Norfloxacin Release from Polymeric Micelle of Poly(γ-benzyl L-glutamate)/Poly (ethylene oxide)/Poly(γ-benzyl L-glutamate) Block Copolymer

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Block copolymers consisting of poly(γ-benzyl L-glutamate) (PBLG) as the hydrophobic part and poly(ethylene oxide) (PEO) as the hydrophilic part were synthesized and characterized. Polymeric micelles of the block copolymers (abbreviated GEG) were prepared by a dialysis method. The GEG block copolymers were associated in water to form polymeric micelles, and the critical micelle concentration (CMC) values of the block copolymers decreased with increasing PBLG chain length in the block copolymers. Transmission electron microscopy (TEM) observations revealed polymeric micelles of spherical shapes. From dynamic light scattering (DLS) study, sizes of polymeric micelles of GEG-1, GEG-2, and GEG-3 copolymer were 106.5± 59.2 nm, 79.4±46.0 nm, and 37.9±13.3 nm, respectively. The drug loading contents of GEG-1, GEG-2 and GEG-3 polymeric micelles were 12.6, 11.9, and 11.0 wt %, respectively. These results indicated that the drug-loading contents were dependent on PBLG chain length in the copolymer; the longer the PBLG chain length, the more the drug-loading contents. Release of norfloxacin (NFX) from the nanoparticles was slower in higher loading contents of NFX than in lower loading contents due to the hydrophobic interaction between PBLG core and NFX.

Introduction

Novel drug carriers such as polymeric micelles, surface-modified particles, liposomes, and nanoparticles have been proposed to obtain effective sustained or targeted drug delivery.[1-8] Several problems, such as biodistribution of drugs, drug solubility, undesirable side effects, rapid clearance by reticuloendothelial system (RES), thermally unstable, short blood circulation, structurally fragile and lower loading efficiency still may exist.

Since block copolymers exhibit surfactant behavior[9] and form micelles due to amphiphilicity, polymeric micelles[9-12] or core-shell type nanoparticles[7] prepared by block copolymers composed of a hydrophobic part and a hydrophilic one is a considerably attracted area. These drug carrier systems with core-shell structure have predominant characteristics that the hydrophobic inner-core acts as a drug incorporation site, especially ease entrapment of hydrophobic drugs by hydrophobic interactions,[13] and the hydrophilic outershell may be cloaked to avoid being quickly uptaked by the RES and clearable organs such as liver, spleen, lungs, and kidneys and then have the potential of long blood circulation times of polymeric carriers. Some reported advantages of these systems include reduced toxic side effects of anticancer drug and selective targeting, solubilization of hydrophobic drugs, stable storage, long blood circulation, biodistribution, and lower interactions with RES.[7,14,15] Thus, this core-shell type nanoparticles may be appropriate vehicles for drug delivery.

Cho *et al.*[16] previously have reported formation of polymer micelles composed of poly(γ-benzyl L-glutamate) (PBLG) and poly(ethylene oxide) (PEO) in aqueous environment.

Successful treatment of some localized infectious diseases is not always achieved due to the low retention or permeability of the drug at the infection site and bacterial resistance against antibiotic treatment. Especially, most of intracellular infections are difficult to eradicate because bacteria protected from antibiotics inside lysosomes.[17] Infected cells may also constitute a reservoir for micro-organisms which are released from time to time causing the recurrence of systemic infections. The need for intracellular chemotherapy has been recognized for many years.[18] Couvreur *et al.*[19,20] reported that the antibiotics loaded nanoparticles were found to improve dramatically the therapeutic efficiency of drug in experimental intracellulat infections of the mouse.

NFX is an fluoroquinolone antibacterial agent which is recently released for the treatment of uncomplicated and complicated urinary tract infections and has hydrophobic character.[21-23] The drug antagonizes DNA gyrase, an enzyme essential for bacterial DNA replication. Na *et al.* reported the polymeric drug of norfloxacin which was prepared by chemically attached to the dextran and has a broad spectrum against gram-positive and negative bacteria same as norfloxacin itself.[24]

For this study, we have synthesized PBLG/PEO/PBLG (GEG) block copolymers and prepared polymeric micelles using dialysis method which was the same as previously reported procedure.[16,25] PBLG acts as a hydrophobic block which is a drug incorporation site as the core and has biodegradability[26] and hydrophilic PEO which has nontoxicity and nonimmunogenic water-soluble polymer as the shell and is known to prevent interactions with proteins and cells.[27] Physicochemical characteristics of GEG copolymeric micelles were investigated. To evaluate the GEG copoly-

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meric micelles as a novel carriers for delivery of NFX as a model drug, NFX-loaded micelles were prepared and drug release was performed *in vitro*.

Experimental

Materials. Bis[poly(ethylene oxide)bis(amine)](BPEOBA: M.W.=20000) and γ-benzyl *L*-glutamate were purchased from Sigma Chem. Co. (St. Louis, MO, USA). The structure of bis[poly(ethylene oxide)bis(amine)] was shown in Figure 1(a) according to the manufacturer's report. Poly (γ-benzyl *L*-glutamate) (PBLG) homopolymer (accoding to the manufacturers data, M.W. of PBLG is 22000) was purchased from Sigma Co.. Triphosgene, N-hexane and dichloromethane were purchased from Aldich Chem. Co. Inc. (Milwaukee, WI, USA). Norfloxacin (NFX) was purchased from Sigma Co. Dimethylformamide (DMF) as reagent grade was used without further purification.

Synthesis of block copolymer. γ-benzyl *L*-glutamate N-carboxyanhydride (γ-BLG NCA) (Figure 1(b)) was prepared according to the method proposed by Goodman and Hutchison.[28]

As reported previously,[25,29-31] GEG block copolymers prepared by polymerization of γ-BLG NCA (b) initiated with BPEOBA (a) in methylene chloride solution at a total concentration of γ-BLG NCA and BPEOBA of 3 wt% for 72 hrs at room temperature is shown in Figure 1(*c*). The reaction was progressed until the characteristic IR peak of γ-BLG NCA (1850 cm^{-1}, 1780 cm^{-1}, 915 cm^{-1}) disappeared.[31] The reaction mixture was poured into a large excess of diethyl ether to precipitate the block copolymer. The reaction mixture may contain unreacted BPEOBA and block copolymers. Since BPEOBA cannot be precipitated from the mixture of dichloromethane and diethyl ether (although the latter is a non-solvent for BPEOBA), the unreacted BPEOBA was removed by filtration with glass filter and the block copolymer was obtained as precipitants. The resulting block copolymer was washed twice with diethyl ether and then dried in vacuo for 48 hrs.

Preparation of NFX-loaded polymeric micelles. Formation of polymeric micelles and drug loading was carried out as follows: 20 mg of PBLG/PEO/PBLG triblock copolymer was dissolved in 10 mL of DMF and subsequently 20-40 mg of NFX was added. The solution was stirred at room temperature and solubilized entirely. To form polymeric micelles and remove free drugs, the solution was dialyzed using molecular cutoff 12,000 g/mol dialysis tube against 1.0 L×3 of distilled water for 3 h and then distilled water exchanged at intervals of 3 h during 9 h (total 12 h). The solution was freeze-dried for storage to perform following experiment and drug release study.

For the measurement of drug-loading content, freeze-dried samples of GEG micelles were suspended into methanol and vigorously stirred for 2 h and sonicating for 15 min. Resulting solution was centrifuged with 20000 g for 30 min and supernatent was taken for measurement of drug concentration using ultraviolet (UV) spectrophotometer (Shimadzu UV-1201) at 322 nm.

FT-Infrared (FT-IR) spectroscopy measurement. FT-IR spectra of the sample of block copolymers were measured by KBr method with Bruker IFS-66 FT-IR spectrometer between 4,000 and 400 cm^{-1}.

Measurement of fluorescence spectroscopy. To investigate the fluorescence spectroscopy characteristics, GEG block copolymer solutions without drug were prepared as follows: 20 mg of GEG block copolymer was dissolved in 10 mL of DMF and dialyzed up to 2 days as the same method described above. Resultant solution was adjusted to the various concentrations of block copolymers.

Critical micelle concentration (CMC) of the GEG block copolymers were estimated to prove the potential of micelle formation by the measurement of fluorescence spectroscopy (JASCO FP 777 spectrofluorometer, Japan) using pyrene as a probe.[32-34] To obtain sample solutions, a known amount of pyrene in acetone was added to each of a series of 20 mL vials and the acetone evaporated. The amount was adjusted to give a pyrene concentration in the final solution of either 6.0×10^{-7} M. First 10 mL of various concentrations of block copolymer solutions was added to each vial and then were heated for 3 h at 65 °C to equilibrate the pyrene and the micelles and left to cool overnight at room temperature. Emission wavelength was 390 nm for excitation spectra. Excitation and emission bandwiths were 3 and 1.5 nm, respectively.

Observation of transmission electron microscope (TEM). The morphology of the polymeric micelles was observed under a TEM (JEOL, JEM-2000 FX II, Japan). A drop of polymeric micelle suspension in aqeous solution was placed on a carbon film coated copper grid and freeze-dried. The specimen on the copper grid was not stained. Observation was done at 80 kV.

Dynamic light scattering (DLS) measurements. DLS was measured with a S4700 (Malvern Instruments, England) with an argon laser beam at the wavelength of 488 nm at 20 °C and the value is expressed in number-averaged scales as a unimode. The scattering angle of 90° was used.

***In vitro* release studies.** The release experiment *in vitro* was carried out as follows: 7 mg of NFX loaded-polymeric micelles and 1 mL phosphate buffered saline (PBS, 0.1 M, pH 7.4) were put into a dialysis tubes and the tube was introduced into a vial with 10 mL PBS.[35-37] At specific time intervals, the medium was taken and replaced with fresh PBS. The concentration of the released NFX was determined by UV spectrophotometer (Shimadzu UV-1201) at 322 nm.

Results and Discussion

Characterization of GEG block copolymers. GEG block copolymers was prepared by polymerization of γ-BLG NCA initiated with amine-terminated PEO in dichloromethane solution as shown in Figure 1(c). Goodman and Hutchison[28] have reported that the polymerization mechanism is the primary-amine mechanism in which the initiator amine undergoes a nucleophilic addition to the C-5 carboxyl group of the NCA, as reported by compositions and molecular weights of the copolymers are listed in Table 1. Copolymer composition and molecular weight were estimated from peak intensities of the methylene proton signal of the PBLG block and the methylene proton signal of the PEO block in the nuclear magnetic resonance (NMR) spectrum as reported previous-

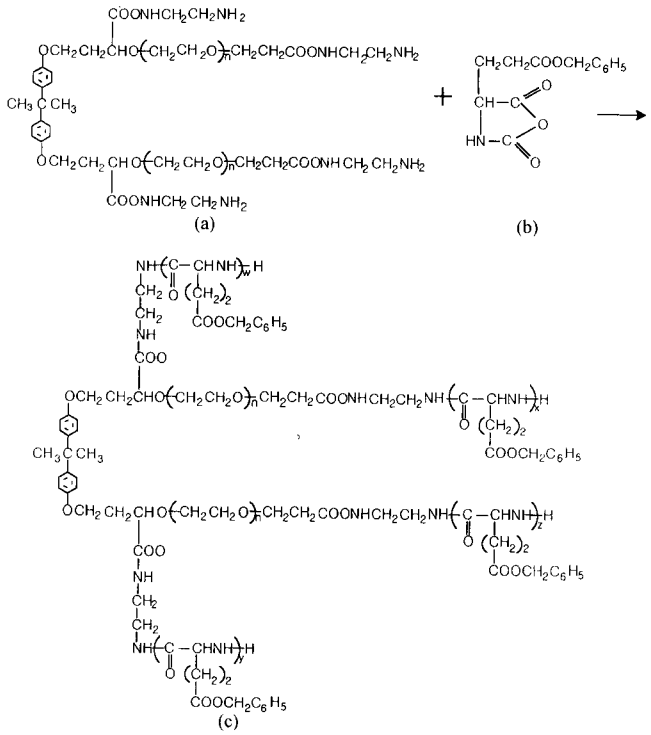

Figure 1. Synthesis scheme of PBLG/PEO/PBLG (GEG) block copolymer: Bis[poly(ethylene oxide)bis(amine)] (a), γ-benzyl *L*-glutamate N-carboxyanhydride (b) and GEG block copolymer (c).

Table 1. Characteristics of PBLG/PEO/PBLG block copolymers

Sample	Content of monomeric units in mol %		M_n
	PBLG	PEO[a]	
GEG-1	54.3	45.7	138500
GEG-2	40.4	59.6	87500
GEG-3	26.5	73.5	55900

[a] M.W. of PEO: 20000.

ly.[29~31] Assuming that all the amine groups of PEO participate in the polymerization, the number-average molecular weight, *Mn* of the copolymer can be calculated from the copolymer composition and the molecular weight of PEO chains.

FT-IR spectra of PBLG homopolymers and GEG block copolymers in the region of 4,000-400 cm^{-1} was shown in Figure 2. The amide I, II and V bands of these GEG block coplymers appear at 1,650 cm^{-1}, 1,550 cm^{-1}, and 615 cm^{-1}, respectively, at the same wavenumbers as for the PBLG homopolymer.[38]

Figure 3(a) shows the excitation spectrum of pyrene in the various concentrations of GEG-3 block copolymers. Pyrene is expected to preferentially partitioned into hydrophobic cores with a change of the photophysical properties of molecules. In the excitation spectrum, a red shift was observed with increasing concentration of GEG-3 block copolymers. The red shift of pyrene has also been observed in the micelle of PS-PEO block copolymers.[33] The (0,0) bands in the pyrene excitation spectra were examined and compared with the intensity ratio $I_{339}/I_{333.5}$; this ratio takes the value characteristic of pyrene in water at low concentrations and the value of pyrene entirely in the

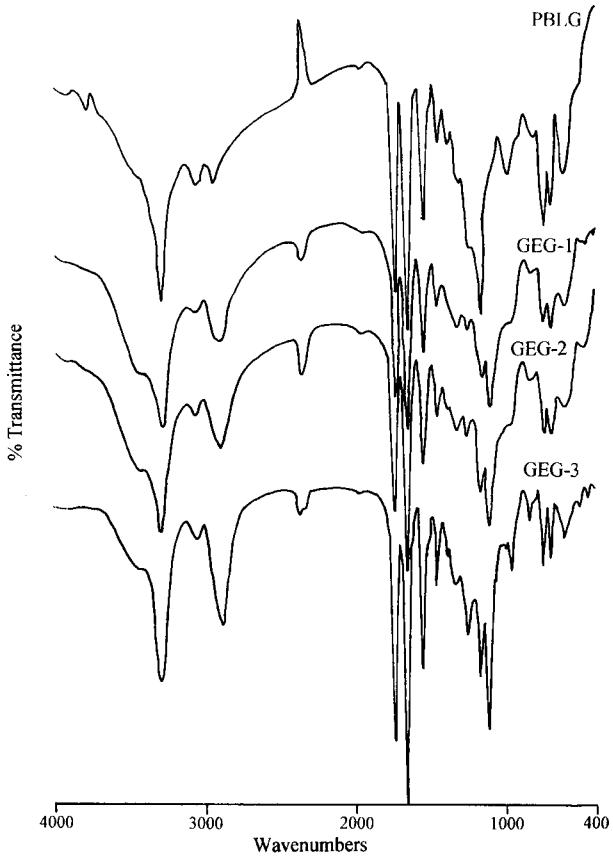

Figure 2. FT-Infrared spectra of PBLG homopolymer and GEG block copolymers.

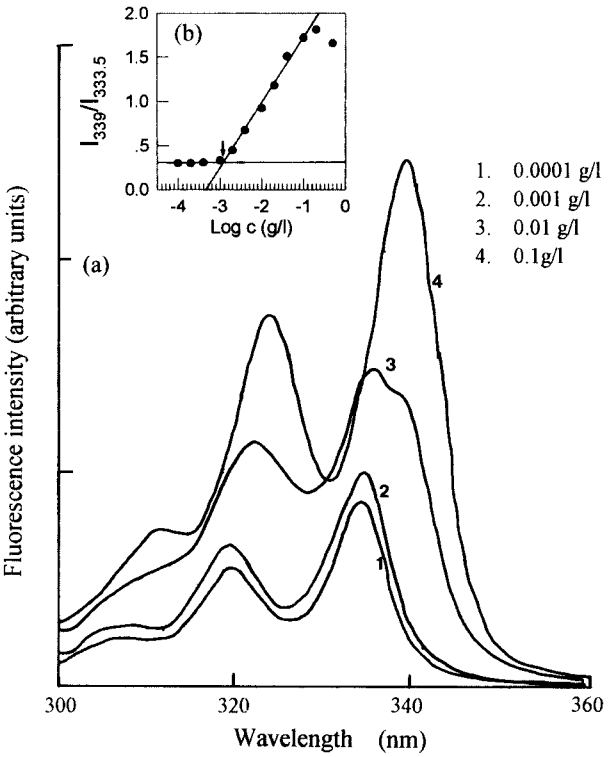

Figure 3. Fluorescence excitation spectra of pyrene/GEG-3 against concentration of GEG-3 in distilled water (Emission wavelength: 390.0 nm) (a) and plot of the intensity ratio $I_{339}/I_{333.5}$ from pyrene excitation spectra *vs.* log *c* for block copolymers against concentration of GEG in distilled water (b).

Table 2. The CMC values of the GEG block copolymers obtained from fluorescence spectroscopy measurement

Sample	PBLG content in mol %	CMC (mol)
GEG-1	54.3	1.2×10^{-8}
GEG-2	40.4	1.7×10^{-8}
GEG-3	26.5	2.0×10^{-8}

hydrophobic domain. A plot of $I_{339}/I_{333.5}$ versus log c is shown in Figure 3(b). A flat region in the low concentration extreme and a sigmoidal region in the crossover region were noted. This crossover region at the concentration of 0.0011 g/L can be evaluated to the CMC values of GEG-3 block copolymer. As shown in Table 2, the CMC values of the GEG block copolymer were decreased with increasing of the PBLG chain length. These tendency were similar with our previous report.[16,25,37]

Morphology and size distribution. Generally, complexes of cells within a number of organs act as major filters. Also, it was reported that size and surface chemistry are the major factors determining the survivability of particulate delivery technologies in the body.[39] For enhancing the survivability of particulate, the presence of a negative charge, overall hydrophilicity, and a low aspect size (> 1 μm) were tried by other workers.[40]

TEM micrographs of GEG-3 polymeric micelle (Figure 4) show sphere like micelles with diameter of approximately 20-100 nm.

Figure 5 shows the particle size distribution of GEG-3 polymeric micelle based on intensity average by DLS. The GEG-3 polymeric micelle has average diameter of 48.8± 25.0 which was almost similar result with TEM observation. The particle size with different PBLG chain length are shown in Table 3. Particle size distribution based on

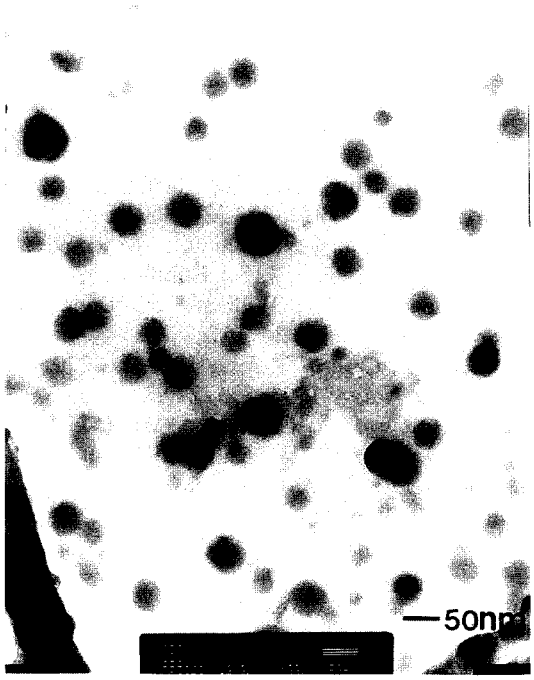

Figure 4. Transmition electron micrograph of GEG-3 polymeric micelles.

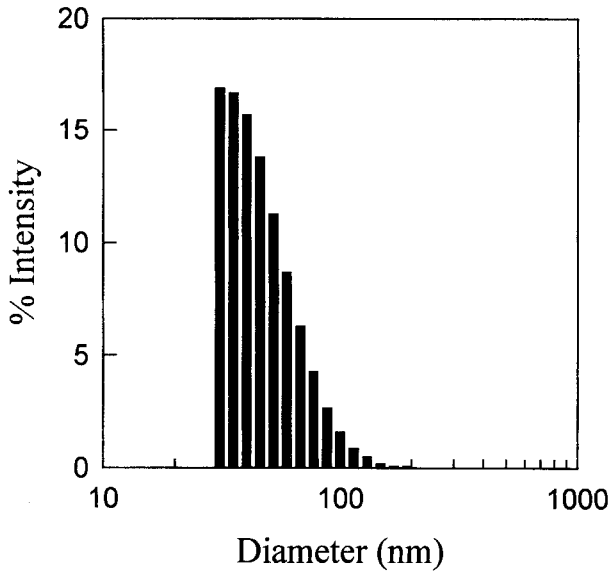

Figure 5. Particle size distribution of GEG-3 polymeric micelle measured by dynamic light scattering.

number average was 106.5±59.2 nm for GEG-1, 79.4±46.0 nm for GEG-2, and 37.9±13.3 nm for GEG-3. Usually, the polymeric micelles should be small and have narrow size distribution.[41] The size distribution of GEG block copolymer micelles was not narrow, which may be due to the secondary aggregation. This phenomena accounted for secondary aggregation during the procedure of polymeric micelle preparation and DLS measurement. The cause of the secondary aggregation is still unclear in spite of the frequently reported phenomena. For the secondary aggregates, several possibilities were envisioned, such as single large core-shell micelles, onionlike particles with alternating concentric layers of solvated and undissolved blocks, loose clusters of small regular micelles, further associate by hydrophobic interactions or by the van der Waals interactions between exposed cores as the same principles for formation of micelles and incomplete dissociation of the bulk polymer, etc.[35,41-43] From the result of Figure 5 and Table 3, the particle sizes were dependent on the PBLG chain length, *i.e.*, the longer the PBLG chain length, the larger the particle size. The nanoparticles formed by dialysis are small and spherical structures. The nanoparticles do not show large asymmetries because large deviations from the spherical shapes are normally thermodynamically unfavored.[4]

Drug loading and release study *in vitro*. The NFX loading contents and loading efficiency onto the GEG block copolymer micelles are shown in Table 4. It was

Table 3. Particle size distribution of GEG block copolymer micelles

Sample	PBLG content in mol %	Particle size (nm)		
		Intensity average	Volume average	Number average
GEG-1	54.3	142.8±93.8	119.1±67.4	106.5±59.2
GEG-2	40.4	82.8±47.6	88.7±54.3	79.4±46.0
GEG-3	26.5	48.8±25.0	44.3±20.4	37.9±13.3

Table 4. Drug-loading contents and loading efficiency onto the polymeric micelles of GEG block copolymers

Sample	Polymer conc. (mg)[a]	Initial drug conc. (mg)[a]	Loading contents (wt %)	Loading efficiency (%)
GEG-1	20	20	12.6	14.4
GEG-2	20	20	11.9	13.5
GEG-3	20	20	11.0	12.4
	20	40	19.1	11.8

[a] in 10 mL of solvent.

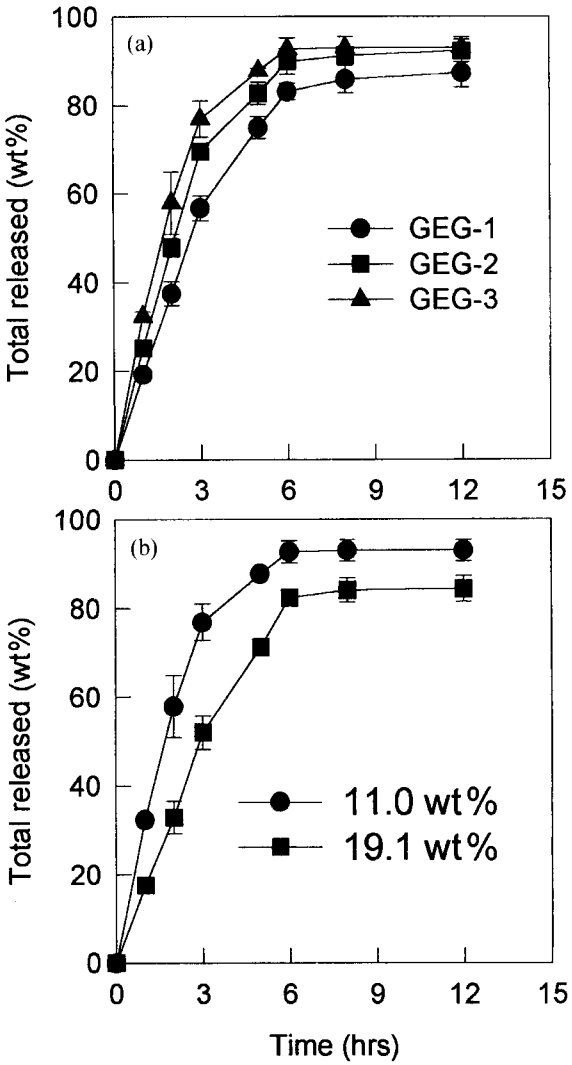

Figure 6. Release of NFX from polymeric micelles as a function of PBLG chain length (drug-loading content: 12.6 wt% for GEG-1, 11.9 wt% for GEG-2 and 11.0 wt% for GEG-3) (a) and against drug-loading contents (b).

found that the drug-loading contents and loading efficiency were dependent on the PBLG chain length, *i.e.*, the longer the PBLG chain length, the more the drug contents and the higher the loading efficiency. And the effect of initial drug concentration on the GEG-3 was shown that the high drug concentration induced the more drug loading. But the loading efficiency was slightly decreased.

Figure 6(a) shows the NFX release from various GEG polymeric micelles with different PBLG chain length. Although the direct comparison of release rate between GEG-1, GEG-2, and GEG-3 cannot be performed due to the different drug-loading contents, NFX release rate was relatively slower in the micelles of longer PBLG chain length than that of shorter PBLG chain length. The more hydrophobic domain of block copolymer should lead to the stronger hydrophobic interaction between hydrophobic block domain and drug.[37] Figure 6(b) shows NFX release from GEG-3 polymeric micelles having different drug-loading content. These results indicate that the more the drug content, the slower the drug releases. At lower loading, NFX may be present as a dispersed state in the core segment whereas a crystallization of drug in the PBLG core occurs at higher loading.[7,37] The crystallized drug should be dissolved more slowly and diffused into the outer aqueous phase. Therefore, the control of drug release kinetics can be achieved by the content of hydrophobic units of block copolymer and drug loading content.

Conclusions

The block copolymer based on PBLG as the hydrophobic part and PEO as hydrophilic was synthesized and characterized. Fluorescence spectroscopy measurement suggested that GEG block copolymers were associated in water to form polymeric micelles and CMC values of the block copolymers decreased with increasing PBLG chain length in the block copolymer. Polymeric micelles were spherically shaped and the size of nanoparticles was dependent on the PBLG chain length in the copolymer. The drug-loading contents and loading efficiency were also dependent on the PBLG chain length. The longer the PBLG chain length, the more the drug contents and the higher the loading efficiency. Release of NFX from the higher loading contents was slower than from lower loading contents due to the hydrophobic interaction between PBLG core and NFX.

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