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Research paper

Development and characterization of a novel Cremophor® EL free liposome-based paclitaxel (LEP-ETU) formulation

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Abstract

Taxol® is a marketed product for the treatment of ovarian, breast, non-small cell lung cancer and AIDS-related Kaposi's Sarcoma. It is thus far one of the most effective anticancer drugs available on the market. However, paclitaxel is only sparingly soluble in water and therefore, intravenous administration depends on the use of the non-ionic surfactant Cremophor® EL (polyethoxylated castor oil) to achieve a clinically relevant concentrated solution. Unfortunately, Cremophor® EL increases toxicity and leads to hypersensitivity reactions in certain individuals. We have developed a well characterized novel lyophilized liposome-based paclitaxel (LEP-ETU) formulation that is sterile, stable and easy-to-use. The mean particle size of the liposomes is about 150 nm before and after lyophilization, and the drug entrapment efficiency is greater than 90%. Stability data indicated that the lyophilized LEP-ETU was physically and chemically stable for at least 12 months at 2–8 and 25 °C. Moreover, the formulation can be diluted to about 0.25 mg/ml without drug precipitation or change in particle size. In vitro drug release study in phosphate-buffered saline (PBS, pH 7.4) showed that less than 6% of the entrapped paclitaxel was released after 120 h, indicating that the drug is highly stable in an entrapped form at physiologic temperature.

Keywords: Liposome-based formulation; Paclitaxel; Liposomes; Stability; Lyophilization; Taxol®; Entrapment

1. Introduction

Paclitaxel (Fig. 1) is a natural product that was first isolated from the bark of the Pacific Yew tree, Taxus brevifolia, by Wani et al. [1]. It is a novel antineoplastic agent that exhibits a unique mechanism of action against advanced ovarian, breast and non-small cell lung cancers [2,3]. The tumoricidal activity of paclitaxel is attributed to its ability to induce irreversible aggregation of microtubules [1,4]. The paclitaxel formulation is marketed by Bristol-Myer Squibb Pharmaceuticals under the trade name of Taxol® for the treatment of ovarian, breast, non-small cell lung cancers and AIDS-related Kaposi's Sarcoma. It is thus far one of the most effective anticancer drugs available on the market. However, because paclitaxel is extremely insoluble in water as well as in other vehicles commonly used in parenteral dosage forms, the current Taxol® formulation consists of paclitaxel solubilized in 50:50 (v/v) Cremophor® EL (polyethoxylated castor oil) and dehydrated alcohol, USP. Taxol® must be diluted to a concentration of 0.3–1.2 mg/ml before use [2]. Despite the dilution, the amount of Cremophor® EL necessary to deliver the required doses of paclitaxel is significantly higher than that administered with any other marketed pharmaceutical injectable drugs and causes serious or fatal hypersensitivity episodes in humans [5]. In order to reduce the intensity and incidence of reactions associated with Cremophor® EL administration, pre-medication with corticosteroids (dexamethasone) and antihistamines (both H_1 and H_2 receptor antagonists) is used clinically [2]. However, the cumulative side effect of dexamethasone may add to Taxol® treatment-related morbidity and in some cases, result in early

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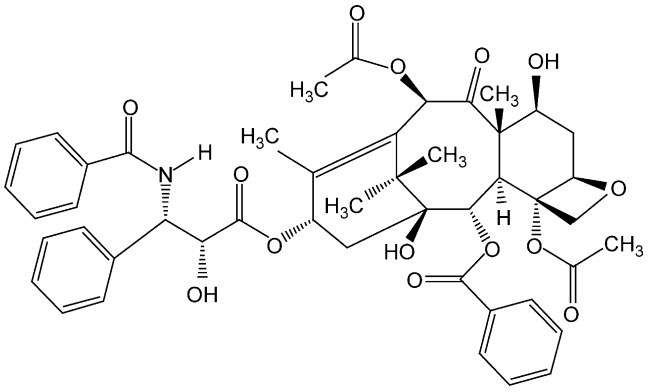

Fig. 1. Chemical structure of paclitaxel.

discontinuation of therapy. It has been shown that premedication regimen can reduce the incidence of serious hypersensitivity reactions to less than 5%; however, milder reactions can still occur in approximately 30% of patients [5]. Hypersensitivity reactions usually occur more frequently with shorter infusion schedules, hence, an infusion time of 3–24 h is recommended. Patients are generally required to be admitted to the hospital overnight because of long infusion time. This causes additional inconvenience to the patients. In addition, Cremophor® EL alters the biodistribution of paclitaxel as a result of entrapment of the drug in circulating Cremophor® EL micelles, thereby reducing the free drug fraction available for cellular partitioning, and consequently, contributes to the non-linear pharmacokinetics of paclitaxel [6–9]. Furthermore, Taxol® is associated with several stability and compatibility issues, such as possibility of drug precipitation upon dilution, leaching of plasticizer from the PVC infusion bag and infusion set, non-specific adsorption to plastic and glass surfaces [10,11]. The aforementioned stability and compatibility issues present a number of practical problems with respect to the special requirement of a filter device and use of non-plasticized containers and infusion sets during drug storage and infusion.

Given all the drawbacks of the commercial product, it is thus apparent that there is a need for a better paclitaxel formulation that is less toxic and may be more efficacious than Taxol®. The primary goal of this work was to develop a paclitaxel formulation using a better-tolerated drug delivery system in lieu of Cremophor® EL to deliver paclitaxel. Various approaches or drug delivery systems of formulating paclitaxel, including emulsions, micelles, microspheres, nanoparticles, cyclodextrins, implants and liposomes, have been reported [12]. Among the drug delivery systems, liposomes represent a mature, versatile technology with considerable potential for entrapment of both lipophilic and hydrophilic drugs [13–15]. There are several commercially available liposomal products for treatment of a number of neoplastic and infectious diseases [16]. Encapsulation and/or entrapment of drugs in liposomes result in distinct changes in the pharmacokinetic and pharmacodynamic properties of the free drugs, and in some cases cause an apparent decrease in toxicity and/or an increase in therapeutic efficacy [16–18].

The objectives of the present study were (1) to develop a pharmaceutically acceptable and better-tolerated liposomal formulation with potential to obviate the need for premedication, shorten infusion time and reduce toxicity; (2) to characterize the formulation fully, and (3) to evaluate the short-term and long-term stability of the formulation at different storage conditions.

2. Materials and methods

Paclitaxel was purchased from Hunde Tech. (Hunde, Tech Development Co., Houston, TX, USA). Purified 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), cardiolipin and cholesterol were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Alpha-tocopheryl acid succinate (TAS) was obtained from Sigma (St Louis, MO, USA). Ethyl alcohol, USP was purchased from Aaper (Shelbyville, KY, USA). Sodium chloride, USP and potassium hydrogen phosphate, USP were obtained from EM Science (Gibbstown, NJ, USA). Sucrose, NF was obtained from Mallickrodt (Baker Inc., Phillipsburg, PA, USA). Pierce Slide-A-Lyzer® dialysis cassettes with a molecular weight cutoff of 10 K were obtained from Pierce Biotechnology (Rockford, IL, USA). Nitrogen gas, NF was procured from BOC Gases (Carol Stream, IL, USA). All chemicals were high purity grade and used as received.

2.1. Solubility studies

Briefly, excess amounts of paclitaxel were added to the appropriate volume of various solvents or purified water in screw capped scintillation vials. The pH of the suspension was then adjusted with 2 M HCl or 0.1 M NaOH, if necessary. The suspension was continuously mixed at ambient temperature until saturation was attained. An aliquot of the sample (5 ml) was withdrawn at 24 and 48 h intervals. Each withdrawn sample was filtered using a 0.45 μm PTFE filter. The first 2 ml of the filtrate was collected for pH measurement. Additional 2 ml of the remaining filtrate was appropriately diluted and subjected to HPLC analysis (see Section 2.4).

2.2. Liposome preparation

LEP-ETU formulations were prepared by the modified thin-film hydration method. Briefly, the hydrophobic excipients, such as lipids (phosphatidylcholine, cholesterol and cardiolipin), paclitaxel and TAS, were dissolved in ethanol and were transferred into a suitable round bottom flask. The flask was then connected to a Büchi R205 rotary

evaporator (Flawil, Switzerland) and water bath (Buchi B-490) with temperature maintained at 35–40 °C. Vacuum was applied to the flask to evaporate the ethanol and form a homogeneous lipid film on the flask wall. The dry lipid film was maintained overnight under vacuum to remove traces of ethanol. The lipid film was then hydrated with a solution containing sucrose and normal saline by rotating the flask at about 200 rpm at ~40 °C until the lipid film was completely hydrated and a homogeneous dispersion was formed (approximately 1 h). The liposome dispersion was then extruded under a nitrogen atmosphere through two stacked of 0.2 and 0.1 μm polycarbonate filters (Whatman, Inc., Clifton, NJ, USA) using a high-pressure extruder (Northern Lipids, Inc., Canada) at room temperature. The mean vesicle size of liposomes was reduced to about 150 nm. The resulting liposome-based drug formulation was then sterile filtered through 0.22 μm filter (PVDF membrane filter, Millipak-20, Millipore Corp, Billerica, MA, USA) and filled into 50-ml clear vials (Schott Schweiz AG, Switzerland). The filtered formulation in vials was lyophilized using VirTis lyophilizer (Advantage, The VirTis Company, Gardiner, NY, USA). The lyophilization cycle consisted of cooling the solution down to −45 °C for 2 h, primary drying for 48 h at −30 °C, ramp from −30 °C to −5 °C for 24 h, and second drying at 25 °C for 8 h. Several formulations with different lipid compositions were prepared to develop an optimum liposomal product.

2.3. Liposome characterization

The extruded pre-lyophilized liposomes and post-lyophilized liposomes were characterized by the following methods.

2.3.1. Vesicle size measurement

Immediately after preparation, LEP-ETU formulations were examined for possible aggregation by visual inspection. Thereafter, the liposome mean diameter and particle size distribution for pre-lyophilized and reconstituted LEP-ETU samples were determined using dynamic light scattering (DLS) technique with a Nicomp 380 Submicron Particle Sizer (Particle Sizing Systems, Santa Barbara, CA, USA) equipped with auto-dilution function. The laser in this equipment was operated at 632.8 nm using a 90° angle between incident and scattered beams. Polystyrene bead standards were used to verify the performance of the instrument prior to sample measurement. Data were analyzed automatically either by Gaussian Analysis (the least-squares quadratic: cumulants) or Nicomp Distribution Analysis (inversion of the Laplace transform) from the Nicomp CW388 software. Data were analyzed in terms of intensity, volume and number distributions assuming that the particles are spheres of uniform density which scatter light according to classical Mie Theory. Data were reported as volume weighted distribution and represented as the mean of at least two measurements.

2.3.2. Freeze-fracture electron microscopy (EM)

Liposome morphologic analysis was carried out by freeze-fracture electron microscopy. Samples were quenched using sandwich technique and liquid nitrogen-cooled propane [19]. During the experiment, a cooling rate of 10,000 K/s was reached to avoid ice crystal formation and artifacts possibly caused by the cryo-fixation process. The fracturing process was carried out in JEOL JED-9000 freeze-etching equipment (JEOL, Ltd, Tokyo, Japan), and the exposed fracture planes were shadowed with platinum for 30 s at an angle of 25–35° and with carbon for 35 s (2 kV/60–70 mA, 1×10^{-5} Torr). The platinum replicas produced this way were cleaned with concentrated, fuming HNO_3 for 24–36 h followed by a repeated agitation with fresh chloroform/methanol (1:1, v/v) for at least five times. Subsequently, these cleaned replicas were examined with a JEOL 100 CX (JEOL, Ltd, Tokyo, Japan) electron microscope.

2.3.3. Negative-stain transmission electron microscopy (TEM)

The morphology of the liposomes in LEP-ETU was studied by a negative staining method following a standard procedure [20]. Briefly, the samples were diluted with normal saline 1:10 and applied to 300 mesh, Formvar-carbon-coated Cu grids. Liposomes were negatively stained with 2% uranyl acetate, pH 4.8, for 30–60 s. Stained samples were characterized using Philips CM 12 TEM (Philips, Ltd., Eindhoven, The Netherlands) at final magnification (TEM and photo-magnification) of 93,960×. The magnification of the microscope was calibrated with standard latex spheres. Three grids were prepared for each sample and the grid openings were randomly selected and viewed.

2.4. Quantification of paclitaxel and lipids

HPLC methods were used for the analysis of paclitaxel and lipid contents of LEP-ETU. Drug content analysis was performed using a Waters μBondapak C18 column (3.9× 300 mm, 10 μm, Waters Corp., Milford, MA, USA) HPLC column at 25 °C with a mobile phase containing a mixture of acetonitrile and water (55/45, v/v) at a flow rate of 1 ml/min. Sample injection volumes were 20 μl and paclitaxel detection was performed using UV detector at a wavelength of 230 nm. DOPC and cardiolipin were analyzed using an ASTEC DIOL HPLC column (Astec Inc., Whippany, NJ, USA) and an ELSD detector (Polymer Laboratories, Amherst, MA, USA) at 40 °C with a chloroform: methanol: ammonium acetate buffer (71:16:3, v/v) mobile phase at a flow rate of 1 ml/min. Sample injection volumes were 50 μl with evaporation and nebulization temperatures of 110 and 80 °C, respectively. Cholesterol was analyzed using Hypersil BDS C18 (250 mm×4 mm, 5 μm, Alltech Associates, Inc., Deerfield, IL, USA) HPLC column with a mobile phase of acetonitrile: isopropanol (75:25, v/v) at 1.5 ml/min flow

rate and 40 °C column temperature. Cholesterol detection was done using UV detector at 205 nm wavelength.

2.5. Entrapment efficiency of paclitaxel

Entrapment efficiency of paclitaxel in liposomes was determined by a modified minicolumn centrifugation method [21] using commercially available Sephadex G-25 columns (Macrospin Column, Harvard Biosciences, Holliston, MA, USA). A typical procedure is shown in Fig. 2. Briefly, Sephadex G-25 gel in column was allowed to swell in 500 μl MilliQ water for 15 min, and then the column was centrifuged for 4 min at 350×*g* using a table-top micro-centrifuge (Sorvall Biofuge fresco) to remove water. The dry column was loaded with 100 μl placebo liposomes to saturate the column and minimize adsorption of actual sample (LEP-ETU) during preparation. The loaded column was then centrifuged for 15 min at 1520×*g* to expel the liposomes. Subsequently, the LEP-ETU sample was introduced into the column and centrifuged at 1520×*g* for 15 min to separate free paclitaxel from the liposome-entrapped drug. The eluted sample, which contained entrapped liposomal paclitaxel, was analyzed for paclitaxel concentration using HPLC method described previously. The minicolumn method was optimized to achieve almost 100% liposome recovery during sample preparation. The entrapment efficiency was determined by comparing the paclitaxel concentration of the eluted sample with that of LEP-ETU sample prior to column chromatography.

The following equation was used to calculate the entrapment efficiency:

$$\%\ \text{Drug Entrapment} = \frac{\text{Drug}_{\text{after column}}}{\text{Drug}_{\text{before column}}} \times 100\% \quad (1)$$

2.6. In vitro drug release study

In vitro drug release study was conducted to examine physical stability of liposomal paclitaxel in LEP-ETU upon dialysis against 400 ml of PBS. Briefly, 2 ml of reconstituted liposomal paclitaxel sample was placed into the dialysis cassette (Pierce Slide-A-Lyzer® dialysis cassettes with a MW cutoff of 10 K) and then suspended in a temperature-controlled jacketed flask containing 400 ml of phosphate-buffered saline (PBS, pH 7.4) at 37 °C. At various time intervals, aliquot samples were withdrawn from the flask and subjected to visual inspection and drug content analysis by a stability-indicating HPLC method.

2.7. Short-term and long-term stability studies of LEP-ETU

The short-term stability was conducted to monitor physical and chemical stabilities of the liquid form of LEP-ETU samples (non-diluted and diluted) using post-lyophilized and reconstituted LEP-ETU formulations at 2–8 °C and room temperature for up to 72 h. The long-term stability study was conducted to evaluate the lyophilized LEP-ETU after storage at 5 °C (±3 °C) and 25 °C (±2 °C)/60% relative humidity (RH ±5%) for an extended period of time. The stability parameters, such as, pH, lipid content, drug concentration, particle size distribution and drug entrapment efficiency were determined as a function of the storage time.

2.8. Residual moisture content determination

The residual moisture content of lyophilized LEP-ETU was determined using Karl Fischer volumetric titrator (DL-38, Mettler Toledo, Columbus, OH, USA) connected with an analytical balance. Approximately 200 mg of

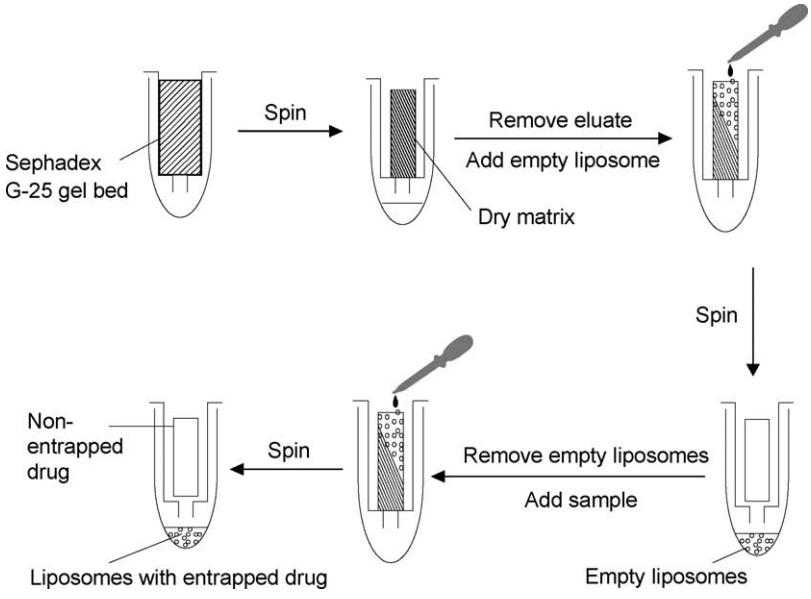

Fig. 2. Determination of drug entrapment efficiency by minicolumn centrifugation method.

the lyophilized powder was taken from each vial and placed directly in the reaction chamber. The exact weight of the sample taken from the vial was recorded for final water content calculation. The percent of moisture content of the lyophilized LEP-ETU was calculated based on the amount of water determined in the pre-weighed sample.

3. Results and discussion

3.1. Pre-formulation

Paclitaxel is poorly soluble in aqueous media. As indicated in the Table 1, the solubility of paclitaxel in aqueous medium at different pH conditions was about 1.0 μg/ml, which is within the range of the reported paclitaxel aqueous solubility of 0.3–30 μg/ml [22,23]. It was not surprising that the aqueous solubility was pH-independent since paclitaxel does not contain groups that may be ionized in an acceptable pH range. Dordunoo and Burt [23], however, reported that the maximum stability of paclitaxel solution was in the pH range of 3–5, thus the new formulations were formulated and maintained at this pH range.

3.2. Formulation development

Formulation development was conducted to identify a liposome-based formulation that possesses the highest drug entrapment efficiency per mole of lipid in the liposomes, exhibits small and uniform particle size and increased physical and chemical stability upon storage as well as upon reconstitution and dilution.

A typical manifestation of liposome formulation instability is an increase in particle size due to aggregation or fusion of unstable liposomes during formulation processing and/or upon storage. As the particle size increases, drug efficacy is reduced due to the decrease in the effective surface area of the liposomes as well as altered disposition of the liposomes in vivo, which results in differences in pharmacokinetic parameters [24]. The general trend for liposomes of similar lipid composition is that an increase in vesicle size translates into more rapid uptake by the reticuloendothelial system (RES) [25–27] with subsequent faster clearance and a shorter half-life ($t_{1/2}$). Taken together, controlling and maintaining liposomes at small and uniform size is critical in developing a viable pharmaceutical product.

Table 1
Solubility of paclitaxel in various solvents at room temperature

Solvent	Solubility (μg/ml)
MilliQ water	1.0
10% Sucrose/0.9% saline, pH 1.5	<1.0
10% Sucrose/0.9% saline, pH 6.0	<1.0
Phosphate saline buffer, pH 7.4	0.95

Several prototype formulations were investigated in terms of different types of lipids, lipid-to-drug ratio, drug concentration, drug entrapment, filterability and short-term physical and chemical stabilities. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), a neutral zwiterionic phospholipid, was first chosen as one of the lipid components in the formulation because it has a low gel to lipid-crystalline phase transition temperature ($T_m \sim -22$ °C), and forms liposomes that are flexible enough to entrap relatively more hydrophobic molecules. Kirby et al. [28] and Senior and Gregoriadis [25] indicated that regardless of the liposome surface charge, cholesterol-rich liposomes were more stable than cholesterol-poor or cholesterol free liposomes both in vitro and in vivo. To increase the liposome stability both in vivo and in vitro, cholesterol was included in the formulations. Liposomes containing cardiolipin, on the other hand, reportedly reduced cardiotoxicity associated with doxorubicin by altering the pharmacokinetics and tissue distribution of the drug [29], and modulated multidrug resistance [30] via the formation of drug–cardiolipin complex through electrostatic interaction. Cardiolipin may also form complexes with lipophilic drugs through hydrophobic interaction to exert similar results. Therefore, cardiolipin was selected for the formulation. Moreover, the addition of negatively charged lipid will increase physical stability by preventing aggregation and fusion of liposomes due to electrostatic repulsion. TAS was added in the formulation as an antioxidant to prevent potential oxidation of the unsaturated lipid (DOPC) and increase product stability. The optimization by varying lipid composition and drug-to-lipid ratios in the formulation was carried out in order to achieve the highest drug loading and maximum physical and chemical stabilities upon storage.

Figs. 3 and 4 show the percent of drug loading as a function of amount of cholesterol and cardiolipin in liposomes. As indicated in these figures, the percent of drug loading decreased as the molar percent of cholesterol and cardiolipin in the formulation increased, provided that the lipid-to-drug molar ratio remained constant. This might be due to the fact that the paclitaxel molecules compete with

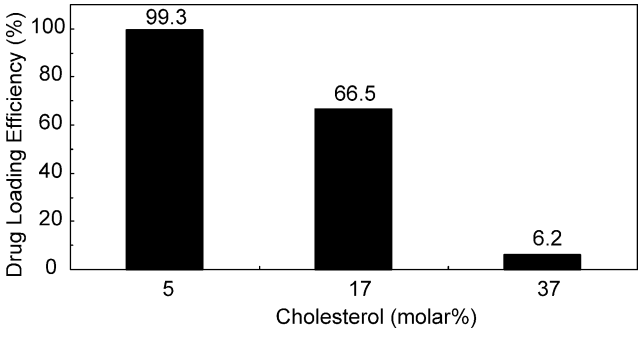

Fig. 3. Drug loading efficiency (%) of paclitaxel as a function of amount of cholesterol in liposomes. DOPC/cholesterol/cardiolipin molar ratio: 90:5:5; 77:17:7 and 58:37:5. All formulations were formulated at about 33:1 lipid-to-drug molar ratio.

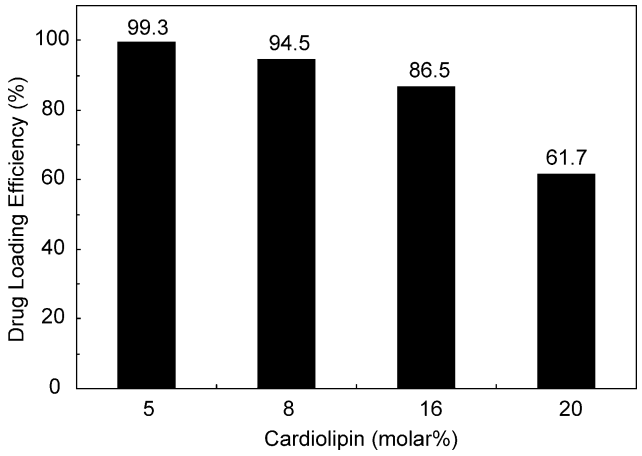

Fig. 4. Drug loading efficiency (%) of paclitaxel as a function of amount of cardiolipin in liposomes. DOPC/cholesterol/cardiolipin molar ratio: 90:5:5; 92:0:8; 84:0:16 and 65:15:20. All formulations were formulated at about 33:1 lipid-to-drug molar ratio.

cardiolipin and cholesterol molecules for the hydrophobic space in the lipid bilayer. The more molecules of cholesterol and cardiolipin each liposome of the same vesicle size has, the less space they leave in the lipid bilayer for paclitaxel molecules to occupy. Moreover, the inclusion of cholesterol in liposomes also restricted the flexibility of the hydrocarbon chains of the lipids, and hence hindered paclitaxel penetration into lipid bilayer.

The maximum mole percent of drug that could be loaded into the liposome bilayer was about 3.5%. Above 3.5 mole%, the formulation was not stable as the drug precipitated during liposome sizing or upon storage. Study revealed that the liposome formulation would be stable so long as the lipid-to-drug molar ratio was maintained at greater than 30 to 1. Other lipids, such as saturated phospholipids, DMPC and DSPC, were also investigated. However, due to the high rigidity of their lipid bilayers, paclitaxel could not be effectively loaded (<1 mole%) as compared to the unsaturated phospholipids bilayer. Furthermore, the paclitaxel formulations containing saturated lipids tended to precipitate more quickly than similar formulations containing unsaturated lipids (less than 24 h upon storage at room temperature).

Upon further optimization, a lead formulation which consisted of DOPC, cholesterol and cardiolipin in molar ratio of 90:5:5, and paclitaxel at lipids-to-drug molar ratio of 33:1 with the paclitaxel concentration of 2 mg/ml was developed. The mean vesicle size of the liposomes was reduced and controlled at about 150 nm by extrusion. The sterility was achieved by filtration of the formulation through a 0.22 μm membrane filter under aseptic condition. No appreciable loss ($<1\%$) of drug and lipids, and change in liposome particle size distribution were observed after filtration.

Freeze-drying or lyophilization of the formulation was performed to enhance the chemical/ physical stabilities of the liposomes and minimize lipid hydrolysis. Phospholipids in liposomes are known to be sensitive to hydrolysis and oxidation in aqueous medium. They can be hydrolyzed to form lysophospholipids and free fatty acids. The lysophospholipids can be further hydrolyzed to glycerophospho compounds and fatty acids [31–35]. The hydrolytic degradation may change the rigidity of liposomal bilayers, retention of entrapped drug, and alter liposome size and distribution. One of the major challenges of freeze-drying of liposomes is the preservation of the structural integrity of liposome during dehydration/rehydration process. In the absence of any protective agents, especially when liposome dispersion is frozen, vesicle fusion and leakage of internal aqueous contents from liposomes can occur [36]. Sugars have been shown to act as protective agents during dehydration/rehydration of liposomes to prevent vesicle fusion and help retention of encapsulated compounds within liposomes [37,38]. The ability of sugars, such as sucrose and trehalose, to prevent vesicle fusion was evaluated by measuring the vesicle size and drug entrapment efficiency after the formulation was lyophilized and reconstituted. The sugar-to-lipid molar ratio was found to be critical in preserving the integrity of the liposomes subjected to freeze-dry cycles. The optimum sugar-to-lipid molar ratio found to be effective in protecting LEP-ETU liposomes from aggregation and fusion ranged from 6:1 to 8:1.

Our study revealed that trehalose was not as effective as sucrose in protecting liposomes of LEP-ETU during lyophilization. Interestingly, with the same amount of sugar in the formulation and the same lyophilization cycle, the particle size of the liposomes containing trehalose increased significantly after lyophilization. By contrast, the particle size of the liposomes containing sucrose remained unchanged before and after lyophilization (Fig. 5). Thereafter, sucrose was chosen as the lyoprotectant in the final formulation.

3.3. Characterization of pre-lyophilized and reconstituted LEP-ETU

Both pre-lyophilized and reconstituted LEP-ETU samples were milky translucent liposome dispersions. The liposome vesicle size of the pre-lyophilized and reconstituted LEP-ETU sample was measured by DLS. The pre-lyophilized LEP-ETU samples were measured directly, whereas the reconstituted LEP-ETU samples were prepared by adding an appropriate volume of water for injection, USP to the lyophilized LEP-ETU cake prior to the measurement. A typical particle size distribution of LEP-ETU is shown in Fig. 5. The liposome particle size distribution of the samples under both conditions was mono-modal (Gaussian) distribution. There was no significant change in liposome vesicle size before and after lyophilization suggesting that the formulation of LEP-ETU contained sufficient lyoprotectant to preserve the integrity of the liposomes, and also the lyophilization cycle used was optimum.

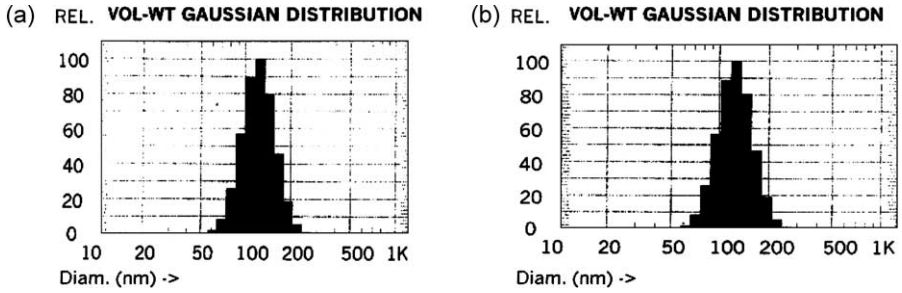

Fig. 5. Typical liposome size distribution of LEP-ETU before lyophilization and after reconstitution. Left (a): before lyophilization; right (b): after lyophilization and reconstitution. (a) Volume weighting: mean diameter = 125.8 nm; SD = 31.3 nm (24.9%); cumulative results: 25% of distribution < 95.8 nm, 50% of distribution < 113.5 nm, 70% of distribution < 134.9 nm, 90% of distribution < 157.5 nm, 99% of distribution < 204.7 nm. (b) Volume weighting: mean diameter = 128.1 nm; SD = 31.9 nm (24.9%); cumulative results: 25% of distribution < 97.5 nm, 50% of distribution < 115.6 nm, 70% of distribution < 137.4 nm; 90% of distribution < 160.4 nm; 99% of distribution < 208.8 nm.

The freeze-fracture electron microscopy and negative-stain transmission electron microscopy images of reconstituted LEP-ETU are presented in Fig. 6. The image examined by freeze-fracture electron microscopy showed that the liposomes were discrete particles with sharp boundaries that range in size from 100 to 160 nm. The particle size data were comparable to the results obtained from particle size measurement using DLS technique. The sharp boundary of the vesicles indicated the unilamellar structure of the LEP-ETU liposomes. The image from negative-staining TEM confirmed that the liposomes were discrete, round structures and free from drug crystals.

3.4. In vitro dialysis study

There was no drug precipitation or formation of paclitaxel crystals or paclitaxel degradant peak observed by visual inspection or HPLC during the course of the study. Fig. 7 shows the drug release profile of LEP-ETU through dialysis. Approximately 5.3% drug was released from LEP-ETU into PBS buffer over 120 h of dialysis, which suggests that the paclitaxel remained associated with the liposomes in the formulation under the condition studied. This also suggests that LEP-ETU would be stable when diluted with either normal saline or PBS buffer during clinical use. The release study conducted here was not intended to establish in vitro/in vivo correlation. Nonetheless, efforts are being made to develop an in vitro drug release method that may predict the liposomal drug retention in vivo.

3.5. Entrapment efficiency of paclitaxel

The percentage of the drug entrapped into liposomes was found to be in the range of 94–100%. The high drug entrapment efficiency further confirmed the high association of drug with liposomes.

3.6. Short-term and long-term stabilities

The short-term stability study showed that the reconstituted LEP-ETU (undiluted formulation) was physically and chemically stable for up to 72 h at both 2–8 °C and room temperature (Table 2). There was no significant change in liposome size, pH, drug entrapment efficiency, lipid

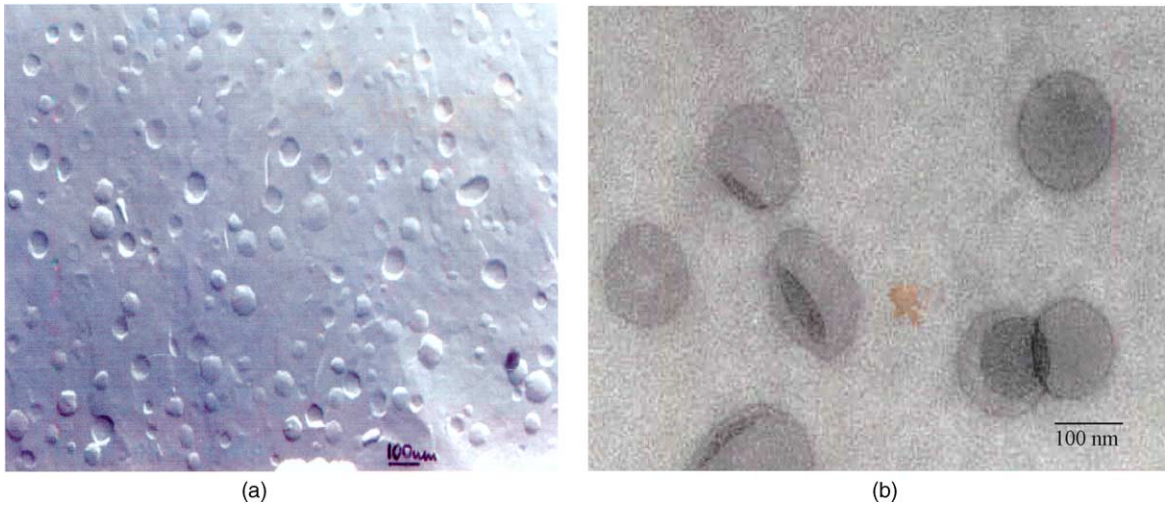

Fig. 6. (a) Freeze-fracture electron micrographs of reconstituted LEP-ETU, (b) negative-staining electron micrographs of reconstituted LEP-ETU.

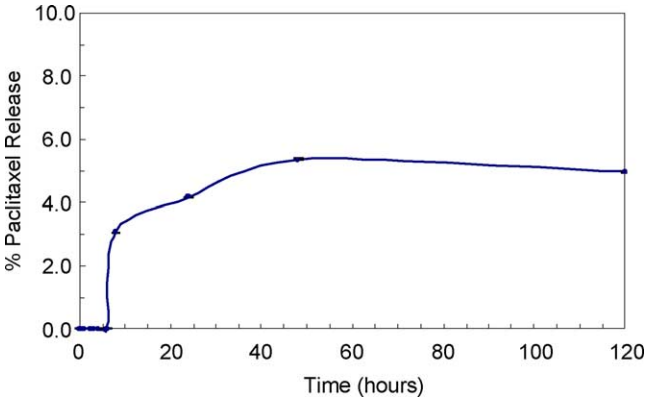

Fig. 7. In vitro paclitaxel release profile of reconstituted LEP-ETU in PBS at 37 °C.

and drug contents for the reconstituted LEP-ETU at both storage conditions.

Since paclitaxel is usually given to the patient as intravenous infusion, further dilution of the product is necessary to adjust the dose and drug infusion rate at a given time. The result of the dilution study demonstrated that 8-fold diluted LEP-ETU product stored at 2–8 °C and room temperature was physically and chemically stable for up to 72 and 48 h, respectively (Table 3). At both temperatures, the mean liposome vesicle diameter remained unchanged at the end of study. No precipitation or drug crystals were observed during and at the end of study. Paclitaxel and lipid concentrations remained unchanged at both temperature conditions over the course of the stability study, except for the cardiolipin concentration. The cardiolipin concentration of LEP-ETU decreased with time. However, the cardiolipin content was still within the specification set for the product (≧90% of initial) up to 72 h at 2–8 °C and 48 h at room temperature, respectively. The entrapment efficiency remained the same regardless of the storage time and condition. There was no appreciable change in pH for the diluted samples over the 48- and 72-h study period at room temperature and 2–8 °C (Table 3). It can be concluded that the reconstituted LEP-ETU product can be further diluted up to 8-fold and be used within 24 h at clinical setting.

The long-term stability showed that LEP-ETU lyophilized cake was physically and chemically stable at both 5 and 25 °C/60%RH for up to 12 months (Table 4). The residual water content may have a significant effect on the solid-state stability of lyophilized liposomes; therefore the residual moisture content of the lyophilized LEP-ETU was monitored. van Winden and Crommelin [39] reported that the lyophilized liposomes consisted of sucrose, showed a size increase, drug degradation and leakage of encapsulated drug after storage at 30 °C when the lyophilized cakes contained circa 3.5% residual water. Our results showed that the residual water content remained unchanged in the range of 0.7–1.2% for up to 12 months at both storage conditions.

The drug and lipid contents were within the specifications (≧90% of initial) set forth for the product for up to 12 months at both storage conditions. No significant changes in mean particle size, pH and drug entrapment were observed during the course of stability study.

A number of liposomal formulations of paclitaxel were reported in the literature; among them, the formulation developed by Sharma and Straubinger [40] showed the reduced toxicity and retained growth-inhibitory activity of the free drug in vitro. Fetterly and Straubinger [41] also reported that in animal models, their liposomal formulation of paclitaxel possessed lower toxicity and equal anti-tumor efficacy compared with Taxol®. However, the formulation process used was complicated and presented difficulties in large scale production. In addition, the particle size of the resulting liposomes was a multi-model distribution containing different size populations of liposomes, which would make the interpretation of pharmacokinetic results more difficult. Crosasso et al. conducted studies to compare PEGylated liposomes with the conventional liposomes of paclitaxel in terms of in vitro drug release rate, physical stability and pharmacokinetics as well as biodistribution

Table 2
Stability data of reconstituted (undiluted) LEP-ETU samples stored at 2–8 °C and room temperature

	Time (h)	Drug or lipid content (% initial)				Paclitaxel entrapment (%)	pH	Size (nm)
		Paclitaxel	Cardiolipin	Cholesterol	DOPC			
2–8 °C	0	100	100	100	100	~100	4.3	135
	8	103	100	99	102	~100	4.3	135
	24	103	100	100	100	~100	4.3	131
	48	102	99	98	99	~100	4.4	129
	72	99	99	98	98	~100	4.3	130
RT[a]	0	100	100	100	100	~100	4.3	135
	8	102	97	99	100	~100	4.3	126
	24	102	99	99	100	~100	4.3	128
	48	104	99	98	100	~100	4.4	131
	72	102	97	98	98	~100	4.3	138

All values are mean values of duplicate samples for each time point. Lipid and drug results are percent of the initial concentrations as measured at time zero.
[a] RT: room temperature (20–25 °C).

Table 3
Stability data of 8-fold diluted and reconstituted LEP-ETU samples stored at 2–8 °C and room temperature

	Time (h)	Drug or lipid content (% initial)				Paclitaxel entrapment (%)	pH	Size (nm)
		Paclitaxel	Cardiolipin	Cholesterol	DOPC			
2–8 °C	0	100	100	100	100	~100	6.0	133
	8	104	100	100	99	~100	5.5	137
	24	103	97	100	100	~100	5.7	128
	48	104	92	101	100	~100	6.2	131
	72	101	93	100	101	~100	5.9	130
RT[a]	0	100	100	100	100	~100	6.1	133
	8	108	98	100	100	~100	5.6	130
	24	102	99	100	101	~100	5.8	131
	48	106	92	100	100	~100	6.3	134

The reconstituted LEP-ETU was diluted 8-fold with normal saline. All values are mean values of duplicate samples for each time point. Lipid and drug results are percent of the initial concentrations as measured at time zero.
[a] RT: room temperature (20–25 °C).

behavior. They found that PEGylated liposomes significantly increased the half-life ($t_{1/2}$ 48.6 h) of paclitaxel in mice as compared to conventional liposomes ($t_{1/2}$ 9.27 h). Biodistribution study in mice showed a considerable decrease in drug uptake in mononuclear phagocytic system (MPS)-containing organs (liver and spleen) with PEGylated liposomes as compared to conventional liposomes [42]. However, both Crosasso et al. and Immordino et al. [43] found that only about 1.7 mole% paclitaxel could be incorporated in the PEGylated liposomes. Moreover, the PEGylated liposomes of paclitaxel were found to be physically unstable. They concluded that to confer clinical advantage to PEGylated liposomal formulation it would be necessary to further increase taxanes' (paclitaxel and docetaxel) concentration inside the lipid bilayer without affecting liposomal stability. Soepenberg et al. reported the result of the phase I study of liposomal paclitaxel that dose limiting toxicity (DLT) was observed at the dose level of 150 mg/m^2/week, less than 70% of the intended cumulative dose [44]. They also observed that the whole blood clearance of total paclitaxel was similar for liposomal paclitaxel (15.3 ± 8.98 l/h/m^2) and Taxol® (17.5 ± 3.43 l/h/m^2). They later concluded that their liposomal paclitaxel formulation is unlikely to provide any improvement over the taxanes currently in clinical use.

We have developed a Cremophor® EL free, sterile, lyophilized LEP-ETU formulation which is a viable alternative to the Taxol®, due to its small and uniform liposome size and easy-to-use character. The formulation process used for LEP-ETU is simple and scalable. As demonstrated, the lyophilized LEP-ETU was physically and chemically stable for at least 12 months at 2–8 and 25 °C (stability study is ongoing, 2-year shelf-life is predicted). The reconstituted, liquid form of LEP-ETU (undiluted as well as 8-fold diluted LEP-ETU) was also found to be physically and chemically stable up to 48 h at both 2–8 °C

Table 4
Lyophilized LEP-ETU stability data at 2–8 and 25 °C

	Time (Mon)	Drug or lipid content (% of initial)				Paclitaxel entrapment (%)	Mean vesicle size (nm)	Moisture (%)	pH
		Paclitaxel	Cardiolipin	Cholesterol	DOPC				
5±3 °C	Initial	100	100	100	100	100	124	0.95	4.5
	1	101	100	104	97	100	124	1.20	4.5
	3	95	97	101	97	101	126	0.85	4.7
	6	99	96	95	98	96	119	1.07	4.5
	9	97	106	101	103	94	118	0.92	4.5
	12	95	109	93	102	98	113	0.84	4.7
	Initial	100	100	100	100	100	124	0.95	4.5
25±2 °C	1	97	100	102	99	100	138	0.88	4.5
60±5% RH[a]	3	92	93	101	95	103	139	0.95	4.6
	6	98	90	101	93	94	129	0.90	4.5
	9	95	102	95	101	94	132	0.72	4.5
	12	92	107	97	101	100	127	NA	4.6

All values are mean values of duplicate samples for each time point. Lipid and drug results are percent of the initial concentrations as measured at time zero.
[a] RH: relative humidity.

and room temperature. The results of these studies clearly demonstrated design and development of a novel liposome-based paclitaxel with desirable physical and chemical characteristics. It is anticipated that hypersensitivity reaction can be avoided because LEP-ETU is not formulated in a Cremophor® EL-containing vehicle. The novel, sterile and lyophilized LEP-ETU appears to offer advantages of better safety (avoidance of hypersensitivity), no morbidity (avoidance of pre-medication) and improved patient compliance (shorter infusion time and less time spent in the treatment center). Preliminary results of on-going phase I trials obtained to date showed that LEP-ETU could be easily administered without pre-medication. Furthermore, it was found that LEP-ETU was well-tolerated at doses up to 325 mg/m^2, which is significantly greater than recommended dose of 175 mg/m^2.

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