Non-specific and Specific Adsorption of Proteins on Langmuir–Blodgett Films of Amino Acid Derivative Polymers

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(Received December 25, 2000)

Polymer LB films consisting of lysine and alanine derivative polymers were prepared to investigate the interfacial recognition between protein and ligand on the LB film. The surface of lysine derivative polymer LB film was modified by the biotin molecule that is the ligand for avidin. The biotinylation was carried out without inducing non-specific adsorption of proteins; avidin specifically adsorbed onto the biotinylated surfaces. Moreover, it was found that there is an optimum molar ratio of biotinylation for the adsorption of avidin onto the surface.

The immobilization of proteins on solid surfaces is an important challenge for the development of sensors[1] and the study of biomolecular recognition at the interface.[2–4] Non-specific adsorption of proteins usually occurs on hydrophobic surfaces and the efforts to modify the surface to make it resistant to the non-specific adsorption of proteins have been carried out.[5–7]

The solid surface is often treated by a self-assembled monolayer of thiol derivatives (SAMs method),[8,9] Langmuir–Blodgett film (LB technique),[10,11] and layer-by-layer deposition.[11] The LB technique provides a biomimetic surface with well-defined molecular orientations and a thickness of nanometer level. However, conventional LB films prepared from low molecular-weight compounds are not mechanically and thermally stable, and have a defect caused by crystallization.

We have continued to investigate the preparation of functional polymer LB films. Through such studies, we found that poly(*N*-alkylacrylamide) structure is suitable for the formation of high quality monolayer where hydrogen bonding of the amide groups plays an important role for self-assembly.[13–16] Moreover, based on this excellent property of the polyacrylamide LB film, we fabricated a device for the molecular chiral recognition using polymer LB film of a copolymer of *N*-dodecylacrylamide (DDA) with (*S*)-2-methoxy-1, 1-binaphthalen-2-yl methacrylate.[17]

In the research reported in this paper, we prepared the polymer LB films consisting of lysine (P-Lys-Ac, Fig. 1) and alanine (P-Ala-Ac, Fig. 1) derivative acrylamide polymers. The amino group in the polymer LB film is exposed to the surface and can be modified by a biotin group (Fig. 1). We investigated the interfacial interaction between the biotin group and avidin on the polymer LB films.

Experimental

Materials. Lysine and alanine derivative polymers (poly{*N*-[1-(tetradecylcarbamoly)-5-aminobutyl]acrylamide} (P-Lys-Ac) and poly {*N*-[1-(tetradecylcarbamoly)ethyl]acrylamide} (P-Ala-Ac), Fig. 1) were prepared by using a conventional radical polymerization of the corresponding monomers; N^{α}-acryloyl-*N*-tetradecyllysinamide and N^{α}-acryloyl-*N*-tetradecylalaninamide, respectively. The monomers were prepared by using general synthetic methods of peptides.[18,19] The molecular weight and dispersion of P-Ala-Ac are 5.60×10^3 and 1.46, respectively. Avidin (67 kD) (Sigma) and bovine serum albumin (69 kD) (Sigma) were all used as received.

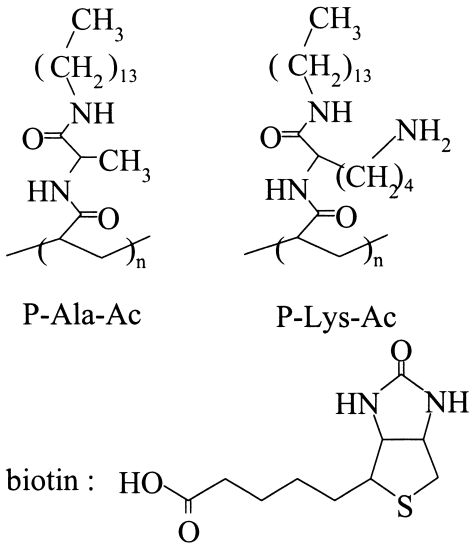

Fig. 1. Chemical structures of P-Lys-Ac, P-Ala-Ac, and biotin.

Measurement. The molecular weights and dispersions of polymers were determined by a Toyo Soda gel permeation chromatography (GPC) using a polystyrene standard. UV-vis absorption measurements were carried out with a Hitachi U-3000 UV-vis spectrophotometer. Measurement of surface pressure–surface area (π–A) isotherms were carried out with an automatic Langmuir trough (Kyowa Kaimen Kagaku, HBM-AP) equipped with a Wilhelmy balance at a compression speed of 14 $cm^2\ min^{-1}$ and at 20 °C. Milli-Q grade water (resistivity $>$ 17 $M\Omega\ cm^{-1}$) was used as the subphase. Ionic strength (I) and pH of subphase were var-

ied with NaCl and NaOH, respectively. The polymers were spread from tetrahydrofuran or chloroform solutions (spectroscopic grade) (10^{-3} M(1 M = 1 mol dm^{-3})) on the water surface. Quartz substrate (40 × 15 × 1 mm) for the deposition of polymer monolayers was made hydrophobic with trichloro(octadecyl)silane.

Results and Discussion

Spreading Behaviors and Formation of Multilayers of Lysine and Alanine Derivative Polymers. Spreading behaviors of P-Lys-Ac and P-Ala-Ac polymers on the water surface were investigated by the measurement of surface pressure – surface area (π–A) isotherms. In the π–A isotherm of P-Lys-Ac on pure water, no increase in the surface pressure was observed, which indicates that the polymer forms an aggregated form on the water. Increase in surface pressure was observed when the subphase conditions (pH and I) were changed. Figure 2 shows the π–A isotherms of P-Lys-Ac (A) and P-Ala-Ac (B) at 20 °C on various subphases (pH = 7 – 13 and ionic strength (I) = 0.1 – 1.5). As shown in Fig. 2A, the average molecular occupied surface area per repeating unit (limiting area, A_{LA}) for the P-Lys-Ac changed very little when the subphase conditions were $I > 1.0$ and pH > 13. The A_{LA} of P-Lys-Ac was 0.370 nm^2/monomer unit at pH = 13 and I = 1.0. Here the A_{LA} are given by extrapolation of the steeply rising part of the π–A curve to zero surface pressure. On the other hand, the A_{LA} of the P-Ala-Ac was 0.434 (nm^2/monomer unit) and this value did not change with the pH or ionic strength of the subphase (Fig. 2B). These results indicate that the coordination of ionic species to the amino group in Lys and deprotonation of the amino group ($-NH_3^+ \rightarrow -NH_2$) weaken the interaction between the amino groups, and prevent the aggregation of the P-Lys-Ac. It is considered that the P-Lys-Ac and P-Ala-Ac form densely packed monolayers on pH = 13, I = 1.0 subphase and neutral subphase, respectively. We previously reported that the amino acid side chain in P-Ala-Ac locates between tetradecyl chains in monolayer.[18] Taking into consideration the results that the monolayer behavior drastically changed by the subphase conditions and that the A_{LA} value was smaller than that of P-Ala-Ac, one can conclude that the amino acid side chain (amino substitute) in the P-Lys-Ac monolayer should exist in the water phase. The P-Lys-Ac monolayer was transferred onto a hydrophobic quartz substrate as Y-type LB film at pH = 13, I = 1.0 under a surface pressure of 28.5 mN m^{-1} with a dipping speed of 5 mm min^{-1}. The P-Ala-Ac monolayer was transferred onto a substrate as Y-type LB film at neutral condition under a surface pressure of 27.5 mN m^{-1} with a dipping speed of 10 mm min^{-1}. The transfer ratios for P-Lys-Ac and P-Ala-Ac were 0.95 and 1.0, respectively.

Modification of the Surface of LB Film and Adsorption Experiment of Proteins on the LB Film. Figure 3 is a schematic representation of the protocol for modifying the surface of LB film and for measuring the amount of protein adsorbed onto the LB films. We deposited P-Ala-Ac LB film with 10 layers (Ala10 LB film) onto a quartz slide and then 1 layer of P-Lys-Ac LB film as shown in Fig. 4a. To modify the surface of P-Lys-Ac LB film with biotin, the LB film was im-

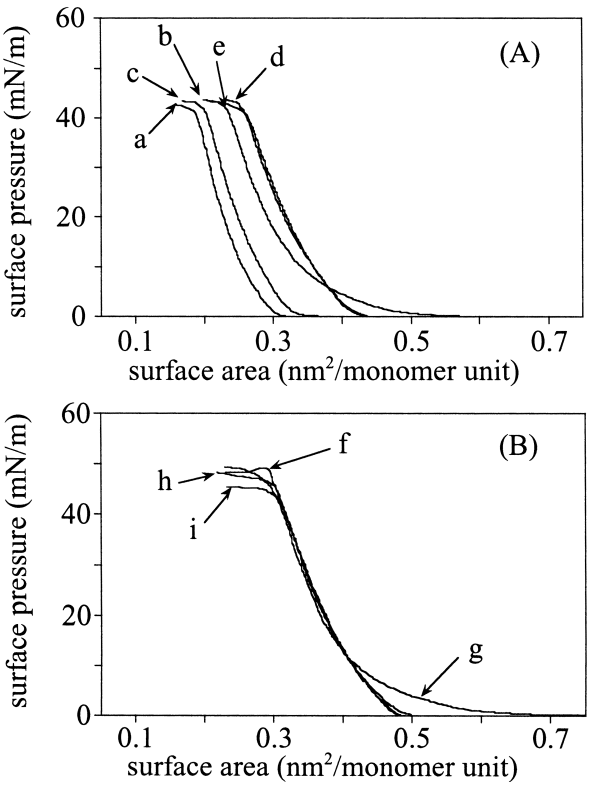

Fig. 2. Surface pressure – surface area isotherms for P-Lys-Ac (A) and P-Ala-Ac (B) at 20 °C on various subphases. (pH, I) = (12, 0.5) (a), (12, 1) (b), (13, 0.5) (c), (13, 1) (d), (13, 1.5) (e), (7, 0) (f), (7, 1) (g), (13, 0.1) (h), (13, 1) (i).

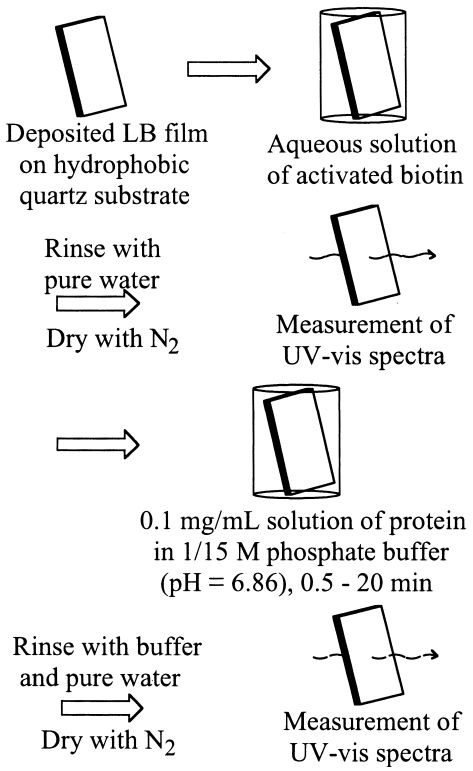

Fig. 3. Schematic representation of the protocol for modifying the surface of P-Lys-Ac LB films and measuring an adsorbed protein on the LB films.

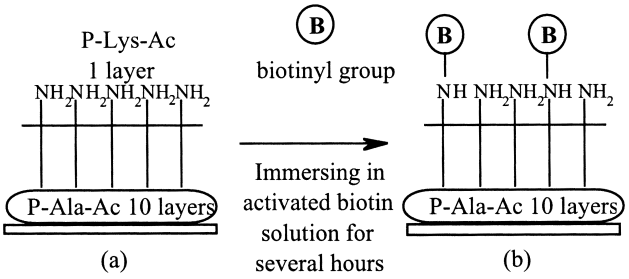

Fig. 4. Schematic illustration of P-Lys-Ac LB film (a) and biotinylated LB film (b).

mersed in an activated biotin (*N*-(2,2-dimethylpropyl)-*N*-carbodiimide) aqueous solution for several hours (0 – 5 h), and then rinsed thoroughly with pure water. The biotinylated LB films were then immersed for several minutes in solutions containing 0.1 mg mL^{-1} of protein (avidin or bovine serum albumin (BSA)) in 1/15 M aqueous phosphate buffer (pH = 6.86) at 25 °C. The amount of proteins adsorbed on LB film was measured by the changes of the absorbance at 195 nm (Δ Abs.).

The molar ratios of biotinyl groups to amino groups on the surface of P-Lys-Ac LB film, B/A, were determined by the absorbance at 193 nm. B/A ratios increased with immersing time of P-Lys-Ac LB film into the activated biotin solution. On the other hand, no change of absorbance was observed when Ala10 LB film was immersed into the same solution. This means that the biotinyl groups are not physically adsorbed on the LB film, but were chemically introduced to the amino groups of the surface of P-Lys-Ac LB film (Fig. 4b).

Adsorption of Proteins on the LB Films. The biotinylated LB films and Ala10 LB film were immersed into bovine serum albumin (BSA) or avidin solutions (0.1 mg mL^{-1}) in 1/15 M phosphate buffer at 25 °C for several minutes. UV-vis spectra of the LB films after they were rinsed with the buffer and pure water show an absorption maximum at 195 nm, and a shoulder around 290 nm, which are assigned to peptide bond, and to the tryptophan and tyrosine in protein, respectively. The amount of adsorbed protein on the LB film was determined from the absorbance gain at 195 nm. Since the molecular weight of BSA is nearly equal to that of avidin and the absorption coefficient at 195 nm (peptide bond) for the two proteins should be almost equivalent, the relative ratio of adsorbed BSA and avidin can be determined from absorbance at 195 nm. Figures 5A (BSA) and 5B (avidin) show the changes in the absorbance at 195 nm as a function of the immersing time. The Δabs values, in all cases, increased and became saturated after about 10 – 20 min. The increase in the absorption at 195 nm for non-biotinylated (amine-terminated surface) and Ala10 (methyl-terminated surface) LB films was very small, which indicates that non-specific adsorption of proteins on the surface occurred.

In the non-specific adsorption of proteins on the surface of the LB films, both avidin and BSA adsorbed on the Ala10 LB film (methyl-terminated surface) a little bit more than on non-biotinylated LB film (amine-terminated surface). This difference is attributable to the difference in hydrophobicity of the LB films. It is reported that the non-specific adsorption of protein on solid surfaces depends on a hydrophobic character of

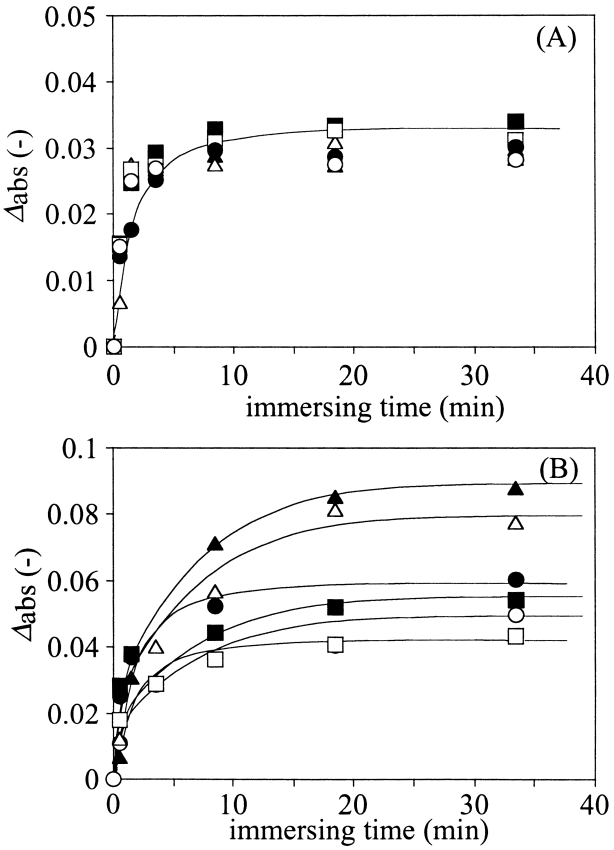

Fig. 5. The changes of absorbace at 195 nm each immersing time of various LB films in protein solution.
(A): BSA = 0.1 mg mL^{-1} [non-biotinylated LB film (○), B/A = 0.13 (●), B/A = 0.26 (△), B/A = 0.31 (▲), B/A = 0.41 (□), Ala10 LB film (■)].
(B): avidin = 0.1 mg mL^{-1} [non-biotinylated LB film (○), B/A = 0.13 (●), B/A = 0.17 (△), B/A = 0.22 (▲), B/A = 0.40 (□), Ala10 LB film (■)].

the surface and the amine-terminated surface is more resistant to the non-specific adsorption of protein than with the methyl-terminated surface.[6–8] On the other hand, the adsorption of avidin onto the non-biotinylated LB film and Ala10 LB film surfaces is larger than that of BSA. This is due to the difference in surface charges between the two proteins. Whitesides et al.[6–8] and Blankenburg et al.[20] demonstrated that the non-specific adsorption of proteins is enhanced by an increase of surface charge of proteins. In our experiments, the surface charge of avidin is larger than that of albumin in aqueous phosphate buffer (pH = 6.86) because isoelectric points for avidin and BSA are 11 and 5, respectively. This result does not conflict with the reports by Whitesides and Blankenburg. However, at the present, the physicochemical effect of surface charge of protein on the adsorption could not be explained. The difference in the adsorption between avidin and BSA may be explained by the difference in the hydration region between avidin and BSA.

Figure 6 shows the relationship between B/A ratio (the molar ratio of biotinyl groups to amino groups) and the saturated values of Δabs. In the case of BSA, the Δabs values have no dependence on B/A ratio, having a constant value. The result

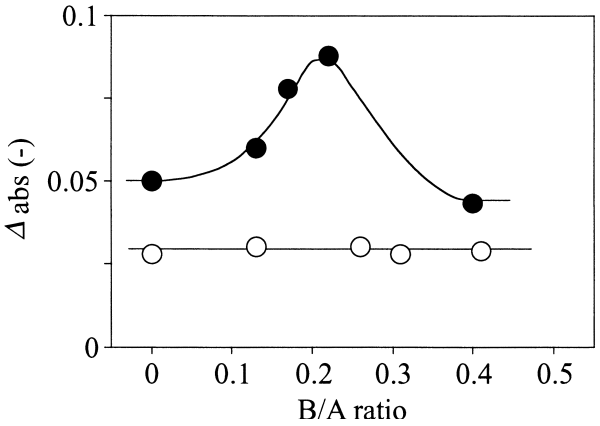

Fig. 6. Relationship between the saturated values of the Δabs (immersing time = 34 min) and the B/A ratios of LB films. Avidin (●), BSA (○).

indicates that there are no specific interactions between the surface of the LB films and BSA. This is compatible with the fact that the BSA does not specifically bind with biotin. Moreover, it is noteworthy that the introduction of biotinyl groups onto the surfaces does not enhance the non-specific adsorption of proteins.

In the adsorption of avidin, different results from that for the adsorption experiment of BSA were obtained. The saturated values of Δabs varied with B/A ratio and the plots of B/A vs Δabs have a maximum at B/A = 0.2 (Fig. 6). In the B/A region of 0 to 0.2, the amount of adsorbed protein (Δabs) increases with increasing B/A ratio, which is definitely caused by the increase of biotinyl groups as ligand molecules for avidin. The results show that the modification of the amine-terminated surface by the biotin molecule has been carried out successfully and, moreover, that the bio-specific recognition between the biotinyl groups and the avidin is carried out on the surface of the modified LB films (Fig. 7B). On the contrary, the amount of adsorbed protein decreased at B/A = 0.4. Such phenomenon might be attributed to the steric hindrance around the binding sites between avidin and biotin, based on excessive introduction of biotinyl groups on the surface as shown in Fig. 7C. We conclude that the nonspecific adsorption of avidin onto the excessive biotinylated surface (B/A = 0.4) has become the same as that onto the amine-terminated surface. Here we consider the structure of avidin. Avidin consists of four protomers, which binds with one molecule of biotin (Fig. 7A); and the size of an avidin molecule is 4.0 × 5.5 × 5.5 nm.[21] However, the biotin-binding sites capable of interacting with the biotinyl group on the LB film are two sites, which have a separation of 2 nm (Fig. 7A).[22] Therefore, when the distance between biotinyl groups on the LB films is about 2 nm, the interfacial interaction between avidin and biotin might be efficiently carried out. The two-dimensional density of amino group on the P-Lys-Ac is calculated from the molecular occupied surface area (A_{LA}) in the π–A isotherms, and the distance between the biotinyl groups ($[B]_D$) on the modified LB film is estimated from B/A ratios. The limiting area of P-Lys-Ac is determined to be 0.37 nm^2/monomer unit from the π–A isotherm. The $[B]_D$ values for the biotinylated LB films of B/A = 0.13, 0.17, 0.22, and 0.40 are obtained to be 1.9, 1.7, 1.5, and 1.1 nm, respectively. The maximum adsorption of avidin on biotinylated surface is expected at B/A ratio = 0.13 ($[B]_D$ = 1.9 nm). However, the actual optimum interaction was observed at B/A = 0.2 ($[B]_D$ = 1.5). The reason why this distance is better will be examined in the future study.

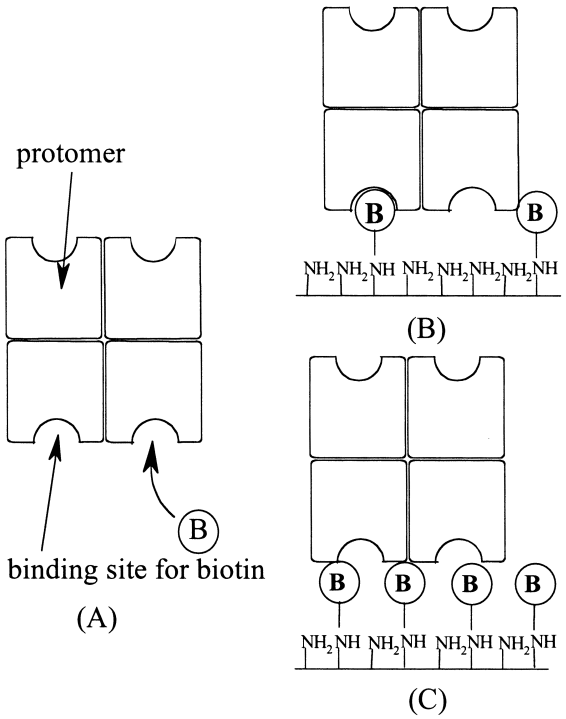

Fig. 7. Illustration of avidin molecule (A) and avidin binding to the surfaces of LB film modified with biotin at low (B) and high (C) densities.

Conclusion

The polymer LB films consisting of lysine and alanine derivative polymers were prepared. The surface of lysine derivative polymer LB film was covered with amino groups and was chemically modified with biotin molecules, without inducing non-specific adsorption of proteins. Moreover, avidin binded strongly with the surface of biotinylated LB film. The application of this method to modifying the surface of sensors is expected in the field of biosensors.

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