International Journal of Pharmaceutics 365 (2009) 150–156

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical nanotechnology

Antitumor activity of adriamycin-incorporated polymeric micelles of poly(γ-benzyl L-glutamate)/poly(ethylene oxide)

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ARTICLE INFO

Article history:
Received 8 April 2008
Received in revised form 12 August 2008
Accepted 13 August 2008
Available online 22 August 2008

Keywords:
Polymeric micelle
Adriamycin
Multiblock copolymer
CT 26 colon carcinoma
Antitumor activity

ABSTRACT

The multiblock copolymer composed of poly(γ-benzyl L-glutamate) (PBLG) and poly(ethylene oxide) (PEO) was synthesized to prepare polymeric micelles as an anticancer drug carrier. Adriamycin (ADR) used as an anticancer drug was incorporated into the polymeric micelles prepared by the multiblock copolymer. The higher the drug feeding ratio, the higher the drug loading contents and the lower the drug loading efficiency. The increased drug feeding ratio resulted in increased particle sizes. At all of the formulations, particle sizes were less than 150 nm. The particles were observed as spherical shapes. ADR release from ADR-loaded polymeric micelles *in vitro* was decreased with an increased drug loading contents. In *in vitro* antitumor activity test using CT 26 tumor cells, polymeric micelles showed almost similar cytotoxicity when compared to ADR itself while polymeric micelles themselves did not affect cytotoxicity. In *in vivo* antitumor activity test using mice tumor xenograft model, the polymeric micelles showed improved survivability of mice with minimized weight changes and excellent tumor growth suppression efficacy. Polymeric micelles of the multiblock copolymer suggested to be a good candidate for anticancer drug delivery carrier.

1. Introduction

A variety of colloidal drug carrier using macromolecules has been investigated as an effective drug delivery system for enhanced therapeutic purposes. Among them, polymeric micelles or core-shell type nanoparticles prepared by block copolymers have been proposed to attain selective drug targeting to desired site of action in recent decades (Gref et al., 1994; Jeong et al., 1998; Yokoyama et al., 1990a, 1991). Amphiphilic macromolecules such as block or graft copolymers and hydophobically modified polysaccharides can form self-assemblies in aqueous environment, i.e. self-aggregates and polymeric micelles (Akiyoshi et al., 1993; Jeong et al., 1999, 2006; Kwon et al., 1993a; La et al., 1996). Especially, polymeric micelles are considerably spotlighted as a colloidal drug delivery system due to predominent characteristic of these systems such as reduced toxic side effects of anticancer drug, passive targeting, solubilization of hydrophobic drugs, stable storage, long blood circulation, favorable biodistribution, reduced particle size, and low interactions with reticuloendothelial system (RES) (Jeong et al., 1999; Kwon et al., 1993b, 1994; Yokoyama et al., 1990b, 1991). Hydrophobic segment of block copolymers is composed of hydrophobic inner-core as a drug incorporation site by hydrophobic interactions (Kwon et al., 1995). Also, hydrophilic segment forms hydrated outer shell which plays a role in avoiding the attack of biological system.

We previously reported flower type polymeric micelle formation by multiblock copolymers and they showed typical core-shell structures in an aqueous solution (Jeong et al., 1999). Furthermore, the polymeric micelles showed small particle sizes around 50 nm in diameter and very low critical micelle concentration (CMC) in aqueous system. Generally, it was reported that anticancer agent-incorporated or conjugated polymeric micelles had enhanced blood circulation time and suppressive effect on the solid tumor growth (Kwon et al., 1994, 1995; Yokoyama et al., 1990a, 1991). Kataoka and collaborators have extensively investigated polymeric micelles

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doi:10.1016/j.ijpharm.2008.08.011

as hydrophobic drug carriers such as anticancer agent, adriamycin. We also reported that core-shell type nanoparticles composed of poly(γ-benzyl L-glutamate) (PBLG) and poly(ethylene oxide) (PEO) physically entrapped hydrophobic drugs with high stability and controlled released as a pseudo zero-order kinetics (Jeong et al., 1999).

In this study, we prepared polymeric micelles using multiblock copolymer composed of PBLG and PEO (abbreviated as GEG) and tested their antitumor effect *in vitro* and *in vivo*. Physicochemical characteristics of ADR-incorporated GEG polymeric micelles were also investigated. To evaluate antitumor activity of the GEG polymeric micelles, CT 26 colon carcinoma cells were used to cell viability test *in vitro* and tumor-induced mice were used as tumor xenograft animal model *in vivo*.

2. Materials and methods

2.1. Materials

Bis[poly(ethylene oxide)bis(amine)] (BPEOBA: M.W. = 20,000), dialysis tube (molecular cut-off 12,000 g/mol), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and γ-benzyl L-glutamate were purchased from Sigma Chem. Co. (St. Louis, MO). Triphosgene was purchased from Aldrich Chem. Co. Inc. (Milwaukee, WI, USA). Adriamycin·HCl was kindly supplied by Dong-A Pharm. Co. Ltd. (Korea). *n*-Hexane, tetrahydrofuran (THF), dimethylformamide (DMF), triethylamine (TEA), and methylene dichloride were purchased from Aldrich Chem. Co. Inc. (Milwaukee, WI, USA). All chemicals used were of reagent or spectrometric grade. *n*-Hexane and methylene dichloride were stored with 4 Å molecular sieves before use.

2.2. Synthesis of multiblock copolymer

γ-Benzyl L-glutamate *N*-carboxyanhydride (BLG-NCA) was prepared by a method described in the literature (Jeong et al., 1999). Briefly, the multiblock copolymer was obtained by polymerization of BLG-NCA initiated by the BPEOBA 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 GEG block copolymers. The initiator (amine-terminated PEO) can not be precipitated from a mixture of methylene chloride and diethyl ether, although the latter is a non-solvent for PEO. By adding large excess of diethyl ether to the reaction mixture, GEG 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.

As reported previously (Jeong et al., 1999), the copolymer compositions and the molecular weights of PBLG block was estimated from the results of ^{1}H NMR spectra using $CDCl_3$ (FT-NMR, Bruker AC-300F, 300 MHz). 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.

2.3. Preparation of ADR-incoporated polymeric micelle

ADR-incoporated polymeric micelle of GEG multiblock copolymer was prepared by similar method with minor modification (Jeong et al., 1999, 2006). To prepare polymeric micelles, 40 mg of GEG multiblock copolymer was dissolved in 4 ml of THF–DMF mixed solvent (3 ml THF + 1 ml DMF). Two (or 5 and 10) milligrams of ADR was separately dissolved in 1 ml of DMF with one drop of TEA. ADR in DMF solution was added to polymer solution and magnetically stirred for 1 h. To form polymeric micelles and ADR entrapment, this solution was slowly dropped into 15 ml of deionized water for 10 min. To evaporate solvent, polymeric micelle aqueous solution was adapted to rotary evaporator (Rotary Vacuum Evaporator, Type N-N, EYELA, Tokyo Rikakikai, Co. Ltd., Japan) under reduced pressure and evaporated THF for at least 30 min. Residual solvent was removed by dialysis (dialysis tube: molecular cut-off 12,000 g/mol) against 1 L deionized water for 12 h. To remove solvent, deionized water was exchanged every 2 h. After 12 h, dialyzed solution was used for analysis or lyophilized.

Polymeric micelles themselves were prepared without addition of ADR and then same procedure was employed to make polymeric micelles.

At all experiment, the amount of ADR was weighed from calculated amount of ADR·HCl.

To determine drug loading contents and loading efficiency, the volume of the dialyzed solution was adjusted to 40 ml with deionized water (i.e. 40 mg of GEG multiblock copolymer/40 ml of water). ADR concentration was measured with UV spectrophotometer at 479 nm (UV-1201, Shimadzu Co., Ltd., Japan). For blank test, empty polymeric micelles were adjusted to 40 ml (i.e. 40 mg of GEG multiblock copolymer/40 ml of water) and 0.1 ml of this solution was diluted with DMF. All experiments were triplicated. The equation of drug loading contents and loading efficiency were as follows:

$$\text{drug loading contents} = \frac{\text{amount of ADR in the polymeric micelles}}{\text{weight of polymeric micelles}} \times 100$$

$$\text{loading efficiency} = \frac{\text{residual amount of ADR in the polymeric micelles}}{\text{feeding amount of ADR}} \times 100$$

2.4. Observation of transmission electron microscope (TEM)

The morphologies of the polymeric micelles were 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. The specimen on the copper grid was not stained. Observation was done at 80 kV.

2.5. Photon correlation spectroscopy (PCS) measurements

PCS was measured with a Zetasizer 3000 (Malvern Instruments, England) with He–Ne laser beam at a wavelength of 633 nm at 25 °C (scattering angle of 90°). Nanoparticle solution prepared by diafiltration method was used for particle size measurement (concentration: 1 mg/ml).

2.6. In vitro release studies

The release experiment *in vitro* was carried out as followed: ADR-incorporated polymeric micelles were prepared as described above and final aqueous solution was adjusted to 40 ml (i.e. 40 mg of polymer/40 ml of water). Two milliliters of adjusted solution were introduced into dialysis tube (M.W. cut-off 12,000 g/mol) and dialysis tube was introduced into a bottle with 98 ml of phosphate buffered saline (PBS, 0.1 M, pH 7.4). Release test was performed at 37 °C with stirring rate of 100 rpm. The whole release medium was exchanged with fresh medium at predetermined time inter-

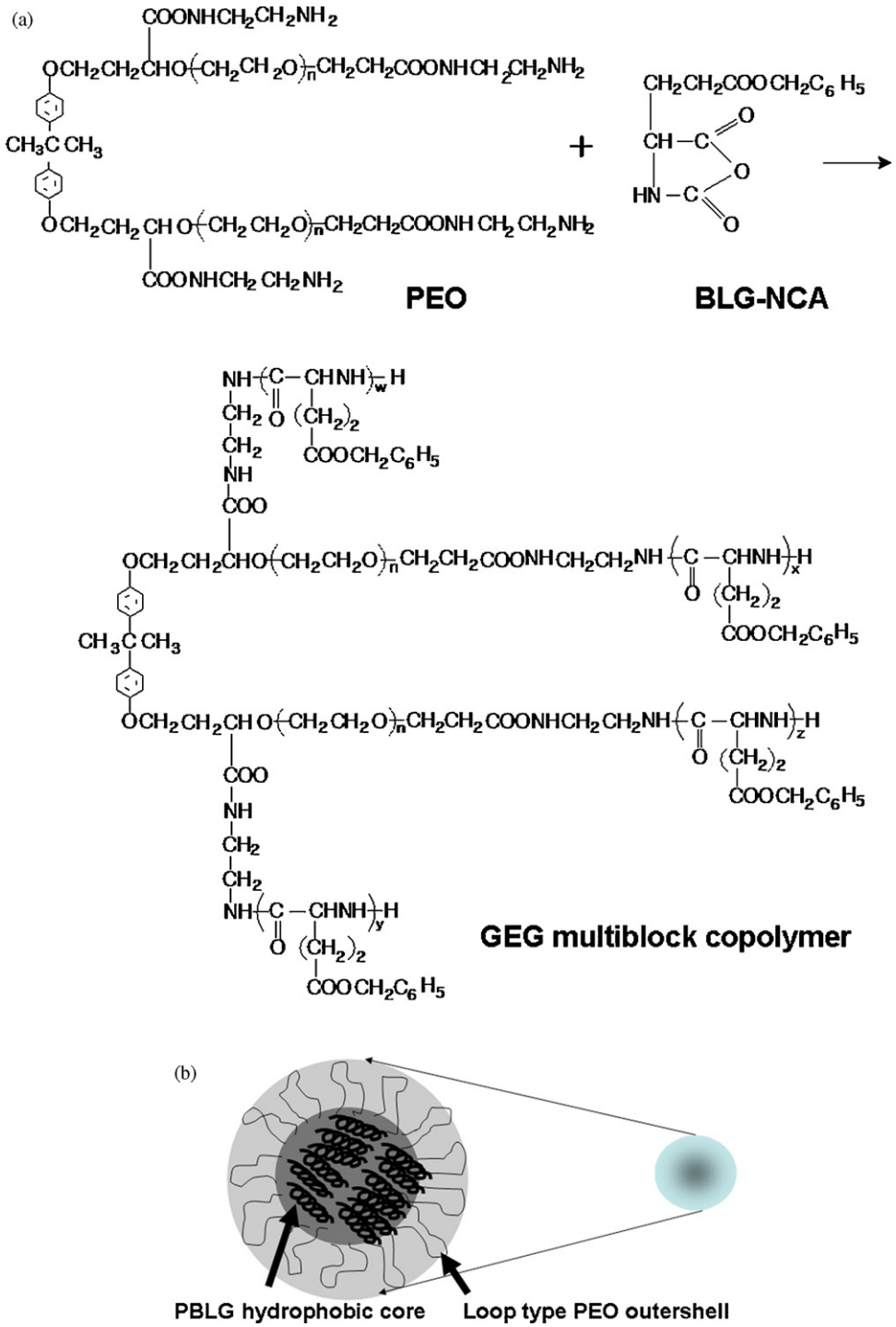

Fig. 1. Schematic illustrations of GEG multiblock copolymer (a) and formation of flower type polymeric micelles (b).

vals. The concentration of the released ADR was determined by UV spectrophotometer (Shimadzu UV-1201) at 479 nm.

2.7. In vitro antitumor activity

To test the antitumor activity of ADR-incorporated polymeric micelles, CT 26 colon cacinama cells were used. CT 26 cell were maintained at 5% CO_2 incubator at 37 °C. The effect of free ADR and ADR-incorporated polymeric micelles on the tumor cell proliferation was determined using an MTT cell proliferation assay. ADR was dissolved in DMSO and diluted 100 times using DMEM (supplemented with 10% serum). ADR-incorporated polymeric micelles were distributed in the DMEM (supplemented with 10% serum) and diluted to adjust the equivalent concentration of the free ADR. The tumor cell lines were seeded at a density of 5×10^3 per well in 96 well plates using 100 μl of DMEM supplemented with 10% serum in a CO_2 incubator (5% CO_2 at 37 °C) for 12 h. After that, 100 μl of DMEM (supplemented with 10% serum) containing free ADR or ADR-incorporated polymeric micelles was added. After 2 days of incubation, MTT was added to the 96 wells and incubated for 4 h

in a CO_2 incubator (5% CO_2 at 37 °C). After that, the supernatant was discarded and 100 μl of DMSO was added to the 96 wells. The absorbance was measured at 560 nm using a microtiter plate reader (Thermomax microplate reader, Molecular Devices).

2.8. In vivo antitumor activity

Antitumor activity against solid tumors was evaluated with CT 26 colon carcinoma cells. CT 26 cell (5×10^4 cell in 0.2 ml of 0.9% NaCl solution) were inoculated s.c. into the backs of mice (BALB/c mice, 5 weeks old, average bodyweight was 20 g). Drug injection was started after 2 days of tumor cells inoculation and first drug injection was determined at beginning of the test, i.e. day 0. Drug was injected into tail vein for four times for 6 days with an interval of 2 days at a volume of 0.1 ml/10 g body weight. Free ADR and ADR-loaded GEG polymeric micelles (GEG–ADR 2) were mixed and redistributed into 0.9% NaCl solution at a dose of 10 mg/kg. The mortality was monitored everyday, and body weight and tumor volume were measured every 2 days. Eight mice were included in each group. Tumor volume was calculated by equation of ($LW^2/2$), where L is the long diameter and W is the short diameter.

3. Results and discussion

3.1. Characterization of polymeric micelle formation of GEG multiblock copolymer

The reaction scheme of multiblock copolymer (abbreviated as GEG) prepared by polymerization of γ-BLG NCA initiated with amine-terminated PEO in methylene chloride solution was shown in Fig. 1 as described previously (Jeong et al., 1999). The copolymer composition and the molecular weight were estimated from the 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 ^{1}H NMR spectrum. Assuming that all the amine groups of PEO participate in the polymerization, the number-average molecular weights, Mn of the copolymer can be calculated from the copolymer composition and the M.W. of PEO chains. Since M.W. of PEO is 20,000, the calculated MW of PBLG block and total M.W. of copolymer was about 8900 and 28,900, respectively.

Aqueous suspension of the reversed block copolymer architecture, poly(propylene oxide) (PPO) -PEO-PPO, showed different phase behavior when compared to PEO-PPO-PEO triblock copolymer (Mortensen and Pedersen, 1993; Mortensen et al., 1994). Mortensen (1997) reported that aqueous solutions of PPO-PEO-PPO triblock copolymer were associated into a homogeneous phase constituting with an interconnected network of micelles in which micellar cores of hydrophobic poly(propylene oxide) were interconnected by hydrophilic poly(ethylene oxide) strands. Since GEG multiblock copolymers have similar molecular architecture, flower type polymeric micelles in aqueous solution are expected as shown in Fig. 1(b).

GEG multiblock copolymer formed stable polymeric micelles in aqueous solution at very low critical micelle concentration, 3.1×10^{-7} mol (Jeong et al., 1999). These polymeric micelles showed small particle sizes around 50 nm in diameter. However, loading efficiency was very low (30%:w/w) because significant amounts of drug were released during dialysis procedure. To improve loading efficiency, preparation method was slightly modified, i.e. polymer/drug was dissolved in mixed solvent, THF/DMF, and volatile solvent was removed by rotary evaporation. After that, dialysis procedure was employed to remove residual solvent.

Characterization of ADR-incorporated polymeric micelles was summarized in Table 1. As shown in Table 1, loading efficiency of ADR was higher than 60% (w/w) at all formulation. The higher the drug feeding amount, the higher the drug loading contents and the lower the loading efficiency. Particle sizes of ADR-incorporated polymeric micelles were lower than 150 nm at all formulation. The higher drug feeding ratio induced larger particle sizes. Especially, polymeric micelles themselves were lower than 40 nm in particle sizes. Fig. 2 showed morphologies of empty or ADR-incorporated polymeric micelles of GEG multiblock copolymer observed by TEM. As shown in Fig. 2, polymeric micelles were observed as spherical shapes and particle sizes were lower than 50 nm of empty polymeric micelles (Fig. 2a), 50–100 nm of GEG-ADR 1 (Fig. 2b) and around 100 nm of GEG-ADR 2 (Fig. 2c), indicating that particle sizes by TEM images showed similar results as PCS ones. Fig. 2d showed typical particle size distribution of ADR-incorporated polymeric micelles (GEG-ADR 2). As shown in Fig. 2d, ADR-incorporated polymeric micelles showed small particle sizes with average size of 96.7 nm (Table 1) with unimodal and narrow size distributions as we expected.

3.2. In vitro drug release behavior

Fig. 3 showed the release behavior of ADR from the ADR-loaded GEG polymeric micelles into outer aqueous phase. As shown in Fig. 3, the higher drug loading contents, the slower the release rate. In the release behavior of GEG polymeric micelles, drug was released fast for first 1 day at all formulation and then released continuously over 1 week. It is thought that hydrophobic drug can be released slowly at higher drug contents and ADR release from ADR-loaded polymeric micelles showed similar behavior since ADR is one of the hydrophobic anticancer agents. The similar phenomena were reported by several authors (Gref et al., 1994; Jeong et al., 1998; Kwon et al., 1995). The crystallization of hydrophobic drug can occur inside the polymeric micelles at higher drug loading contents while the drug can be existed as a molecular dispersion at low drug contents. Therefore, ADR release from the ADR-loaded polymeric micelles with higher drug loading contents showed slower release kinetics than low drug loading contents.

3.3. In vitro and in vivo antitumor activity

To assess the antitumor activity of ADR-incorporated polymeric micelles, CT 26 colon carcinoma cells were used and tested by MTT method at 96 well multi plate. As shown in Fig. 4, ADR (ADR itself) showed distinct cytotoxicity at higher than 1.0 μg/ml

Table 1
Characterization of ADR-incorporated polymeric micelles of GEG multiblock copolymer

Sample	Weight ratio (mg) (polymer/ADR)	Drug contents (%, w/w)		oading efficiency (%, w/w)	Particle size (nm)		
		Theoretical	Experimental		Intensity average (% in area)	Volume average (% in area)	Number average (% in area)
Empty	40/0	–	–	–	43.2 ± 19.3	37.8 ± 12.0	31.9 ± 10.8
GEG-ADR 1	40/2	4.8	3.6	74.7	72.3 ± 29.0	65.8 ± 31.0	49.6 ± 16.5
GEG-ADR 2	40/5	11.1	7.9	68.6	96.7 ± 38.9	83.9 ± 25.8	81.8 ± 27.6
GEG-ADR 3	40/10	20.0	13.2	60.8	138.1 ± 57.2	131.1 ± 38.4	119.8 ± 29.3

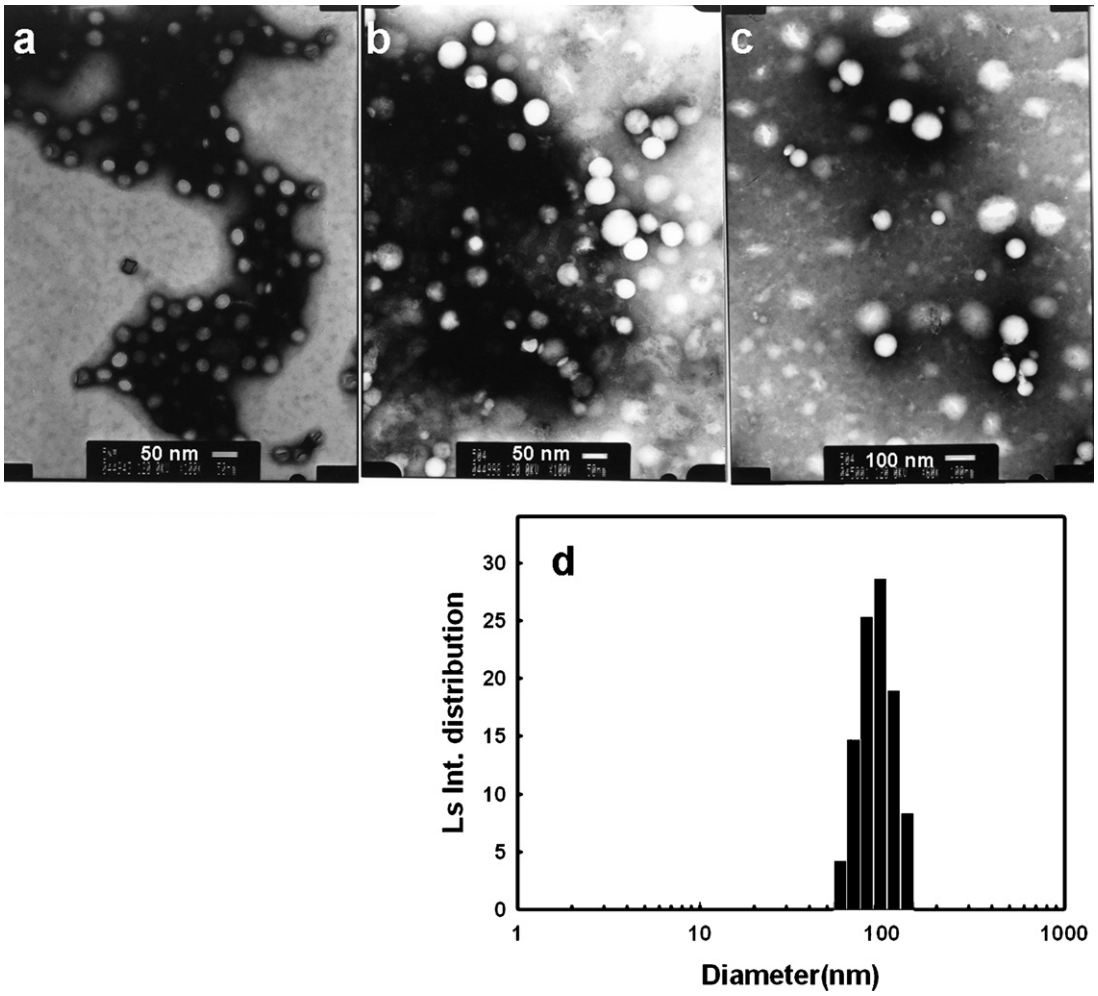

Fig. 2. TEM images of ADR-incorporated polymeric micelles of GEG multiblock copolymer. Empty polymeric micelles (a); ADR-incorporated polymeric micelles (GEG-ADR 1) (b); GEG-ADR 2 (c) and typical particle size distribution of ADR-incorporated polymeric micelles (GEG-ADR 2) (d).

of ADR concentration. At 10 μg/ml, survived cells were less than 20%. At higher ADR concentration (100 μg/ml), survived cells were not significantly changed. The ADR-incorporated polymeric micelles (GEG-ADR 2) showed lower cytotoxic behavior when compared with ADR itself, because sustained release behavior of the ADR from the polymeric micells might be the reason of lower toxicity. However, polymeric micelles themselves did not significantly affect the survivability of tumor cells. Almost 90% of tumor cells were survived at highest concentration of polymeric micelles.

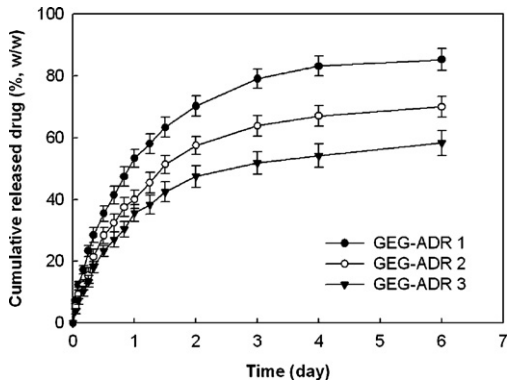

Fig. 3. The effect of drug contents on the ADR release from polymeric micelles.

In vivo anticancer activity was tested using mice tumor xenograft model. 5×10^4 CT 26 murine tumor cells were inoculated into each case of the mice. Fig. 5 shows survival ratio of mice treated with ADR itself and ADR-loaded polymeric micelles (GEG-ADR 2). ADR itself at a high dose of 10 mg/kg mice resulted in toxic death of all mice within 3 weeks while half of total mice were survived for 6 weeks in the case of control, indicating that ADR itself has significant cytotoxicity and survivability of mice were not improved. However, ADR-loaded polymeric micelles showed distinct survivability of mice. All of the treated mice were survived until 8 weeks and 5 mice were survived for 10 weeks. The results indicated that polymeric micelles significantly increased survivability of mice. Furthermore, weight changes of mice were smaller than control mice as shown in Fig. 6. Weight of mice treated with ADR itself decreased for 3weeks due to the cytotoxicity of drug. Also, the drug cytotoxicity led to reduced survivability as shown in Fig. 5. The results also indicated that ADR-loaded polymeric micelles at equivalent dose of 10 mg ADR/kg mice were less toxic than ADR itself. The *in vivo* antitumor activity of ADR itself and ADR-loaded polymeric micelles against CT 26 murine tumor was assessed as a tumor growth as shown in Fig. 7. Tumor volume (mm^3) measurement started at 2 weeks from the first injection. As shown in Fig. 7, tumor growth in control mice was distinct at 3 weeks and tumor

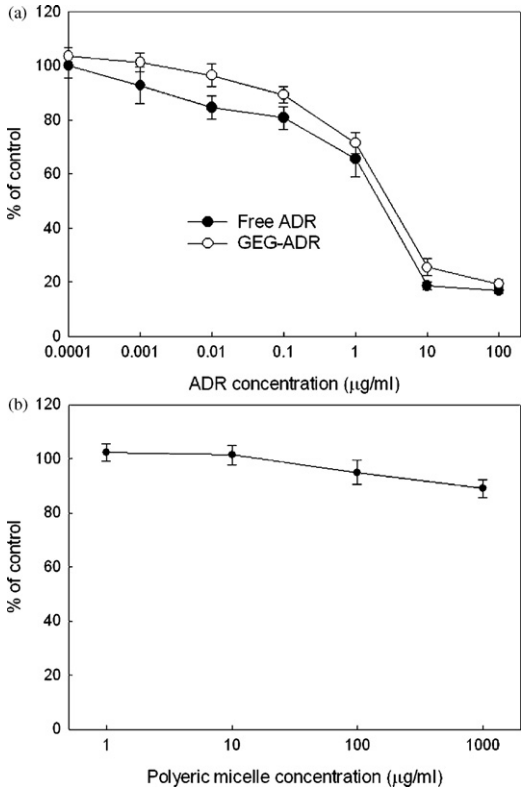

Fig. 4. Cytotoxicity of ADR-incorporated polymeric micelle of GEG multiblock copolymer against CT 26 colon carcinoma cells. ADR or ADR-incorporated polymeric micelle (GEG-ADR 2, drug contents: 7.9% (w/w)) was treated 96 well plate with 5×10^3 cells/well for 1 or 2 days.

volume was rapidly increased according to the time course. On the other hand, tumor volume was significantly suppressed by treatment of ADR-loaded polymeric micelles when compared to control mice. Although mice treated with free ADR resulted in highest suppression on the tumor growth, ADR itself resulted in toxic death within 3 weeks. The results indicated that ADR-loaded polymeric micelles had high anticancer activity with lower drug cytotoxicity. Resultantly, decreased drug cytotoxicity and increased anticancer activity of polymeric micelles might induce higher survivability of

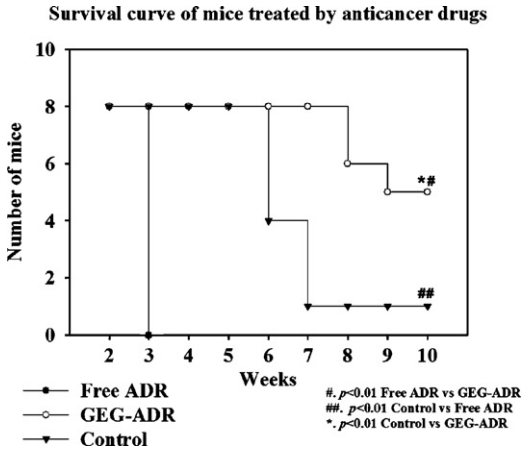

Fig. 5. Survival ratio of mice after drug injection. Mice were treated with free ADR (10 mg ADR/kg mice) and polymeric micelle (10 mg equivalent ADR/kg mice). Survived mice were checked everyday. 8 mice were used for test of each case. PBS (pH 7.4) was used for control and GEG-ADR 2 (drug contents: 7.9% (w/w)) was used for the treatment of polymeric micelle.

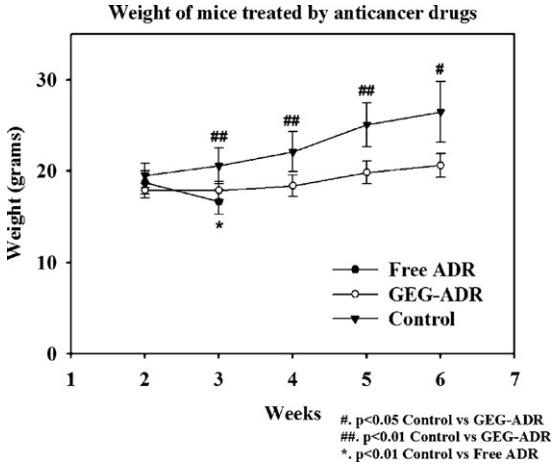

Fig. 6. Body weight changes of the mice treated with free ADR (10 mg ADR/kg mice) and GEG-ADR 2 (10 mg equivalent ADR/kg mice). PBS (pH 7.4) was used for control and GEG-ADR 2 (drug contents: 7.9% (w/w)) was used for the treatment of polymeric micelles.

mice with minimized weight changes and high suppression ratio of tumor growth. When ADR-loaded polymeric micelles were used, the reason of increased survivability and favorable tumor suppression of mice might be due to the enhanced vascular permeability and retention (EPR) effect of polymeric micelles or polymeric conjugates (Greish et al., 2005; Maeda et al., 2000; Noguchi et al., 1998). Noguchi et al. (1998) reported that *N*-(2-hydroxypropyl) methacrylamide (HPMA) copolymers were accumulated in the solid tumor and higher M.W. copolymers showed more effectiveness in the tumor accumulation of polymers. Furthermore, Greish et al. reported that pirarubicin-incorporated polymeric micelles of styrene-maleic acid (SMA) copolymer showed marked antitumor activity with low cytotoxicity and high tumor targeting efficiency. They insisted that marked antitumor activity of this polymeric micelles can be attributed to the EPR effect of macromolecular drugs seen in solid tumors. The enhanced antitumor activity and reduced cytotoxicity of polymeric micelles were also reported by other researchers (Yokoyama et al., 1990a,b, 1991). It was suggested that efficient tumor accumulation of polymeric micelles must be due to the EPR effect and passive targeting function although they do not have active targeting function.

Resultantly, we suggest that ADR-loaded polymeric micelles is a superior candidate for antitumor drug delivery.

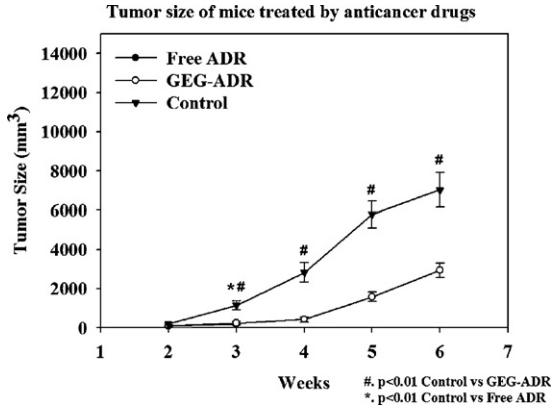

Fig. 7. Tumor growth after drug injection. Tumor cells were inoculated s.c. into the backs of mice and drug injection was started after 2 days of tumor cell inoculation. PBS (pH 7.4) was used for control and GEG-ADR 2 (drug contents: 7.9% (w/w)) was used for the treatment of polymeric micelles.

4. Conclusions

The multiblock copolymer composed of PBLG as the hydrophobic part and PEO as the hydrophilic one was synthesized to prepare anticancer drug delivery carrier. ADR was incorporated into the polymeric micelles of GEG multiblock copolymer. The higher the drug feeding ratio, the higher the drug contents and the lower the loading efficiency. The increased drug feeding ratio resulted in increased particle sizes. At all of the formulations, particle sizes were less than 150 nm. Morphologies of the polymeric micelles were observed as spherical shapes. ADR release from ADR-loaded polymeric micelles *in vitro* was decreased with an increased drug loading contents. *In vitro* antitumor activity test using CT 26 tumor cells, ADR-loaded polymeric micelles showed almost similar cytotoxicity when compared to ADR itself while polymeric micelles themselves did not affect cell survivability. In *in vivo* antitumor activity test using mice tumor xenograft model, ADR-loaded polymeric micelles showed improved survivability of mice with minimized weight changes and excellent tumor growth suppression efficacy. The polymeric micelles of GEG multiblock copolymer suggested to be a good candidate for anticancer drug delivery carrier.

Acknowledgement

This study was financially supported by Chonnam National University.

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