Self-assembled Soft Nanofibrils of Amphipathic Polypeptides and Their Morphological Transformation

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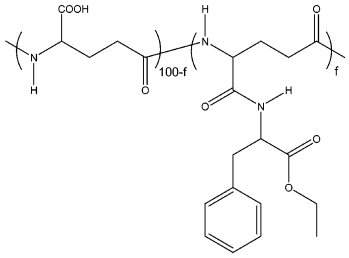
In living systems, water-dispersed particles are widely present as proteins, viruses, organelles, etc., and they have beautiful and cleverly designed morphologies.[1] Since their morphologies play a crucial role in their individual functions,[2] the morphological control of self-assembled nanoparticles will be important in the development of bioconjugate materials. However, there have been no morphological studies on self-assembled nanoparticles from biologically occurring polymers or their derivatives, despite their promised biocompatibility. In addition, morphological transformation is an attractive phenomenon to induce stimuli-responsiveness, as represented by the centrosome transformation in cell division.[2] Here we report a morphological transformation study on self-assembled bio-nanoparticles comprised of biologically occurring polypeptides.

Polypeptide chains with peculiar structures often result in an amphipathic protein, effectively inducing a segregation between the hydrophobic and hydrophilic moieties in water.[1] We mimicked this amphipathic design; a hydrophilic polypeptide backbone was modified by an aromatic amino acid side chain, which would be expected to show strong hydrophobic and π-stacking interactions.[3] We selected a bacterial-secreted polypeptide poly(γ-glutamic acid) sodium salt (γ-PGA; L-Glu/D-Glu = 40/60) since it has already been confirmed to be biocompatible[4] and biodegradable.[5,6] Moreover, the weight-average molecular weight of γ-PGA is 30 kDa and the polydispersity is so low (1.2) that the polymer chains can efficiently self-assemble. We constructed an amide linkage between γ-PGA and L-phenylalanine ethylester (L-PAE) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) according to a previously reported procedure.[3] By controlling the molar ratio of EDC to L-PAE and/or the ratio of L-PAE to the γ-PGA carboxyls in the feed, we obtained γ-PGA-*g*-L-PAE containing 11−79 mol % of L-PAE-modified units to the total units (the *f* value; see the chemical structures shown in Table 1), where *f* was determined from the integral intensity ratio of the proton peaks of the phenyl group to the total proton peaks of the alkylene backbone. We summarized the results of solubility tests using γ-PGA-*g*-L-PAE with various *f* compositions in Table 1. γ-PGA and those polymers with low *f* values of 11−20 mol % dissolved easily in water. Those polymers with *f* values between 36 and 41 mol % were dispersible in water to yield stable colloidal translucent solutions where no precipitate appeared until they settled for more than a week. Polymers with *f* values over 58 mol % were not water-dispersible. In organic solvents, the polymers became soluble in dimethyl sulfoxide (DMSO), dimethylformamide, and chloroform beyond a critical value if the *f* value was increased. These results indicate that this increased modification of L-PAE may enhance the hydrophobicity of the polymer chains. To observe the morphology of the colloidal particles, we performed field-emission scanning electron microscopy (FE-SEM; JEOL JSM-6700F; acceleration voltage: 3kV) of samples spontaneously dried from the dispersion state on glass plates. The copolymers with *f* values between 36 and 41 mol % formed the nanospheres,[3] and also formed a small amount of fibrous nanomatter on rare occasions. Then we attempted to perform a morphological study of the nanoparticles with *f* values more than 41 mol %, which were not water-dispersible. We can assume that the γ-PGA-*g*-L-PAE chains may adopt a conformation

Table 1. Solubility and Dispersity of γ-PGA-*g*-L-PAE[a]



Poly{γ-glutamic acid-*g*-(L-phenylalanine ethyl)}
γ-PGA-*g*-L-PAE

f (mol %)[b]	11	20	36	41	58	72	74	79
water	+	+	±	±	−	−	−	−
chloroform	−	−	−	−	−	−	−	+
dimethyl formamide	−	−	+	+	+	+	+	+
dimethyl sulfoxide	−	+	+	+	+	+	+	+

[a] Solubility test was made at a polymer concentration of 50 mg mL^{-1}. +, soluble; ±, dispersed; −, insoluble. [b] Mark "*f*" refers to the molar fraction percentage of L-PAE units to the total copolymer units measured by ^{1}H NMR.

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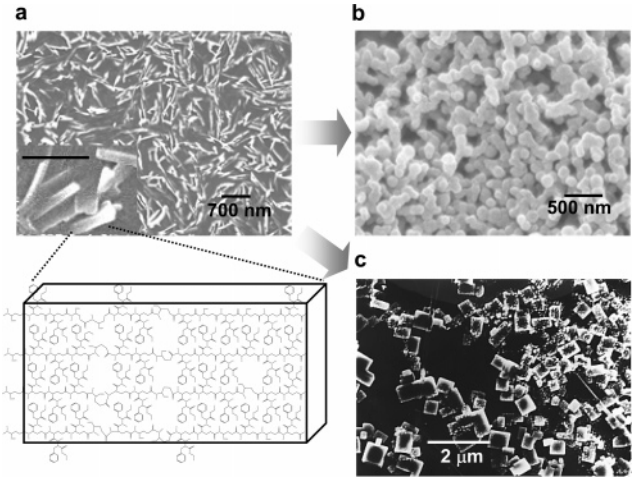

Figure 1. SEM images of γ-PGA-*g*-L-PAE of $f = 74$ mol %. (a) The nanoparticles formed from the DMSO/H_2O (10/1, v/v) solution with a polymer concentration of 5 g L^{-1}. The close-up view is shown in the top right side. Schematic illustration of the molecular arrangement in the nanoparticle is shown below it. (b) The nanoparticles transformed (a) by dialysis in water for 3 days. (c) The nanoparticles formed from the DMSO/H_2O (1/1, v/v) solution with a polymer concentration of 5 g L^{-1}.

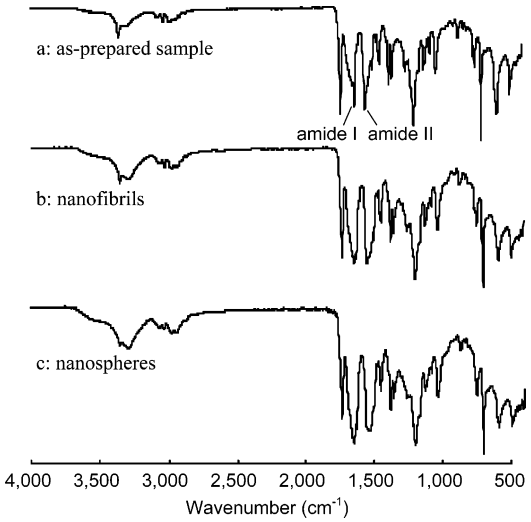

Figure 2. FT-IR/ATR spectra of the as-prepared γ-PGA-g-L-PAE with *f* of 74 mol %, the nanofibrils, and the nanospheres transformed from the nanofibrils.

appropriate for water-dispersion under certain conditions, based on the fact that hydrophobic proteins such as bovine serum albumin yield a colloidal solution in their natural state, although the denaturated ones adopt a random conformation precipitate. We made slow environmental changes around the polymer chains; several tens of small water drops (total volume: 0.5 and 0.1 mL) were slowly added into the DMSO solution (1 mL), with a polymer concentration of 5 mg·mL^{-1} under vigorous agitation. This treatment gave a translucent or almost transparent solution. γ-PGA-*g*-L-PAE with *f* values of 58, 72, and 74 mol % gave translucent colloidal solutions stable for 3 days, whereas polymers of $f = 79$ mol % precipitated. The colloidal solution was centrifuged (14500 rpm, 10 min) to sediment the white matter, followed by three rinses and repeated centrifugation in water to give an aqueous colloid solution stable for more than 3 h. This colloid was spontaneously dried on the glass plate in order to investigate the morphology by scanning electron microscopy (SEM, Figure 1a−c). γ-PGA-*g*-L-PAE with $f = 58$ mol % still formed spherical particles. On the other hand, polypeptides with $f = 72$ and 74 mol % formed fibrous nanoparticles. Figure 1a shows nanofibrils of the $f = 74\%$ polypeptide (100 nm thick and more than 1 mm long) formed from the DMSO/H_2O (10/1 v/v) solution. The close-up view shown in the bottom left-hand side indicates that the edge of the nanofibrils was squarish. Some of the fibrils are branched. If the fibrils adopt a β-amyloid structure, they should have a binding site for Congo red, showing apple-green birefringence under the cross-nicols.[7] However, no fibrils stained by Congo red showed such a specific birefringence. Neither the X-ray diffraction nor the electronic diffraction patterns of these fibrils showed any distinct diffraction, indicating that the fibrils formed an amorphous structure at the molecular level. The polymer chains could not be crystallized due to the random sequence of L-/D- residues. The infrared (IR, Spectrum One Perkin-Elmer) spectra of the as-prepared samples showed vibration peaks from amide I at 1645 cm^{-1} and amide II at 1510 cm^{-1} (Figure 2a). When the nanofibrils were formed, these peaks were shifted into 1651 cm^{-1} and 1502 cm^{1}, respectively (Figure 2b), which indicated that the amide hydrogen bonds became weaker. We can presume the hydrogen bonding works interchain because we previously found that the γ-PGA propyl ester having the same backbone as γ-PGA-*g*-L-PAE formed the interchain amide hydrogen bonds.[8] In the fibrils, the polymer chains may be weakly associated, thus resulting in soft fibrils. The optical crossed-polarizing microscopic image showed strong birefringence, indicating the molecular orientation. Although X-ray sensitive moiety of the electron-rich L-PAE side chains adopted no regular arrangement, the polymer chains oriented themselves inside the fibrils, as shown in the illustration of Figure 1a. As shown in the previous report,[3] the phenyl ring of L-PAE may show a π-stacking behavior which can induce oriented self-assembly with the aid of chain length monodispersity. Although the mechanism of orientation is difficult to explain, the cooperation of the phenyl plane stacking with interchain hydrogen bonding of amide linkage may extend the polymer backbone to induce the orientation.

The fibril morphology was stable under repeated centrifugation in water, but after dialysis in water for 3 days they disappeared completely. Instead, nanospheres with a diameter of ca. 200 nm appeared (Figure 1b). The volume of one fibril corresponded approximately to that of one sphere, indicating that the nanofibrils transformed into the nanospheres. Since the nanosphere showed IR peaks of amide I and II at the same wavenumbers as those of the nanofibriles (Figure 2c), the strength of the hydrogen bonding may be kept during the morphology change from the fibril to the sphere. This transformation may be due to a minimization of the surface area and the nanoparticle softness.

On the other hand, when we prepared a polymer colloidal aqueous solution in the DMSO/water mixed solvent with a further increase in the water composition (DMSO/H_2O, 1/1),

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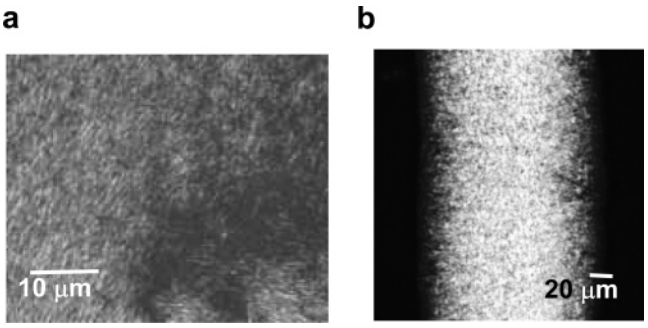

Figure 3. Crossed-polarizing microscopic images of γ-PGA-*g*-L-PAE with $f = 72$ mol % (a) in a concentration of 40 wt %. (b) The fiber spun from the dispersion state (a).

rectangular platelet-like particles appeared (Figure 1c). The fibrils grew into these platelets, presumably through additional self-assembly with the polymer chains dissolved by an increase in the water composition. If additional polymer chains attached onto the preformed fibrils, then the matching at the attachment face may be important because even slight mismatching may induce disconnection due to weak interactions, whereas good matching contributes to growth into the platelets. These are the first observation of morphological transformation for the polypeptide nanoparticles.

Figure 3a shows a crossed-polarizing microscopic photograph of the fibrils from a polymer of $f = 72$ mol %, which was concentrated up to 40 wt %. We can observe many bright fibrils with a sub-micrometer-sized width and micrometer-sized length, suggesting the presence of fibril bundles. In addition, the black blush appearing in the bottom-right of this figure corresponds to the polarizing direction of the cross-nicols. This finding indicates that the fibrils were automatically oriented like liquid crystals of Tobacco mosaic virus (TMV)[9,10] presumably due to excluded volume effects at the fiber-bundle level. The dispersion of the fibrils showed a high viscosity at this concentration, and we could spin the macroscopic fiber (several centimeters long), resulting in enhanced brightness under the crossed-polarizer (Figure 3b). The nanofibrils were aligned in the macroscopic fiber. Thus, amphipathic polypeptide chains with aromatic amino acid pendant groups self-assembled in an appropriate interaction to yield transformable nanofibrils. These dynamic nanofibrils may lead to the development of novel, stimuli-responsive bionanoparticles for controlled drug release and vaccines.

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