Polypeptide Spheres. VII. Macroreticulation of Spherical Poly(γ-methyl-L-glutamate) Beads

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SYNOPSIS

Spherical beads from poly(γ-methyl-L-glutamatate) alone were prepared using various diluents for macroreticulating by the "suspension and evaporation" method. The porosity and the pore size of the beads obtained were remarkably dependent on the chemical structure of a diluent. This is related to the specific miscibility of a diluent with poly(γ-methyl-L-glutamate). For example, decahydronaphthalene made the beads much more macroporous than tetrahydronaphthalene with similar hydrophobisity: the aromaticity of diluent increases the miscibility. However, it was found that long-chain alkyl compounds with a miscible group such as methyl dodecanoate, oleic acid, linoleic acid, and dialkyl-phthalate were not only very effective for macroreticulating, but also induced narrow pore-size distribution. In addition, the diluent influenced physicochemical properties of the beads to cause specific affinity to proteins.

INTRODUCTION

Recently, nonionic polymer packings with large pores have been preferred for gel permeation chromatographic separation for aqueous macromolecules such as proteins and nucleic acids. However, most hydrophilic porous polymer packings from nonionic saccharides cannot be used at high flow rate conditions because the pressure resistance decreases with increase in pore size.[1] Also inorganic packings from silica gels are very rigid but ionic and susceptible to hydrolyzation by acids and bases.[2]

On the other hand, macroporous spheres from poly(γ-methyl-L-glutamate) (PMLG) developed by us are rigid and show higher flow rate resistance than any other available hydrophilic polymer gels because of intermolecular hydrogen bonding between peptide chains.[3,4] In this communication, we report that porous PMLG spheres with a series of porosity and pore sizes could be prepared by modification of a previous technique based on the proper selection of a diluent as a macroreticulating reagent. In addition, it is shown that the diluent influences chemical properties such as hydrophobicity as well as pore size.

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EXPERIMENTAL

Sphering Procedure

Porous PMLG spheres with diameters from 44 to 105 μm were prepared by our previous technique.[3,4] Dialkyl-phthalate, decahydronaphthalene, tetrahydronaphthalene, methyl dodecanoate, oleic acid, linoleic acid, and others were used as diluents for macroreticulation.

Gel Permeation Chromatography (GPC)

The prepared PMLG spheres were packed into a glass column (150–300 × 5 mm inner diameter). The chromatograph included a JASCO 880-PU pump and a Shodex refracto monitor SE-51. The exclusion molecular weight and the porosity of packings were estimated by calibration curves made using a homogeneous series of dextran and maltose as a permeable substance. The linear part corresponding to the coverage of the GPC separation is shown in Eq. (1).[3,5]

$$\log M = \beta - \alpha_{\text{eff}}(V_e/V_t) \qquad (1)$$

where V_t is the total volume of the gel bed and V_e is the eluting volume of a substance with molecular weight M.

Other Measurements

The α_{BM} value related to the hydrophilicity of packing was estimated by the separation factor of 1-butanol (k'_{BuOH}) to methanol (k'_{MeOH}) in an aqueous system.

$$\alpha_{BM} = \frac{k'_{BuOH}}{k'_{MeOH}} = \frac{(V_{BuOH} - V_0)/(V_{D_2O} - V_0)}{(V_{MeOH} - V_0)/(V_{D_2O} - V_0)} \quad (2)$$

where V_{BuOH}, V_{MeOH}, and V_{D_2O} are elution volumes for 1-butanol, methanol, and D_2O, respectively, and V_0 is a void volume. The degree of swelling was measured by a previous method.[3] The conformation of peptide chains in the sphere was determined from diffusion reflection spectra using a Perkin-Elmer FT-IR Model 1630.

RESULTS AND DISCUSSION

Macroreticulation

In general, poly(α-amino acid)s can be obtained by polymerization of *N*-carboxy anhydrides of α-amino acid (NCA). However, it is difficult to prepare their spherical particles by means of conventional suspension polymerization because NCAs are too reactive and unstable. Therefore, we have developed the "suspension and evaporation" method for sphering poly(α-amino acid) directly.[3] The method is based on insolubility due to the formation of secondary structures when poly(α-amino acid)s are reprecipitated from the solution: the method includes the preparation of suspension particles and gradual removal of solvent from them. Figure 1 shows electron micrographs of typical examples prepared from poly(γ-methyl-L-glutamate) (PMLG) using this method. The spherical PMLG particles were macroreticulated by adding a diluent in the process of sphering and then removing the diluent with a proper solvent. To be effective, most of the diluent must remain within the suspension particles of the PMLG solution during the sphering process, so hydrophobic liquids with boiling points higher than that of the suspension medium were usually selected. However, we have observed that conventional diluents such as toluene and xylene used in suspension polymerization were not efficient for macroreticulation of PMLG spheres.[3] In the present study, the relationship between the chemical structure of the diluent and the pore size produced

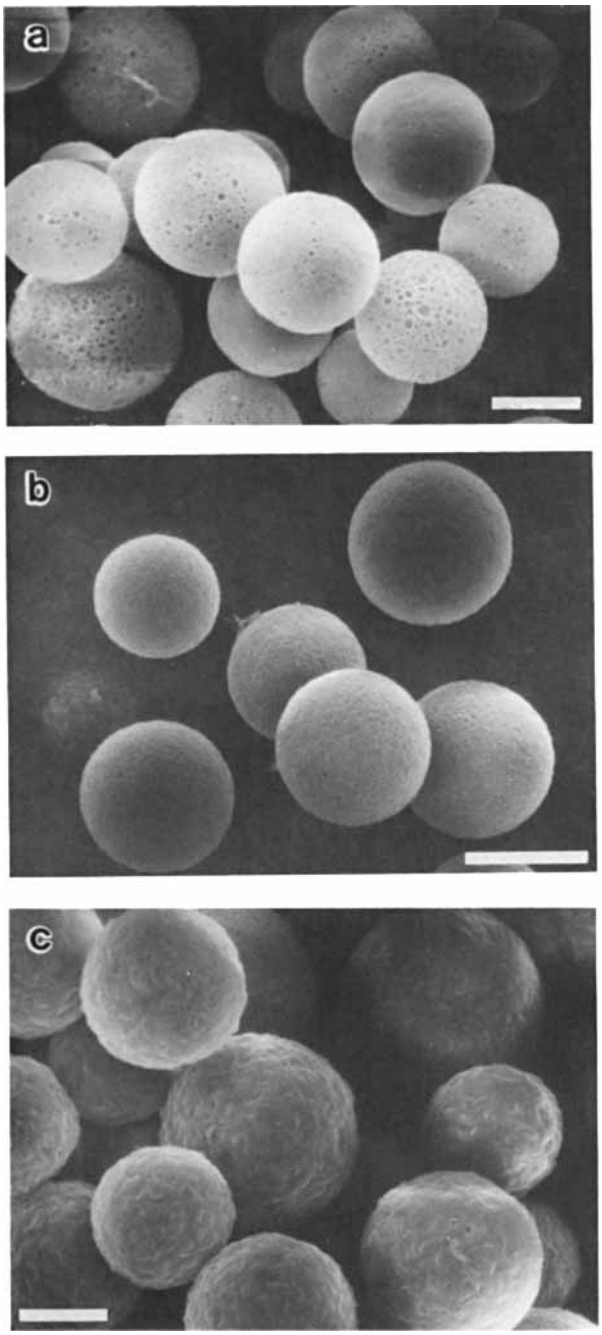

Figure 1 Typical electron micrographs of porous PMLG spheres. (*a*) DHN-2; (*b*) THN-2; (*c*) MD-2. Scale bars show 20 μm.

was investigated. None of the diluents selected for this study are conventional ones.

As shown in Table I, the kind and amount of diluent directly affects the value of M_{lim} and the porosity. In the absence of a diluent, these values are only 500 and 14%, respectively (an M_{lim} value of 60 was reported in our previous study,[3] but the differ-

Table I Values of M_{lim} and Porosity of PMLG Spheres[a] Prepared with Use of Various Diluents

No.	Diluent		M_{lim}[b]	Porosity (%)	α_{eff}	α_{BM}
	Kind	mL/g				
NONE	None	0	500	14	22.1	8.9
DMP-1	Dimethyl phthalate	1.0	1,000	25	12.4	6.1
DMP-2	Dimethyl phthalate	2.0	1,000	34	8.9	5.5
DMP-4	Dimethyl phthalate	4.0	30,000	73	4.8	2.3
DEHP-1	Di(2-ethylhexyl)phthalate	1.0	50,000	73	1.2	2.2
DEHP-2	Di(2-ethylhexyl)phthalate	2.0	500,000	85	2.0	1.7
DEHP-4	Di(2-ethylhexyl)phthalate	4.0	1,000,000	88	2.2	1.6[c]
DOP-2	Dioctyl phthalate	2.0	1,500,000	81	3.6	
MD-2	Methyl dodecanoate	2.0	2,000,000	86	2.8	1.5[c]
OLE-2	Oleic acid	2.0	> 2,000,000	> 81		1.7[c]
LIN-2	Linoleic acid	2.0	> 2,000,000	> 86		1.6[c]
DHN-1	Decahydronaphthalene	1.0	100,000	63	3.6	2.6
DHN-2	Decahydronaphthalene	2.0	1,000,000	84	3.0	1.6[c]
DHN-4	Decahydronaphthalene	4.0	1,500,000	90	3.0	
THN-2	Tetrahydronaphthalene	2.0	20,000	65	4.8	2.9
TCB-2	Trichlorobenzene	2.0	1,500	46	6.6	3.6

[a] Prepared using the PMLG solution incubated at 100°C for 6 h. Suspension medium: 2.5 wt % PVA aqueous solution; solvent for PMLG: dichloroethane (2.0 wt %); ratio of suspension medium to PMLG solution = 6 : 1, sphering temperature 45°C.
[b] Value reduced as a molecular weight of dextran.
[c] Calculated with a V_0 value of 45%.

ence is due to the incubation treatment[4] of PMLG material solution. Details will be described elsewhere). These small values are attributable to the strong cohesion based on hydrogen bonding between peptide chains. However, M_{lim} and porosity increased remarkably when decahydronaphthalene (DHN) and di(2-ethylhexyl)phthalate (DEHP) were added as a diluent. However, such a large increase was not observed if tetrahydronaphthalene (THN) and dimethyl phthalate (DMP) were used instead of DHN and DEHP, respectively. The degree of swelling (S_d) of the PMLG spheres prepared using 2 mL/g of DEHP was examined for each diluent. The values of S_d were 9.1, 11.6, 12.4, and 23.6 for DHN, DEHP, dibutyl phthalate, and DMP, respectively: the higher the aromaticity of diluent, the higher the S_d. This is similar to the case of toluene and xylene, when the aromaticity of diluent increases the miscibility with PMLG to suppress the growth of diluent areas in the PMLG suspension particles. This suspension causes the smaller increase of M_{lim} because the diluent areas become pores in the particles.

The miscibility of a diluent was not always disadvantageous for macroreticulating. For example, DEHP with hydrophobic long-alkyl chain and aromatic groups produced a α_{eff} and a large M_{lim}. The value of α_{eff} is an indication for pore size homogeneity, and the smaller the value, the more homogeneous the pore. In addition, it was found that aliphatic compounds with a polar group such as methyl dodecanoate (MD), oleic acid (OLE), and linoleic acid (LIN) were also very effective for macroreticulating. Consequently, it is considered that partial polar moieties such as carboxylic groups, ester bonding, and aromatic groups suppress excessive cohesion among peptide chains and promote sufficient and homogeneous phase separation of diluent, although less miscible diluents are basically required for macroreticulating the PMLG spheres.

Chemical Affinities

We have previously shown that porous PMLG spheres were applicable to GPC in both aqueous and organic systems because of the chromatographycally amphiphilic properties of PMLG.[3,6] In addition, it has also been reported that methanol and *sec*-butanol were reversed phase chromatographycally separated from ethanol and *tert*-butanol, respectively, when an aqueous solution was used as an eluent.[7,8] This separation mode is related to enhancement of hydrophobicity due to the formation of secondary structure in the peptide chains. The separation factor (α_{BM}) of 1-butanol to methanol was examined for evaluating the hydrophobicity of

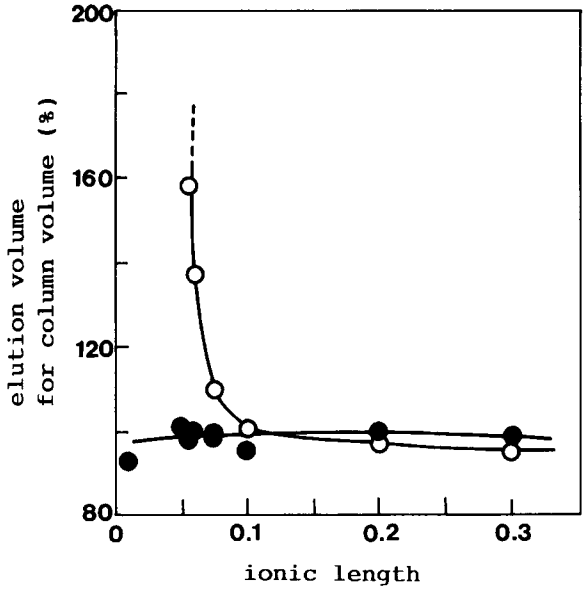

Figure 2 Relationship between the retention volume and the ionic strength of eluent in the liquid chromatography using PMLG spheres as packing. (— ○ —) cytochrome C (M_n = 13000); (— ● —) poly(L-lysine) (M_n = 25000). PMLG spheres: DHN-2, eluent: pH 7.0 phosphate buffer.

each PMLG sphere. As shown in Table I, the α_{BM} is higher in spheres prepared with more miscible diluents, such as DMP, trichlorobenzene (TCB), and THN, than in those prepared with less miscible ones: this shows that the spheres prepared using more miscible diluents are more hydrophobic. The FT-IR spectra of the PMLG spheres showed that the secondary structure of peptide chains was mainly α-helical regardless of the kind of diluent (amid I, 1650 cm^{-1}; amide II, 1550 cm^{-1}; amide V, 620 cm^{-1} with the shoulders based on the β-structural I, 1630 cm^{-1}; II, 1530 cm^{-1}; V, 700 cm^{-1}). Therefore, the difference in hydrophobicity (α_{BM}) cannot be explained only by the conformation of matrix peptide chains. It is considered that the conformation of surface chains is important for hydrophobicity, and that DMP, TCB, and THN promote the formation of hydrophobic ordered structures (such as α-helix and β-structure) and OLE, LIN, MD, DEHP, and DHN do disordering; thus the surface conformation depends on the chemical properties of diluent domains.

It was also found that the PMLG spheres adsorbed some basic proteins in low ion strength conditions. However, the adsorption mechanism seem to differ from that of usual cation exchangers because (1) the retention time was dependent on the kind of diluent used, (2) the retention time was independent of the order of the isoelectric point of proteins [the retention order is as follows: protamine (pI = 10 − 12) > chymotripsinogen (pI = 9.1) > cytochrome C (pI = 10.6) ≫ poly(L-lysine) (pI = 10.5)], (3) as shown in Figure 2, basic amino acids such as lysine and ornithine and their polymers were not adsorbed, and (4) the cation and anion exchange capacities are below 0.1 meq/g. Therefore, it is considered that the specific affinity for basic proteins is also related to the surface structure of PMLG spheres. The details are under further investigation.

In conclusion, the use of a diluent for macroreticulation of PMLG spheres changes the chemical affinity as well as the pore size distribution.

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REFERENCES

1. C. Hirayama, H. Ihara, M. Shiba, M. Nakamura, and Y. Motozato, *J. Chromatogr.*, **409,** 175 (1987).
2. For example, J. H. Knox, B. Kauer, and G. R. Millward, *J. Chromatogr.*, **352,** 3 (1986); J. J. Pesek and S. A. Swedberg, *J. Chromatogr.*, **361,** 83 (1986).
3. H. Ihara, T. Yoshinaga, and C. Hirayama, *J. Chromatogr.*, **362,** 197 (1986).
4. C. Hirayama, H. Ihara, S. Nagaoka, H. Furusawa, and S. Tsuruta, *Polym. J.*, **22,** 614 (1990).
5. K. A. Granth and P. Flodin, *Makromol. Chem.*, **48,** 160 (1961).
6. H. Ihara, T. Yoshinaga, Y. Motozato, and C. Hirayama, *Polym. J.*, **17,** 1301 (1985).
7. H. Ihara, T. Yoshinaga, and C. Hirayama, *Polym. Preprints Jpn.*, **35,** 2010 (1986).
8. C. Hirayama and H. Ihara, *Polym. Appl.*, **37,** 18 (1987).

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