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Adriamycin release from flower-type polymeric micelle based on star-block copolymer composed of poly(γ-benzyl L-glutamate) as the hydrophobic part and poly(ethylene oxide) as the hydrophilic part

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Abstract

Star-block copolymer based on PBLG as the hydrophobic part and PEO as the hydrophilic one (as abbreviated GEG) was synthesized and characterized. Polymeric micelle was prepared by the diafiltration method. From the measurement of photon correlation spectroscopy, the nanoparticle sizes of GEG-1, GEG-2 and GEG-3 were 106.5 ± 59.2, 43.8 ± 0.7 and 13.5 ± 1.0 nm in number average, respectively, indicating of the formation of polymeric micelle. Also, the nanoparticle sizes were dependent on the PBLG chain length, i.e. the more PBLG content in the copolymer, the larger the particle size. From the observation of transmission electron microscope(TEM), GEG-2 block copolymer had almost spherical shapes with size range about 20–70 nm, that was similar to particle size measurement. Fluorescence spectroscopy measurement indicated that GEG block copolymers associated in water to form polymeric micelles and critical micelle concentration (CMC) values of the block copolymers decreased with increasing PBLG chain length in the block copolymer. Characteristic peaks of the protons of the benzyl group in the PBLG and the methylene protons adjacent to the benzyl group of the PBLG segment in the GEG-2 nanoparticles appeared in $7.2 \sim 7.4$ and $5.0 \sim 5.2$ ppm, respectively, and disappeared in D_2O, indicating the restricted motions of these protons within the micellar core and the very rigid structure of the PBLG core in the GEG polymeric micelles. Release of ADR from the polymeric micelles in vitro was slower in longer PBLG chain length and higher loading contents of ADR. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Flower type micelle; Polymeric micelle; Star-block copolymer; Nanoparticle; Adriamycin release

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1. Introduction

As novel drug carriers, polymeric micelles or core-shell type nanoparticles prepared by block copolymers have been proposed to attain effective drug targeting to desired site of action in recent decades (Yokoyama et al., 1990a; Malmsten and Lindman, 1992; Gref et al., 1994; Jeong et al., 1998; Nah et al., 1998). Due to amphiphilicity, block or graft copolymers self-assemble into polymeric micelles with core-shell structure and slowly dissociate to free polymeric chain in contrast with micelle of low molecular weight surfactants (Yokoyama et al., 1990a; Malmsten and Lindman, 1992). Polymeric micelles based on block copolymers have interesting structural characteristics, i.e. hydrophobic inner-core and hydrophilic outer shell. Hydrophobic block forms the inner-core of the structure which acts as a drug incorporation site, especially for the hydrophobic drugs by hydrophobic interactions (Kwon et al., 1995) and hydrophilic block forms the hydrated outer shell which plays a role in avoiding the uptake by the reticuloendothelial system (RES). Also, the predominant characteristics of this system have been reported such as reduced toxic side effects of anticancer drug by micelle formation with block copolymer and selective targeting, solubilization of hydrophobic drugs, stable storage, long blood circulation, favorable biodistribution and lower interactions with RES (Yokoyama et al., 1990b, 1991; Gref et al., 1994; Kwon et al., 1993a, 1994, 1995). In contrast with conventional microspheres or nanoparticles, polymeric micelles generally have a reduced small particle size as a similar tendency with natural biomolecule vehicles, virus. Since the major factors to determine the fate of nanoparticulate carriers in the blood circulation are particle size and surface chemistry (Kreuter, 1991), polymeric micelles may be appropriate vehicles for drug delivery such as anticancer agents or other hydrophobic drugs.

In previous studies, we reported that core-shell type polymeric micelle based on linear block copolymer composed of poly(γ-benzyl L-glutamate) (PBLG) and poly(ethylene oxide) (PEO) have physically entrapped hydrophobic drugs with high stability and controlled release as a pseudo zero-order kinetics (Jeong et al., 1998). Also, we report that polymeric micelles from the star-block copolymer composed of PBLG as the hydrophobic part and PEO as the hydrophilic one could be formed and clonazepam as the hydrophobic model drug could be loaded onto the polymeric micelles (Nah et al., 1998). Kwon et al. (1995) already reported that linear block copolymer composed of poly(β-benzyl L-aspartate) (PBLA) and PEO formed micelles through self-association in water.

In this study, we are interested in smaller nanoparticle size based on polymeric micelles from star-block copolymer having a small amount of hydrophobic part. The small nanoparticle sizes will be important from the practical viewpoint of delivery of anticancer drugs. The star-block copolymer had peculiar properties such as lower glass transition temperature, smaller hydrodynamic radii, less crystallinity and slower biodegradability than linear block copolymer (Li and Kissel, 1998). Therefore, it may be expected that the shape and physicochemical character of polymeric micelles obtained by star-block copolymer will be different from the polymeric one obtained by linear block copolymer.

2. Materials and methods

2.1. Materials

Bis[poly(ethylene oxide) bis(amine)] (BPEOBA: M.W. = 20 000) and γ-benzyl L-glutamate were purchased from Sigma (St Louis, MO). Triphosgene was purchased from Aldrich (Milwaukee, WI). Adriamycin HCl was kindly supplied by Dong-A Pharm. Co. Ltd. (South Korea). All chemicals used were of reagent or spectrometric grade. n-Hexane and methylene dichloride were stored with 4 Å molecular sieves and used without further purification.

2.2. Synthesis of star-block copolymer

γ-Benzyl L-glutamate *N*-carboxyanhydride (BLG-NCA) was prepared by a method described in the literature (Goodman and Hutchison, 1966).

The star-block copolymer (c) was synthesized by a similar method to that previously reported (Cho et al., 1995; Nah et al., 1998). Briefly, the multi-block copolymer was obtained by polymerization of BLG-NCA (b) initiated by the BPEOBA (a) in methylene chloride, at a total concentration of BLG-NCA and BPEOBA of 3% (w/v), at room temperature for 72 h. The reaction mixture may contain unreacted amine-terminated PEO, and the desired block copolymers. The initiator (amine-terminated PEO) cannot be precipitated from a mixture of methylene chloride and diethyl ether, although the latter is a non-solvent for PEO. By adding a large excess of diethyl ether to the reaction mixture, the block copolymers precipitated were collected on a filter, while the unreacted amine-terminated PEO was removed as a filtrate. The resulting copolymer was washed with diethyl ether and then dried in vacuo. The reaction scheme is shown in Fig. 1.

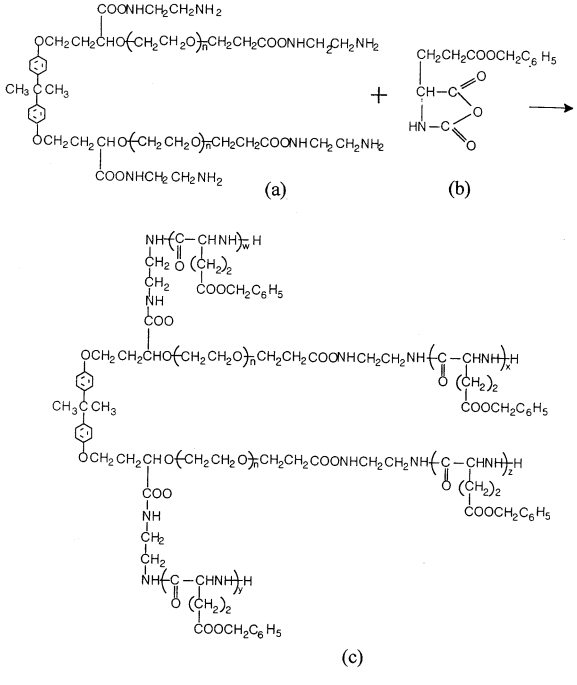

Fig. 1. Synthesis scheme of star-block copolymer (abbreviated as GEG) (c).

2.3. ^1H NMR measurement

^{1}H NMR spectra of the copolymers were measured in $CDCl_3$ to estimate the copolymer compositions and the molecular weights of PBLG blocks, using a JEOL FX 90Q NMR spectrometer. As the number-average molecular weight (20 000) of PEO is known, one can estimate the number-average molecular weights of the PBLG block and the copolymer composition calculated from the peak intensities in the spectrum assigned to both polymers, respectively (Cho et al., 1990; Nah et al., 1998).

For approved micellar structure of the block copolymer micelles, ^{1}H NMR spectra were measured in $CDCl_3$ and D_2O. The concentration of the polymeric micelle concentration was 0.5 wt. % in $CDCl_3$ and D_2O (Kwon et al., 1993b).

2.4. Measurement of fluorescence spectroscopy

To investigate the fluorescence spectroscopy characteristics, the block copolymer solutions without drug were prepared as follows; 20 mg of GEG block copolymer was dissolved in 5 ml of DMF and dialyzed using a molecular cut-off 12 000 g/mol dialysis tube (Sigma, St Louis, MO) against 3×1 litre of distilled water for 3 h and then 3–4 h for 2 days. The resultant solution was adjusted to the various concentrations of block copolymers.

Critical micelle concentrations (CMC) of the block copolymers were estimated to prove the potential of micelle formation by the measurement of fluorescence spectroscopy (Shimadzu F-7000 spectrofluorimeter, Shimadzu, Tokyo, Japan) using pyrene as a probe (Kalyanasundaram and Thomas, 1977; Wilhelm et al., 1991; Kwon et al., 1993b; Marctic and Nair, 1994). 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 6.0×10^{-7} M. Various concentrations (10 ml) of block copolymer solutions were added to each vial and then they were heated for 3 h at 65°C to equilibrate the pyrene and the micelles and left to cool overnight

at room temperature. The emission wavelength was 390 nm for excitation spectra. Excitation and emission bandwidths were 1.5 and 1.5 nm, respectively.

2.5. Observation of transmission electron microscope (TEM)

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

2.6. Photon correlation spectroscopy (PCS) measurements

PCS was measured with a Zetasizer 3000 (Malvern Instruments, UK) with He–Ne laser beam at a wavelength of 633 nm at 25°C (scattering angle of 90°). Nanoparticle solution prepared by the diafiltration method was used for particle size measurement (concentration: 1 mg/ml) and measured without filtering.

2.7. Preparation of adriamycin (ADR)-loaded polymeric micelles

Formation of polymeric micelles and drug loading were carried out as follows: 20 mg of block copolymer was dissolved in 4 ml of DMF and subsequently 25 mg of ADR in 1 ml DMF with 1.3 equivalent of triethylamine added. The solution was stirred at room temperature and solubilized entirely. To form polymeric micelles and remove free-drugs, the solution was dialyzed using a molecular cut-off 12 000 g/mol dialysis tube against 1.0 litre of acetate buffer (pH 5.5, 0.1 M) for 2 h and then 4×1 litre of distilled water for 12 h. Then, the solution was analyzed or freeze-dried.

For evaluation of drug loading content, ADR-loaded GEG polymeric micelles were measured using UV spectrophotometer (Shimadzu UV-1201) at 479 nm.

Table 1
Characterization of PBLG/PEO/PBLG block copolymers[a]

Sample	Content of monomeric units (mol %)		$\overline{Mn}$
	PBLG	PEO	
GEG-1	14.9	85.1	37 400
GEG-2	8.2	91.8	28 900
GEG-3	3.8	96.2	24 000

[a] M.W. of PEO is 20 000.

2.8. In vitro release studies

The release experiment in vitro was carried out as follows: 5 mg of ADR loaded-polymeric micelles and 1 ml of phosphate buffered saline (PBS 0.1 M, pH 7.4) were put into a dialysis tube and then this was introduced into a vial with 10 ml of PBS. At specific time intervals, the whole medium was taken and replaced with fresh PBS. The concentration of the released ADR was determined by UV spectrophotometer (Shimadzu UV-1201) at 479 nm.

3. Results and discussion

3.1. Characterization of block copolymers and their polymeric micelles

Block copolymers (abbreviated as GEG) prepared by polymerization of γ-BLG NCA initiated with amine-terminated PEO in methylene chloride solution are shown in Fig. 1. It may be assumed 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 Goodman and Hutchison (1966). Compositions and molecular weights of the copolymers are listed in Table 1. The copolymer composition and the molecular weight were estimated from peak intensities of the methylene proton signal (5.0 ppm) of the PBLG block and the methylene proton signal (3.7 ppm) of the PEO block in the nuclear magnetic resonance (NMR) spectrum. Assuming that all the amine groups of PEO partici-

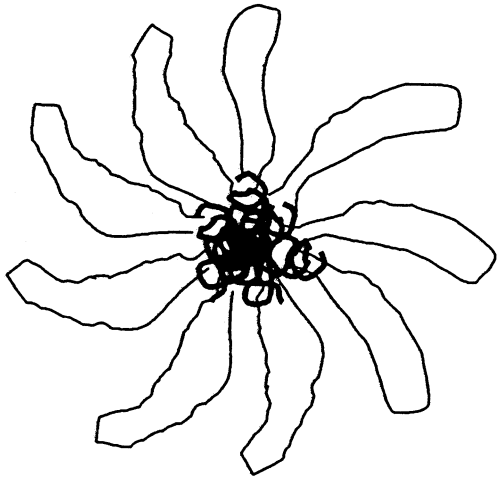

Fig. 2. Schematic drawing of flower-type polymeric micelle structure of GEG block copolymer.

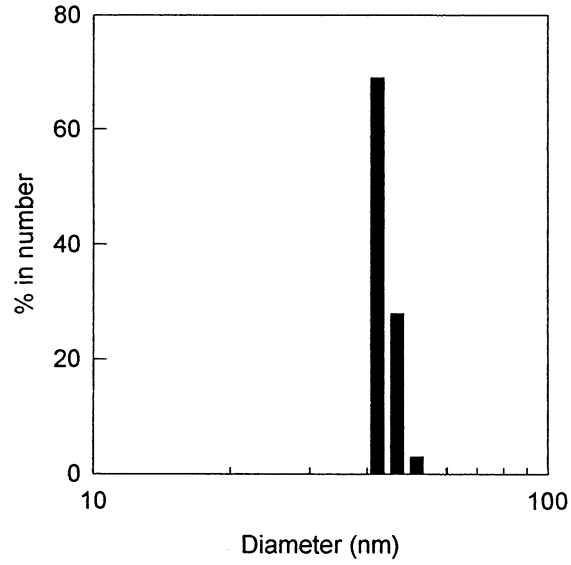

Fig. 3. Particle size distribution of GEG-2 by photon correlation spectroscopy. Concentration of GEG-2 was 1.0 mg/ml and measured at 25°C.

pate in the polymerization, the number-average molecular weights, Mn of the copolymer can be calculated from the copolymer composition and the molecular weight of PEO chains.

3.2. Morphology and size distribution of polymeric micelles

Fig. 2 shows the particle size distribution of GEG-3 polymeric micelle based on number average by PCS. As expected, GEG block copolymers result in small and narrow size distributed particles with a similar size range to other block copolymeric micelles such as PBLA (or poly(aspartate))–PEO diblock copolymer (Yokoyama et al., 1990a,b; Kwon et al., 1993b) and poly(styrene)–PEO (Wilhelm et al., 1991). The particle size against PBLG chain length is shown in Table 2. Particle size distributions for GEG-1, GEG-2 and GEG-3 were 106.5 ± 59.2, 43.8 ± 0.7 and 13.5 ± 1.0 nm, respectively, based on number average. From the values in Fig. 3 and Table 2, the particle sizes were dependent on the PBLG chain length, i.e. the longer the PBLG chain length, the larger the particle size. The polymeric micelles formed by dialysis method are small, spherical structures and the polymeric micelles do not show large asymmetries because large deviations from the spherical shapes are normally energetically and entropically unfavored.

Table 2
Particle size distribution of GEG block copolymer nanoparticles measured by photon correlation spectroscopy

Sample	PBLG content (mol %)	Particle size (nm) (area: %)		
		Intensity average	Volume average	Number average
GEG-1	14.9	142.8 ± 93.8	119.1 ± 67.4	106.5 ± 59.2
GEG-2	8.2	43.1 ± 1.5 (91.2)	43.9 ± 0.6 (96.3)	43.8 ± 0.7
		152.0 ± 38.9 (8.8)*	149.7 ± 45.8 (3.7)*	
GEG-3	3.8	18.2 ± 11.6 (98.6)	15.1 ± 4.3	13.5 ± 1.0
		148.2 ± 95.1 (1.4)*		

* Secondary aggregation.

Fig. 3 shows TEM photographs of GEG-3 polymeric micelle. They showed almost spherical shapes and the size ranges is about 20 ~ 70 nm in diameter, which compares with the dimension of viruses and thus they may be able to penetrate the sinusoidal and fenestrated capillaries that have pores approximately 100 nm in size. Generally, linear AB diblock copolymers consisting of a hydrophobic and hydrophilic group aggregated into core-shell micelles without the macroscopic phase separation. These consist of an inner core, comprising insoluble blocks, and an exterior shell of soluble blocks swollen by the solvent. On the other hand, Halperin (1991) reported that flower-type micelles were formed by the linear multiblock copolymers. In this star-block copolymer, it is thought that flower-type spherical micelles are formed from the star-block copolymers as shown in Fig. 4. But the micelles are phase-separated if the water-insoluble chains become longer (data not shown).

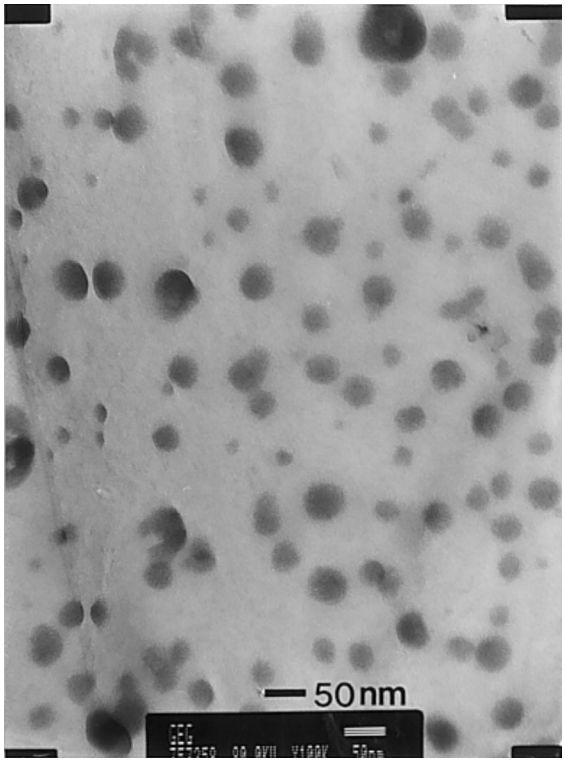

Fig. 4. Transmission electron microscopy (TEM) photograph of GEG-2 micelles.

3.3. Fluorescence study of pyrene in GEG polymeric micelles

Critical micelle concentrations (CMC) were estimated to prove the potential of micelle formation of GEG block copolymers using pyrene as a hydrophobic probe (Kalyanasundaram and Thomas, 1977; Wilhelm et al., 1991; Kwon et al., 1993b; Marctic and Nair, 1994).

Fig. 5(a) shows the excitation spectrum of pyrene in the various concentrations of GEG-2 block copolymers. Pyrene is preferentially partitioned into the hydrophobic core with a change of the photophysical properties of molecules. In the excitation spectrum, a red shift was observed with increasing concentration of GEG-2 block copolymers. A red shift of pyrene in the excitation spectrum was observed in the study of micelle formation of PS-PEO block copolymers (Wilhelm et al., 1991). Plots of I_{340}/I_{335} vs log C are shown in Fig. 5(b). I_{340} and I_{335} were chosen as the value of the wavelength of the (0,0) band in the pyrene excitation spectra and the value of pyrene entirely in the hydrophobic domain of polymeric micelle, respectively. A flat region existed in the low concentration extreme and a sigmoidal region in the crossover region. This result indicated that change in signal in the region of 0.009 g/l for GEG-2 can be evaluated for the CMC values of GEG block copolymer. As shown in Table 3, the estimation of CMC values for the GEG block copolymer decreased with increasing PBLG chain length. A similar tendency was reported previously (Kim et al., 1997; Jeong et al., 1998; Nah et al., 1998).

Further evidence of polymeric micelle formation of GEG block copolymer and limited mobility of the PBLG chain in the core of the micelle was obtained with ^{1}H NMR in $CDCl_3$ and D_2O. As shown in Fig. 6(a) of $CDCl_3$ where micelle formation is not expected, the characteristic peak of the protons of the benzyl group and the methylene protons adjacent to the benzyl group of the PBLG segment was shown in 7.2 ~ 7.4 and 5.0 ~ 5.2 ppm, respectively. But in D_2O (Fig. 6(b)), these peaks disappeared. These results indicated restricted motions of these protons within the micellar core and rigid structure of PBLG core of the GEG polymeric micelles. This behav-

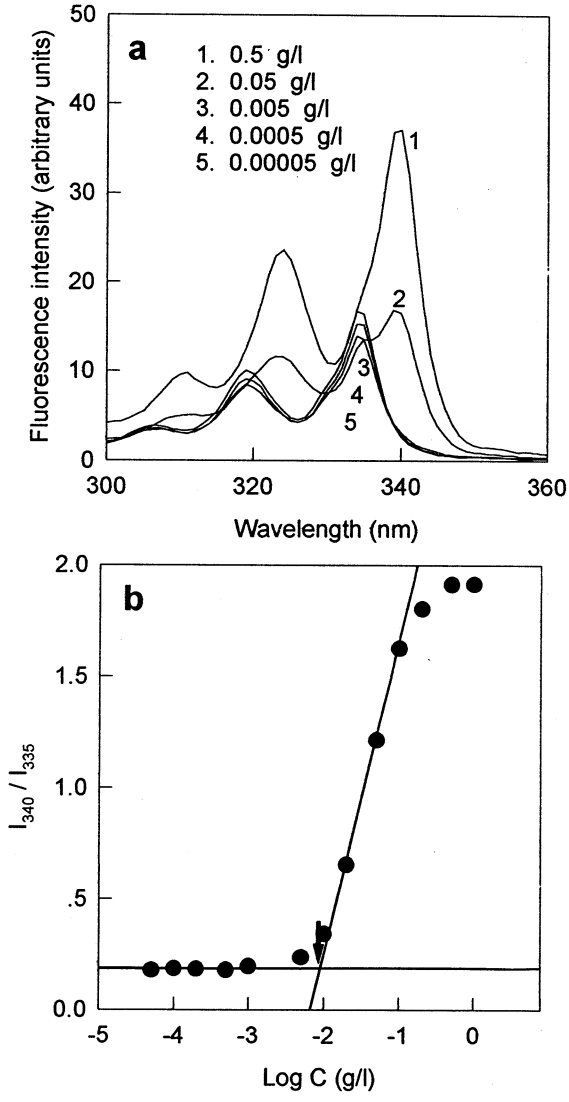

Fig. 5. Fluorescence excitation spectra of pyrene/GEG-2 against concentration of GEG-2 in distilled water (emission wavelength: 390.0 nm) (a) and plots of the intensity ratio of I_{340}/I_{335} from pyrene excitation spectra vs. log C for block copolymer against concentration of GEG in distilled water (b).

ior of GEG micelles is in contrast with low molecular amphiphiles and PEO–PPO–PEO block copolymers which typically exhibit liquid-like cores and relatively higher mobility. Kwon et al. (1993b) also reported that poly(β-benzyl L-aspartate) (PBLA)/PEO diblock copolymer has a rigid PBLA core and hydrated PEO outer shell in the

Table 3
The CMC values for the GEG block copolymers by fluorescence spectroscopy measurement

Sample	PBLG content (mol %)	CMC (mol)
GEG-1	14.9	2.1×10^{-7}
GEG-2	8.2	3.1×10^{-7}
GEG-3	3.8	8.3×10^{-7}

^{1}H NMR study of polymeric micelles of diblock copolymers which was similar to our results.

3.4. Drug loading and release study in vitro

The ADR loading contents and loading efficiency onto the GEG polymeric micelles are shown in Table 4. It was found that drug loading contents and loading efficiency increased with an increase of PBLG chain length in the copolymer. Total release of ADR from the polymeric micelle according to the PBLG chain length is shown in

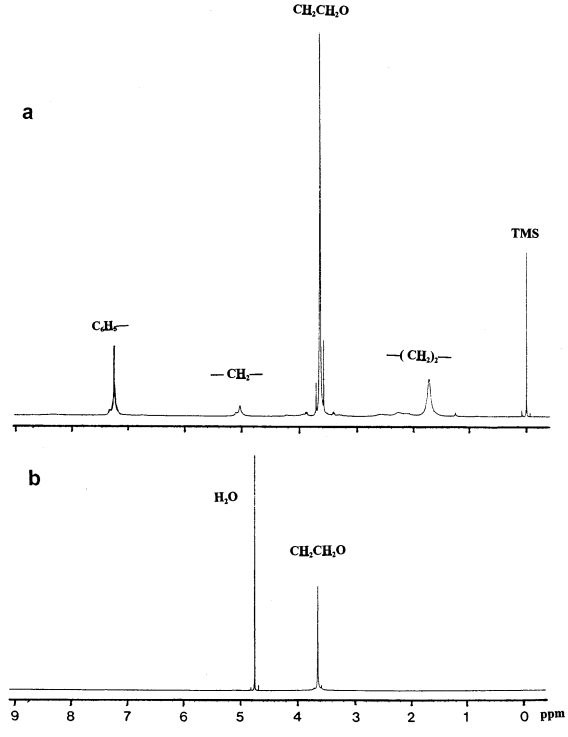

Fig. 6. ^{1}H NMR spectra of GEG-2 polymeric micelle in $CDCl_3$ (a) and D_2O (b).

Table 4
ADR loading content and loading efficiency onto the GEG polymeric micelles

Sample	Initial amount of polymer (mg)	Initial amount of drug (mg)	Drug loading content (wt. %)	Loading efficiency (wt. %)
GEG-1	20	12.5	17.5	33.0
GEG-2	20	12.5	15.4	29.1
	20	20	19.1	23.6
GEG-3	20	12.5	11.5	20.8

Fig. 7. These results indicated that the release of ADR from the micelle decreased with an increase of PBLG content between GEG-1 and GEG-2, which had almost the same drug loading contents. The slower release in the micelles of longer PBLG chain length can be attributed to the stronger hydrophobic interaction between hydrophobic domain and drug. Fig. 8 shows ADR release from GEG-2 polymeric micelles having different drug-loading contents. It was found that the more the drug content, the slower the drug release. It may be supposed that at lower loading, ADR is present as a dispersed state in the core segment whereas a crystallization of drug in the PBLG core occurs at higher loading (Jeong et al., 1998).

In the release pattern of all the samples, we observed pseudo zero-order release after an initial burst release effect, indicating entrapping of moderate lipophilic ADR in the hydrophobic core of the micelles. It is generally assumed that a drug is released by several processes (Gref et al., 1996) such as diffusion through the polymer matrix, release by polymer degradation and solubilisation and diffusion through microchannels that exist in the polymer matrix or are formed by erosion. In this system, polymer degradation did not occur within a few days due to the hydrophobic properties of PBLG; accordingly, we can consider that the drug is released from the micelles through the diffusion mechanism in vitro.

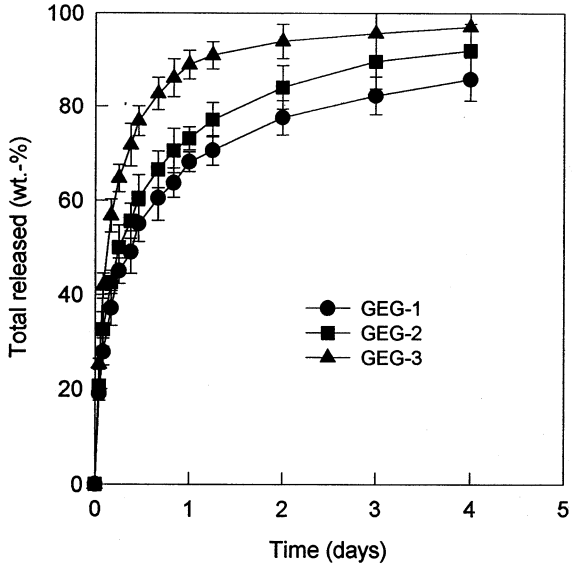

Fig. 7. Release of ADR from GEG polymeric micelle against PBLG content: drug loading content of GEG-1, GEG-2 and GEG-3 was 17.1, 15.4 and 11.5 wt. %, respectively.

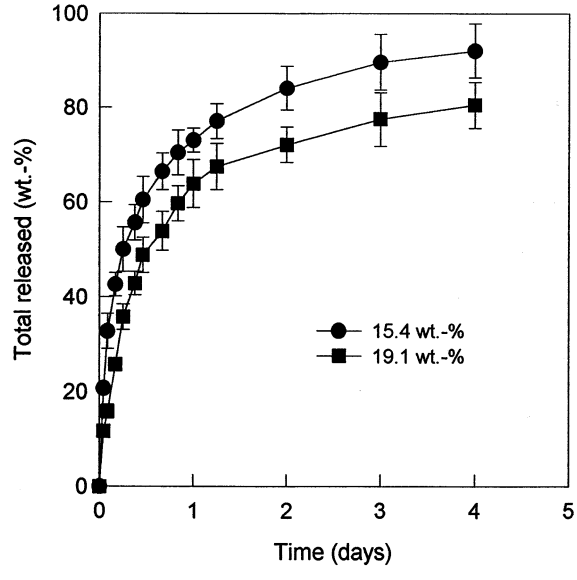

Fig. 8. Release of ADR from GEG-2 polymeric micelle against drug loading content.

In conclusion, flower-type polymeric micelles were formed from the star-block copolymer based on PBLG as the hydrophobic part and PEO as the hydrophilic one. In the particle size measurement using photon correlation spectroscopy, particle sizes of GEG-1, GEG-2 and GEG-3 were 106.5 ± 59.2, 43.8 ± 0.7 and 13.5 ± 1.0 nm in number average, respectively, indicating formation of polymeric micelle of GEG block copolymer. Also, nanoparticle sizes were dependent on the PBLG chain length, i.e. the higher the PBLG block content, the larger the particle size of GEG block copolymer. From TEM observations, GEG-2 block copolymer resulted in almost spherical particles with a size range of about 20–70 nm which was similar to particle size measurements. Fluorescence spectroscopy measurements suggested that GEG block copolymers were associated in water to form polymeric micelles and the CMC values of the block copolymers decreased with increasing PBLG chain length in the block copolymer. The characteristic peak of the protons of the benzyl group and the methylene protons adjacent to the benzyl group of the PBLG segment shown in $7.2 \sim 7.4$ and $5.0 \sim 5.2$ ppm disappeared in D_2O, indicating restricted motions of these protons within the micellar core and rigid structure of PBLG core of the GEG polymeric micelles. Release of ADR from the polymeric micelles in vitro was slower in longer PBLG chain lengths and higher loading contents of ADR.

Acknowledgements

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