Novel Polyion Complex Micelles Entrapping Enzyme Molecules in the Core: Preparation of Narrowly-Distributed Micelles from Lysozyme and Poly(ethylene glycol)−Poly(aspartic acid) Block Copolymer in Aqueous Medium

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Received August 25, 1997; Revised Manuscript Received November 14, 1997

ABSTRACT: A core−shell-type supramolecular assembly, a polyion complex micelle, was prepared in this study from chicken egg white lysozyme and poly(ethylene glycol)−poly(aspartic acid) block copolymer (PEG-P(Asp)) through electrostatic interaction in aqueous medium. Lysozyme/PEG-P(Asp) micelles thus prepared had an extremely narrow distribution ($\mu_2/\bar{\Gamma}^2 < 0.04$) with an average diameter of 47 nm in dynamic light scattering measurements. No precipitate formation was observed even after 1 month standing at ambient temperature, suggesting that the system is in a thermodynamic equilibrium state. The stoichiometry in terms of the molar ratio of Lys and Arg residues in lysozyme and Asp residues in PEG-P(Asp) was confirmed by dynamic and static light scattering as well as by laser-Doppler electrophoresis measurements. A change in the apparent molar mass of the micelle with varying PEG-P(Asp)/lysozyme ratio in the region with excess lysozyme agreed well with calculated values if a cooperative association mechanism is assumed to occur. The diffusion coefficient of lysozyme/PEG-P(Asp) micelles prepared at a stoichiometric mixing ratio showed neither angular nor concentration dependence, indicating their spherical shape with no secondary aggregate formation. A core−shell structure with a polyion complex core and a PEG corona was suggested from an extremely low absolute value of zeta-potential. The association numbers of lysozyme and PEG-P(Asp) in the stoichiometric micelle were calculated from the apparent molar mass and were determined to be 36 and 42 for lysozyme and PEG-P(Asp), respectively. Such PIC micelles entrapping enzymes in the core are expected to be useful as functional materials including carrier systems in drug delivery applications and a nanometric-scale reactor for enzymes.

Introduction

A supramolecular structure formed through macromolecular assembly has recently received considerable attention from various aspects in both fundamental and applied fields of polymer science.[1,2] A block copolymer micelle is one on which intensive studies have been carried out by many research groups including ourselves.[3−13] Relevant size (several tens of nanometers) and structure (core−shell morphology) characteristics of block copolymer micelles lend themselves for use in many applications, including drug delivery systems,[14−18] separation technologies,[19,20] and optoelectronic devices.[21] Further, in line with an increased interest in block copolymer micelles as functional materials, a remarkable impetus has arisen to design new types of micelles having purposeful functionalities in their structure.

Recently, a new concept emerged in block copolymer micelle formation in an aqueous medium. This involves the spontaneous formation of a macromolecular assembly, "polyion complex (PIC) micelles", driven through electrostatic interaction in an aqueous medium of charged block copolymers with poly(ethylene glycol) (PEG) segments.[22] Unlike polyion complexes formed from an oppositely charged pair of simple homopolymers or statistical copolymers, PIC micelles from charged block copolymers are totally water-soluble and narrowly distributed. This was evidenced for both combinations of a pair of oppositely charged block copolymers[22] and an oppositely charged pair of block copolymer with a simple polyelectrolyte, including poly(amino acid) and oligo DNA.[23,24] A similar associate formation was recently found for vinyl polymer-based systems by Kabanov et al.[25]

A remarkable feature of PIC micelles as functional materials is that the PIC core can serve as a microreservoir of charged compounds, e.g., DNA and enzyme, allowing modulation of their inherent properties such as stability, solubility, and reactivity.[26−29] This motivated us to the present study of preparing a new type of PIC micelle composed of a pair of oppositely-charged block copolymers and proteins. It should be noted that the narrowly distributed protein/block copolymer micelle itself is a totally new entity of polyion complexes and is of great interest from the basic standpoint of supramolecular assembly of macromolecules. Further, the core of the PIC micelles may provide a unique field for enzymatic reaction because it forms a separated phase from the outer aqueous phase. The enzyme in the core might be active even under the conditions where the enzyme is usually inactive, e.g., high temperature and organic media, because of the segregation from the outer phase. In this sense, the core of the micelles is regarded as a nano-reactor.

We report the first preparation of water-soluble and narrowly distributed PIC micelles from chicken egg white lysozyme and poly(ethylene glycol)−poly(aspartic acid) block copolymer (PEG-P(Asp)). Lysozyme was selected as a model protein to incorporate into the micelle because it has a high isoelectric point (pI = 11),

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S0024-9297(97)01277-1 CCC: $15.00
Published on Web 01/27/1998

is positively charged over a wide range of pH, and has practical usage in drug delivery application as a lytic enzyme. Detailed physicochemical characteristics are also available for this protein which can be used as a component of the PIC micelle system to gain insight into the complexation mechanisms.

Experimental Section

Materials. α-Methoxy-ω-aminopoly(ethylene glycol) (M_w = 12 000) was a kind gift from Nippon Oil & Fats Co., Ltd., Japan. β-Benzyl L-aspartate *N*-carboxyanhydride (BLA-NCA) was synthesized from β-benzyl L-aspartate by the Fuchs−Farthing method using triphosgene.[30,31] Chicken egg white lysozyme was purchased from Sigma, St. Louis, MO, and used without further purification.

Synthesis of Poly(Ethylene Glycol)−Poly(α,β-aspartic Acid) (PEG-P(Asp)) Block Copolymers. PEG-P(Asp) block copolymers were prepared by alkali hydrolysis of the side chain benzyl groups of poly(ethylene glycol)−poly(β-benzyl L-aspartate) (PEG-PBLA) block copolymers which were synthesized by BLA-NCA polymerization initiated by the terminal primary amino group of α-methoxy-ω-aminopoly(ethylene glycol) under argon atmosphere in dimethylformamide (DMF).[22,30,31] The composition of the obtained PEG-PBLA was determined from 400 MHz ^{1}H NMR measurement (JEOL EX400, Japan) carried out in dimethyl sulfoxide (DMSO)-d_6 at 80 °C. The polymerization degree of BLA units was calculated to be 15 from the peak intensity ratio of the methylene protons of PEG (OCH_2CH_2: δ = 3.5 ppm) and the phenyl protons of the BLA unit ($COOCH_2C_6H_5$: δ = 7.3 ppm). Gel permeation chromatography (GPC) measurement was carried out in DMF including 10 mM LiCl at 40 °C using the combination of columns of TSK gel G4000H_{HR} and G3000H_{HR} (Tosoh, Japan). The GPC chromatogram of the obtained block copolymer was unimodal, and M_w/M_n was determined to be 1.03 using the calibration curve of PEG. Derivatization of the PEG-PBLA block copolymer to the PEG-P(Asp) block copolymer was carried out by alkali hydrolysis using 0.5 N NaOH. To check the degree of debenzylation as well as the purity of the obtained polymer, ^{1}H NMR measurement in D_2O was carried out. Quantitative debenzylation was confirmed from the disappearance of the peaks corresponding to the benzyl group. Peaks in the 400 MHz ^{1}H NMR spectrum were all assignable to protons derived from PEG-P(Asp), indicating a high purity of the polymer sample thus obtained.

Preparation of Polyion Complex Micelle. Given amounts of chicken egg white lysozyme and PEG-P(Asp) were dissolved in phosphate buffer solution (PBS, 10 mM, pH 7.4, $Na_2HPO_4\cdot 12H_2O$; 2.865 g/L, $NaH_2PO_4\cdot 2H_2O$; 0.312 g/L). After filtration through a 0.1 mm filter to remove dust, these solutions were mixed in various mixing ratios at ambient temperature to form PIC micelles. The concentrations of the solutions used in each series of experiments are given in the figure legends. The number of aspartic acid residues in PEG-P(Asp) against the total number of lysine and arginine residues in chicken egg white lysozyme (r = [Asp in PEG-P(Asp)]/[Lys and Arg in lysozyme]) was used as a major parameter in these experiments.

Light Scattering Measurements. Light scattering measurements were carried out using a DLS-700 instrument (Otsuka Electronics Co., Ltd., 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 25.0 ± 0.2 °C.

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

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

where $g^{(2)}(\tau)$ is the normalized second order correlation function, β is a parameter of the optical system (constant), $g^{(1)}(\tau)$ is the normalized first order correlation function, τ is the delay time, and $\bar{\Gamma}$ is the average characteristic line width. $g^{(1)}(\tau)$ can be expressed by the following equation:

$$g^{(1)}(\tau) = \int G(\Gamma) \exp(-\Gamma\tau)\, d\Gamma \qquad (2)$$

where $G(\Gamma)$ is a distribution function of Γ. The autocorrelation functions were analyzed using the method of cumulants in which

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

yielding an average line width $\bar{\Gamma}$, and a variance (polydispersity index) $\mu_2/\bar{\Gamma}^2$. In the cumulant approach, the Z-averaged diffusion coefficient was obtained from the average line width, $\bar{\Gamma}$, based on the following equation:

$$\bar{\Gamma} = Dq^2 \qquad (4)$$

$$q = 4\pi n \sin(\theta/2)/\lambda \qquad (5)$$

where q is the magnitude of the scattering vector and q is the detection angle. The corresponding hydrodynamic radius, R_h, can then be calculated using the Stokes−Einstein equation:

$$R_h = k_B T/(6\pi\eta D) \qquad (6)$$

where k_B is the Boltzmann constant, T is the absolute temperature, and η is the solvent viscosity. Also, the size distribution was estimated from the correlation function profile using the histogram method.[32] The diameter range of the size distribution was held from 1 to 200 nm.

In the static light scattering (SLS) measurements, the light scattered by a dilute polymer solution may be expressed as

$$KC/\Delta R(\theta) = 1/M_{w,app}\,[1 + q^2 R_g^2/3] + 2A_2C \qquad (7)$$

where C is the polymer concentration, $\Delta R(\theta)$ is the difference between the Rayleigh ratio of the solution and that of the solvent, $M_{w,app}$ is the apparent weight average molar mass, R_g^2 is the mean square radius of gyration, A_2 is the second virial coefficient, and $K = (4\pi^2 n^2 (dn/dc)^2)/(N_A\lambda^4)$ (N_A is Avogadro's number). The known Rayleigh ratio of benzene was used as a calibration standard.

The refractive index increments, dn/dc, of solutions were measured using a DRM-1020 double-beam differential refractometer (Otsuka Electronics Co.). The K in eq 7 is a function of the refractive index, dn/dc. The dn/dc of the system having several components as block copolymer micelles is expressed as a summation of the refractive index increments of each component:[33,34]

$$(dn/dc) = W_A(dn/dc)_A + W_B(dn/dc)_B + W_C(dn/dc)_C + \ldots \qquad (8)$$

where $(dn/dc)_A$, $(dn/dc)_B$ and $(dn/dc)_C$ are the refractive index increments and W_A, W_B, and W_C are the weight fractions of A, B, and C components, respectively. The dn/dc values for poly(ethylene glycol) homopolymer, poly(α,β-aspartic acid) homopolymer (DP = 18), PEG-P(Asp), lysozyme, and the associates of lysozyme and PEG-P(Asp) (PIC micelle) at r = 1.00, 1.60, 2.00, 2.667, and 4.00 in 10 mM PBS were measured, respectively, and are summarized in Table 1. The calculated values of dn/dc based on eq 8 were also given for PEG-P(Asp) and PIC micelles. The use of the $(dn/dc)_{cal}$ for the mixture solutions prepared at various mixing ratios seems to be suitable in the analysis of SLS, because the experimental values ($(dn/dc)_{exp}$) of PEG-P(Asp) and PIC micelles agree well with the calculated values ($(dn/dc)_{cal}$) based on eq 8.

Laser-Doppler Electrophoresis Measurement. Laser-Doppler electrophoresis measurement was carried out using an ELS-800 instrument (Otsuka Electronics Co.,Ltd.). The measurement was performed at 25.0 ± 0.2 °C with an electrical field strength of 30−35 V/cm. This instrument measures the particle velocity using a laser light scattering technique. Because of the Doppler effect, the frequency of the scattered

Table 1. The dn/dc Values of PEG, P)(Asp), PEG-P(Asp), Lysozyme, and Lysozyme/(PEG-P(Asp) Mixtures with Varying Composition

					lysome/PEG-P(Asp) mixtures			
	PEG	P(Asp)	PEG-P(Asp)	lysozyme	r=1.00	r=2.00	r=2.667	r=4.00
$(dn/dc)_{exp}$	0.1378	0.1559	0.1419	0.1927	0.1668	0.1513	0.1478	0.1437
$(dn/dc)_{cal}$[a]			0.1404		0.1660	0.1511	0.1477	0.1431

[a] The $(dn/dc)_{cal}$ was calculated from the $(dn/dc)_{exp}$ of PEG, P(Asp), and lysozyme using eq 8.

laser light is different from the frequency of the original laser beam. This frequency shift, the Doppler frequency, is related to the particle velocity. The relationship between the frequency shift and the electrophoretic mobility is expressed by the following equation:

$$u = (v_d\lambda)/[2En\sin(\theta/2)] \quad (9)$$

where v_d is the Doppler frequency, u is the electrophoretic mobility, E is the electrical field strength, n is the refractive index, λ is the wavelength of the original laser beam, and θ is the scattering angle.

From the determined electrophoretic mobility, the zeta-potential (ζ) was calculated by the Smoluchouski equation as follows:

$$\zeta = 4\pi\eta u/\epsilon \quad (10)$$

where η is the viscosity of the solution and ϵ is the dielectric constant of the solvent.

Results and Discussion

Preparation of Water-Soluble Polyion Complex Micelles. DLS measurements were performed for the solutions of lysozyme/PEG-P(Asp) prepared at various mixing ratios of r in the range between 0.125 and 4.00. As described in the experimental section, r is the ratio of the number of aspartic acid residues in PEG-P(Asp) to the total number of lysine and arginine residues in lysozyme (r = [Asp in PEG-P(Asp)]/[Lys and Arg in lysozyme]). The concentration of lysozyme in the solution was held constant (2.0 mg/mL), changing the PEG-P(Asp) concentration to modulate the r values. It should be noted that these solutions showed no precipitate formation and were optically clear even after 1 month storage at room temperature, which is in sharp contrast to the obvious and prompt precipitation observed in the mixture of lysozyme with P(Asp) homopolymer solutions. This apparent transparency of the lysozyme/PEG-P(Asp) system is due to the formation of water-soluble PIC micelles detectable through dynamic light scattering as described below.

Figure 1 shows the change in the cumulant diameter and polydispersity index ($\mu_2/\bar{\Gamma}^2$) with mixing ratio (r). The cumulant diameter remained constant (ca. 47 nm) in the range of $0.125 \le r \le 1.00$ and increased linearly from 47 to 80 nm at $1.00 \le r \le 4.00$. On the other hand, a decrease was observed in the polydispersity index from 0.10 to 0.05 with an increment in r from 0.125 to 1.00, which then remained constant with an extremely low value of 0.05 in the region of $1.00 \le r \le 4.00$. The narrowly distributed feature ($\mu_2/\bar{\Gamma}^2 < 0.1$) of the associates and the long-term stability over a wide range of mixing ratios are quite unique, suggesting that the associates may have some ordered structure, presumably, a core−shell structure where PEG segments form the corona and surround a core composed of ion-complexed lysozyme with P(Asp) segments of the block copolymers (PIC micelles), as is the case with the narrowly distributed micelle formation from PEG-P(Asp) and P(Lys).[24] Our previous study confirmed that

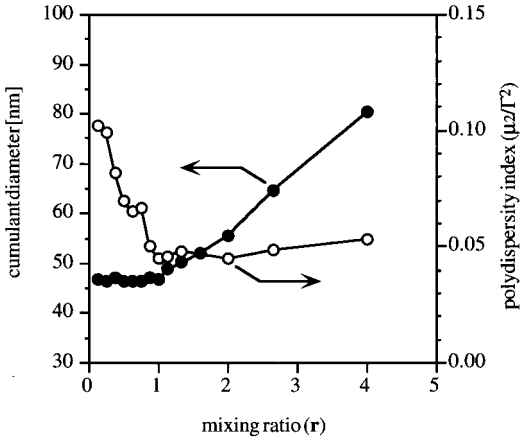

Figure 1. Change in the cumulant diameter (●) and the polydispersity index (○) with mixing ratio r for lysozyme/PEG-P(Asp) micelles. (Detection angle 90°; temperature 25.0 ± 0.2 °C; lysozyme concentration 2.0 mg/mL; variable with mixing ratio in the range of 0.28−8.91 mg/mL.)

mixing of P(Lys) with PEG-P(Asp) in aqueous medium led to the spontaneous formation of PIC micelles ($\mu_2/\bar{\Gamma}^2 \sim 0.05$) with extremely narrow distribution. Steric stabilization by the PEG corona was suggested for the P(Lys)/PEG-P(Asp) micelle system because of the very low absolute value of the zeta-potential. The diameter as well as the polydispersity index profiles shown in Figure 1 further indicated the existence of two regions with a boundary at r = 1.00, which may produce a substantial difference in the micelle properties. Thus, in this paper, the characteristics of micelles prepared in the region of $r \le 1.00$ are discussed with a special focus on the cooperative feature of PIC formation. Details of the micelles formed in the region of $r > 1.00$ will be reported in a forthcoming paper.[35]

Although the polydispersity indices at $0.125 \le r \le 1.00$ are sufficiently small to assume a narrow distribution, there is an obvious trend of a decrease in the polydispersity index with an increment in r in this region. This suggests that the distribution becomes narrower when r reaches 1.00. In line with this trend of the polydispersity index estimated from the cumulant analysis, the histogram analysis of the DLS data shown in Figure 2 gives a bimodal distribution at $r < 1.00$. Other than a main fraction with a diameter of ca. 50 nm, a minor fraction with a smaller diameter (4.5 nm) is observed. At r = 1.00, the distribution became unimodal with an average diameter of 52 nm.

It is worth noting that the major fraction in the z-weighted distribution obtained by the histogram method has a constant diameter of ca. 50 nm regardless of the r value in this region ($r < 1.00$), suggesting that the composition of these major fractions may be unchanged with the mixing ratio. The results of zeta-potential measurement further support this assumption. As summarized in Table 2, the zeta-potentials of the associates have a very small absolute value regardless of the mixing ratio (r), suggesting the compositional

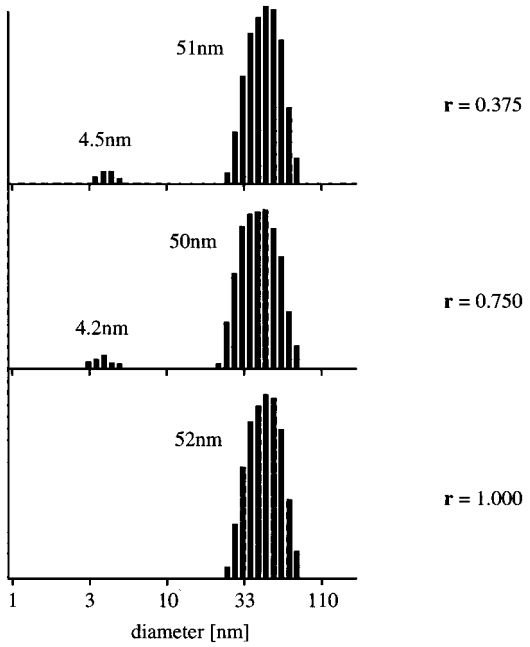

Figure 2. Change in z-weighted size distribution obtained from the histogram analysis of DLS with the mixing ratio *r*. (Temperature 25.0 ± 0.2 °C; total concentration 2.5 mg/mL; detection angle 90°; size range of analysis 1−200 nm.)

Table 2. Zeta-Potential of Lysozyme/PEG-P(Asp) Micelles Prepared at Various Mixing Ratios

mixing ratio (*r*)	zeta-potential [mV]
0.125	0.87 ± 0.65
0.250	0.83 ± 0.48
0.375	0.73 ± 0.59
0.500	0.99 ± 0.55
0.625	1.00 ± 0.78
0.750	0.99 ± 0.82
0.875	0.15 ± 0.60
1.000	−0.06 ± 0.58

similarities of these associates. Further, these small absolute values of the zeta-potential suggest that these micelles are likely to have a PEG corona to sterically stabilize their dispersion in aqueous medium. Lysozymes should be embedded in the core of the micelle mainly through electrostatic interaction with the P(Asp) segments of the block copolymers. This observation of the charge neutrality of the micelles is in good accordance with our previous results on PIC micelles prepared from charged block copolymers and oligomers of natural or synthetic origin, in which core−shell architecture was proposed.[22−24]

It should be noted that in the DLS histogram shown in Figure 2 there is a fraction with a small size (4.5 nm). Presumably, this fraction is attributed to free lysozyme in the solution because the size of lysozyme given in the literature is ca. 4 nm,[36] which agreed with the average diameter of the smaller fraction in the DLS histogram.

These results of DLS as well as zeta-potential measurements strongly suggest that the micelle formation between lysozyme and PEG-P(Asp) in the range of $r \leq 1.00$ proceeds in a cooperative manner: All of the PEG-P(Asp) in the solution form a charge-neutralized micelle ($r = 1.00$) with lysozyme, with the excess lysozyme remaining in the solution as the free form. With an increase in the *r* value approaching 1.00, the fraction of free lysozyme in the system should decrease providing a cooperative complexation mechanism, which is in line with a linear decrease in the polydispersity index with

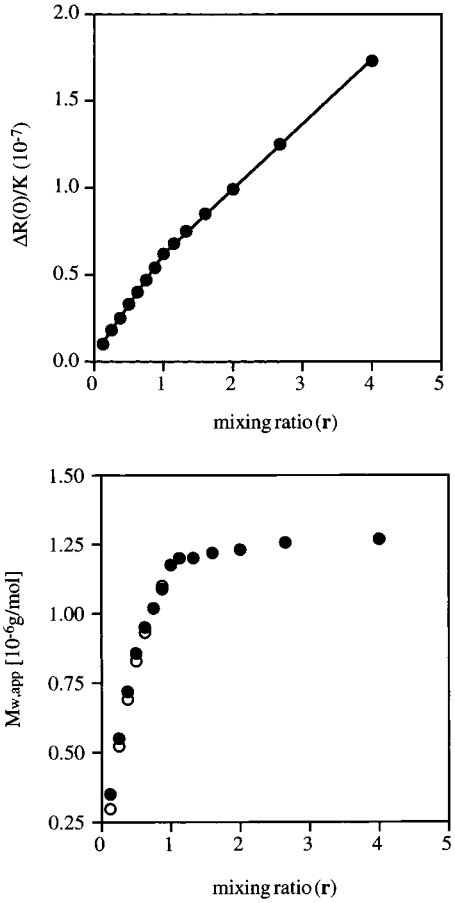

Figure 3. Change in $\Delta R(0)/K$ (a) and $M_{w,app}$ (b) with the mixing ratio *r* obtained from SLS. In (b), closed symbols are experimental values and open symbols are theoretical values. (Temperature 25.0 ± 0.2 °C; detection angle 30, 45, 60, 75, 90, 105, 120, 135, and 150°; lysozyme concentration 2.0 mg/mL; PEG-P(Asp) concentration, variable with mixing ratio in the range of 0.28−8.91 mg/mL.)

r determined from cumulant analysis of the DLS data (Figure 1).

SLS Measurements. The cooperative feature of PIC micelle formation in this region of $r \leq 1.00$ was further supported from SLS measurements. The same samples used in DLS measurements were subjected to SLS measurements with detection angles from 30 to 150°. The dn/dc values calculated from eq 8 were applied to analyze the data. When the data are extrapolated to 0°, the scattering vector, *q*, in eq 7 becomes zero. Equation 7 can then be expressed by the following equation (Debye plots);

$$KC/\Delta R(0) = 1/M_{w,app} + 2A_2C \qquad (11)$$

The second virial coefficient, A_2, in eq 11 might be neglected for polymeric micellar systems in a selective solvent, because they generally have quite small A_2 values.[37,38] Consequently, eq 11 may be expressed as follows:

$$KC/\Delta R(0) \approx 1/M_{w,app} \qquad (12)$$

In this manner, the apparent weight average molar mass, $M_{w,app}$, can be estimated from SLS measurements using eq 12.

Figure 3 shows the change in $\Delta R(0)/K$ (Figure 3a) and $M_{w,app}$ (Figure 3b) with the mixing ratio. The $\Delta R(0)/K$

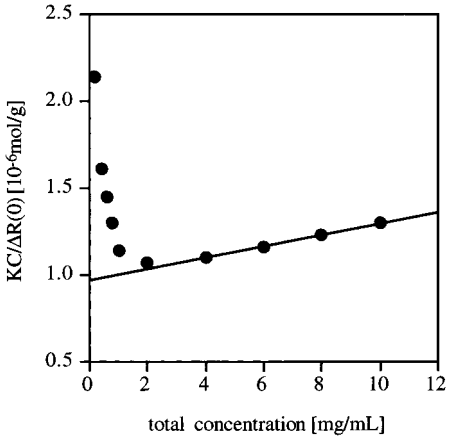

Figure 4. Estimation of the critical association behavior of lysozyme/PEG-P(Asp) micelles prepared at the stoichiometric mixing ratio (r = 1.00). (temperature 25.0 ± 0.2 °C; detection angles, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, and 150°.)

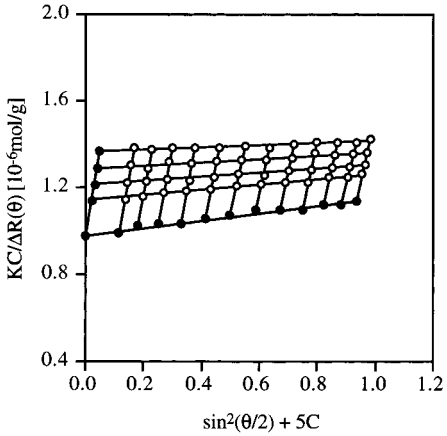

Figure 5. Zimm plots for lysozyme/PEG-P(Asp) micelles prepared at the stoichiometric mixing ratio (r = 1.00). (temperature, 25.0 ± 0.2 °C; detection angles, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, and 150°; concentration 4.0, 6.0, 8.0, and 10.0 mg/mL.)

was determined by extrapolating the linear plots of $\Delta R(\theta)/K$ vs $\sin^2(\theta/2)$ to the intercept. In this way, the variance in dn/dc with r can be corrected. As is the case with the cumulant analysis of DLS data shown in Figure 1, $\Delta R(0)/K$ vs r plots (Figure 3a) were discriminated into two linear regions with a boundary at $r = 1.00$. Correlation coefficients were 0.998 for $0 \le r \le 1.00$ and 1.000 for $1.00 \le r \le 4.00$. This change in the slope again indicates that the manner of the association is different in these two regions. The $\Delta R(0)/K$ vs r plots in Figure 3a were then converted into $M_{w,app}$ using eq 12 (Figure 3b). Experimental values of $M_{w,app}$ at each mixing ratio (r) in the region of $0 \le r \le 1.00$ are in good agreement with the calculated $M_{w,app}$ assuming a cooperative association mechanism (open symbols in Figure 3b), in which PIC micelles coexist with free lysozyme molecules. Complex formation in a cooperative manner is consistent with previous studies on lysozyme complexation with anionic polyelectrolytes, including potassium poly(vinyl alcohol) sulfate,[36] poly(acrylic acid),[39,40] and carboxymethyl cellulose,[41] in which cooperative precipitation was evidenced. Indeed, the stoichiometric precipitation was also confirmed by us for the mixed solutions of lysozyme with P(Asp) homopolymer (DP = 18) and α-methoxy ω-hydroxypoly(ethylene glycol) (M_w = 12 000) through the measurements of lysozyme concentration in the supernatant (data not shown). It was indicated that block copolymerization of PEG with the P(Asp) segment is essential to obtain water-soluble and narrowly distributed micelles entrapping lysozymes in a cooperative manner.

Providing that the micelles form so as to neutralize the Lys and Arg residues in lysozyme with Asp residues in PEG-P(Asp), the association numbers of lysozyme and PEG-P(Asp) in the micelle can be estimated from $M_{w,app}$. This is a reasonable assumption considering the good agreement in the measured and calculated values of $M_{w,app}$ shown in Figure 3b. Note that this calculation was based on the assumption that the Lys and Arg residues in lysozyme should be neutralized with Asp residues in PEG-P(Asp). The concentration dependence of the molecular weight of PIC micelles was then estimated under stoichiometric condition ($r = 1.00$) by extrapolating $KC/\Delta R(0)$ vs C plots to infinite dilution according to eq 12. Figure 4 shows the relationship between $KC/\Delta R(0)$ and the total concentration (C). Although the plot follows a straight line nicely with a moderate slope (correlation coefficient: 0.995), corresponding to A_2 in eq 11, in the region higher than 2.0 mg/mL, a steep increase in the $KC/\Delta R(0)$ value was observed below this critical concentration. Obviously, there is a remarkable decrease in $M_{w,app}$ around this concentration. A similar phenomenon of the critical concentration in $KC/\Delta R(0)$ vs C plots was previously reported by Eisenberg et al. for polymeric micelles from polystyrene−poly(sodium acrylate) block copolymer in tetrahydrofuran (THF), polystyrene−poly(4-vinylpyridine) block copolymer in toluene, and polystyrene−polyisoprene block copolymer in *n*-hexadecane and was attributed to the critical association concentration (c.a.c.) by considering the polydispersity of the block copolymer.[42] Note that PIC micelles prepared from lysozyme and PEG-P(Asp) showed c.a.c. behavior similar to that observed for polymeric micelles prepared in selective solvents. Nevertheless, the c.a.c. was not precisely identified from this plot because the $KC/\Delta R(0)$ value still increased under the most diluted experimental condition. Zimm plots shown in Figure 5 were made from the data of 4.0, 6.0, 8.0, and 10.0 mg/mL to determine $M_{w,app}$ and A_2. Because of an uncertainty in the c.a.c. value, correction based on the c.a.c. was not done in these plots. The $M_{w,app}$ and A_2 values thus obtained are summarized in Table 3. The A_2 has a considerably small absolute value. To provide the charge stoichiometry ($r = 1.00$) of the PIC micelle, the association number was calculated from the $M_{w,app}$ of PIC micelles using the molecular weights of lysozyme (14 300 g/mol) and PEG-P(Asp) (14 600 g/mol). It was revealed that the PIC micelles formed from lysozyme and PEG-P(Asp) consist of 36 molecules of lysozyme and 42 chains of PEG-P(Asp) in the charge-neutralized condition.

Table 3. Light Scattering Data of Lysozyme/PEG-P(Asp) Micelles

	association number[b]					
$M_{w,app}$[a] [g/mol]	PEG-P(Asp)	lysozyme	R_g[a] [nm]	R_h[c] [nm]	R_g/R_h	A_2[a] [mol·mL/g²]
1 082 200	42	36	17.1	24.1	0.710	$1.927 \cdot 10^{-5}$

[a] These values were determined from Zimm plots. [b] The association number was calculated from $M_{w,app}$ and average molecular weight of lysozyme and PEG-P(Asp). [c] The R_h was calculated from a dependence of the diffusion coefficient on concentration by using Stokes−Einstein equation.

Estimation of Narrowly Distributed PIC Micelles Prepared at a Stoichiometric Mixing Ratio.

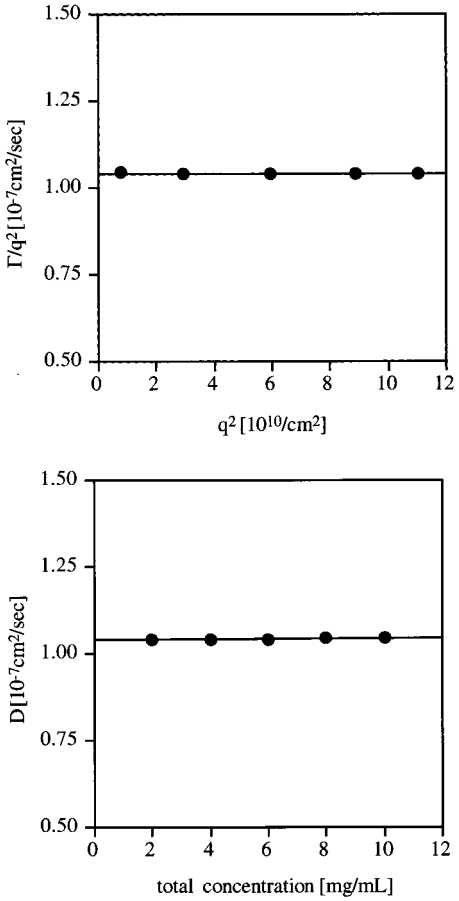

Figure 6. Dependence of diffusion coefficient on detection angle (a) and total concentration (b) of lysozyme/PEG-P(Asp) micelles prepared at the stoichiometric mixing ratio (r = 1.00). The angular dependence was confirmed for the solutions prepared at 5.0 mg/mL and the detection angles were 30, 60, 90, 120, and 150°. The measurements for concentration dependence were carried out at a 90°detection angle. (Temperature 25.0 ± 0.2 °C.)

Detailed analysis based on a cumulant approach was then carried out for the micelle prepared at $r = 1.00$. Figure 6 shows the dependence of the diffusion coefficient (D) on detection angles (Figure 6a as well as concentrations (Figure 6b) for lysozyme/PEG-P(Asp) micelles. For spherical particles, the Γ/q^2 values ($= D$) should be independent of the scattering vector (detection angle) because of the undetectable rotational motion.[33] The plots presented in Figure 6a clearly showed that the Γ/q^2 values were independent of the scattering vector, suggesting that the micelle may have a spherical shape. Further, the D values were also independent of the concentration (Figure 6b), indicating that the increment in total concentration induces no formation of secondary aggregates (the cluster of micelles). It is likely that the PEG corona may work effectively to prevent micelle aggregation because of a steric replusion mechanism, which is consistent with the formation of a core−shell structure. From Figure 6b, the diffusion coefficient at infinite dilution was determined to be 1.0423×10^{-7} cm^2/sec, and then the hydrodynamic radius (R_h) was calculated to be 23.63 nm using eq 6. As summarized in Table 3, the ratio of the R_g obtained from SLS and the R_h obtained from DLS, R_g/R_h, is calculated to be 0.710, which is close to the theoretical value (0.776) of R_g/R_h for a hard sphere.[43] Yet, the smaller experimental value compared to the theoretical value may reflect a variance in segment densities in a radial direction. On the other hand, it should be noted that a star molecule having no core is calculated to have an R_g/R_h ratio of 0.707,[44] very close to the data ($R_g/R_h = 0.710$) obtained for lysozyme/PEG-P(Asp) micelle systems. A starlike micelle model with a small condensed core surrounded by expanded chains of PEG may then be adopted in this case. For a "meander model", in which the chain is twisted into an expanded helical coil, the end-to-end distance should be 34.4−38.3 nm for PEG with M_w of 12 000 (DP = 273).[45] Another extreme conformation for an elongated PEG chain is a "zigzag conformation" where the distance should be 95.5 nm.[45] On the other hand, a value of 5.8 nm is calculated for the end-to-end distance of the PEG chain in a "random coil conformation".[45] The experimental value of R_h (24 nm) in Table 1 suggests that PEG chains in the micelle may not take a fully elongated conformation, which is in line with results reported for other block copolymer micelles with core−shell architecture.[33]

Conclusions

In this study, PIC micelles entrapping peptidyl compounds were prepared through electrostatic interactions between chicken egg white lysozyme as a cationic peptide and poly(ethylene glycol)-poly(aspartic acid) block copolymer as the anionic counterpart. In a sharp contrast with the immediate precipitation that follows the mixing of lysozyme and P(Asp) homopolymer solutions, the mixture of lysozyme and PEG-P(Asp) solutions spontaneously formed water-soluble and narrowly distributed PIC micelles. Micellization proceeds in a cooperative manner at $r \leq 1.00$, and stoichiometry was confirmed by both dynamic and static light scattering measurements. Lysozyme/PEG-P(Asp) micelles thus prepared had a spherical shape and were narrowly distributed with an average hydrodynamic diameter of 47 nm. Further, the micelles had an extremely low absolute value of zeta-potential, suggesting that the PIC core was covered with non-ionic PEG segments to form a core−shell architecture. Thirty-six molecules of lysozyme with 42 chains of PEG-P(Asp) were estimated to be packed in the condensed core, which is certainly of great interest from the viewpoint of applying this system as a nano-reactor. Indeed, an accelerated enzymatic reaction was recently observed for this lysozyme/PEG-(Asp) micelle system, the details of which will be reported in our forthcoming paper.[46]

Acknowledgment. The authors would like to thank Dr. Takao Aoyagi, Tokyo Women's Medical College, for valuable discussions and Mr. Tsuyoshi Matsumoto, Science University of Tokyo, for his assistance in polymer synthesis. Financial support by the Ministry of Education, Science, and Culture, Japan (a Grant-in Aid for Scientific Research: Priority Area Research Program−Supramolecular Structures) is highly appreciated.

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MA971277V