimmobilized gold surfaces, which were prepared by immediate PEGylation after Fab′ immobilization, was carried out. Figure 5 shows the changes in the AFM topography images of the Fab′/mixed-PEG surfaces as a function of the length of the time after the

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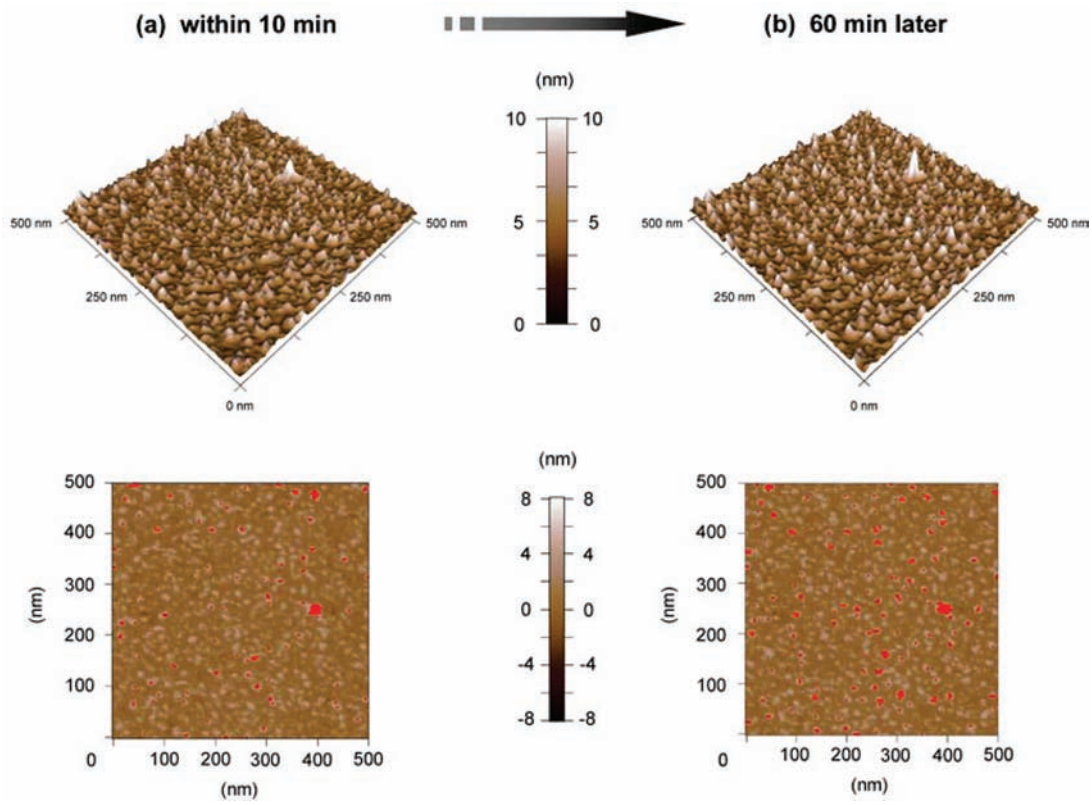

Figure 5. Changes in the AFM topography images in 3D (above) and height images in 2D (bottom) of the Fab′/mixed-PEG coimmobilized gold surface as a function of time after mixed-PEG immobilization. AFM measurements were carried out (a) within 10 min and (b) at 60 min after the end of Fab′ immobilization, followed by PEGylation. In the 2D images, the regions with a height of more than 4 nm are distinguished by red coloring. Tapping-mode (AC-mode) AFM in phosphate buffer solution at pH 7.0 was employed. The scan was performed in 1000 × 1000 nm^2, and images were extracted in 500 × 500 nm^2.

coimmobilization of mixed-PEGs. Figure 5a shows the Fab′/mixed-PEG surfaces within 10 min after PEGylation, while Figure 5b shows the surface at 60 min after PEGylation. The rms roughness in Figure 5, panels a and b, was 1.15 ± 0.09 and 1.28 ± 0.09, respectively. The rms roughnesses of the mixed-PEG-modified gold surface within 10 min and at 60 min after PEGylation were almost the same (0.29 ± 0.04 and 0.27 ± 0.03, respectively), and the negligible change in rms roughness was due to the retention of the conformation of the immobilized Fab′, made possible by the surrounding mixed-PEG adlayer. The height plots of the Fab′/mixed-PEG coimmobilized surface is shown in Figure S2b in Supporting Information, where the heights of the surface deposits were overall lower than those on the Fab′ immobilized surface shown in Figure 4 and Figure S2a, presumably due to the coimmobilized PEG(5k) layer, whose thickness was previously reported about 3 nm.[45] In the 2D images in Figure 5, the regions that have a height of more than 4 nm are distinguished by red coloring. As compared with Figure 5a, a large number of surface deposits with a height of more than 4 nm were observed in Figure 5b, indicating that the height of the immobilized Fab′ was maintained and increased as the length of time after the mixed-PEG immobilization increased. These results suggest that the coimmobilized mixed-PEG layer prevented the surface-induced denaturation of Fab′ on the gold surface. The densely packed tethered PEG chains might decrease the interaction between the gold surface and the intractable residues of Fab′.

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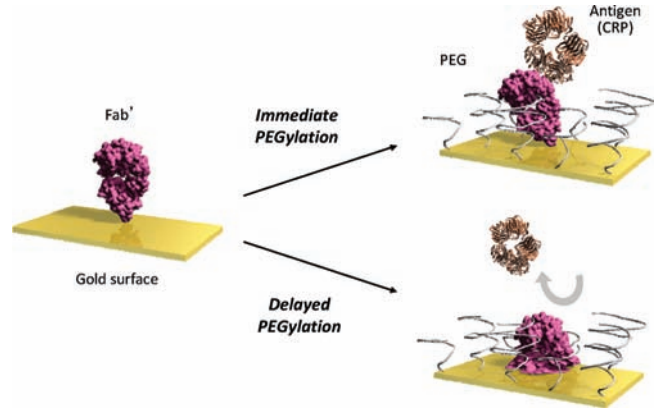

Figure 6. Schematic illustration of time-dependent inactivation of immobilized Fab′ surrounded by mixed-PEG layer on gold surface. Two types of Fab′/mixed-PEG coimmobilized surfaces were described. Immediate PEGylation after Fab′ immobilization (above) prevents the conformational and extensive orientation change of Fab′ on gold surface, resulting in the effective recognition of antigen (CRP). In contrast, delayed PEGylation after Fab′ immobilization (bottom) does not prevent the conformational and orientation change of Fab′ on gold surface.

Simplified illustrations of the Fab′/mixed-PEG coimmobilized gold surfaces may be drawn to describe the obtained results of SPR, fluorometry and AFM as shown in Figure 6. Initial adsorption of Fab′ onto the gold surface takes place due to S−Au bond formation, and most of its native structure is retained. Immediate PEGylation prevent the reorientation of adsorbed Fab′ on the gold surface and the antigen can be bound

to the Fab′ surrounded by mixed-PEG layer. In contrast, delayed PEGylation induced the reorientation and the tendency to increase surface interaction of Fab′, which leads to the conformational change of Fab′ including the antigen-binding site. Consequently, the antigen cannot bind to Fab′ surrounded by mixed-PEG layer. The immediate formation of coimmobilized PEG layer may play an important role in the prevention of denaturation processes of Fab′ on the gold surface.

Conclusions

The adsorption behavior of Fab′ with PEG layer on a gold surface was investigated from the perspective of surface-induced inactivation and structure stabilization. SPR studies using the mixed-PEG blocking technique revealed that the antigen-binding function of the immobilized Fab′ was inactivated time-dependently and disappeared almost completely within 60 min after Fab immobilization. The results of the fluorescence-based and AFM analyses suggest the time-dependent adsorption-induced conformational and/or orientation change of the immobilized Fab′. On the other hand, the prevention effect of the coimmobilized mixed-PEG layer on the surface-induced denaturation of Fab′ is interesting and worthy of further study, because the main role of the tethered PEG chains on the surface was, as is generally recognized, to prevent nonspecific adsorption onto the surface. Since several groups have reported the inactivation of Fab′ on a substrate surface by conventional methods;[37,46] our new findings are useful for improving the strategy of immobilization of antigen fragments onto surfaces. A similar effect of coimmobilized PEG derivatives on single-stranded DNA (ssDNA) on a gold surface has been reported,[47] where a nanolevel phase-separated layer[48,49] fabricated by PEG-polyamine block copolymer improved the binding activity of the ssDNA immobilized on the gold surface. The development of an advanced coimmobilization methodology for biomacromolecules with functional synthetic polymers and further understanding of the macromolecular behavior on these interfaces will hopefully result in a new strategy for the construction of high-performance bioinspired substrates.

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Supporting Information Available: Binding responses of CRP and BSA on Fab′/PEG(5k), Fab′/PEG(2k) and Fab′/mixed-PEG coimmobilized gold surfaces, as determined by the SPR sensor (Table S1). Surface density of Fab′ on the gold surface, determined by radiometric assay (Figure S1). Changes in the height of Fab′ immobilized and Fab′/mixed-PEG coimmobilized gold surface as a function of time after surface preparation, as measured by AFM analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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