PREPARATION OF pH-SENSITIVE CORE-SHELL TYPE POLYMERIC MICELLE FROM POLY(PEPTIDE-*b*-LACTIDE) DIBLOCK COPLYMERS AS BIODEGRADABLE MIOMEDICAL MATERIAL

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Introduction

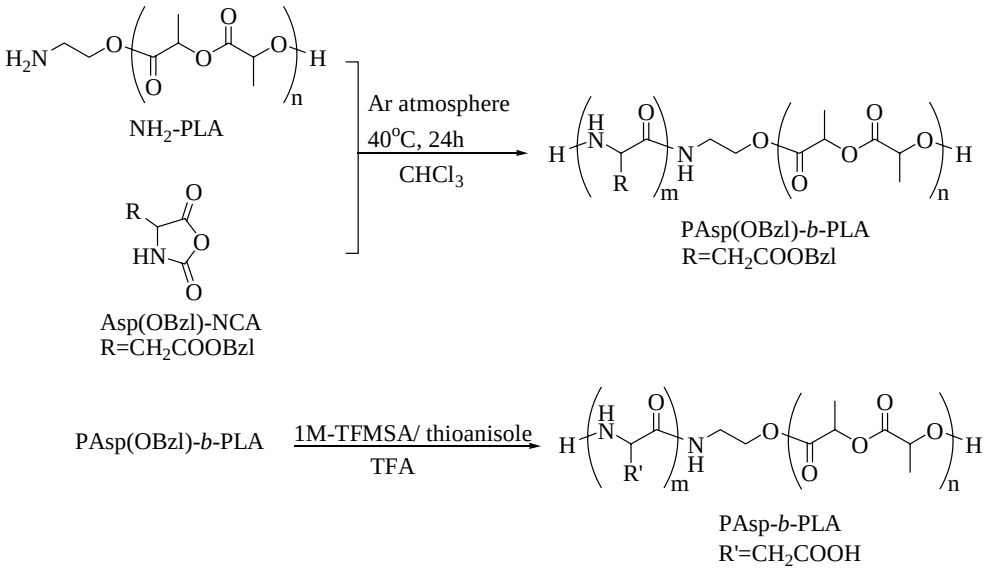
The formation of polymeric micelles by the self-association of diblock copolymers consisting of hydrophilic and hydrophobic segments in aqueous medium is currently a topic of great interest. This interest is motivated by the attractive applications of polymeric micelles to various research areas such as drug delivery carrier technology and nanotechnology. In the biomedical materials field, polymeric micelles must possess several specific properties to be of use. These include biocompatibility, biodegradability, target specificity, and stability in the body. Polylactide (PLA) is one of the most commonly used relatively hydrophobic and biodegradable polyesters that possess some of the above qualities. A number of polymeric micelles formed from PLA-based amphiphilic diblock copolymers have been investigated in terms of various biomedical applications. Poly(ethylene glycol) (PEG) is frequently chosen as a hydrophilic segment to complement PLA because of its biocompatibility. Kataoka *et al*. reported the formation of PEG-*b*-PLA diblock copolymer micelles with modified PEG chains and the application of this micelle as a drug carrier for active targeting[1]. They successfully modified the outer PEG layer of the micelles using an aldehyde group introduced at the PEG terminal. Polypeptides consisting of α-amino acids are synthetically intriguing as polymeric micelles due to their biodegradability and variation in functionality that results from the differences in side chain groups. Previously, we synthesized poly(aspartic acid)-*block*-polylactide (PAsp-*b*-PLA) diblock copolymer consisting of a hydrophilic/anionic PAsp segment and a hydrophobic PLA segment, and observed polymeric micelle formation in aqueous solution using a dialysis method.[2] In this study, details regarding the formation of these polymeric micelles are reported.[3] It is well known that the shape and size of polymeric micelles are affected by the ratio of the length of the hydrophilic and hydrophobic segments. To establish the relationship between this factor and polymeric micelle formation in this study, several kinds of PAsp-*b*-PLA copolymers having various hydrophilic/hydrophobic chain length ratios were synthesized. In addition, the sensitivity of the carboxylic acid groups of the PAsp segment to changes in the pH and the resulting effects on the PAsp-*b*-PLA polymeric micelles in aqueous solution were observed.

Experimental

Block Copolymer Synthesis

PLA having a primary amino group at the terminal (NH_2-PLA) was synthesized by the method reported previously.[4] Poly(aspartic acid)-*block*-polylactide (PAsp-*b*-PLA) having various peptide and PLA chain lengths were synthesized using NH_2-PLA as a macroinitiator according to the method reported in a previous paper.[2] Briefly, the polymerization of *N*-Carboxy-β-benzyl-L-aspartate anhydride,

Asp(OBzl)-NCA, using NH_2-PLA as a macroinitiator was carried out in chloroform at 40°C for 24 h under an Ar atmosphere (Scheme 1). Upon completion, the reaction mixture was poured into cooled methanol, which resulted in the precipitation of a white solid. The obtained PAsp(OBzl)-*b*-PLA was characterized by ^{1}H- and ^{13}C-NMR. The degree of polymerization of each segment was estimated from ^{1}H-NMR spectra reported previously.[2] The removal of the benzyl group was carried out by treatment with 1M-trifluoromethane sulfonic acid (TFMSA) /thioanisole/ trifluoroacetic acid (TFA). PAsp(OBzl)-*b*-PLA was dissolved in TFA followed by the addition of 1M-TFMSA/thioanisole. This mixture was then gently stirred at 0°C for 1 h. The reaction mixture was poured into cooled diethyl ether, which resulted in the precipitation of a white solid. The obtained polymer (PAsp-*b*-PLA) was dried under vacuum. Removal of the benzyl group was confirmed by ^{1}H NMR.



Scheme 1. Synthetic route of PAsp-b-PLA.

Preparation and Characterization of Polymeric Micelles

PAsp-*b*-PLA (20 mg) was dissolved in DMSO (3 ml) and placed in a permeable dialysis membrane (MW cut off: 500). The permeable membrane was immersed in pure water (Milli-Q grade) or various pH phosphate buffer solutions (PBS) and dialyzed for 17 h. Following the dialysis, the solution was centrifuged (12000 rpm) to separate the small amount of precipitate from the supernatant. The supernatant (3 ml) was then used for the dynamic light scattering (DLS) measurements, atomic force microscopy (AFM) observations and critical micelle concentration (CMC) estimations. DLS measurements were carried out on a DLS-7000 apparatus (Otsuka Electronics Co.) with vertically polarized incident light with a wavelength of 488 nm supplied by an argon laser operated at 15 mW. The AFM images were obtained in the tapping mode in air with a SPI 3800N/SPA400 (Seiko Instruments Inc.) using a scan speed of 1 Hz and data collection at 256×256 pixels. The AFM images were taken at a scan size of 1 μm × 1 μm in air. The pKa values of the carboxyl groups of the PAsp segments were determined by neutralization titrations. The polymeric micelle solutions were titrated with a 1.0×10^{-4} M NaOH solution. The pH of the solution was monitored using a pH meter (HORIBA 50-H).

Determination of Critical Micelle Concentration

The critical micelle concentration (CMC) was determined using pyrene as a fluorescence probe. Pyrene, a hydrophobic molecule, was preferentially distributed in the micelle core, causing changes in the photophysical properties. A saturated aqueous solution of pyrene (6×10^{-7} mol/L) was used for these experiments. The micelle concentration in these experiments varied from 1.0×10^{-5} mg/ml to 1.0 mg/ml. Fluorescence intensities of the pyrene entrapped in the micelle core were determined by an F-4010 fluorescence spectrophotometer (λ_{ex}=330 nm, Hitachi Co. LTD, Japan) at room temperature.

Cell Viability Test

Mouse fibroblast L929 cells were subcultured in E-MEM with 10% (v/v) fetal calf serum (FCS, JRH Bioscience), and were harvested using [PBS (-)] (Nissui pharmaceutical Co.) containing 0.025% (w/v) trypsin and 0.01% ethylenediaminetetraacetic acid. The cell viability after 48 h was evaluated by the following method: A suspension of the L929 cells (100 μl, 1.0×10^5 cells/ml) in E-MEM medium containing 10% FCS was added to a 96-well microplate and cultured in a humidified atmosphere containing 5% CO_2 at 37°C. After pre-incubation for 48 h, aliquots of a micelle solution were added to the cells, which were then further incubated for 48 h. At certain points during the incubation, the number of cells was measured using a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. An aqueous MTT solution (20 μl) was added to the well followed by incubation at 37°C. The cell viability was calculated using the following equation:

$$\text{Cell viability (\%)} = 100 - \frac{Nc - Nt}{Nc} \times 100$$

Where *Nc* and *Nt* are the number of cells without polymeric micelles and with polymeric micelles after 48 h of incubation, respectively.

Results and Discussions

To investigate the effects of the chain lengths on the formation of the polymeric micelles in aqueous solution, three kinds of PAsp-*b*-PLA-[m, n] with various chain lengths were synthesized according to Scheme 1, where m and n refer to the degree of polymerization of the amino acid unit and lactide unit, respectively. The results of the block copolymerization of Asp(OBzl)-NCA using NH_2-PLA as a macroinitiator are summarized in Table 1. In this reaction, the molecular weight of the macroinitiator did not affect the yield or degree of polymerization of the amino acid unit. The chain length of the PLA segment in PAsp-*b*-PLA-[270, 95] and PAsp-*b*-PLA-[70, 95] was the same, while that of the PAsp segment was different. Although the PLA segment in PAsp-*b*-PLA-[70, 95] is comparative with PAsp segments, the PLA segment in PAsp-*b*-PLA-[47, 180] is much longer than its PAsp segment.

Polymeric micelles were prepared from PAsp-*b*-PLA-[70, 95], PAsp-*b*-PLA-[270, 95] and PAsp-*b*-PLA-[47, 180] by dialysis. Figure 1 shows the results of the DLS observations of the polymeric micelles dialyzed against pure water. In all cases, the polymeric micelles were 20 – 45 nm in diameter with a narrow distribution. Compared with the diameter of PAsp-*b*-PLA-[70, 95] polymeric micelles, PAsp-*b*-PLA-[270, 95] polymeric micelles and PAsp-*b*-PLA-[47, 180] polymeric micelles had somewhat larger diameters. The AFM observations revealed that PAsp-*b*-PLA polymeric micelles are spherical in shape. The diameters of the polymeric micelles observed by AFM were in good agreement with the results of the DLS observations. These results indicate that the size of the polymeric micelles reflects the chain length of both types of segments in the copolymer.

The polymeric micelle prepared from PAsp-*b*-PLA diblock copolymer was consisted of hydrophobic PLA core and a hydrophilic PAsp shell. PAsp segment has carboxyl side chain groups, so dissociation constant of proton of carboxyl group (pKa) is a very important factor for the size and pH-sensitivity of the PAsp-*b*-PLA polymeric micelles. The pKa values for the carboxyl group in the shell layer of the polymeric micelles were measured by neutralization titrations. In all cases, the pKa values of the carboxyl groups in the shell layer were around 7. This value is higher than normal pKa values of carboxyl groups. PAsp-*b*-PLA-[270, 95], having the longest PAsp segment, showed the highest pKa value (7.21). The integration of the PAsp segments in the shell layer of the polymeric micelles resulted in a very close proximity among the carboxyl groups, which would promote hydrogen bond formation between the carboxyl groups and other carboxyl or amide bonds of the polymer backbone. This hydrogen bond formation may be the reason for the higher pKa values of the carboxyl groups in the shell layer.

Table 1. Results of polymerization of Asp(OBzl)-NCA[a)] [3]

NH_2-PLA		Asp(OBzl)-NCA		yield		
Mn[b)]	mg (μmol)	g (mmol)	M/ I[c)]	(%)	Mn[d)]	code-[m, n][e)]
13,500	200 (15)	0.3 (1.4)	95	68	28,000	[70, 95]
13,500	200 (15)	1.4 (5.5)	380	71	69,000	[270, 95]
25,900	500 (19)	0.4 (1.7)	90	78	35,500	[47, 180]

a) Polymerization was carried out in chloroform at 40°C for 24h under Ar atmosphere.

b) Estimated by GPC (eluent: THF, standard: PS).

c) Molar ratio of Asp(OBzl)-NCA to the amino group of NH_2-PLA.

d) Estimated by ^{1}H-NMR (DMSO-d_6, 70°C).

e) m and n mean degree of polymerization of amino acid unit and lactide unit, respectively.

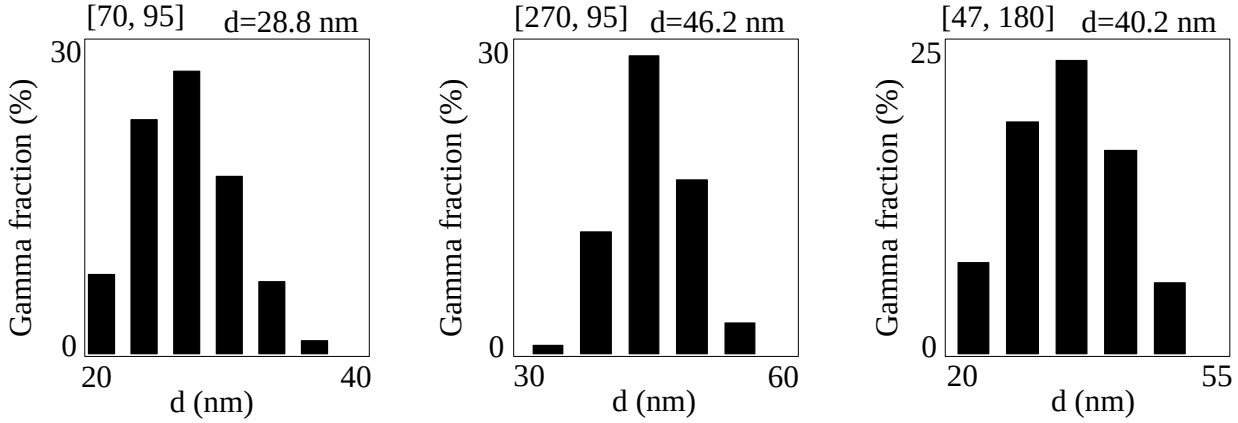


Figure 1. DLS data for the size distributions of PAsp-*b*-PLA polymeric micelles prepared in water.[3]

Figure 2 shows the average diameters of polymeric micelles observed by DLS as a function of pH. In the case of the PAsp-*b*-PLA-[70, 95] polymeric micelles, the average diameter changed drastically between pH 6 and pH 7. Below pH 6, deprotonation of the carboxyl groups of the PAsp segments was inhibited. So, PAsp-*b*-PLA polymeric micelles can be thought of as more tightly aggregated and integrated under these conditions. Above pH 7, however, electrostatic repulsion between the PAsp segments becomes a factor, directly affecting the average size of the polymeric micelles. The average diameter of the PAsp-*b*-PLA-[270, 95] polymeric micelles changed in the pH range from 5 to 6. The PAsp segment of PAsp-*b*-PLA-[270, 95] is about four times longer than that of PAsp-*b*-PLA-[70, 95]. By stronger electrostatic repulsion of PAsp segment of PAsp-*b*-PLA-[270, 95] compared to that of PAsp-*b*-PLA-[70, 95], particle size seemed to increase in low pH. PAsp-*b*-PLA-[47, 180] polymeric micelles, which have the shortest PAsp segment, did not exhibit a significant change in average diameter over the entire pH range. PLA segment of [47, 180] is very long compared to the PAsp segment. This micelle should be a crew-cut micelle and its size governed by the dimensions of the core. These results suggest that hydrophobic interactions among the PLA segments in the PAsp-*b*-PLA-[47, 180] polymeric micelle are dominant to the electrostatic repulsion between the carboxylic groups in the PAsp segments.

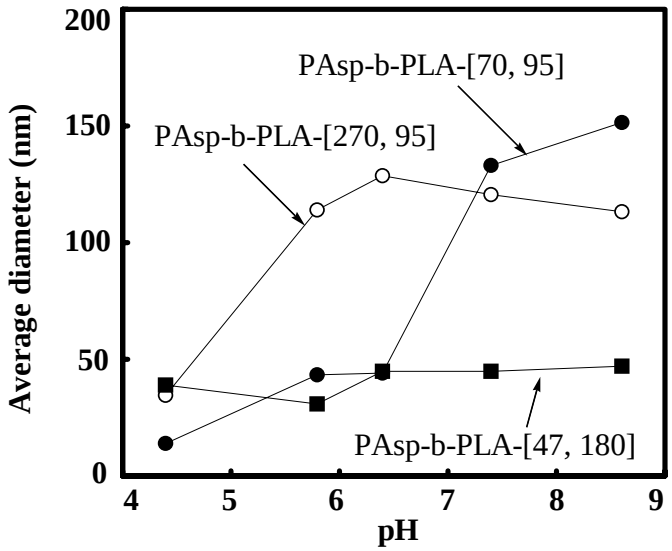


Figure 2. Average diameter of PAsp-*b*-PLA polymeric micelles as a function of pH, closed circle: PAsp-*b*-PLA-[70, 95] polymeric micelles, open circle: PAsp-*b*-PLA-[270, 95] polymeric micelles, and closed square: PAsp-*b*-PLA-[47, 180] polymeric micelles.[3]

Table 2. CMCs of PAsp-*b*-PLA polymeric micelles[3]

	[70, 95]	[270, 95]	[47, 180]
pH = 4.4	6.4×10^{-2} mg / ml	8.3×10^{-2} mg / ml	7.2×10^{-2} mg / ml
pH = 8.6	1.3×10^{-1} mg / ml	7.0×10^{-2} mg / ml	3.6×10^{-1} mg / ml

The shapes of polymeric micelles prepared in phosphate buffer solution (PBS, ionic strength: 0.2 M) at various pH values were observed by AFM under dry conditions. In all cases, spherical shapes were observed. As for the diameter of the polymeric micelles under dry conditions, PAsp-*b*-PLA-[70, 95] and PAsp-*b*-PLA-[47, 180] polymeric micelles had almost the same average diameter of DLS observations. However, PAsp-*b*-PLA-[270, 95] polymeric micelles showed a different tendency at higher pH values. At higher pH values, the diameter of the PAsp-*b*-PLA-[270, 95] polymeric micelles observed by AFM was smaller than that measured by DLS. These results suggest that the shell layer of these polymeric micelles, having expanded due to electrostatic repulsion at higher pH, might shrink during the drying process. Above pH 6, the carboxyl groups of the PAsp segments are almost all deprotonated, which causes the formation of loose-type core-shell polymeric micelles through electrostatic repulsion in the shell layer. These results indicated that the pH value and charge density affect the aggregation state, leading to 'loose' or 'tight' polymeric micelles. Under basic condition, the polymer should form loose aggregation by electrostatic repulsion. The aggregation number should be different from the micelle prepared acidic condition. These may be the reason of the shrinkage observed for the PAsp-*b*-PLA-[270, 95] polymeric micelles.

The critical micelle concentration (CMC) was determined using pyrene as a fluorescent probe. The results of the CMC experiments at pH 4.4 and pH 8.6 are summarized in Table 2. In the case of the PAsp-*b*-PLA-[70, 95] and PAsp-*b*-PLA-[47, 180] polymeric micelles, the CMC values were higher at a higher pH because of the increase in hydrophilicity (deprotonation of carboxyl groups) of the block copolymer. In contrast, the CMC values of the PAsp-*b*-PLA-[270, 95] polymeric micelles were higher and remained almost constant at both pH values because PAsp-*b*-PLA-[270, 95] is primarily hydrophilic in both cases.

These results in total (DLS, AFM, and CMC) indicate that the size and stability of PAsp-*b*-PLA polymeric micelles are determined by electrostatic repulsion, hydrogen bond formation between the PAsp segments, and hydrophobic interactions between the PLA segments.

Finally, we investigated the toxicity of the PAsp-*b*-PLA polymeric micelles. The cell viability slightly decreased as the polymeric micelle concentration increased. However, at high concentrations (0.1mg/ml), the cell viability was still about 80%. These results show that none of the PAsp-*b*-PLA polymeric micelles exhibited serious toxicity against L929 fibroblast cells. This is one of the examples that the PAsp-*b*-PLA polymeric micelles may have biocompatibility. These polymeric micelles can therefore be expected to be put to biomedical use in applications such as a drug delivery carrier.

References

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