Adsorption of Trypsin onto Poly(2-Hydroxyethyl Methacrylate)/Polystyrene Composite Microspheres and Its Enzymatic Activity*

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Synopsis

Poly(2-hydroxyethyl methacrylate)/polystyrene (PHEMA/PS) composite microspheres were produced by emulsifier-free seeded emulsion polymerization for styrene in the presence of PHEMA seed particles. Effects of the surface characteristics of the PHEMA/PS composite microspheres on the adsorption immobilization of trypsin and on its enzymatic activity were discussed. Above 5 mol% of HEMA content, trypsin molecules adsorbed had high activity, 65–100% of the activity of free trypsin. The excellence of the composite microspheres as a carrier for trypsin seems to be closely related with the surface heterogeneity consisting of both hydrophilic and hydrophobic parts.

INTRODUCTION

In preceding articles,[1,2] effects of the surface characteristics, especially the surface hydrophilicity of various polymer microspheres on the adsorption immobilization of trypsin and on its enzymatic activity were examined. In a series of homopolymer microspheres such as polystyrene (PS), poly(methyl methacrylate), and poly(methyl acrylate), as hydrophilicity of the microsphere surfaces increased, the amount of adsorption decreased and the enzymatic activity increased. Thus high adsorption and high activity could not be attained at the same time. However, in the case of styrene-2-hydroxyethyl methacrylate (S-HEMA) copolymer microspheres, their compatibility was attained in the optimum region of the polymer composition. In addition, the copolymers differed clearly from the homopolymers in the relationship between the amount of adsorption and the specific activity. In all cases of the above-mentioned homopolymers, the specific activity linearly increased with the increase in the amount of adsorption. Thus, activity was very low in the low adsorption region, whereas in the case of the copolymers, the activity was high even in the low adsorption region. These desirable results in the copolymers were explained reasonably by assuming that the surface of the copolymer microspheres have a hydrophilic-hydrophobic heterogeneous structure

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consisting of HEMA-rich parts and S-rich parts on the basis of the results in a series of studies.[3,4]

Throughout this article, such an assumption will be clarified more in details from the experiment of the adsorption immobilization of trypsin onto PHEMA/PS composite microspheres which should have the clear heterogeneous surface structure according to our other series of articles on composite polymer microspheres consisting two kinds of polymers.[5,6]

EXPERIMENTAL

Materials

Styrene (S) and 2-hydroxyethyl methacrylate (HEMA) monomers were purified by distillation under the reduced pressure of a nitrogen atmosphere and stored in a refrigerator. Potassium persulfate (KPS) was of reagent grade. The deionized water was distilled with a Pyrex distillator. Trypsin (Type I, crystallized twice) and *N*-α-benzoyl-L-arginine ethyl ester (BAEE) were commercial products (Sigma/Nakarai Chemicals). Commercial-grade sodium dodecyl benzene sulfonate (DBS) was used after drying.

Polymer Microspheres as Carrier

PS and poly(2-hydroxyethyl methacrylate) (PHEMA) emulsions were prepared, respectively, by emulsifier-free emulsion polymerization technique.[7,8] A series of PHEMA/PS composite emulsions were prepared by emulsifier-free, two-stage (seeded) emulsion polymerizations: S was absorbed into the PHEMA seed microspheres produced in the first stage at 3°C for 24 h before the second stage emulsion polymerization was started. Polymerization conditions were listed in Table I. All polymerizations were continued until the conversion exceeded 98%. It was confirmed by TEM observation that S was polymerized in the PHEMA seed microspheres without forming new PS microspheres in each second-stage emulsion polymerization.

Each polymer emulsion was purified by centrifugal washing twice with 10^{-3} N HCl and twice with distilled water, followed by dialysis with poly(vinyl alcohol) hollow fiber dialyzer (Kuraray, Co. Ltd. KL-2) against distilled deionized water for 7 days. PHEMA emulsion was purified by dialysis alone because the microspheres coagulated each other under the centrifugation.

TABLE I
Recipes of Two-Stages (Seeded) Emulsion Polymerization[a]

Sample no.		1	2	3	4	5	6	7	8
1st stage	HEMA[b] (mL)	—	0.3	1.0	1.6	3.5	30	35	40
	KPS[c] (mg)	—	2	5	9	19	160	187	214
	Water (mL)	—	270	270	270	270	1855	1890	1980
	S[d] (mL)	225	30	30	30	30	62	33	—
2nd stage	KPS (mg)	1000	137	137	137	137	200	100	—
	Water (mL)	1175	13.7	13.7	13.7	13.7	20	10	—

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

The specific surface area of each microsphere was measured by the BET method and/or calculated from the average diameter measured by TEM.

According to the previous articles,[5,9] the maximum amount of DBS adsorbed onto a microsphere was measured.

The numbers of surface charges of these microspheres were measured by conductometric titration with 0.2*N* KOH aqueous solution.

Adsorption Immobilization of Trypsin

10 mL solution of trypsin was mixed with 10 mL of the purified emulsion (solid content, 19.4–33.4 g/L). The pH value of mixed solution was adjusted immediately to 8 with 0.5*N* KOH solution. After standing at 3°C for 2–3 h, the mixed solution was centrifuged at 10,000–15,000 g, 3°C and the supernatant was centrifuged twice at 50,000 g, 3°C in order to remove completely a very small amount of wafting microspheres therein. The amount of trypsin adsorbed was calculated from the difference between the initial concentration and the residual concentration in the last supernatant. Trypsin concentration was measured by ultraviolet (UV) spectrophotometry at 280 nm.

Determination of Enzymatic Activity

The enzymatic activities of the free and adsorbed trypsin were determined at 25°C by the pH-stat method,[10] using BAEE as a substrate according to the following procedure.

A known amount of the free or adsorbed trypsin was added to 100 mL of 10^{-3} *M* BAEE. Then the pH was adjusted to 8 and maintained using a Toa Electronics HSM-10A pH-stat with 0.2*N* KOH under stirring for 10 min. The activity was calculated from the amount of KOH consumed. The units of total and specific activities were expressed as μmole/min/m^2 and μmole/min/mg, respectively. Since PHEMA microspheres coagulated each other under the centrifugation, the activity of trypsin adsorbed onto PHEMA was calculated by deducting the activity of free trypsin in the supernatant from the total activity of free and adsorbed trypsins in the emulsion. Since PHEMA microspheres also coagulated each other above about 2 g/L-emulsion of the trypsin concentration, the activity of trypsin adsorbed onto PHEMA above the concentration could not be measured.

Observation of Microsphere Morphology

The purified emulsion was spread on a polyethylene terephthalate (PET) film and dried in a desiccator at room temperature. The PET film was kept under OsO_4 vapor for 12 h and cured in epoxy resin at 50°C for 48 h. The cured block was sectioned into about 800 Å in thickness with a Sorvall MT-6000 ultramicrotome. The thin cross-sections were mounted on copper grids, dried in a desiccator, and observed with a TEM (Hitachi H-800).

RESULTS AND DISCUSSION

The hydrophilicity of the surface of each polymer microsphere used in this study was estimated by measuring the amount of DBS adsorbed thereon with the soap titration method.[11] It is well known that the amount of DBS adsorbed increases with an increase in hydrophobicity of the surface.[5,12] As is

TABLE II
Characteristics of PHEMA/PS Composite Microspheres

HEMA content (mol%)	Specific surface area (m^2/g)	Maximum amount of DBS adsorbed[c] (mg/m^2)	Surface charge density[d] $\times 10^7$	
			Strong acid (mol/m^2)	Weak acid (mol/m^2)
0	6.2[a]	1.12	2.11	4.42
1	15.5[b]	0.72	2.11	3.82
3	19.6[b]	0.66	2.08	4.55
5	34.6[b]	0.49	1.83	5.28
10	16.0[b]	0.46	1.72	5.88
30	7.4[b]	0.25	1.86	6.94
50	8.8[b]	0.18	1.80	7.19
100	12.0[a]	—	—	—

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

obvious from the third column in Table II, the PS microsphere had the most hydrophobic surface and the surface hydrophilicity of PHEMA/PS composite microspheres increased with an increase in HEMA content. In the case of the PHEMA microsphere, the soap titration was not carried out because the PHEMA emulsion could not be concentrated to the required concentration (ca. 20 wt%) for the reliable titration.

Figure 1 shows the adsorption isotherms of trypsin onto the PHEMA/PS composite microspheres at pH 8, 3°C. The initial risings of the curves in the low trypsin concentration region decreased with the increase in HEMA content in the composite microspheres and approached that of the PHEMA. The two-step isotherms, which seem to be based on a rearrangement[1,13] of the

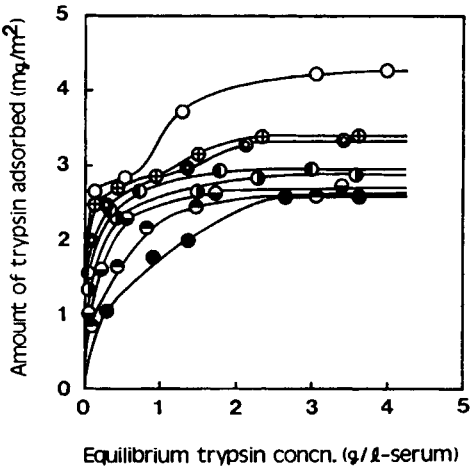

Fig. 1. Adsorption isotherms of trypsin onto PHEMA/PS composite microspheres at pH 8, 3°C; HEMA content (mol%): ○, 0; ⊕, 1; ◎, 3; ◐, 5; ◑, 10; ◒, 30; ◓, 50; ●, 100. Emulsion solid, 9.7 ~ 16.2 g/L serum.

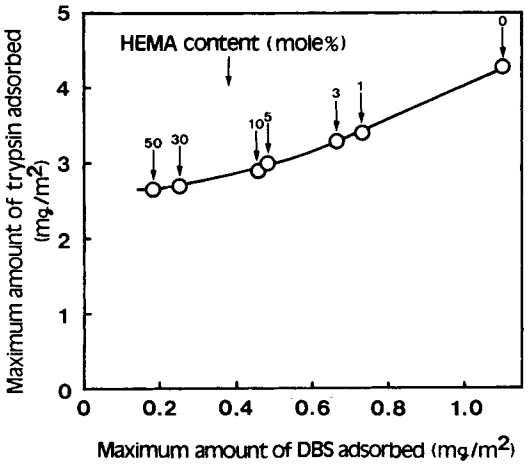

Fig. 2. Relationship between the maximum amount of DBS adsorbed and the maximum amount of trypsin adsorbed onto PHEMA/PS composite microspheres at pH 8, 3°C. Emulsion solid, 9.7 ~ 16.7 g/L serum. Initial trypsin concn., 4 g/L serum.

trypsin molecules adsorbed, became indistinct with the increase in HEMA content.

Figure 2 shows the relationship between the maximum amount of DBS adsorbed and the maximum amount of trypsin adsorbed onto the PHEMA/PS composite microspheres. The maximum amount of trypsin adsorbed decreased with a decrease in the maximum amount of DBS adsorbed, that is, with an increase in the surface hydrophilicity, and leveled off to that in the case of the PHEMA microsphere (cf., symbol ● in Fig. 1). Here, since total negative charges tended to increase with increases in HEMA content in the composite microspheres as shown in the fourth column in Table II, we must point out that the above result of adsorption at pH 8 may involve the effect of surface charges beside that of surface hydrophilicity.

Figure 3 shows the relationship between the amount of trypsin adsorbed and the total activity. In all the microspheres used in this study, total activities increased with increase in the amount of trypsin adsorbed. In the cases of PS and PHEMA/PS (1/99, mole ratio), there was an "induction period" where trypsin molecules adsorbed had no activity. This phenomenon has already been discussed in the preceding article.[1] Above 3 mol% of HEMA content the phenomenon was not observed and above 5 mol% the maximum total activities were higher than that for PS.

Figure 4 shows the relationship between the amount of trypsin adsorbed and the specific activity. In the cases of 1 and 3 mol% of HEMA contents of composite microspheres, the specific activities increased with the increase in the amount of adsorption as well as the case of PS. On the other hand, in the cases of 5, 10, and 30 mol% of HEMA contents of the composite microspheres, there was an amount of trypsin adsorbed at which the specific activity was maximum, respectively. The maximum point was shifted to a lower amount of adsorption with the increase in HEMA content in the PHEMA/PS composite microspheres as well as the series of S-HEMA copolymer microspheres reported in the preceding article,[2] where this phenomenon has already been

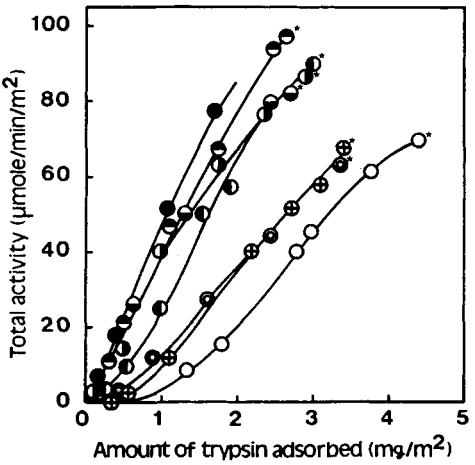

Fig. 3. Relationship between the amount of trypsin adsorbed onto PHEMA/PS composite microspheres and the total activity; HEMA content (mol%): ○, 0; ⊕, 1; ◎, 3; ◐, 5; ◑, 10; ◒, 30; ◓, 50; ●, 100. Enzymatic reaction conditions: pH 8; temp., 25°C; BAEE, 10^{-3}M. Symbol (*) indicates the total activity at the maximum amount of trypsin adsorbed.

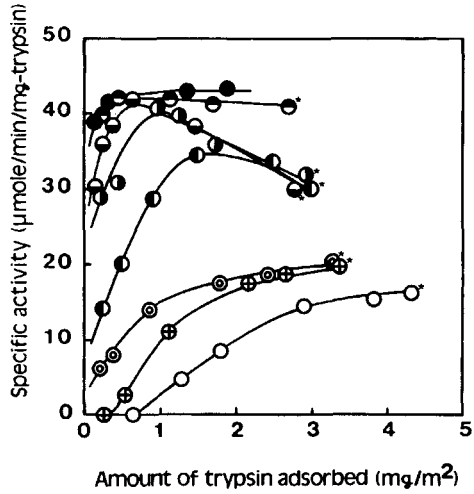

Fig. 4. Relationship between the amount of trypsin adsorbed onto PHEMA/PS composite microspheres and the specific activity; HEMA content (mol%): ○, 0; ⊕, 1; ◎, 3; ◐, 5; ◑, 10; ◒, 30; ◓, 50; ●, 100. Enzymatic reaction conditions: pH 8, temp., 25°C; BAEE, 10^{-3}M. Symbol (*) indicates the specific activity at the maximum amount of trypsin adsorbed.

discussed. Above 5 mol% of HEMA content, trypsin molecules adsorbed had high activity, 65–100% of the activity of free trypsin (42 μmol/min/mg), even in the low adsorption region where they lost activity completely in the case of the PS microsphere.

Figure 5 shows the relationship between the maximum amount of DBS adsorbed and the maximum specific activity of trypsin adsorbed. The maximum specific activity increased stepwise with the decrease in the maximum amount of DBS adsorbed. In particular, it increased remarkably with the increase in the surface hydrophilicity between 3 and 5 mol% of HEMA

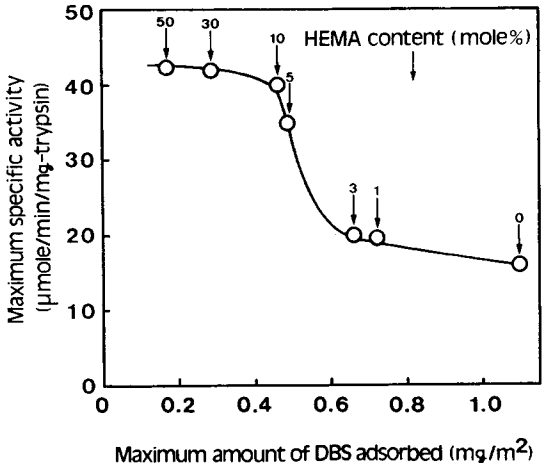

Fig. 5. Relationship between the maximum amount of DBS adsorbed and the maximum specific activity of trypsin adsorbed. Enzymatic reaction conditions: pH 8; temp., 25°C; BAEE, 10^{-3}M.

contents. This phenomenon seems to be due to the variation of the surface heterogeneity produced in this region of polymer composition, as discussed in another article.[3] Above 10 mol% of HEMA content, each maximum specific activity was nearly equal to that of free trypsin and 2.5 times the maximum specific activity of PS-adsorbed trypsin. This indicates that trypsin molecules adsorbed onto the surfaces of the PHEMA/PS composite microspheres

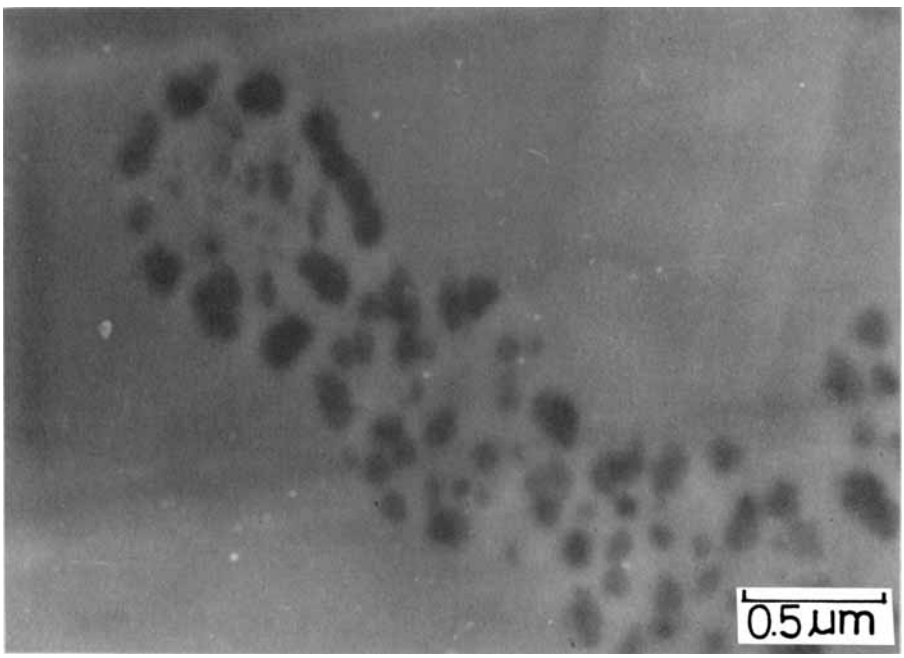

Fig. 6. Electron micrograph of the thin cross-section of PHEMA/PS (30/70, mole ratio) composite microspheres.

without heavy denaturation. In this way, PHEMA/PS composite microspheres were excellent as a carrier for trypsin.

Figure 6 shows the electron micrograph of the thin cross-section of the PHEMA/PS (30/70, mole ratio) composite microspheres. It is obvious that the microsphere had a heterogeneous structure where PHEMA parts were observed as black parts and PS parts as white parts in the electron micrograph, because PHEMA is stained selectively[14] by OsO_4. A similar phase-separated morphology was also observed in the cases of 5 and 10 mol% of HEMA content (data not shown). Such a heterogeneous structure should be formed at the surface of the microsphere, though HEMA component tends to accumulate at the surface more than S component as reported in the preceding article.[2]

From the above results, it is concluded that the PHEMA/PS composite microspheres are excellent carriers due to their surface heterogeneity consisting of both hydrophilic and hydrophobic parts.

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