

## Preparation of Porous Spherical Poly( $\gamma$ -methyl L-glutamate) Particles Containing $\beta$ -Structure

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**ABSTRACT:** Porous poly( $\gamma$ -methyl L-glutamate) spherical particles underwent transition  $\alpha$ -helix-to- $\beta$ -structure in the main chain as a result of heat treatment in water or methanol. The particles obtained maintained their sphericity and characteristic porosity after the heat treatment, while the transition increased insolubility against organic solvents. The amount of  $\beta$ -structure depended on the temperature. The transition in water included hydrolysis of the residual carbomethoxy groups. Adsorption capacity of the particles for cytochrome C depended remarkably on the content of  $\beta$ -structure as well as the amount of carboxyl groups produced.

**KEY WORDS** L-Glutamate /  $\alpha$ -Helix /  $\beta$ -Structure / Liquid Chromatography  
/ Adsorption / Cytocrome C /

Synthetic polypeptides have been utilized as functional polymer materials for high performance liquid chromatography. For example, it is known that resin-supported poly(L-leucine)<sup>1</sup> and poly( $\gamma$ -benzyl L-glutamine)<sup>2</sup> show enantioselective retention behaviors for amino acid derivatives. We have reported that porous spherical particles prepared from poly( $\gamma$ -methyl L-glutamine) (PMLG) alone produced  $\alpha$ -helical conformation in the main chain and showed unique affinities (*e.g.*, for proteins and endotoxins) in the liquid chromatography.<sup>3-7</sup> It is assumed that these affinities of the polypeptides are closely related to their secondary structures as well as their primary structures. Therefore, it is expected that the mode of affinity can be varied by controlling the secondary structures. On the other hand, it is known that  $\beta$ -structure formation was effectively formed in poly( $\alpha$ -amino acid) films under formic acid vapor.<sup>8,9</sup> This transition occurs due to their partial solubilization and

following reprecipitation. If this method is used for  $\beta$ -structure formation in porous spherical particles, their original morphology and porosity also will be modified. Therefore, it is necessary to develop a new approach for  $\beta$ -structure formation. In this paper, we report that porous and spherical PMLG particles undergo an  $\alpha$ -helix-to- $\beta$ -structure transition in the main chain with heat treatment in water or methanol. This procedure assures their sphericity and porosity. In addition, it was observed that with transition in water, hydrolysis of residual carbomethoxy groups occurred which showed an adsorption capacity for cytochrome C different from that of the  $\alpha$ -helix-rich particles.

### EXPERIMENTAL

PMG F-8000 (a degree of polymerization of 1000, manufactured by Ajinomoto Co., Ltd.) was used as a material poly( $\gamma$ -methyl L-

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glutamate). Porous PMLG spherical particles were prepared by the *suspension evaporation* method reported previously.<sup>10</sup> Particles with average diameters of 44–105  $\mu\text{m}$  were obtained by adjusting the stirring speed and sieving. Di(2-ethylhexyl)phthalate was used as a diluent for macroreticulation<sup>4</sup> in the sphering process. The porous PMLG particles obtained were packed into a stainless steel column (15  $\times$  0.46 cm i.d.) with water. The calibration curves of the column in size exclusion chromatography (SEC) were determined using pullulan standards, and the porosity and exclusion molecular weight ( $M_{\text{lim}}$ ) of the column were determined as 67% and  $5 \times 10^4$ , respectively, using these calibration curves.<sup>11</sup> The porosity corresponds to the elution volume of  $\text{D}_2\text{O}$  which is the smallest molecule permeable within particles. The  $M_{\text{lim}}$  corresponds to the molecular weight of the largest molecule (pullulan) within the particles.

Adsorption for cytochrome *C* was examined by a batch method as follows: PMLG particles were washed and equilibrated in 0.02 M-phosphate buffer (pH 7.0,  $\mu=0.05$ ). One ml of the wet particles was diluted in buffer solution to 5 ml. Five ml of cytochrome *C* solution (10  $\text{mg l}^{-1}$ , 0.02 M-phosphate buffer, pH 7.0,  $\mu=0.05$ ) was added to the PMLG suspension. The suspension was shaken at 25°C for 2 h and filtrated through a Millipore filter (0.8  $\mu\text{m}$ ) in order to remove the particles. The remaining cytochrome *C* concentration of the filtrate was

determined using a Shimadzu UV160-A spectrophotometer.

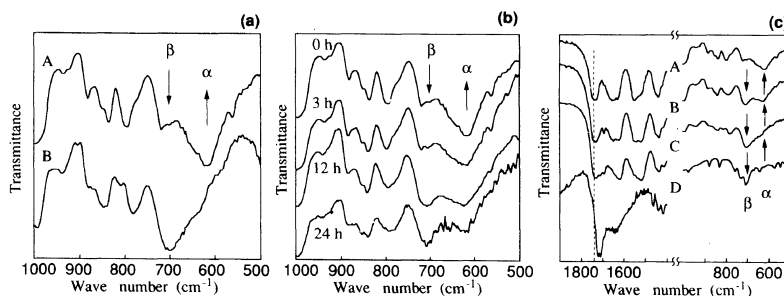
The swelling degree ( $S_d$ , wet-ml dry  $\cdot \text{g}^{-1}$ ) of the PMLG particles was determined as follows: PMLG particles were suspended in a methanol-dioxane mixture. After degassing, the suspension was aged at 25°C for 12 h.  $S_d$  is the amount of the wet volume divided by the weight of particles dried *in vacuo*.

Electron microscopic observations were performed without drying or staining PMLG particles, using a JEOL JSM-5400 LV. DSC thermograms were obtained using a heating rate of 2°C  $\text{min}^{-1}$  by a Seiko I & E SSC-580 with a DSC-10 instrument. FT-IR spectroscopy was carried out using a Perkin-Elmer 1640 IR spectrophotometer.

## RESULTS AND DISCUSSION

### *Thermally Induced Phase Transition in Methanol*

The PMLG particles ( $M_{\text{lim}}$  50000, porosity 65%) showed an IR spectrum with absorption band characteristic of amide V at 620  $\text{cm}^{-1}$  (Figure 1a). This indicates that the PMLG particles are mainly composed of  $\alpha$ -helical conformation in the main chain. When the PMLG particles were suspended in methanol and incubated at 220°C for 70 min, IR spectra showed significant change. As shown in Figure 1a, absorption at 620  $\text{cm}^{-1}$  disappeared but reappeared at 700  $\text{cm}^{-1}$ . This new band is



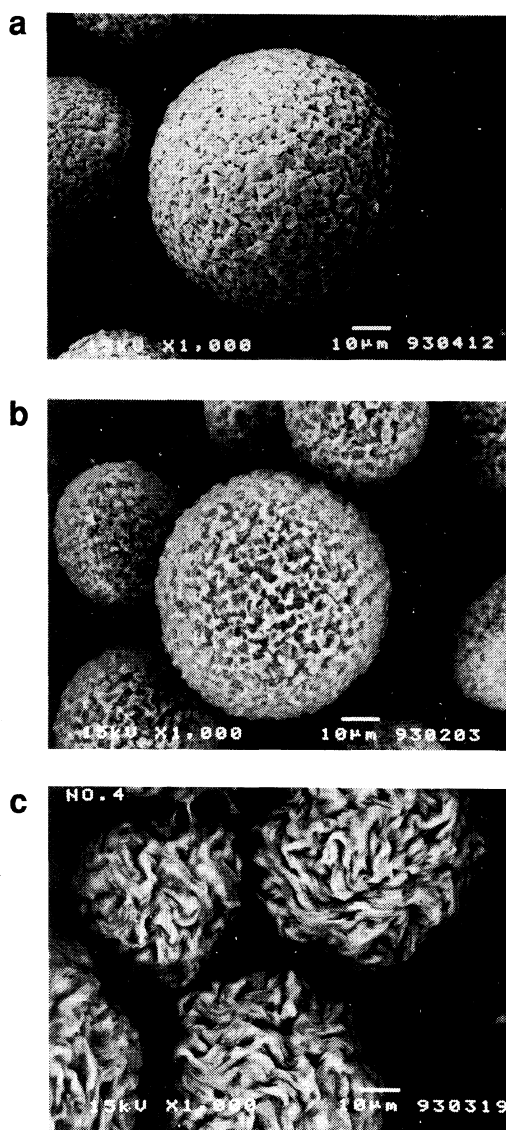
**Figure 1.** FT-IR spectra of spherical PMLG particles. (a) Before (A) and after (B) heat treatment at 220°C for 70 min in methanol. (b) The particles exposed to formic acid vapor at 30°C for given time. (c) The particles (B, C, and D) heated at 220°C for given time in water.

ascribed to  $\beta$ -structural amide V. Such transition was not observed either in incubation with methanol at room temperature or without methanol at 220°C.

$\beta$ -Structure formation is triggered by formic acid vapor treatment in poly( $\alpha$ -amino acid) films.<sup>8,9</sup> In this study, similar  $\beta$ -structure formation in the PMLG particles was observed by incubating at 30°C under formic acid vapor and washing with methanol. As shown in Figure 1b, the  $\alpha$ -helix-to- $\beta$ -structure transition progressed with increase of treatment time. However, the formic acid vapor treatment produced undesirable modifications in particle properties. First, the sphericity disappeared as shown in an electron micrograph (Figure 2c). Secondly, both the  $M_{lim}$  and porosity decreased remarkably (Figure 3b). These modifications cause lowering of column efficiency in their liquid chromatography use. On the contrary, the PMLG particles after heat treatment in methanol showed no significant change in sphericity (Figure 2b) and the  $M_{lim}$  and porosity were almost completely maintained (Figure 3a). This difference between the two methods is explained as follows: the transition is induced by lowering of hydrogen bonding interaction between amide bondings. Formic acid strongly prevents this interaction, causing partial solubilization with  $\alpha$ -helix-to-random coil transition. The removal of formic acid reprecipitates PMLG and is accompanied by random coil-to- $\beta$ -structure transition. During this process, the PMLG particles shrink remarkably. On the other hand, the heat treatment alone in the presence of methanol reduces hydrogen bonding interactions (most likely due to the increasing temperature). The increase of molecular motion due to lowering of hydrogen bonding interaction promotes the transition to stable conformation ( $\beta$ -structure).

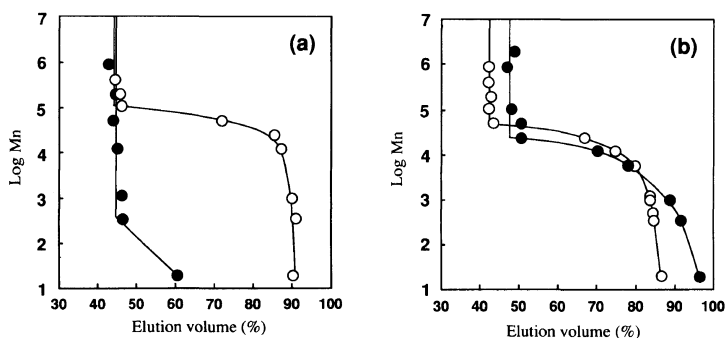
#### *Thermally Induced Phase Transition in Water*

Differential scanning calorimetry (DSC) was carried out using the porous PMLG particles dispersed in water. As shown in Figure 4, this

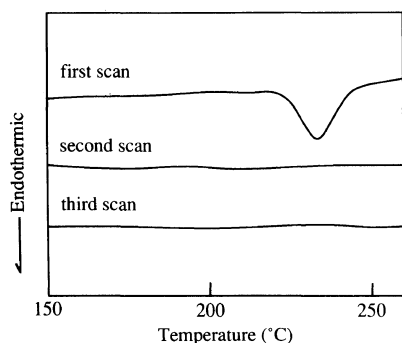


**Figure 2.** Scanning electron micrographs of PMLG particles under vacuum condition (0.6 torr). Scale bars indicate 10  $\mu$ m. (a) Non-treated. (b) Heated at 220°C for 70 min in methanol. (c) Exposed to formic acid vapor at 30°C for 24 h.

dispersion provided a DSC thermogram with an endothermic peak at temperatures between 190 and 240°C (peak-top temperature,  $T_c = 220^\circ\text{C}$ ). This peak was not observed in the second or third scans. Therefore, this phase transition is thermally irreversible. The IR



**Figure 3.** Calibration curves of columns packed with PMLG particles in aqueous SEC. (a): ○, non-treated; ●, exposed to formic acid vapor at 30°C, 24 h. (b): ○, non-treated; ●, heated at 220°C, 70 min in methanol.



**Figure 4.** DSC thermograms of PMLG particles in water.

spectra of the particles showed two significant changes after treatment at 220°C for 70 min. First, the absorption of  $1740\text{ cm}^{-1}$  decreased while that of  $1710\text{ cm}^{-1}$  increased (Figure 1c). By further treatment at 220°C, the particles swelled remarkably and increased in transparency. The IR spectra of the resulting product showed that the absorption of  $1740\text{ cm}^{-1}$  completely disappeared (Figure 1c). pH-titration of the products showed a cation-exchange capacity of  $6.99\text{ meq g}^{-1}$ . This value corresponds to 100% of the residual carboxylic groups in poly(L-glutamic acid). These results indicate that the residual carbomethoxy groups of PMLG were hydrolyzed due to the heat treatment in water. Secondly, a significant spectral change was observed at about  $600\text{--}800\text{ cm}^{-1}$ , assigned to amide V (Figure 1c). This shows that the conversion includes

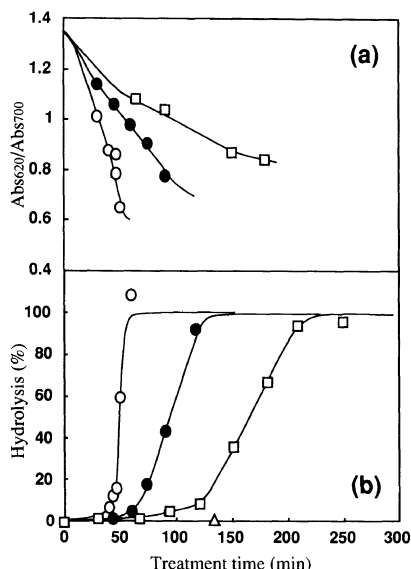
$\alpha$ -helix-to- $\beta$ -structure transition. Therefore, the endothermic peak in the DSC includes the conformation change in the main chain as well as the hydrolysis of the side groups.

The partially hydrolyzed PMLG particles in water at 220°C were readily esterified in methanol containing sulfuric acid.<sup>12</sup> The IR spectra showed no significant spectral change in the conformation of the main chain: the  $\beta$ -structure was maintained after esterification. In addition, the SEC calibration curve of the column with the esterified particles provided porosity (64%) and  $M_{\text{lim}}$  ( $3 \times 10^4$ ) similar to the original PMLG particles.

#### *Time Courses of Transition in Water*

Figure 5a shows time courses of the  $\alpha$ -helix-to- $\beta$ -structure transition in water at various treatment temperatures. The degree of the transition was estimated by the absorbance ratio based on the amide V absorptions. As shown in Figure 5a, the rate and degree of the transition increased with increase of temperature.

Figure 5b shows that the heat treatment in water was accompanied by hydrolysis of residual groups. The degree of hydrolysis was estimated by pH-titration of resulting products. Figure 5b shows that the conversion was composed of slow and rapid hydrolysis processes and the rapid hydrolysis occurred after the conformational change. The lag time



**Figure 5.** Time courses of (a) conformation change (the absorbance ratio at 700 to 620 cm<sup>-1</sup>) and (b) conversion (hydrolysis) of PMLG particles in water. ○, Treatment at 270°C; ●, Treatment at 240°C; □, Treatment at 200°C; △, Treatment at 150°C.

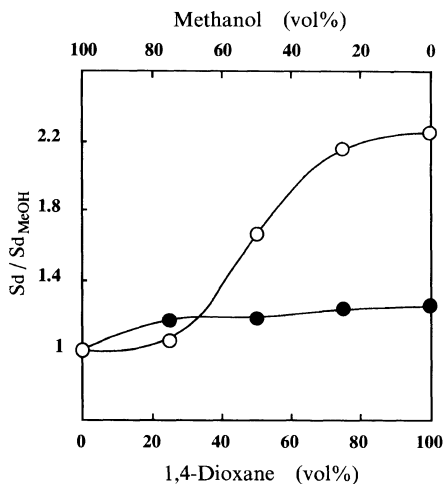
up to the rapid hydrolysis was strongly dependent on the treatment temperatures.

On the other hand, the hydrolysis of the residual groups proceeded readily in an aqueous NaOH solution. In this case, there was no observable  $\alpha$ -helix-to- $\beta$ -structure transition. The DSC thermogram showed no significant endothermic peak.

These results indicate that the peptide moieties partially hydrolyzed residual groups by hydroxyl ions no longer undergo  $\beta$ -structure formation in the main chain. Therefore,  $\alpha$ -helix-to- $\beta$ -structure transition is preferable at higher temperatures (for example, at 220°C), as the treatment at lower temperatures (for example, at room temperature) causes only slow hydrolysis of residual groups without  $\beta$ -structure formation.

#### *Effect of the Conformational Change*

The degree of swelling ( $S_d$ ) was compared in PMLG particles with and without heat treatment in methanol. Figure 6 shows the

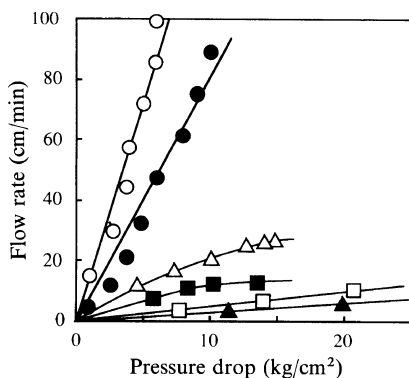


**Figure 6.** Degree of swelling for PMLG particles. ○, non-treated; ●, heated at 220°C, 70 min in methanol.

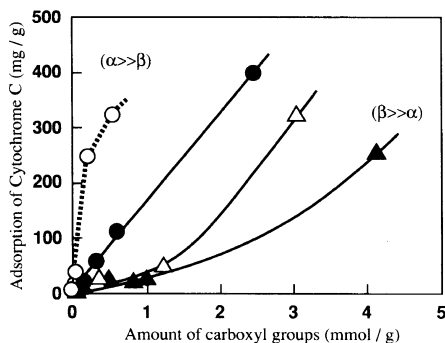
relationship between the composition of solvents and  $S_d$ . It is clear that the particles with heat treatment maintain small and constant  $S_d$  values independent of the composition. This is attributable to the fact that  $\beta$ -structural conformation based on intermolecular hydrogen bonding shows a cross-linking effect, while  $\alpha$ -helical conformation is based on intramolecular interaction.

A constant degree of swelling in particles can be an important characteristics for their chromatography use. PMLG particles were packed into a stainless steel column and their flow-rate properties were examined. As shown in Figure 7, the column with  $\beta$ -structure-rich particles showed a smaller pressure drop in the plots of the flow-rate vs. the pressure drop than the column with the  $\alpha$ -helix-rich particles. This high flow-rate resistance is also attributed to crosslinking effects due to  $\beta$ -structural conformation.

Such crosslinking effects are especially important for partially hydrolyzed particles. For example, particles (prepared by hydrolysis with aqueous NaOH) containing 3.0 meq g<sup>-1</sup> of carboxyl groups were completely dissolved in water at pH 11. However, the particles (prepared by heat treatment) containing



**Figure 7.** Relationship between the flow-rate and the pressure drop in the aqueous SEC. Column: spherical particles with diameters of 44 to 105  $\mu\text{m}$  were packed into a 0.46  $\times$  15 cm stainless steel column. Mobile phase, water;  $\circ$ , PMLG particles with heat treatment at 220°C, 70 min in methanol;  $\bullet$ , PMLG particles without heat treatment;  $\triangle$ , cellulose gel ( $M_{lim}=800000$ );  $\blacktriangle$ , dextran gel (Sephadex G-50,  $M_{lim}=10000$ );  $\square$ , dextran gel (Sephadex G-25,  $M_{lim}=5000$ );  $\blacksquare$ , dextran gel (Sephacryl S-400,  $M_{lim}=500000$ ).



**Figure 8.** Effect of carboxyl groups on the adsorption of cytochrome C.  $\circ$ , treated with NaOH aq at 45°C;  $\bullet$ , treated at 200°C in water;  $\triangle$ , treated at 240°C in water;  $\blacktriangle$ , treated at 270°C in water.

4.0 meq g<sup>-1</sup> of carboxyl groups maintained their spherical morphology under the same condition.

It was also clarified that the formation of  $\beta$ -structure altered the adsorption capacity in chromatography use. Figure 8 shows the relationship between the amount of produced carboxyl groups and the adsorption capacity for a basic protein, cytochrome C. Due to increased production of carboxyl groups, the

adsorption capacity for cytochrome C increased, as the adsorption included ionic interaction. However, the adsorption capacity decreased with increase of the content of  $\beta$ -structure. These results indicate that the formation of  $\beta$ -structure influences the dissociation of residual carboxyl groups because of their highly-oriented structure.

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12. The estrification of partially hydrolyzed PMLG particles was performed as follows: 5 wet-ml of the particles with 0.91 mmol g<sup>-1</sup> of carboxyl groups were suspended in 100 ml of methanol containing of 45 mg of sulfuric acid. The mixture was stirred at 60°C for 48 h, then the complete disappearance of carboxyl groups was confirmed by IR spectroscopy.