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Atsushi Harada[ab]; Kazunori Kataoka[a]
[a] Department of Materials Science & Technology, Science University of Tokyo, Noda, Chiba, Japan [b] Research Institute for Biosciences, Science University of Tokyo, Noda, Chiba, Japan

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FORMATION OF STABLE AND MONODISPERSIVE POLYION COMPLEX MICELLES IN AQUEOUS MEDIUM FROM POLY(L-LYSINE) AND POLY(ETHYLENE GLYCOL)-POLY(ASPARTIC ACID) BLOCK COPOLYMER

Atsushi Harada[1,2] **and Kazunori Kataoka** *,[1]
[1]Department of Materials Science & Technology
and [2]Research Institute for Biosciences
Science University of Tokyo
2641 Yamazaki, Noda
Chiba 278, Japan

ABSTRACT

In this study, the formation of polyion complex micelles from a pair of poly(L-lysine) homopolymers (P(Lys)) and poly(ethylene glycol)-poly(aspartic acid) block copolymers (PEG-P(Asp)) with varying chain length was demonstrated in aqueous medium. There exists the lower critical chain length in the charged segments of both P(Lys) and PEG-P(Asp) to form stable polyion complex micelles in nanometric scale. The scaled average characteristic line width ($\bar{\Gamma}K^2$) was independent on the detection angles for all combinations, suggesting that the formed polyion complex micelles may have a spherical shape. Furthermore, the transitional diffusion coefficient (D_T) had no concentration dependence, indicating the micelle system was free from secondary aggregates (the cluster of micelles). It is of interest that the micellar size was almost constant (ca. 50 nm) regardless of the change in the chain length of the charged segments. Size distribution was extremely narrow, and the values of variance ($\mu_2/\bar{\Gamma}^2$) were always less than 0.1. Laser-Doppler electrophoresis measurements revealed that the polyion complex micelles were electrically neutral, suggesting that the PEG corona surrounding the polyion complex core may contribute to their stable dispersion in an aqueous medium through steric repulsion of the tethered hydrophilic chain, in this case, PEG. This sys-

tem was considerably stable against the change in ionic strength, and it maintained a constant diameter in the region below 0.4 M NaCl. However, they dissociated under high ionic strength condition as 0.6 M NaCl. The system may have potential utility to include charged peptides and nucleotides in the core, delivering these biologically useful substances into a target site in the body.

INTRODUCTION

The self-assembly of block copolymers to form polymeric micelle in aqueous medium has recently received considerable attention from both fundamental and practical viewpoints, and the detailed studies on their physicochemical charac-teristics including size distribution, determination of thermodynamical parameters (i.e. critical micelle concentration, etc.) and the kinetics of association have been carried out by many research groups including ourselves using techniques such as TEM, SEM, light scattering and fluorescence [1-17]. The most thoroughly surveyed system of polymeric micelles in aqueous medium is the one composed of a hydrophobic core and hydrophilic corona with ionic or nonionic character. Such polymeric micelles prevent themselves from aggregation due to the repulsive nature of the hydrophilic corona surrounding the water-incompatible core. Obviously, an interaction other than the hydrophobic interaction might be applied as the driving force for the formation of polymeric micelles in aqueous medium. We have recently found the formation of spherical polyion complex micelles with extremely narrow size distribution based on the electrostatic interaction of a pair of oppositely charged block copolymers, poly(ethylene glycol)-poly(L-lysine) block copolymer (PEG-P(Lys)) and poly(ethylene glycol)-poly(aspartic acid) block copolymer (PEG-P(Asp)) [18]. It is worth noticing that similar monodispersive associates were found to form when even one of the pair was charged from a block copolymer to changed oligomers with synthetic or natural origin [19].

In this paper, we report a detailed study on the formation of polyion complex micelles from poly(L-lysine) homopolymer (P(Lys)) and PEG-P(Asp). It was confirmed from this study that the polyion complex micelles thus prepared have extremely narrow size distribution and a spherical shape, although there exists the lower critical chain length of P(Asp) and P(Lys) to form stable polyion complex micelles. The size of polyion complex micelles thus formed maintained an almost constant value (the diameter of ca. 50 nm) even if the chain length of P(Asp) and P(Lys) varied in the range of DP (degree of polymerization) of P(Asp) = 18 to 78 and DP of P(Lys) = 20 to 45.

EXPERIMENTAL

Materials

ε-(Benzyloxycarbonyl)-L-lysine and ß-benzyl L-aspartate were purchased from Peptide Institute, Inc., Japan, and used without further purification. Bis(trichloromethyl) carbonate (triphosgene) and 30% hydrobromide in acetic acid (HBr/AcCOOH) were purchased from Tokyo Kasei Kogyo Co., Ltd., Japan, and used without further purification. α-Methoxy-ω-aminopoly(ethylene glycol) (Mw = 5000) was a generous gift from Nippon Oil & Fats Co., Ltd., Japan. n-Butylamine was purchased from Wako Pure Chemical Industries, Ltd., Japan, and was distilled by a general method. N, N-dimethylformamide (DMF) and chloroform ($CHCl_3$) were doubly distilled by general methods. Lys-Lys-Lys-Lys-Lys was purchased from SIGMA Chemical Co. and used without further purification.

Synthesis of Poly(L-lysine) Homopolymer (P(Lys))

Poly(L-lysine) homopolymer (P(Lys)) was prepared by the ring-opening polymerization of ε–(benzyloxycarbonyl)-L-lysine-N-carboxyanhydride (Lys(Z)-NCA), which was synthesized by the Fuchs-Farthing method using triphosgene [18], initiated by the *n*-butylamine. Lys(Z)-NCA was dissolved in doubly distilled DMF followed by the addition of *n*-butylamine. The mixture was stirred for 24 hours at 40°C under a dry argon atmosphere and then precipitated into diethyl ether. The polymerization degree (DP) of P(Lys(Z)) was estimated by 400-MHz ^{1}H-NMR (JEOL EX400) in DMSO-*d6* from the peak intensity ratio of the methyl protons of *n*-butylamine ($CH_3(CH_2)_3NH$: δ = 0.8 ppm) and the phenyl protons of ε-(benzyloxycarbonyl) group of Lys(Z) ($CH_2C_6H_5$: δ = 7.3 ppm). In this study, two P(Lys(Z)) with a different polymerization degree, i.e., 20 and 45, were prepared. The obtained P(Lys(Z)) was dissolved in HBr/AcCOOH and stirred for 2 hours to remove the ε-(benzyloxycarbonyl) group. After precipitation into diethyl ether, the obtained product was redissolved in distilled water and dialyzed against distilled water using a Spectropor 6 dialysis membrane (molecular weight cut off = 1000). Finally, a dried powdered sample of P(Lys) was obtained by lyophilization. The DP of the obtained P(Lys)s was confirmed to be unchanged through the deprotection process from the peak intensity ratio of the methyl protons of *n*-butylamine ($CH_3(CH_2)_3NH$: δ = 0.8 ppm) and the ε-methylene protons of Lys ($(CH_2)_3CH_2NH_3$: δ = 3.0 ppm) in ^{1}H-NMR spectra in D_2O at room temperature.

Synthesis of Poly(Ethylene Gycol)-Poly(Aspartic Acid) Block Copolymer (PEG-P(Asp))

The synthetic procedure of PEG-P(Asp) was previously reported [20, 21]. Briefly, poly(ethylene glycol)-poly(ß-benzyl L-aspartate) block copolymer was synthesized by the ring-opening polymerization of ß-benzyl L-aspartate-N-carboxyanhydride initiated by α-methoxy-ω-aminopoly(ethylene glycol) (PEG). The polymerization was carried out in the mixture of doubly distilled $CHCl_3$ and DMF at 40°C. The obtained PEG-PBLA was debenzylated using 0.5 N NaOH. ^{1}H-NMR measurements in D_2O were carried out to determine the composition. From the peak intensity ratio of the methylene protons of PEG (OCH_2CH_2: δ = 3.7 ppm) and the methylene protons of α,ß-amide of P(Asp) (δ = 2.7 ppm), the compositions of prepared samples were 8, 18 and 78, respectively, which are abbreviated as 5-8, 5-18 and 5-78. It should be noted that the intramolecular isomerization of aspartic acid units in the block copolymer taking place to form ß-aspartate units occurred during the deprotection process. The ratio of α– and ß-aspartate units in the P(Asp) segment was determined to be 1:3 by ^{1}H-NMR.

Preparation of Polyion Complex Micelle

Given amounts of P(Lys) and PEG-P(Asp) were separately dissolved in a phosphate buffer (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). Polyion complex micelle solution was then prepared by mixing these two solutions in an equal molar ratio of L-lysine and aspartic acid residues. The solutions were stored overnight at room temperature before carrying out the characterization.

Dynamic Light Scattering Measurements

Dynamic light scattering (DLS) measurements were carried out using a DLS-700 instrument (Otsuka Electronics Co., Ltd.), equipped with an Ar ion laser (λ_0 = 488nm) as an incident beam. All measurements were performed at 23.6 ± 0.1 °C.

The general formula for the photoelectron count time correlation function has the form:

$$g^{(2)}(\tau) = 1 + ß \mid g^{(1)}(\tau) \mid^2 = 1 + ß \exp(-2 \bar{\Gamma}\tau) \quad (1)$$

where $g^{(2)}(\tau)$ is the normalized second-order correlation function, ß 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. In the

case of polydisperse systems, $g^{(1)}(\tau)$ can be expressed by the following equation:

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

where $G(\Gamma)$ is a distribution function of Γ. In the present analysis, the auto-correlation functions were analyzed using the method of cumulants in which

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

yielding an average line width, $\bar{\Gamma}$, and a variance (polydispersity index), $\mu_2/\bar{\Gamma}^2$.

For spherical particles, $\bar{\Gamma}$, is related to the transitional diffusion coefficient, D_T, provided that the internal motions are negligible. In the cumulant approach, the z-weighted D_T was obtained from $\bar{\Gamma}$ based on the following equation:

$$\bar{\Gamma} = D_T K^2 \quad (4)$$

$$K = (4\pi n/\lambda)\sin(\theta/2) \quad (5)$$

where n is the refractive index of the solvent, K is the magnitude of the scattering vector.

In the diluted concentration region, the concentration dependence of the D_T can be expressed by a first-order expansion:

$$D_T = D_0(1 + k_d C) \quad (6)$$

where D_0 is the D_T at infinite dilution, k_d is the diffusion second virial coefficient, and C is the concentration. The corresponding hydrodynamic diameter, d_h, can then be calculated using the Stokes-Einstein equation:

$$d_h = k_b T/3\pi\eta D_0 \quad (7)$$

where k_b is the Boltzmann constant, T is the absolute temperature, and η is the solvent viscosity.

The estimation of the size distribution was carried out from the correlation function profile by the use of histogram analysis software. In the histogram method [22], Equation 2 is replaced by

$$g^{(1)}(\tau) = \Sigma G(\Gamma_i) \exp(-\Gamma\tau)\Delta\Gamma \quad (8)$$

and $G(\Gamma_i)$ was determined using the Marquart nonlinear least squares routine. $G(\Gamma_i)$, which is the distribution according to the ratio of light scattering by the particles with Γ, was then converted into the particle size distribution, G(d), using

Equations 4 and 7. The size distribution according to the weight and number ratios was then determined from G(d).

Laser-Doppler Electrophoresis Measurements

Laser-Doppler electrophoresis measurements were carried out using an ELS-800 instrument (Otsuka Electronics Co., Ltd.), which is equipped with a He/Ne laser (λ_0 = 632.8 nm) as an incident beam. The measurements were performed at 25.0 ± 0.2°C with an electrical field strength of 32-36 V/cm. This instrument measures the particle velocity using a laser light scattering technique. Due to the Doppler effect, the frequency of the scattered 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:

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

where ν_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 detection angle.

From the electrophoretic mobility, the zeta-potential was calculated by the Smouchouski equation as follows:

$$\xi = 4\pi\eta u / \varepsilon \quad (10)$$

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

RESULTS AND DISCUSSION

Formation of Polyion Complex Micelles

Polyion complexes were formed from various combinations of P(Lys) and PEG-P(Asp) with different chain length under stoichiometric conditions where the unit ratio of Lys in P(Lys) and Asp in PEG-P(Asp) is equal. The samples were prepared by mixing the phosphate buffer (10 mM, pH 7.4) of PEG-P(Asp) and P(Lys) at room temperature and allowing them to stand overnight before dynamic light scattering measurements. In any case, using either PEG-P(Asp) (5-8) or P(Lys) (5), no obvious increment in the scattering intensity was observed. On the other hand, considerable increment in light scattering intensity was obtained when the constituent polymer chains, either block copolymer or homopolymer, have a

polymerization degree higher than ca. 20, although the solution was visibly transparent. This result clearly indicates that there is a lower critical chain length to form polymer associates with nanometric size scale (polyion complex micelle). A similar threshold effect in terms of chain length was observed in general for coacervate or precipitate formation of polyion complexes made from a pair of various types of oppositely-charged polyelectrolytes [23]. Polyelectrolytes should need to have a certain number of charged groups along their chain to express cooperative polymer effect, allowing the formation of stable polyion complexes with cooperative and stoichiometric features. Polyelectrolytes with a DP shorter than the critical length behave like a low molecular weight counter-ion without showing any cooperative effect, thus, the system consisting of such shorter polyelectrolytes only yields weak associates in a non-stoichiometric manner. Obviously, the association force of such a system is too weak to form the micelle structure with a large association number. Worth noticing for the system consisting of PEG-P(Asp) and P(Lys) with sufficient chain length to show cooperative effect is that it never gave apparent turbidity even though a considerable increase in light scattering intensity was observed under a laser beam (488 nm). This is in sharp contract with the well-known phenomenon of precipitate or coacervate formation observed in the mixed solution of two oppositely-charged polyelectrolytes, and suggests the formation of associates in nanometric scale (polyion complex micelle) which was previously confirmed in the system consisting of a pair of oppositely charged block copolymers having a poly(ethylene glycol) chain (PEG-P(Asp) / PEG-P(Lys)). A combination of PEG-P(Asp) with P(Lys) having sufficient chain length led to the formation of polyion complex micelles with narrow distribution as described in the next section.

Dependence of the Diffusion Coefficient of Polyion Complex Micelles on the Detection Angle and the Concentration

In order to confirm whether the formed micelles have a spherical shape, angle-trace DLS measurements were performed at 30°, 60°, 90°, 120° and 150° of detection angles. For spherical particles, the scaled characteristic line width ($\bar{\Gamma}/K^2$) should be independent of the detection angle due to the undetectable rotational motion. Figure 1 shows the result of the angle-trace DLS measurements for P(Lys) (20)/ PEG-P(Asp) (5-78). Obviously, $\bar{\Gamma}/K^2$ has no angular dependence, suggesting that the formed associates had spherical shape without no clustering of micelles. Because the angular dependence of $\bar{\Gamma}/K^2$ is negligible, the following DLS measurements were performed at 90° of detection angle. Furthermore, we studied the dependence of the transitional diffusion coefficient (D_T) on the polymer

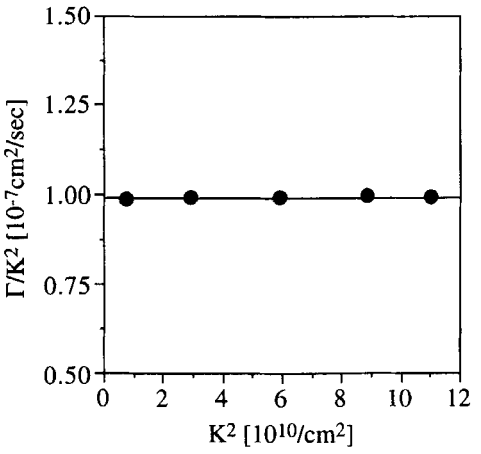

Figure 1. Relationship between the scaled average characteristic line width ($\bar{\Gamma}$ /K^2) and the magnitude of the scattering vector (K^2) for P(Lys) (20)/PEG-P(Asp) (5-78) (total concentration, 5.0 mg/mL; temperature, 23.6 ± 0.1°C; detection angle, 30°, 60°, 90°, 120° and 150°).

concentration. The DLS measurements were carried out for the solutions prepared at 1.0, 2.0, 5.0 and 10.0 mg/mL. Figure 2 shows the relationship between the D_T and the polymer concentration for P(Lys) (20) / PEG-P(Asp) (5-78). It was obvious that the D_T was independent of the polymer concentration and the diffusion second virial coefficient (k_d) in Equation 6 was almost zero. This suggests that the increment of polymer concentration did not induce the change in the diameter, i.e., no formation of the cluster of micelles. In line with this consideration, the size distribution obtained by histogram analysis maintained a unimodal profile in the measured concentration range. Figure 3 shows the weight- and number-converted size distribution of P(Lys) (20) / PEG-P(Asp) (5-78). The size distribution (d_w/d_n) is as narrow as 1.04. The diffusion coefficient at infinite dilution (D_0) was then obtained from Figure 2 using Equation 6. The hydrodynamic diameter (d_h) was then calculated using the Stokes-Einstein equation (Equation (7)). These values for all combinations are summarized in Table 1. The d_h determined for all combinations was almost constant and is ca. 50nm.

The coefficient of τ^2 in Equation 3 in the Experimental section gives the width of the distribution. The normalized coefficient of $\tau^2,\mu_2/\ \bar{\Gamma}/2$, is called variance and is used as an indication of the degree of polydispersity. The values of the variance for all the combinations are also summarized in Table 1. Obviously, these

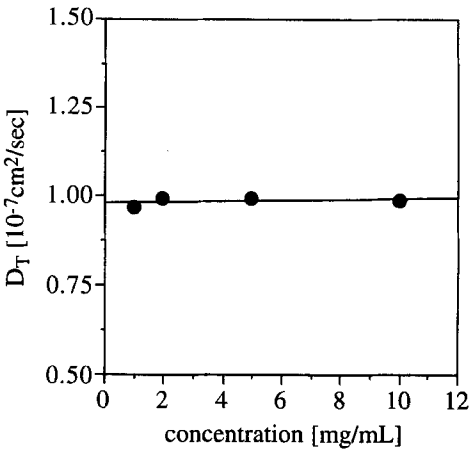

Figure 2. Plots of the transitional diffusion coefficient (D_T) against the total concentration for P(Lys) (20)/PEG-P(Asp) (5-78) (temperature, 23.6 ± 0.1°C; detection angle, 90°).

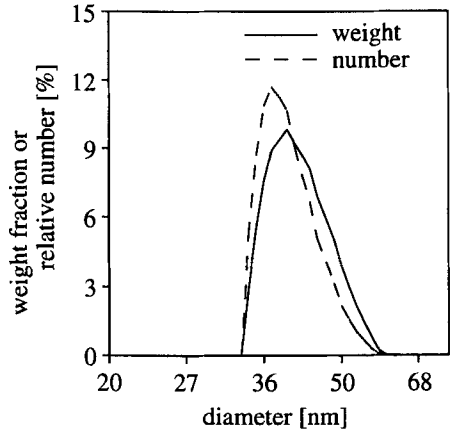

Figure 3. Size distribution for P(Lys) (20)/PEG-P(Asp) (5-78) obtained by histogram analysis of DLS measurement (total concentration, 2.0 mg/mL; temperature, 23.7° C; detection angle, 90°). Weight- and number-averaged diameters were 43.7 nm (d_w) and 41.9 nm (d_n), respectively, with the polydispersity (d_w/d_n) of 1.04.

TABLE 1. Dynamic Light Scattering Date of Polyion Complex Micelles

	P(Lys) / PEG-P(Asp)			
	20/5-18	20/5-78	45/5-18	45/5-78
D_0 [$10^{-7}cm^2/sec$]	0.9756	0.9860	0.9523	0.9591
d_h [nm]	48.64	48.04	49.64	49.18
μ_2/Γ^2 *	0.045	0.012	0.014	0.023

* These values were determined from the measurements at 5.0mg/mL.

values were all below 0.1, indicating that the system is regarded as essentially monodispersive without any formation of larger aggregates (the cluster of micelles). It is interesting to note that such a monodispersive polyion complex micelle can be prepared by just a simple mixing of solutions of oppositely-charged polyelectrolytes. Although there have been many attempts to obtain monodispersive polymeric micelles with a core surrounded by a palisade of tethered polymer chain (polymer brushes) in an aqueous medium from amphiphilic block copolymer, only a few examples have been known to have a narrow size distribution with a variance of below 0.1 [24]. Furthermore, in the process of micellization, a complicated procedure of solvent exchange from good solvent to selective solvent is always required. On the other hand, the micellization through polyion complex formation is surely a simple and reproducible way to construct a supramolecular assembly with a definite size and a palisade of tethered polymer chains. The formation of the core-shell structure of the polyion complex micelles was strongly indicated from zeta-potential measurement as described below.

Zeta-Potentials of Polyion Complex Micelles

The laser-Doppler electrophoresis measurements were performed in order to estimate the zeta-potential of polyion complex micelles. In this measurement, the electrophoretic mobility of polyion complex micelles, EPM, were determined based on the Doppler effect of the frequency of the scattered light, which is related to the particle velocity under an electric field. The zeta-potentials of polyion complex micelles were then calculated from the EMP using the Smouchouski equation

TABLE 2. Zeta-Potential of Polyion Complex Micelles

	P(Lys) / PEG-P(Asp)			
	20/5-18	20/5-78	45/5-18	45/5-78
EPM	-0.024	0.085	0.061	-0.042
zeta-potential [mV]	-0.364	1.314	0.951	-0.647

(Equation 10). The EMP and the zeta-potentials of polyion complex micelles are summarized in Table 2. The zeta-potentials for all combinations had an extremely small absolute value, consistent with the formation of a core-shell structure where the PEG corona blocks the micelle aggregation through steric repulsion.

Stability of Polyion Complex Micelles Against Ionic Strength Change

It is known that the stability of the polyion complex is strongly affected by the ionic strength of the medium: being destabilized with an increase in ionic strength due to electrostatic shielding [25, 26]. We estimated the effect of ionic strength on the stability of polyion complex micelles by measuring the DLS of polyion complex micelle solutions (4.0 mg/mL) after the addition of the phosphate buffer including sodium chloride at various concentrations. The samples were stored overnight before measurements. Figure 4 shows the change in the transitional diffusion coefficient (D_T) with ionic strength for P(Lys) (20)/PEG-P(Asp) (5-78). Similar trends were observed for all combinations. The D_T remained constant up to 0.4 M NaCl, indicating that the polyion complex micelles have a constant size in this region. The size distribution in the histogram profile remained unimodal as well. However, there was observed a considerable decrease in D_T, i.e., an increase in diameter, when NaCl concentration increased to 0.5 M. This may be due to a rearrangement of the micelle structure through the dehydration of the PEG corona induced by an increase in ionic strength. It should be noted that the cloud point of PEG in aqueous medium is known to be quite sensitive to the kind and concentration of co-existing electrolytes and monotonously decreases with NaCl concentration due to a change in the water structure around PEG [27]. Further increase in NaCl concentration to 0.6 M NaCl resulted in a steep decrease in light scattering intensity, suggesting the dissociation of the polyion complex micelles to

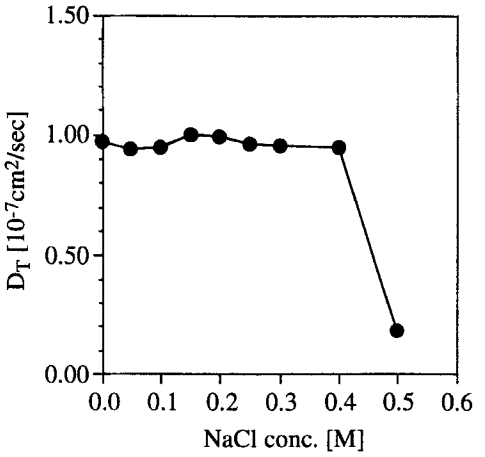

Figure 4. Change in the transitional diffusion coefficient with the concentration of sodium chloride for P(Lys) (20)/PEG-P(Asp) (5-78) (total concentration, 2.0 mg/mL; temperature 23.6 ± 0.1°C; detection angle, 90°).

constituent polyelectrolytes due to electrostatic shielding. To confirm the dissociation of polyion complexes under a high ionic strength condition, a similar experiment was carried out using the mixture of poly(L-lysine) and poly(α,β-aspartic acid) homopolymers. In 10 mM phosphate buffer, the mixture of these homopolymers induced turbidity. This was due to the formation of complex coacervates, which can be observed under the microscope to have diameter of ca. 10 mm. An increase in NaCl concentration to 0.6 M by the addition of NaCl in the system caused the solution to become transparent, which is in good agreement with a decease in light scattering intensity for the P(Lys)/PEG-P(Asp) system at this salt concentration. It should be noted that this change in the association is completely reversible; the micelle recovered its initial diameter and dispersity through a decrease in salt concentration by dilution. This result suggests that micelles are in thermodynamically equilibrium state.

CONCLUSIONS

In this study, we demonstrated by dynamic light scattering measurements the formation of polyion complex micelles having a narrow size distribution and spherical shape from a pair of oppositely charged block copolymer (PEG-P(Asp))

and homopolymer (P(Lys)). We also investigated the effect of the chain length of the charged segments on the stability and the diameter of polyion complex micelles. The chain length had a crucial influence on the stability of polyion complex micelles, and the use of P(Lys) and P(Asp) with a DP less than ca. 20 results in no detection of micelles having the diameter of several tens nm, indicating that there exists the critical chain length to form stable micelles. On the other hand, micelles formed from a pair having charged segments with a higher DP than the critical value always gave a diameter around 50 nm regardless of their chain length. Furthermore, the diameter remained constant even at a relatively high concentration (10.0 mg/mL). The formed micelles were confirmed to have a very small absolute value of zeta-potential, suggesting that micelles were sterically stabilized through the PEG palisade surrounding the polyion complex core.

This type of polyion complex micelle system might be useful as vehicles for charged oligopeptides and oligonucleotides in the field of drug targeting, because they have charged moieties in their backbone and may form polyion complex micelles with charged block copolymers with a PEG segment including PEG-P(Asp) and PEG-P(Lys). Their biologically-derived substances are segregated in the core of the micelles from the outer aqueous entity and, thus, may be prevented from enzymatic degradation. Indeed, as reported elsewhere [19], we have recently confirmed the formation of polyion complex micelles composed of antisense-oligonucleotides and cationic block copolymers.

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