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PREPARATION AND GEL PERMEATION CHROMATOGRAPHIC PROPERTIES OF POROUS SPHERES FROM POLY(γ-METHYL OR γ-BENZYL L-GLUTAMATE)

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SUMMARY

Porous spheres with average diameters of 5–300 μm have been prepared for the first time from poly(γ-methyl or γ-benzyl L-glutamate) alone, and their gel permeation chromatographic properties, both without further modification and after cross-linking with appropriate agents, have been examined. The polypeptide spheres were prepared by gradually removing the solvent from the suspension particles containing polypeptide. The particle size and porosity were controlled with ease by adjusting the viscosity of the peptide solution and suspension medium, and by selecting additional diluents in the sphering (solidification) process, respectively. The spheres of poly(γ-methyl L-glutamate) can not only withstand a remarkably high flow-rate, but also show typical gel permeation chromatographic behaviours in both aqueous and organic systems. These good properties are attributable to inter- and intramolecular hydrogen bonding based on the formation of specific polypeptide conformations.

INTRODUCTION

Packing materials for gel permeation chromatography (GPC) are classified into those for aqueous and organic media, according to whether they are made from neutral polysaccharides or water-soluble synthetic polymers and polystyrene, respectively. The former type have been widely used for the analysis and separation not only of water-soluble polymer samples, but also of biological materials, *e.g.* proteins, nucleic acids and viruses.

Since it was first reported in 1959 that hydrophilic gels can be synthesized from dextran[1], many gels for aqueous GPC have been prepared from a variety of hydrophilic polymers, such as agar[2,3], cellulose[4], poly(acrylamide)[5,6] and poly(vinyl alcohol)[7,8]. However, they are not free of defects: for example, most of the hydrophilic gels with a large exclusion molecular weight (M_{lim}) cannot be used at high flow-rate condition, because the larger the value of M_{lim} the worse the pressure resistance. This is an unavoidable defect of gel(swelling)-type packings. On the other hand, we have succeeded in making porous and spherical particles of cellulose by a unique

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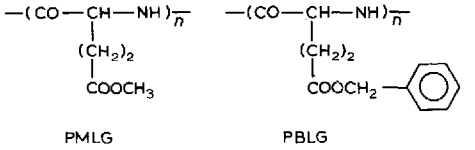
method[9–12]. This packing is very rigid and shows a higher pressure-resistance than any other available product. We consider that these excellent properties are due to intermolecular hydrogen bonding in cellulose.

This paper reports the production of novel packings for GPC from poly(γ-methyl or γ-benzyl L-glutamate), abbreviated as PMLG and PBLG. The former in particular can not only withstand a surprisingly high flow-rate, but also shows typical GPC behaviour in both aqueous and organic systems. These properties are attributed to the fact that the polypeptide is hydrophilic in chemical structure, but shows affinity and insolubility for most organic solvents by forming a specific conformation, such as β-structure and α-helix.



PMLG PBLG

EXPERIMENTAL

Preparation of polypeptide spheres

PMLG and PBLG were synthesized by the polymerization of carboxyanhydrides of γ-methyl and γ-benzyl L-glutamate in 1,2-dichloroethane and tetrahydrofuran, respectively. Commercial PMLG solution was also used as received.

Porous and spherical particles of these polypeptides were prepared as follows. A 1,2-dichloroethane solution of PMLG obtained by polymerization was diluted with 1,2-dichloroethane containing 2–10 wt.% of decahydronaphthalene (or diethylbenzene, 1-octanol, etc.) to give a 2.0–3.5 wt.% solution. This solution was suspended in a 0.5–5.0 wt.% solution of aqueous poly(vinyl alcohol) (viscosity, 40–46 cP; degree of saponification, 86.5–89 mol%). The mixture was stirred at a fixed speed (500–1000 rpm) at 40°C for 12–24 h to remove the dichloroethane. The spherical particles produced after filtration were washed successively with water, hot water, ethanol, and ether, to give a 50–90% yield of PMLG spheres of average diameter 5–300 μm. PBLG particles were obtained by a similar procedure from a dichloromethane solution.

These polypeptide spheres were cross-linked as required by transesterification with diol. The spheres were placed in a three-necked flask fitted with a stirrer, a condenser for azeotropic distillation and a thermometer. Decahydronaphthalene–chloroform (3:1), 0.2 equiv. of sulphuric acid and 0.5 equiv. of triethylene glycol were added and the mixture was stirred at 65°C with addition of fresh chloroform as necessary. Spheres cross-linked by triethylene glycol were obtained by filtering and washing with water and methanol. The yield was 90–100%.

Gel permeation chromatography

PMLG spheres prepared by our procedure and commercial packings from Sephadex, Sepharose and Bio Gel-P (for comparison) were packed into glass columns (15 × 0.5 cm I.D.) and stainless-steel columns (15 × 0.8 cm I.D.). The chromatograph included a Waters Assoc. 6000 p.s.i. pump (Model 510) and a Shodex refracto

monitor (SE-11). A homologous series of dextran and maltose in water and polystyrene in tetrahydrofuran was used as permeable substances. The calibration curves were obtained by plotting the average molecular weight against the peak elution volumes. The slope of the centre part of the curve is given by the equation[13]:

$$\log M = \beta - \alpha (V_e/V_t) \quad (1)$$

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. The excluded molecular weight, M_{lim}, was obtained by extrapolating the linear part of the log M *versus* V_e curve to $V_e = V_0$, where V_0 is outer volume of the gel bed.

Other measurements

Differential scanning calorimetry (DSC) of wet particles from which free water (or medium) is removed by centrifuging (200 *g*, 3 min), was carried out using a Seiko I & E SSC-580/DSC-10 instrument. The PMLG particles were sealed in an Al sample pan and DSC thermograms were obtained at a heating rate of 2°C min^{-1}.

The measurement of the degree of swelling (wet ml/dry ml) and solvent was carried out as previously reported[14]. Solvents used in the measurements were the same as those in the GPC operation.

RESULTS AND DISCUSSION

Polypeptide spheres

The polypeptide spheres are produced by the gradual evaporation of the solvent from the suspension (or emulsion) containing the polypeptide and the diluent. Consequently, the size of the spheres depends on the volume of the size of suspension and the concentration of polypeptide. These factors can be controlled by adjusting the initial concentrations of polypeptide and poly(vinyl alcohol) in the aqueous solution, and the stirring speed. In this study, they were controlled mainly by varying the concentration of the suspension media. For example, PMLG spheres with the average diameters of 44–105 μm and 5–10 μm were obtained from 2 wt.% and 5 wt.% of poly(vinyl alcohol) aqueous solution, respectively. As a result, we succeeded in preparing polypeptide spheres with average diameters of 5–300 μm. These sizes are suitable for chromatography.

Typical optical photographs and electron micrographs of PMLG and PBLG particles prepared by this procedure are shown in Fig. 1. The shape is spherical and uneven. This unevenness of the surface indicates partial crystallization of polypeptide in the sphering process from suspension. The existence of polypeptide crystals is indicated by the glittering observed under a polarization microscope.

The properties of PMLG spheres were investigated. These particles were insoluble in the usual chromatographic solvents without further treatment such as cross-linking: *e.g.*, water, methanol, ethanol, 2-propanol, acetonitrile, tetrahydrofuran, benzene, ether, hexane and hexane mixtures (but they swelled remarkably and because distorted in chloroform, 1,2-dichloroethane and dioxane). This insolubility is a characteristic property of polypeptides. Generally, polypeptides of random coil and α-helical form dissolve in polar solvents, but polypeptides with the β-structure

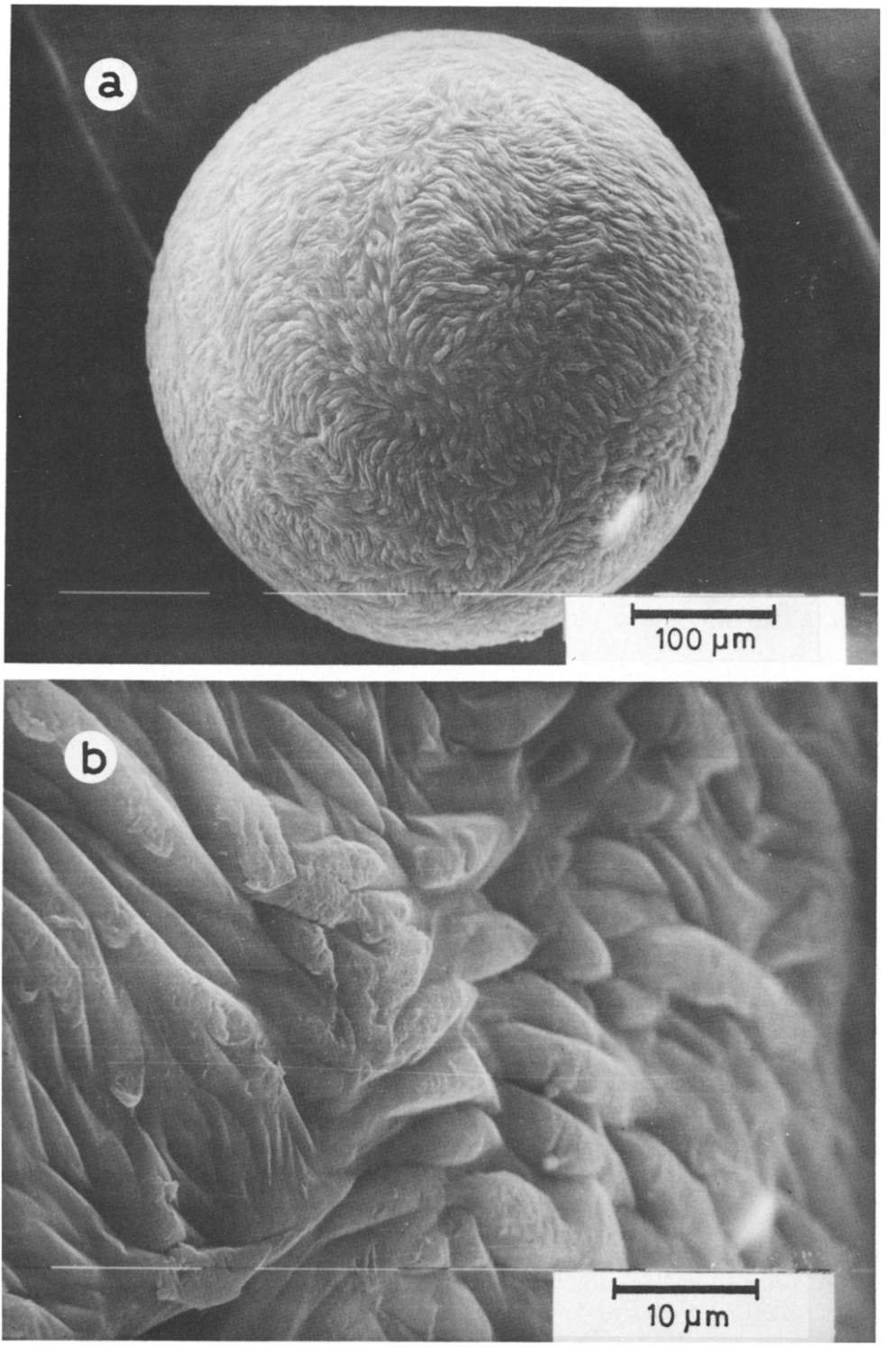


Fig. 1.

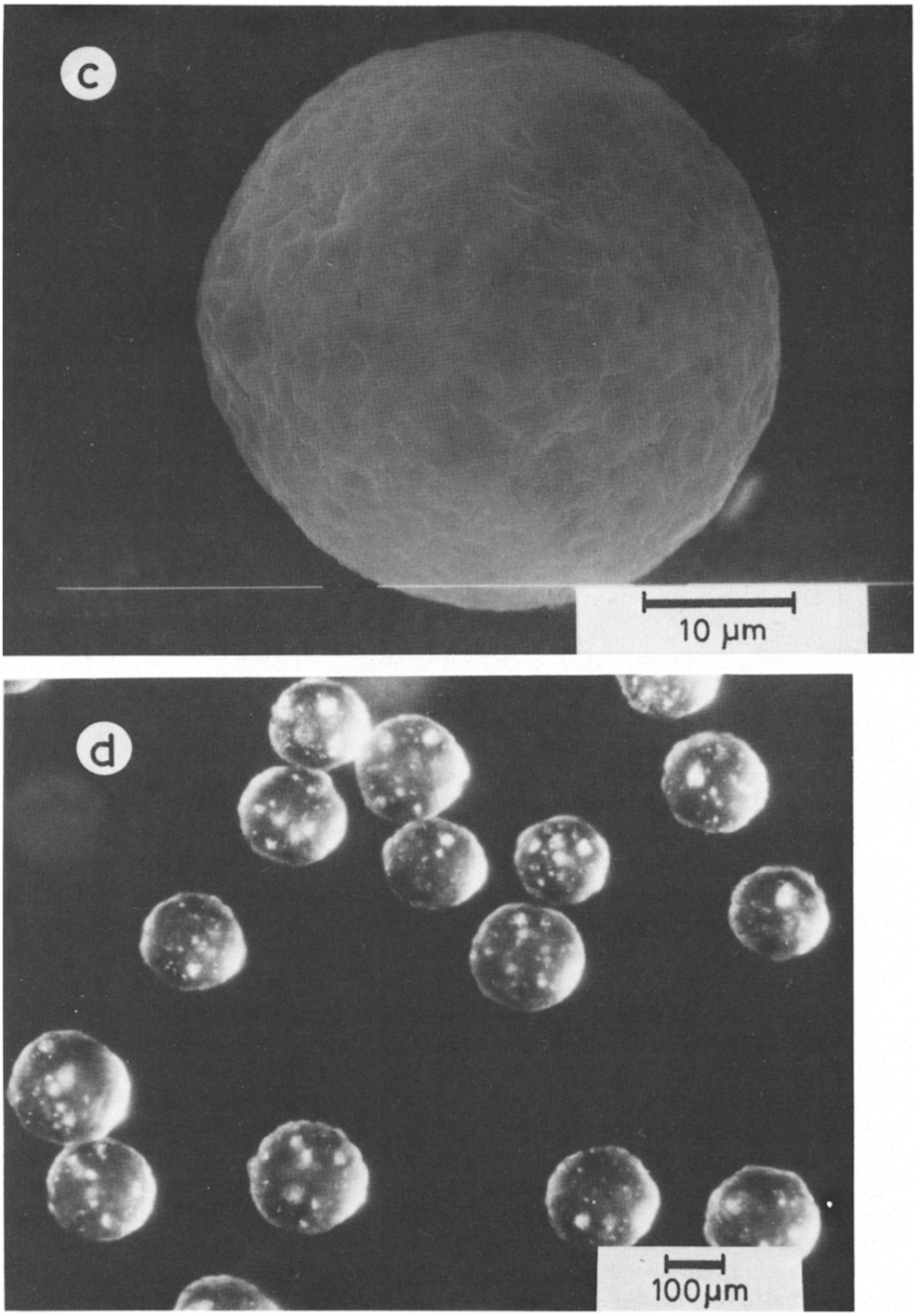


Fig. 1.

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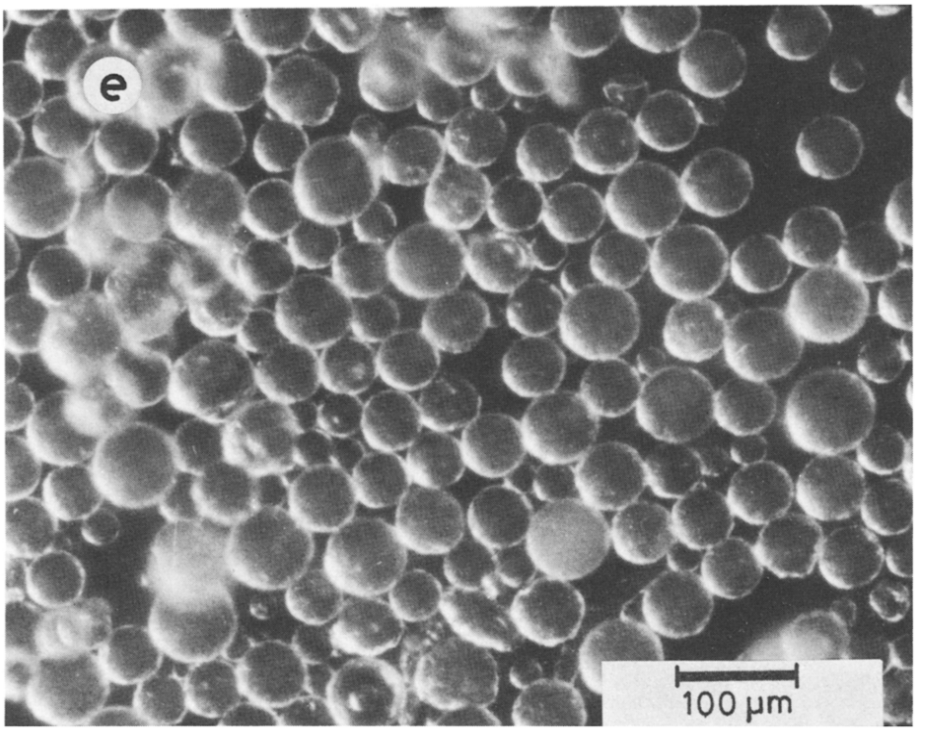


Fig. 1. Electron micrographs (a, b and c) and optical photographs (d and e) of polypeptide spheres: (a), (b) and (d) are pictures of non-porous PBLG spheres; (c) and (e) show non-cross-linked PMLG spheres B-13 and B-3, respectively.

are insoluble in most solvents because of strong intermolecular hydrogen bonding. This hydrogen bonding of the β-structure cannot be destroyed by the usual solvent[15]. As shown in Fig. 2, the production of β-structure was confirmed by IR spectroscopy. PMLG spheres showed absorption bands characteristic of the carbonyl group at 1685 cm^{-1} (anti-parallel β-structure) and 1630 cm^{-1} (parallel β-structure) as well as at 1650 cm^{-1} (α-helix)[16]. Thus, the insolubility of PMLG spheres is attributable to the formation of β-structure.

PMLG spheres were cross-linked as required by transesterification with diol. The resulting particles show different solubility from non-cross-linked particles, although the exact degree of cross-linking is difficult to determine because of the contamination of mono-substituent. The cross-linked particles are insoluble even in hot chloroform (Table I). The insolubility of the cross-linked particles seems to be unrelated to β-structure, because the absorption bands due to β-structures decreased or disappeared (*cf.* Fig. 2c). The β-structure may have disappeared in the cross-linking process because the cross-linking reaction was carried out in a chloroform mixture, which is predisposed to the α-helix form[15].

The cross-linking process is significant not only because the spheres are insolubilized, but also because the general properties are altered. For example, the PMLG spheres became more hydrophilic, after being cross-linked by poly(ethylene glycol). The exclusion molecular weight and the porosity became very small, after complete

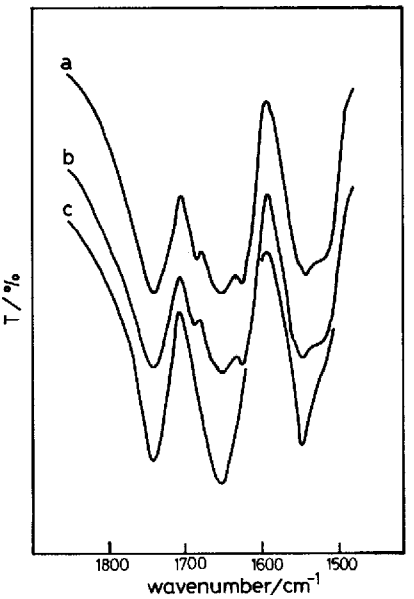


Fig. 2. IR spectra of PMLG spheres: (a) material PMLG (powder after reprecipitation from methanol); (b) porous PMLG spheres (B-9); (c) cross-linked PMLG spheres (C).

cross-linking by an equimolar solution of ethylene glycol for the methoxycarbonyl group of PMLG. Therefore, the GPC ability of the PMLG spheres was examined without prior cross-linking treatment, unless stated otherwise.

Gel permeation chromatography in aqueous solution

The chromatographic properties were investigated using PMLG spheres with diameters of 25–44 μm because of the ease of packing into a column. We examined PMLG particles that were sphered in the absence or the presence of a diluent, *e.g.* decahydronaphthalene or diethylbenzene. Fig. 3 shows typical calibration graphs for GPC in aqueous solution. Table II summarizes the exclusion molecular weights (M_{lim}) and porosities, which were estimated from the graphs of Fig. 3 and some physical properties of PMLG spheres. The porosity was determined[8,9] from eqn. 2:

TABLE I

DEGREE OF SWELLING (*Sd*) OF PMLG SPHERES

Sphere B-12 was prepared using 100% of decahydronaphthalene; sphere C was cross-linked using triethylene glycol.

Sphere No.	*Cross-linking*	*Porosity (%)*	*Sd (wet ml/dry ml)*			
			Water	*Ethanol*	*Chloroform*	*Hexane*
A	No	12	1.0	1.2	Distorted*	1.0
B-12	No	61	2.1	2.4	Distorted*	2.6
C	Yes	60	2.4	2.8	3.1	2.0

* Remarkably large degree of swelling.

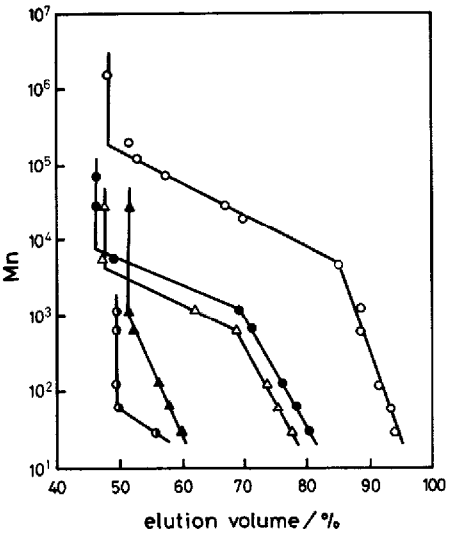


Fig. 3. Typical calibration curves of GPC for PMLG spheres in aqueous solution: ○ = B-15; ● = B-12; △ = B-10; ▲ = B-2; ◑ = A.

TABLE II

VALUES OF M_{lim}, POROSITY AND *Sd* OF PMLG SPHERES IN AQUEOUS SOLUTION

Sphere No.	*Diluent*		*Sd for water (wet ml/dry ml)*	M_{lim} *for dextran*	*Porosity (%)*
	Type	*wt.%*			
A	None	0	1.0	60	12
B-1	Butyl acetate	100	1.1	100	19
B-2	1-Hexanol	100	1.0	1000	16
B-3	1-Octanol	100	1.2	1000	37
B-4	1-Octanol	300	1.3	10 000	59
B-5	Toluene	100	1.1	150	14
B-6	*o*-Xylene	100	1.2	150	17
B-7	*m*-Xylene	100	1.2	150	32
B-8	*p*-Xylene	100	1.2	150	20
B-9	Diethylbenzene	100	1.5	6000	60
B-10	Diethylbenzene	200	1.8	6000	55
B-11	Diethylbenzene	300	1.7	6500	60
B-12	Decahydro-naphthalene	100	1.9	8100	61
B-13	Decahydro-naphthalene	200	2.1	40 000	74
B-14	Decahydro-naphthalene	300	2.1	120 000	83
B-15	Methyl dode-canoate	100	2.1	200 000–2 000 000	82

$$\text{Porosity} = \frac{V_{^2H_2O} - V_0}{V_t - V_0} \times 100\ (\%) \quad (2)$$

where $V_{^2H_2O}$ is the elution volume of ^2H_2O.

The type and the amount of a diluent directly affect the value of M_{lim} and the porosity. In the absence of a diluent, these values are only 60 and 12%, respectively. These spheres can be assumed to be non-porous, in contrast to the triacetates of cellulose[9,10] and pullulan[17], which have porosities of 40% and 90% even in the absence of a diluent. This is probably due to the strong cohesion of PMLG in the sphering process.

The effects of a diluent are remarkable in decahydronaphthalene, diethylbenzene, 1-octanol and methyl dodecanoate. For example, when 100–300% of decahydronaphthalene was used as a diluent, M_{lim} and porosity increased from 8100 to 120 000 and from 61% to 83%, respectively. The largest value of M_{lim}, 10^6, was obtained by the use of methyl dodecanoate, although values of M_{lim} were difficult to reproduce. In any case, the role of methyl dodecanoate as a diluent should be clarified by further studies because the diluent effects are surprising and significant.

On the contrary, when the diluent is butyl acetate, 1-hexanol, toluene or xylene, the effect is scarcely observable. These solvents increase the value M_{lim} and the porosity to some degree in the case of the sphering of cellulose triacetate[9,10]. This indicates that the PMLG spheres are reticulated only by specific diluents because of the strong cohesion effect.

The different diluents produce different effects on the degree of swelling of PMLG spheres. The non-porous spheres did not swell in water ($Sd = 1$), but the porous spheres prepared with the use of decahydronaphthalene doubled in size on swelling. In contrast, in the case of porous spheres prepared with the use of 1-octanol the value of Sd is only 1.3. Therefore, it is clear that two different types of sphere, swelling gel-type and permanent porous resin-type, can be produced. However, the formation mechanism is not yet clear.

As a result, we succeeded in the preparation of non-porous and porous PMLG spheres with a series of M_{lim} values from 10^3 to 10^6. These values are suitable to GPC.

Amphiphilicity

Fig. 4 shows calibration graphs for GPC in water and tetrahydrofuran. As shown in figure, PMLG spheres give typical GPC behaviour even in the organic system, and the calibration pattern is closely similar to that in the aqueous system. This indicates that PMLG spheres are amphiphilic and the reticular structure does not change even in organic systems.

This amphiphilicity was shown calorimetrically. As shown in Fig. 5, the melting behaviour of solvent containing in the spheres was examined by DSC. The DSC thermogram of the PMLG spheres containing water gives only a melting peak due to water (Fig. 5a). When these particles were washed with methanol in a column for 10 min, this peak disappeared (Fig. 5b). After the particles were washed with benzene, a peak due to benzene was observed (Fig. 5c). Even when water was replaced by dioxane, such peak-transfer was observed. Thus it is clear that the PMLG spheres are chromatographically amphiphilic also.

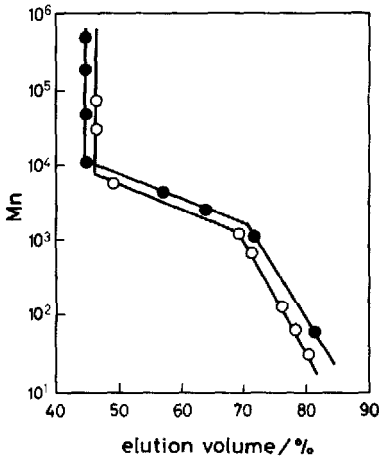
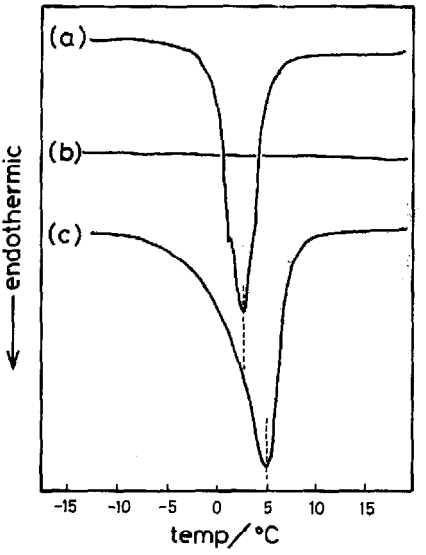


Fig. 4. Calibration graphs of GPC of PMLG sphere B-12 in tetrahydrofuran and water: ● = tetrahydrofuran; ○ = water.

Flow-rate properties

The PMLG spheres are insoluble in the usual chromatographic solvents. This property produces a remarkably high flow-rate for the chromatographic process. Fig. 6 shows the relationship between the pressure drop and the flow-rate for the PMLG spheres prepared using 100% of decahydronaphthalene as a diluent. The PMLG spheres easily reached 30 ml min^{-1} cm^{-2} (corresponding to *ca.* 6 ml min^{-1} at 5 mm I.D.)



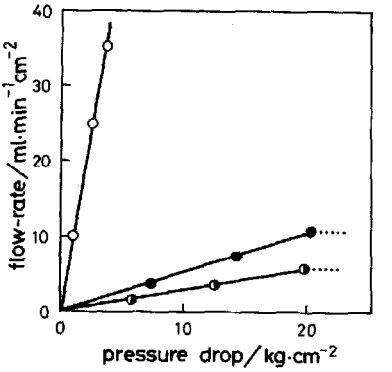


Fig. 5. DSC thermograms of PMLG spheres containing the medium for GPC: (a) water; (b) methanol; (c) benzene.

Fig. 6. Relationship between flow-rate and pressure drop in aqueous solution: ○ = PMLG spheres (B-12, size 25–44 μm, M_{lim} 8100); ● = Sephadex G-25 (size 44–105 μm, M_{lim} 5000); ◑ = Sephadex G-50 (size 44–105 μm, M_{lim} 10 000).

in water, but the corresponding commercial packings reached a limit at 2 ml min^{-1} (at 5 mm I.D.). This lower pressure-resistance of the commercial products is caused by their large degree of swelling. The PMLG spheres are rigid and the degree of swelling is small, regardless of whether they are cross-linked.

CONCLUSION

Porous spheres from synthetic polypeptides alone were prepared for the first time by our unique method. The particle size, the porosity and the pore size are controlled with ease by controlling the preparation conditions. The polypeptide spheres obtained are very rigid, insoluble in most chromatographic solvents and tolerate high flow-rates without further treatment.

If the spheres are cross-linked by transesterification with diol compounds the solvent-resistance increases.

In addition, it was confirmed that the spheres can be used in both aqueous and organic systems for GPC, and the eluent replacement was very easy.

These properties are attributable to the fact that the polypeptide shows amphiphilicity and insolubility, due to the formation specific conformations such as β-structure and α-helix.

Research into the application of polypeptide spheres to high-performance liquid chromatography has just begun, and we shall investigate further modifications and applications to affinity chromatography for biochemical and medical research as well as GPC.

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