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Adsorption of Plasma Proteins on Arg–Gly–Asp–Ser Peptide-Immobilized Poly(vinyl alcohol) and Ethylene–Acrylic Acid Copolymer Films

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ABSTRACT: Tetrapeptide Arg–Gly–Asp–Ser (RGDS) exhibiting cell-attachment activity was immobilized to poly(vinyl alcohol) (PVA), and to ethylene–acrylic acid copolymer (PEA) conaining 2.84 mol% acrylic acid. RGDS was also immobilized to PEA *via* Gly–Gly–Gly (GGG) as a spacer. Adsorption behaviors of plasma proteins, albumin and γ-globulin, on these peptide-immobilized PVA and PEA surfaces were examined in phosphate buffer solution by means of interfacial pressure. It was found that the interfacial pressures of these polymer surfaces were drastically lowered by immobilizing the peptides, RGDS and GGGRGDS.

KEY WORDS Plasma Proteins Adsorption / Arg–Gly–Asp–Ser (RGDS) peptide / RGDS-Immobilized Polymer Film / Surface Pressure / Surface Area / Interfacial Pressure / Ethylene–Acrylic Acid Copolymer / Poly(vinyl alcohol) /

Along with the recent progress in structure elucidation on cell-adhesive proteins such as fibronectin, vitronectin, and laminin, some specified amino acid sequences in these proteins were presumed to act as the cell-attachment determinant of the proteins. An amino acid sequence, –Arg–Gly–Asp–Ser– (–RGDS–), is said to afford the cell-attachment site of fibronectin which locates in the blood plasma and on the cell surface. Piershbacher and co-workers[1,2] have pointed out that synthetic tetrapeptide Arg–Gly–Asp–Ser (RGDS) exhibits cell-attachment activity. In a separate paper,[3] authors examined the cell-attachment activity toward RGDS-immobilized poly(vinyl alcohol) film by using L-929 cells. The aim of this paper is to discuss adsorption of plasma proteins represented by albumin and γ-globulin onto RGDS-immobilized polymer films, because, in the application of materials exhibiting cell-attachment activity, for example, as a cell cultivation substrate, cell cultures are carried out in the presence of serum.

In this paper, RGDS was immobilized to poly(vinyl alcohol) (PVA) and to ethylene–acrylic acid (2.84 mol%) copolymer (PEA) films. Besides, Gly–Gly–Gly–Arg–Gly–Asp–Ser (GGGRGDS) peptide immobilized to PEA film was also examined to investigate the effect of GGG sequence introduced as a spacer. The protein adsorption behaviors were examined after the procedures and theoretical treatments reported earlier by us.[4]

EXPERIMENTAL

Materials

The protein samples used for adsorption

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were bovine serum albumin (BSA) and bovine serum γ-globulin (IgG), both purchased from Sigma. These proteins were dissolved in a phosphate buffer solution (PBS) composed of Na_2HPO_4, NaH_2PO_4, and water (pH = 7.4).

The polymers used for immobilization of oligopeptides were poly(vinyl alcohol) (PVA) purchased from Aicello Chemical Inc., and ethylene–acrylic acid (acrylic acid content, 2.84 mol%) copolymer (PEA) contributed from Teijino Co., Ltd. PVA and PEA films were prepared by casting from 5 wt% dimethyl sulfoxide solution and from 2 wt% cyclohexane–dioxane (8 : 2, v/v) solution, respectively, on glass plates (38 × 13 × 1 mm). Finally films were dried *in vacuo* for 5 days at 40°C. Oligopeptides RGDS and GGGRGDS used were synthesized by liquid-phase procedure.[5] Immobilization on of oligopeptides is as follows.

Immobilization of Oligopeptides

In a glass vessel, 2 g *p*-toluene sulfonyl chloride was dissolved in 7 ml diethylether, to which two PVA films were introduced and allowed to stand for 2 h at 20°C. Whereby, OH residues locating on the film surface were activated. Then, 80 mg RGDS peptide was reacted with the activated PVA films for 72 h at 30°C in phosphate buffer solution adjusted to pH 4.5, filled in a glass vessel. Thus, the RGDS peptide was immobilized to PVA film at its N-terminal.

Immobilizations of RGDS and GGGRGDS peptides to PEA were performed as follows. The –COOH residues locating on the PEA film surface were activated at 0°C for 30 min with 4 g water-soluble carbodiimide dissolved in 400 ml phosphate buffer solution (pH 4.5). Then 80 mg RGDS peptide was reacted with the activated PEA film for 4 h at 0°C. Thus, the RGDS peptide was immobilized to PEA film at its N-terminal. The immobilized film was rinsed with pure water for 72 h, and then dried *in vacuo* for 4 days. With respect to GGGRGDS peptide, the same procedures were carried out to immobilize the peptide.

Surface characterization of immobilized films was carried out by means of C1s and N1s spectra measured with a Shimadzu 750 ESCA spectrometer using $MgK_{\alpha 1,2}$ exciting radiation.

Procedures to Determine Surface Pressure and Surface Area of Adsorbed Proteins, and Critical Surface Tension of Films

Adsorption kinetics of protein on polymer film surface in phosphate buffer solution (PBS) was quantitatively described elsewhere,[4] *i.e.*, the surface pressure Π is related to the surface area A of the adsorbed protein by:

$$\frac{d\Pi}{dt} = k_1 \left\{ c_0 \left(\frac{d\Gamma}{d\Pi} \right)^{-1} - \frac{1}{\sqrt{\pi D}} \int_0^t \frac{\Pi'(\tau)}{\sqrt{t-\tau}} d\tau \right\} \times \exp\left(-\frac{\Pi A}{kT} \right) - k_2 \Pi \tag{1}$$

where Γ is the number of protein molecules adsorbed per unit area, t is the time, $\Pi' = \Pi/dt$, D is the diffusion constant of protein, C_0 is the bulk concentration of protein, k_1 and k_2 are the rate constants for adsorption and desorption, respectively, and k is the Boltzman constant. If the desorption constant k_2 is assumed to be 0, then A is estimated from $\log(d\Pi/dt)$ *vs.* Π curve.

The surface pressure Π, as a function of time, was determined from measurements on contact angles developed by us,[4] and the interfacial tensions by Wilhelmy Plate method. The contact angles, $\theta'(t)$, of protein solution and θ of reference solution (PBS) on polymer film surface were measured in *n*-hexane. The equation[4] used is;

$$\Pi = \gamma'_{HW}(t) \cos\theta'(t) - \gamma_{HW} \cos\theta \tag{2}$$

where, γ_{HW} is the interfacial tension between *n*-hexane (H) and reference solution (W), and $\gamma'_{HW}(t)$ is that between *n*-hexane and protein solution (W'). Π, $\gamma'_{HW}(t)$ and $\cos\theta'(t)$ depend on time, but γ_{HW} and $\cos\theta$ are independent of time. Protein solutions used were 0.1 wt% BSA

and 0.1 wt% IgG solutions dissolved in PBS throughout the experiment.

The critical surface tensions γ_c of films used were derermined from Zisman plots[6] by using 10 liquids of surface tensions covering 36.23 to 72.30 dyn cm^{-1}.

RESULTS AND DISCUSSION

Characterization of Oligopeptide-Immobilized Polymer Surface

Figures 1 and 2 illustrate C1s and N1s ESCA spectra for PVA and RGDS-PVA, respectively. Figures 3 and 4 are those for PEA, RGDS-PEA, and GGGRGDS-PEA. The numerical ESCA data are tabulated together with the critical surface tension γ_c of the films in Table I.

Oxygen to carbon ratio (O/C ratio) in % unit of bulk PVA is 50. The obtained value 35.06 mean that about 70% of the OH residues locates on the surface and other residues are buried in the film interior. For PEA, the O/C

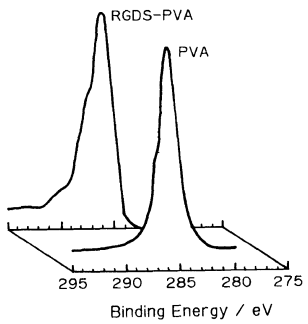

Figure 1. C1s ESCA spectra of PVA and RGDS-PVA surfaces.

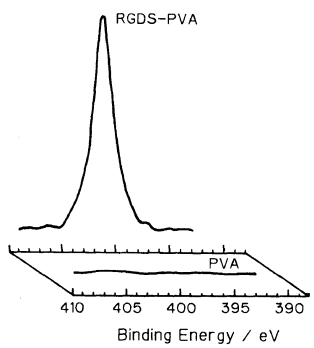

Figure 2. N1s ESCA spectra of PVA and RGDS-PVA surfaces.

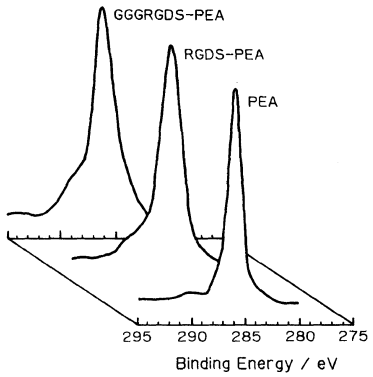

Figure 3. C1s ESCA spectra of PEA, RDGS-PEA, and GGGRGDS-PEA surfaces.

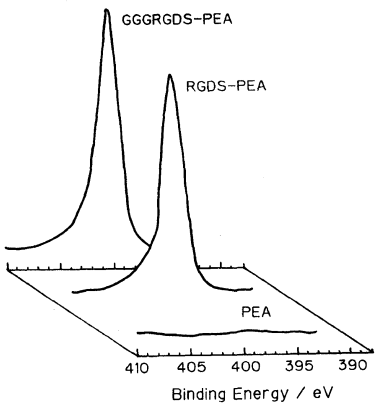

Figure 4. N1s ESCA spectra of PEA, RGDS-PEA, and GGGRGDS-PEA sirfaces.

Table I. Surface composition and critical surface tension

Designation	Elemental ratios		Critical surface tension
	O:C/%	N:C/%	γ_c/dyn cm^{-1}
PVA	35.21	0	39.05
RGDS-PVA	35.92	10.28	41.09
PEA	2.12	0	35.15
RGDS-PEA	12.03	5.35	37.31
GGGRGDS-PEA	13.99	5.60	38.58

% of bulk PEA is 3.7%, so, about 57% of the COOH residues locate on the surface. Distributions of hydrophilic residues such as OH and COOH on the film surface are strongly dependent on the procedure to prepare films. Obviously from the figures, C1s peak of the oligopeptide-immobilized films exhibits a shoulder at 289—291 eV characteristics to carbon atom of the amide bond, and N1s peaks of immobilized films are remarkable, in contrast to the absence of peak for PVA and PEA. Thus, we confirmed that the oligopeptides were really immobilized to substrate polymer films. The critical surface tension γ_c ($=39.05$ dyn cm^{-1}) of PVA is quite large. γ_c ($=35.15$ dyn cm^{-1}) of PEA, whose acrylic acid content is 2.84 mol% is compared with γ_c ($=31$) of polyethylene.[6] The critical surface tensions of oligopeptide-immobilized polymers, RGDS-PVA, RGDS-PEA, and GGGRGDS-PEA, are larger than those of respective substrate polymers, PVA and PEA. This fact means that the hydrophilicity of the surface is increased by immobilizing oligopeptides carrying polar amino acid residues such as Arg, Asp, and Ser.

Adsorption Behaviors of BSA and IgG on Oligopeptide-Immobilized PVA and PEA

In Figure 5, the interfacial tension γ'_{HW} between *n*-hexane and protein solution (Protein-PBS) is plotted against time. The interfacial tension γ_{HW} between *n*-hexane and PBS was 49.9 dyn cm^{-1} independent of time, though not shown in the figure. The reason why *n*-hexane was used is that the interfacial tension (51.0 dyn cm^{-1}) of *n*-hexane against water is the largest among various organic liquids. $\gamma_{HW}=49.9$ dyn cm^{-1} obtained for PBS was slightly lower than the value for water. As shown in the figure, γ'_{HW} for both BSA and IgG decreases with time, and approaches to an equilibrium value after about 3 h. The curve for IgG is on higher level than for BSA. This means that protein molecules are adsorbed at the interface with time, and reduce the

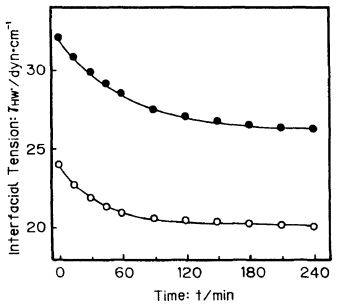

Figure 5. Time dependence of interfacial tentions γ'_{HW} at *n*-hexane/protein solution interface for 0.1 wt% BSA (○), and 0.1 wt% IgG (●).

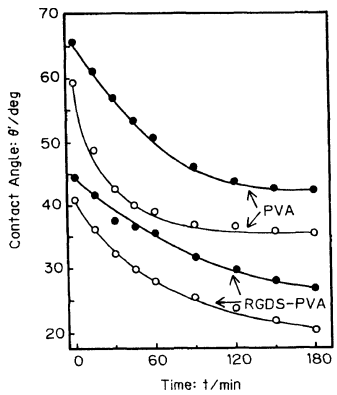

Figure 6. Contact angles of 0.1 wt% BSA solution (○), and 0.1 wt% IgG solution (●) on PVA and RGDS-PVA surfaces plotted against time.

interfacial tension; mutual miscibility of IgG solution with *n*-hexane is smaller than that for BSA solution. In IgG molecule, hydrophilic domain (F_{ab}) and hydrophobic domain (F_c) are localized, thus the F_c portion may orient toward *n*-hexane phase at the interface. The contact angles θ of PBS solution in the absence of proteins were 58.7° for PVA, 154.0° for PEA, 18.9° for RGDS-PVA, 118.0° for RGDS-PEA, and 96.7° for GGGRGDS-PEA, respectively, independent of time. The contact angles θ' of 0.1% BSA solution and of 0.1% IgG solution on PVA and RGDS-PVA films in *h*-hexane were plotted against time in Figure 6. The curves indicate that equilibrium is attained after about 3 h. The θ' value for BSA solution is

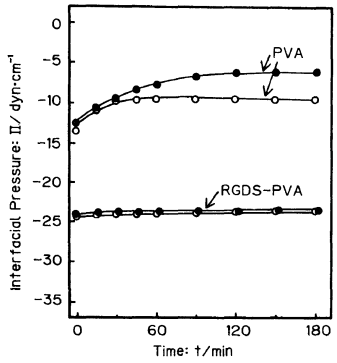

Figure 7. Time dependence of interfacial pressures of 0.1 wt% BSA solution (○), and 0.1 wt% IgG solution (●) on PVA and RGDS-PVA surfaces.

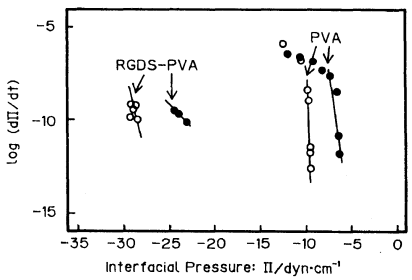

Figure 8. Log(dΠ/dt) plotted against Π for adsorptions of 0.1 wt% BSA solution (○) and 0.1 wt% IgG solution (●) on PVA and RGDS-PVA surfaces.

higher than that for IgG solution owing to rather hydrophilic nature of BSA molecule. Introduction of RGDS peptide to PVA considerably reduces the contact angle for both BSA and IgG solution, because of the presence of polar groups in the peptide.

Figure 7 shows the interfacial pressure Π obtained from eq 2. Π indicates the extent of stabilization of interfacial energy between polymer film and protein solution. That is, a larger Π value means that more stable interface is formed by the adsorption of protein molecules. It is pointed out that Π is decreased by introducing RGDS to PVA. An important result shown in the figure is that the interfacial pressure is always minus. This fact indicates that surface of hydrophilic polymer, such as PVA, is difficult to adsorb the prtoein molecules BSA and IgG. In Figure 8, log(dΠ/dt) is plotted against Π, from which the surface area A of protein molecule is obtained from eq 1.

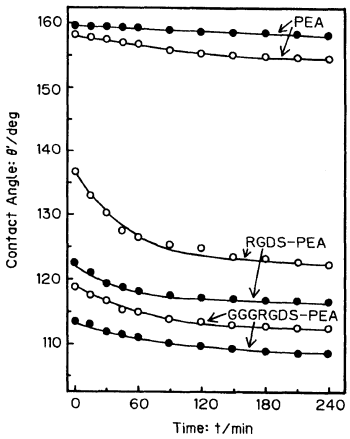

Figure 9. Contact angles of 0.1 wt% BSA solution (○), and 0.1 wt% IgG solution (●) on PEA, RGDS-PEA, and GGGRGDS-PEA surfaces plotted against time.

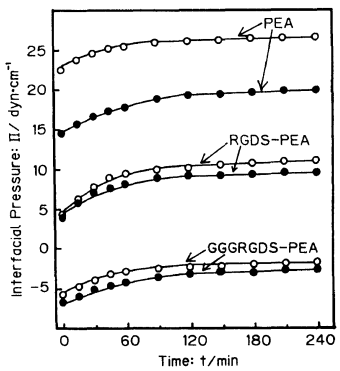

Figure 10. Time dependence of interfacial pressures of 0.1 wt% BSA solution (○), and 0.1 wt% IgG solution (●) on PEA, RGDS-PEA, and GGGRGDS-PVA surfaces.

Next, experimental results on PEA, RGDS-PEA, and GGGRGDS-PEA are given in Figures 9, 10, and 11. As mentioned before, PEA polymer is a copolymer of ethylene–acrylic acid, containing only 2.84 mol% acrylic acid. So, PEA is said to be rather hydrophobic polymer: contact angle θ' of PEA is considerably higher than that of PVA (Figure 6), and interfacial pressure Π of PEA is positive (Figure 10). These values were, however,

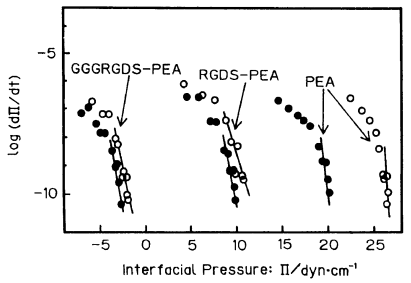

Figure 11. Log($d\Pi/dt$) plotted against Π for adsorptions of 0.1 wt% BSA solution (○) and 0.1 wt% IgG solution (●) on PEA, RGDS-PEA , and GGGRGDS-PEA surfaces.

Table II. Surface pressure Π and surface area A of BSA and IgG on peptide-immobilized PVA and PEA surfaces

Designation	Π/dyn/cm^{-1}		A/Å	
	BSA	IgG	BSA	IgG
PVA	−9.4	−6.3	980	1070
PGDS-PVA	−23.5	−23.7	410	500
PEA	26.7	20.3	940	630
PGDS-PEA	11.1	10.0	510	460
GGGRGDS-PEA	−1.9	−2.6	580	430

drastically lowered by immobilization of RGDS and GGGRGDS.

Finally, Π values at 3 h, and A values are summarized in Table II. As obvious from the table, for hydrophilic PVA surface, adsorption of BSA and IgG is not easy, and this tendency is increased by introducing RGDS peptide to PVA. For rather hydrophobic PEA surface, Π (26.7, 20.3 dyn cm^{-1}) are positive values and not so large. But these are small positive values for RGDS-PEA, and finally suppressed to minus values for GGGRGDS-PEA. The effect of spacer, GGG, is distinguishable. Regarding the surface area, it is difficult to draw explicit explanation, but it seems that the surface areas are reduced by the immobilization of peptides to polymer surfaces. Dimensions of A shown in the table are not largely conflict with those estimated from molecular dimensions of albumin and γ-globulin.

As mentioned earlier, this work was designed to elucidate the effects of serum proteins during cell attachment to peptide-immobilized polymer surface. The interaction of cells with peptide exhibiting cell-adhesion activity would be very specific. The results obtained in this paper may suggest that serum proteins BSA and IgG do not largely affect the cell-attachment to the peptides, especially by the use of rather hydrophilic polymer surface.

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