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Polyethylene glycol-complexed cationic liposome for enhanced cellular uptake and anticancer activity

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ABSTRACT

Liposomes as one of the efficient drug carriers have some shortcomings such as their relatively short blood circulation time, fast clearance from human body by reticuloendothelial system (RES) and limited intracellular uptake to target cells. In this study, polyethylene glycol (PEG)-complexed cationic liposomes (PCL) were prepared by ionic complex of cationically charged liposomes with carboxylated polyethylene glycol (mPEG-COOH). The cationic liposomes had approximately 98.6 ± 1.0 nm of mean particle diameter and 45.5 ± 1.1 mV of zeta potential value. While, the PCL had 110.1 ± 1.2 nm of mean particle diameter and 18.4 ± 0.8 mV of zeta potential value as a result of the ionic complex of mPEG-COOH with cationic liposomes. Loading efficiency of model drug, doxorubicin, into cationic liposomes or PCL was about 96.0 ± 0.7%. Results of intracellular uptake evaluated by flow cytometry and fluorescence microscopy studies showed higher intracellular uptake of PCL than that of Doxil®. In addition, *in vitro* cytotoxicity of PCL was comparable to cationic liposomes. In pharmacokinetic study in rats, PCL showed slightly lower plasma level of DOX than that of Doxil®. *In vivo* antitumor activity of DOX-loaded PCL was comparable to that of Doxil® against human SKOV-3 ovarian adenocarcinoma xenograft rat model. Consequently, the PCL, of which surface was complexed with PEG by ionic complex may be applicable as drug delivery carriers for increasing therapeutic efficacy of anticancer drugs.

1. Introduction

Liposomes are spherical vesicle composed of phospholipid bilayer membranes. Liposomes have been extensively studied in an attempt to enhance the therapeutic efficacy of various drugs in the field of drug delivery system (Drummond et al., 1999; Sharma and Sharma, 1997; Bajoria and Sooranna, 1998). However, liposomal drugs for intravenous injection have been found to be plagued by their rapid opsonization and by being taken up by the reticuloendothelial system (RES) located mainly in the liver and spleen (Andresen et al., 2005; Moghimi and Patel, 1998). In general, this rapid uptake of drug carriers leads to a short blood circulation time of the incorporated drug. Notably, this problem has been resolved by modification of the surface of liposomes with polyethylene glycol (PEG) or covalent conjugation of PEG to drug carrying molecule (Moghimi and Szebeni, 2003; Torchilin and Trubetskoy, 1995). PEG possesses ideal properties for modulating pharmacokinetic behavior of drug; non-toxicity, good solubility in aqueous solution, low immunogenicity and antigenicity (Gabizon and Papahadjopoulos, 1988; Klibanov et al., 1990). Also, PEG conjugation to bioactive molecules has no deleterious effect on conformation or activity of them. These properties of PEG have been explained by its high mobility associated with conformational flexibility and water-binding ability (Ceh et al., 1997; Shimada et al., 2000). Despite a remarkable prolongation of circulation half-life of the incorporated drug by the PEG-modified liposomes, intracellular uptake of the PEG-liposomes which were delivered to the target cells could be lowered by electrostatic repulsion between negatively charged surface of PEG-liposomes and cell membranes (Chandaroy et al., 2002).

Cationic liposomes have been studied as drug carriers because of their selective accumulation in tumor endothelial cells (Wu et al., 2007). Enhancement of cellular uptake as well as *in vitro* cytotoxicity has been demonstrated and those results were attributed to the ability of cationic liposomes to interact with cells via electrostatic interaction, which could induce endocytosis of the liposomes and also facilitate drug release to the cytosol by endosomal escape (Dass, 2003). These properties of cationic liposomes have been applied to the delivery of nucleic acid, in spite of cytotoxicity of cationic lipids themselves at high concentration and rapid clearance of cationic liposomes from circulation. Thus, we hypothesized that liposomes modified with PEG and cationic lipid could prolong

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the circulation time in bloodstream and enhance cellular uptake of the liposomes to target cells. In this study, PEG-complexed cationic liposomes (PCL) were prepared by ionic complexation of cationically charged liposomes with anionic PEG derivative, mPEG-COOH. The complexed amount of PEG in PCL was determined by ^{1}H NMR analysis. Intracellular uptake of doxorubicin (DOX)-loaded PCL was investigated by using flow cytometry assay and fluorescence microscopic observation. *In vitro* cytotoxicity of PCL was evaluated by MTT assay. Dissociation of PEG from PCL was evaluated by ^{1}H NMR analysis. Pharmacokinetic behavior of various liposomal formulation of DOX was investigated after intravenous (i.v.) injection of each formulation into SD rats. *In vivo* antitumor activity was evaluated after i.v. injection of each formulation into human ovarian adenocarcinoma xenograft tumor-bearing mice.

2. Materials and methods

2.1. Materials

L-α-Phosphatidylcholine (soy hydrogenated, HSPC), cholesterol (CHOL) and 1,2-distearoyl-*sn*-glycero-3-trimethylammonium-propane (DSTAP) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Doxil® was purchased from ALZA Corporation (Mountain View, CA, USA). Doxorubicin hydrochloride (DOX), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and daunorubicin hydrochloride were purchased from Sigma–Aldrich Chemical Co (St. Louis, MO, USA); fetal bovine serum (FBS), penicillin–streptomycin, paraformaldehyde and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Gibco BRL/Life Technologies (New York, NY, USA). Poly(ethylene glycol) methylether (mPEG2000) was purchased from Sigma Chemical Company (St. Louis, MO, USA). All other materials were of analytical grade and used without further purification.

2.2. Preparation of DOX-loaded cationic liposomes

Cationic liposomes were prepared by thin film-hydration method and their lipid composition was as follows: HSPC:CHOL:DSTAP = 12.6:8.3:4.5 (mass ratio, total 15.95 mg/ml). DOX loading into inner core of the cationic liposomes was carried out by using the remote loading method (Han et al., 2006, 2007). Briefly, the lipids with the above composition were dissolved in 5 ml of chloroform, dried to a thin film in a rotary evaporator (Buchi Rotavapor R-200, Switzerland) and then suspended in 10 ml of 250 mM ammonium sulfate solution. The liposomal solution was extruded five times through a polycarbonate filter (pore size; 200, 100 and 80 nm, Whatman, USA) with a high pressure extruder (Northern Lipids Inc., USA). Unloaded ammonium sulfate was removed through dialysis in distilled water for 48 h at 4 °C by using cellulose dialysis tubing (MWCO 12000–14000, Viskase Co., IL, USA). Liposomal solution and DOX solution (5 mg/ml) (1:1, v/v) were mixed and then incubated for 2 h at 60 °C. The mixture was dialyzed to remove the unloaded DOX for 48 h at 4 °C. The DOX-loaded liposomes were stored at 4 °C until use. The concentration of DOX in the liposomes was measured by using UV–vis spectrophotometry at 497 nm (UV-mini, Shimadzu, Japan) and the loading efficiency was calculated according to the following equation:

$$\text{loading efficiency}\,(\%) = \frac{F_t}{F_i} \times 100 \quad (1)$$

where F_t is the concentration of DOX in the liposomes after their dissolution in organic solvent mixture consisting of chloroform:methanol:distilled water (2:1:0.05, v/v) and F_i is the initial concentration of DOX. The particle size and zeta potential of the liposomes were measured by using light scattering with a particle diameter analyzer (ELS-Z, Otuska, Japan).

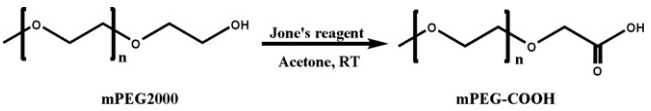

Fig. 1. Synthesis of mPEG-COOH.

2.3. Synthesis of mPEG2000-COOH

mPEG2000-COOH was synthesized as shown in Fig. 1. Poly(ethylene glycol) methylether (10 g, 5 mmol) was dissolved in 100 ml of acetone at room temperature. The solution was cooled to 0 °C by placing the flask in ice bath. Later, 5 ml of Jones reagent (containing 0.02 M of CrO_3) was added to the flask drop by drop over a 15 min period. The flask was removed from ice bath and then the reaction mixture was stirred for 20 h at room temperature. The reaction was quenched by adding 3 ml of isopropyl alcohol. Subsequently, 1 g of finely activated carbon powder was added and stirred for 2 h. The reaction products were filtered with wet celite, and the filtrate was evaporated in a rotary evaporator. The viscous liquid was dissolved in 50 ml of water and extracted with chloroform. The extracts were evaporated to obtain the product and dried under vacuum for 48 h. Yield: 98%; ^{1}H NMR (δ, ppm, $CDCl_3$): 3.38 (s, 3H, CH_3), 3.66 (m, ~44H, OCH_2CH_2O), 4.16 (s, 2H, CH_2) (Dhanikula and Hildgen, 2006; Nakazono et al., 2002).

2.4. Preparation of PEG-complexed cationic liposomes (PCL)

To complex the mPEG2000-COOH on the surface of cationic liposomes, pH of the mPEG2000-COOH solution was adjusted up to 10 by using 1 M of sodium hydroxide solution for 30 min at room temperature. The alkaline mPEG2000-COOH solution was added to cationic liposome solution (1:1, v/v) and the mixture was incubated for 2 h. The mixture was dialyzed by using dialysis tubing (MWCO 100000, Spectrum Laboratories, Inc., CA, USA) to remove the uncomplexed mPEG2000-COOH. Amount of the complexed PEG on the liposomal surface was measured by using ^{1}H NMR analysis (Automated NMR System, Buruker, Germany) of PCL.

2.5. Cell line and animals

B16F10, a murine melanoma cell line, and SKOV-3, human ovarian adenocarcinoma were cultured in DMEM added with 10% (v/v) heat-inactivated FBS and 10 μl/ml penicillin–streptomycin. The cultures were sustained at 37 °C in a humidified incubator containing 5% CO_2. The cells were maintained within their exponential growth phase.

Female BALB/c mice (5–6 weeks old, 18–22 g) and SD rats (7 weeks old, 200–220 g) were purchased from Harlan Int. (IN, USA). All of the procedures involved in the animal experiments were performed according to approved protocols and in accordance with the recommendations of the NIH guideline for the proper use and care of laboratory animals.

2.6. Cytotoxicity study

Cytotoxicity of liposomes was determined by MTT assay. Murine melanoma cells, B16F10 cells, were transferred to 96-well tissue culture plates at 1×10^4 cells per each well and incubated for 24 h at 37 °C prior to drug treatment. The culture medium was replaced with medium containing serial dilutions of various liposomes. Then, 20 μl of MTT stock solutions (5 mg/ml) were added to each well and the plates were incubated for 4 h at 37 °C. The

Table 1
Physical properties of liposomal DOX.

Liposomes	Mean particle diameter (nm)	Zeta potential (mV)	DOX loading efficiency (%)
Cationic liposome	98.6 ± 1.0	45.5 ± 1.1	96.8 ± 0.5
Doxil®	99.2 ± 0.8	−21.2 ± 0.7	95.7 ± 0.4
PCL	110.1 ± 1.2	18.4 ± 0.8	95.2 ± 0.8

culture medium was then removed and dimethyl sulfoxide was added in order to dissolve the blue formazan crystals converted from MTT. Cell viability was assessed by measuring absorbance at 590 nm with microplate reader (SpectraMax 190, Molecular Device, USA). The viability was expressed as percentage with respect to the absorbance obtained from control well without drug treatment. The percent viability was calculated according to the following equation:

$$\text{viability}\,(\%) = \frac{\text{Abs}_t}{\text{Abs}_c} \times 100 \quad (2)$$

where Abs_t is the absorbance of drug-treated well and Abs_c is the absorbance of control well without drug treatment.

2.7. Intracellular uptake of liposomes

Intracellular uptake of liposomes was determined by flow cytometry assay (Hwang et al., 2007). Murine melanoma cells, B16F10 cells, were transferred to 24-well tissue culture plates at 1×10^4 cells per each well and incubated for 12 h at 37 °C. The culture medium was replaced with 15 μg DOX/ml of liposomal solution and then incubated for 2 h in DMEM medium. The culture medium was then removed and then each well was washed with phosphate buffered saline (PBS, pH 7.4) solution. Three hundred microliters of paraformaldehyde (5%, v/v) was added to each well to fix the cells. The samples were determined by flow cytometry with a FACScan (Becton Dickinson, San Jose, CA, USA). Cell-associated DOX was excited with an argon laser (488 nm) and fluorescence was detected at 560 nm. Data were collected of 10,000 gated events and analyzed with the CELL Quest software program.

2.8. Fluorescence microscopy

After an initial passage in tissue culture flasks, murine melanoma, B16F10, cells were grown in 24-well tissue culture plates. After reaching 70–80% of confluence, the plates were washed with PBS (pH 7.4), treated with MEM-α (1 ml/well) and then incubated for 1 h at 37 °C. Liposomal solutions were added and incubated for 15 min at 37 °C and 5% CO_2. After incubation, the cells were washed twice with PBS (pH 7.4). Three hundred microliters of paraformaldehyde (5%, v/v) was added to fix the cells. Cells were observed with a fluorescence microscope (Ckx41, Olympus, Japan) under bright light or fluorescence with green filter (excitation/emission: 480/590) (Xiong et al., 2005).

2.9. Release of dissociated PEG from PCL

Release of dissociated PEG from the surface of PCL was investigated in PBS (pH 7.4). Five milliliters of plain PCL solution was sealed into cellulose dialysis tubes (MWCO 100,000, Whatman, USA) and those were incubated in 1 L of PBS for 24 h at 37 °C with continuous stirring. At predetermined time points, aliquots were withdrawn from inner dialysis tubes. The amount of PEG was measured by using ^{1}H NMR (Automated NMR System, Buruker, Germany).

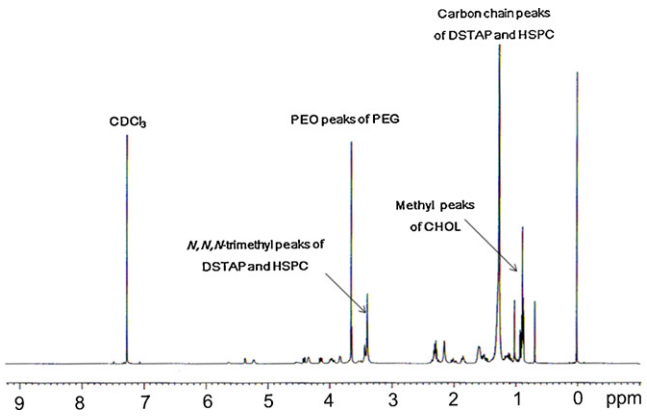

Fig. 2. ^{1}H NMR spectrum of PCL.

2.10. Pharmacokinetic study

In order to monitor the plasma levels of DOX in SD rats, DOX-loaded liposomes were injected via a tail vein of SD rats at a dose of 6.0 mg DOX/kg body weight. Seven hundred microliters of plasma was collected from a tail vein of SD rats. The concentration of DOX in plasma was determined by high performance liquid chromatography (NS-3000i, Futecs Co. Ltd., South Korea). Four hundred microliters of plasma was deproteinized by vortexing them with 0.6 ml of methanol containing 5 μg/ml daunorubicin hydrochloride as internal standard. After centrifugation at 13,000 rpm for 5 min, the supernatant was obtained and injected into a reverse-phase column (Symmetry®, 4.6 mm × 250 mm, Waters, MC, USA). The mobile phase was a mixture of 0.2 M potassium phosphate buffer, acetonitrile and triethylamine, pH 4.0 (70:30:0.2, v/v). Flow rate was 1.0 mL/min and absorbance of the eluent was monitored at 234 nm.

The pharmacokinetic parameters were calculated from the average DOX concentrations in the bloodstream by using a pharmacokinetic software, WinNolin calculator (Seoul National

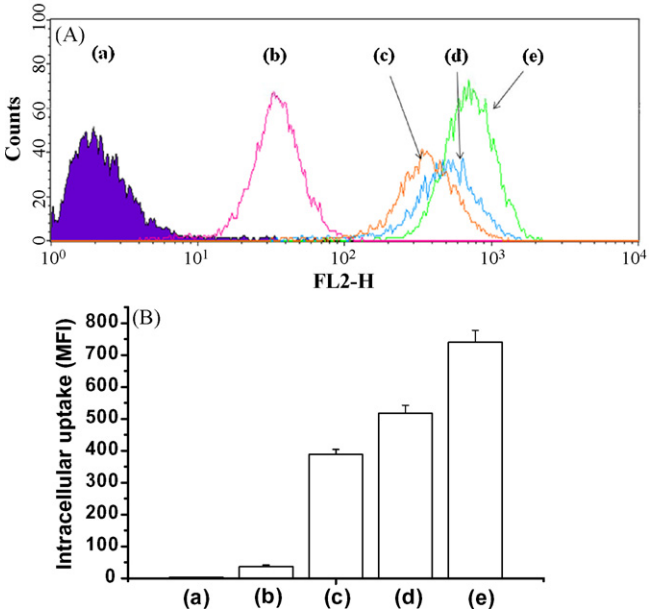

Fig. 3. (A) Cellular uptake of DOX-loaded liposomes by flow cytometry assay. (B) Mean fluorescence intensity (MFI) of DOX-loaded liposomes by flow cytometry assay. (a) Control, (b) Doxil®, (c) PCL, (d) cationic liposome and (e) free DOX. Control is a background of B16F10 cells without DOX. Mean and S.D. are shown ($n = 3$).

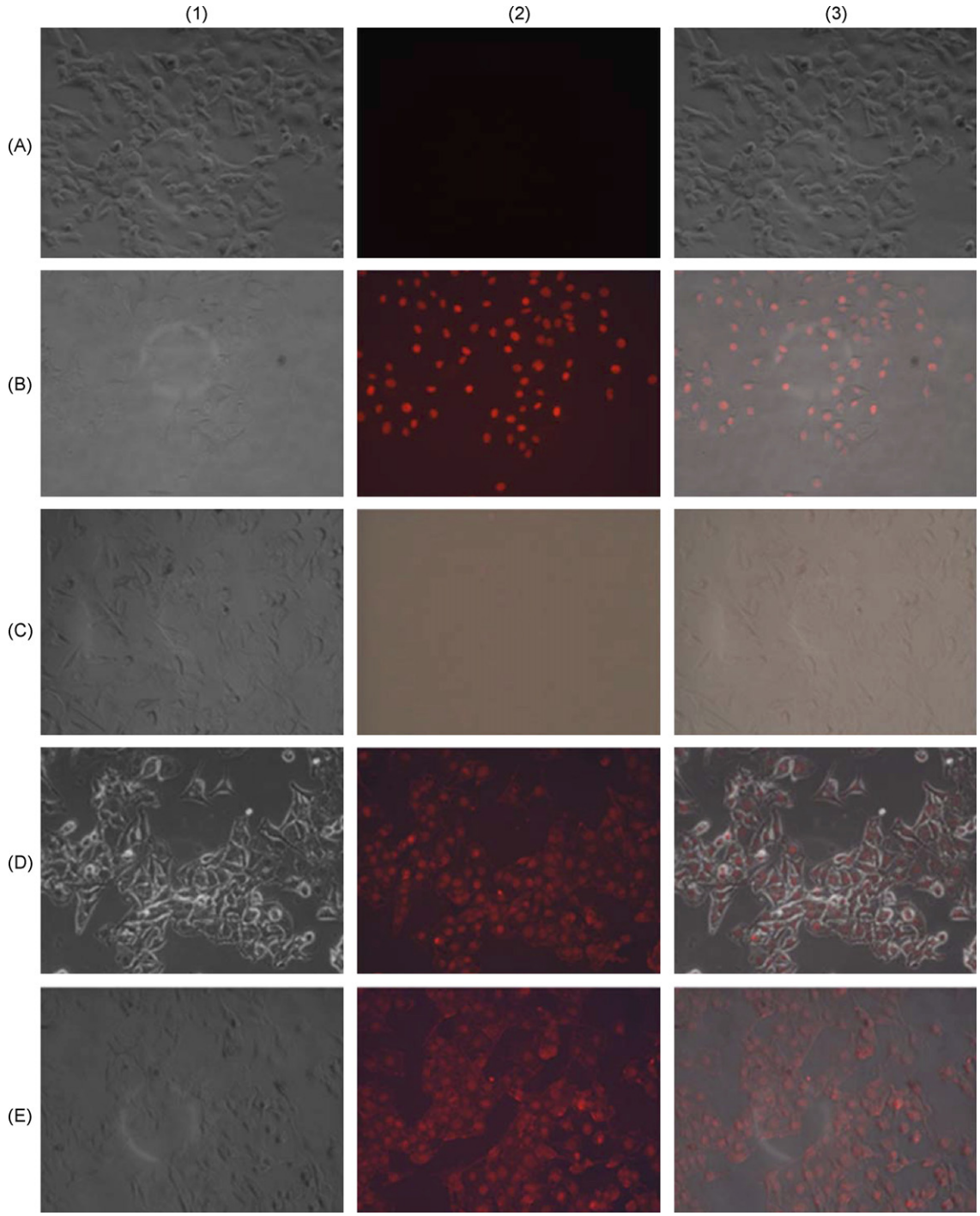

Fig. 4. Fluorescence microscopy images of B16F10 cells (murine melanoma cells) incubated for 15 min at 37 °C. Row (A): PBS; row (B): free DOX; row (C): Doxil®; row (D): cationic liposome and row (E): PCL. Column (1): phase contrast image; column (2): fluorescence image and column (3): overlaid image of (1) and (2).

University, South Korea). Significance of the data was evaluated by using *t*-test and $P < 0.001$ was considered statistically significant.

2.11. In vivo antitumor activity

5×10^5 cells of human ovarian adenocarcinoma, SKOV-3 (ATCC, American type culture collection, USA), in 20 μl were carefully inoculated into the right limb armpits of female BALB/c mice. Six days after the tumor inoculation, free DOX solution or liposomal DOX suspension was injected intravenously via a tail vein at a dose of 6 mg DOX/kg body weight. The tumor volume was monitored for 16 days after a single i.v. injection of various liposomal formulations. In order to determine the tumor volume, each individual tumor size was measured with a caliper and the tumor volume was calculated using the following equation:

$$\text{tumor volume}\,(\text{mm}^3) = \frac{\text{width} \times \text{length}^2}{2} \quad (3)$$

3. Results and discussion

3.1. Physical properties and PEG amount of PCL

The physical properties of various liposomes were evaluated by measuring their mean particle diameter, zeta potential and DOX loading efficiency. Those results are summarized in Table 1. The loading efficiency of DOX was 95–97%. The mean particle diameter of cationic liposome and Doxil® was approximately 98–100 nm and particle diameter of PCL increased to approximately 110 nm as a result of ionic complex of cationic liposome with negatively charged mPEG2000-COO^-. Brush-form of PEG on the surface of liposome has generally known to have length of approximately 50 Å (Ceh et al., 1997). Thus, increase of particle diameter of PCL by approximately 10 nm indicates that mPEG2000-COO^- was complexed on the surface of cationic liposome. In addition, the zeta potential value of cationic liposome was 45.5 ± 1.1 mV and this value changed to 18.4 ± 0.8 mV as a result of ionic complex of cationic liposome with negatively charged mPEG2000-COO^-. These results confirmed that mPEG2000-COO^- could be fixed on the surface of cationic liposomes. Fig. 2 shows a ^{1}H NMR spectrum of PCL. Polyethylene oxide (PEO) peaks of PEG were observed at 3.6 ppm. And *N*,*N*,*N*-trimethyl peaks of DSTAP and HSPC were observed at 3.4 and 3.3 ppm, respectively. Also, originated peaks from carbon chains of DSTAP and HSPC were observed at 1.2 ppm. These results show that surface of PCL was coated by mPEG2000-COOH. The fixed amount of PEG in PCL was 7.40 mM when it was calculated from peak areas in ^{1}H NMR spectrum.

3.2. Intracellular uptake of PCL evaluated by flow cytometry

To investigate the intercellular uptake of DOX transfected by liposomes, the amount of DOX uptake into the B16F10 cells were evaluated by flow cytometry and the results are shown in Fig. 3. Intracellular uptake of PCL was higher than Doxil® and slightly lower than cationic liposomes even though it was lower than free DOX as shown in Fig. 3A. Also, Fig. 3B showed that mean fluorescence intensity (MFI) value of PCL was approximately eightfold higher than that of Doxil®. These results indicate that cationic charge of cationic liposome and PCL can induce greater electrostatic interaction with B16F10 cells and hence induce internalization by endocytosis (Dass, 2003; Krasnici et al., 2003). Though cationic liposomes showed the highest cellular uptake among liposome, it is generally known that cationic liposomes cleared rapidly from circulation (Wu et al., 2007; Dass, 2003). In case of Doxil®, cellular uptake was much lower compared to PCL and cationic liposome and it could be attributed to anionic charge (approximately −21 mV) of the surface of Doxil®. Anionic charges of liposomal surface lead to electrostatic repulsive-force with cellular membrane which can inhibit cellular uptake of liposome by endocytosis (Chandaroy et al., 2002). Therefore, it is regarded that surface charge of liposomal surface is the important factor for liposome-mediated transfection of the loaded drug.

3.3. Intracellular uptake of PCL observed by fluorescence microscopy

Cellular uptake of free DOX or DOX-loaded liposomes was observed by fluorescence microscopy. Fig. 4 shows B16F10 cells visualized after 15 min of incubation with PBS, free DOX and DOX-loaded liposomes. Free DOX is known to have high cellular uptake that can deliver DOX to the nucleus of tumor cells via endocytosis (Hwang et al., 2007). Though free DOX have good cellular uptake property, free DOX is difficult to reach to the tumor site because free DOX disappears by rapid opsonization and being taken up by the RES of liver and spleen. In case of Doxil®, cellular uptake to B16F10 cells cannot be observed because anionic surface charges of the surface Doxil® could induce electrostatic repulsive-force with cellular membrane. Cationic liposome and PCL demonstrated enhanced cellular uptake to B16F10 cells compared to Doxil®. This might be due to cationic charge of cationic liposome and PCL which can induce electrostatic interaction with cells and facilitate DOX release into the cytosol by endosomal escape (Wu et al., 2007; Carmona-Ribeiro et al., 1997; Carvalho and Carmona-Ribeiro, 1998). At cytoplasm of cells, PCL showed higher fluorescence than cationic liposome. This result indicates that PCL might be adhered to the surface of cell membrane or accumulated in cytoplasm before the uptake of DOX escaped from endosome to cellular nucleus (Fenske et al., 2001). This slower nucleus uptake of DOX by PCL compared to that of cationic liposome may be attributable to relatively low cationic surface charge of PCL. Meanwhile, the cationic liposome and PCL showed higher fluorescence image of cellular nucleus than Doxil®. These results indicate that PCL and cationic liposome are more efficient for delivering DOX to the nucleus of tumor cell by their enhanced intracellular uptake.

3.4. In vitro antitumor activity of PCL

Cytotoxicity of Doxil®, cationic liposome and PCL against murine melanoma cells, B16F10 cells, was evaluated by MTT assay and the result is shown in Fig. 5. Cell viability was dependent on the concentration of DOX. Free DOX showed low cell viability values compared to the liposomal DOXs. The free DOX is known to have high cellular uptake property to tumor cell (Hwang et al., 2007). Cationic liposome and PCL showed low cell viability compared to Doxil®. The low cytotoxicity of Doxil® might be due to low intracellular uptake of Doxil® which was originated from the inhibition of cellular uptake by PEG on the surface of Doxil® as shown in flow cytometry (Fig. 3) and fluorescence microscopy (Fig. 4) studies. Cationic lipid, DSTAP, incorporated in cationic liposome or PCL, however, can induce high electrostatic interaction with cell wall of B16F10 cells. Moreover cationic lipids are known to have cytotoxicity themselves (Wu et al., 2007; Torchilin and Trubetskoy, 1995; Hirofumi et al., 1999; Lv et al., 2006; Romoren et al., 2004). Therefore, it is considered that cytotoxicity of PCL or cationic liposome results from the combination of cytotoxicities of cationic lipid and DOX. The cytotoxicity of cationic liposome having strong cationic charge was slightly higher than that of PCL having weak cationic charge.

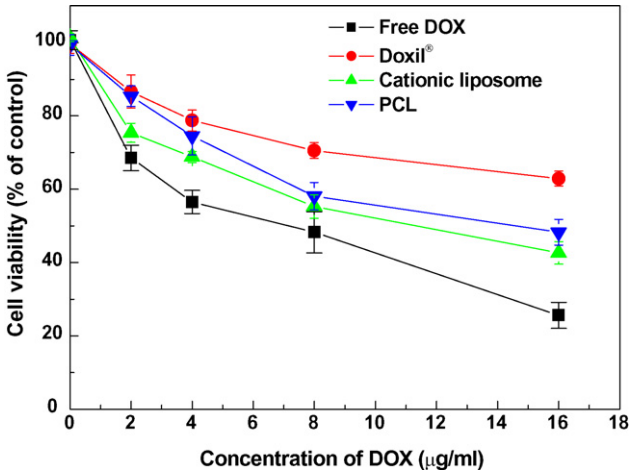

Fig. 5. Cytotoxicity of free DOX, Doxil®, cationic liposomes and PCL against B16F10 cells (murine melanoma cells) assayed by using MTT assay method after 4 h incubation at 37 °C. Mean and S.D. are shown ($n = 3$).

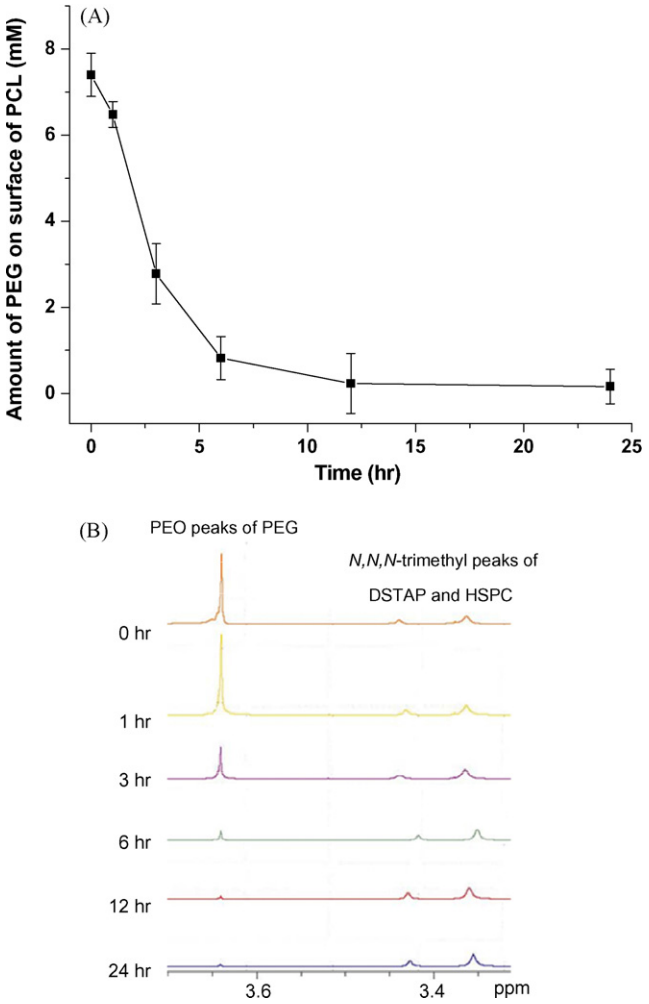

Fig. 6. (A) Amount of PEG on the surface of PCL during incubation at 37 °C. Mean and S.D. are shown ($n = 3$). (B) ^{1}H NMR spectrum of PCL incubated at 37 °C. Mean and S.D. are shown ($n = 3$).

3.5. Release of dissociated mPEG from PCL

In our preliminary experiment, mPEG complex on the surface of PCL was stable against dialysis of PCL in distilled water over 48 h (data not shown). However, mPEG was dissociated from PCL in PBS (pH 7.4) at 37 °C. Amount of complexed mPEG on the surface of PCL in PBS (pH 7.4) at 37 °C was measured by using ^{1}H NMR analysis and the results are shown in Fig. 6A and B. The amount of complexed mPEG decreased gradually and approximately 90% of mPEG was dissociated from PCL in 12 h. These results indicate that mPEG-COO$^-$ could be dissociated from PCL and the surface of PCL could become to be charged cationically. The fact that the surface of PCL could be changed from mPEG-complexed liposome to cationically charged liposome *in vitro* suggests a novel targetability of PCL *in vivo* as shown in Fig. 7. After intravenous (i.v.) injection of PCL, mPEG on PCL may protect the PCL from interacting with plasma proteins in blood stream and macrophages in RES. Also, the PCL which can reach to tumor cells by enhanced permeability and retention (EPR) effect of neovascular endothelial cell may increase intracellular uptake of the PCL to tumor cells due to the exposed cationic charges of the liposomes after dissociation of mPEG from the surface of PCL.

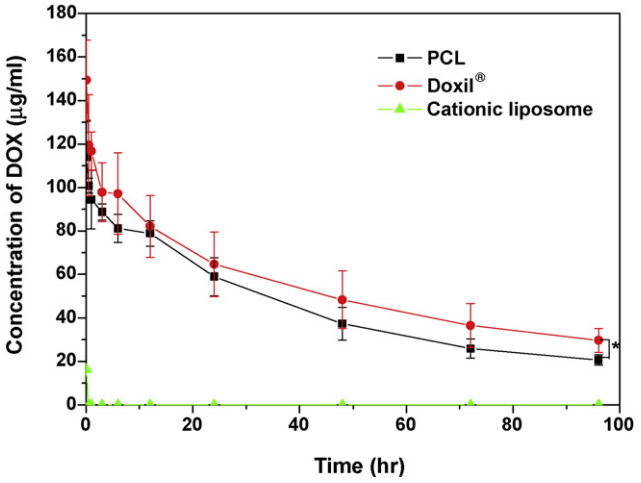

Fig. 8. Plasma concentration of DOX after intravenous injection of cationic liposome, Doxil® and PCL formulations at a dose of 6.0 mg/kg to rats (*$P < 0.001$ vs. Doxil®). The data represents the mean ± S.D. of three rats.

3.6. Pharmacokinetic characteristics of PCL

The pharmacokinetic profiles of DOX in bloodstream after i.v. injection of cationic liposome, Doxil® and PCL at a dose of 6.0 mg DOX/kg body weight are shown in Fig. 8. Plasma DOX of cationic liposome disappeared rapidly from the circulation. In contrast, Doxil® and PCL showed a long circulation up to 98 h. Cationic liposome disappeared from the circulation by rapid opsonization and being taken up by the RES of liver and spleen because of cationic surface charge could increase interaction of vesicles with hemocytes or plasma proteins in blood stream and macrophages in RES (Wu et al., 2007; Carvalho and Carmona-Ribeiro, 1998). On the contrary, PCL showed prolonged blood circulation though concentration of DOX was slightly lower compared to that of Doxil®. PCL and Doxil® have PEG on surface of the liposomes. Modification of liposomal surface with a hydrophilic and flexible polymer such as PEG is widely used to prolong circulation time (Torchilin and Trubetskoy, 1995). It is well accepted that the PEG layer formed on the liposomal surface may effectively protect the liposomes from interacting with plasma proteins in the blood stream, leading to reduction in RES uptake and prolongation of circulation time in blood stream (Hirofumi

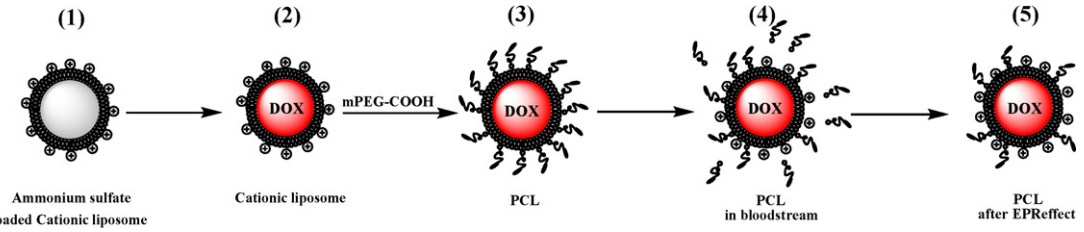

Fig. 7. Schematic representation of PCL. (1) Ammonium sulfate-loaded cationic liposome, (2) DOX-loaded cationic liposome, (3) PCL complexed with mPEG-COO$^-$ by electrostatic interaction, (4) dissociation of mPEG-COO$^-$ from the surface of PCL in bloodstream and (5) exposure of cationic charges on PCL by dissociation of mPEG-COO$^-$ after EPR effect.

Table 2
Pharmacokinetic parameters of DOX after intravenous injection of the various liposomal formulations in rat at a dose of 6.0 mg DOX/kg ($n = 3$).

	$AUC_{0-96\,h}$ (μg h/ml)	C_{max} (μg/ml)	$t_{1/2\,(0-96\,h)}$ (h)
Cationic liposome	0.675	16.270	–[a]
PCL	4282.388	113.917	39.900
Doxil®	5192.992	149.363	45.638

AUC, area under the curve; C_{max}, maximum concentration of DOX; $t_{1/2}$, half-life time.
[a] Half-life time could not be evaluated.

et al., 1999). Though PCL have PEG layer on the liposomal surface, its surface was charged cationically with 18.4 ± 0.8 mV of zeta potential value. PCL might be easily attacked by plasma proteins compared to Doxil® of which the surface was charged anionically with −21.2 ± 0.7 mV of zeta potential value. The slight difference of DOX concentration between PCL and Doxil® might be attributed to the difference in surface charge of the liposomes. The pharmacokinetic parameters of cationic liposome, Doxil® and PCL are summarized in Table 2. PCL showed high area under the curve (AUC, 4282.388 μg h/ml) of DOX over four thousand times against that of cationic liposome (0.675 μg h/ml), though it has slightly lower than that of Doxil® (5192.992 μg h/ml). These results indicate that PCL can circulate for a longer time in the blood circulation system than cationic liposomes.

3.7. *In vivo* antitumor activity of PCL

Antitumor activities of DOX-loaded liposomes (PCL and Doxil®) *in vivo* were evaluated in human ovarian adenocarcinoma, SKOV-3 cells, tumor-bearing BALB/c mice at a dose of 6 mg DOX/kg body weight. As shown in Fig. 9, liposomal DOX formulations suppressed the growth of tumor as compared with the PBS control group. These results indicate that DOX loaded in liposomes could be released from the liposome and then diffused passively into the tumor cells or the DOX-loaded liposomes might be directly internalized by endocytosis after delivery to target cells by EPR effect. Inhibition of tumor growth by PCL was similar to Doxil®, although PCL showed slightly lower concentration of DOX in blood stream than Doxil® as shown in Fig. 8. The similar therapeutic efficacy of PCL to that of Doxil® in spite of slightly lower DOX concentration of PCL in circulation suggests that the cationic surface charge of PCL exposed by dissociation of PEG from PCL may enhance intracellular uptake of PCL to tumor cells (Krasnici et al., 2003; Carmona-Ribeiro et al., 1997; Carvalho and Carmona-Ribeiro, 1998). The higher cellular uptake of PCL to cancer cells than Doxil® *in vitro* as shown in Figs. 3 and 4 in this study can be one reason to support the antitumor activity *in vivo* comparable to that of Doxil® despite of lower AUC value of PCL. Another reason for the antitumor activity of PCL *in vivo* may be attributable to the faster and higher localization of cationic liposomes to tumor tissue as suggested by other previous reports (Wu et al., 2007; Dass, 2003). It is reasonable to suggest that the high DOX levels of the PCL in the tumor would result in high antitumor activity. To elucidate major factor that determine antitumor activity of PCL, *in vivo* biodistribution behavior including tumor localization and elimination of PCL needs to be studied and it will be the subject of our further study. All mice survived till the end of test period and no severe sign of toxicity was observed in the mice treated with the liposomal DOX.

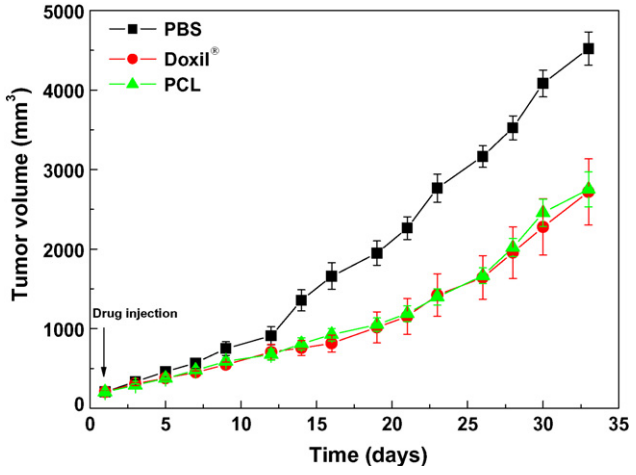

Fig. 9. Tumor growth inhibition by injection of PBS control, Doxil® and PCL formulations in tumor-bearing BALB/c mice at a dose of 6 mg DOX/kg body weight. 5×10^5 cells of human ovarian adenocarcinoma, SKOV-3 were inoculated into the armpits of the mice and the animals were treated with an intravenous injection of PBS control, Doxil® and PCL solution. The arrow indicates the number of days after the implantation of the SKOV-3 tumor cells that the free DOX, Doxil® and PCL formulations were injected. The data represents the mean ± S.D. of three mice.

4. Conclusion

PEG-complexed cationic liposomes (PCL) have been developed and *in vitro* cellular uptake and *in vivo* anticancer activity were evaluated. Mean particle diameter and zeta potential of PCL were approximately 110 nm and 18 mV, respectively. PCL showed higher cellular uptake and cytotoxicity against B16F10 cells compared to Doxil®. These results indicate that cationic charge of PCL can induce electrostatic interaction with cell wall of B16F10 cells and hence induce internalization by endocytosis. Cationic liposome and PCL could successfully deliver the DOX to the tumor cells via enhanced cellular uptake property as observed in fluorescence microscopy study. Amount of the complexed mPEG decreased gradually and approximately 90% of mPEG was dissociated from PCL in 12 h. Pharmacokinetic characteristics indicate that PCL can circulate for a longer time in the blood circulation system after their intravenous (i.v.) injection than cationic liposome. Antitumor activity *in vivo* of DOX-loaded PCL against human SKOV-3 ovarian adenocarcinoma xenograft model was comparable to that of Doxil®. Antitumor activity of PCL was similar to Doxil®, in spite of PCL showed lower concentration of DOX in blood stream than that of Doxil® in pharmacokinetic characteristics study. The high therapeutic efficacy of the PCL could be attributed to the enhanced cellular uptake effect due to the increased cationic charge characteristics of PCL. Thus, our study suggests the potential of PEG-complexed cationic liposome for use as drug delivery systems to enhance the therapeutic efficacy of anticancer drugs administered by intravenous injection.

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