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Controlled release of paclitaxel from microemulsion containing PLGA and evaluation of anti-tumor activity in vitro and in vivo

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Abstract

The main objective of this study was to develop an optimal paclitaxel microemulsion prepared by self-microemulsifying drug delivery system (SMEDDS) which is a mixture of paclitaxel, tetraglycol, Cremophor ELP, and Labrafil 1944 and a paclitaxel microemulsion containing poly(D,L-lactide-*co*-glycolide) (PLGA) in order to offer controlled release of paclitaxel. To achieve this goal, paclitaxel and PLGA were dissolved by solubilizer like tetraglycol. There was not observed any change in molecular weight of PLGA after being solubilized by tetraglycol. The droplet size for all of the formulation of microemulsion was found in the range of 45–270 nm by dynamic light scattering (DLS). It was observed that the droplet size of microemulsion without PLGA was smaller than that of microemulsion containing PLGA by transmission electron microscopy (TEM). The droplet of microemulsion containing PLGA was almost of spherical shape with smooth surface and there was no aggregation or adhesion among droplet of microemulsion by atomic force microscopy (AFM). The release behaviour of paclitaxel from microemulsion containing PLGA having various molecular weights (8K, 33K, and 90K) exhibited a biphasic pattern characterized by a fast initial release during the first 48 h, followed by a slower and continuous release for 144 h, in contrast that the release of paclitaxel from microemulsion without PLGA was finished during 24 h. This result was identical with the result of anti-tumor activity in vitro of paclitaxel from microemulsion containing PLGA against human breast cancer cell line MCF7 and this formulation enhanced anti-tumor activity in vivo compared with microemulsion without PLGA against SKOV-3 human ovarian cancer cells bearing nude mice model.

Keywords: Paclitaxel; Microemulsion; SMEDDS; PLGA; Controlled release; Anti-tumor activity

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1. Introduction

Paclitaxel has shown significant anti-tumor activity against various tumors such as breast cancer, advanced ovarian carcinoma, lung cancer, head and neck carcinoma, and acute leukemias when administered systemically (Rowinsky and Donehow, 1995; Holmes et al., 1991; Lokich et al., 1998; Forastiere, 1994; Rowinsky et al., 1989). Paclitaxel injection for clinical application is presently only dosage form (Taxol®) of a solution of paclitaxel in 50% of Cremophor EL® and 50% of alcohol. Because application of paclitaxel is limited by low solubility in water due to low therapeutic index using acceptable solvents pharmaceutically. However, Cremophor EL® as a solubilizer used in paclitaxel injection (Taxol®) has severe side effects, such as hyperlipidaemmia, abnormal lipoprotein, and aggregation of erythrocytes, on humans (Weiss et al., 1990), and paclitaxel injection has been accompanied with patient's incompliance by daily injection.

A variety of researches to avoid using Cremophor EL® were investigated to deliver paclitaxel with high therapeutic efficacy such as liposome (Sharma et al., 1993; Crosasso et al., 2000), nanosphere (Bartoli et al., 1990; Feng and Huang, 2001), and parenteral emulsions (Tarr et al., 1987; He et al., 2003). These drug delivery systems have following disadvantages: liposome has poor shelf stability and insufficient (for lipophilic drugs) loading; nanosphere has poor loading efficiency and the problem of elimination of residual solvents; emulsion is not proper to deliver anti-cancer drugs parenterally because of rapid release. Among these, emulsion may be a promising alternative way because it provides good biocompatibility, longer shelf life, good solubilization of poorly water-soluble drugs, and high concentration of lipophilic drugs in aqueous media if it could offer controlled release of anti-cancer drugs. Among the emulsifying methods, the self-microemulsifying drug delivery systems (SMEDDS) are worthy of notice. SMEDDS are isotropic mixtures of oil, a surfactant, and possibly one or more hydrophilic solvents or cosurfactants, which form fine oil-in-water emulsions or microemulsions when exposed to aqueous media under condition of gentle agitation (Holm et al., 2003). A kind of the ways to control the release from emulsion is using biodegradable polymer as a matrix in solubilizer.

One of the biodegradable polymer that has been used widely is poly(D,L-lactide-*co*-glycolide) (PLGA). PLGA has been approved for drug delivery use by the Food and Drug Administration, so it has been used for the study of a controlled release system over the past decade. It provides many advantages such as regulating varying degradation period according to molecular weight of PLGA and producing biocompatible and toxicologically safe by-products that are further eliminated by the normal metabolic pathways. It has been widely used as carriers in controlled release delivery systems (Choi et al., 2002; Fonseca et al., 2002; Husmann et al., 2002; Mu and Feng, 2003; Seo et al., 2002; Seong et al., 2003; Wang et al., 2003) due to above reasons.

The main objective of this study was to develop the optimal formulation of SMEDDS containing paclitaxel and PLGA for the improvement of release characteristics without any property change, such as chemical property of paclitaxel and weight loss of PLGA. In our effort, it was found that tetraglycol or PEG 400 was a good solubilizer for PLGA as well as paclitaxel and tetraglycol was much better solubilizer than PEG 400 with respect to droplet size and shelf stability of emulsion. From this result, we developed an optimal paclitaxel microemulsion prepared by SMEDDS and a paclitaxel microemulsion containing PLGA having various molecular weights (8K, 33K, and 90K) and their characterization were conducted by dynamic light scattering (DLS) for droplet size and transmission electron microscopy (TEM) and atomic force microscopy (AFM) for their surface morphology. The in vitro release profiles of paclitaxel from microemulsion without PLGA and miroemulsion containing PLGA was compared by high-performance liquid chromatography (HPLC). In vitro and in vivo anti-tumor activity of paclitaxel, released from microemulsion without PLGA and miroemulsion containing PLGA against human breast cancer cell line MCF7 and SKOV-3 human ovarian cancer cells were compared, respectively.

2. Materials and methods

2.1. Materials

Paclitaxel (Genexol®) was purchased from Samyang Genex Co. (Seoul, Korea). Polyglycolyzed

glycerides (Labrafill M-1944CS) were obtained from Gattefosse (Westwood, NJ). Cremophor ELP was purchased from BASF (Germany). Tetraglycol was purchased from Sigma Chemical Co. (St. Louis, MO, USA). PLGA having molecular weight of 8K, 33K, and 90K (mole ratio of lactide to glycolide, 50/50, 50/50, and 75/25; Resomer® RG 502H, 503H, and 756H, respectively) were purchased from Boehringer Ingelheim (Germany). Acetonitrile, methanol, chloroform (HPLC grade, Hurdick & Jackson, USA), and methylene chloride (MC, Tedia, Japan) were used as received. All other chemicals were of reagent grade. Deionized water was prepared by a Milli-Q purification system from Millipore (Molsheim, France). Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS), antibiotic–antimycotic, phosphate buffered saline (PBS), and trypsin/1,2-ethylenediaminetetraacetic (EDTA) were purchased from Gibco BRL® (Grand Island, NY, USA). MTT was purchased from Sigma Chemical Co. (St Louis, MO, USA). T-75, 24-well, and 96-well plates were purchased from Falcon Co. (Lincoln Park, NJ, USA). Female nude (nu/nu) athymic mice were purchased from Charles River France Inc. (Saint Aubin Les Elbeuf, France).

2.2. Phase diagram study

The pseudo-ternary phase diagrams of solubilizer, surfactant/cosurfactant, and water were developed using water titration method; the mixtures of solubilizer and surfactant/cosurfactant at certain weight ratios were diluted with water in a drop-wise manner. For each phase diagram, at a specific ratio of surfactant/cosurfactant, 0.5:1, 1:1, 1.5:1, and 2:1 (w/w), transparent and homogenous mixture of solubilizer, drug, and PLGA was formed under the mixing of a mixture (magnetic stirrer). Then each mixture was titrated with water and visually observed for phase clarity and flow ability. After the identification of microemulsion region in the phase diagrams, the microemulsion formulations were selected at desired component ratios. The paclitaxel-incorporating microemulsions were prepared by dissolving the drug powder into the microemulsion system. In order to confirm the microemulsion formulation, the selected microemulsion was prepared as the following.

2.3. Preparation of microemulsion containing PLGA

A series of SMEDDS was prepared in each of the eight formulas (Table 1) with various PLGA and/or paclitaxel, solubilizer, surfactant, and cosurfactant. Briefly, PLGA and/or paclitaxel were dissolved, respectively, by solubilizer such as tetraglycol in glass vials. Surfactant and cosurfactant were accurately weighed into glass vials. Then the components were mixed by gentle stirring and vortex mixing until paclitaxel had perfectly dissolved. The mixture was stored at room temperature until used. Before using, microemulsion was formed from SMEDDS with contacting aqueous phase.

2.4. Molecular weight analysis of solubilzed PLGA in SMEDDS

Molecular weight of PLGA before and after being solubilized by solubilizer was determined, respectively, by gel permeation chromatography (GPC), equipped with a differential refractive index detector (Shodex® RI-71) and three columns (Shodex® GPC K-802, Asahipak GF-510 HQ, and GF-7M HQ) in series maintained 35 °C. Sample molecular weight averages were determined relative to polystyrene monodisperse standards with molecular weights ranging from 1270 to 1,950,000 g/mol (Shodex® SM-105). Chloroform was used as mobile phase at 0.6 ml/min.

2.5. Microemulsion characterization

2.5.1. Emulsion droplet size analysis

Formulation (50 μl) of SMEDDS was diluted with water to 50 ml in a volumetric flask and gently mixed by inverting the flask. The droplet size distributions of resultant emulsions were determined by dynamic light scattering (DLS, ELS-8000, Photal, Japan) at room temperature. The emulsion droplet diameters were analyzed and the value of mean emulsion droplet diameters were compared with the each other's formulation.

2.5.2. Morphology observation by TEM and AFM

The shape and surface morphology of microemulsion were examined by the transmission electron microscopy (TEM, CM-12, Philips, Netherlands) and the

Table 1
Formulation of paclitaxel SMEDDS containing PLGA and the emulsion droplet size

Composition (g, w/w)	Formulation			
	A	B	C	D
Drug				
Paclitaxel		0.01		
Solubilizer				
Tetraglycol		1.0		
PLGA				
8K g/mol	–	0.1	–	–
33K g/mol	–	–	0.1	–
90K g/mol	–	–	–	0.1
Cosurfactant				
Labrafil 1944	0.7	0.7	0.7	0.7
Surfactant (Cremophor)				
ELP	0.8	0.8	0.8	0.8
Mean partcle size (nm)	45.1 ± 0.6	125.4 ± 0.2	145.2 ± 0.7	194.3 ± 0.4

atomic force microscopy (AFM, XE-100, PSIA, Korea). For observation by TEM, a drop of microemulsion (1 ml) was placed on copper electron microscopy grids (Formvar filmed) and the sample was quickly frozen at liquid nitrogen, followed by freeze-dried at $-55\,^{\circ}C$ (Kim et al., 2003; Montagne et al., 2002). The sample for AFM image has been handled in same manner at TEM analysis. AFM analysis was conducted with Nanoscope IIIa in the non-contact mode. Before observation, the lyophilized sample was fixed on a double-sided sticky tape that was stuck on the standard sample stand.

2.6. In vitro release study

The release study of paclitaxel was performed in five-fold. The in vitro release profile of paclitaxel from microemulsion was assessed by determination of the residual amount of paclitaxel present in microemulsion. SMEDDS containing paclitaxel 0.01 g was put into 50 ml vials in phosphate buffered saline (PBS, pH 7.4). The vials were monitored for a period of incubation at $37\,^{\circ}C$ and constant shaking at 160 strokes/min. At specific time following incubation, vials were withdrawn and SMEDDS was retrieved by ultracentrifugation. After removing the supernatant, the pellet was washed twice with distilled water and freeze dried. The paclitaxel content in microemulsion was assayed by HPLC. Briefly, a specific amount of lyophilized SMEDDS was dissolved in 1 ml MC and mixed with 5 ml of an acetonitrile:MeOH (60:40, v/v). The mixture was then vortexed for 5 min and phase separation occurred. Twenty microliters of aliquots of supernatant (acetonitrile phase) were analyzed by HPLC. The HPLC analysis system consisted of tsp-P2000 pump, tsp-UV2000 ultraviolet detector, and tsp-AS3000 autosampler. The chromatographic column was a Fluofix 12W425 4.6 mm × 250 mm (Neos, Japan) and the guard column 1EWDG1 4 mm × 10 mm (Neos, Japan). A mobile phase of acetonitrile:water (60:40, v/v) was pumped isocratically at a flow rate of 1 ml/min. The effluent was monitored at 227 nm.

2.7. Cell culture

For in vitro and in vivo anti-tumor activity test, human breast cancer cell line MCF7 and SKOV-3 human ovarian cancer cell line were used, respectively. The experimental cells were grown in RPMI 1640 medium, antibiotic–antimycotic 1% (v/v) was added and supplemented with 10% (v/v) FBS. The cells were cultured in a 95% air/5% CO_2 atmosphere at $37\,^{\circ}C$ in a humidified incubator, and were dissociated with 0.05%

trypsin-EDTA, in case of transferring or dispensing before experiment.

2.8. In vitro anti-tumor activity test

The experimental cells were counted in a hemacytometer (Reichert Co., USA) using the trypan blue exclusion method. The cells were plated on 24-well flat-bottomed plates, with each well at a density of 1×10^5 cells/well, and incubated for 24 h at 37 °C in the CO_2 incubator. After the cells were attached on 24-well flat-bottomed plates, microemulsion without paclitaxel and microemulsion at concentration (0.0025 μg/ml) of paclitaxel was directly added to cell culture medium of 500 μl, respectively. The cells were incubated with microemulsion for measurement duration, 1, 3, and 6 days, at 37 °C in the CO_2 incubator. The cytotoxicity following the above-mentioned treatments was evaluated by 3-[4,5-dimethylthiazol-2-yl]-3,5-diphenyltetrazolium bromide dye (MTT) assay. The MTT assay is an indirect measure of cell density or number of living cells attached to the culture plate by formation of colored formazan crystals. Briefly, the culture medium was aspirated and cells were washed with PBS (pH 7.4) at the end of the incubation period with different formulations. Cells were then incubated with 500 μl of the MTT solution (0.5 mg/ml) in RPMI 1640 medium without FBS for 4 h at 37 °C. The MTT solution with culture medium was removed without the formazan crystals formed. Five hundred microliters of an isopropanol acidic solution (isopropanol–HCl 0.04N) was then added in order to dissolve the formazan crystals formed. After solubilizing, the absorbance was measured with the ELISA microplate reader (Emax, Molecular Devices Co., USA) at a wavelength of 520 nm. The results of two independent experiments were expressed as treatment over control (T/C) values; cell viability (%) = T/C × 100. All experiments were performed in triplicate.

2.9. In vivo anti-tumor activity test

Briefly, a solid tumor was established upon S.C. injection of 0.1 ml of SKOV-3 human ovarian cancer cells suspension (7×10^6 cells per mice) to the right flank of female nude (nu/nu) athymic mice (6–8 weeks, 22–34 g). Treatments were started when tumor in the nude mice reached a tumor volume of 48–100 mm^3 and this day was designated as day 0. On day 0, they were randomly divided into groups of five mice. A single i.v. dose of microemulsion prepared by formulation A and microemulsion containing PLGA prepared by formulation B was administered intravenously at concentrations (0.0025 μg/ml) of paclitaxel on initial treatment day. In control experiments, saline and paclitaxel-free microemulsion vehicle prepared by formulation B were used. Tumor growth was documented by measuring the length and width of the tumors with dial calipers twice weekly. Tumor volume was calculated by formula for the approximation of the volume of a spheroid; tumor volume = length × $width^2/2$. Statistical analysis of the tumor volume changes was evaluated by two-sample *t*-test. The serial measurement of tumor volume was followed until the day at which the animals were sacrificed.

3. Results and discussion

3.1. Phase behavior

Phase diagrams were constructed in the presence of paclitaxel. SMEDDS formed fine solubilizer-water emulsions with only gentle agitation, upon their introduction into aqueous media. Since the free energy required to form an emulsion is very low, the formation is thermodynamically spontaneous (Craig et al., 1995). Surfactants form a layer around the emulsion droplets, reduce the interfacial energy as well as provide a mechanical barrier to coalescence. The visual test is measured the apparent spontaneity of emulsion formation. The series of SMEDDS were prepared and their self-emulsifying properties were observed visually. Pseudo-ternary phase diagrams were constructed to identify the self-emulsifying regions and to optimize the concentration of surfactant and cosurfactant (Fig. 1). The efficiency of emulsification was good when the S/CoS concentration was more than 40% of SMEDDS formulation. It was observed that increasing the concentration of the surfactant within self-emulsifying regions increased the spontaneity of the self-emulsification region. Therefore, the formulation of SMEDDS in this study was established (Table 1). In this study, S/CoS ratio 1:1 was used because this ratio is the most stable after formation of microemulsion and has the broadest

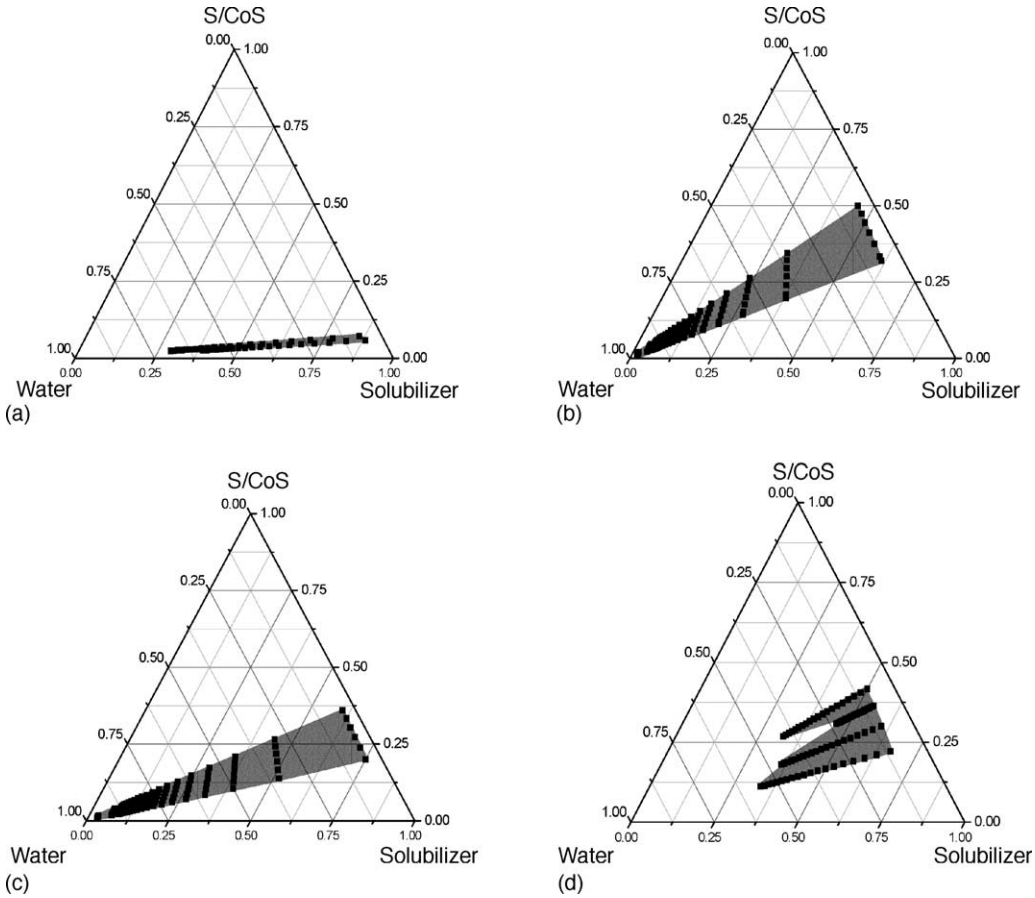

Fig. 1. Pseudo-ternary phase diagrams indicating the efficient self-emulsification region (S/CoS: (a) 0.5:1; (b) 1:1; (c) 1.5:1; (d) 2:1, the dot area represents O/W microemulsion existence range).

self-emulsifying regions with using small amount of surfactant, which is required in paclitaxel bio application.

3.2. Molecular weight analysis of solubilzed PLGA in SMEDDS

Generally, molecular weight of PLGA is an important role to control the release profile of drug as a matrix. Therefore, change of molecular weight of PLGA used in SMEDDS has to be checked. Change of molecular weight of PLGA before and after being solubilized by solubilizer. There was no change in molecular weight of PLGA after being solubilized by solubilizer like tetraglycol.

3.3. Droplet size analysis and morphology observation

The effect of the formulation of SMEDDS on droplet size of microemulsion is shown in Table 1. The droplet size for all formulations was found in the range of 45–195 nm. The formulation of microemulsion could affect droplet size by following factors such as including PLGA, the molecular weight of PLGA, and type of cosurfactant and surfactant. The microemulsion prepared by formulation A showed the smallest droplet size of 45.1 nm. The formulation A was formatted by Labrafil 1944 and Cremophor ELP as cosurfactant and surfactant, not contained PLGA. It could suggest that this is the most optimal formulation

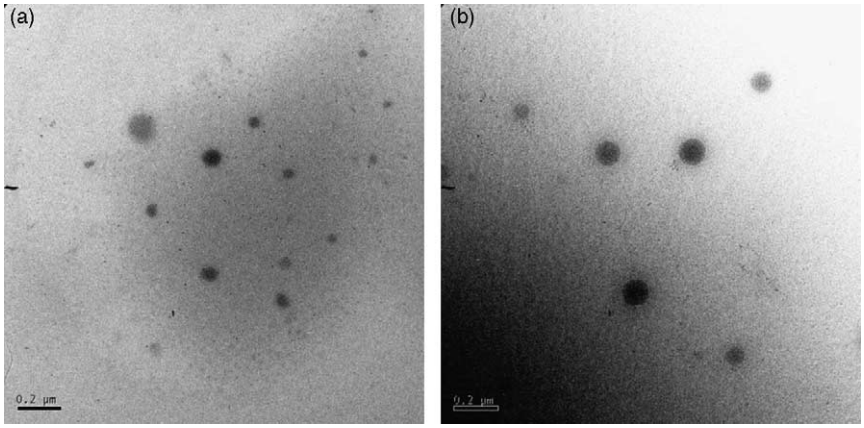

Fig. 2. Transmission electron microphotographs of a paclitaxel emulsion containing PLGA: (a) microemulsion without PLGA prepared by formulation A and (b) microemulsion containing PLGA prepared by formulation B (the scale bar for all images represents 0.2 μm and ×50,000).

to create the smallest droplet size. And it also could be observed that droplet size was small by using smaller molecular weight of PLGA (formulation B–D). The same results were shown from the morphology of microemulsion by TEM and AFM analysis.

The morphology of microemulsion by TEM analysis was shown in Fig. 2. It showed the spherical shape and uniform droplet size of microemulsion. It was also observed that the droplet size of microemulsion prepared by formulation A (a) was smaller than that of microemulsion containing PLGA prepared by formulation B (b). It could be suggested that the presence of PLGA in microemulsion affected the droplet size when the same formulation was used.

The image of shape and surface characteristics of microemulsion containing PLGA prepared by formulation B was obtained by applying non-contact mode AFM (Fig. 3). It was shown that droplet of microemulsion was almost of spherical shape with smooth surface and there was no aggregation or adhesion among droplet of microemulsion.

3.4. *In vitro release study*

The in vitro release behaviour of paclitaxel from microemulsion ($n = 5$) without PLGA and microemulsion containing PLGA having various molecular weights is shown in Fig. 4. As can be observed, the release behaviour of paclitaxel from microemulsion containing various PLGA having various molecular weights (formulations of B, C, and D) exhibited a biphasic pattern characterised by a fast initial release during the first 48 h, followed by a slower and continuous release for 144 h in contrast that the release of paclitaxel from microemulsion prepared by formulation A was finished during 24 h. It could offer that PLGA dissolved in microemulsion made paclitaxel release slowly, continued up to 168 h. As we expected, the release pattern was identical, irrespective of the molecular weight of PLGA, but the release rate of paclitaxel from microemulsion was related to the molecular

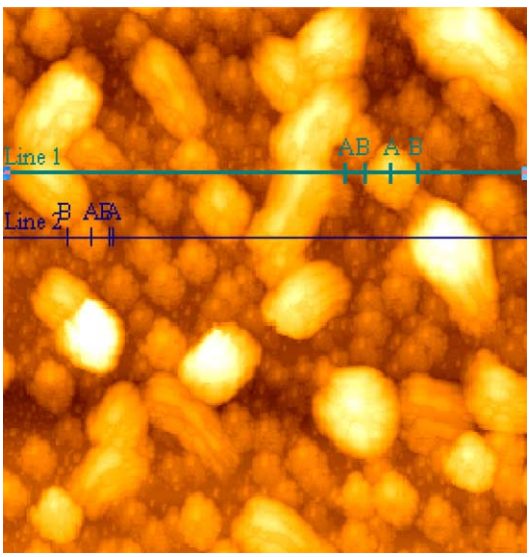

Fig. 3. AFM image of a paclitaxel microemulsion containing PLGA prepared by formulation B (the line 3 μm image).

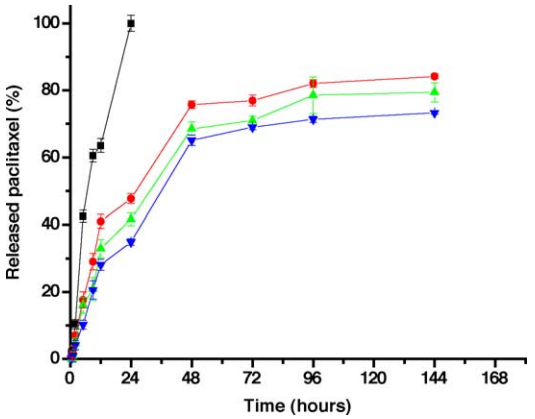

Fig. 4. Release profiles of paclitaxel from a paclitaxel microemulsion prepared by formulation A (■); B (●); C (▲); D (▼), respectively ($n = 5$). Each value represents the mean ± S.D.

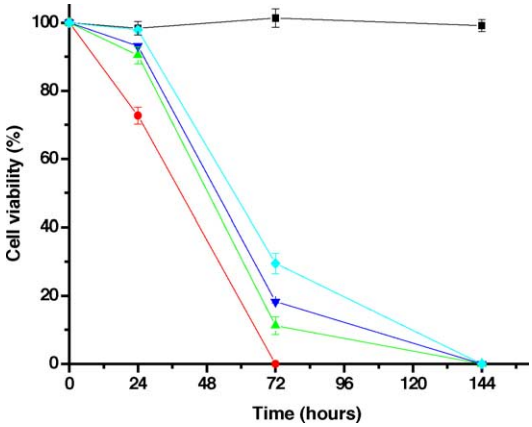

Fig. 5. Cytotoxicity of paclitaxel from a paclitaxel microemulsion containing PLGA against human breast cancer cell line MCF7 in vitro. The cells were incubated with microemulsion for measurement duration at 37 °C in the CO_2 incubator. Cell survival fractions were assessed by MTT assay. Paclitaxel-free microemulsion prepared by formulation B (■), paclitaxel microemulsion prepared by formulation A (●); B (▲); C (▼); D (♦), respectively. Each value represents the mean ± S.D.

weight of PLGA. The release rate was fast with decreasing molecular weight of PLGA and was fast in order: PLGA 8K, PLGA 33K, and PLGA 90K g/mol. It might suggest that the molecular weight of PLGA in microemulsion could control the release rate of paclitaxel from microemulsion.

3.5. *In vitro anti-tumor activity*

The cytotoxicity of paclitaxel released from microemulsion, prepared by various formulations in vitro against human breast cancer cell line MCF7 is shown in Fig. 5. In the case of paclitaxel-free microemulsion prepared by formulation B, put in culture medium, all the samples did not reveal cytotoxicity to the MCF7 cell line during test period. Paclitaxel-free microemulsion vehicle prepared by formulation B also did not effect on the MCF7 cell line growth and survival in the direct and continuous exposure to the cell for test period. This cell line was sensitive to the released drug when it was exposed continuously to paclitaxel for 144 h. For microemulsion without PLGA prepared by formulation A, the above 28% of cells were dead, for 24 h of incubation in contrast that a reduction of less than 10% in cell viability for all of the microemulsion containing PLGA (formulation B–D) was observed. Cell growth for microemulsion without PLGA prepared by formulation A was almost totally inhibited at 72 h of incubation time but all of the microemulsion containing PLGA almost inhibited the cell growth totally at 144 h of incubation. The reduction rate of cell viability for formulations B, C, and D could be explained with respect to the dependence of release rate of paclitaxel from microemulsion containing PLGA on the molecular weight of PLGA. As a result of these, we supposed that paclitaxel was released from microemulsion containing PLGA continuously and the cytotoxicity against the MCF7 cell line was affected significantly by the released amount of paclitaxel. This result of cell test was accomplished with the release profile of microemulsion containing PLGA.

3.6. *In vivo anti-tumor activity*

In our in vivo anti-tumor activity, three groups ($n = 5$) according to the formulation in Table 1 including saline and paclitaxel-free microemulsion prepared by formulation B were subjected to the experiment. In vivo anti-tumor activity was investigated by measuring tumor volume changes of SKOV-3 human ovarian cancer cell bearing nude mice after treating with microemulsion (Fig. 6). The tumor growth was delayed after treating with microemulsion without PLGA prepared by formulation A and microemulsion containing PLGA prepared by formulation B in contrast that saline and paclitaxel-free microemulsion prepared

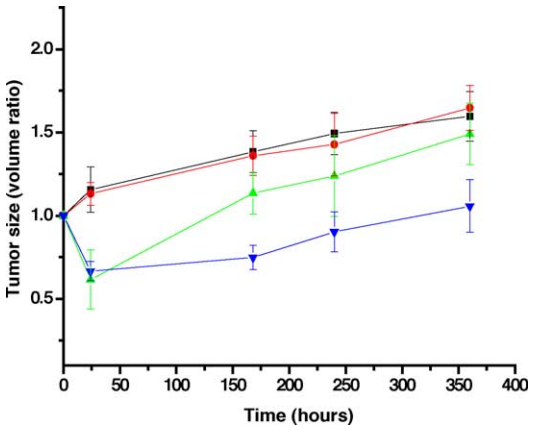

Fig. 6. Tumor volume changes of animals treated with saline (●), paclitaxel-free microemulsion prepared by formulation B (■), paclitaxel microemulsion prepared by formulation A (▲); B (▼), respectively ($n = 5$). Each value represents the mean ± S.D.

by formulation B did not inhibit tumor growth. Paclitaxel microemulsion seems to contribute to the overall tumor-size reduction. But tumors treated with microemulsion without PLGA prepared by formulation A was regrown faster than treatment of microemulsion containing PLGA prepared by formulation B after 24 h. Statistical analysis demonstrated a significant ($P > 0.05$) difference in both control and microemulsion contained paclitaxel below 168 h. After 168 h, the microemulsion without PLGA demonstrated not a significant ($P > 0.05$) difference in control. This result revealed that microemulsion containing PLGA prepared by formulation B released paclitaxel slowly and continuously in contrast that microemulsion without PLGA prepared by formulation A released paclitaxel very fast.

4. Conclusions

The obtained results indicated that the potential use of microemulsion using PLGA for sustained release of lipophilic drugs such as paclitaxel was established. The release behaviour of paclitaxel from microemulsion containing PLGA having various molecular weights exhibited sustained release in contrast that the release of paclitaxel from microemulsion without PLGA was very rapid. Moreover, it was shown that microemulsion containing PLGA enhances its anti-tumor activity as compared to microemulsion without PLGA. These results strongly prove that the formulations developed in this study could be used for clinical trials.

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