Effect of the Secondary Structure of Poly(L-lysine) Segments on the Micellization in Aqueous Milieu of Poly(ethylene glycol)−Poly(L-lysine) Block Copolymer Partially Substituted with a Hydrocinnamoyl Group at the N^{ϵ}-Position

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ABSTRACT: A series of poly(ethylene glycol)−poly(L-lysine) block copolymers partially substituted in varying degrees with a hydrocinnamoyl group at the N^{ϵ}-position was prepared by a coupling reaction of hydrocinnamic acid to the ϵ-amino group of the poly(L-lysine) segment of the block copolymer using (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP Reagent) as a condensation reagent. The N^{ϵ}-substituted block copolymers with less than 90% substitution formed clear solutions in 0.1 M phosphate buffer (pH 7.4) by dialyzing from DMSO. Light scattering measurements revealed multimolecular micelle formation for the block copolymers with 65−70% of the substitution degree. The cumulant diameter of the micelles was approximately 40 nm with a moderate polydispersity ($\mu_2/\Gamma^2 \sim 0.15$). A fairly low critical association concentration (~40 mg/L) was estimated using pyrene as a fluorescence probe molecule. At a pH as high as 11, the block copolymer with a substitution degree even as low as 50%, which gave only a subtle indication of micellization at pH 7.4, clearly formed multimolecular micelles, suggesting that the balance between the side-chain (hydrocinnamoyl group) interaction and electrostatic repulsion of the protonated ϵ-amino groups may play a substantial role in the micelle stabilization. Furthermore, in the circular dichroism spectra of the solutions, a remarkable change in the secondary structure of the copolymers was observed, that is, a change from random coil to β-sheet structure, either with an increased substitution degree or with a pH increment. Multimolecular micellization concomitantly occurred with β-sheet formation. Presumably, the layered packing of β-sheets through the side-chain interaction of aromatic groups and intermolecular hydrogen bonding may stabilize the micelle structure. It is of interest that β-sheet structures were preserved even below the critical association concentration, suggesting the existence of intra- and/or interstrand interaction of the block copolymers under highly diluted conditions.

Introduction

Recently, a growing interest has been shown in the formation of multimolecular micelles with core−shell architecture in aqueous milieu through the association of amphiphilic block copolymers.[1−12] These micelles are of interest not only from a basic standpoint of macromolecular association in selective solvents but also from the development of functional materials in which the size and the core−shell architecture of block copolymer micelles play a substantial role. The latter includes materials used in such fields as drug delivery,[13−15] diagnosis,[16] separation technonogy,[17−19] and optotechnology.[20] A variety of hydrophilic polymers with charged or uncharged nature have been selected as shell-forming segments of the block copolymers, while vinyl or polycondensated polymers with hydrophobic side chains have been used as core-forming segments. Among these diverse combinations, the system involving poly(ethylene glycol) (PEG) as the hydrophilic segment has been most extensively studied mainly due to the flexibility and the hydrophilicity of the PEG chain, allowing one to achieve the effective steric stabilization propensities.[21] Furthermore, the nontoxicity as well as nonimmunogenicity of PEG[22] will be additional advantages when the micelle system is utilized in the medicinal field as a carrier of therapeutic and diagnostic drugs.

We have been working on the block copolymer micelles based on the block copolymers of PEG and poly(amino acid)s as a novel carrier system for drug targeting.[13,23−27] Poly(amino acid)s were chosen as the counterpart of PEG in the block copolymer, because of their biocompatibility as well as the ease of engineering the structures through copolymerization and side-chain derivatization. Indeed, successful targeting of an anticancer drug to solid tumor and, eventually, complete tumor regression were achieved by PEG−poly(amino acid) block copolymer micelles entrapping doxorubicin in the core.[27,28]

From a different viewpoint, the micelle system based on PEG−poly(amino acid) block copolymers brings a novel issue to the basic field of macromolecular association. That is the effect of the secondary structure of poly(amino acid) segments on micellization. Recently, we have found that the PEG−poly(L-lysine) block copolymer (PEG−P(Lys)) in aqueous milieu at pH > 10.8

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undergoes a selective dimer formation with a micelle like structure, in which P(Lys) segments of two polymer strands were segregated from the aqueous exterior by PEG palisades.[29] It is worth noticing that this dimerization of PEG−P(Lys) was synchronized with the conformational transition of P(Lys) segments from random-coil to α-helix structure, suggesting an important correlation between the micellization and the conformation of poly(amino acid) segments. It is of further interest to get insight into this correlation more deeply by modulating the side-chain structure of the P(Lys) segments in the PEG−P(Lys) block copolymers. In this study, varying fractions of ϵ-amino groups of the P(Lys) segment in the block copolymer were substituted with hydrocinnamoyl groups to modulate the hydrophobic/hydrophilic balance of the block copolymer as well as to emphasize side-chain association through the $\pi-\pi$ interaction of the aromatic rings of the hydrocinnamoyl groups, inducing a substantial change in the secondary structure of the P(Lys) segments. Thus, clear correlation was obtained between the multimolecular micellization of the block copolymer and the conformational transition of the P(Lys) segments.

Experimental Section

Materials. [(ϵ-Benzyloxy)carbonyl]-L-lysine (Lys(Z)) was purchased from Peptide Institute, Inc., Osaka, Japan, and used without further purification. Bis(trichloromethyl)carbonate (triphosgene), hydrocinnamic acid (HCA), and *N*,*N*-diisopropylethylamine were purchased from Tokyo Chemical Industry Co., Ltd., Tokyo, Japan, and used without further purification. α-Methoxy-ω-aminopoly(ethylene glycol) (PEG; M_w = 5900, M_w/M_n = 1.05, functionality of amino group = 0.956) was a gift from Nippon Oil & Fats Co., Ltd., Tokyo, Japan. PEG was reprecipitated into a 100-fold excess of diethyl ether from a chloroform solution (0.1 g/mL). The reprecipitated PEG was then dissolved in benzene (0.1 g/mL), followed by freeze-drying to obtain the sample for the block copolymer synthesis. Trifluoroacetic acid, anisole, methanesulfonic acid, lithium chloride, 1-methyl-2-pyrrolidone (NMP), dimethyl sulfoxide (DMSO), and pyrene were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan, and used without further purification. (Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) was purchased from Aldrich Chemical Co., Inc., Milwaukee, WI, and used without further purification.

Synthesis of Poly(ethylene glycol)−Poly(L-lysine) Block Copolymer (PEG−P(Lys)). Poly(ethylene glycol)−poly[[(ϵ-benzyloxycarbonyl]-L-lysine] block copolymer (PEG−P(Lys(Z)) was synthesized according to our previous report[30] by the ring-opening polymerization of Lys(Z) *N*-carboxyanhydride, prepared from [(ϵ-benzyloxy)carbonyl]-L-lysine by the Fuchs−Farthing method, initiated from the primary amino group of PEG. The 400-MHz ^{1}H NMR measurement (EX400, JEOL, Tokyo, Japan) in DMSO-d_6 at 80 °C was carried out for the obtained PEG−P(Lys(Z)) sample, confirming that all of the observed peaks were assignable to the expected structure. The polymerization degree (DP) of the P(Lys(Z)) segment was calculated to be 18 from the peak intensity ratio of the methylene protons of PEG (OCH_2CH_2; 3.6 ppm) and the phenyl protons of P(Lys(Z)) ($CH_2C_6H_5$; 7.3 ppm). The GPC measurement in THF at 40 °C was also carried out in order to determine the molecular weight distribution (M_w/M_n) using an HLC-8020 system (Tosoh, Tokyo, Japan) equipped with a column combination of TSK gel G4000H_{XL}, G3000H_{XL}, and G2000H_{XL} columns. The M_w/M_n was determined to be 1.09 using the calibration curve based on standard PEG samples.

The removal of the protection group, (ϵ-benzyloxy)carbonyl group, was carried out in trifluoroacetic acid using methanesulfonic acid and anisol. After 3 h of reaction at room temperature, the reaction mixture was neutralized by the addition of triethylamine, followed by dialysis against water using a Spectrapor 6 membrane (molecular weight cutoff = 1000). Through lyophilization from water, PEG−P(Lys) was obtained as a white powder. The 400-MHz ^{1}H NMR measurement in D_2O was carried out to confirm the complete deprotection of the $N^ϵ$-position of PEG−P(Lys) through the disappearance of the peaks corresponding to the (ϵ-benzyloxy)carbonyl group.

Introduction of Hydrocinnamoyl Groups to PEG−P(Lys). The conjugation of the hydrocinnamoyl group to the $N^ϵ$-position of PEG−P(Lys) was carried out using BOP Reagent.[31,32] Given amounts of PEG−P(Lys), hydrocinnamic acid, and BOP Reagent were dissolved in NMP with 5 wt % LiCl. To the solution was then added *N*,*N*-diisopropylethylamine. The mixture was stirred for 24 h at 25 °C to proceed the condensation reaction, followed by dialysis against DMSO to remove excess BOP Reagent, unreacted hydrocinnamic acid, and other impurities. Subsequently, the DMSO solution was dialyzed against distilled water, followed by lyophilization.

The purity of the obtained sample was checked by reversed-phase liquid chromatography using an HPLC system (Jasco, Tokyo, Japan) equipped with a μBondasphere 5μ CN 100-Å column (Water, Co., Milford, MA) and UV detector (UV-970; Jasco, Tokyo, Japan) as well as by ^{1}H NMR measurement in DMSO-d_6. The coupling degree was determined by two different methods: (1) ^{1}H NMR measurement in DMSO-d_6 and (2) the fourth differential UV spectrum in $CHCl_3$. In the ^{1}H NMR measurement, the substitution degree of the $N^ϵ$-position of PEG−P(Lys) was determined from the peak intensity ratio of the phenyl protons of the hydrocinnamoyl group ($COCH_2CH_2C_6H_5$; 7.2 ppm) and the α-, β-, and γ-trimethylene protons of the side-chain alkyl group of the lysine residue ($CH_2CH_2CH_2CH_2NH$; 1.2−2.2 ppm). In the fourth differential UV spectrum, the substitution degree was determined from the intensity difference between the peak maximum at 268 nm and the peak minimum at 273 nm using the calibration curve based on hydrocinnamic acid.

Preparation of Multimolecular Micelles from PEG−P(Lys) Partially Substituted with A Hydrocinnamoyl Group at the $N^ϵ$-position. Hydrocinnamoyl-substituted PEG−P(Lys)s with varying degrees of substitution were dissolved in DMSO at 5.0 mg/mL. These solutions were dialyzed against 200-fold excess of distilled water using a Spectrapor 6 membrane (molecular weight cutoff = 1000) at ambient temperature to prepare multimolecular micelles. The pH of the micelle solution was adjusted by the addition of 0.1 N HCl or 0.1 N NaOH. The exchange of the medium to 0.1 M phosphate buffer (pH 7.4; PBS) ($Na_2HPO_4·12H_2O$, 29.009 g/L; $NaH_2PO_4·2H_2O$, 2.964 g/L) was accomplished by dialysis.

Light Scattering Measurements. Light scattering measurements were carried out using a DLS-700 in-

strument (Otsuka Electronics Co., Ltd., Osaka, Japan). Vertically polarized light of 488-nm wavelength from an Ar ion laser (15 mW) was used as the incident beam. All measurements were performed at 23.5 °C, and sample solutions were purified by passage through a 0.45-μm filter (Millex-VV, Millipore, Bedford, MA).

In the dynamic light scattering measurements, the general formula for the photoelectron count time correlation function has the form

$$g^{(2)}(\tau) = 1 + \beta|g^{(1)}(\tau)|^2 = 1 + \beta \exp(-2\bar{\Gamma}\tau)$$

where $g^{(2)}(\tau)$ is the normalized second-order correlation function, β is a parameter of the optical system, $g^{(1)}(\tau)$ is the normalized first-order correlation function, τ is delay time, and $\bar{\Gamma}$ is the average characteristic line width. By the cumulant approach,[33] $g^{(1)}(\tau)$ can be expressed as the equation

$$g^{(1)}(\tau) = \exp[-\bar{\Gamma}\tau + (\mu_2/2!)\tau^2 - (\mu_3/3!)\tau^3 + \cdots]$$

yielding $\bar{\Gamma}$ and variance (polydiseprsity index), $\mu_2/\bar{\Gamma}^2$. Also, the *Z*-averaged diffusion coefficient, *D*, and hydrodynamic diameter, *d*, can be obtained from $\bar{\Gamma}$ using the following equations:

$$\bar{\Gamma} = DK^2 = D[(4\pi n_0/\lambda_0) \sin(\theta/2)]^2$$

$$d = k_B T/(3\pi\eta_0 D) \quad \text{(Stokes–Einstein equation)}$$

The time-averaged scattering intensities, I, were also measured using a DLS-700 instrument. The total number of counts per constant time which is proportional to the intensity was measured under constant optical conditions. The scattering intensity of the sample is evaluated from the equation

$$I = I_{\text{solution}} - I_{\text{solvent}}$$

where I_{solution} and I_{solvent} are the measured scattering intensity of polymer solution and solvent, respectively.

Determination of Critical Association Concentration (cac). The cac of the micelle was determined from the fluorescence method using pyrene as a probe molecule. Probe dissolution was accomplished by a protocol developed for the poly(ethylene glycol)–polystyrene block copolymer.[5] A pyrene concentration of 6.0×10^{-7} M was used. Fluorescence measurements were carried out using an FP-770F fluorometer (Jasco, Tokyo, Japan) at 25.0 °C, and the emission spectra excited at 339 nm were monitored over the range between 350 and 450 nm. The polymer solutions were deoxygenated with nitrogen gas prior to measurements, and experiments were carried out at 25 °C.

Circular Dichroism Measurements. Circular dichroism (CD) measurements were carried out at ambient temperature using J-600 and J-700 spectropolarimeters (Jasco, Tokyo, Japan) equipped with 1 mm and 3 mm path length cells (GL Science Co., Ltd., Tokyo, Japan) at room temperature. The sample concentrations were adjusted in order to have the same residual molarity of the lysine unit.

Results and Discussion

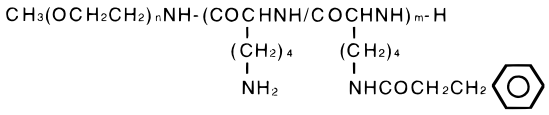
Coupling of Hydrocinnamic Acid to the ϵ-Amino Group of the Poly(ethylene glycol)–Poly(L-lysine) block copolymer (PEG–P(Lys)). The coupling degrees of hydrocinnamic acid to PEG–P(Lys) were determined from ^{1}H NMR and the fourth differential UV spectrum as described in Experimental Section and are summarized in Table 1 for the samples reacted under different feed ratios of HCA to the lysine residue in PEG–P(Lys). Obviously, NMR and UV measurements gave concordant results. The values thus obtained are also in good agreement with the calculation assuming a quantitative reaction, indicating the high efficiency of the coupling reaction using the BOP Reagent.

Table 1. Conjugation of a Hydrocinnamoyl Group to PEG–P(Lys)

	coupling degree [mol %]		
feed ratio [mol %]	UV method[a]	NMR method[b]	yield [%]
30	24	25	80
40	35	32	73
50	43	46	75
55	51	48	70
60	54	54	72
70	65	68	70
80	70	74	70
100	90	96	55

[a] These values were determined from the fourth differential UV spectra in $CHCl_3$. [b] These values were determined from ^{1}H NMR measurements in DMSO-d_6.

Samples with different coupling degrees of HCA are coded as P-HC-*X*, where *X* stands for the coupling degree of HCA to the ϵ-amino group of the lysine residue in PEG–P(Lys) expressed in mol percent. The structural formula of P-HC-*X* is shown as follows:



Associate Formation of P-HC-X in Aqueous Milieu. As previously reported, PEG–P(Lys) in aqueous milieu gradually changes its conformation from a random coil under acidic conditions to an α-helix in an alkali environment, resulting in the supramolecular association to form a dimer.[29] A pH as high as 11 is required for all of the PEG–P(Lys) in milieu to be in the dimer form due to complete deprotonation of the ϵ-amino groups of the P(Lys) segment. The introduction of the hydrophobic hydrocinnamoyl (HC) units to PEG–P(Lys) is expected to increase the cohesive force of the block copolymer to form associates even in an aqueous milieu with relatively low pH.

All of the P-HC-*X*, except $X = 90$, gave transparent aqueous solutions by dialyzing from DMSO to acidic water (pH 3.65) or 0.1 M PBS (pH 7.4). Yet, as shown in Figure 1, scattering intensity measurements of these solutions revealed a remarkable effect of the coupling degree of HC on the scattering intensities, indicating HC-dependent multimolecular micelle formation. The light scattering intensity of PEG–P(Lys) (I_0) was taken as the standard for the relative scattering intensity (I/I_0) in Figure 1. It is obvious from this figure that approximately 50 mol % substitution by HC is the critical coupling degree to induce multimolecular association of the block copolymers both in acidic water (pH 3.65) and in the buffer (pH 7.4).

Dynamic light scattering (DLS) measurements were then carried out based on the cumulant approach for P-HC-65 and -70, which gave sufficient scattering intensities. As summarized in Table 2, the multimolecular micelles in the size range of approximately

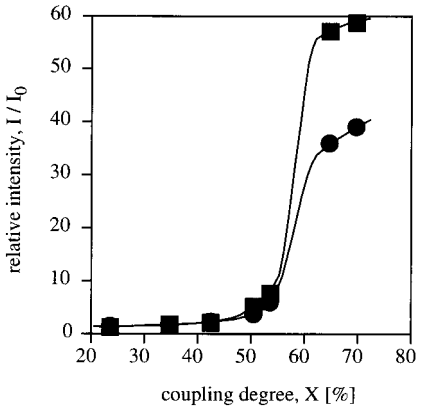


Figure 1. Change in the relative scattering intensity (I/I_0) with the coupling degree (X) of P-HC-X. I and I_0 were scattering intensities of P-HC-X and PEG−P(Lys) (P-HC-0), respectively. (■, pH 7.4 (0.1 M phosphate buffer); ●, pH 3.65; detection angle, 90°; polymer concentration, 0.18 mg/mL; temperature, 23.5 °C).

Table 2. Properties of P-HC-*X* Multimolecular Micelles in Phosphate Buffer

coupling degree[a] [mol %]	average diameter[b] [nm]	polydispersity[b] ($\mu_2\Gamma/^2$)	cac[c] [mg/L]
65	37.7	0.13	37
70	29.7	0.18	43

[a] These values were determined from the fourth differential UV spectra in $CHCl_3$. [b] These values were obtained from cumulant analysis of dynamic light scattering. [c] These values were determined by using pyrene as the fluorescence probe.

30−40 nm were observed in the buffer (pH 7.4) with a moderate polydispersity ($\mu_2/\Gamma^2 < 0.2$). The cac's are also given in Table 2, which were determined using pyrene as a fluorescence probe to monitor the change in the polarity of the microenvironment in the multimolecular micelle. Pyrene will preferentially partition into hydrophobic microdomains (e.g., interior of micelles) with a concurrent change in the molecule's photophysical properties.[34] A change in the vibrational structure of pyrene monomer emission, specifically, the intensity ratio of the (0, 0) band, I, to the (0, 2) band, III, with local polarity was used in this study to determine the cac.

The cac values are low enough to be consistent with the known low cac values for multimolecular micelle systems prepared from amphiphilic block copolymers in aqueous milieu.[4,5,9−12] Yet, in comparison with our previous cac data obtained for the micelle prepared from the PEG−poly(β-benzyl L-aspartate) block copolymer (PEG−PBLA; M_w of PEG = 5000, polymerization degree of PBLA = 19)[11,12] with a segment length similar to that of P-HC-X, a 1 order of magnitude increase in cac was observed for P-HC-65 and -70. This may be due to the presence of unsubstituted ϵ-amino groups in the interior of the multimolecular micelle, contributing to the decrease in the cohesive force of the core-forming segments, in this case HC-modified polylysine, to destabilize the micelle structure. Indeed, the protonation of the ϵ-amino groups seems to have a crucial effect on the formation of multimolecular micelles from P-HC-X with a relatively low substitution degree of HC.

Figure 2a shows a change in the scattering intensity of the aqueous solution of P-HC-51 with pH. Although the P-HC-51 solution gave only a low scattering intensity at pH 7.4 as shown in Figure 1, a remarkable increase in the scattering intensity in the higher pH

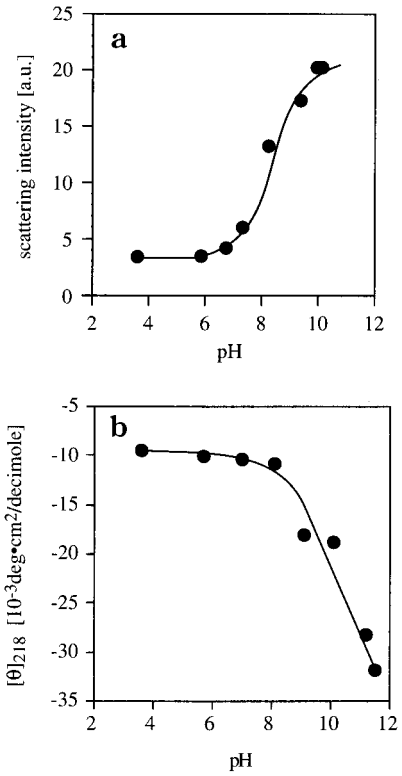


Figure 2. Change in the scattering intensity (a) and the mean residual ellipticity at 218 nm ($[\theta]_{218}$) (b) with pH for P-HC-51. In a: detection angle, 90°; polymer concentration, 0.26 mg/mL (0.49 mmol of lysine residue/L); temperature, 23.5 °C. In b: polymer concentration, 0.26 mg/mL (0.49 mmol of lysine residue/L); room temperature.

region is observed, suggesting the formation of the multimolecular micelles. This result suggests that the multimolecular association of P-HC-51 is modulated through the balance between the hydrophobicity of HC units and the protonation degree of the ϵ-amino groups in the polylysine chain. Furthermore, it should be noted that PEG−P(Lys), the block copolymer without HC units, only forms a dimer with an α-helix conformation even at a pH as high as 11.[29] The obvious increase in the associate size and, consequently, the association number of P-HC-51 compared to PEG−P(Lys) indicates that the introduction of the hydrophobic HC unit into the P(Lys) segment of the block copolymer led to an increase in the interchain association force to form multimolecular micelle structures. It is worth noticing that this interchain association seems to have a close correlation with a change in the secondary structure of the P(Lys) segment in the block copolymer.

Change in the Secondary Structure of P(Lys) Segments in P-HC-*X* Micelles. The secondary structures of P(Lys) segments in P-HC-X with varying degrees of substitution were estimated by circular dichroism (CD) in the buffer at pH 7.4. As shown in Figure 3, an obvious change in the secondary structure was observed with the substitution degree. The P(Lys) segment of P-HC-0 took a random-coil conformation, since more than 90% of the ϵ-amino groups were protonated at pH 7.4.[29] CD spectra gradually changed with an increase in the substitution degree (X) from 0 to 54. The crossover wavelength remained at 204 nm. It should be noted that this crossover wavelength well-agreed with that of the pH-induced helix−coil transition for poly(L-lysine) and PEG−P(Lys) reported in the literature.[29,35] Further, the block copolymers with a

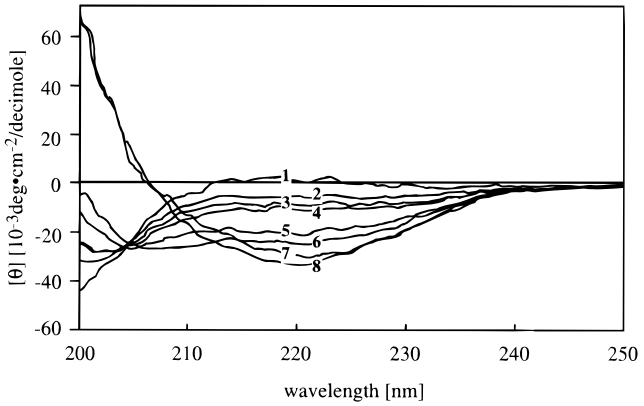


Figure 3. Circular dichroism spectra for P-HC-*X*. ($X = 0$ (1), 24 (2), 35 (**3**), 43 (4), 51 (5), 54 (6), 65 (7), 70 (8); concentration of lysine residue, 0.49 mmol/L; cell length, 1 mm; room temperature).

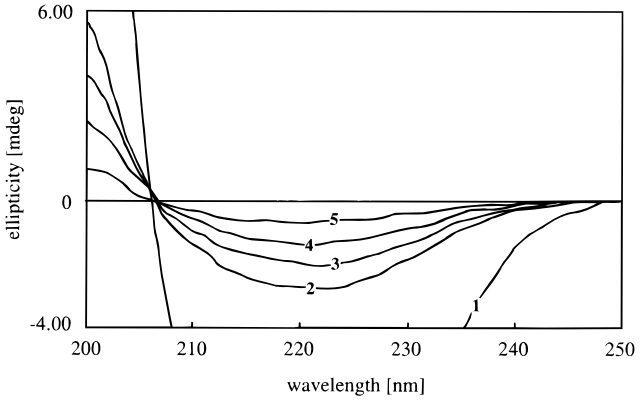


Figure 4. Influence of the critical association concentration on the secondary structure of P-HC-70 (1, 216 mg/L (390 μmol/L); 2, 43.2 mg/L (78 μmol/L); 3, 32.4 mg/L (54 μmol/L); 4, 21.6 mg/L (39 μmol/L); 5, 10.8 mg/L (19 μmol/L); cell length, 3 mm; room temperature).

substitution degree of around 50% (P-HC-51 and -54) had CD spectra with two minima around 209 ($\pi-\pi^*$) and 222 nm ($n-\pi^*$), suggesting the formation of an α-helix structure. Also, the α-helix formation of a phenylalanine-modified P(Lys) homopolymer ($M_w = 20{,}000$) in a neutral aqueous solution was previously reported by Anand et al.,[36] which is consistent with our present results, except that in our case P(Lys) with a smaller molecular weight ($M_w = 2400$) was used as the block copolymer. On the other hand, the spectra of P-HC-65 and -70 have typical characteristics of β-sheets with a minimum at 218 nm ($n-\pi^*$ transition). Consequently, by increasing the HC contents, the preferential secondary structure of the P(Lys) segments in the block copolymer gradually changes from random coil to α-helix in the region of $0 \leq X \leq 54$ and becomes a β-sheet at $X \geq 65$.

A particularly impressive point of the previous order is that it seems to correlate closely with the association behavior of the block copolymer as shown in Figure 1. β-Sheet formation in P-HC-65 and -70 is clearly in line with their multimolecular association. It should be noted that aromatic amino acids, including phenylalanine, tend to be the best β-sheet-forming residues in proteins because of their high intrinsic β-sheet-forming propensities and side-chain interaction energies.[37] Lysine itself is categorized into a group of amino acids with a moderate tendency to form β-sheet structures. The heat treatment is known to be required to transform the secondary structure of P(Lys) from a α-helix to β-sheet structure.[35] Yet, the introduction of an aromatic group to the side chain of the lysine residue through HC substitution may increase the β-sheet-forming propensities as well as the cross-strand side-chain interactions of the block copolymers, allowing the inducement of multimolecular association through β-sheet packing. Multimolecular micelles should be stabilized by the aligned side-chain interaction of aromatic groups as well as by interstrand hydrogen bonding in β-sheet structures. Note that β-sheet side chains should alternate above and below the plane of the sheet along each strand, allowing the inducement of the layered packing of the β-sheet to form the core of the multimolecular micelle structure.

A concomitant change in the secondary structure of the HC-modified P(Lys) segment in the block copolymer with multimolecular micelle formation was also confirmed in the pH-dependent micellization of P-HC-51 shown in Figure 2. That is, the $[\theta]_{218}$ vs pH curve shown in Figure 2b well-correlates with the light scattering intensity vs pH curve in Figure 2a. CD spectra of P-HC-51 at pH ≤8 have two minima as shown in Figure 3. Then, at pH ≥9, the spectra underwent a considerable change due to the formation of a β-sheet structure having a minimum at 218 nm. Consequently, the residual ellipticities were significantly decreased in this region as shown in Figure 2b. This indicates a conformational transition of the HC-modified P(Lys) segments to the β-sheet-enriched structure with a decrease in the protonation degree of the ϵ-amino groups, allowing the formation of multimolecular micelles through interstrand interactions. Note that P-HC-70 had a considerably high scattering intensity throughout the measured pH and its CD spectrum had a pattern typical of a β-sheet-enriched conformation even at a lower pH than 3 (data not shown), indicating the cohesive force is strong enough to form a multimolecular micelle in this case even under acidic conditions.

An important issue to be considered is whether the conformational transition to the β-sheet structure plays a key role in the multimolecular association of the block copolymer to form micelles, because there is another possibility that the hydrophobic association of the HC-modified P(Lys) segments forms the local environment with low polarity, facilitating the alignment of P(Lys) strands to form the β-sheet structure through hydrogen bonding. To gain insight into this cause-and-effect relationship, CD measurements of P-HC-70 were carried out in the concentration range across the cac.

Secondary Structure of P-HC-70 Across cac. The secondary structures of P-HC-70 were then estimated from CD spectra across the cac. The concentration-dependent change in the CD spectra for the P-HC-70 system in very diluted conditions below the cac (43 mg/L) is shown in Figure 4. Obviously, the spectra of P-HC-70 had typical characteristics of the β-sheet structure, indicating that it keeps the β-enriched conformation even below the cac. In other words, multimolecular micellization may proceed through the association of polymer strands with the β-sheet conformation. Given that micelles may dissociate into unimer molecules below the cac, the HC-modified P(Lys) strand should fold back itself to form an intramolecular β-sheet structure which may be surrounded by a PEG chain to be segregated from the aqueous phase. However, this kind of unimolecular micelle might be hard to form

because the chain length of HC−P(Lys) is significantly short (DP = 18). Another possibility, which seems to be more likely, is that, even below the cac, P-HC-70 may associate through intermolecular β-sheet formation. Thus, the results of CD measurements across the cac support our contention that β-sheet formation plays a crucial role in the association of the block copolymers into a multimolecular micelle structure.

Finally, it should be noted that in the medicinal field the targeted delivery of proteins, including cytokines and enzymes, has become one of the emergent subjects for the therapy of various life-threatening diseases and the development of novel carrier systems of these proteinous drugs has received considerable attention.[38] As β-strands have a strong tendency to associate with each other, the amphiphilic block copolymer with a segment to have a high propensity to form β-structure, i.e., P-HC-*X*, might be able to stabilize β-sheet-forming peptides and proteins under physiological conditions through incorporation into the core of the micelle structures. Such a demonstration is now underway in our laboratory, and the results will be reported elsewhere.

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References and Notes

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