Direct observation of interaction between proteins and blood-compatible polymer surfaces

Tomohiro Hayashi[a)]
Department of Electronic Chemistry, Interdisciplinary Graduate School of Science and Engineering, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama, Kanagawa 226-8502, Japan and Local Spatio-Temporal Functions Laboratory, Frontier Research System, RIKEN (The Institute of Physical and Chemical Research), 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

Masaru Tanaka[b)]
Nanotechnology Research Center, Research Institute for Electronic Science, Hokkaido University, N21W10, Sapporo, 001-0021, Japan

Sadaaki Yamamoto
Core Research Initiative "Sousei", Hokkaido University, N21W10, Sapporo, 001-0021, Japan

Masatsugu Shimomura[b)]
Nanotechnology Research Center, Research Institute for Electronic Science, Hokkaido University, N21W10, Sapporo, 001-0021, Japan

Masahiko Hara
Department of Electronic Chemistry, Interdisciplinary Graduate School of Science and Engineering, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama, Kanagawa 226-8502, Japan and Local Spatio-Temporal Functions Laboratory, Frontier Research System, RIKEN (The Institute of Physical and Chemical Research), 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

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The adhesion force between blood-compatible polymer (poly(2-methoxyethyl acrylate: PMEA) and proteins (fibrinogen and bovine serum albumin (BSA)) were measured by atomic force microscopy. The PMEA surface showed almost no adhesion to native protein molecules, whereas non-blood-compatible poly(*n*-butyl acrylate): PBA strongly adhered to proteins. Interestingly, adhesion did appear between PMEA and proteins when the proteins were denatured. In all cases, these trends were not affected by the conditions of the solution. Combining the results with previous reports, the authors conclude that interfacial water molecules play a critical role in the protein resistance of PMEA. © *2007 American Vacuum Society.* [DOI: 10.1116/1.2794712]

I. INTRODUCTION

Design of blood-compatible materials is one of the most important and urgent research topics in the medical field responding to requests for implanting materials and materials for regenerative therapy.[1] Poly(2-methoxyethyl acrylate) (PMEA) [Fig. 1(a)] is one of the best blood-compatible polymers,[2] and its blood compatibility has been characterized by using various approaches.[3–6] Although PMEA is already being used for practical applications such as artificial lungs,[7] the mechanism of the blood compatibility of PMEA is still not fully understood.

The difference between PMEA and non-blood-compatible polymers evidently appeared in the results of differential scanning calorimetry (DSC) measurements. The mixtures of PMEA and water showed the cold crystallization of water at around −50 °C, whereas mixtures of non-blood-compatible polymers and water did not display this feature, indicating that water hydrating PMEA may give rise to its blood compatibility.[4,5] This idea was supported by the results of attenuated total-reflection infrared (ATR-IR) spectroscopy. Ide and coauthors reported that water molecules weakly bound to the primary hydration water may correspond to the cold-crystallizable water.[8] Recently, based on ATR-IR measurements and *ab initio* calculations, Morita and co-workers suggested that water molecules, which may be responsible for the blood compatibility, interact weakly with the methoxy moiety of PMEA in an intermediate way.[9]

Although many have suggested that weakly bound hydrating water plays an important role in blood compatibility, there are only two studies on the strength of the interaction between PMEA and biomolecules. In these reports, the protein resistance of PMEA was revealed by an adsorption experiment of bovine serum albumin (BSA) and fibrinogen onto PMEA surfaces using a quartz-crystal microbalance (QCM) technique.[6] In a comparison of PMEA with hydrophobic polypropylene (PP) and hydrophilic poly(2-hydroxyethyl methacrylate) (PHEMA), PMEA exhibited smaller adsorption amounts of BSA and fibrinogen and higher detachment rate constants for these two proteins, indicating that the blood compatibility cannot be explained simply by the affinity of the polymer surface to water.

[a)]Author to whom correspondence should be addressed; electronic mail: hayashi@echem.titech.ac.jp
[b)]Present address: Institute of Multidisciplinary Research for Advanced Materials (IMRAM), Tohoku University, 2-1-1 Katahira, Aoba-ku, Sendai, 980-8577, Japan.

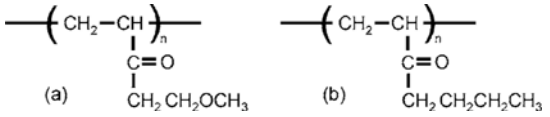

FIG. 1. Chemical structures of (a) poly(2-methoxyethyl acrylate): PMEA, and (b) poly(*n*-butyl acrylate): PBA.

To understand the mechanism of the blood compatibility of PMEA, the interaction between PMEA and biomolecules in water must be explored intensively. In general, interactions in water may stem from the interplay of several different kinds of forces, such as electrostatic interaction, van der Waals interaction, water-mediated force, and steric force.[10,11] Therefore, to elucidate which force is responsible for the blood compatibility, we need direct observation of the force operating between PMEA and biomolecules.

In this work, for the first time, we performed, a direct observation of the interaction of protein molecules with both blood-compatible and non-blood-compatible polymers using atomic force microscopy (AFM), which has generally been applied to measure the interactions in water.[12] Our focus was on the adhesion between polymer and protein molecules. We employed poly(*n*-butyl acrylate) (PBA) [Fig. 1(b)] as a contrastive example of non-blood-compatible polymer, because PBA does not exhibit blood compatibility and cold crystallization in DSC, although PBA does show a similar glass transition temperature as PMEA, indicating that their chain mobility is similar.[5] Based on the results of the adhesion experiment with AFM and the adsorption experiment with QCM, we discuss the mechanisms of polymer-protein interactions and blood compatibility.

II. EXPERIMENTAL DETAILS

Both PMEA and PBA were prepared by radical polymerization and their weight-average weights are 85 000 and 105 000, respectively. These polymers were dissolved in toluene at a concentration of 1 wt % and spin-coated on freshly cleaved highly oriented pyrolytic graphite (HOPG) substrates at 3000 rpm. Using ellipsometry, the thicknesses of the polymer films were found to range from 500 to 800 nm. The prepared polymer surface was kept under the same solution used for the measurement for 20 min prior to the experiment.

The AFM system used in this study was the commercially available NanoScope IV with a PicoForce unit that has a closed-loop feedback system for the z direction (Veeco, Inc., Santa Barbara, CA). Two kinds of probes were employed. One is a Si_3N_4 AFM tip (nominal tip curvature and spring constant are 50 nm and 0.01 N/m, respectively). The other probe is a so-called colloid probe that has a silica sphere (4 μm in diameter) glued at the end of the tip-less cantilever (nominal spring constant is 0.06 N/m). The spring constant of each cantilever was calibrated by measuring its thermal fluctuations.[13] The probes were cleaned by ultraviolet (UV)-ozone exposure for 15 min just prior to the experiments to remove any organic contaminants adsorbed on the tip surface.

Bovine serum albumin (BSA) and human fibrinogen active fragment were purchased from Nakaraitesque, Inc. and Peptide Institute, Inc., respectively. Fibrinogen was hydrated in PBS buffer solution (pH 7.4, 50 mM). Its final concentration was 2 mg/mL. Adsorption of the proteins onto AFM probes was carried out based on the method of Feldman *et al.* with slight modification.[14] First, the AFM probes were immersed in deionized water in a vial. Water containing the proteins was then added to the vial up to a concentration of 1 mg/mL. After 1 h of adsorption, the air-water interface was aspirated to remove the film of denatured protein formed at the air-water interface and the buffer solution was added. After several cycles of this procedure, the AFM tip was immediately transferred into the liquid cell of the AFM system. The stability of fibrinogen molecules on a silica substrate was already checked by Tunc *et al.* In their report, fibrinogen maintained its native form on the hydrophilic silica substrate as confirmed by AFM.[15] The slight conformational change of a BSA molecule on silica was observed by circular dichroism.[16] However, the conformational change was fully recovered after desorption into solution. We therefore concluded that there is no significant structural change of the proteins after adsorption onto a silica surface. As described later, the adhesion force critically depends on whether or not there are proteins on the probe. Moreover, we changed the measuring positions every five or six measurements, we found no significant change in the adhesion force. Therefore, we think that proteins were immobilized on the probe surface during our measurements.

Denaturation of the proteins was carried out in two ways: (1) AFM probes overlaid with the proteins were dried under dry nitrogen for 20 min at room temperature. (2) Proteins in solution were heated at 80 °C for 30 min. The AFM probes were then immersed in the solution containing denatured proteins for 1 h.

AFM force curve measurements were performed in the usual contact mode with a loading rate of 200 nm/s. Maximum loading force was kept under 20 nN to avoid mechanical damage to the protein molecules. All force curves presented in this work were the first force curves taken just after approaching. Note that there is no significant difference between the first and five subsequent curves in most cases. Solution conditions were pure water (pH was around 5.8 due to dissolved carbon dioxide), phosphate buffer (50 mM, pH 6.5 and 7.4), and phosphate buffer (50 mM, pH 6.5 and 7.4) +NaCl(0.1 M). The pH value of the buffer solution was tuned by adding HCl.

QCM measurements were performed using QCM-D (*Q*-Sense AB, Vaestra Froelunda, Sweden) with AT-cut quartz sensors coated with gold. The resonant frequency of the sensors is 5 MHz, and the third overtone (15 MHz) was used to monitor the adsorption. Based on the simple Sauerbrey equation, a shift of 1 Hz in the figures corresponds to 5.9 ng/cm^2. Prior to the preparation of PMEA and PBA, the

TABLE I. Average maximum adhesion force between probes (bare silica and decorated with proteins) and polymer surfaces in pure water normalized by the tip radius (mN/m). n_{tot} and n_f are the total number of measured force curves and the number of freshly prepared samples, respectively. $F_{ad\text{-}tot}$ is the average of maximum adhesion force obtained from all force curves. F_{ad} was obtained by averaging only first force curves in repetition of approach-retract cycles. SD is the standard deviation of $F_{ad\text{-}tot}$.

Surface-tip combination	$n_{tot}(n_f)$	$F_{ad\text{-}tot}(F_{ad})$ (mN/m)	SD
Silica-polymer interactions			
PMEA-silica	74 (5)	24 (28)	7.6
PMEA-Si_3N_4	92 (7)	9.3 (9.6)	3.8
PBA-silica	54 (5)	7.0 (7.5)	1.8
PBA-Si_3N_4	103 (8)	4.9 (5.3)	1.2
Protein-polymer interactions			
PMEA-BSA	98 (7)	0.06 (0)	0.06
PMEA-fibrinogen	107 (6)	0.10 (0)	0.10
PBA-BSA	58 (5)	2.8 (2.2)	2.2
PBA-fibrinogen	51 (5)	11 (7.5)	2.7
Denatured protein (dried)-polymer interactions			
PMEA-BSA	60 (5)	0.38 (0.34)	0.22
PMEA-fibrinogen	49 (5)	0.95 (0.60)	0.34
PBA-BSA	40 (5)	4.8 (5.0)	1.9
PBA-fibrinogen	44 (5)	7.8 (6.1)	4.5
Denatured protein (heated)-polymer interactions			
PMEA-BSA	26 (3)	1.1 (1.5)	0.22
PMEA-fibrinogen	33 (3)	3.0 (2.1)	0.23
PBA-BSA	29 (3)	4.1 (5.8)	1.3
PBA-fibrinogen	33 (3)	11 (9.8)	4.7

gold surface was modified with the self-assembled monolayer (SAM) of *n*-octadecane alkanethiol (ODT) to avoid the dewetting behavior of the polymers in water. The formation of the SAM was done by immersing the sensor into an ethanol solution containing ODT at a concentration of 1 mM for 24 h. Polymers were spin-coated on the modified sensor according to the same procedure as in the case of the HOPG substrates. The sensors were stabilized in water or buffer solution for 20 min prior to the injection of the protein solution.

III. RESULTS AND DISCUSSION

First, the interaction between a bare silica sphere and the polymer surface in pure water was measured. Number of samplings, averaged maximum forces, and standard deviations in the cases of pure water are summarized in Table I. As seen from the retract traces of the force curves [Fig. 2(a)] and average maximum force observed in the force curves [Fig. 2(b)], it is clear that both polymers show strong adhesion to silica and that PMEA is more adhesive to silica than PBA.

The difference between the adhesion of PMEA and PBA might stem from the amount of hydrogen bonding between polymer and silica surfaces. A silica surface possesses a considerable number of silanol groups. These silanol groups strongly interact with polar groups of the polymers via hydrogen bondings. In the case of PBA, the silanol groups form hydrogen bondings with carbonyl groups. As for PMEA, in addition to carbonyl groups, methoxy groups can form the hydrogen bondings, resulting in stronger adhesion than PBA.

Contrary to the above results, there was a large difference in the strength of the adhesion of the polymers to the proteins. As seen in Figs. 3(a) and 3(b), there was only small adhesion between PMEA and the silica probe overlaid with the proteins. This suggests, first, that protein molecules were adsorbed on the silica bead; this was also confirmed by the QCM measurements, observing the adsorption of the proteins onto SiO_2 substrates (data not shown), and second, that the silica surface did not interact with the polymer surface during the measurements; i.e., only adsorbed protein molecules interacted with the polymer surfaces. Contrary to PMEA, PBA interacted strongly with these proteins, as presented in Figs. 4(a) and 4(b). The strong adhesion between PBA and the proteins was demonstrated by the stretching behavior of either protein or polymer in the force curve in Fig. 4(a).

An important finding here is that there is no drastic change in strength of the adhesion depending on the solution conditions. Under all conditions, PMEA showed weak adhesion with the proteins we used, whereas strong adhesion was always observed between PBA and proteins. What should be noted here is that the interaction between PBA and fibrinogen was always much stronger than that between PBA and

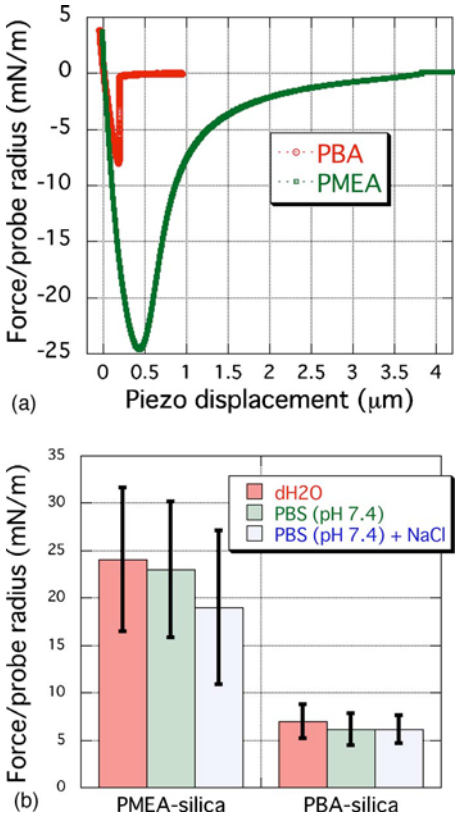

FIG. 2. (a) Force profiles on receding taken in the first scan as a function of *z*-piezo displacement. These curves were taken with the systems of PMEA-silica (green) and PBA-silica (red) in pure water. The zero position in the *x*-axis was defined arbitrarily at the position where the force curve crosses the zero of force. (b) Averaged maximum force observed for the systems of PMEA- and PBA-silica with different solution conditions. Error bars are standard deviations.

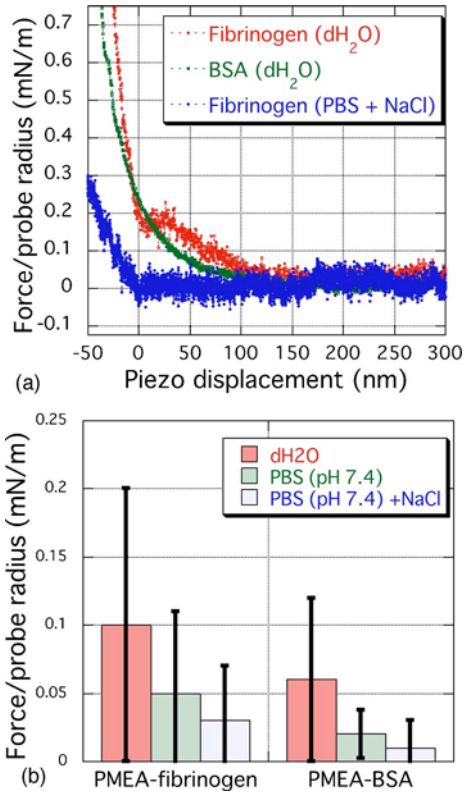

FIG. 3. (a) Force profiles on retracting taken in the first scan taken with the combination of the PMEA and proteins [fibrinogen (red) and BSA (green)] adsorbed on the silica sphere in pure water. A blue curve in (a) was measured in PBS buffer (*p*H 7.4) containing NaCl at a concentration of 0.1 M. (b) Averaged maximum force observed for the combinations of PMEA and proteins with different solution conditions. Error bars are standard deviations.

BSA [Fig. 4(b)]. This result can be explained by the fact that the sticking efficiency of fibrinogen is higher than that of BSA.[17]

For PMEA, force curves both on approaching and receding in pure water were almost identical because of the absence of the adhesion. There was long-range repulsion observed between the protein and the PMEA surface. This repulsion disappeared at a high salt concentration; therefore, the origin of the repulsion is electrostatic interaction between the proteins and the PMEA surface. With our experimental condition, fibrinogen and BSA bear negative charge because the isoelectric points of fibrinogen and BSA are 4.7 (Ref. 18) and 5.5,[19] respectively.

To elucidate whether or not electrostatic repulsion is responsible for blood compatibility, the surface charge of PMEA and PBA was characterized by measuring the force between these polymers and Si_3N_4 tips, which are negatively charged in pure water.[20] Long-range repulsion was observed between the Si_3N_4 tip and the polymers, indicating that both are negatively charged in pure water (Fig. 5). So far, we speculate that the negative charge is due to the preferential adsorption of negative ions such as hydroxyl ions near the surface.[21] In a previous report, Herrwerth *et al.* studied the protein resistance of the gold surface that was modified with SAMs of oligo(ethyleneglycol)-terminated alkane thiol.[22] Their finding was that the protein resistance of the SAMs is obviously associated with the ability of water trapping in the SAM (interior hydrophilicity and lateral density of the SAM on the substrate) and with the accessibility of water molecules into the SAM (hydrophilicity of the terminal groups). They speculated that the negative ions immobilized by the trapped water molecules at the SAM-water interface repel negatively charged proteins. In contrast with the case of the SAM, our results indicate that long-range electrostatic repulsion does not play a major role in the protein resistance of PMEA, because both polymer surfaces bear negative charge in water, as shown in Fig. 5. This is also supported by the fact that the strength of the adhesion does not depend on the ion concentration of the solution, which critically governs the Debye decay length [Fig. 3(b) and Fig. 4(b)]. Our results of force measurements suggest that the inertness of PMEA to the protein molecule stems from the short-range repulsion between PMEA and the proteins and not from long-range electrostatic repulsion.

Next, we discuss the interaction between PMEA and the denatured proteins. The striking fact here is that adhesion between the denatured protein and PMEA [Figs. 6(a)–6(c)] was observed, whereas there was very weak adhesion ob-

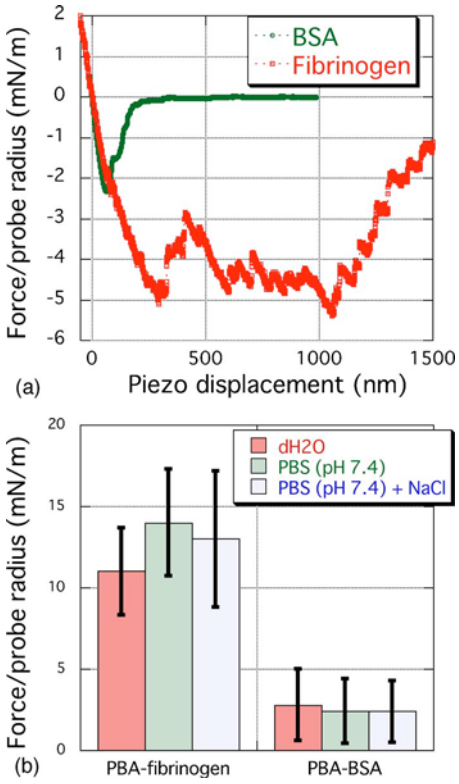

FIG. 4. (a) Force profiles on retracting taken in the first scan taken with the combination of the PBA and proteins [fibrinogen (red) and BSA (green)] adsorbed on the silica sphere in pure water. (b) Averaged maximum force observed for the combinations of PBA and proteins with different solution conditions. Error bars are standard deviations.

served between PMEA and native proteins. As in the previous cases, the adhesion did not depend on the condition of the solution. Similar to the interaction between PBA and proteins, denatured fibrinogen showed a stronger adhesion than BSA.

To confirm the above finding, adsorption experiments of proteins onto the polymer surfaces were performed using QCM [Figs. 7(a) and 7(b)]. A PBA film always adsorbed protein molecules independent of solution conditions, and

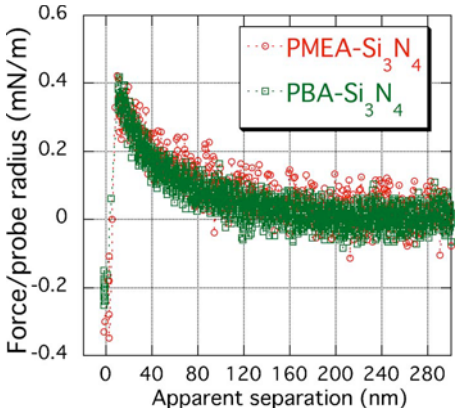

FIG. 5. Force plots on approaching as a function of apparent tip-surface separation measured with the systems of polymer surfaces [PMEA (red) and PBA (green)] and a silicon nitride (Si_3N_4) AFM tip in pure water.

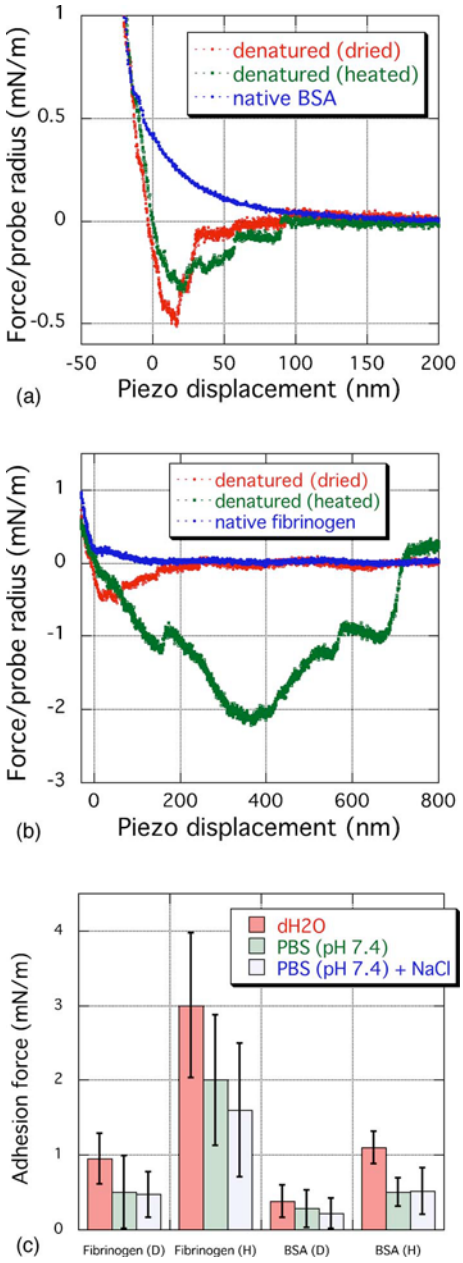

FIG. 6. Force profiles on retracting taken in the first scan taken with the combination of the PMEA and denatured proteins [(a) BSA and (b) fibrinogen] adsorbed on the silica sphere in pure water. (c) Averaged maximum force observed for the combinations of PMEA and denatured proteins with different solution conditions. Error bars are standard deviations.

the protein did not desorb but rather remained on the polymer surface. [Data are presented in the supporting material; solution conditions are pure water, PBS buffer (*p*H 7.4, 50 mM), and PBS buffer (*p*H 7.4, 50 mM) +NaCl 0.1 M.][23] For PMEA, after injecting the native protein molecules, a resonant frequency shift was observed due to the adsorption of the proteins and/or the change in the density of the solution around the QCM sensor. As clearly seen in Fig. 7(a), native protein molecules did not remain on the PMEA surface after rinsing. By contrast, QCM measurement revealed that denatured protein molecules remained adjacent to the

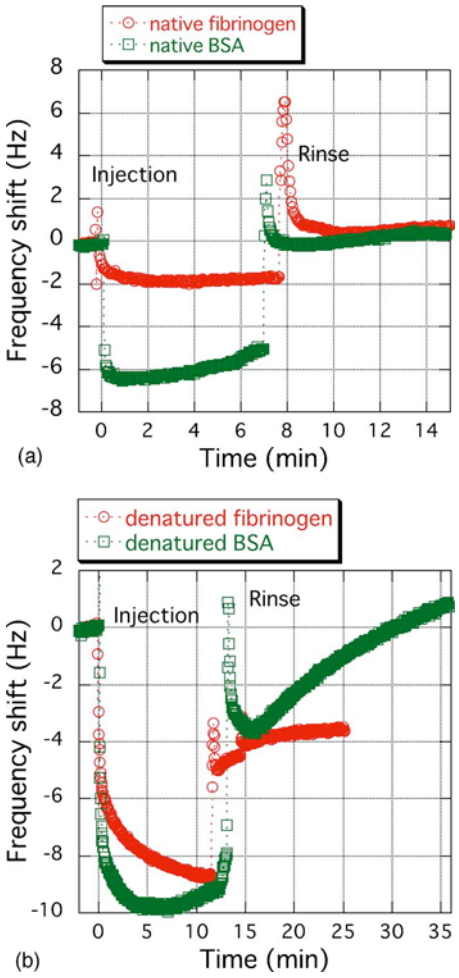

FIG. 7. QCM charts of adsorption of fibrinogen and BSA proteins [(a) native and (b) denatured] onto PMEA surfaces. The solution condition is PBS buffer (*p*H 7.4, 50 mM) containing NaCl at a concentration of 0.1 M.

PMEA surface even after rinsing [Fig. 7(b)]. What should be noted here is that denatured BSA gradually desorbed from the PMEA surface after rinsing, whereas denatured fibrinogen remained on the surface. This difference may be explained by the fact that heat-denatured fibrinogen adhered to PMEA more strongly than heat-denatured BSA. Adhesion between PMEA and fibrinogen denatured by heating is stronger than that between PMEA and BSA denatured by heating [Fig. 6(c)]. To conclude, the interaction between PMEA and denatured protein is stronger than that between PMEA and native proteins, supporting the results of the AFM measurements.

Finally, we discuss the mechanism of protein resistance of PMEA. To explain the mechanism of protein resistance of poly(ethylene oxide)(PEO), which is the most-studied protein-resistant material, steric repulsion has often been proposed.[24] In this idea, the approach of protein molecules to PEO-grafted substrates is unfavorable from the standpoint of conformational entropy, when the polymer is fully hydrated. However, with the idea of the steric repulsion only, we cannot explain the following three findings obtained by our experiments: (1) Although both PMEA and PBA are fully hydrated in water, the adhesion between PBA and the proteins was 100–300 times stronger than that between PMEA and the proteins. (2) PMEA strongly adhered to hydrophilic silica probes, whereas PMEA showed very weak adhesion to water-soluble proteins. (3) When the proteins were denatured, the adhesion between PMEA and the protein became stronger. To answer these questions, we need to take into account the behavior of molecules at polymer-water and protein-water interfaces.

Since the trend of the adhesion did not critically depend on the solution condition, indicating that electrostatic force may not be responsible for the protein resistance of PMEA, we focus our attention on the interfacial behavior of water. As mentioned in the Introduction, based on the results of DSC, water in the matrix of PMEA is categorized into three types: (1) nonfreezing water that is strongly bound to the polymer. (2) Freezing bound water that interacts with polymer in an intermediate way and crystallizes at around 230 K (cold crystallization). (3) Freezing water that interacts very weakly with the polymer and crystallizes at around 273 K. This classification was well supported by the results of ATR-IR and quantum-chemical calculations performed by Morita *et al.* in terms of the ratio of each water and vibrational frequencies.[9] According to the systematic DSC measurements of poly(meth)acrylate polymers by Tanaka *et al.*, only PMEA showed cold crystallization.[5] The cold crystallization was found only for some kinds of natural and synthetic biocompatible polymers, such as polysaccharides, gelatin, and poly(ethylene glycol),[25] whereas nonfreezing and freezing water were generally observed in common polymers, including PBA. Recently, the cold crystallization of water was observed for BSA in DSC measurements.[26] The authors speculated that water molecules in the second hydration layer, which exists outside of a primary hydration shell, shows cold crystallization. Contrary to PMEA, the mixture of silica powder and water did not show the cold crystallization, indicating that freezing bound water does not exist.[27]

Analyzing our results, very weak adhesion was observed only for the combination of PMEA and native proteins. On the other hand, strong adhesion was obviously observed for the other combinations. Therefore, it can be concluded that objects surrounded by freezing bound water adhere to each other very weakly. Although the mechanism of the decrease in the adhesion force due to the presence of the freezing bound water is not clear, we speculate that the layer of the freezing bound water may prevent short-range attraction such as electrostatic interaction between the polar or charged groups and van der Waals interactions. It should be noted that there is small dependence of adhesion force on the solution conditions. This may stem from the change in the hydration state of the proteins due to a slight conformational change in the protein structure. This result also supports the correlation of the adhesion force and hydration states of proteins and polymers.

In the case of the combination of silica and PMEA, the hydrogen bondings between the oxygen atom of PMEA and silanol groups on the silica surface are the source of the

strong adhesion. Because the adhesion in water was much weaker than that under air (data not shown), interfacial water tends to weaken the adhesion between PMEA and silica. However, the interaction between silica and PMEA was much stronger than that between native proteins and PMEA, implying that the layer of freezing bound water with a certain thickness is responsible for the drastic decrease in the adhesion.

Based on this idea, we can explain the origin of the adhesion between PMEA and denatured proteins. When proteins are denatured, the hydrophobic part of the amino acid chain, which is folded inside in the native state, will be exposed to the protein-water interface. The hydration state of the protein then changes and the layer of freezing bound water may be affected, resulting in the adhesion due to short-range van der Waals and electrostatic interactions. This might be the reason for the adhesion between PMEA and denatured proteins.

In this paper, we discussed the mechanism of protein resistance of PMEA based on the results of AFM force curve measurements. Our results strongly indicate that the interfacial water (in particular, freezing bound water) plays an important role in the blood compatibility of PMEA. This idea is somewhat consistent with previous reports on the protein resistance of oligo (ethylene glycol)-terminated SAMs and PEO-grafted surfaces, suggesting that hydration and conformational flexibility of the molecules are important factors to govern the protein resistance.[28] On the other hand, Chen *et al.* reported that the surface with balanced charge and crystalline structure showed strong resistance to protein adsorption, indicating that the surface charge is responsible for the protein resistance.[29] Though there are many ideas proposed to explain the mechanism of protein resistance of various systems, there has been no conclusive work so far. Therefore, further experimental and theoretical studies will be required to give insight to the mechanism of protein resistance.

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