Preferential Adsorption of Bovine Fibrinogen Dimer onto Polymer Microspheres Having Heterogeneous Surfaces Consisting of Hydrophobic and Hydrophilic Parts*

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SYNOPSIS

The adsorption onto polymer microspheres of bovine fibrinogen (BFb) containing both dimeric and monomeric species was examined using various homopolymer, copolymer, and poly(2-hydroxyethyl methacrylate)/polystyrene composite microspheres which were produced by the emulsifier-free (seeded) emulsion polymerization technique. The preferential adsorption of the BFb dimer was clearly observed in an optimum region of the surface hydrophilicities of the polymer microspheres. The preferential adsorption of the BFb dimer onto the composite polymer microspheres having heterogeneous surfaces with both hydrophilic and hydrophobic parts was more remarkable than that onto the homopolymer and copolymer microspheres having homogeneous surfaces. The behavior is discussed in comparison with those for bovine serum albumin and human γ-globulin.

INTRODUCTION

In recent years, proteins (enzymes, antibodies, etc.) have been used in chemical processes as bioreactors, biosensors, and bioseparators after being bound to appropriate carriers.[1,2] We have been trying to immobilize enzyme[3-5] and antibody[6-8] molecules onto submicron-size monodisperse polymer microspheres produced by emulsifier-free emulsion polymerization.[9,10] Our results have shown that the surface properties, especially surface hydrophilicities and surface heterogeneities, greatly affected the amount of immobilization and the bioactivities.

Adsorption chromatography is a powerful means in the field of biotechnology for the high efficiency separation of biomolecules. Since polymer microspheres produced by emulsifier-free emulsion polymerization have clean surfaces and large specific surface areas, they are expected to form an excellent material for the carriers of adsorption chromatography.

Recently, Zsom[11] reported that bovine serum albumin (BSA) dimers (polymers) adsorbed preferentially compared to BSA monomers onto polystyrene (PS) microspheres having anionic surface charges. This result gives useful information on the "surface design" of the carrier for adsorption chromatography.

In previous articles, we have examined the preferential adsorption behaviors of BSA[12] and human γ-globulin (HGG),[13] containing both dimeric and monomeric species, onto various homopolymer, copolymer, and poly(2-hydroxyethyl methacrylate)/polystyrene (PHEMA/PS) composite microspheres. The results show that there were surface hydrophilicities whose heterogeneities were suitable for the preferential adsorptions of BSA and HGG dimers over the corresponding monomers.

The purposes of this article are to clarify whether there is a surface hydrophilicity whose heterogeneity is such as to allow preferential adsorption onto various polymer microspheres of bovine fibrinogen (BFb) dimer over the monomer and to compare the results with those for BSA and HGG.

EXPERIMENTAL

Materials

Styrene (S), ethyl methacrylate (EMA), methyl methacrylate (MMA), methyl acrylate (MA), and

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Table I Conditions of Emulsion Polymerization[a]

Sample No.	1	2	3	4	5
S[b] (mL)	255	—	—	—	—
EMA[c] (mL)	—	100	—	—	—
MMA[d] (mL)	—	—	100	—	50
MA[e] (mL)	—	—	—	100	50
KPS[f] (mg)	1000	100	28	28	28
Water (mL)	1175	900	900	900	900

[a] Atmosphere, N_2; polym. temp., 70°C.
[b] S, styrene.
[c] EMA, ethyl methacrylate.
[d] MMA, methyl methacrylate.
[e] MA, methyl acrylate.
[f] KPS, potassium persulfate.

2-hydroxyethyl methacrylate (HEMA) were purified by distillation under reduced pressure in a nitrogen atmosphere. Potassium persulfate (KPS) was of a reagent grade. The bovine fibrinogen (BFb) was a commercial product (MILES). Commercial grade sodium dodecyl benzene sulfonate (DBS) was dried before use.

Polymer Microspheres

Polystyrene (PS), poly(2-hydroxyethyl methacrylate) (PHEMA), poly(ethyl methacrylate) (PEMA), poly(methyl methacrylate) (PMMA), poly(methyl acrylate) (PMA), and MMA-MA copolymer (P(MMA-MA)) emulsions were prepared by emulsifier-free emulsion polymerization[8] under the conditions listed in Table I. PHEMA/PS composite emulsions having different compositions were prepared by emulsifier-free seeded emulsion polymerization of S with PHEMA seed particles under the conditions listed in Table II. Each polymer emulsion was purified by dialysis after having been centrifugally washed, twice with 10^{-3} N HCl, and twice with distilled water.

The specific surface area of each microsphere was measured by the BET method using nitrogen gas and/or calculated from the number-average particle diameter measured by transmission electron microscopy.

The occupied area (A_m) of each DBS molecule adsorbed onto each emulsifier-free microsphere at the saturation adsorption was determined using the soap titration method with DBS.[14-16]

The ζ-potential of each microsphere at pH 7.4 (0.1 M Tris buffer) was measured using a PEN KEM Laser-Zee Model 500.

Adsorption of BFb

Five milliliters of BFb phosphate buffer solution was mixed with 5 mL of purified emulsion (polymer content, 15 g/L). The pH value of the mixed so-

Table II Recipes for Two-Stage (Seeded) Emulsion Polymerization[a]

HEMA Content (mol %)	3	5	10	15	17.5	20	30	50
1st Stage								
HEMA[b] (mL)	1.0	1.6	3.5	8.4	7.5	7.9	15	35
KPS[c] (mg)	5	9	19	42	40	42	80	187
Water (mL)	270	270	270	900	928	920	920	1890
2nd Stage								
S[d] (mL)	30	30	30	45	33.4	30	31	33
KPS (mg)	137	137	137	206	200	200	200	100
Water (mL)	13.7	13.7	13.7	20.6	20	20	20	10

[a] Atmosphere, N_2; polym. temp., 70°C
[b] HEMA, 2-hydroxyethyl methacrylate.
[c] KPS, potassium persulfate.
[d] S, styrene.

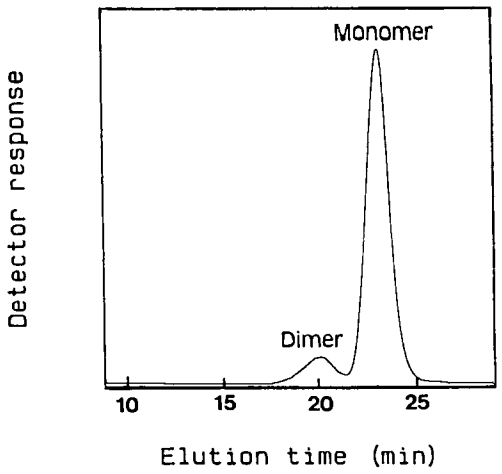

Figure 1 A typical HPLC chart of BFb solution. Elution conditions: 0.1*M* phosphate buffer (pH 7.4); 0.6 mL/min; column, TSK-GEL 4000SW; UV detection, 280 nm.

lution was adjusted to 7.4 with a 0.1*M* phosphate buffer. After standing at 3°C for 3 h, the mixed solution was centrifuged at 15,000 rpm (40,000 g) and the supernatant was filtrated using a Millipore cellulose acetate filter (pore size, 0.22 μm).

The amount of the BFb adsorbed was calculated from the difference between the initial BFb concentration and the residual BFb concentration in the supernatant. The BFb concentration was determined by measuring the absorbance using the calibration curve obtained preliminarily at 280 nm.

The dimer/monomer ratio in the BFb solution before and after the adsorption was measured by high performance liquid chromatography (HPLC) (Tosoh, TSK-GEL G4000SW) using UV spectroscopy as the detection method.[17] The decrease in solution concentration of each protein was determined by measuring the specific peak areas of the HPLC chromatograms using a curve resolver.

RESULTS AND DISCUSSION

Figure 1 shows a typical HPLC chromatogram of the BFb used in this experiment. The HPLC chromatogram had two peaks, at about 20 min and 23 min of retention, which are due to the dimeric and monomeric species, respectively. The dimeric and monomeric species could be easily distinguished. Though the dimeric component may contain polymeric species, it is treated collectively as dimeric species in this article. Good linear relationships were established between the total BFb concentration and the peak areas due to the BFb dimer and monomer. The dimer/monomer ratio calculated from the ratio of these slopes was 0.10 regardless of the BFb concentration.

Tables III and IV show the surface characterizations of all the polymer microspheres used in this study. There was some difference in the ζ-potential values due to $-SO_4^-$ groups as initiator fragment and $-COO^-$ groups derived from hydrolysis of the fragment. Zsom[11] reported that PS microspheres having high anionic surface charges adsorbed BSA dimers preferentially over the monomers, but those having low charges did not do so. Since the surface charges of the microspheres calculated from the ζ-potentials shown in Tables III and IV were low, their influence will be negligible in the following discussion. Similarly, the influence of —OH groups given by hydrolysis of $-SO_4^-$ groups is also negligible.

The surface hydrophilicity of each microsphere was estimated from the A_m value. It is well known that the A_m value increases with an increase in the surface hydrophilicity of polymer microsphere.[14-16]

Table III Characteristics of Various Polymer Microspheres

Microspheres	Diameter[a] (μm)	Specific Surface Area[b] (m^2/g)	Occupied Area of DBS Molecule[c] (Å^2)	Zeta-Potential[d] (mV)
PS	0.60	9.44	53	−41.4
PEMA	0.45	14.6	108	−28.6
PMMA	0.55	9.24	133	−29.5
P(MMA-MA)	0.36	10.7	176	−25.0
PMA	0.57	9.98	214	−43.8

[a] Measured by TEM.
[b] Calculated from diameter measured by TEM.
[c] Measured by the soap titration method.
[d] Measured by the electrophoretic method.

Table IV Characteristics of PHEMA/PS Composite Microspheres

HEMA Content (mol %)	Specific Surface Area[a] (m^2/g)	Occupied Area of DBS Molecule[b] (Å^2)	Zeta-Potential[c] (mV)
0	9.4	53	−41.4
3	19.6	88	−44.8
5	14.6	118	−41.6
10	16.0	126	−35.1
15	17.2	152	−43.7
17.5	22.7	165	−44.2
20	13.8	180	−36.2
30	7.4	232	−42.0
50	8.8	322	−41.0

[a] Measured by the BET method.
[b] Measured by the soap titration method.
[c] Measured by the electrophoretic method.

The results in Table III indicate that the surface hydrophilicity increased in order PS < PEMA < PMMA < P(MMA-MA) < PMA microspheres, and with an increase in the HEMA content of PHEMA/PS composite microspheres.

Figure 2 shows the adsorption isotherms of BFb onto various microspheres having homogeneous surfaces at pH 7.4 and at 3°C for 3 h. The amount of the BFb adsorbed was the sum of those of the dimer and monomer adsorbed. The maximum amount of BFb adsorbed decreased with an increase in the A_m value of the polymer microspheres. This indicates that the interaction between BFb and the polymer microspheres decreased with an increase in the surface hydrophilicity.[17]

Figure 3 shows the relationships between the amount of BFb adsorbed onto the polymer microspheres having homogeneous surfaces and the A_m values; this is compared with those of BSA[12] and HGG.[13] The adsorption experiments with BSA and HGG were carried out at isoelectric points of the two proteins i.e. at pH 4.7 and 7.5, respectively, in order to neglect the influence of the ionic interaction between the proteins and microspheres on their adsorption behaviors. Since BFb was not sufficiently soluble in water at pH at its isoelectric point (pH

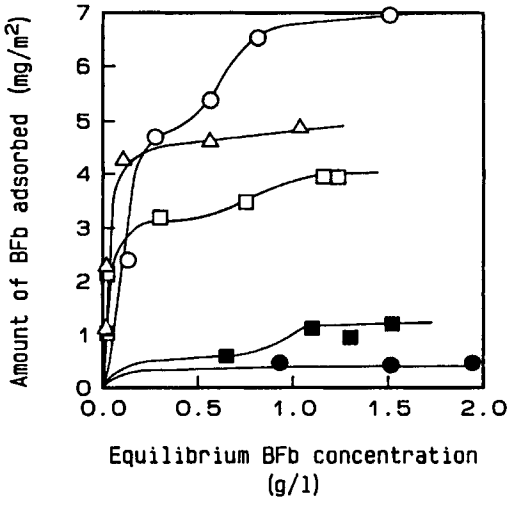

Figure 2 Adsorption isotherms of BFb onto (○) PS, (△) PEMA, (□) PMMA, (■) P(MMA-MA), and (●) PMA microspheres at 18°C, pH 7.4 (0.05M phosphate buffer) for 3 h. Solid emulsion, 15 g/L.

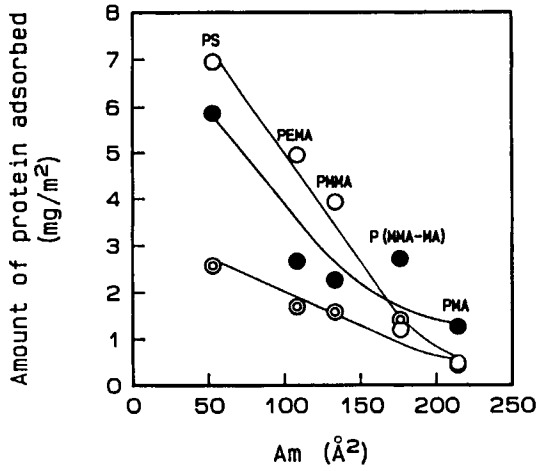

Figure 3 Relationships between the amounts of (○) BFb (pH 7.4), (●) HGG (pH 7.5), and (◎) BSA (pH 4.7) adsorbed and A_m values of the polymer microspheres having homogeneous surfaces. Solid emulsion, 15 g/L.

5.5),[19] the adsorption experiments were carried out at pH 7.4 where BFb bears negative charges. Therefore the maximum amounts of BFb adsorbed at pH 7.4 onto various microspheres having negative charges may be lower than those at pH 5.5, the isoelectric point of BFb. Nevertheless, the amounts of BFb adsorbed were the largest of the three proteins. This indicates that the interaction between BFb and the microspheres is the strongest in them.

The preferential adsorption of the dimer was estimated as follows. The dimer/monomer ratio in the aqueous solution before the adsorption is represented by the D_0/M_0 ratio, where D_0 and M_0 are peak areas of the dimer and monomer in the chromatograms, respectively. The dimer/monomer ratio in the BFb adsorbed, D_a/M_a, is represented by $(D_0\text{-}D)/(M_0\text{-}M)$, where D and M are peak areas of dimer and monomer in the chromatogram of the supernatant after the adsorption, respectively. The preferential adsorption was evaluated as the $(D_a/M_a)/(D_0/M_0)$ ratio. When the preferential adsorption does not occur, the $(D_a/M_a)/(D_0/M_0)$ ratio is just one. An increase in this ratio indicates an increase in the preferential adsorption of the dimer.

Figure 4 shows the relationships between the $(D_a/M_a)/(D_0/M_0)$ ratios and the A_m values of the polymer microspheres having homogeneous surfaces at high equilibrium BFb concentration. At the BFb equilibrium concentration, in the cases of PS and PEMA, whose the A_m values were below 108 Å^2, the ratios were almost one. That is, preferential adsorption was not observed. However, in the cases of PMMA, P(MMA-MA), and PMA, whose the A_m values were in the range from 133 Å^2 to 214 Å^2, the ratios were above one. That is, preferential adsorptions of the BFb dimer were observed in these cases. These results suggest that there is an appropriate surface hydrophilicity for preferential adsorption of the BFb dimer onto the polymer microspheres. In other words, preferential adsorption of the BFb dimer does not occur under conditions where either strong or weak interaction operates between the BFb and the microspheres. At present, we cannot give a clear explanation for this.

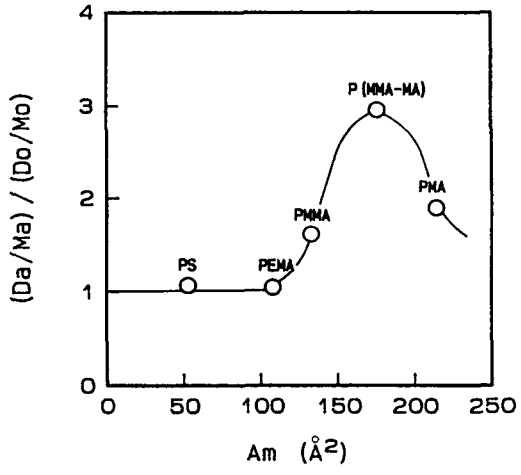

Figure 4 Relationships between $(D_a/M_a)/(D_0/M_0)$ ratios of BFb and A_m values of polymer microspheres having homogeneous surfaces. Solid emulsion, 15 g/L.

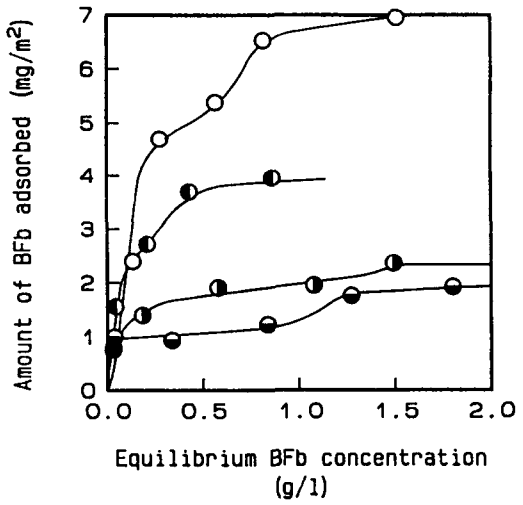

Figure 5 Adsorption isotherms of BFb onto PHEMA/PS composite microspheres at 3°C, pH 7.4 (0.05*M* phosphate buffer) for 3 h. HEMA content (mol %): ○, 0; ◐, 3; ◑, 5; ◒, 10. Solid emulsion, 15 g/L.

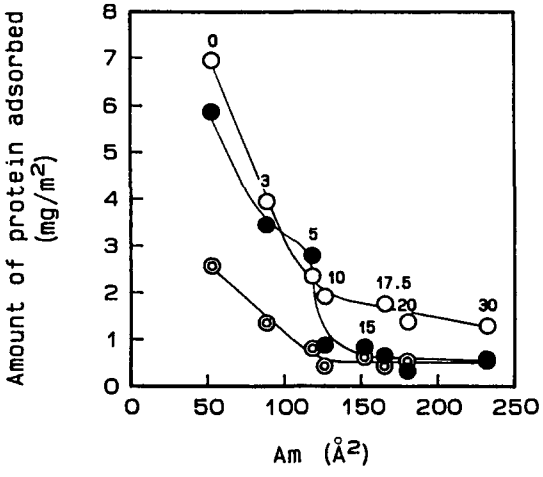

Figure 6 Relationships between the amounts of (○) BFb (pH 7.4), (●) HGG (pH 7.5), and (◎) BSA (pH 4.7) and A_m values of polymer microspheres having heterogeneous surfaces. Numbers indicate HEMA content (mol %). Solid emulsion, 15 g/L.

Similar adsorption experiments were carried out using PHEMA/PS composite microspheres. The PHEMA/PS composite microspheres have heterogeneous surfaces consisting of both hydrophilic and hydrophobic parts. The formation of heterogeneous surface has been estimated from electron microscopic observation of an ultrathin section of the particles,[20] the adsorption behavior of various proteins onto the particles in comparison with that onto homopolymer particles,[21] and surface composition determined by X-ray photoelectron spectroscopic analysis.[22] Such a heterogeneous surface was suitable for protein adsorption without denaturation.[4-6,10]

Figure 5 shows the adsorption isotherms of BFb onto PHEMA/PS composite microspheres having different compositions. The amount of the BFb adsorbed decreased with an increase in the HEMA content.

Figure 6 shows the relationships between the maximum amount of BFb adsorbed onto the PHEMA/PS composite microspheres and the A_m values compared with those of BSA and HGG. The relationships were similar for all three proteins. As in the case of microspheres having homogeneous surfaces, the amount of protein adsorbed increased in the order of BSA < HGG < BFb.

Figure 7 shows the relationships between the $(D_a/M_a)/(D_0/M_0)$ ratios under high BFb equilibrium concentration and the A_m values of the PHEMA/PS composite microspheres. The ratio increased with the increase in the HEMA content, and the maximum value was obtained at 20 mol % of the HEMA content. Where the A_m values ranged from 170 Å^2 to 250 Å^2, the ratios were remarkably high. The maximum ratio for the PHEMA/PS composite microspheres was much greater than those for polymer microspheres having homogeneous surfaces. This difference seems to be due to the surface heterogeneity, but the reason is not yet clear. In the case of 50 mol % of HEMA, the microspheres were

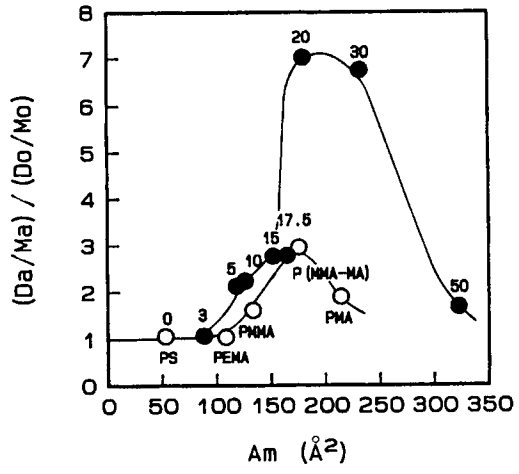

Figure 7 Relationships between $(D_a/M_a)/(D_0/M_0)$ ratios of BFb and A_m values of the polymer microspheres having the (○) homogeneous and (●) heterogeneous surfaces. Numbers indicate HEMA content (mol %). Solid emulsion, 15 g/L.

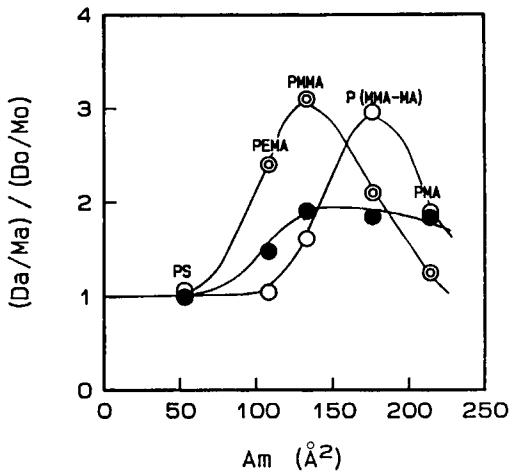

Figure 8 Relationships between $(D_a/M_a)/(D_0/M_0)$ ratios of (○) BFb, (●) HGG, and (◎) BSA, and A_m values of the polymer microspheres having homogeneous surfaces. Solid emulsion, 15 g/L.

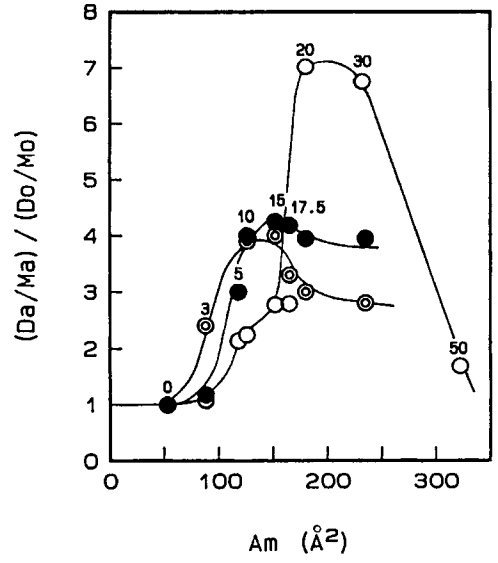

Figure 9 Relationships between $(D_a/M_a)/(D_0/M_0)$ ratios of (○) BFb, (●) HGG, and (◎) BSA, and A_m values of polymer microspheres having heterogeneous surfaces. Numbers indicate HEMA content (mol %). Solid emulsion, 15 g/L.

aggregated each other after being mixed with the BFb solution.

Figure 8 shows the relationships between the $(D_a/M_a)/(D_0/M_0)$ ratios and the A_m values of the polymer microspheres having homogeneous surfaces for BSA, HGG, and BFb. The range of surface hydrophilicities at which the preferential adsorption of each dimer occurred shifted to higher A_m value as the hydrophobicity of the proteins increased in the order of BSA < HGG < BFb. A similar tendency was observed in their preferential adsorption behaviors on PHEMA/PS composite microspheres having heterogeneous surfaces as shown in Figure 9.

From the above results, it is concluded that there are certain regions of surface hydrophilicities whose heterogeneities are suitable for the preferential adsorption of serum protein dimers over corresponding monomers.

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