Covalent immobilization of antibody fragments on well-defined polymer brushes via site-directed method

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Abstract

Well-defined polymer brushes and block copolymer brushes consisting of 2-methacryloyloxyethyl phosphorylcholine (MPC) and glycidyl methacrylate (GMA) were prepared by surface-initiated atom transfer radical polymerization (ATRP). The polymer brushes were used for the immobilization of antibody fragments in a defined orientation. Pyridyl disulfide moieties were introduced to the polymer brushes via a reaction of epoxy groups in GMA units. Fab' fragments were then immobilized onto these surfaces via a thiol-disulfide interchange reaction and the reactivity of antibodies with antigens was investigated. Antigen/antibody binding on the polymer brushes was more preferable than that on epoxysilane films as a control surface. Furthermore, the activity of the antibodies immobilized on the block copolymer brushes having biocompatible PMPC was greater than that on other surfaces that did not have PMPC in their structures.

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1. Introduction

There has been great interest in the development of protein biochips because they would be powerful tools for proteomic and diagnostic investigation in obtaining information about protein functions and interactions, and for screening complex protein samples [1,2]. Protein immobilization is a key technology for the successful development of a protein microarray. Strategies for immobilizing proteins on substrates have been studied but considerable development is still required. Amine-, aldehyde-, and epoxide-derivatized glass slides are often used as protein microarray platforms [3]. On these surfaces, proteins are immobilized via a reaction with the amine and carboxyl groups of amino acid residues such as lysine and glutamic acid. However, since these amino acids are usually abundant in proteins, the attachment may occur simultaneously through the many residues that enhance heterogeneity in the population of immobilized proteins [1]. The multipoint attachment also causes structural deformation of the proteins resulting in a partial or total loss of activity [4]. Furthermore, the random orientation of the immobilized proteins decreases the accessibility of the active site. To immobilize proteins in a defined orientation according to site is therefore very important for maintaining the biological activities of proteins. Peluso et al. performed oriented immobilization of antibodies by a specific reaction between biotin and streptavidin using a site-specifically biotinylated antibody [5]. They compared the activity of full-sized antibodies and Fab' fragments immobilized in both a random and oriented state. The increased analyte binding capacity of the surfaces with oriented capture agents was consistently observed over surfaces with randomly oriented capture agents, with improvements up to 10 times greater. In addition, it was demonstrated that Fab’ fragments retained 90% of normal activity on average when specifically oriented. Chen and co-workers controlled the orientation of antibodies by changing the surface charge [6,7]. They also utilized the charge distributions of the antibodies. On positively charged surfaces, IgG 1 type antibodies oriented with the antigen-binding domain directed to the liquid phase showing higher reactivity with antigens compared to that of antibodies immobilized on negatively charged surfaces.
In addition to oriented immobilization of proteins, suppressing nonspecific interactions with biomolecules is crucial for developing highly sensitive protein biochips. Nagasaki et al. performed the co-immobilization of both antibody and PEG on magnetic bead surfaces and showed that the nonspecific adsorption of proteins from cell lysates could easily be reduced [8]. Furthermore, a 20-fold higher S/N ratio was achieved with the antibody/PEG co-immobilized surface compared to a surface treated with bovine serum albumin, a conventional blocking reagent.

Surface modification at the molecular level is then required to control the function of the biomolecules on the surface. In recent years, living radical polymerization has been used for surface-initiated polymerization and well-defined polymer brushes were successfully produced [9–12]. This method is called the “grafting from” system and can be used to prepare dense polymer brushes as compared with the adsorption of functionalized polymers to solid/liquid interfaces, which is called the “grafting to” system due to steric hindrance of the polymers [11]. Atom transfer radical polymerization (ATRP) is one of the methods of living radical polymerization and is studied widely because a wide range of monomers can be used in the process.

In contrast, we have been studying 2-methacryloyloxyethyl phosphorylcholine (MPC) polymers synthesized as biomimetics in biomembrane structures [13–16]. The MPC polymers (PMPC) exhibit a property that resists nonspecific interaction with plasma proteins and cells showing excellent biocompatibility [17,18]. In addition, it has been confirmed that no activation and inflammatory response of cells in contact with PMPC are induced [19,20]. To control the surface structures of PMPC on a submolecular scale for investigating the effect on biofouling, we have prepared PMPC brushes by ATRP. On well-defined PMPC brushes, protein adsorption was effectively reduced. Furthermore, a 20-fold higher S/N ratio was achieved with the antibody/PEG co-immobilized surface compared to a surface treated with bovine serum albumin, a conventional blocking reagent.

2. Materials and methods

2.1. Materials

Silicon wafers (100 orientation, P/B doped) were purchased from Yamanaka Semiconductor Co., Ltd., Tokyo, Japan. MPC was synthesized by previously reported methods [29]. GMA and ethyl-2-bromoisobutyrate were purchased from Tokyo Chemical Industry Co., Ltd., Tokyo, Japan, and Sigma-Aldrich, Japan, respectively, and purified by distillation before use. 3-(2-Bromoisobuteryl)propyl dimethylchlorosilane (BDCS) was synthesized as previously described [30,31]. Purified water (reverse osmosis) was further purified on a Millipore Milli-Q system that involves reverse osmosis, ion exchange, and filtration steps (18.2 MΩ cm). Goat anti-mouse IgG F(ab’)_2 fragment and goat anti-mouse IgG fluorescein isothiocyanate (FITC) conjugate F(ab’)_2 fragment were purchased from Sigma-Aldrich, Japan. Mouse anti-rat IgG FITC conjugate was obtained from Zymed Laboratories, Inc., California, USA, and donkey anti-rabbit IgG rhodamine conjugate was purchased from Cosmo Bio Co., Ltd., Tokyo, Japan. Other chemicals were used as received without further purification.

2.2. BDCS monolayers on silicon wafer

Silicon wafers were cut into 1.2 cm × 1.2 cm pieces, cleaned before use by ultrasound in toluene for 5 min, and rinsed with toluene, absolute acetone, and absolute ethanol. After being dried in an argon gas stream, the wafers were washed by O2 plasma for 30 min and placed in a clean oven at 120 °C for 2 h. Silanization was immediately performed after treatment of the plates.

The BDCS monolayer on the silicon wafer was prepared by the method previously reported [21,31]. Briefly, cleaned silicon wafers were held in a custom-designed holder and placed in a dry flask to which 30 mL of dry toluene, triethylamine (21 μL, 0.15 mmol), and BDCS (33 μL, 0.15 mmol) were added under an argon gas atmosphere. The flask was allowed to stand for 72 h. The wafers were then removed from the solution, rinsed with toluene, absolute acetone, and absolute ethanol, and dried in an argon gas stream.

2.3. Preparation of well-defined polymer brushes

Methanol was used as a solvent for the atom transfer radical polymerization of MPC. The solvent was purged with argon at an elevated temperature, which was higher than the boiling point of methanol. After boiling for 5 min, the solvent was cooled to room temperature under argon to eliminate any oxygen before the polymerization. Copper(I) bromide (CuBr) (29 mg,
0.20 mmol) and 2,2′-dipyridyl (bpy) (63 mg, 0.41 mmol) were dissolved in 9 mL of methanol, and ethyl-2-bromoisobutyrate (EBIB) (30 μL, 0.20 mmol) was added as a sacrificial initiator. After being stirred for 30 min under an argon gas atmosphere, the BDCS-immobilized silicon wafers were then submerged into the flask. MPC (12.0 g, 41 mmol) was separately dissolved in 21 mL of methanol and the solution was purged with argon for at least 30 min before use. The MPC solution was then added to the flask and polymerization occurred at room temperature with stirring under an argon gas atmosphere. After polymerization for 30 min and 1 h, the silicon wafers were removed from the polymerization mixture and immediately submerged into the solution for GMA polymerization.

GMA was polymerized from PMPC brushes immediately after the MPC polymerization. A mixed solvent of 7 parts methyl–ethyl–ketone (MEK) and 3 parts ethanol was used as the solvent. CuBr (8.6 mg, 0.060 mmol), bpy (19 mg, 0.12 mmol), and EBIB (9 μL, 0.060 mmol) were dissolved in 21 mL of MEK and 9 mL of ethanol in a new flask with stirring under an argon gas atmosphere during the polymerization of MPC. The silicon wafers with the PMPC brushes were submerged into this solution. GMA (1.6 mL, 0.012 mol) purged with argon for 30 min was added and polymerization occurred at room temperature with stirring under an argon gas atmosphere. The silicon wafers were periodically removed from the polymerization mixture and rinsed with THF, acetone, and ethanol. Subsequently, the wafers were extracted with a Soxhlet apparatus in THF overnight and dried in an argon gas stream. They were then washed by ultrasound in water, rinsed with ethanol, and dried in an argon gas stream.

A PMPC brush and a PGMA brush were prepared using the same method as described above. After polymerization, the PMPC brush was washed by extraction with a Soxhlet apparatus in methanol and subsequent ultrasound in water. The PGMA brush was washed using the Soxhlet apparatus in THF and sonication in water.

The number-average molecular weight of free MPC polymer in solution was measured with a Tosoh GPC system with a refractive index detector and size-exclusion columns, Shodex SB-804 HQ and SB-806HQ, with a poly(ethylene glycol) (Tosoh standard sample) standard in distilled water containing 10 mM LiBr. For measurement of the number-average molecular weight of free GMA polymer, KF-803 (Shodex) was used with the Tosoh standard sample. Data were collected at 1H NMR (α-500, JEOL, Tokyo, Japan).

2.4. Preparation of epoxysilane films on silicon wafer

Epoxysilane films were prepared according to the method previously described [32]. Briefly, silicon wafers were cut and cleaned as described above. The cleaned silicon wafers were held in a custom-designed holder and placed in a dry flask to which 29.7 mL of dry toluene and (3-glycidoxypropyl) trimethoxysilane (Gelest, Inc.) (0.3 mL, 1.4 mmol) were added under an argon gas atmosphere. The flask was allowed to stand for 24 h. The wafers were then removed from the solution, rinsed with toluene, absolute acetone, and absolute ethanol. After subsequent cleaning by ultrasound in absolute ethanol and rinsing with ethanol, the wafers were dried in an argon gas stream.

2.5. Introduction of pyridyl disulfide moieties

Pyridyl disulfide moieties were introduced to the polymer brushes via a reaction of epoxy groups in GMA units. The silicon wafers on which the polymer brushes were prepared were submerged into diithiothreitol (DTT) solution (3 mM DTT, 0.1 M potassium bicarbonate pH 8.5, 0.5 mM EDTA) and allowed to react at room temperature for 15 h with stirring. The wafers were then rinsed with 0.1 M potassium bicarbonate pH 8.5, deionized water, 0.2 M sodium acetate pH 5.0, and deionized water. After being dried with an argon gas stream, the wafers were soaked in a solution of 2,2-dithiodipyridine (2PDS) and 2-thiopyridine (2TP) (34 mM 2PDS, 8 mM 2TP, 50 mM sodium bicarbonate, 45% ethanol) and allowed to react at room temperature for 1.5 h. The wafers were rinsed with 50% ethanol and dried in vacuo.

2.6. Surface analysis

The dynamic contact angles for the sample plates were recorded by use of a probe fluid, deionized water (18.2 MΩ), Gilmont syringes, and a First Ten Angstroms FTÅ125 goniometer. The advancing (θA) and receding (θR) contact angles were measured with addition to and withdrawal from the drop, respectively.

The thickness of the polymer brush was measured on an auto ellipsometer (DVA-36L3, The Optronics Co., Ltd., Tokyo, Japan) operating with a 632.8 nm He–Ne laser at a 70° incident angle.

X-ray photoelectron spectroscopy (XPS) was performed using a magnesium anode non-monochromatic source (Kratos- Shimadzu, Kanagawa, Japan). Survey scans spectra of C1s, O1s, N1s, P2p, Br3d, and S2p were obtained. Data were collected at take off angles of 15° and 90°.

2.7. Immobilization of Fab′ fragments on activated polymer brushes

The F(ab′)2 fragments were split into Fab′ fragments with 2 mM 2-mercaptoethanolamine in 0.1 M sodium phosphate, 5 mM EDTA, pH 6.0 for 1 h at 37°C. After reaction, excess 2-mercaptoethanolamine was removed by running the sample through a column (NAP-25, Amersham) with 0.1 M sodium phosphate, 5 mM EDTA, pH 6.0, as the eluent. The prepared sample was analyzed using 5%–20% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (samples were diluted with nonreducing protein loading buffer). A ~50 kDa band corresponding to the Fab′ fragment [33] was observed. There were few contaminants corresponding to the sizes of the free light chains and the cleaved heavy chains. This antibody fragment solution was used for immobilization to the surfaces.
To determine the area in contacted with the antibody solution, we used silicone rubber and acrylic plates with holes. The silicone rubber was washed by ultrasound in acetone and ethanol before use. The acrylic plates were also cleaned by ultrasound in ethanol. The diameters of the holes were 7.5 mm. The silicon wafers were first covered with silicone rubber, and then put between two acrylic plates, one of which had a hole. They were then fixed in place with clips. A 50 μL solution of 2.6 mg/mL Fab’ fragments was added and placed in contact with each surface. Reaction occurred at room temperature for 21 h under humid conditions. The wafers were rinsed with 0.1 M sodium phosphate, 5 mM EDTA, pH 6.0, by changing the contacting conditions. After being rinsed with PBS, the silicone rubber and the acrylic plates were removed. The samples were rinsed again with PBS and deionized water. After being dried in an argon gas stream, the surfaces were observed with a fluorescent microscope and the fluorescence intensity was analyzed.

3. Results and discussion

3.1. Preparation of well-defined polymer brushes

The block copolymer brushes consisting of PMPC and poly(GMA) (PGMA) were prepared on silicon wafers by surface-initiated ATRP (Scheme 1). First, MPC was polymerized from a BDCS monolayer and GMA was then polymerized from the PMPC brushes immediately after the MPC polymerization.

The polymerization of MPC was performed by the same basic method previously described [21]. We used methanol as the solvent instead of a mixture of water and methanol because we have found that the polymer radical concentration was more constant in methanol than in a mixture of water and methanol (data not shown). We obtained about 7–8 nm thick PMPC brushes with a theoretical molecular weight of 15,000–19,000 for 30 min or 1 h of polymerization time (Table 1). In previous work, we have clarified that serum protein adsorption and fibroblast adhesion could be effectively reduced on PMPC brushes when the brush thickness was above about 5 nm [21]. Thus, we chose PMPC brushes with a brush thickness of about 7–8 nm, which is above 5 nm, and used them for the block copolymerization with GMA.

Fig. 1 shows the kinetics of ATRP of GMA from the end of PMPC. The semilogarithmic plot of monomer concentration vs. time was linear up to 71% conversion. The linearity of the first-order plot of the monomer concentration suggested that the polymer radical concentration remained constant and that polymerization could be controlled on a polymerization time scale. GMA could be polymerized from PMPC brushes and the thickness of PGMA was increased with an increase in polymerization time, as described previously [24]. Block copolymer brushes with a PGMA layer thickness of 4.3 and 7.7 nm, and theoretical
molecular weights of 10,100 and 18,100 were obtained for polymerization times of 6 and 18 h, respectively. The formation of block copolymer brushes confirmed the living character of this polymerization and showed that at least a considerable fraction of the chain ends was still active for initiation of further film growth.

Because the molecular weight of free polymers produced in solution is assumed to be the same as that of polymer chains on the surface [34], we analyzed free polymers by GPC. The $M_n$ obtained by polystyrene calibration increased with an increase in the polymerization time, but there was some deviation from the theoretical value (data not shown). Thus, we used theoretical $M_n$ in the following discussions. The graft density $\sigma$ (chains/nm²) of each polymer brush was calculated according to the following equation:

$$\sigma = \frac{h \rho N_A}{M_n}$$

where $h$ is the layer thickness of each polymer layer determined by ellipsometry, $\rho$ is the density of the dry polymer layer (1.30 g/cm³ for PMPC, 1.0 g/cm³ for PGMA), $N_A$ is Avogadro’s number, and $M_n$ is the number-average molecular weight of the graft polymers (theoretical value). It is reported that the bulkiness of the monomer unit has a steric effect on the behavior of graft polymerization [35] resulting in the lower graft density of the polymer brush consisting of a large-sized monomer than that consisting of a small-sized monomer. This behavior was also observed in this study. A PGMA brush showed a higher graft density than did a PMPC brush consisting of a large-sized monomer compared with a GMA monomer (Table 1). The graft density of the PGMA brush layer in the block copolymer brush was lower than that of the PMPC brush, which was directly prepared from the BDCS monolayer without the PMPC brush layer. In the block copolymer brush, the graft density of the PGMA unit is regulated by that of the PMPC brush. The lower graft density of the PGMA unit in the block copolymer brush is thus reasonable. This result demonstrates that GMA was indeed polymerized from the ends of the PMPC brushes. We prepared PGMA brushes for comparison with block copolymer brushes in the following experiments. Since PGMA brushes were more densely polymerized than the PGMA layer in the block copolymer brushes, the thickness of the PGMA brushes was higher than that of the PGMA layer in the block copolymer brushes for the same polymerization time. We prepared PGMA brushes having

![Scheme 1. Synthetic route of block copolymer brushes on silicon wafer by ATRP.](image)

Table 1

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Polymer thickness (nm)</th>
<th>Graft density^b,c (chains/nm²)</th>
<th>Water contact angle (^c)</th>
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<tr>
<td></td>
<td>PMPC</td>
<td>PGMA</td>
<td>$\theta_A$</td>
</tr>
<tr>
<td>PMPC18.7K^d</td>
<td>8.1 ± 1.2</td>
<td>–</td>
<td>0.35 ± 0.0</td>
</tr>
<tr>
<td>PGMA11.0K</td>
<td>–</td>
<td>8.2 ± 0.9</td>
<td>0.43 ± 0.1</td>
</tr>
<tr>
<td>PMPC14.9K-δ-PGMA10.1K</td>
<td>7.0 ± 0.4</td>
<td>4.3 ± 0.7</td>
<td>0.26 ± 0.05^e</td>
</tr>
<tr>
<td>PMPC17.5K-δ-PGMA18.1K</td>
<td>7.9 ± 0.6</td>
<td>7.7 ± 0.7</td>
<td>0.26 ± 0.04^e</td>
</tr>
</tbody>
</table>


^b Mean ± S.D., n ≥ 4.

c Calculated according to Eq. (1) using theoretical $M_n$.

d Theoretical $M_n$ of polymer chain is 18,700 g/mol; theoretical $M_n$ was determined by conversion $\times$ targeted DP (200) $\times$ MW of monomer (MPC: 295.3 g/mol, GMA: 142.15 g/mol).

e Graft density of PGMA brush layer in the block copolymer brush.
a thickness similar to the PGMA unit in the block copolymer brushes. 8.2 nm thick PGMA brushes with a theoretical molecular weight of 11,000 were obtained for a polymerization time of 6 h.

The PMPC brushes showed an extremely low water contact angle because of the hydrophilic nature of PMPC. This value increased after the copolymerization with GMA but it was still lower than that of the PGMA brushes, especially in the receding contact angle. This result implies that the surface property of a block copolymer brush reflected the properties of both PMPC and PGMA. To understand the condition and the conformation of block copolymer brushes precisely, more detailed experiments are needed such as responses to solvents.

Table 1 summarizes the characteristics of each polymer brush prepared. We used these polymer brushes in the following experiments.

### 3.2. Introduction of pyridyl disulfide moieties

Pyridyl disulfide has been used for covalent immobilization of proteins on supports [36–38] and conjugation of biomolecules [39,40] by a thiol-disulfide interchange reaction. This reaction proceeds under mild conditions and specifically occurs between thiol-disulfide bonds. Since the occurrence of exposed thiols in proteins is usually very low, this method can be used for site-directed immobilization or conjugation of proteins. A Fab’ fragment is an antibody fragment that has a thiol group in the opposite side of the antigen-binding domain. Therefore, when we use thiol groups for immobilization, we can immobilize the Fab’ fragments in an ordered orientation on substrates without affecting the antigen-binding domain [5]. We introduced pyridyl disulfide moieties onto the polymer brushes to immobilize the Fab’ fragments in an ordered orientation. The introduction of pyridyl disulfide moieties was performed according to the method previously described (Scheme 2) [38,41]. Grazì et al. used two reaction steps, the thiolation of epoxy groups with DTT and the activation of thiolated supports with 2-PDS. In the first reaction, they succeeded in controlling the degree of substitution of the epoxy groups by changing the concentration of DTT. They achieved 6.7% and 12.5% conversion when the molar ratio of DTT to epoxy group was 1.8:1 and 3.7:1, respectively, and the reaction time was 1 h at room temperature. In this study, we reacted excess DTT with polymer brushes because the molar ratio of DTT to epoxy group is about 1800:1. Furthermore, the reaction lasted for 15 h. This concentration of DTT and the reaction time seems to be sufficient to substitute a considerable amount of epoxy groups in the polymer brushes. We used 2TP in addition to 2PDS in the second reaction to effectively introduce pyridyl disulfide moieties. 2TP can split the nonreactive disulfide bond formed between two adjacent thiols in the polymer brushes by forming a reactive thiopyridyl group and a free thiol, which is immediately converted to another thiopyridyl group by reaction with 2PDS [41].

After the reaction with 2PDS and 2TP, the introduction of pyridyl disulfide moieties was confirmed by XPS analysis. Fig. 2 shows the $S_{2p}$ spectrum of each surface. An $S_{2p}$ signal at approximately 163 eV was observed on PMPC-$b$-PGMA brushes and PGMA brushes; this signal could be assigned to neutral sulfur species (e.g., disulfide) [42]. On the PMPC-$b$-PGMA brushes, there was a nitrogen peak at approximately 400 eV in addition to that from phosphorylcholine in MPC at about 403 eV (data not shown). We also observed a $N_{1s}$ signal at 406.6 eV on the PGMA brush, which has no nitrogen atom itself. These signals can be attributed to nitrogen in the pyridyl disulfide moieties. As a control, PMPC brushes with no epoxy groups were subjected to the same reaction, and there was no $S_{2p}$ signal. It was
also confirmed that no $S_{2p}$ signal was present on the PMPC-$b$-PGMA brushes without the introduction reaction. These results suggest that pyridyl disulfide moieties could be introduced in polymer brushes for reaction via the epoxy groups in the GMA units. Table 2 shows the composition of each surface determined by XPS analysis at a take-off angle of 15°. The sulfur ratio of PMPC14.9K-$b$-PGMA10.1K and PMPC17.5K-$b$-PGMA18.1K was 0.7% and 1.2%, respectively, also confirming that the introduction of pyridyl disulfide moieties could be performed via GMA units.

### 3.3. Immobilization of Fab' fragments on activated polymer brushes

We immobilized Fab' fragments on polymer brushes by the thiol-disulfide interchange reaction via thiol groups in Fab' fragments and pyridyl disulfide moieties in polymer brushes defining the orientation of the antibodies (Fig. 3). The polymer brushes without pyridyl disulfide moieties were also used as control surfaces to investigate the effect of the moieties on the immobilization of antibody fragments. Fig. 4 shows the XPS spectra of polymer brushes after contact with the Fab' fragments. First, the spectra of the polymer brushes with pyridyl disulfide moieties will be discussed. In the PMPC-$b$-PGMA brush, $N_{1s}$ signals at a binding energy of 399.8 and 402.5 eV were observed. These signals could be attributed to the amine group in the Fab' fragment and the choline group in the MPC unit, respectively. This signal assigned to the amine group in the antibody was also detected in the PGMA brush at 400.0 eV. $S_{2p}$ signals at a binding energy of 163.3 and 163.5 eV were observed in the PMPC-$b$-PGMA brush and the PGMA brush, respectively, indicating the existence of disulfide. In contrast, in the control surfaces without the pyridyl disulfide moieties, nitrogen signals from the antibody fragments were detected but the intensity was lower than that from the surfaces having the pyridyl disulfide moieties. Furthermore, no $S_{2p}$ signal was observed in these non-activated polymer brushes. The experimental atomic ratios were calculated from the atomic percentages and compared between polymer brushes consisting of the same composition. $N/Si$ was 1.6 and 0.4 for the activated PMPC-$b$-PGMA brush and the non-activated PMPC-$b$-PGMA brush, respectively. The ratio of nitrogen from antibody and MPC was also calculated giving 1.4 for the activated PMPC-$b$-PGMA brush and 0.9 for the non-activated PMPC-$b$-PGMA brush. On the PGMA brushes, the activated PGMA brush showed higher a ratio of $N/Si$ (1.1) than did that of the PGMA brush without the pyridyl disulfide moieties (0.4). The relatively large amount of nitrogen in the activated polymer brushes suggests that the Fab' fragments could be preferentially immobilized via a thiol-disulfide interchange reaction. It is thought that Fab' fragments on non-activated polymer brushes were immobilized via nucleophilic reactions involving amine groups of antibody fragments and

### Table 2
Surface composition of polymer brushes determined by XPS analysis

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>XPS data (take off angle 15°) (%)</th>
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<tr>
<td></td>
<td>C</td>
</tr>
<tr>
<td>PMPC14.9K-$b$-PGMA10.1K</td>
<td>60.2</td>
</tr>
<tr>
<td>PMPC17.5K-$b$-PGMA18.1K</td>
<td>63.4</td>
</tr>
<tr>
<td>PGMA11.0K</td>
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</tr>
<tr>
<td>PMPC18.7K</td>
<td>32.2</td>
</tr>
<tr>
<td>PMPC17.5K-$b$-PGMA18.1K*</td>
<td>67.6</td>
</tr>
</tbody>
</table>

* Sample not treated with pyridyl disulfide.
epoxy groups of the polymer brushes in random orientation, or adsorbed nonspecifically. The occurrence of nonspecific adsorption of antibody fragments must be also considered in the case of using activated surfaces. The PMPC brush was also placed in contact with the Fab’ fragment solution as a control, and the nitrogen atom from the antibody was not detected, thereby confirming the ability of PMPC to suppress biofouling.

To compare the amount of immobilized antibody, FITC-labeled Fab’ fragments were reacted with each surface with pyridyl disulfide moieties and the fluorescence intensity was analyzed. We prepared an organosilane monolayer having epoxy groups (epoxysilane films) as a control surface because it is often used as a substrate for biosensors to immobilize biomolecules such as DNA and protein. Fig. 5 is a comparison of the fluorescence intensity. The amount of immobilized antibody in the PMPC-b-PGMA brushes changed with the length of the PGMA unit. PMPC-b-PGMA brushes with longer PGMA units and PGMA brushes could immobilize larger amounts of Fab’ fragments compared with epoxysilane films, showing 2.3 times and 4.1 times larger fluorescence intensity than that of the epoxysilane films, respectively.

3.4. Reaction with antigen of Fab’ fragment-immobilized surfaces

The activity of immobilized antibody fragments was investigated by the reaction with an FITC-labeled antigen (Fig. 6). Fig. 7 shows the ratio of the fluorescence intensity of each surface. The fluorescence intensity of the epoxysilane films was considered as a standard for the ratio. The values of the polymer brushes was higher than that of the epoxysilane films, suggesting that the polymer brush surfaces were effective for

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**Fig. 3.** Scheme of Fab’ fragment-immobilization in defined area of surface.

**Fig. 4.** XPS spectra of polymer brushes treated and not treated with pyridyl disulfide after contact with Fab’ fragments.
reaction with the antigen compared with the epoxysilane films. It was shown that Fab’ fragment-immobilized PMPC14.9K-b-PGMA10.1K, PMPC17.5K-b-PGMA18.1K, and PGMA11.0K had 1.9-, 6.2-, and 6.1-fold higher reactivity with the antigen than did that of epoxysilane films, respectively. In PMPC-b-PGMA brushes, the fluorescence intensity increased with an increase in the thickness of the PGMA unit, indicating that we could control the reaction with an antigen by changing the length of the PGMA unit. When we compared Fig. 5 with Fig. 7, it was obvious that antibody fragments on PGMA11.0K and PMPC17.5K-b-PGMA18.1K showed similar reactivity with antigens (Fig. 7) although PGMA11.0K immobilized larger amounts of Fab’ fragments than did that of PMPC17.5K-b-PGMA18.1K (Fig. 5). This result implied that Fab’ fragments immobilized on PMPC17.5K-b-PGMA18.1K could react with antigens more effectively than that on PGMA11.0K. While the molecular weight of the PGMA unit in PMPC17.5K-b-PGMA18.1K is larger than that of PGMA11.0K, PGMA11.0K grafted to a silicon wafer more densely than did a PGMA unit to PMPC17.5K-b-PGMA18.1K (Table 1). Then, the number of GMA monomer units in the defined area as \( N \) (U/nm\(^2\)) was calculated according to the value in Table 1 and the following equation:

\[
N = \frac{M_n \sigma}{F_w}
\]

where \( M_n \) is the theoretical number-average molecular weight of each polymer brush, \( \sigma \) is the graft density of PGMA, and \( F_w \) is the molecular weight of the GMA monomer (142.15 g/mol). \( N \) of PGMA11.0K and PMPC17.5K-b-PGMA18.1K were 33.3 and 33.1, respectively, showing almost the same value for these polymer brushes. This implies that the capacity of immobilizing Fab’ fragments is the same in these two kinds of polymer brushes. Therefore, it can be assumed that the difference in the amount of immobilized antibody fragments is due to the differences between the polymer brushes such as the conformation, accessibility by Fab’ fragments, and influence of nonspecific adsorption. For the reason of achieving an efficient reaction with the antigens of the antibody fragments immobilized on
PMPC$_{17.5K}$-$b$-PGMA$_{18.1K}$, it is thought that the condition of the Fab’ fragments is different between the PGMA brush and the PMPC-$b$-PGMA brush, which has biocompatible PMPC under PGMA [43]. It was demonstrated that the control of surface chemistry and structure at the molecular level influenced the activity of the immobilized antibody.

The effect of the orientation of the immobilized antibody was also investigated. Polymer brushes without pyridyl disulfide moieties were used for the immobilization of Fab’ fragments and subsequent reaction with FITC-labeled antigens in the same experiment with the activated polymer brushes. A fluorescence intensity about 20 times greater was observed on the PMPC-$b$-PGMA brush with the pyridyl disulfide moieties than that on the non-activated PMPC-$b$-PGMA brush (data not shown). Photographs of the observations with the fluorescent microscope are shown in Fig. 8. In the activated polymer brushes, the fluorescence intensity in the Fab’ fragment-immobilized and antigen-contacted area was greater than that of the non-contacted area, and the boundary could be observed. In contrast, on the polymer brushes without the pyridyl disulfide moieties, almost no difference between the areas where the antigen was contacted and not contacted was seen. These results demonstrated the effectiveness of oriented immobilization of antibody fragments without affecting the antigen-binding domain.

3.5. Nonspecific protein adsorption on Fab’ fragment-immobilized polymer brushes

To reduce nonspecific interactions with biomolecules that are not analytes is crucial for achieving highly sensitive biocognition. Therefore, the ability to suppress nonspecific adsorption of proteins on antibody fragment-immobilized surfaces was examined. Fab’ fragments were immobilized on activated surfaces and subsequently rhodamine-labeled IgG, which is not an antigen of the immobilized antibody fragments was contacted. Fig. 9 compares the fluorescence intensity after contact with the rhodamine-labeled IgG. A larger amount of protein was adsorbed on the polymer brushes than on the epoxysilane films. The adsorption of IgG was increased with the length of the PGMA unit in the block copolymer brushes, suggesting that IgG was mainly adsorbed on the PGMA unit. Although the number of GMA monomer units in the defined area is the same between PGMA$_{11.0K}$ and PMPC$_{17.5K}$-$b$-PGMA$_{18.1K}$ as described above, relatively low protein adsorption was observed in PMPC$_{17.5K}$-$b$-PGMA$_{18.1K}$ compared to that in PGMA$_{11.0K}$. It is thought that the suppressed adsorption of proteins on PMPC$_{17.5K}$-$b$-PGMA$_{18.1K}$ is because the PMPC unit has a property to suppress nonspecific adsorption of proteins. This result indicates that further optimization of brush structure to reduce nonspecific protein adsorption is required.
4. Conclusion

In this study, we have demonstrated the oriented immobilization of antibody fragments onto polymer brushes consisting of PMPC and PGMA having epoxy groups that can be used to immobilize biomolecules. Covalent immobilization of antibody fragments in an oriented state on well-defined polymer brushes was performed via a thiol-disulfide interchange reaction. Changing the thickness of the PMPC unit enabled control of the amount of immobilized Fab' fragments and subsequent reaction with antigens. The effectiveness of polymer brushes for reaction with antigens compared to that of the epoxysilane films was shown. In addition, the structural differences of the polymer brushes influenced the biological reactions, showing higher reactivity of antigens with antibody fragments on PMPC-PGMA brushes. Further investigation of polymer brush structures is needed. However, the characteristics of PMPC and the dense immobilization of antibodies in defined orientation are attractive for developing highly sensitive biorecognition surfaces.

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