Polymer Journal, Vol. 26, No. 5, pp 561—569 (1994)

Surface Modified Polymer Microspheres Obtained by the Emulsion Copolymerization of 2-Methacryloyloxyethyl Phosphorylcholine with Various Vinyl Monomers

Kazuo SUGIYAMA* and Hitoshi AOKI

Department of Industrial Chemistry, Faculty of Engineering, Kinki University, Takaya, Higashihiroshima, 729-17 Japan

(Received September 6, 1993)

ABSTRACT: A series of copolymer microspheres of 2-methacryloyloxyethyl phosphorylcholine (MPC) with comonomers (M) such as methyl-(MMA), ethyl-(EMA), *n*-butyl-(BMA), hexyl methacrylate (HMA), and styrene (St), poly(MPC-*co*-M), was prepared from the emulsifier-free emulsion copolymerization. From the kinetics of copolymerization of MPC and MMA, it was found that the initial rate of polymerization of MMA increased drastically in the presence of small amounts of MPC. A decrease in the yield of poly(MPC-*co*-MMA) microspheres and increase in that of its aggregates were observed with increasing content of the MPC moiety from 0 to 10 mol% in feed. The diameters of poly(MPC-*co*-M) microspheres were much smaller than those of corresponding poly(M) microspheres except for poly(MMA) microspheres. It was confirmed from XPS measurements that the MPC moiety is localized on the surface of particles. A series of poly(MPC-*co*-M) microspheres was found to absorb bovine serum albumin (BSA) less than the corresponding poly(M) microspheres as the control. It was concluded that the reduction of adsorbtion of BSA on poly(MPC-*co*-M) is dependent on the hydrophilicity of comonomer M and the MPC composition on the surface of particles.

KEY WORDS Emulsion Copolymerization / 2-Methacryloyloxyethyl Phosphorylcholine / Vinyl Monomers / Surface Modified Polymer Microspheres / XPS / Albumin Adsorption /

Considerable attention has been paid to surface modified polymers by phosphorylcholine moieties, which exist on the extracellular surface of the lipid bilayer, in respect to biocompatibility and other properties.[1-3] Nakabayashi's group reported that potential blood biocompatible copolymers, comprising 2-methacryloyloxyethyl phosphorylcholine (MPC) and *n*-butyl methacrylate (BMA) moieties, reduce protein adsorption from human plasma[4] and the activation of platelets.[5,6] There is thus no doubt that the introduction of the zwitterionic head group of phosphatidylcholine into polymer is useful for improving thrombo-resistance.[4-9]

On the other hand, well-controlled emulsion polymerization of vinyl monomers gives polymer microspheres with highly uniform size, extremely large surface area, and controlled surface charge density. Hence, the particles may be used as medical diagnostics,[10] protein separators,[11] and enzyme-immobilized materials.[12] The particles are also employed to evaluate interactions between plasma proteins and polymer surfaces, using the polymer microsphere-column method[5] other than polymer-coated glass beads.[13] The polymer microspheres whose surfaces are modified with an analogous structure to biomembrane are also anticipated to be important polymer materials

* To whom correspondence should be addressed.

for various biomedical uses. Yamaguchi *et al.* reported surface modified polystyrene microspheres using the emulsion polymerization of styrene (St) in the presence of polymerizable and non-polymerizable phospholipids.[14] In a previous paper[15] we also reported the interactions between BSA and the copolymer microspheres obtained from the emulsion copolymerization of methyl methacrylate (MMA) and 2-[2-(methacryloyloxy)ethyldimethylammonio]-6-[4-(4-methoxyphenylazo)-phenoxy]hexyloxyphosphate. In the course of our study as far as the functionalized polymer microspheres were concerned, our interest was directed to the immobilization of phosphorylcholine moiety on the surface of polymer microspheres. The present paper describes the kinetics for the emulsifier-free emulsion copolymerization of MPC with various vinyl monomers (M) and characterization of copolymer microspheres [poly(MPC-*co*-M)] as well as the interactions between BSA and poly(-MPC-*co*-M) microspheres.

EXPERIMENTAL

Reagents

2-Hydroxyethyl-(HEMA), ethyl-(EMA), hexyl methacrylate (HMA), MMA, BMA, and St were purified by standard methods and distilled under reduced pressure of nitrogen.

Potassium persulfate (KPS) was recrystallized from water. Cation-(PK-212) and anion-exchange resin (PA-312) used for purifying polymer microspheres were obtained from Mitsubishi Chemical Industries and cleaned according to the procedure described in the literature.[16] Crystallized and lyophilized BSA was purchased from Sigma Chemical Co. and was used as obtained. Distilled and deionized water was used throughout the experiments.

Preparation of MPC

2-Chloro-1,3,2-dioxaphospholane (CDP), bp 42—43°C/13 mmHg, was prepared by the method of Lucas *et al.*[17] 2-Chloro-2-oxo-1,3,2-

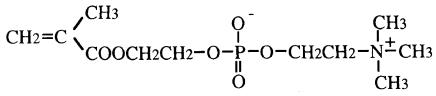

Figure 1. Structure of MPC.

λ^5-dioxaphospholane (COP), bp 91—92°C/2 mmHg, was prepared in 65% yield by oxidation of CDP with oxygen following the method of Edmundson.[18] MPC (Figure 1) was prepared by modifying the method described in literatures[7,19] as follows: Into a 300 ml three-necked round bottomed flask equipped with a magnetic stirrer, drying tube, N_2 inlet tube, and dropping funnel, were placed 19.5 g (0.15 mol) of HEMA in 100 ml of dry tetrahydrofuran (THF). After cooling with dry-ice/methanol at −5°C, 20.8 g (0.15 mol) of COP were added dropwise to the stirred solution under a stream of nitrogen for 1 h. In our method, the reaction of HEMA with COP was performed in the absence of any acceptor for HCl evolved in the course of reaction in THF. The reaction was continued below 5°C while introducing nitrogen until evolution of HCl gas ceased. After reaction for 48 h, the solution was evaporated under reduced pressure to give a colorless viscous oil, 2-(2-oxo-1,3,2-dioxaphospholoyloxy)ethyl methacryate, followed by dissolving in 50 ml of dry THF. After the solution was transferred to a 200 ml glass pressure bottle at −5°C, 16.9 g (0.30 mol) of trimethylamine in 50 ml of dry THF were rapidly added at once. The closed glass bottle was shaken in a thermostat maintained at 40°C for 24 h and then allowed to stand in a refrigerator for 10 h. The precipitate formed was filtered off and washed with dry THF to give white crystalline MPC in 29.8 g (67.3%) yield. mp. 137—140°C. ^{1}H NMR spectra data of MPC obtained here were the same as those of MPC described in the literature.[7] *Anal.* C:H:N = 43.91:7.94:4.44%. Calcd for $C_{11}H_{22}NO_6P$ (M = 295.273), C:H:N = 44.75:7.72:4.74%.

Emulsion Polymerization

Emulsifier-free emulsion polymerization was carried out according to the method in a previous paper.[15] In brief, known amounts of MPC and comonomer were copolymerized by KPS as an initiator in water at 70°C under nitrogen stream for a given time, stirring mechanically with 350 rpm speed of agitation. Polymer microspheres were purified as follows: The coarse particles and aggregates as non-dispersed mass products were filtered through a glass filter (17G2), dried, and weighed. The filtrate was centrifuged at 12500 rpm using a Sigma Laborzentrifugen 2–15, decanted and redispersed in water. After repeating this procedure three times, polymer microspheres were purified by treatment with the mixture of cation- and anion exchange resins.

Measurements of Particle Size

Particle size of polymer microspheres was determined by TEM at 180 kV (5×10^{-7} Torr) with a Topcon EM002B: A very dilute dispersion of polymer microspheres was dropped on a carbon-mounted copper sheet mesh (VECOGRID 400 mesh) and air-dried. Particle size was estimated by comparing TEM with polystyrene latex standard (Dow-Diagnostics, Uniform Latex Particle, ϕ600 nm, Lot No. 2F8E). Size distribution of particles was measured with a Pacific Scientific NICOMP 370.

X-Ray Photoelectron Spectroscopy (XPS)

The surface of particles was analyzed by XPS using a Shimadzu ESCA 750: The emulsion of polymer microspheres was dropped on a stainless-steel holder and dried for a few hours. Measurements were made using Mg-K_α in a vacuum of less than 5×10^{-7} Torr. The ratio of the number of atoms for nitrogen and phosphorus to carbon was obtained based on relative sensitivity factors, 1.77 and 1.25 for N_{1s}/C_{1s} and P_{2p}/C_{1s}, respectively. The spectra of N_{1s} and P_{2p} were measured for 25 and 16 scans, respectively.

Measurements of Equilibrium Water Content

A polymer membrane was prepared from casting a 25 ml of THF solution containing 200 mg of dry polymer microspheres on mercury bath with 9.27 cm of diameter at room temperature. The membrane was dried *in vacuo* at 40°C for 10 h, followed by immersing in pH 7.40 of phosphates buffer solution at 25°C. After being in equilibrium for 24 h, the membrane was taken off and excess solution was removed by light tamping between filter papers. The measurement was repeated three times for each sample. Equilibrium water content was calculated by the following equation[7]:

$$\text{Equilibrium water content} = \frac{\text{weight of water in the membrane}}{\text{weight of membrane swollen}}$$

Adsorption Procedure

Interaction between BSA and polymer microspheres was measured at 0.01 of ionic strength adjusted with NaCl at pH 5.6: The specific surface area of polymer microspheres was calculated from the diameter determined by transmission electron microscopy (TEM) and the emulsion containing a definite concentration of the polymer microspheres was prepared for the adsorption experiment. A mixture of a known concentration of BSA aqueous solution and 40 $m^2 l^{-1}$ of polymer microspheres was equilibrated at 25°C for 2 h, followed by centrifugation at 12500 rpm for 15 min. The amount of BSA adsorbed on polymer microspheres was determined by the Lowry method.[20] The amount of BSA adsorbed was calculated from the content of free BSA in water, measuring the absorbance at 750 nm of BSA by UV measurements with a Shimadzu UV-160A spectrophotometer.

RESULTS AND DISCUSSION

Copolymerization of MPC with Various Vinyl Monomers

Prior to the preparation of copolymer

Table I. Condition for the emulsion copolymerization of MPC and various vinyl monomers

MPC	0—30 mmol
Comonomer[a]	300 mmol
KPS	0.3 mmol
Water	300 ml
Speed of agitation	350 rpm
Temperature	70 °C
Time	5—24 h

[a] Comonomers used were methyl-(MMA), ethyl-(EMA), butyl-(BMA), hexyl methacrylate (HMA), and styrene (St).

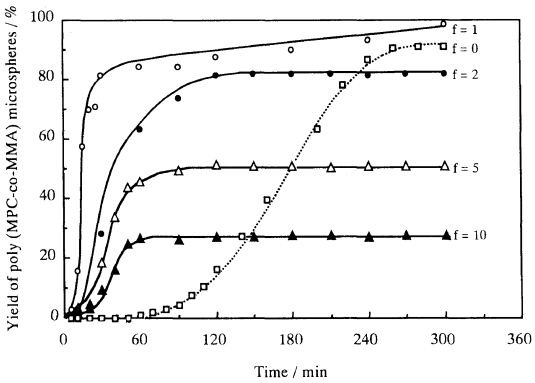

Figure 2. Polymer microspheres yield-time curves for the emulsion copolymerization of MPC and MMA at 70°C. MMA, 300 mmol; KPS, 0.3 mmol; water, 300 ml. f represents the mol% of MPC to MMA monomer.

microspheres, poly(MPC-*co*-M), the emulsion copolymerization of MPC and MMA initiated by KPS as an initiator was carried out in the absence of emulsifier at 70°C, varying the mol% of MPC to MMA comonomer (f) in feed from $f=1$ to $f=10$. The polymerization condition is given in Table I. For kinetics, polymerization was followed by sampling an aliquot of the emulsion from the stirred emulsion at prescribed time intervals. Poly(MPC-*co*-MMA) microspheres-time curves for the copolymerization are plotted in Figure 2, together with those of poly(M) microspheres for the homopolymerization of MMA ($f=0$). It was found that the addition of small amounts of MPC resulted in drastic increase in the rate of polymerization of MMA at an earlier stage.

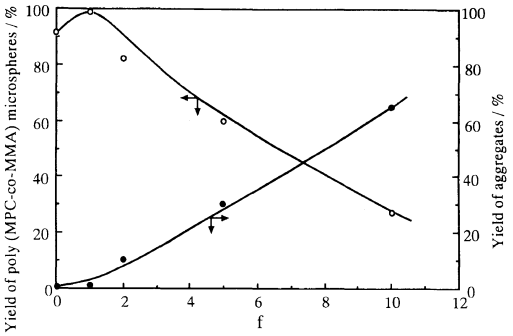

Figure 3. Effects of concentration of MPC (f) on yields of poly(MPC-*co*-MMA) microspheres and aggregates in the emulsion copolymerization of MPC and MMA at 70°C. f represents mol% of MPC to MMA monomer.

This means that a larger number of particles was produced by increasing the solubility of MMA to water when MPC is present, and the Trommsdorff gel effect due to the increase in viscosity. These effects were described in the case of emulsifier-free emulsion copolymerization of St and sodium styrene sulfonate.[21] A decrease in poly(MPC-*co*-MMA) microspheres yield and extensive increase in yield of coarse particles and its aggregates were also observed with increasing MPC concentration in the feed as shown in Figure 3. There are two possible reasons to explain the formation of aggregates: the aggregates probably originated from bridging flocculation in the presence of large amounts of MPC homopolymer, and by interacting the charged groups derived from KPS fragments with MPC moiety to reduce stability to the dispersion. Emulsifier-free emulsion copolymerization of MPC with various vinyl monomers was also carried out at 70°C with constant mol% of MPC, $f=1$. The polymerization was continued under introducing nitrogen in the reaction vessel until the characteristic odor of comonomer was completely absent from mixture. Table II shows the results of characterization for a series of poly(MPC-*co*-M) microspheres. It was found that polymerization time to reach a final stage increases with decreasing hydrophilic nature of the comonomers.

Table II. Characterization of poly(MPC-*co*-M) microspheres obtained from the emulsion copolymerization of MPC and various vinyl monomers

Microspheres	f[a]	Time	Yield	Diameter[b]	U[c]	In feed[d]		XPS analysis[e]	
		h	%	nm		10^3 P/C	10^3 N/C	10^3 P/C	10^3 N/C
Poly(MPC-*co*-MMA)	1	5	98	160 ±34.0	1.122	1.97	1.97	3.24	3.66
Poly(MPC-*co*-MMA)	2	5	82	210 ±56.7	1.348	3.83	3.83	(5.80)	(5.16)
Poly(MPC-*co*-MMA)	5	5	60	—	—	9.01	9.01	(9.11)	(9.50)
Poly(MPC-*co*-MMA)	10	5	28	—	—	16.39	16.39	(11.33)	(10.31)
Poly(MPC-*co*-EMA)	1	10	96	133 ±17.6	1.055	1.64	1.64	2.09	2.16
Poly(MPC-*co*-BMA)	1	10	90.6	123 ±18.7	1.074	1.23	1.23	(4.10)	(4.37)
Poly(MPC-*co*-HMA)	1	12	87.8	84.4±26.7	1.399	0.99	0.99	(2.73)	(3.72)
Poly(MPC-*co*-St)	1	24	95	92.7±22.9	1.210	1.23	1.23	3.88	4.11

[a] f represents mol% of MPC to comonomer.
[b] Calculated from size distribution.
[c] $U=D_W/D_N$, where D_W and D_N are the weight-average and number-average diameters of microspheres, respectively.
[d] P/C and N/C are the ratios of atoms for P and N to C calculated in the feed. The composition in the bulk copolymer could not determine, because MPC content in the feed was too small to measure.
[e] P/C and N/C are the ratios of atoms for P and N to C obtained by XPS. Values in parenthesis are obtained from the fused particles samples.

TEM Measurernts and Particle Size Distribution

Figure 4 shows TEM pictures of poly(MPC-*co*-M) microspheres at $f=1$, together with those of corresponding poly(M) microspheres. TEM pictures for the microspheres of poly(MPC-*co*-MMA) at $f\geqq 2$, poly(MPC-*co*-BMA), and poly(MPC-*co*-HMA) could not be taken under the same conditions because the particles fuse with each other to form membranes in the course of drying. Particle size distribution of poly(MPC-*co*-M) microspheres was also measured as shown in Figure 5. The results are shown in Table II. It is apparent from the TEM pictures that particle sizes of poly(MPC-*co*-EMA) microspheres and poly(MPC-*co*-St) microspheres are much smaller than those of poly(M) microspheres, while the copolymerization of MPC and MMA gives slightly larger size of particles than poly(MMA) microspheres. Increase in the diameter of poly(MPC-*co*-M) microspheres except for the case of St comonomer was also noted with decreasing hydrophilic nature of M comonomers, whose hydrophilicity decreased in the order of MMA > EMA > BMA > HMA > St. The fact that the diameter of poly(MPC-*co*-St) microspheres is larger than that of poly(MPC-*co*-HMA) microspheres is in conflict with the order of hydrophilicity of comonomers. It is well known that the solubility, polarity, and hydrophile–lipophile balance (HLB) of monomers, play important roles in emulsion polymerization. These differences in diameter of poly(MPC-*co*-M) microspheres and poly(M) microspheres are, therefore, accounted for by the difference in increase in solubility of comonomer when hydrophilic MPC is present. On comparing St and HMA comonomers, the addition of MPC may cause increase in solubility of the former much more than that of latter. It is concluded that particle diameter depends on the generation of charged stable oligomeric radicals which are converted to primary growing particles.

The uniformity ratio (U) is defined by $U=D_W/D_N$, where D_W and D_N represent the weight-average and number-average diameter of polymer microspheres, respectively. Since in general polymer microspheres would be considered monodispersed when $U\leqq 1.01$,[22,23] poly(MPC-*co*-EMA) microspheres and poly(-

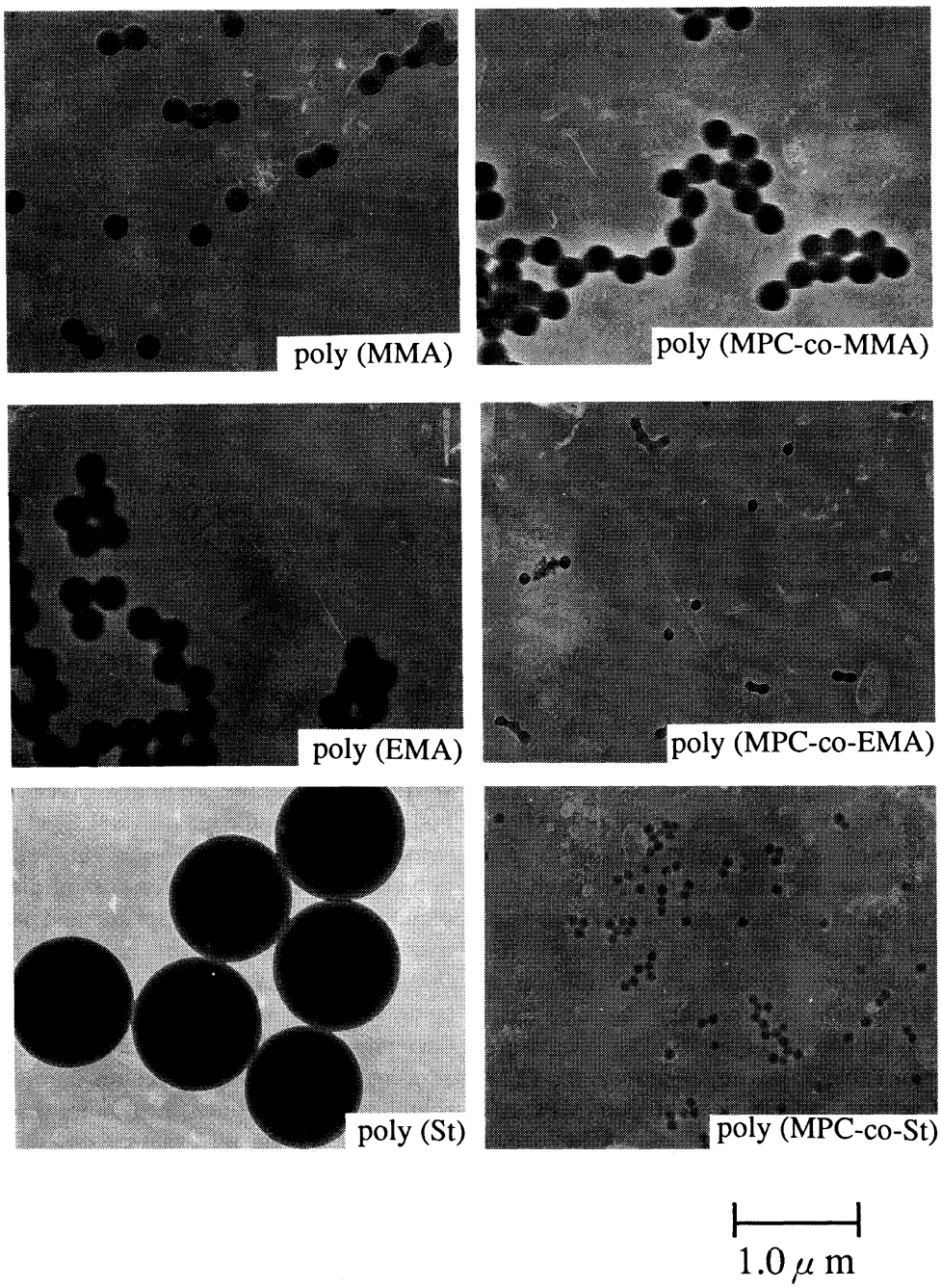

Figure 4. TEM micrographs of poly(MPC-*co*-M) microspheres at $f=1$ and homopolymer microspheres. ×20000

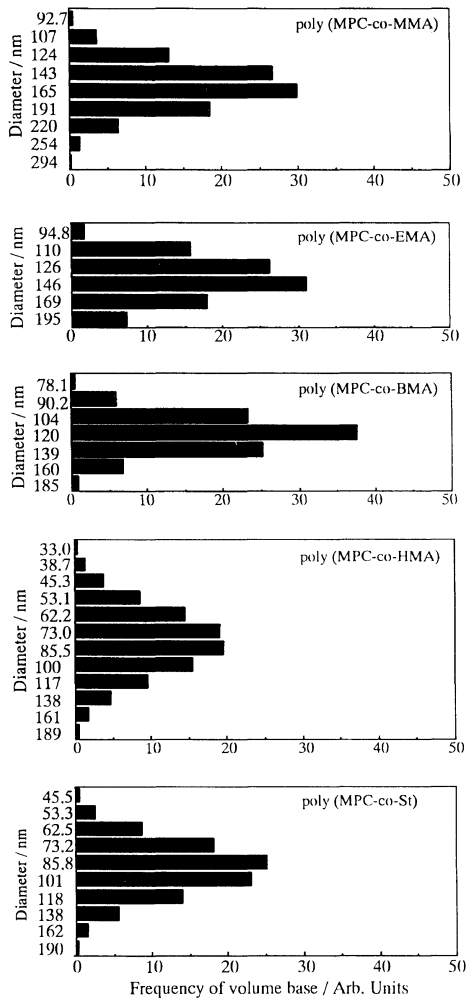

Figure 5. Particle size distribution for poly(MPC-*co*-M) microspheres at $f=1$.

MPC-*co*-BMA) microspheres were found to be particles close to monodispersed.

XPS Measurement of Particles

The surface of poly(MPC-*co*-M) microspheres was analyzed quantitatively by XPS. XPS was taken in the fused particles for the microspheres of poly(MPC-*co*-MMA) at $f \geqq 2$, poly(MPC-*co*-BMA), and poly(MPC-*co*-HMA). The signals of N_{1s} and P_{2p} of poly(MPC-*co*-MMA) microspheres at $f=1$ as a typical instance are illustrated in Figure 6. The peaks for the nitrogen signal at 402 eV and phosphorus one at 133 eV were assigned to quarternary nitrogen and phosphorus in phosphate group of the MPC moiety, respectively. The ratios of the number of atoms for phosphorus and nitrogen to carbon (P/C and N/C) of samples are presented in Table II. Both P/C and N/C of samples except for poly(MPC-*co*-MMA) microspheres at $f=10$ were observed to be larger than those calculated in the feed, though N/P varied from 0.89 to 1.36 for MPC that of N/P = 1 as calculated for the feed. Such difference in the ratio of N/P may be attributed to the liberation of phosphate groups from the surface by hydrolysis other than error in measurements. In the case of poly(MPC-*co*-MMA) microspheres prepared at $f=10$, it is considered that a large amount of MPC in the feed is consumed not to produce poly(MPC-*co*-M) microspheres but to form its aggregates.

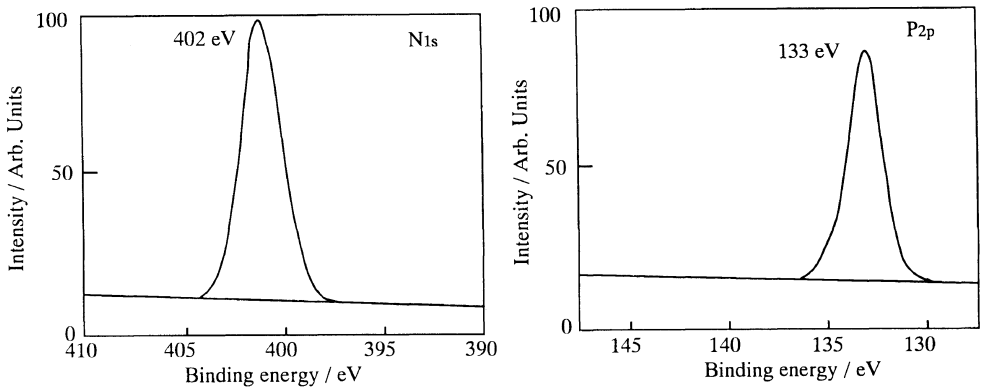

Figure 6. XPS for N_{1s} and P_{2p} spectra of poly(MPC-*co*-MMA) microspheres at $f=1$.

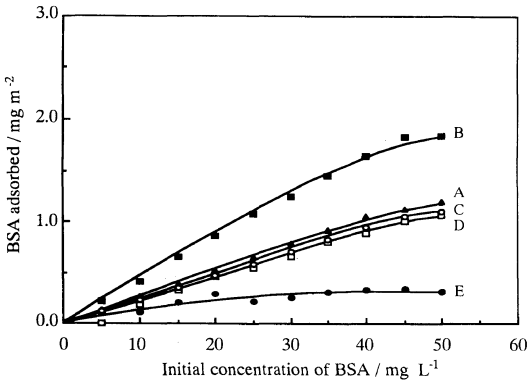

Figure 7. Adsorption of BSA onto various poly(MPC-*co*-M) microspheres, pH 5.6, ionic strength 0.01, at 25°C for 2 h: A, poly(MPC-*co*-St); B, poly(MPC-*co*-HMA); C, poly(MPC-*co*-BMA); D, poly(MPC-*co*-EMA); and E, poly(MPC-*co*-MMA).

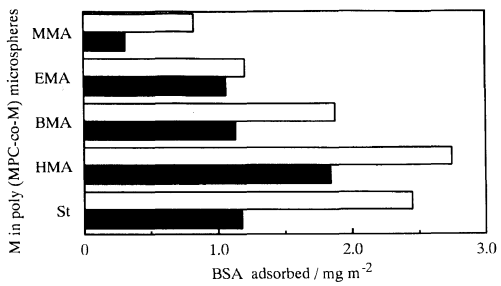

Figure 8. Comparison of adsorption of BSA onto poly(MPC-*co*-M) microspheres at $f=1$ and poly (M) microspheres, initial concentration of BSA 50 mg l^{-1}, pH 5.6, ionic strength 0.01, at 25°C for 2 h. □, poly(M) microspheres; ■, poly(MPC-*co*-M) microspheres.

It may be concluded that the MPC moiety is localized on the surface of particles.

BSA Adsorption on Poly(MPC-co-M) Microspheres

Interaction between poly(MPC-*co*-M) microspheres at $f=1$ and BSA was studied in order to obtain basic information for biomedical applications. The adsorption of BSA on poly(MPC-*co*-M) microspheres was examined in water at pH 5.6, ionic strength 0.01 adjusted with NaCl aqueous solution, together with the adsorption experiments for poly(M) microspheres as control. This pH is close to the isoelectric point of BSA, at which molecules form compact structures.[24] Figure 7 shows the results of BSA adsorption on poly(MPC-*co*-M) microspheres. Figure 8 describes BSA adsorption on a series of microspheres of poly(MPC-*co*-M) and poly(M) at 50 mg l^{-1} of initial concentration of BSA. It can be said from Figure 8 that the introduction of phosphatidylcholine analogous moieties onto the surface of particles results in a decrease in BSA adsorption. Ishihara *et al.* reported that the membranes of poly(MPC-*co*-BMA), obtained by the solution copolymerization of MPC and BMA, absorb water well and become a hydrogel structure even MPC mole fraction in poly(MPC-*co*-BMA) is 0.04.[7] This membrane was found to reduce protein adsorption from human plasma[4] and activation of platelets.[5,6] Table III shows the equilibrium water content of a series of membranes prepared from polymer microspheres in pH 7.40 at 25°C. Comparing the membranes prepared from poly(MPC-*co*-M) microspheres at $f=1$ with corresponding poly(M) membranes, the former samples show larger equilibrium water content than the latter. The surface layer of the poly(MPC-*co*-M) microspheres is presumed to absorb water much more than membrane because the MPC moiety is concentrated on the surface of the particles. Taking into account

Table III. Equilibrium water content for membranes obtained from polymer microspheres

Membranes	f[a]	Equilibrium water content[b] × 100
Poly(MPC-*co*-MMA)	1	1.31
Poly(MPC-*co*-EMA)	1	1.17
Poly(MPC-*co*-BMA)	1	0.93
Poly(MPC-*co*-HMA)	1	0.53
Poly(MPC-*co*-St)	1	0.57
Poly(MMA)	0	0.33
Poly(EMA)	0	0.29
Poly(BMA)	0	0.06
Poly(HMA)	0	0.05
Poly(St)	0	0.04

[a] f represants mol% of MPC to comonomer.
[b] At 25°C, pH 7.40.

the results reported by Ishihara,[7] the surface of a series of poly(MPC-*co*-M) microspheres obtained here seems to form a partial hydrogel structure on the surface of the particles. In conclusion, reduction of adsorption of BSA on poly(MPC-*co*-M) microspheres is dependent on the hydrophilicity of M comonomer and MPC composition on the surface of particles.

Acknowledgments. The authors thank Mr. T. Ohashi of Western Industrial Research Institute of Hiroshima for measurement of XPS. Thanks are also due to Mr. T. Yoshida of Nippon Oil & Fats Co., Ltd. Tsukuba Research Laboratory for the measurement of particle size distribution. The authors are grateful to the Environmental Science Institute of Kinki University for financial support. This study was also supported by a Grant-in-Aid for Science Research from Japan Private School Promotion Foundation.

REFERENCES AND NOTES

1. Z. Foltynowicz, K. Yamaguchi, B. Czajka, and S. L. Regen, *Macromolecules*, **18**, 1394 (1985).
2. A. A. Durrani, J. A. Hayward, and D. Chapman, *Biomaterials*, **7**, 121 (1986).
3. D. Letourneur, C. Douzon, and M. Jozefowicz, *J. Polym. Sci., A, Polym. Chem. Ed.*, **29**, 1367 (1991).
4. K. Ishihara, N. P. Ziats, B. P. Tierney, N. Nakabayashi, and J. M. Anderson, *J. Biomed. Mater. Res.*, **25**, 1397 (1991).
5. K. Ishihara, R. Aragaki, T. Ueda, A. Watanabe, and N. Nakabayashi, *J. Biomed. Mater. Res.*, **24**, 1069 (1990).
6. T. Ueda, K. Ishihara, and N. Nakabayashi, *Kobunshi Ronbunshu*, **48**, 289 (1991).
7. K. Ishihara, T. Ueda, and N. Nakabayashi, *Polym. J.*, **22**, 355 (1990).
8. T. Ueda, K. Ishihara, and N. Nakabayashi, *Makromol. Chem., Rapid Commun.*, **11**, 345 (1990).
9. T. Ueda, H. Oshida, K. Kurita, K. Ishihara, and N. Nakabayashi, *Polym. J.*, **24**, 1259 (1992).
10. J. L. Robbins, G. A. Hill, B. N. Carle, J. H. Carlquist, and S. Marcus, Proceedings of the Society of Experiment Bio Medicine, Vol. 109, 1992, p 321.
11. A. Rembaum, S. P. S. Yen, and R. S. Molday, *J. Macromol. Sci.-Chem.*, **A13**, 603 (1979).
12. H. Kawaguchi, N. Koiwai, and Y. Ohtsuka, *J. Appl. Polym. Sci.*, **35**, 743 (1988).
13. H. Sato, H. Morimoto, A. Nakajima, and Y. Noishiki, *Polym. J.*, **16**, 1 (1984).
14. K. Yamaguchi, S. Watanabe, and S. Nakahama, *Makromol. Chem.*, **190**, 1195 (1989).
15. K. Sugiyama, K. Shiraishi, K. Ohga, H. Shirahama, H. Tamai, H. Yasuda, and K. Kikukawa, *Polym. J.*, **25**, 521 (1993).
16. H. J. van den Hul and J. W. Vanderhoff, *J. Colloid Interface Sci.*, **28**, 336 (1968).
17. H. J. Lucas, F. W. Mitchell, and C. N. Scully, *J. Am. Chem. Soc.*, **72**, 5491 (1950).
18. R. E. Edmundson, *Chem. Ind.* (London), 1828 (1962).
19. T. Umeda, T. Nakaya, and M. Imoto, *Makromol. Chem., Rapid Commun.*, **3**, 457 (1982).
20. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
21. J. H. Kim, M. Chainey, M. S. El-Asser, and J. W. Vanderhoff, *J. Polym. Sci., A, Polym. Chem.*, **30**, 171 (1992).
22. H. Shirahama, H. Ohno, and T. Suzawa, *Colloids and Surfaces*, **60**, 1 (1991).
23. A. Homola and R. O. James, *J. Colloid Interface Sci.*, **59**, 123 (1977).
24. T. Suzawa, H. Shirahama, and T. Fujimoto, *J. Colloid Interface Sci.*, **86**, 144 (1982).