Scanning Force Microscopic Study of Protein Adsorption on the Surface of Organosilane Monolayers Prepared by the Langmuir-Blodgett Method

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SUMMARY: The *n*-octadecyltrichlorosilane (OTS, $CH_3(CH_2)_{17}SiCl_3$), 18-nonadecenyltrichlorosilane (NTS, $CH_2=CH(CH_2)_{17}SiCl_3$), [2-(perfluorooctyl)ethyl] trichlorosilane (FOETS, $CF_3(CF_2)_7CH_2CH_2SiCl_3$) monolayers, and their mixed monolayers were used as the model substrates for the study of protein adsorption mechanism. Surface plasmon resonance (SPR) spectroscopy was applied to analyze the protein adsorption behavior onto the surface of the monolayers. Atomic force microscope (AFM) was used to observe the monolayer surfaces after exposure of these monolayers to bovine serum albumin (BSA) and γ-globulin(IgG) solution. AFM observation revealed that the charged protein either below or above the isoelectric point was preferentially adsorbed onto the FOETS phase of the (OTS/FOETS) mixed monolayer. SPR revealed that the amount of adsorbed protein in the charged state was lower than that in the neutral state. These results indicate that the preferential adsorption of protein onto the FOETS phase for the mixed monolayer systems at either below or above pI is due to (1) the minimization of interfacial free energy between the monolayer surface and the buffer solution, and (2) the electrostatic repulsion among protein molecules bearing charges.

Introduction

The adsorption of proteins onto an artificial surface is a central concern for system in which man-made materials contact biological fluids such as biosensors and artificial organs[1,2]. The adsorption amount and the conformation of adsorbed proteins play a key role in biological response[1,2]. The protein adsorption at solid-liquid interfaces has been investigated by various techniques such as ellipsometry[3], fluorescence spectroscopy[4], radiotracers[5], surface-enhanced Raman spectroscopy[6,7], attenuated total reflection Fourier transform infrared (ATR-FT-IR) spectroscopic flow cell method[8,9], and atomic force microscopy (AFM)[10-12]. AFM has a high resolution, and also, has a potential ability of real time imaging during adsorption process in an aqueous media.

It has been reported that the domain of multiphase polymer plays an important role in the mechanism of biocompatibility[13 - 15]. The role of microphase separation concept on biocompatibility of multiphase polymer system has been discussed in terms of presence of organized adsorbed plasma protein layer, which reflected the surface structure of multiphase

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polymers. However, since multiphase polymer shows the surface structure reorganization upon exposure to a water[15], it is necessary to employ a well-defined surface as a model system in order to undestand the mechanism of protein adsorption. The organosilane monolayer is a stable monolayer system in an aqueous environment[16-28]. The mixed monolayer prepared by the Langmuir-Blodgett(LB) methods showed the phase separation when one component was amorphous state. The phase-separated organosilane monolayer can be used as a model system for protein adsorption.

In this study, the (alkyltrichlorosilane/ fluoroalkyltrichlorosilane) mixed monolayers with various surface chemistry were prepared as a model surface for protein-surface interaction studies. Bovine serum albumin (BSA) and γ-globulin (IgG) were chosen to study the interaction of protein at the solid-liquid interface. The protein-monolayer surface interaction was investigated on the basis of SPR measurements and AFM observations.

Experimental

The *n*-octadecyltrichlorosilane (OTS, $CH_3(CH_2)_{17}SiCl_3$), 18-nonadecenyltrichlorosilane (NTS, CH_2=$CH(CH_2)_{17}SiCl_3$), [2-(perfluorooctyl)ethyl]trichlorosilane (FOETS, $CF_3(CF_2)_7CH_2CH_2SiCl_3$) monolayers, and their mixed monolayers were used as the model substrates for the study of protein adsorption mechanism. Fig.1 shows the schematic representation of preparation of organosilane monolayers by the LB method. These monolayers were polymerized on the water subphase and were subsequently immobilized onto the silicon

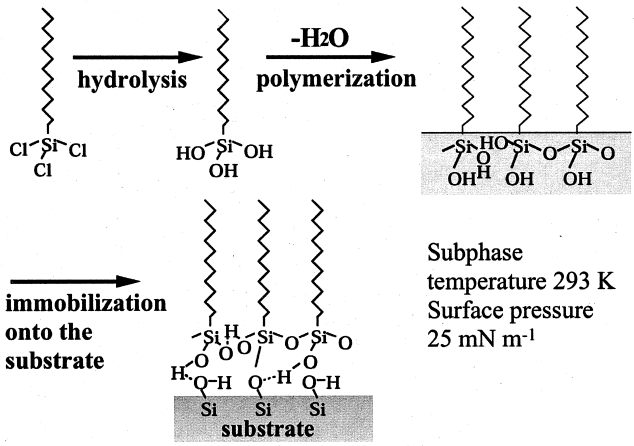


Fig.1 Schematic representation of formation of organosilane monolayers by the LB method

wafer surface by the upward drawing method[16-28]. The (carboxylated NTS(NTS_{COOH})/FOETS) mixed monolayer was prepared through an oxidation of vinyl group of the NTS phase in the (NTS/FOETS) mixed monolayer[28,29]. The NTS_{COOH} monolayer showed high surface free energy, where the magnitude was comparable to that of water.

The adsorption behavior of bovine serum albumin (BSA) and γ-globulin(IgG) on the monolayer surface was studied by surface plasmon resonance spectroscopy(SPR). The details on the principles of SPR can be found elsewhere[30]. Substrates for SPR were prepared by evaporation of chromium (ca.1-2nm in thickness) and gold (ca.50nm in thickness) onto glass cover slips. The metalized substrates were immersed in the γ-mercaptopropyltriethoxysilane (MTS) ethanol solution for 1 hour. The MTS coated substrates were subsequently exposed to the 0.1M hydrochloric acid solution to obtain hydroxyl group-modified gold surface. The mixed organosilane monolayers were deposited on the hydroxyl group-modified gold surface by the LB method. The substrate was glued to the SPR prism. The special care was taken to prevent the noises due to the accumulation of air bubbles. The concentrations of BSA and IgG for SPR measurement were 0.1g l^{-1}. The flow rate of protein solution was 11μl s^{-1}.

The morphology of adsorbed BSA and IgG on the monolayer surface was observed by AFM. The observation was carried out under non-contact mode. Rectangular Si-cantilever with a spring constant of 47-63 N m^{-1} was used for the AFM observation. The monolayer was immersed in the protein solution with a concentration of 0.1g l^{-1} for 10 mins. The immersed specimen was rinsed with phosphate buffer solution (PBS) and dried under an atmospheric pressure. A comparison of the AFM images of the state of adsorbed protein before and after drying revealed that the morphology of the adsorbed protein did not change after drying.

The protein-surface interaction was studied on the basis of force-distance curve measurement. Triangular Si_3N_4-cantilever with a spring constant of 0.1 N m^{-1} was used for the AFM observation. At first, a BSA immobilized-probe was used for the measurement of force-

Table 1 The water contact angle, surface free energy and aggregation state of the monolayers

Organosilane compound	θ_{water}/deg	γ_{SV}^{*}/mN m^{-1}	Aggregation structure **
$CF_3(CF_2)_7CH_2CH_2SiCl_3$ (FOETS)	110	13.4	amorphous
$CH_3(CH_2)_{17}SiCl_3$ (OTS)	109	22.7	crystalline
CH_2=CH $(CH_2)_{17}SiCl_3$ (NTS)	95	26.9	crystalline
$HOOC(CH_2)_{17}SiCl_3$ (NTS_{COOH})	24	68.8	crystalline

*Owens's method, **at 293K by electron diffraction[17,28]

distance curve between BSA and (OTS/FOETS) mixed monolayer surface. However, the denaturation of BSA seems to occur when the tip approaches to the surface and the reproducible data were not obtained. Then, the AFM probe with well-defined surface composition to mimic the nonspecific interaction between BSA molecules and mixed monolayer was prepared. Since BSA shows isoelectric point, pI=4.7, the $HOOC(CH_2)_9SH$ chemisorbed Au-coated probe was employed for the force-distance curve measurement. Force-distance curve measurement was performed in PBS with pH=7.4.

Results and Discussion

Aggregation structure of mixed organotrichlorosilane monolayer

The OTS, FOETS, NTS, mixed (OTS/FOETS)(50/50mol/mol), and (NTS/FOETS) (50/50mol/mol) monolayers were prepared at the water surface and subsequently immobilized onto the silicon wafer substrate. Table 1 summarizes the aggregation state and surface free energy of the monolayers. The OTS and NTS were in a crystalline state at room temperature, whereas FOETS was amorphous at 293K. NTS_{COOH} also showed a crystalline state at 293K. The surface free energy of FOETS, OTS, NTS, and NTS_{COOH} was 13.4, 22.7, 26.9 and 68.8mNm^{-1}, respectively. Fig.2 shows the AFM images of the mixed (OTS/FOETS), (NTS/FOETS), and (NTS_{COOH}/FOETS) monolayers. AFM observation revealed that the mixed (OTS/FOETS), and (NTS/FOETS) monolayers were in a phase-separated state, and circular flat-topped domains of ca. 1-2 μm in a diameter were surrounded by a sea-like and flat region. The

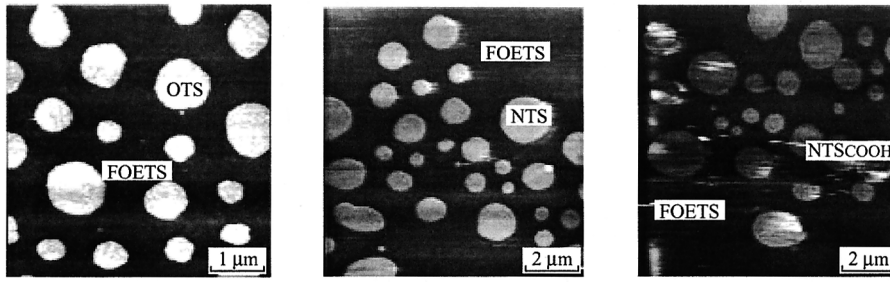


Fig.2: AFM images of the mixed (OTS/FOETS) , (NTS/FOETS) and (NTS_{COOH}/FOETS) monolayers.

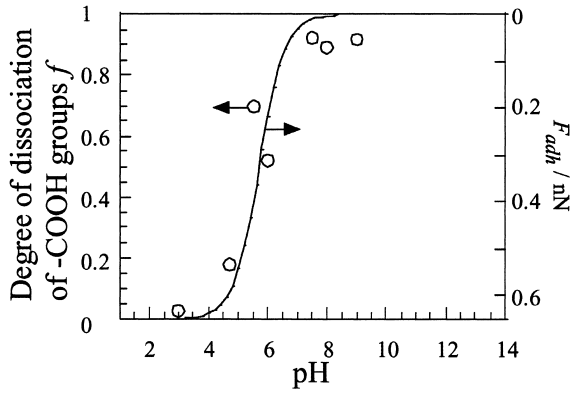


Fig.3: Force titration curves between the $HOOC(CH_2)_9SH$ chemisorbed Au-coated probe and NTS_{COOH} monolayer surface.

phase separation in the mixed (OTS/FOETS) and (NTS/FOETS) monolayers might be arisen from the crystallizability of the OTS or NTS component. Even though the oxidation treatment was carried out to the NTS phase for the preparation of the mixed (NTS_{COOH} /FOETS) monolayer, the monolayer remained the phase-separated structure in a similar fashion to that before an oxidation treatment.

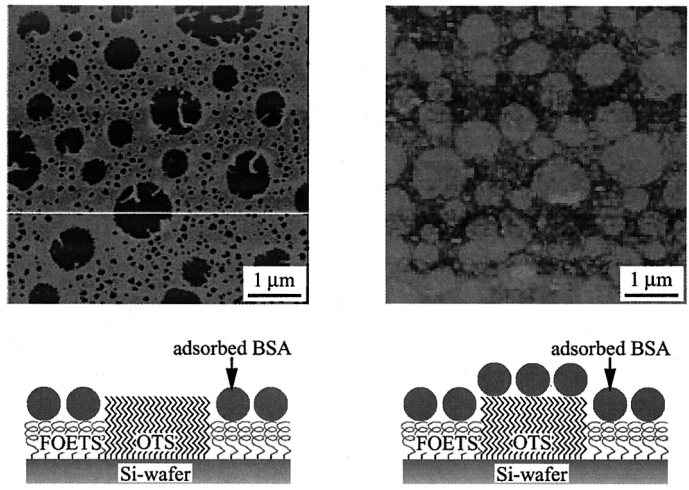
In order to characterize the surface on NTS_{COOH} in aqueous environment at pH=7.5, the force-titration[31] between the $HOOC(CH_2)_9SH$ chemisorbed Au-coated probe and NTS_{COOH} monolayer surface was carried out. In general, adhesion force was observed depending on the chemical identity of the tip and sample surface and was highly sensitive to the ionization state of the terminal functionality induced by varying the solution pH. Fig.3 shows the force titration curves between COOH-modified surfaces. Adhesion measurements as a function of pH on COOH-terminated monolayer exhibited a large drop in adhesion force for pH greater than 5.0. If one assumes the complete ionization occurred at high pH and no ionization at pH=2.0, the fraction of dissociated COOH group can be calculated. The $pKa^{surface}$ was evaluated from following equation,

$$log[f(1-f)] = pH - pKa$$

where f is the degree of ionization at the surface. The magnitude of $pKa^{surface}$ estimated from this curve, 5.5 is in good agreement with the reported value[32] and the free aqueous solution value.

Adsorption behavior of BSA onto (OTS/FOETS) mixed monolayers

The protein adsorption behavior of organosilane monolayers has been studied on the basis of ATR-IR flow cell experiment[20,23]. BSA solution was exposed to organosilane monolayers under flow. BSA showed a steep increase of an adsorbed amount and showed an equilibrium value within 5 mins. It was revealed that the amount of adsorbed BSA on the mixed (OTS/FOETS) monolayer was lower than that on OTS and FOETS monolayers. This result suggests that the adsorption state of BSA onto the mixed (OTS/FOETS) monolayer is different from that onto the OTS and FOETS monolayers. In order to clarify the reason for this adsorption behavior, the morphological observation of the adsorbed BSA was carried out by AFM. The surface of the mixed (OTS/FOETS) monolayer after exposure to a BSA solution was observed under non contact mode AFM. Fig.4(a) shows the AFM image of the mixed (OTS/FOETS) monolayer surface after its exposure to a BSA 0.1g l^{-1} solution in PBS (pH=7.5, 0.01M) at 293 K for 10mins[23]. The bright adsorbed layer of BSA was only observed on the FOETS phase. Even though the scanning was repeated, the destruction of the adsorbed BSA layer was not observed during scanning. The observed AFM images under *in situ* observation were similar to those observed for a dehydrated specimen after BSA exposure[24].



(a) (b)

Fig. 4: AFM images of the mixed (OTS/FOETS) monolayer surfaces after its exposure to a BSA 0.1g l^{-1} solution in PBS with (a) pH=7.5 and (b) pH=4.7 at 293 K for 10mins.

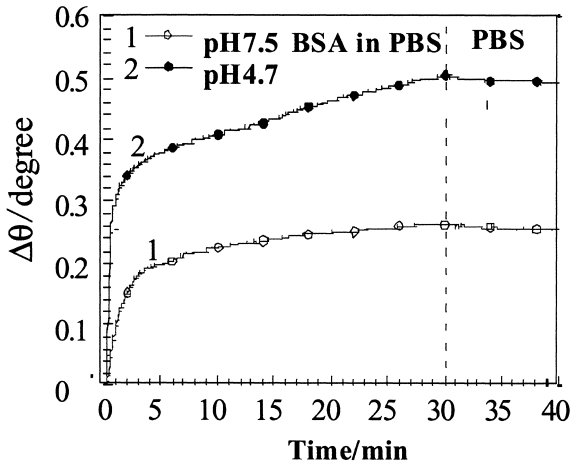


Fig. 5: Δθ versus time for the mixed (OTS/FOETS) monolayer after exposure to the BSA solution with pH=7.5 and 4.7.

The magnitude of the interaction between the protein molecules and the monolayer surface strongly depends on the hydrophobic interaction in the case of the hydrophobic monolayer surface. Since BSA molecules in the buffer solution have negative electric charges at pH=7.5, the electrostatic repulsion among BSA molecules may significantly influence the state of adsorbed BSA. As the surface free energy of the FOETS phase is lower than that of the OTS one, the magnitude of interfacial free energy at the FOETS-water interface is higher than that at the OTS-water one. In the case of the mixed (OTS/FOETS) monolayer, the BSA adsorption onto the OTS phase or onto the FOETS one depends on the magnitude of both the interfacial interaction between BSA molecules and the monolayer surface and the electrostatic repulsion between the adsorbed BSA's and BSA molecules in the solution. In the initial stage of the adsorption, the preferential BSA adsorption onto the FOETS phase may occur because adsorbed BSA onto the FOETS phase would lead to a remarkable decrease in the interfacial free energy. As the BSA adsorption progressed, the adsorbed BSA onto the FOETS phase changed their conformation to attain a steady-state structure and form a stable adsorption layer. On the OTS phase, because of the weak hydrophobic interaction between BSA and the OTS surface and also, the electrostatic repulsion between adsorbed BSA on the FOETS phase and BSA molecules in the solution, their adsorption interaction with the OTS phase would be weak compared with that of FOETS.

In order to confirm the influence of charge of BSA on the adsorption behavior, the adsorption of BSA onto the mixed (OTS/FOETS) monolayer was carried out at the isoelectric point (pI=4.7) of BSA. Fig.4(b) shows the AFM image of the mixed (OTS/FOETS) monolayer surface after its exposure to a BSA 0.1 g l^{-1} solution in PBS (pH=4.7, 0.01M) at 293 K for 10 mins. At this pH, the BSA molecules homogeneously adsorbed onto both the OTS and FOETS phases. This corresponds to the absence of electrostatic repulsion at the isoelectric point.

The amount of adsorbed BSA on the mixed (OTS/FOETS) monolayers was measured with SPR. Fig.5 shows the change in resonance angle, $\Delta\theta$ versus time for the mixed (OTS/FOETS) monolayer after exposure to the BSA solution with pH=7.5 and 4.7. For the protein adsorption study, a solution of buffer was flowed through the cell, substituted with a solution of protein and the replace with the buffer. The change in the refractive index of monolayer surface due to the protein adsorption causes a change in resonance angle. Thus the large shift in resonance angle, $\Delta\theta$ corresponds to the large amount of adsorption. The curves in Fig. 5 indicate that the BSA adsorption at the isoelectric point is greater than that in the charged state. These results correspond to the state of adsorbed BSA observed by AFM. Also, the SPR curve suggested that the desorption of BSA was not observed when the BSA solution was replaced with PBS. This suggests a strong interaction between BSA and monolayer surface. The results obtained here indicate that the electrostatic repulsion among BSA molecules is an important factor how the BSA molecules adsorbed on these surfaces. Fig.6 shows the schematic representation of the state of adsorbed BSA on the mixed (OTS/FOETS) monolayers at pH=7.5. It can be concluded from these results that the preferential adsorption of BSA onto the FOETS phase of the mixed (OTS/FOETS) monolayer system at pH=7.5 is due to (1) the minimization of the interfacial free energy between the monolayer surface and an aqueous solution, and (2) the electrostatic repulsion among BSA molecules bearing negative charges.

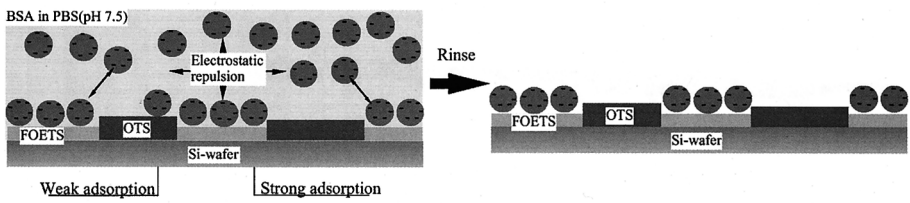
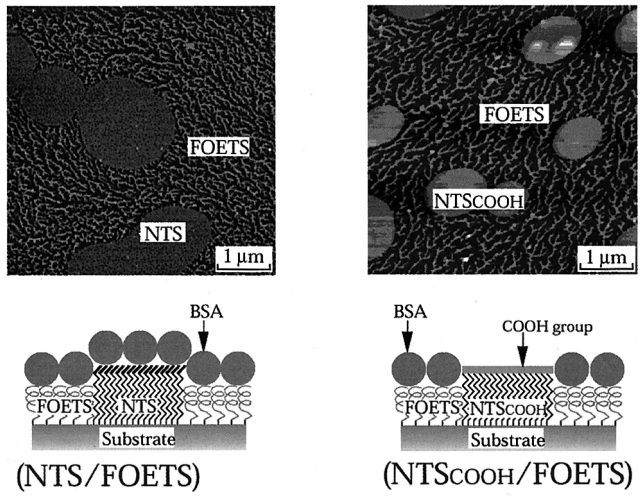


Fig.6: Schematic representation of the state of adsorbed BSA on the mixed (OTS/FOETS) monolayers at pH=7.5

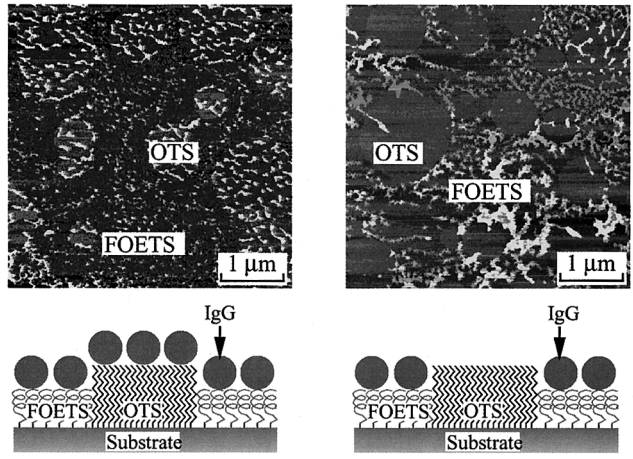


(a) (b)

Fig.7: AFM images of the mixed (NTS/FOETS) and (NTS_{COOH}/FOETS) surface after its exposure to a BSA 0.1g l^{-1} solution in PBS (pH=7.5, 0.01M) at 293 K for 10min

BSA adsorption behavior onto the mixed (NTS/FOETS) and (NTS_{COOH}/FOETS) monolayers

The mixed (NTS/FOETS) and (NTS_{COOH}/FOETS) monolayers were prepared in order to study the influence of the hydrophilic group on the BSA adsorption behavior. Fig.7 shows the AFM images of the mixed (NTS/FOETS) and (NTS_{COOH}/FOETS) surfaces after their exposure to a BSA 0.1g l^{-1} solution in PBS (pH=7.5, 0.01M) at 293 K for 10 mins. The presence of adsorbed BSA was confirmed by the height difference after shaving the adsorbed layer by AFM tip. The NTS phase has a higher amount of adsorbed BSA compared with that of the OTS one because of the presence of C=C bond at the surface. In the case of introduction of carboxylate group on the NTS_{COOH} phase, the BSA adsorption was hardly observed for the NTS_{COOH} phase, because of the electrostatic repulsion between negatively charged BSA and dissociated carboxyl groups of NTS_{COOH}.



(a) (b)

Fig.8: AFM images of the mixed (OTS/FOETS) monolayer surface after its exposure to a IgG 0.1g l^{-1} solution in PBS with (a) pH=7.5 and (b) pH=4.7 at 293 K for 10 mins.

Adsorption behavior of IgG onto the (OTS/FOETS) mixed monolayers

In order to discuss the influence of the charge of protein on adsorption behavior, IgG which has a higher pI than BSA was used. Fig.8(a) shows the AFM image of the mixed (OTS/FOETS) monolayer surface after its exposure to an IgG solution in PBS (pH=7.5, 0.01M) at 293 K for 10mins. At this pH, the IgG molecules are homogeneously adsorbed onto both the OTS and FOETS phases. Since IgG is in an isoelectric point at this pH, the charge repulsion between adsorbed IgG and IgG molecule in solution is weak. Fig.8(b) shows the AFM image of the mixed (OTS/FOETS) monolayer surface after its exposure to an IgG solution in PBS (pH=4.7, 0.01M) at 293 K for 10 mins. The bright adsorbed layer of IgG was only observed on the FOETS phase. This adsorption state of IgG is very similar to that observed for BSA at pH=7.5.

The amount of adsorbed IgG on the mixed (OTS/FOETS) monolayers was measured with SPR. $\Delta\theta$ versus time for the mixed (OTS/FOETS) monolayer after exposure to an IgG solution with pH=7.5 and 4.7 revealed that the IgG adsorption at isoelectric point(pI=7.5) is greater than that in the charged state(pH=4.7). This result correspond to the state of adsorbed IgG by AFM. The

results obtained here indicate that the electrostatic repulsion among IgG molecules is an important factor how the IgG molecules adsorbed at these surfaces.

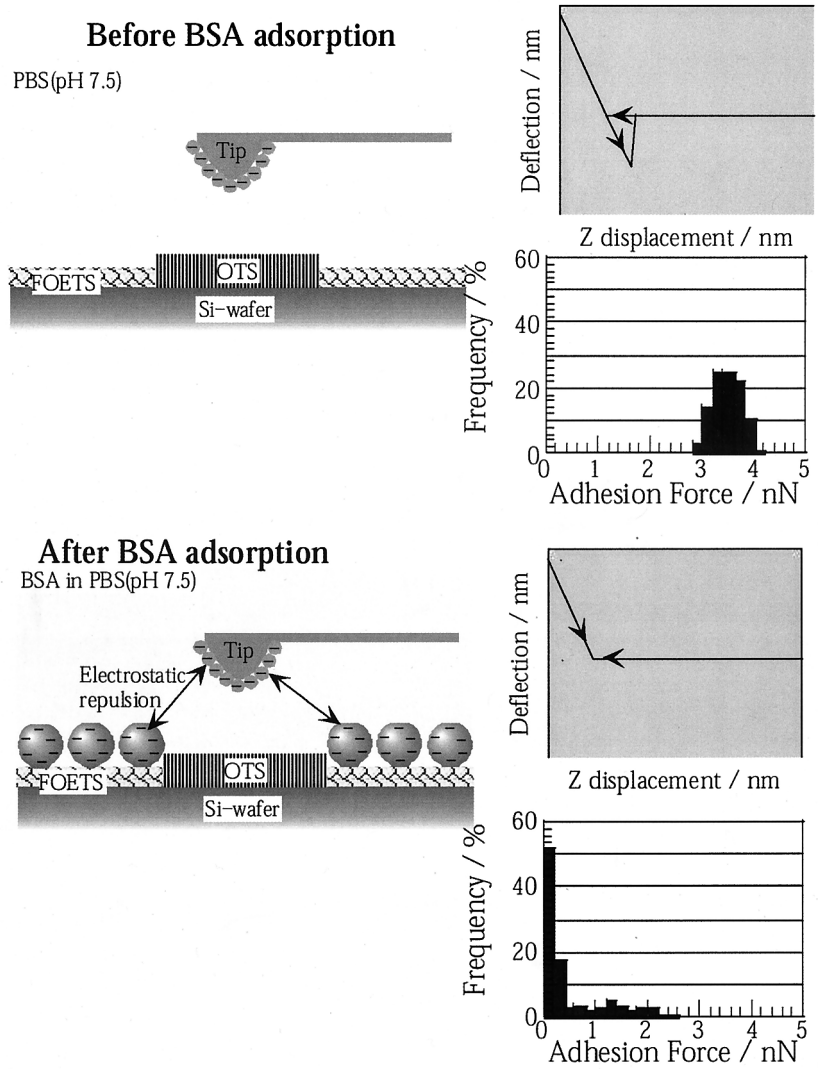


Fig.9: Schematic representation of modified probe tip-monolayer surface and force-distance curves between modified probe and OTS phase in the (OTS/FOETS) mixed monolayers before and after adsorption of BSA at pH=7.5. Histograms showing the distribution of adhesion force derived from force-distance measurements before and after adsorption of BSA are also shown.

From these results, it can be concluded that the preferential adsorption of IgG onto the FOETS phase of the mixed (OTS/FOETS) monolayer system at pH=4.7 is due to (1) the minimization of the interfacial free energy between the monolayer surface and an aqueous solution, and (2) the electrostatic repulsion among IgG molecules bearing positive charges.

Force acting between $HOOC(CH_2)_{10}SH$-modified Au-coated probe and monolayer surface

Force-distance curves between a $HOOC(CH_2)_9SH$-chemisorbed Au-coated probe and the mixed monolayer were measured at pH=7.5. The force titration curve between the $HOOC(CH_2)_9SH$-chemisorbed Au-coated probe and the NTS_{COOH} monolayer gave the pKa of 5.5. Therefore , in the case of pH=7.5, COOH groups at the surface were dissociated. Fig.9 shows schematic representation of modified probe tip-monolayer surface and force-distance curves between modified probe and OTS phase in the (OTS/FOETS) mixed monolayers before and after adsorption of BSA at pH=7.5. The histograms showing the distribution of adhesion force derived from force-distance measurements before and after adsorption of BSA are also shown in Fig. 9. The histograms show the distribution of adhesion force obtained from more than 300 force curve data. In the case of before BSA adsorption, a large adhesion force was observed in the force-distance curve. In order to confirm the decrease in adhesion force due to the electrostatic repulsion among BSA molecules, the force distance curves were measured between COOH-modified probe and the mixed monolayer after exposure to the BSA solution. AFM observation revealed the preferential adsorption of BSA onto the FOETS phase in the mixed monolayer. At pH=7.5, the COOH groups on the tip are dissociated. Since the negatively charged BSAs were selectively adsorbed on the FOETS phase, the adhesion force between modified probe and the OTS phase became lower in comparison with the case before exposure to the BSA solution. The origin of this decrease in adhesion force can be ascribed to the large contribution of electrostatic repulsion between ionized COOH groups on the tip surface and negatively charged BSA adsorbed on the FOETS phase. The results seem to be consistent with the above mentioned model on the selective adsorption of BSA onto FOETS phase in the (OTS/FOETS) mixed monolayer.

Conclusions

AFM and SPR were applied for the characterization of protein adsorption behavior onto phase-separated (OTS/FOETS) monolayer. The preferential adsorption of BSA at pH=7.5 and IgG at pH=4.7 was observed on the FOETS phase having a higher interfacial free energy against water than OTS one. The preferential adsorption of the protein onto the FOETS phase in mixed

(OTS/FOETS) monolayers was not observed at the isoelectric point of protein. These results indicate that the minimization of the interfacial free energy between the monolayer surface and solution as well as the electrostatic repulsion among charged protein molecules are important factors that lead to the preferential adsorption of protein onto the FOETS phase in the mixed (OTS/FOETS) monolayers. The absence of adhesion force between negatively-charged cantilever tip and OTS phase in the presence of adsorbed BSA on FOETS phase of mixed monolayer supports above hypothesis.

Acknowledgment

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