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Enhanced solubility and stability of PEGylated liposomal paclitaxel: *In vitro* and *in vivo* evaluation

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Abstract

An improved PEGylated liposomal formulation of paclitaxel has been developed with the purpose of improving the solubility of paclitaxel as well as the physicochemical stability of liposome in comparison to the current Taxol® formulation. The use of 3% (v/v) Tween 80 in the hydration media was able to increase the solubility of drug. The addition of sucrose as a lyoprotectant in the freeze-drying process increased the stability of the liposome particles. There was no significant difference in the entrapment efficiency of paclitaxel between the conventional non-PEGylated liposomes and our PEGylated liposomes. Cytotoxicity in human breast cancer cell lines (MDA-MB-231 and SK-BR-3) of our paclitaxel formulation was less potent compared to Taxol® after 24 h incubation, but was equipotent after 72 h due to the slower release of drug from the liposome. Our PEGylated liposomes increased the biological half-life of paclitaxel from 5.05 (±1.52) h to 17.8 (±2.35) h compared to the conventional liposomes in rats. Biodistribution studies in breast cancer xenografted nude mouse model showed that our liposomes significantly decreased the uptake in reticuloendothelial system (RES)-containing organs (liver, spleen and lung) while increasing the uptake in tumor tissues after injection compared to Taxol® or the conventional liposomal formulation. Moreover, the PEGylated liposome showed greater tumor growth inhibition effect in *in vivo* studies. Therefore, our PEGylated liposomal formulation of paclitaxel could serve as a better alternative for the passive targeting of human breast tumors.

Keywords: Paclitaxel; PEGylated liposome; Cytotoxicity; Pharmacokinetics; Biodistribution

1. Introduction

Paclitaxel is known as one of the most effective anticancer drugs in the market today. Significant antitumor activity has been demonstrated in clinical trials against a wide variety of tumors, including ovarian carcinoma, breast cancer, head and neck cancers and non-small cell lung cancer (Rowinsky and Donehower, 1995). One of the biggest shortcomings of this drug, however, is its low aqueous solubility.

The current clinical dosage form of paclitaxel, which is parenteral, is dissolved in a mixture of Cremophor® EL (polyoxyethylated caster oil) and ethanol (50:50, v/v) and needs to be diluted right before injection. However, Cremophor® EL has been associated with serious side-effects and leads to hypersensitivity, nephrotoxicity and neurotoxicity in many patients (Singla et al., 2002). Although a premedication regimen with corticosteroids and antihistamine reduces the incidence of serious hypersensitivity, milder reactions have still occurred in 5–30% of patients (Weiss et al., 1990).

In order to increase the therapeutic efficiency and reduce the side-effects caused by these vehicles, much effort has been devoted to improving the aqueous solubility of paclitaxel without using Cremophor® EL. These alternatives include the use of solubilizing agents (*e.g.*, Tween 80) (Singla et al., 2002) and the liposome-based formulations (Crosasso et al., 2000). A vehicle composed of ethanol and Tween 80 (50:50, v/v), to

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be diluted in glucose solution before use, was attempted to be developed for administration of paclitaxel (Singla et al., 2002) and docetaxel (Sparreboom et al., 1998; Immordino et al., 2003). However, in both cases, the precipitation of paclitaxel upon dilution with the aqueous media posed a major problem. On the other hand, liposomes have been used to formulate a variety of hydrophobic, poorly water soluble drugs. It was reported that liposomal paclitaxel improved its solubility and showed similar *in vitro* cytotoxicity against a variety of tumor cell lines compared to that of the free paclitaxel in Cremophor® EL based vehicle (Taxol®) (Sharma et al., 1996). However, one of the major drawbacks of the liposomal formulation was its rapid clearance from blood due to the adsorption of plasma protein to the phospholipid membrane of the liposomes, thereby triggering the recognition and uptake of the liposomes by the mononuclear phagocytic system (MPS) (Schnyer and Huwyler, 2005).

Fortunately, when the surface of the liposomes was modified with a flexible hydrophilic polymer such as polyethylene glycol (PEG), the uptake by MPS could be retarded (Torchilin and Trubetskoy, 1995). This resulted in the increase of the biological half-life and the spontaneous accumulation of liposomes in solid tumor *via* the "enhanced permeability and retention (EPR)" effect (Yuan et al., 1995; Laginha et al., 2005). Nevertheless, studies with PEGylated liposomes showed low concentration of paclitaxel in solution (0.5–0.8 mg/mL) and poor physical stability (<1 week).

Further increasing the concentration of paclitaxel inside the lipid bilayer without affecting the stability of liposomes would confer clinical advantage to the PEGylated liposomal formulation (Crosasso et al., 2000). Therefore, herein, we report on the development of a PEGylated liposomal formulation of paclitaxel by using Tween 80 and a lyoprotectant. The PEGylated liposome has been systematically evaluated for its stability, solubility as well as *in vitro* and *in vivo* activity.

2. Materials and methods

2.1. Materials

Paclitaxel was purchased from Taihua Corporation (Xi'an, China). Soybean phosphatidylcholine (S_{100}PC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine [methoxy (polyethyleneglycol)-2000] ($MPEG_{2000}$-DSPE) were generous gifts from Lipoid Company (Ludwigshafen, Germany). Cholesterol (CH) and Tween 80 were bought from Tokyo Kasei Co. Ltd. (Tokyo, Japan). 3-(4,5-Dimethyltiazol-2-ly)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Millipore polycarbonate membranes (1.2 μm, 0.4 μm and 0.2 μm pore size) were purchased from Millipore Corporation (Isopore™, County Cork, Ireland). Dulbecco's modified eagle medium (DMEM), modified eagle medium (MEM), penicillin-streptomycin and fetal bovine serum were obtained from Invitrogen (Ontario, Canada). All other chemicals were of the highest grade possible and obtained from commercial sources.

2.2. Cancer cell lines

SK-BR-3 and MDA-MB-231 human breast cancer cell lines were purchased from the American Type Culture Collection (Rockville, MD, USA). SK-BR-3 cells were cultured in MEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotics (100 U/mL penicillin G and 0.1 mg/mL streptomycin). MDA-MB-231 human breast cancer cells were cultured in DMEM supplemented with 10% heat-inactivated FBS and 1% antibiotics (100 U/mL penicillin G and 0.1 mg/mL streptomycin). Cells were maintained at 37 °C in a humidified incubator containing 5% CO_2.

2.3. Preparation of liposomes

Both the conventional liposome composed of S_{100}PC/CH (90:10, molar ratio) and the PEGylated liposome composed of S_{100}PC/CH/$MPEG_{2000}$-DSPE (90:10:5 as a molar ratio) were prepared by the modified thin-film hydration method. Briefly, the hydrophobic excipients, paclitaxel (3.5 mg/mL), CH and lipids [10% (w/v) S_{100}PC and $MPEG_{2000}$-DSPE], were dissolved in chloroform and transferred into a suitable conical flask. The flask was then connected to a BÜCHI R-200 rotary evaporator (Flawil, Switzerland) and water bath (BÜCHI B-490) with temperature maintained at 40 °C under the aspirate vacuum. The thin-film layer formed was flushed with nitrogen gas for 5 min and maintained overnight under vacuum to remove traces of chloroform. The thin-film was re-suspended in phosphate buffer saline (PBS, pH 4.0) with or without 3% (v/v) Tween 80 by rotating the flask at about 300 rpm until the lipid film was completely hydrated. Then, the liposome dispersion was serially passed through 1.2, 0.4 and finally 0.2 μm pore size filters (Isopore™) under nitrogen gas with an extruder (Northern Lipids, Inc., Canada). Un-entrapped paclitaxel was removed from the liposome suspensions by centrifuging at 1000 rpm for 10 min, after which the supernatant liposomal dispersion was centrifuged at 50,000 rpm for 30 min to precipitate the liposomes. Complete precipitation of liposomes was confirmed by observing the absence of particles in the supernatant using a NICOMP 370 Submicron Particle Sizer. The supernatant was discarded, and the liposome pellet was washed twice with PBS (pH 7.4). The pellet was then suspended in distilled water containing sucrose (molar ratio of sugar-to-lipid = 2.3), and freeze-dried (Laboratory Floor Model Freeze-dryer FD5512, Ilshin, Seoul, Korea). The final liposome particles were stored in tight containers at 4 °C for further experiments.

2.4. Physicochemical characteristics of liposomes

2.4.1. Morphology of liposomes

The morphology of the conventional and the PEGylated liposomes was observed by transmission electron microscopy (TEM). For negative staining, liposomes were diluted with distilled water and dropped on a formvar-coated copper grid (300-mesh, hexagonal fields) and air-dried for 1 min at room temperature after removing the excessive sample with filter paper. After adhesion of liposomes, 10 μL of 2% uranyl acetate

solution was dropped onto the grid as a staining solution. The excess staining solution was removed with filter paper in 30 s. In order to eliminate impurity, the uranyl acetate solution was filtered by polycarbonate 0.2 μm filters before deposition. At the end, the sample was air-dried for about 10 min at room temperature, and then observed by TEM (JEM-1010, JEOL Ltd., Tokyo, Japan).

2.4.2. Entrapment efficiency of paclitaxel in liposomes

The entrapment efficiency (EE) is defined as the ratio of the amount of the paclitaxel encapsulated in liposome to that of the total paclitaxel in liposomal dispersion. The amount of paclitaxel encapsulated in liposomes was measured following the method in the literature with slight modification (Shieh et al., 1997). Briefly, aliquots (0.1 mL each) of liposomal dispersion diluted to 1.1 mL by PBS (pH 7.4) immediately after preparation was centrifuged at 1000 rpm for 10 min to remove any paclitaxel particle already released from the liposomes. Then, 1.0 mL of the liposome supernatant was centrifuged at 50,000 rpm for 30 min (Beckman, XL-100, Fullerton, CA, USA). After removing the supernatant by aspiration, the precipitate (*i.e.*, liposome pellet) was washed twice with PBS (pH 7.4). The liposome pellet was dissolved in 6 mL of water and organic solvents mixture (50:50, v/v), of which the latter was composed of isopropanol, ether and ethanol (2:1:2, v/v/v). The concentration of paclitaxel was determined by high performance liquid chromatography (HPLC) after appropriate dilution with the mixed solvent that destroyed the liposome pellet. An aliquot (0.1 mL each) of the liposome suspension was also dissolved with the same mixed solvent to determine the total amount of paclitaxel in the liposome suspension, after which the EE was calculated from the following equation:

$$\mathrm{EE}(\%) = \frac{\text{amount of paclitaxel in liposome pellet (}\mu\text{g)}}{\text{amount of paclitaxel in liposomal dispersion (}\mu\text{g)}} \times 100$$

An aliquot (50 mg each) of the freeze-dried liposome powder was dissolved with the same mixed solvent (4 mL) to determine the content of paclitaxel in the freeze-dried liposome powder using the following equation after appropriate dilution with the same mixed solvent:

$$\mathrm{Content} = \frac{\text{amount of paclitaxel in freeze-dried liposome (}\mu\text{g)}}{\text{amount of freeze-dried liposome (mg)}}$$

The EE and paclitaxel content were determined from three separately prepared liposome suspensions, and were expressed as the mean ± standard deviation.

2.4.3. Particle size distribution and zeta-potential

The mean particle size and particle size distribution of the liposomes were determined using a NICOMP 370 Submicron Particle Sizer (Particle Sizing Systems, Santa Barbara, CA, USA). The change of particle size was determined at room temperature for 24 h to observe the aggregation of liposomes. The zeta potential of the liposomes was measured by an electrophoretic light scattering spectrophotometer (ELS-8000, OTSUKA Electronics Co. Ltd., Japan) at room temperature after appropriate dilution with distilled deionized water.

2.5. In vitro release of paclitaxel from liposomes

Release of paclitaxel from conventional and PEGylated liposome was observed using the dialysis method at room temperature, and was compared with that from Taxol®. After reconstituting the freeze-dried liposomes in PBS (pH 7.4) to make 1.5 mg/mL of paclitaxel, an aliquot of each liposomal dispersion (0.1 mL) was placed in a dialysis tube (MWCO 6000–8000, Gene Bio-Application Ltd., Israel) and was tightly sealed. Then, the tube was immersed in 200 mL of release medium, *i.e.*, PBS (pH 7.4) containing 0.1% (v/v) Tween 80 to maintain sink condition (Koziara et al., 2004; Zhang et al., 2004). While stirring the release medium using the magnetic stirrer at 300 rpm, samples (0.5 mL) were taken at predetermined time intervals from the release medium for 24 h, which was refilled with the same volume of fresh medium. Concentration of paclitaxel was determined by HPLC after appropriate dilution with acetonitrile without further treatment.

2.6. In vitro cytotoxicity assay

The cytotoxicity of paclitaxel, loaded in liposomes (conventional and PEGylated liposome), against MDA-MB-231 and SK-BR-3 breast cancer cells was determined by using the MTT dye reduction assay (Twentyman and Luscombe, 1987), and was compared with that of Taxol®. Briefly, 2.0×10^4 cells/well in its exponential growth phase was plated in 96-well flat-bottom tissue-culture plates. The cells were incubated at 37 °C in a 5% CO_2 incubator for 24 h, during which cells were attached and resumed to grow. Freeze-dried liposomes were diluted with culture media to make various concentrations of paclitaxel, and were added in triplicate (200 μL each). Control wells were treated with equivalent volumes of paclitaxel-free media. After 24 h or 72 h, the supernatant was removed. MTT (0.5 mg/mL) in PBS (pH 7.4) and culture medium (100 μL each) was added to each well and incubated for 4 h. The unreduced MTT and medium were then discarded. Each well was washed with 200 μL of PBS after which was added 200 μL of DMSO to dissolve the MTT formazan crystals. Plates were shaken for 20 min and absorbance was read at 560 nm using the microplate reader (Molecular Devices Corporation, USA). The IC_{50} values (*i.e.*, concentration resulting in 50% growth inhibition) of paclitaxel were graphically calculated from concentration-effect curves, considering the optical density of the control well as 100% (Sharma et al., 1996).

2.7. Pharmacokinetics study

Pharmacokinetics studies of paclitaxel were performed in male Sprague–Dawley rats (200–250 g) as described elsewhere with slight modification (Kim et al., 2001; Aliabadi et al., 2005). For intravenous administration, freeze-dried liposomes were suspended in PBS (pH 7.4) to make 1.5 mg/mL paclitaxel solution. Taxol® (6 mg/mL paclitaxel in Cremophor® EL and ethanol

mixture) was diluted with PBS (pH 7.4) to 1.5 mg/mL, as a control. Femoral arteries and veins of the rats were cannulated with polyethylene tubing (PE-50, Clay Adams, Parsippany, NJ, USA) under ketamine anesthesia at supine position. After complete recovery (1 h) from the anesthesia, paclitaxel (7.5 mg/kg) was administered *via* femoral vein. Blood samples (<0.3 mL) were withdrawn from the femoral artery at appropriate time intervals for 24 h. Plasma samples (100 μL) were obtained by immediately centrifuging the blood samples at 7000 × *g* for 5 min and stored at −20 °C until analyzed by HPLC. The plasma concentration profiles of paclitaxel after intravenous administration were fitted to the conventional two-compartment model using the WinNonlin® program (Version 3.1, Pharsight Co., Mountainview, CA, USA).

2.8. Tissue distribution study

The human breast cancer cell line, MDA-MB-231, in its exponential growth was inoculated into 4-week-old (18–20 g) female BALB/C nu/nu athymic (nude) mice (Charles River, Korea). Cancer cells at a number of 4 to 5×10^6 were suspended in 0.2 mL of culture medium and subcutaneously inoculated at the right flank of mice using a 1.0 mL syringe. Animals were kept in a SPF facility and had free access to food and water. When the tumor volumes became 100–200 mm^3 after 2–3 weeks of inoculation, paclitaxel (7.5 mg/kg) was administered *via* tail vein. For intravenous administration, freeze-dried liposomes were suspended in PBS (pH 7.4) to make 1.5 mg/mL paclitaxel solution. Taxol® was diluted with PBS (pH 7.4) to 1.5 mg/mL, as a control. After 0.5, 6, and 24 h of injection, blood samples were collected from the eyes of three to five mice in each group, after which the mice were sacrificed by cervical dislocation in order to obtain tissue samples. The organs (liver, spleen, lung, heart, kidney, brain and tumor) were removed and washed twice with physiological solution (0.9% NaCl), weighed and stored at −20 °C until analyzed by HPLC. Tissue distribution of paclitaxel was expressed as the amount of paclitaxel per gram of tissues.

2.9. In vivo tumor growth inhibition study

The nude mice xenograft model was prepared as described in Section 2.8 and the animals were kept in a SPF facility to observe the tumor growth. When the tumor volume became about 50 mm^3, liposomes in PBS (pH 7.4, 1.5 mg/mL of paclitaxel) or Taxol® (diluted to 1.5 mg/mL with PBS, pH 7.4) was intravenously administered (7.5 mg/kg) by tail vein three times at days 0, 4 and 8 (total 22.5 mg/kg). Normal saline was injected for the control group. The tumor volumes of nude mice were monitored twice a week for up to 60 days. The tumor volume and calculation was performed using the formula $0.4(a \times b^2)$, where a is the largest and b is the smallest diameter (Maeda et al., 2004).

2.10. HPLC analysis of paclitaxel

HPLC method was used for the analysis of paclitaxel concentration in all samples. For EE and *in vitro* release studies, the samples (50 μL) were directly injected into the HPLC system without further treatment, while plasma samples were extracted with ethyl acetate before injection, as previously reported with minor modification (Song and Au, 1995).

For plasma samples, 100 μL of plasma was spiked with 50 μL of *n*-butyl *p*-hydroxy-benzoate (5 μg/mL) as an internal standard, and was extracted with 2 mL of ethyl acetate with vigorous mixing for 5 min. After centrifugation at 3000 rpm for 5 min, the organic phase was collected. The extraction procedure was repeated with 2 mL of ethyl acetate, and total organic phase was combined and dried under nitrogen gas. The residue was then dissolved in 100 μL of zinc sulfate solution (0.5 g of zinc sulfate and 1.0 mL of ethylene glycol in 100 mL of methanol) and was mixed for 5 min. The solution was centrifuged for 5 min at 3000 rpm, and 50 μL of the supernatant was injected into the HPLC system.

For tissue samples, they were homogenized with 10 times the volume of water, containing 4% (w/v) bovine serum albumin (Sparreboom et al., 1995), using a tissue homogenizer (IKA-Ultra-Turrax® T25 basic, Germany) for 5 min at 4 °C. The tissue homogenate (1.0 mL) was spiked with 50 μL of internal standard (*n*-butyl *p*-hydroxy-benzoate, 5 μg/mL), and was extracted twice with 2 mL of ethyl acetate, as described for the plasma sample. The ethyl acetate fractions were combined and were dried under nitrogen gas. The residue was then dissolved with 100 μL of zinc sulfate solution, and was mixed for 5 min. The solution was centrifuged at 3000 rpm for 5 min, and 50 μL of the supernatant was injected into the HPLC system.

The HPLC system was equipped with a Waters 2487 Dual λ Absorbance Detector, 717 plus Autosampler and 515 HPLC dual pumps. A reverse phase LiChrospher® 100 RP-18 column (250 mm × 4.6 mm, 5 μm, Merck, Germany) was used at room temperature and the detector wavelength was set at 227 nm. Mixture of acetonitrile:water (50:50, v/v) was used as the mobile phase at a flow rate of 1.0 mL/min.

2.11. Statistical analysis

All data were expressed in the form of the mean ± standard deviation. For comparison of mean values between the formulations, the Student's t-test was used. In all cases, $p < 0.05$ was accepted as denoting a statistical difference.

3. Results and discussion

3.1. Formulation development

Among the different drug delivery systems, the liposomal formulation is considered to be a relatively non-toxic technology with considerable potential for encapsulating both lipophilic and hydrophilic drugs. Despite the many benefits of the paclitaxel liposomes, the solubility and stability problems have been a hindrance to further develop them for clinical applications. One of the approaches taken in this report was therefore to investigate the effect of surfactants in the liposomal formulation to increase the paclitaxel content.

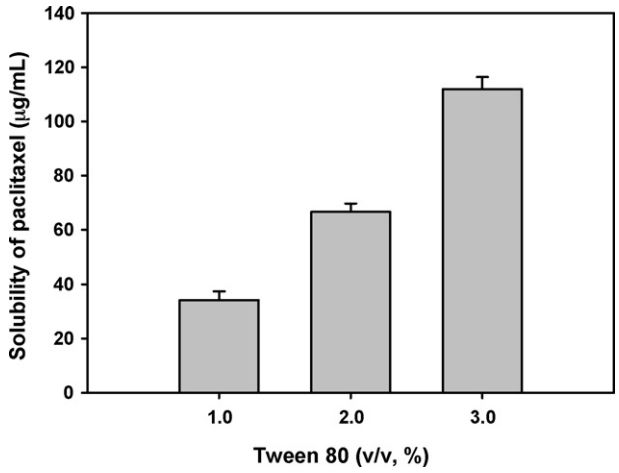

Fig. 1. The effect of Tween 80 concentration on the solubility of paclitaxel in the hydyration medium (phosphate buffer saline, pH 7.4).

Among the surfactants available for clinical use, Tween 80 had been previously reported to enhance the entrapment efficiency and the stability of the liposomes (Gu et al., 1982; Zou et al., 1989; Kronberg et al., 1990). As shown in Fig. 1, the solubility of paclitaxel increased as the content of Tween 80 increased in a concentration-dependent manner, and reached up to 120 μg/mL with 3% (v/v) Tween 80. However, it was also reported that the addition of 6% Tween 80 resulted in significant liposome destruction (Zou et al., 1996). In our preliminary study, the addition of 3% (v/v) Tween 80 in the hydration medium completely hydrated the dry lipid film within 2 h as well as stabilized the particle size of the liposome for at least 24 h. Thus, 3% (v/v) Tween 80 was selected as the hydration medium for the preparation of liposomes in this study.

Once un-entrapped paclitaxel was separated from the liposome suspension by ultracentrifugation, liposome particles were freeze-dried to enhance their physicochemical stabilities during the storage. One of the major challenges of freeze-drying the liposomes is the preservation of the structural integrity of the liposome during the dehydration/reconstitution process. Sugars have been reported to act as protective agents during the dehydration/reconstitution of liposomes by preventing vesicle fusion and helping retention of the encapsulated compounds within the liposomes (Crowe et al., 1985; Madden et al., 1985). Based on our previous results (Yang et al., submitted for publication), sucrose (molar ratio of sugar-to-lipid = 2.3) was added as a lyoprotectant before freeze-drying the liposome particles.

3.2. Characterization of paclitaxel liposomes

3.2.1. Morphology

The image from negative-staining TEM (Fig. 2) showed that both the conventional and PEGylated liposomes were of discrete and round structure ranging in size from 100 to 200 nm, which were consistent with the results obtained from the particle size measurement shown in Table 1.

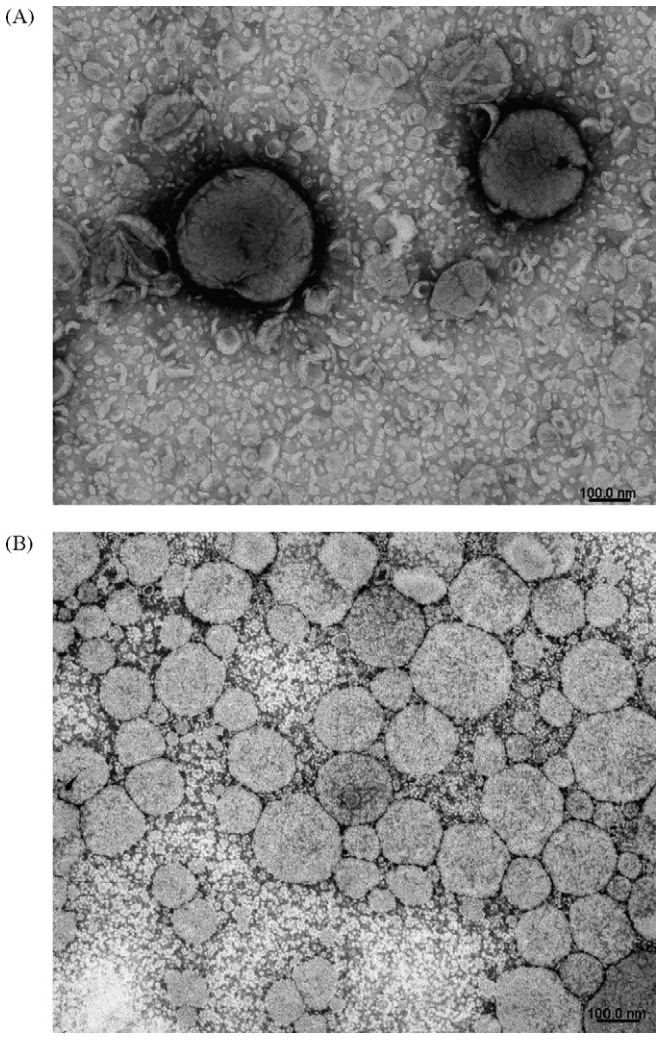

Fig. 2. Transmission electron micrograph of conventional liposome (A) and PEGylated liposome (B) of paclitaxel.

3.2.2. Paclitaxel content and entrapment efficiency

The different formulations of paclitaxel-loaded conventional and PEGylated liposomes are summarized in Table 2 together with their physicochemical characteristics. When 3% (v/v) of Tween 80 was added in the hydration medium of liposomes, the solubility of paclitaxel in both conventional and PEGylated liposomal dispersion increased up to 3.39 mg/mL, which is significantly higher than that of liposomal formulations without Tween 80. This value is higher than that reported by other researchers (Crosasso et al., 2000; Immordino et al., 2003; Zhang et al., 2005) and is high enough to be used in clinical studies (*i.e.*, 0.3–1.2 mg/mL). Moreover, the addition of 3% (v/v) Tween 80 also significantly increased the entrapment efficiency of paclitaxel in the liposomes ($p < 0.01$). Tween 80 seemed to have increased the content of paclitaxel in the inner water phase due to its solubilizing effect (Fig. 1) and decreased the leakage of paclitaxel from the lipid bilayer. The amount of paclitaxel in PEGylated liposomes (10.78 μg/mg) was lower than that in the conventional liposome (12.39 μg/mg) since $MPEG_{2000}$-DSPE increased the total weight of PEGylated liposomes.

Table 1
Effect of 3% (v/v) Tween 80 and freeze-drying on the change of particle size of liposomes[a]

	Time (h)	Conventional liposome		PEGylated liposome	
		Without Tween 80	With Tween 80	Without Tween 80	With Tween 80
Before freeze-dry	0	185.57 ± 5.64	182.37 ± 3.45	186.00 ± 7.08	188.70 ± 3.86
	12	275.27 ± 7.54	187.07 ± 3.91*	280.07 ± 9.73	190.13 ± 5.20*
	24	524.53 ± 47.19	190.03 ± 4.22*	688.10 ± 27.47	191.37 ± 4.25*
Reconstituted liposome	0	184.77 ± 5.35	177.40 ± 4.93	175.03 ± 6.08	172.17 ± 4.21
	12	252.50 ± 12.30	184.27 ± 8.30*	227.63 ± 9.02	178.33 ± 4.48*
	24	411.93 ± 18.69	182.77 ± 10.14*	312.20 ± 14.46	168.83 ± 8.57*

[a] All data are expressed the means ± standard deviation ($n = 3$).
* $p < 0.01$ compared to liposome without Tween 80.

3.2.3. Zeta potential and particle size

The zeta potential of the conventional liposome was almost neutral as expected since S_{100}PC and cholesterol do not bear a charge. With the addition of 3% (v/v) Tween 80 in the hydration medium, the mean zeta potential of conventional liposome dispersion was more negative (Table 2), which is consistent with previous reports (Lee et al., 2005; Yang et al., submitted for publication). The reason for the lower zeta potential could be due to the partial hydrolysis of Tween 80.

The zeta potential of PEGylated liposomes was more negative than that of conventional liposomes due to the negatively charged phosphate group of MPEG-DSPE, which is also in accordance with the result reported in literature (Hinrichs et al., 2006). In this case, the effect of Tween 80 on zeta-potential seems to be negligible since the negative charge due to the PEGylation is so much larger.

The particle size distribution of the liposomes prepared in this study showed a mono-modal distribution (data not shown) with the mean particle sizes at about 185 nm (Table 1). There was no significant change in liposome particle size and zeta potential before and after freeze-drying (at 0 h in Table 1), suggesting that the freeze-drying cycle used was optimum and the formulation contained sufficient amount of lyoprotectant to preserve the integrity of the liposomes.

A typical phenomenon of instability in the liposome formulation is the increase in particle size due to the aggregation or the fusion of unstable liposomes during the formulation processing and/or upon storage. An increase in particle size of liposomes generally results in rapid uptake by the reticuloendothelial system (RES) with subsequent rapid clearance and a short half-life. Thus, controlling and maintaining liposomes at small and uniform sizes are critical in developing a viable pharmaceutical product. It is interesting to note that the particle sizes of liposomes before and after freeze-drying were almost constant for up to 24 h when 3% (v/v) Tween 80 was added in the hydration medium, while an increase up to 600 nm without Tween 80 (Table 1) was observed. These results suggested that the incorporation of Tween 80 resulted in the increase of the liposome stability in the solution. Currently, the mechanism of Tween 80 on the liposome stability is unknown. It is speculated that the hydrocarbon tail of Tween 80 might be able to penetrate into the lipid bilayer, thus leaving the polyethylene oxide groups on the surface of the liposomes thereby introducing a steric barrier on the surface of the liposomes, which might decrease liposome fusion and consequently decrease lipid and paclitaxel exchange upon collision of the liposome particle (Gu et al., 1982).

3.3. In vitro release studies

In order to maintain sink condition during the release study, 0.1% (v/v) of Tween 80 was added in the release medium (PBS,

Table 2
The physicochemical properties of different paclitaxel liposome formulations[a]

Property	Conventional liposome		PEGylated liposome	
	Without Tween 80	With Tween 80	Without Tween 80	With Tween 80
Paclitaxel in liposomal dispersion[b] (mg/mL)	2.28 ± 0.07	3.39 ± 0.27*	2.15 ± 0.07	3.28 ± 0.07*
Entrapment efficiency[c] (%)	61.02 ± 1.61	70.83 ± 2.75*	57.44 ± 2.60	70.88 ± 2.81*
Paclitaxel content in liposome powder[d] (μg/mg)	8.57 ± 0.37	12.39 ± 0.07*	5.74 ± 0.52	10.78 ± 0.18*
Zeta-potential (mV)				
Before freeze-dry	−1.23 ± 0.64	−7.93 ± 0.78*	−16.25 ± 2.15	−20.31 ± 4.60
After freeze-dry	−0.42 ± 2.72	−9.31 ± 1.24*	−20.82 ± 1.26	−18.02 ± 3.34

[a] All data are expressed the means ± standard deviation ($n = 3$).
[b] Paclitaxel concentration after extrusion was determined immediately by HPLC analysis through liquid extraction and the value included the amount of free paclitaxel in the liposomal dispersion.
[c] $\mathrm{EE}(\%) = \frac{\text{amount of paclitaxel in liposome pellet (μg)}}{\text{amount of paclitaxel in liposomal dispersion (μg)}} \times 100$.
[d] $\text{Content} = \frac{\text{amount of paclitaxelin freeze-dried liposome (μg)}}{\text{amount of freeze-dried liposome (mg)}}$.
* $p < 0.01$ compared to liposome without Tween 80.

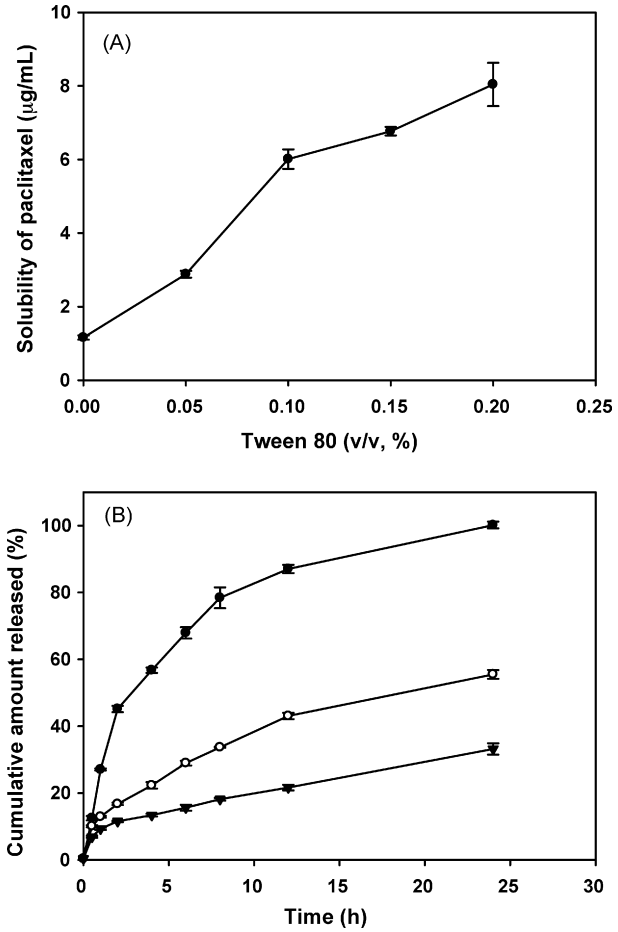

Fig. 3. (A) Effect of Tween 80 on the solubility of paclitaxel in the release medium (phosphate buffer saline, pH 7.4). (B) Release of paclitaxel from Taxol® (●), conventional liposome (◯) and PEGylated liposome (▼) into the release medium containing 0.1% (v/v) of Tween 80 at room temperature. Each data represents the mean ± standard deviation ($n = 3$).

pH 7.4). Although the solubility of paclitaxel increased up to 8.75 μg/mL with the addition of 0.2% (v/v) Tween 80 in the medium, 0.1% (v/v) Tween 80 already achieved high solubility (6.32 μg/mL) of paclitaxel enough to maintain the sink condition (Fig. 3A). In the *in vitro* release study, paclitaxel in a mixture of Cremophor® EL and ethanol (50:50, v/v) (*i.e.*, Taxol® formulation) released rapidly and was almost completed within 24 h. However, the conventional and PEGylated liposomes released 55% and 33% of paclitaxel within 24 h of dialysis at room temperature, respectively (Fig. 3B). The release of paclitaxel showed an initial burst release phase, releasing approximately 15% of paclitaxel during the first 2 h, and the release rate was reduced thereafter, indicating that the release of paclitaxel reached a slow release status.

This result suggests that it takes time for paclitaxel to be released once encapsulated in the liposomes because lipid bilayers are stabilized by cholesterol and/or Tween 80. Thus a depot effect could be achieved using liposomes, especially in the PEGylated liposomal formulation. The above results, which suggest that the drug would be stable in the blood circulation and would be released slowly at the tumor site, are indications that our PEGylated liposomal formulation meets the requirement for an effective drug delivery system (Song et al., 2006).

Table 3
Cytotoxicity of paclitaxel in Cremophor® EL-based vehicle (Taxol®), conventional and PEGylated liposome against breast cancer cells[a]

Cell line	Formulation	IC_{50} (nM)	
		24 h	72 h
MDA-MB-231	Taxol®	133.57 ± 10.12	72.16 ± 9.54
	Conventional liposome	168.33 ± 6.34*	65.92 ± 8.03
	PEGylated liposome	249.71 ± 26.38*,†	75.04 ± 7.31
SK-BR-3	Taxol®	173.64 ± 22.56	74.32 ± 6.68
	Conventional liposome	264.49 ± 20.62*	80.17 ± 5.78
	PEGylated liposome	472.17 ± 44.57*,†	94.85 ± 12.76

[a] All data are expressed the means ± standard deviation ($n = 4$).
* $p < 0.01$ compared with Taxol®.
† $p < 0.01$ compared with plain liposome.

3.4. In vitro cytotoxicity study

The cytotoxicity of paclitaxel in conventional and PEGylated liposomes against two breast cancer cells, MDA-MB-231 and SK-BR-3, was compared with that of Taxol® by MTT assay. Table 3 summarizes the IC_{50} values of Taxol® and paclitaxel-liposomes at two different incubation times. After 24 h incubation, significantly higher IC_{50} values compared to that of Taxol® were observed for both liposomal formulations. The PEGylated liposomal formulation was even less toxic than the conventional liposomes after 24 h incubation, which is probably related to the steric effect of the $MPEG_{2000}$-DSPE in the bilayers forming a barrier on the surface of the liposomes. Therefore, steric hindrance of PEGylated liposomes may increase the stability of liposomes and may reduce the release of paclitaxel when the liposomes come into contact with the medium and cells (Crosasso et al., 2000). However, it is interesting to note that the paclitaxel-loaded liposomal formulations, whether PEGylated or not, were almost equipotent with Taxol® after 72 h incubation. This is probably due to the slower release of paclitaxel from the liposomes, and is consistent with the results of the release study (Fig. 3).

3.5. Pharmacokinetics study

To assess the pharmacokinetic behavior of paclitaxel loaded in the conventional and PEGylated liposomes, each formulation at a dose of 7.5 mg/kg as paclitaxel was intravenously administrated in Sprague–Dawley rats. The plasma concentration profiles of paclitaxel after intravenous injection of Taxol®, the conventional liposomes and the PEGylated liposomes are shown in Fig. 4. Table 4 shows the pharmacokinetic parameters of paclitaxel, obtained by fitting the data to a two-compartment model. Free paclitaxel in Cremophor® EL-based formulation (*i.e.*, Taxol®) was quickly removed from the circulating sys-

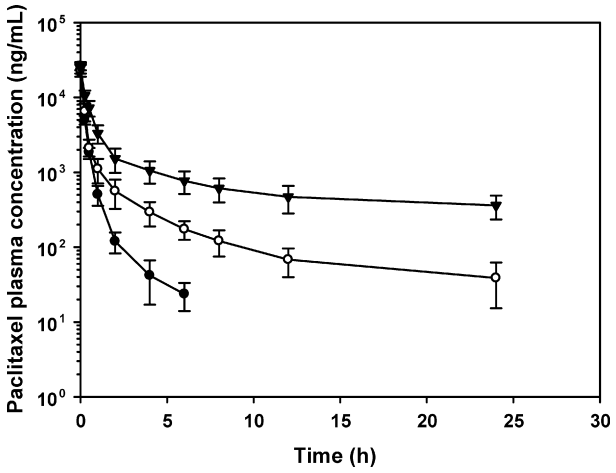

Fig. 4. Plasma concentration profiles of paclitaxel after intravenous administration (7.5 mg/kg as paclitaxel) in rats. Taxol® (●), paclitaxel in conventional liposome (○) and PEGylated liposome (▼). Each data represents the mean ± standard deviation ($n = 3$–5).

tem after intravenous injection, showing a biphasic pattern with a rapid distribution phase ($t_{1/2\alpha} = 9.6$ min) and a rapid terminal elimination phase ($t_{1/2\beta} = 1.65$ h), and was below the HPLC detection limit after 6 h. However, liposomal formulations significantly changed the paclitaxel pharmacokinetic parameters in comparison with Taxol®, as expected. The values of $t_{1/2\beta}$, MRT and AUC were found to be much higher for paclitaxel loaded in liposomes than those of Taxol®. On the other hand, total body clearance of paclitaxel incorporated in conventional and PEGylated liposomes decreased 1.6- and 7.1-fold, respectively, compared to that of free paclitaxel in the Taxol® formulation. Furthermore, the AUC, MRT and $t_{1/2\beta}$ of paclitaxel incorporated in PEGylated liposomes significantly increased compared with those of the conventional liposomes ($p < 0.05$) (4.4-, 6.0-, and 3.5-fold increase, respectively). The longer $t_{1/2\beta}$ in the PEGylated liposomes than in the conventional liposomes appeared to be related to the reduced uptake of liposomal drug by the elements of the mononuclear phagocytic system (MPS) and hence to the reduced clearance (Crosasso et al., 2000).

3.6. Tissue distribution study

In vivo tissue uptake of paclitaxel was evaluated after intravenous injection of each formulation (*i.e.*, Taxol®, conventional and PEGylated liposome) in breast carcinoma xenografted mouse model. Breast carcinoma was successfully xenografted on nude mice after 2–3 weeks of inoculating MDA-MB-231 cells. Fig. 5 shows the distribution of paclitaxel into various organs after intravenous administration of Taxol® or liposome formulations (7.5 mg/kg as paclitaxel) *via* tail vein.

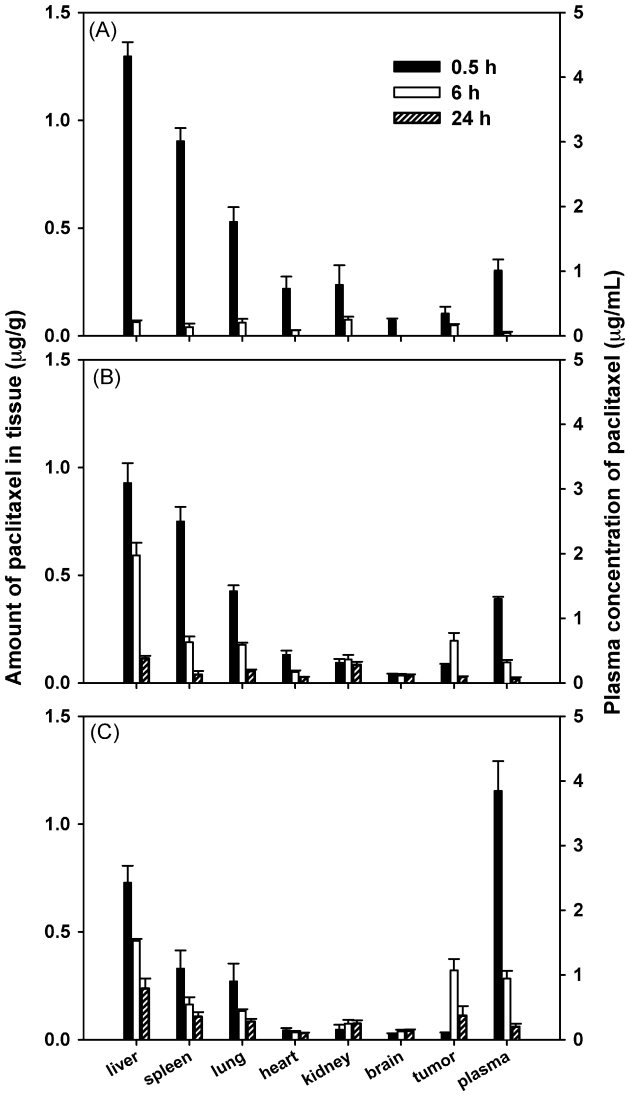

Fig. 5. Biodistribution of paclitaxel after 0.5 h, 6 h, and 24 h of intravenous injection of (A) Taxol®, (B) conventional liposome, or (C) PEGylated liposome (7.5 mg/kg as paclitaxel) in nude mice bearing MDA-MB-231 human breast cancer xenografts. Each data represents the mean ± standard deviation ($n = 3$–5).

In case of Taxol®, plasma concentration of paclitaxel was almost negligible at 6 h, and it was rapidly uptaken and cleared by the liver, spleen and lung (Fig. 5). However, when paclitaxel was encapsulated in liposomes, the plasma concentration

Table 4
Pharmacokinetic parameters of paclitaxel after intravenous administration of Taxol®, plain and PEGylated liposome in rats (7.5 mg/kg as paclitaxel)[a]

	AUC (μg/mL h)	MRT (h)	$t_{1/2\alpha}$ (h)	$t_{1/2\beta}$ (h)	CL_t (mL h^{-1})
Taxol®	4.47 ± 0.59	0.52 ± 0.11	0.16 ± 0.03	1.65 ± 0.29	1.70 ± 0.21
Conventional liposome	7.17 ± 0.17*	3.15 ± 1.00*	0.17 ± 0.05	5.05 ± 1.52*	1.05 ± 0.02*
PEGylated liposome	31.86 ± 4.37*,†	18.75 ± 2.44*,†	0.34 ± 0.04	17.80 ± 2.35*†	0.24 ± 0.03*,†

[a] All data are expressed the means ± standard deviation ($n = 3$–5).
* $p < 0.05$ compared with Taxol®.
† $p < 0.05$ compared with plain liposome.

was maintained for up to 24 h. Moreover, PEGylated liposomes showed higher plasma level than that of conventional liposomes, which is consistent with the results from the pharmacokinetic study in rats (Fig. 4).

In tumor tissue, paclitaxel concentration in PEGylated liposomes was significantly higher than that in conventional liposomes and in Taxol® at 6 and 24 h. Also, in the case of PEGylated liposomes, the paclitaxel concentration in tumor was higher than that in spleen, lung, heart, kidney and brain tissues from 6 h. These results suggested that PEGylated liposomes were distinctly localized in the tumor tissues. It seemed that long-circulating time and slow release of PEGylated liposomes might offer enough chance for paclitaxel to be attained at the tumor site through the EPR effect and maintain the effective therapeutic concentration for a long period of time through the depot effect. Therefore, these results indicate that our PEGylated liposomal formulation effectively increased the antitumor efficiency while lessening the potential side-effects.

3.7. Inhibition of tumor growth

Since the paclitaxel loaded conventional liposomes and PEGylated liposomes were highly accumulated in the tumor tissues of MDA-MB-231 human breast cancer xenograft model (Fig. 5), the tumor growth inhibition effect was further evaluated. The study on the control (saline) group ended on the 35th day because the tumor volume was excessively enlarged (about 2000 mm^3), while other groups lasted until the 60th day. As shown in Fig. 6, the PEGylated liposomes suppressed tumor growth most efficiently, followed by the conventional liposomes and Taxol® ($p < 0.05$). This enhanced anti-tumor activity of the PEGylated liposomes can be explained by the increased local concentration of pacltiaxel near the tumor *via* EPR effect, which is supported by our present data.

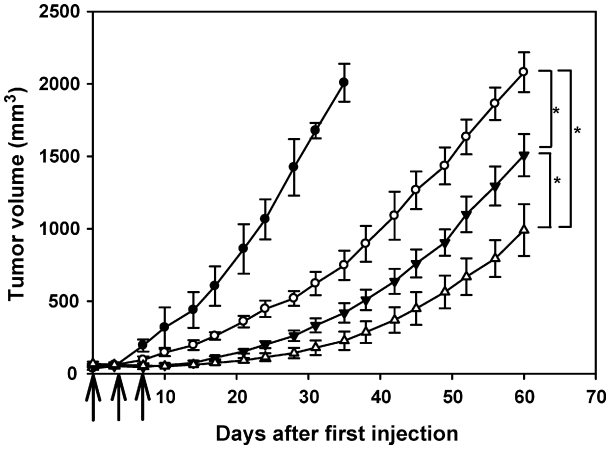

Fig. 6. The effect of paclitaxel on the inhibition of tumor growth in nude mice inoculated with MDA-MB-231 human breast cancer cells. When tumor volume became about 50 mm^3, Taxol® (◯), conventional liposome (▼) or PEGylated liposome (△) was intravenously injected (7.5 mg/kg as paclitaxel each time) on days 0, 4, and 8 (arrows). Normal saline (●) was injected as a control. The zero point of *X*-axis indicates the first day of paclitaxel injection. Each data represents the mean ± standard deviation ($n = 4$–6). Tumor volume of each group was statistically different after 60 days ($^{*}p < 0.05$).

4. Conclusions

The most significant finding of this study is that PEGylated liposomes with high paclitaxel content and good stability were successfully developed by incorporating 3% (v/v) Tween 80 and by freeze-drying with sucrose as a lyoprotectant. The solubility of paclitaxel increased from 1.6 μg/mL in aqueous solution to 3.39 mg/mL in the conventional type liposomal dispersion, which increased the possibility of its clinical use. The PEGylated formulation also showed similar solubility. Moreover, the freeze-drying procedure significantly increased the stability of liposomes allowing long-term storage. The PEGylated liposomes significantly increased the biological half-life of paclitaxel after intravenous injection in rat, and showed high accumulation of the drug in tumor tissue, thereby more effectively inhibiting the tumor growth in mice. Therefore, this PEGylated liposome formulation of paclitaxel could serve as a better alternative for treating human breast cancer.

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