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pH-Induced Micellization of Biodegradable Block Copolymers Containing Sulfamethazine

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Abstract: pH-sensitive block copolymers were synthesized by coupling reaction of sulfamethazine and amphiphilic diblock copolymer, and their micellization-demicellization behavior was investigated. Sulfamethazine (SM), a derivative of sulfonamide, was introduced as a pH responsive moiety while methoxy poly(ethylene glycol)-poly(D,L-lactide) (MPEG-PDLLA) and methoxy poly(ethylene glycol)-poly(D,L-lactide-*co*-ε-caprolactone) (MPEG-PCLA) were used as biodegradable amphiphilic diblock copolymers. After the sulfamethazine was carboxylated by the reaction with succinic anhydride, the diblock copolymer was conjugated with sulfamethazine by coupling reaction in the presence of DCC. The critical micelle concentration (CMC) and mean diameter of the micelles were examined at various pH conditions through fluorescence spectroscopy, dynamic light scattering and transmission electron microscopy. For MPEG-PDLLA-SM and MPEG-PCLA-SM solutions, the pH-dependent micellization-demicellization was achieved within a narrow pH band, which was not observed in the MPEG-PDLLA and MPEG-PCLA solutions. The micelle showed a spherical morphology and had a very narrow size distribution. This pH-sensitive block copolymer shows potential as a site-targeted drug carrier.

Keywords: pH-sensitive, sulfamethazine, micelle-demicelle transition, block copolymer.

Introduction

Over the last few decades, polymeric micelles have attracted considerable attention as a drug carrier in pharmaceutical and biomedical application.[1-4] Many researches have extensively focused on the development of novel drug delivery system to improve the efficiency and reduce the undesirable denaturalization of drugs. The polymeric micelle formed by the self-assembly of amphiphilic block copolymer, has core-corona structure, hydrophobic core and hydrophilic corona. Inner core acts as a depot to accommodate the hydrophobic drug whereas the hydrophilic corona protects the micelle from the intermicellar association, protein adsorption and cell adhesion. Moreover the micelle size ranging several tens of nanometers enabled to prevent from the renal exclusion and nonselective uptake by reticuloendothelial system after intravenous injection. From these characteristics of the micelles, the long-term circulation of polymeric micelles and enhanced permeation and retention effect can be realized.[5,6] Recently, site-targeted drug delivery has been a major concern to achieve enhanced therapeutic effect and minimize the harmful side effect of the drug. For this purpose the micelle of stimuli-responsive block polymer was designed. The stimuli-responsive micelles undergo structural change in response to the particular environmental change, such as temperature,[7,8] pH,[9,10] and electric field,[11,12] which make possible controlled release on a target site. Especially, pH-sensitive micelles have an advantage that any external stimulus was not required for site-specific release because each organ has its own pH condition. In addition it is revealed that the extracellular pH of tumor, pH 7.0, is lower than that of normal tissue, pH 7.4.[13,14] Once drug-loaded micelles of pH-sensitivity were administrated into the body, the micelle dissociation occurs at the site where the pH condition induces the structural change of micelles and drug release. Generally, in order to endow pH sensitivity on the micelle, the amphiphilic block copolymer forming the micelle should be incorporated with ionizable group, for example carboxylic acid[15,16] or amine group.[17,18] The ionized moiety in the block copolymer at a certain pH condition disturbs the intermolecular hydrophobic interaction and consequently destroys the micelle structure.

In this study, we prepared a novel pH-sensitive block copolymer by coupling of sulfamethazine, a derivative of sulfonamide, and amphiphilic diblock copolymer of poly (ethylene glycol) and biodegradable polyesters. Sulfonamide group in sulfamethazine was expected to serve as a pH-sensitive moiety in the physiological pH condition.[19,20]

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The physicochemical properties of the micelle were investigated in terms of micelle size, critical micelle concentration (CMC) and pH-sensitivity by monitoring the fluorescence of pyrene at different pH.

Experimental

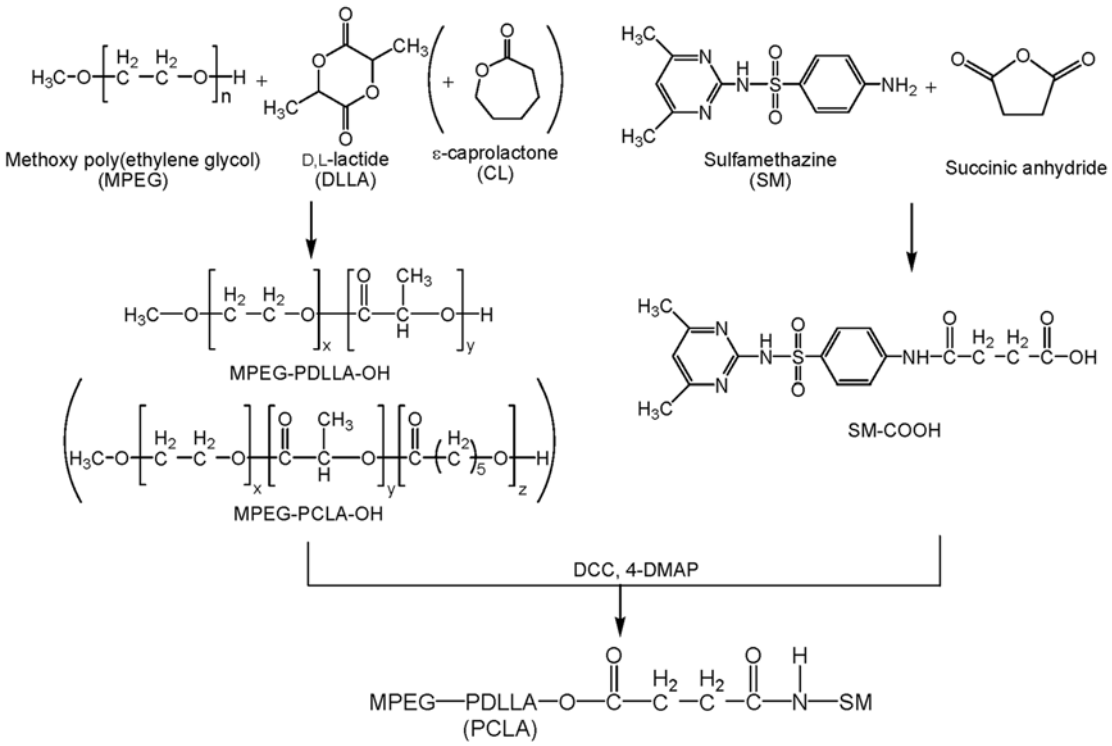
Materials. Methoxy poly(ethylene glycol) (MPEG) (M_n of 2,000), D,L-lactide (LA), ε-caprolactone (CL), stannous 2-ethyl-hexanoate ($Sn(Oct)_2$), anhydrous 1,4-dioxane, anhydrous methylene chloride (MC), methacryloyl chloride, 3-mercaptopropionic acid (MPA), dicyclohexyl carboimide (DCC), succinic anhydride, 4-(dimethyl amino) pyridine (DMAP) were used as received from Aldrich. Sulfamethazine and 2,2′-azobisisobutyronitrile (AIBN) were supplied from Sigma and Junsei Co, respectively. AIBN was recrystallized from methanol twice prior to use. Unless stated otherwise, other reagents and solvents have been used as received.

Synthesis of Biodegradable pH-Sensitive Block Copolymer. Sulfamethazine Carboxylation: Sulfamethazine (SM) was carboxylated using succinic anhydride. SM (0.1 mol) and anhydrous 1,4-dioxane were mixed for 30 min at 50 °C in the two-neck flask with reflux condenser, and then succinic anhydride (0.12 mol) and DMAP (0.012 mol) were added in this flask under dry nitrogen atmosphere. The reactants were heated slowly to 85 °C, and the reaction was carried out for 12 hrs. The carboxylated SM (CSM) was precipitated during the reaction and the CSM was obtained by filtration. In acidic condition, only SM was dissolved owing to amine next to the phenyl group while both SM and CSM were dissolved in basic condition. The CSM is precipitated in the acidic condition because the amine is changed to amide group by carboxylation with succinic anhydride. Therefore, after the filtered CSM was dissolved in 0.1 N NaOH solution, the HCl solution was add to CSM solution to precipitate the CSM only. The precipitated CSM was filtrated, washed with distilled water, and then dried under vacuum at 40 °C for 48 hrs. The yield of CSM was around 95% after drying.

Biodegradable Block Copolymer: The synthesis of methoxy poly(ethylene glycol)-poly(D,L-lactide) (MPEG-PDLLA) block copolymer was performed through a ring-opening copolymerization with the MPEG as an initiator. Stannous 2-ethyl-hexanoate ($Sn(Oct)_2$) was used as a catalyst. The composition of MPEG/PDLLA was adjusted by the feed ratios of MPEG and DLLA. MPEG and $Sn(Oct)_2$ were added to a two-neck round-bottom flask, and were dried for 4 hrs under vacuum at 110 °C. After cooled to room temperature, LA was added under a dry nitrogen atmosphere. The reactant mixture was dried for 1 hr under vacuum at 60 °C, and slowly heated to 130 °C under dry nitrogen atmosphere. The reaction was carried out for 18 hrs. After the reaction, the products were cooled to room temperature, dissolved in the methylene chloride (MC), and then precipitated in excess diethyl ether. The precipitated products were dried under vacuum at 40 °C over 48 hrs and MPEG-PDLLA was finally obtained. The methoxy poly(ethylene glycol)-poly(ε-caprolactone-*co*-D,L-lactide) (MPEG-PCLA) was prepared in the same way as in MPEG-PDLLA synthesis except that ε-caprolactone and D,L-lactide were used in MPEG-PCLA synthesis instead of D,L-lactide in MPEG-PDLLA synthesis. The yield of these block copolymers was over 80%.

Coupling CSM with Biodegradable Block Copolymer: The biodegradable amphiphilic block copolymer (MPEG-PDLLA, MPEG-PCLA) were coupled with CSM using DCC and DMAP as a coupling reagent and catalyst, respectively. The coupling reaction process is as follow: The MPEG-PDLLA block copolymer (4 g) was weighed into a two-neck flask and dried under vacuum at 85 °C for 2 hrs. After the CSM was added under dry nitrogen atmosphere, the reactants were dried again under vacuum at 85 °C for 1 hr in order to remove the moisture perfectly. The reactants were cooled to room temperature under dry nitrogen atmosphere, and then the DCC and DMAP solution in anhydrous MC (40 mL) was added into flask using a glass syringe. The feed mol ratio of MPEG-PDLLA, CSM, DCC and DMAP was 1.0/1.2/1.4/0.14. The reaction was carried out at room temperature for 48 hrs. The CSM was not dissolved in MC. But, as the coupling reaction proceeded, the CSM coupled with MPEG-PDLLA became soluble in MC. In the other hands, DCC was converted into dicyclohexylurea (DCU) during the reaction and residual DCC after reaction was changed to DCU by adding the two or three drops of water. Precipitated DCU and residual CSM were removed by filtering with 0.4 μm filter paper. The final product was precipitated by pouring the filtrated mixture in excess diethyl ether. The precipitated final products were dried under vacuum at 40 °C for over 48 hrs, and the yield of final product was over 70%. The MPEG-PCLA was coupled with CSM in the same manner as the coupling reaction of MPEG-PDLLA and CSM. The entire synthetic route was shown in Scheme I.

Characterization of Biodegradable pH-Sensitive Block Copolymer. ^{1}H-NMR spectra was recorded on a Varian-Unity Inova 500NB operated at 500 MHz, and was used to determine the molecular structure and composition of each block in the block copolymer. DMSO-d_6 and $CDCl_3$ were used as solvents, which contained 0.03 v/v% tetramethylsilane (TMS). The composition of each block was calculated on the basis of the typical proton peak integration of MPEG, CL and LA from ^{1}H-NMR spectra. To measure molecular weight and molecular weight distribution, gel permeation chromatography (GPC) with two stryragel columns (Shodex-KF 802.5, KF 803L) was performed. Tetrahydrofuran (THF) was used as an eluent at a flow rate of 1 mL/min. Calibration was carried out using poly(ethylene glycol) (Waters Co.) with the molecular weight ranging from 420 to 22,100.



Scheme I. Synthesis of pH-sensitive MPEG-PCLA-SM and MPEG-PDLLA-SM.

With a refractive-index detector (Shodex, RI-101), the retention volume was measured at 45 °C.

CMC and Micelle-Demicelle Transition - Fluorescence. The critical micelle concentration (CMC) was determined by a fluorescence probe technique using pyrene.[21] The pyrene solution in THF was poured in the PBS buffer solution, and THF was eliminated by stirring at 40 °C for 4 hrs. The final concentration of pyrene in buffer solution was 1.0×10^{-6} M. The block copolymer solutions were prepared with concentration ranging from 5.6×10^{-5} to 10 mg/mL. A 10 mg/mL stock solution was prepared and solutions with various concentrations were made by dilution. The emission spectra of pyrene were measured at a fixed excitation wavelength of 334 nm by fluorescence spectrometer (AMINCO·BOWMAN® Series2). Emission spectra of pyrene were recorded at 350 to 540 nm. In order to investigate the micelle-demicelle transition, the block copolymer solutions of 5 mg/mL were prepared in the different pH conditions. The pH of block copolymer solution was adjusted stepwise at interval of 0.2 from pH 6.0 to 8.6 with the 5 M NaOH solution, and then pH was reversely adjusted from pH 8.6 to 6.0 with the 5 M HCl solution. All the fluorescence spectroscopy was carried out at 25 °C.

Micelle Size-DLS & TEM. The size of block copolymer micelles was measured by dynamic light scattering (DLS) using Malvern PCS100 spectrogoniometer and Brookhaven BI-9000AT digital autocorrelator at a wavelength of 633 nm. For DLS measurement, the polymer solution in PBS solution was fixed as 2 mg/mL. Before transferred into the light scattering cells, the polymer solution was passed through a 0.45 μm filter. The intensity autocorrelation was recorded at a scattering angle of 90°. The CONTIN algorithms were used in the Laplace inversion of the autocorrelation function to obtain micelle size. The mean diameter was evaluated from the Stokes-Einstein equation. The DLS experiments were performed at 25 °C. In addition, aqueous dispersion of micelles was examined by transmission electron microscopy (TEM, JEOL). Specimens were prepared by dropping the amphiphilic block copolymer aqueous solution onto carbon coated EM grids. The solutions on the grid was refrigerated in liquid nitrogen and lyophilized by a freeze dryer. The micelles on the grid were stained by 20 wt% phosphotungstic acid aqueous solution for 1 sec. Specimens were vacuum-dried before examination using a TEM instrument.

Results and Discussion

Synthesis of pH-Sensitive Biodegradable Block Copolymer. The carboxylated sulfamethazine was prepared by the reaction of amine in sulfamethazine and succinic anhydride, and confirmed by FTIR (Figure 1) and ^{1}H-NMR (Figure 2(a) and (b)). Figure 1 shows clearly that, after carboxylation the absorption for carbonyl group was observed at 1730 cm^{-1} whereas the sharp peaks for primary amine (3300~3500 cm^{-1}) disappeared. In ^{1}H-NMR spectrum of Figure 2, the peak for proton of amino group in sulfa-

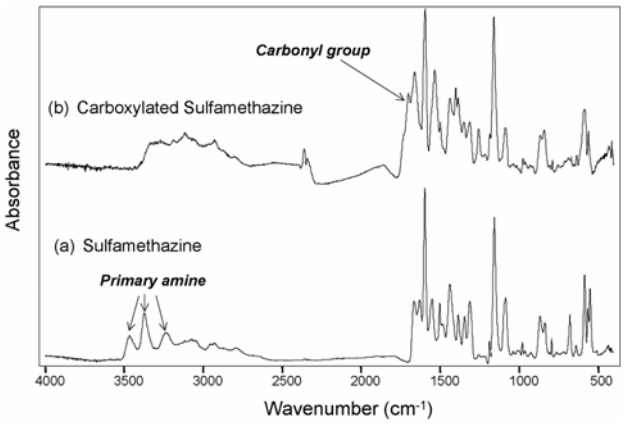


Figure 1. FTIR spectra of sulfamethazine (a) and carboxylated sulfamethazine (b).

methazine (***e*** at 6.0 ppm in (a)) is shifted downfield to 10.4 ppm (***e*** in (b)) and peak for protons in methyl group (***f, g*** in (b)) appeared. From these results we ascertained the successful synthesis of carboxylated sulfamethazine.

In this study, the MPEG-PDLLA and MPEG-PCLA block copolymers were synthesized from the ring opening polymerization. The number average molecular weight (M_n) of block copolymers can be calculated by the ^{1}H-NMR spectrum from known MPEG molecular weight. Figure 3 shows the representative ^{1}H-NMR spectrum of MPEG-PCLA block copolymer and its chemical structure. The assignment of ^{1}H-NMR spectrum for MPEG-PDLLA and

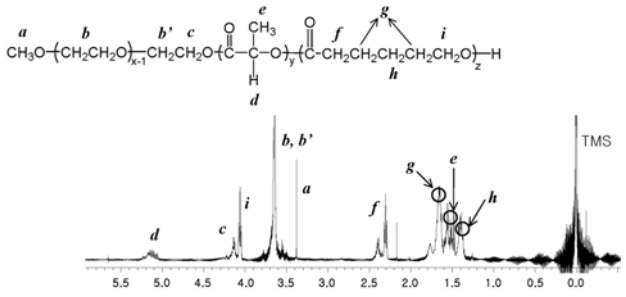


Figure 3. ^{1}H-NMR spectrum of MPEG-PCLA block copolymer in $CDCl_3$.

calculation of molecular weight of block copolymer was reported in our previous paper.[22] The characteristic peaks of methylene proton of MPEG (***b, b'***, 3.5 ppm), methine proton of LA unit (***d***, 5.1 ppm) and methylene proton of CL unit (***f***, 2.3 ppm) were used for calculating M_n and composition of block copolymer with the following equations.

$$\frac{y}{4(x-1)+2} = \frac{I_d}{I_b + I_{b'}}, \quad \frac{2z}{4(x-1)+2} = \frac{I_f}{I_b + I_{b'}}$$

The number of oxyethylene unit, x, was calculated to be 45 from the molecular weight of MPEG (M_n of 2,000). Information on the molecular weight of synthesized copolymer and the ratio of each block was listed in Table I. And the GPC trace for the synthesized diblock copolymer showed unimodal and narrow peak (Figure 4). These characterization

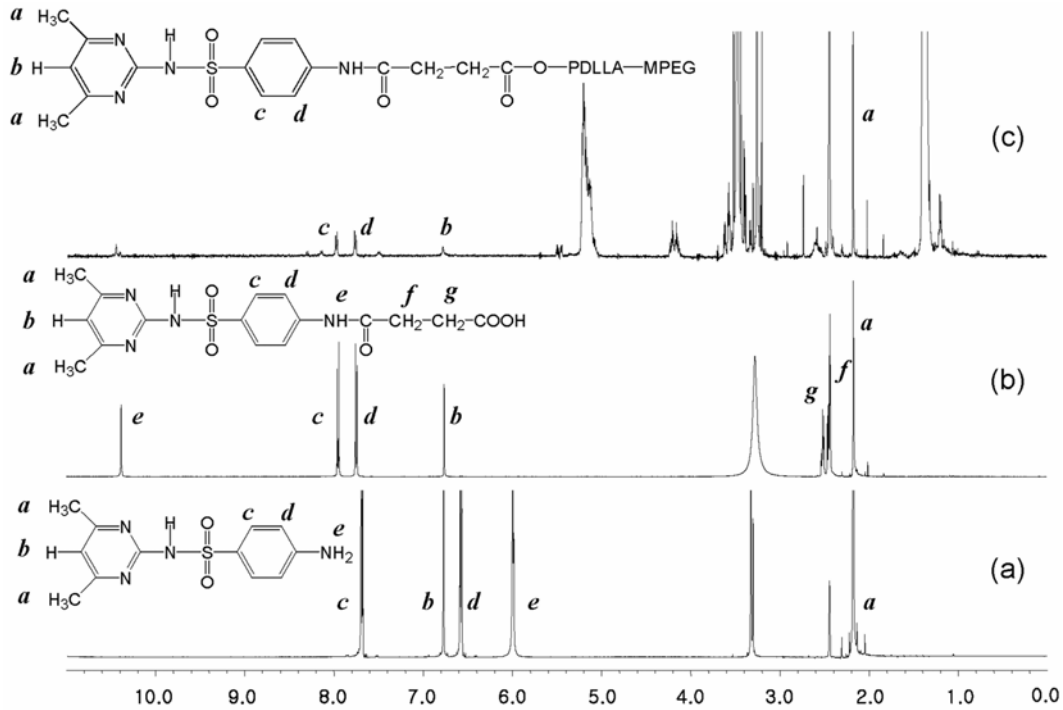


Figure 2. ^{1}H-NMR spectra of sulfamethazine (a), carboxylated sulfamethazine (b), and MPEG-PDLLA-SM (c).

Table I. Molecular Characteristics of Diblock Copolymers

	M_n (MPEG-biodegradable polyester)		M_w/M_n[a]
	Calculated by feed ratio	Calculated by ^{1}H-NMR	
MPEG-PDLLA (1.2k)	2000-1200	2000-1167	1.15
MPEG-PDLLA (1.6k)	2000-1600	2000-1574	1.16
MPEG-PDLLA (2.0k)	2000-2000	2000-1922	1.19
MPEG-PCLA (1.2k)	2000-1200	2000-1030 (CL:LA=1.6:1)	1.21

[a]Calculated from GPC.

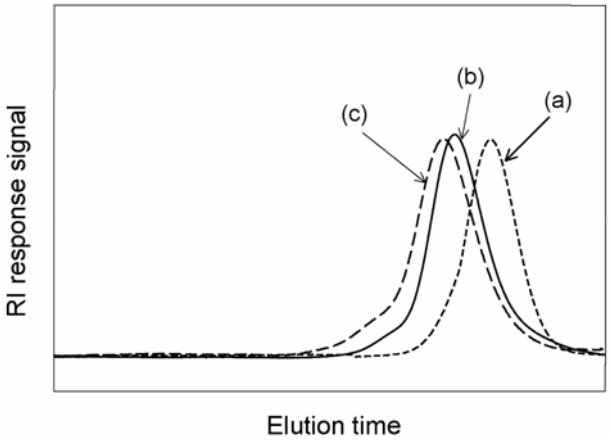


Figure 4. GPC trace of MPEG (M_n of 2,000) (a), MPEG-PDLLA(1.2k) (b), and MPEG-PDLLA(1.2k)-SM (c).

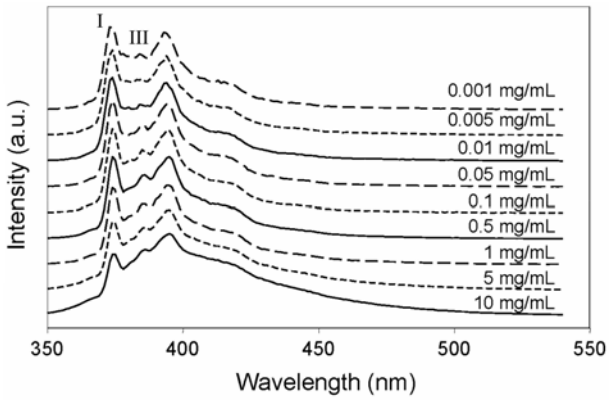


Figure 5. Emission spectra of pyrene in MPEG-PDLLA(1.6k)-SM block copolymer solution at pH 7.0. The concentration of pyrene was 1.0×10^{-6} M.

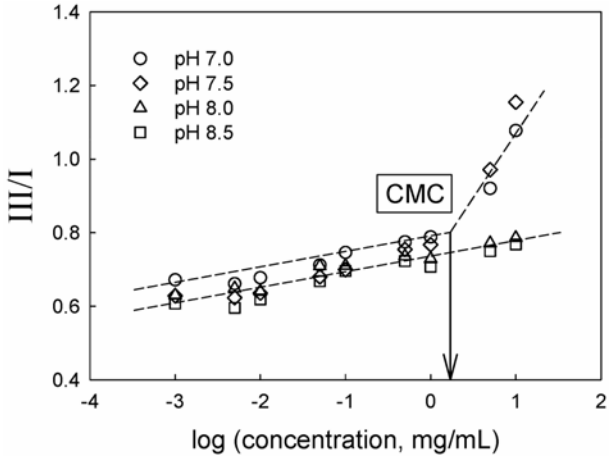


Figure 6. Intensity ratio of III/I in the emission spectra as a function of MPEG-PDLLA(1.6k)-SM concentration at various pH conditions.

results indicate that the block copolymer with narrow molecular weight distribution have been successfully prepared.

The biodegradable block copolymer (MPEG-PCLA and MPEG-PDLLA) was coupled with CSM by DCC and DMAP. The synthesized MPEG-PDLLA-SM and MPEG-PCLA-SM block copolymer was characterized by ^{1}H-NMR and GPC. In Figure 2(c), ^{1}H-NMR spectrum of MPEG-PDLLA(1.2k)-OSM shows the aromatic protons in CSM (***c, d***) as well as imidazole ring proton in CSM (***a, b***). It was confirmed from GPC that the molecular weight was increased after coupling reaction (Figure 4).

pH-Dependence of Critical Micelle Concentration (CMC). Fluorescence spectroscopy was performed to investigate the micellization behavior of the synthesized block copolymer. The pyrene was used as a fluorescence probe to detect the micropolarity change. The ratio of the intensity of the third vibrational peak (III, λ=384 nm) to that of the first vibrational peak (I, λ=374 nm) of emission spectrum of pyrene is known as a very sensitive indicator for micropolarity of the local environment. When the block copolymers form micelle structure, the pyrene is arrested in the hydrophobic core of the micelle and subsequently the III/I value increases. Moreover, the intensity ratio III/I provides qualitative information on the number of micelles in a given system. The typical emission spectra of pyrene in MPEG-PDLLA(1.6 k)-SM copolymer solution are shown in Figure 5. As the polymer concentration was increased from 0.001 to 10 mg/mL, the peak at 384 nm underwent a noticeable increase compared to that of 374 nm, which indicates the micelle formation and increment of number of micelles.

Figure 6 shows the dependence of III/I on the block copolymer concentration at various pH conditions. Below pH 7.5, MPEG-PDLLA(1.6 k)-SM molecules readily self-assembled and formed micelles because the sulfamethazine was not ionized and acted as hydrophobic part with PDLLA block. On the other hand, any micellization behavior was

Table II. Critical Micelle Concentration (CMC) of the pH-sensitive Block Copolymer Solutions

	Critical Micelle Concentration (CMC), mg/mL			
	pH 7.0	pH 7.5	pH 8.0[a]	pH 8.5[a]
MPEG-PDLLA(1.2k)-SM	2.09	2.24	N/A	N/A
MPEG-PDLLA(1.6k)-SM	1.65	1.84	N/A	N/A
MPEG-PDLLA(2.0k)-SM	1.01	1.42	N/A	N/A
MPEG-PCLA(1.2k)-SM	1.12	1.40	N/A	N/A

[a]CMC was not observed at pH 8.0 and 8.5 by fluorescence spectroscopy.

not observed through fluorescence spectroscopy at pH 8.0 and 8.5. It can be inferred that, in these pH conditions, the block copolymer could be fully solubilized due to the fact that the ionization of nitrogen atoms in sulfonamide group (SO_2NH) of sulfamethazine already ionized and electrostatically repelled themselves, which suppress the self-assembly of polymer chain and consequently endowed more hydrophilicity on the block copolymer. As the polymer concentration was increased, the micellization started and III/I ratio showed sudden increase at a certain concentration, which was defined as a critical micelle concentration (CMC). The CMC values for the synthesized block copolymer summarized in Table II. It clearly showed the effects of pH and the hydrophobicity of block copolymer on the micellization behavior. At pH 8.0 and 8.5, any CMC value could not be observed for all the copolymers coupled with sulfamethazine because of ionization of sulfonamide group as stated above.

In case of MPEG-PDLLA-SM, as the molecular weight of hydrophobic PDLLA was increased, the CMC shifted to the lower concentration as expected. The increment in the molecular weight of hydrophobic block induced higher hydrophobic character of the block copolymer, which allowed self-assembly of polymer chain at the lower concentration in aqueous buffer solution. The CMC was slightly decreased at lower pH condition, for example, CMC of MPEG-PDLLA(2.0k)-SM was 1.01 and 1.42 at pH 7.0 and 7.5 respectively. It demonstrates that CMC and micellization behavior was under the influence of the extent of ionization of sulfonamide group. In other words, as the solution became acidic (lower pH), the proportion of ionized sulfonamide group in sulfamethazine decreased and the block copolymer carried rather hydrophobic character, resulting in the lower CMC value. When MPEG-PDLLA(1.2k)-SM was compared to MPEG-PCLA(1.2k)-SM, the block copolymer containing PCLA started to micellize at lower concentration. It could be explained by the fact that, though the molecular weight of PCLA is similar that of PDLLA, the PCLA block has more hydrophobicity than PDLLA block.

Micellization-Demicellization Transition. The pH-induced micellization-demicellization was examined at constant concentration (5 mg/mL) of polymer solution by fluorescence spectrometry. The intensity ratio of III/I in the emission spectrum was plotted as a function of pH in Figure 7. As shown in Figure 7(a), the intensity ratio, III/I, in MPEG-PDLLA (1.6k) solution was nearly constant on the pH change. Besides, III/I values determined during pH increase and decrease coincided closely with each other. It was confirmed that the MPEG-PDLLA block copolymer was fully solubilized regardless of pH condition and showed no structure change with pH change. But, MPEG-PDLLA (1.6k)-SM molecules formed a micellar structure at pH 6.0 and number of the micelles were decreased with pH increase. This demicellizaiton phenomenon was mainly attributed to the electrostatic repulsion between ionized sulfonamide groups, which disturbed the hydrophobic interaction of

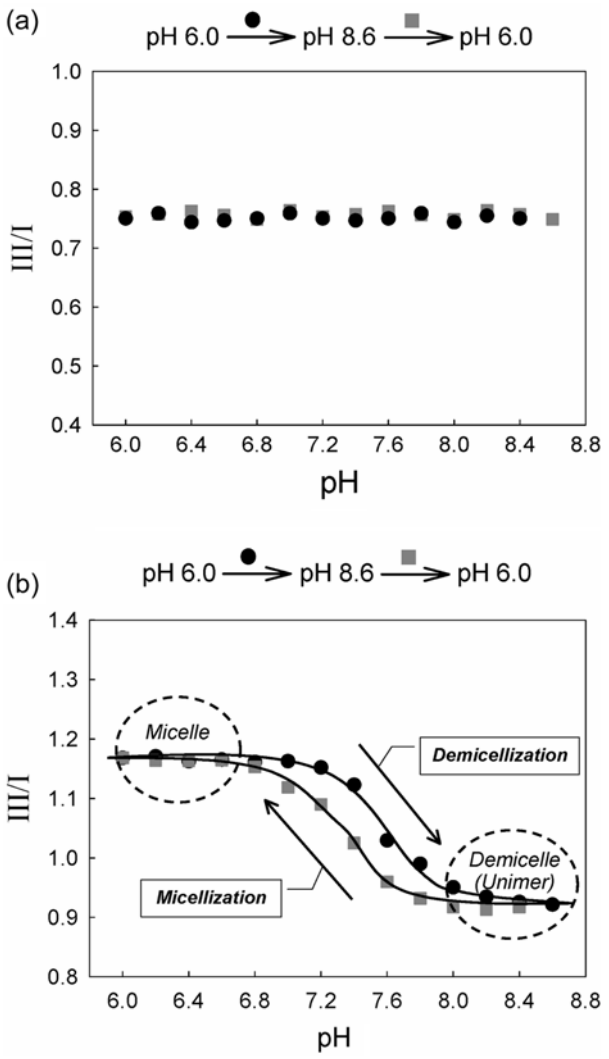


Figure 7. Intensity ratio of III/I in the emission spectra as a function of pH at constant polymer concentration of 5mg/mL; MPEG-PDLLA(1.6k) (a) and MPEG-PDLLA(1.6k)-SM (b).

PDLLA or PCLA, therefore, facilitated the dissociation of the micelle. When the NaOH solution was added to pH 8.6, it seemed that all the micelle structure was destroyed and MPEG-PDLLA-SM molecules were in so-called unimer state. When pH of solution was decreased from pH 8.6 to 6.0, the MPEG-PDLLA-SM experienced structure transition from unimer to self-assembled micelle. With the decrement in pH, deionized sulfonamide group enabled the polymer chain to associate between themselves and form micelle structure. It is remarkable that the synthesized MPEG-PDLLA-SM and MPEG-PCLA-SM block copoly-mers showed reversible micellization-demicellizaiton behavior within narrow pH range, pH 7.0 ~ 8.0.

Micelle Size. The micelle size was determined by dynamic light scattering (DLS) and transmission electron microscopy (TEM) (Figure 8). DLS results showed that the micelle size increased with increasing the molecular weight of hydrophobic PDLLA block. For MPEG-PDLLA-SM block copoly-mers, the mean diameter of micelle increased from 20.2 to 32.5 nm when the molecular weight of PDLLA was increased from 1,167 to 1,922 g/mol, which was resulted from the expanded PDLLA core because of longer hydrophobic PDLLA block. Micelles in TEM images were spherical and its size was similar to that from DLS study.

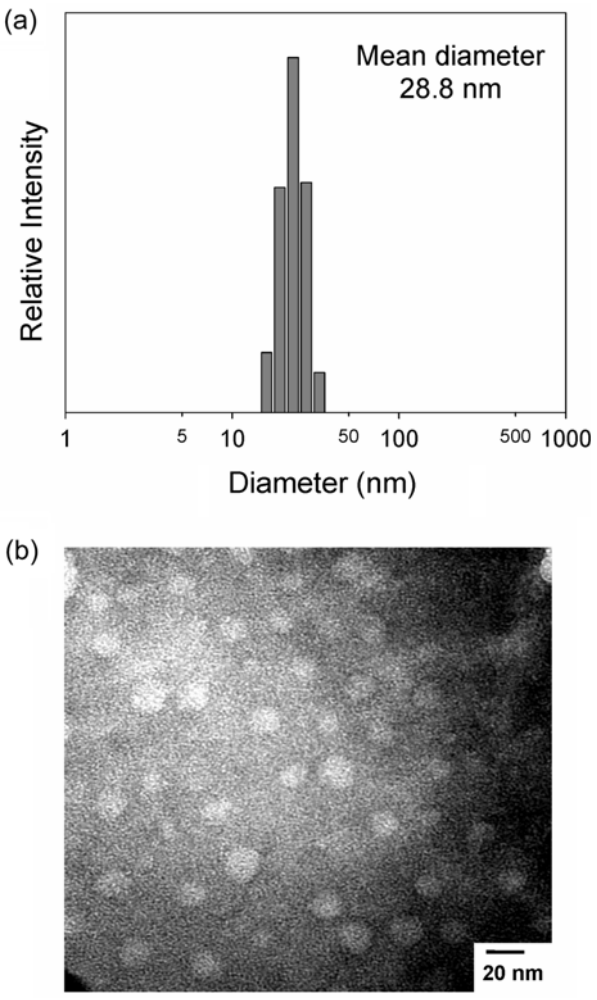


Figure 8. Micelle size and size distribution of MPEG-PDLLA (1.6k)-SM in buffer solution of pH 7.0; DLS results (a) and TEM image (b).

Conclusions

We synthesized successfully the pH-sensitive block copoly-mers, MPEG-PDLLA-SM and MPEG-PCLA-SM. They form a micelle structure below pH 7.5 but the micelle structure was destroyed at rather higher pH condition. For MPEG-PDLLA-SM solution, the higher CMC values were observed at the more alkaline condition due to the degree of ionization of sulfonamide group. When the hydrophobic PDLLA block became longer, the CMC shifted to the lower concentration. Compared to MPEG-PDLLA-SM molecules with the similar molecular weight of hydrophobic block, the MPEG-PCLA-SM system shows lower CMC because the hydrophobicity of PCLA is stronger than that of PDLLA. The SM-coupled block copolymers showed the micellization-demicellization behavior on the pH increase and decrease. It occurs reversibly within narrow pH range. The pH-sensitive MPEG-PDLLA-SM and MPEG-PCLA-SM block copolymer would be a good candidate for site-targeted drug deliver carrier.

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