Aggregation of Polypeptide-Based Amphiphiles in Water

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ABSTRACT: The amphiphile, poly(γ -methyl L-glutamate) with a phosphate head group at the terminal (PMG₁₀-P) was prepared. PMG₁₀-P was dispersed in aqueous solution to form a globular assembly after sonication. One hour later, globular assemblies had joined each other to transform into fibrous aggregates. At 24 h, the fibrils assembled to form twisted ribbon-like aggregate. The thickness of the ribbon-like sheet was *ca.* 4.0 nm which is compatible with the chain length of PMG₁₀-P when in the β -form conformation. The β -form conformation of PMG₁₀-P was confirmed by circular dichroism and fourier transform infrared spectrometry (FT-IR) measurements. The ribbon-like aggregate is β_1 at room temperature. The phase transition from β_1 to β_{II} above *ca.* 35°C was confirmed by differential scanning calorimetry (DSC) measurements. Further increase in temperature induced the disaggregation of PMG₁₀-P whose conformation is in random coil with some α -helix. This conformational behavior is summarized in a temperature-concentration phase diagram.

KEY WORDS Amphiphilic Polypeptide / Ribbon-Like Molecular Assembly / β -Form / Phase Transition / Temperature Concentration Diagram/

In the organization of a cell, the lipid bilayer is a very important structure.¹ The double layer structure composed of lipid molecule forms spontaneously in water.² Kunitake *et al.* demonstrated the formation of a synthetic bilayer membrane and vesicle constructed with dodecyltrimethylammonium bromide,³ with similar characteristics to lipid bilayers. Their discovery led to studies on molecular assemblies and various of artificial amphiphilic molecules have been prepared.^{4–17} These molecules form not only bilayer structures comparable to the lipid bilayer but also another superstructures in water.^{4–13} A synthetic amphiphile containing an amino acid residue aggregates to form the helical and/or cylindrical structure.^{4–17} These structures are well-defined. The interior of assembly may be rather statistical, because these assemblies are composed by amphiphiles containing hydrocarbons as a hydrophobic part. There are a few studies on amphiphiles consisting of non-hydrocarbon tails.^{18–25} In order to construct a molecular assembly having well-defined structure, peptide derivatives may be a useful.

In this study, we prepared an amphiphile, poly(γ -methyl L-glutamate) with a phosphate head group at the terminal (PMG₁₀-P), containing polypeptide as the hydrophobic part instead of hydrocarbons and studied aggregation in aqueous solution and also temperature and concentration effects on PMG₁₀-P aggregates.

EXPERIMENTAL

Sample Preparation

The amphiphile, poly(γ -methyl L-glutamate) with a phosphate moiety at the terminal (PMG_n-P; Scheme 1), was obtained by the polymerization of the *N*-carboxy anhydride of L-glutamic acid γ -methyl ester (MG-NCA) with O-phosphorylethanolamine as initiator. MG-NCA (1.0 g) was dissolved in ethyl acetate (50 mL; Katayama

Chemical Co., Ltd., Japan). The solution was added dropwise to aqueous solution (pH 9.0) containing the initiator and the mixture was vigorously stirred for two hours at room temperature. The mixture was evaporated at 60°C to give a white powder. The white powder was washed with water several times and dried *in vacuo*. Gel permeation chromatographic (GPC) analysis of the sample obtained was performed using a G 4000 HXL column (Tosoh Ltd., Japan) and *N,N*-dimethylformamide as elution solvent. The highly-purified sample was confirmed by a single peak in the GPC trace. Average degree of polymerization, *n*, of 10 (PMG₁₀-P; $M_n = 1571$) was estimated from ¹H NMR analysis of trifluoroacetic acid solution of the polypeptide. The distribution of molecular weight ($\overline{M}_w/\overline{M}_n$) of PMG₁₀-P, 1.10, was found by GPC analysis.

Aqueous solutions of PMG₁₀-P for various measurements below were prepared by sonication with the probe type sonifier (Biomic model 7250 B, SEIKO I&E Co. Ltd., Japan) at 40 W and 0°C for 1–2 min.

Circular Dichroism (CD) Spectroscopy

Circular dichroism (CD) spectra of PMG₁₀-P in aque-

Scheme 1. Chemical structure of the amphiphilic polypeptide (PMG_n-P).

ous solution at several temperatures were recorded on a JASCO J-820 K equipped with temperature control system (PTC-423 L). CD spectra of the PMG₁₀-P aqueous solution were expressed in terms of molar ellipticity, $[\theta]$, based on the residue molecular weight.

Transmission Electron Microscopy (TEM)

The morphology of the PMG₁₀-P assembly in aqueous solution was observed by transmission electron microscopy (TEM) on a JEOL-100 CXII. Samples of TEM observation were prepared by applying a drop of PMG₁₀-P aqueous solution (6.4×10^{-5} M) onto carbon-coated 400 mesh copper grids, which were then negatively stained by the dropwise addition of 2% aqueous ammonium molybdate.

Fourier Transform Infrared Spectroscopy (FT-IR)

The FT-IR spectrum of PMG₁₀-P cast film from the aqueous solution (6.4×10^{-5} M) was measured by a Perkin-Elmer Spectrum 2000.

Transmittance Measurements

To estimate the aggregation of PMG₁₀-P in aqueous solution, we measured the transmittance ($T\%$) at 700 nm on spectrophotometer (JASCO, UVDEC-670). When the concentration of PMG₁₀-P aqueous solution is high, the formation of an assembly induces decrease in transmittance owing to scattering of light in solution. Conversely, the transmittance of the PMG₁₀-P aqueous solution was similar to that of pure solvent at low concentration in which the assembly did not form.

Fluorescence Polarization Measurements

Polarization, $P = (I_{//} - I_{\perp}) / (I_{//} + I_{\perp})$, of diphenylhexatrien (DPH) in the PMG₁₀-P assembly was determined with a fluorescence polarization spectrophotometer FS-501 A (Union Giken). $I_{//}$ and $I_{\perp}$ are fluorescence intensities polarized parallel and perpendicularly to the direction of polarized excitation beam. The concentration of PMG₁₀-P was 6.4×10^{-5} M. The concentration of DPH was 3.4×10^{-6} M. The excitation wavelength of DPH was 375 nm, and fluorescence was observed at 430 nm.

Differential Scanning Calorimeter (DSC)

The DSC curve was obtained for PMG₁₀-P solution with a differential scanning calorimeter DSC 7 (Perkin-Elmer Co., Ltd., Japan). 60 μ L of aqueous solution of PMG₁₀-P (6.4×10^{-5} M) was sealed in a silver sample pan. The pan was placed in the DSC cell compartment and cooled to ca. 0°C. Measurements were started from 10°C, and temperature rose at a rate of 5°C min⁻¹ up to 50°C.

RESULTS AND DISCUSSION

Secondary Structure of PMG₁₀-P in Aqueous Solution

To confirm the conformation of PMG₁₀-P in aqueous solution, CD spectra of PMG₁₀-P were obtained at room temperature (Figure 1). The CD spectra were strongly dependent on PMG₁₀-P concentration. The CD spectrum of PMG₁₀-P at 1.0×10^{-4} M (Figure 1a) exhibited a positive band at 198 nm and negative band at 218 nm, respectively, indicating the polypeptide to be in the β -form

Figure 1. CD spectra of PMG₁₀-P in aqueous solution. Concentration of PMG₁₀-P (a) 1.0×10^{-4} M, (b) 3.0×10^{-5} M, and (c) 1.0×10^{-5} M.

Figure 2. Plots of fluorescence intensity of pyrene as a function of the concentration of PMG₁₀-P.

conformation at 1.0×10^{-4} M. At PMG₁₀-P of 1.0×10^{-5} M the spectrum (Figure 1c) showed a CD pattern indicating the polypeptide to consist of random coil with same α -helix structure. The CD spectrum at 3.0×10^{-5} M (Figure 1b) was between the spectra of Figure 1a and 1c. The amphiphilic polypeptide, PMG₁₀-P, had a critical aggregate concentration (CAC) similar to other amphiphiles. We used fluorescence²⁶ with pyrene as probe for CAC determination, as a result, CAC of PMG₁₀-P was estimated to be 2.3×10^{-5} M by the concentration dependence of the fluorescence intensity of pyrene in the aqueous system (Figure 2). At 1.0×10^{-4} M above CAC, PMG₁₀-P aggregates. This intermolecular interaction may induce β -sheet conformation (Figure 1a). PMG₁₀-P dispersed individually at 1.0×10^{-5} M below the CAC to exist in the random coil rich conformation (Figure 1c).

Figure 3. Transmission electron microscopy of PMG₁₀-P assembly in aqueous solution (a) after sonication and (b) aging 1 h.

Just above CAC, 3.0×10^{-5} M, the β -sheet aggregate co-exist with the molecularly dispersed PMG₁₀-P in random coil conformation and the contribution of both conformations to the CD spectrum is roughly equivalent owing to same concentration. This is the reason why the spectrum of Figure 1b is located between the spectra of Figure 1a and 1c.

Molecular Assembly of PMG₁₀-P in Aqueous Solution

PMG₁₀-P was soluble in aqueous solution and formed β -sheet assembly above CAC (6.4×10^{-5} M). We observed directly the morphology of this molecular assembly by transmission electron microscopy (TEM). PMG₁₀-P dissolved in water by sonication to form globular assemblies with diameter of 50–100 nm (Figure 3a). At 1 h, the globular assemblies joined to transform fibrous aggregates (Figure 3b). The fibrils assembled to form twisted ribbon-like aggregates as shown elsewhere.²⁴ Morphological changes of the aggregates are shown in Figure 4. The thickness of the ribbon-like sheet in Figure 4 was estimated to be *ca.* 4.0 nm.²⁴ This assembly may form by monolayer in water, because the thickness of the ribbon-like sheet was too close to chain length (*ca.* 3.9 nm) of PMG₁₀-P when in the β -form conformation.

To confirm the conformation of PMG₁₀-P assembly, the FT-IR spectrum of PMG₁₀-P cast film from aqueous solution (6.4×10^{-5} M) was measured (Figure 5). The spectrum exhibited bands at 1624 and 1689 cm^{-1} (Amide I) and 1530 cm^{-1} (Amide II), typical for anti-parallel β -sheet conformation. Thus, PMG₁₀-P was soluble in aqueous solution to form ribbon-like assemblies having highly ordered interiors as shown by the structural

Figure 4. Schematic illustration of an aggregate of PMG₁₀-P in aqueous solution.²⁴

Figure 5. FT-IR spectrum of PMG₁₀-P cast film from aqueous solution.

model in Figure 4.

Temperature-Concentration Diagram

To estimate the temperature dependence of the polypeptide conformation, we measured the CD spectra of PMG₁₀-P in aqueous solution (1×10^{-4} M) at several temperatures (Figure 6). At 25°C, the spectrum of

Figure 6. Temperature dependence of CD spectra of PMG₁₀-P in aqueous solution. [PMG₁₀-P] = 1.0×10^{-4} M.

Figure 7. Temperature dependence of transmittance at 700 nm of PMG₁₀-P in aqueous solution at various concentrations.

PMG₁₀-P exhibited a positive band at 198 nm and negative band at 218 nm typical of β -sheet conformation. As the temperature increased, is thus $[\theta]_{198}$ gradually decreased. The β -sheet conformation transformed to random coil and α -helix mixed conformation. This indicates the disaggregation of PMG₁₀-P ribbon-like sheet by increasing the temperature. We confirmed this by temperature dependence of transmittance (Figure 7). At the PMG₁₀-P concentration below CAC, 6.40×10^{-6} and 1.28×10^{-5} M, the transmittance of aqueous solution, ca. 90%, was almost the same as that of pure water from 10°C to 50°C, reflecting the molecularly dispersed PMG₁₀-P. Above CAC, the transmittance of aqueous PMG₁₀-P solutions was low at room temperature but increased with temperature owing to disaggregation of the polypeptide above a certain temperature depending on concentration. This temperature was defined as the ag-

Figure 8. Temperature-concentration phase diagram for PMG₁₀-P in aqueous solution.

Figure 9. (a) Temperature dependence of degree of polarization, P , of DPH in PMG₁₀-P aqueous solution. [PMG₁₀-P] = 6.4×10^{-5} M. [DPH] = 3.4×10^{-6} M. (b) DSC curve of PMG₁₀-P aqueous solution. [PMG₁₀-P] = 6.4×10^{-5} M.

gregation to disaggregation temperature where the transmittance of each concentration is 90% of molecularly dispersed solution. At higher concentrations this temperature was found by extrapolating the temperature dependence curve to 90% transmittance as shown in Figure 7. From these results we got the line (a) in the temperature-concentration diagram in Figure 8.

To investigate the structure of the PMG₁₀-P assembly in more detail the temperature dependence of fluorescence polarization, P , of DPH in the assembly was observed (Figure 9a). P of DPH reflects the microscopic viscosity of a molecular aggregate. Fluorescence polariza-

tion exhibits remarkable temperature dependence. P of DPH embedded in the assembly was *ca.* 0.3 at low temperature and gradually decreased with temperature rise up to *ca.* 34°C. It decreased drastically around 34°C to 0.1 at high temperature. This indicates that the interior of the aggregate is rigid at low temperature and large structure change occurred around 34°C. The DSC curve of PMG₁₀-P aqueous solution is shown in Figure 9b. The endothermic peak supports that a first order phase transition of the aggregate occurs at $T_c = 34^\circ\text{C}$ which corresponds to the temperature observed as large decrease in fluorescence polarization (Figure 9a). These results show the β_1 to β_{II} phase transition similar to bilayer systems, even though the molecular assembly is composed of polypeptide amphiphiles without hydrocarbon chains. From peak area, transition enthalpy was estimated as *ca.* 10 kJ/residue mol. This value is in the range of hydrogen-bond strength. The phase transition may thus be attributed to deformation of intermolecular hydrogen bonding between polypeptide molecules in the β -sheet aggregate. From DSC measurements at several PMG₁₀-P concentrations we got the line (b) in Figure 8. A temperature-concentration diagram for aqueous PMG₁₀-P solution was obtained. The PMG₁₀-P molecule was isolated in solution as a random coil rich conformation in low concentration and high temperature (region III). In region β_I , PMG₁₀-P molecules spontaneously aggregate to form a ribbon-like sheet structure. The interior of the assembly was well-defined and rigid structure based on the β -sheet conformation. The PMG₁₀-P aggregate took on a fluid interior above *ca.* 35°C in the region β_{II} .

CONCLUSION

The amphiphilic polypeptide, poly(γ -methyl L-glutamate) with a phosphate moiety at the terminal, was soluble in water and spontaneously assembled to form ribbon-like molecular aggregates, above CAC, each composed of an anti-parallel β -sheet structure. The assembly formation is closely related to the conformation of PMG₁₀-P, which depends on concentration and temperature of aqueous solution. PMG₁₀-P forms a unique aggregate having well-defined interior and characteristics such as self-organization and thermal transition similar to the lipid bilayer.

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