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Freeze Drying of Double Emulsions to Prepare Topotecan-Entrapping Liposomes Featuring Controlled Release

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Topotecan-entrapping liposomes were prepared by freeze drying double emulsions with hydrogenated soy phosphatidylcholine, N-(carbonyl-methoxypolyethyleneglycol2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine, and cholesterol. Different inner aqueous phases of different pH values containing topotecan together with different chemicals, such as citrate and sulfate, were used to modify the physicochemical state of the drug to prepare W1/O/W2 double emulsions that were then freeze dried to obtain dry products. Upon rehydration, the dry products, which were stable for at least 6 months, formed into unilamellar liposomes with a high encapsulation efficiency of up to 80% and a mean diameter below 200 nm. The in vitro release experiments demonstrated that different formulations displayed different drug release properties. Thus, stable submicron unilamellar topotecan-entrapping liposomes can be prepared by freeze drying double emulsions, and the drug release can be successfully controlled by altering the physicochemical state of the incorporated drug.

Keywords vesicle; lyoprotectant; rehydration; physicochemical property; encapsulation efficiency

INTRODUCTION

Topotecan, a water-soluble analogue of camptothecin, is a topoisomerase I inhibitor, and is one of two camptothecin analogues currently in clinical use for the treatment of ovarian cancer and small-cell lung cancer (Ulukan & Swaan, 2002). The primary dose-limiting toxicity for topotecan is the noncumulative toxicity of myelosuppression regardless of the clinical treatment schedule (Takimoto, Wright, & Arbuck, 1998). Camptothecins possess an α-hydroxy-δ-lactone ring which appears to be structurally important for both passive diffusion of topotecan into cancer cells (Hertzberg, Caranfa, & Hecht, 1989) and successful interaction with the topoisomerase target (Burke, 1996). They also undergo reversible hydrolysis at physiological pH (pH ≈ 7) to give an open ring carboxylate form (Fassberg & Stella, 1992).

Investigations have shown that encapsulation of topotecan in a low pH environment inside liposomes increases the drug stability (Burke & Gao, 1994), enhances its therapeutic index, and reduces its toxicity combined with reduced blood elimination (Tardi et al., 2000). Topotecan displays low affinity for lipids (Burke, Mishra, Wani, & Wall, 1993) and only a very small amount can be intercalated into a bilayer of liposomes. Passive encapsulation techniques have been used to encapsulate topotecan in liposomes, but the products are multilamellar vesicles (MLV) and the encapsulation efficiency (EE) is low (Burke & Gao, 1994). Also, using active loading, topotecan has been encapsulated into unilamellar liposomes with a high drug-to-lipid ratio (0.3, w/w) and a high EE (> 90%), yet the drug was released rapidly (Tardi et al., 2000; Abraham et al., 2004). In addition, the reported liposomal topotecan preparations are aqueous suspensions of liposomes, which often suffer from stability problems, such as hydrolysis, leakage, and aggregation (Sharma & Sharma, 1997).

For liposomal formulations of anticancer agents, two important parameters that affect the therapeutic benefits of liposome delivery are the circulation lifetimes of the carriers following intravenous (iv) injection and the rate of drug release from the liposomes (Drummond, Meyer, Hong, Kirpotin, & Papahadjopoulos,1999; Charrois & Allen, 2004). It has been established that liposomal formulations of certain anticancer agents are extremely sensitive to drug release rates, with the slowest releasing systems exhibiting the best efficacy profiles (Drummond et al., 1999; Charrois & Allen, 2004).

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Recently, based on transmembrane ion gradient loading, different methods, such as induction of precipitation of amphipathic drugs in the liposomal interior and complex formation between amphipathic drug and transition metal within liposomes, have been used to entrap drugs, and this has led to improved drug retention (Cheung, Sun, Leenhouts, & Cullis, 1998; Abraham et al., 2002; Taggar et al., 2006; Zhigaltsev, Maurer, Edwards, Karlsson, & Cullis, 2006). However, these preparation processes involve several steps, such as preparation of multilamellar vesicles (MLVs) and subsequent large unilamellar vesicles (LUVs), establishment of transmembrane ion gradient, and active loading, and are, therefore, rather complex and time consuming.

In a previous report, a novel procedure involving freeze drying double emulsions (FDE) was developed to prepare unilamellar liposomes with desirable properties, such as high encapsulation efficiency, a mean diameter OF less than 200 nm, complete removal of organic solvents, and long-term stability (Wang et al., 2006). However, in that report, the liposomes were mainly made of unsaturated and/or charged phospholipids, such as soy phosphatidylcholine (PC) and soy phosphatidylserine (PS), and, therefore, are not suitable carriers for systemic applications of water-soluble agents (Cevc, 1993; Sharma & Sharma, 1997). In this report, the FDE procedure was used to prepare liposomes with saturated hydrogenated soy phosphatidylcholine (HSPC), N-(Carbonyl-methoxypolyethyleneglycol2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (PEG2000-PE), and cholesterol (CHO), for the encapsulation of topotecan. Also, the vesicles have the advantages, as previously reported (Wang et al., 2006). Different inner aqueous phases of different pH values containing topotecan together with different chemicals, such as citrate and sulfate, used to manipulate the physicochemical state of the drug, were used to prepare the FDE liposomes in the hope of regulating drug release. Such efforts proved successful with different formulations exhibiting different drug release profiles.

MATERIALS AND METHODS

HSPC (Epikuron 200SH) was purchased from Degussa (Freising, Germany). PEG2000-PE was purchased from Lipoid (Ludwigshafen, Germany). Topotecan hydrochloride for injection was a product of Foreignder Pharmaceuticals (Chengdu, China). Sephadex G-50 (medium) size exclusion gel was purchased from Pharmacia (Piscataway, NJ, USA). CHO and all other chemicals (analytical grade) were purchased from Tianjin Chemical Reagent Co. Inc. (Tianjin, China).

Preparation of Topotecan-Entrapping Liposomes by the FDE Procedure

Distilled water was used as the solvent of the aqueous phase (W) and cyclohexane/chloroform (5:1, v/v) for the oil phase (O). Different aqueous solutions (all containing 1 mg/ml topotecan, 150 mM sucrose) were used, respectively, as the inner aqueous phase (W1): (a) 300mM citrate with a pH of 4, (bi) 300 mM citrate with a pH of 5, (c) 300 mM Na_2SO_4 with a pH of 4, and (d) 300 mM Na_2SO_4 with a pH of 5 (the pH of c and d adjusted with a solution of 300 mM H_2SO_4). The outer aqueous phase (W2) contained 150 mM sucrose and an appropriate amount of NaCl to give isotonic solution as W1. The lipids of HSPC/CHO/PEG-PE (3:1:1 mass ratio) were dissolved in cyclohexane/chloroform to produce an organic solution of 10 mg/ml total lipids to be used as the oil phase (O).

The topotecan-entrapping liposomes were prepared by the FDE procedure (Wang et al., 2006), with some modification. The W1/O/W2 double emulsions were prepared at 35°C, and the temperature was controlled using a recycling water bath throughout the process. Briefly, 3 ml O and 2 ml W1 were added to a 10-ml ampoule, and this mixture was emulsified with a homomixer (Ultra Turrax T18, USA) at 22,000 rpm for 30 seconds to form approximately 5 ml W1/O type emulsion (primary emulsion). Then the primary emulsions were sonicated with a probe-type sonicator until the mixture became uniformly opalescent and did not separate for at least 30 minutes after sonication. Following this, the emulsions were mixed with 10 ml W2, and emulsified at 18,000 rpm for 30 seconds to form mostly type A of a W1/O/W2 double emulsion, containing only one internal aqueous droplet (Florence & Whitehill, 1982). The double emulsions were immediately transferred to 3-ml freeze-drying vials with a fill volume of 1 ml, and at once put into liquid nitrogen to freeze them.

The freeze drying process was as follows: (a) prefreezing in liquid nitrogen for 4 hours; (b) primary drying at −37°C for 8 hours; −30°C, −25°C, and −15°C for 2 hours, respectively; and (c) secondary drying at 20°C for 4 hours. The chamber pressure was maintained at 13 pascals during the drying process. When the freeze drying process was over, the vials were immediately filled with dry nitrogen gas, sealed, and protected from light of 4°C.

When needed, aqueous suspensions of topotecan-entrapping liposomes were immediately formed upon rehydration of the lyophilized products with pure water to give the original volume at 60°C.

Characterization of Double Emulsions and Liposomes

Dynamic Light Scattering (DLS) Technique

The mean diameters of the FDE liposomes in aqueous solution were determined by the dynamic light scattering technique (DLS) with a submicron particle size analyzer (Ls23, Beckman, USA).

Freeze-Fracture Electron Microscopy

The freeze-fracture electron micrographs (FEM) were obtained as described elsewhere (Sternberg, 1993), and a freeze-fracture device (Balzers BAF 400D) was used for fracturing.

Small Angle X-Ray Scattering (SAXS)

SAXS measurements were carried out in an evacuated Kratky compact camera (Anton Paar, Graz, Austria) at a temperature of

25°C (Skalko et al., 1998). The camera was mounted on a PW-1700X generator (Philips, Netherlands). Cu K_α X-rays with a wavelength of $\lambda = 0.1542$ nm were emitted, and the K_β emission line was filtered out by a nickel foil. The scattered intensity was determined with a position-sensitive detector PSD-50M (MBraun, Germany). The sample holder was equipped with two mica windows. The X-ray path length through the sample was approximately 1 mm, and the resulted scattering vector Q range was between 0.1 and 2.0 nm^{-1}. Scattering intensities were plotted as a function of the scattering vector Q defined as $4\pi\sin\theta/\lambda$, where θ is the Bragg angle equal to one half of the scattering angle θ_s, and λ is the wavelength.

Three samples were analyzed and all composed of HSPC/CHO/PEG-PE (3:1:1 mass ratio) with a lyoprotectant of sucrose. They were prepared as follows (Wang et al., 2006): sample 1 and sample 2 were lyophilized MLVs and LUVs prepared by hydration of the lipid film and subsequent serial extrusion. Sample 3 was lyophilized FDE liposomes that had been reconstituted by rehydration of the FDE products containing topotecan and sulfate (pH of 4).

Determination of Encapsulation Efficiency

The EE for topotecan was calculated from the drug-to-lipid ratio of liposomes and the total drug-to-lipid ratio. One hundred µl aliquots of liposome suspension were placed on 1 ml Sephadex G-50 (medium) spin minicolumns to remove unencapsulated drug from the liposomes. The amount of topotecan in the excluded fraction was determined by high performance liquid chromatography (LC-10AT liquid chromatograph, SPD-10A UV detector, Shimadzu) at a UV detection wavelength of 228 nm after vesicles were dissolved in an appropriate amount of methanol and diluted to 5 ml with the mobile phase of water/acetonitrile/trifluoroacetic acid (80:20:0.1 v/v/v).

The phospholipid was quantitated using the modified Fiske-Subbarow phosphate assay (Barenholz & Amselem, 1993). Briefly, 1 ml of 70% perchloric acid was added to lipid samples (< 200 µl) followed by heating to approximately 180°C to 200°C for 2 hours or until the samples were colorless. Samples were then cooled, and 750 µl of Fiske-Subbarow solution and 7 ml of 0.22% ammonium molybdate solution containing 2% sulfuric acid were added, followed by heating for 15 minutes in a boiling water bath. After samples were cooled to room temperature, the absorbance was read at 820 nm.

In Vitro Drug Release Assay

The free drug in the topotecan-entrapping FDE liposomes was removed using Sephadex G-50 (medium) spin minicolumns, and experiments were carried out in triplicate. An aliquot of 200 µl liposome suspension (0.5 mg/ml lipid) was incubated with 1800 µl of human blood plasma (HBP) at 37°C. At various time points (0, 1, 4, 8, 16, 24, 36, 48, and hours), 100 µl aliquots were withdrawn and transferred onto 1-ml spin columns to remove the free topotecan. The encapsulated topotecan and liposomal lipid were quantitated as described above.

Detection of Solvent Residue

Analyses of the solvent residue in the freeze-dried products were carried out on a gas chromatograph (GC-2010, Shimadzu) with an hydrogen flame ionization detector (FID) detector.

Stability Test

The stability of the dried products was assessed by determination of the liposomes, which were reconstituted from freeze-dried products protected from light at 4°C for different periods of time, using the changes in encapsulation efficiency and mean diameters as described above.

RESULTS AND DISCUSSION

Size and Encapsulation Efficiency

Examination by DLS revealed that the mean diameters of the FDE liposomes were under 200 nm. The encapsulation efficiency of FDE liposomes for topotecan was up to 80%. Table 1 provides details of the test results.

FEM

As shown by the FEM in Figure 1, the FDE liposomes containing topotecan were unilamellar vesicles with a size under 200 nm. Only the formulation of 300 mM Na_2SO_4 (pH of 5) is shown.

SAXS

The SAXS curves are presented in Figure 2. Characteristically different SAXS curves can be obtained from the

TABLE 1
The Size and EE of the Topotecan-Entrapping FDE Liposomes with Composition of HSPC/CHO/PEG2000-PE (3:1:1 Mass Ratio; $n = 3$)

Formulation[a]	MD[b] ± *SD*[c] (nm)	EE (%)
300 mM citrate (pH 4)	195 ± 77	78.3
300 mM citrate (pH 5)	181 ± 62	80.2
300 mM Na_2SO_4 (pH 4)	189 ± 73	76.5
300 mM Na_2SO_4 (pH 5)	186 ± 69	77.9

[a]The chemicals contained in the inner phase and the pH value of the inner phase. All the formulations contained topotecan and sucrose.

[b]MD = mean diameter.

[c]The *SD* was the largest value from the three replicates.

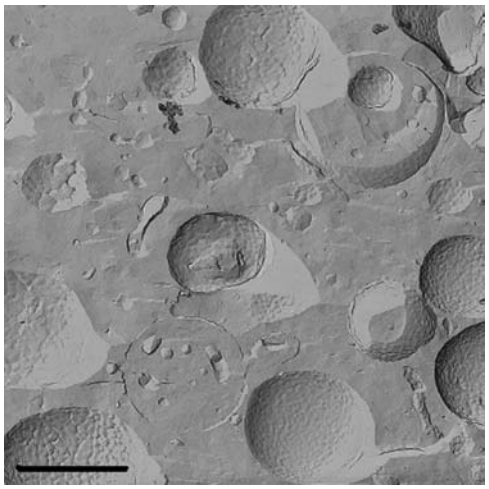

FIGURE 1. Typical freeze-fracture electron micrograph of the topotecan-entrapping FDE liposomes composed of HSPC/CHO/PEG2000-PE (3:1:1 mass ratio) with the inner phase containing 300 mM Na_2SO_4 formulation (pH 5).

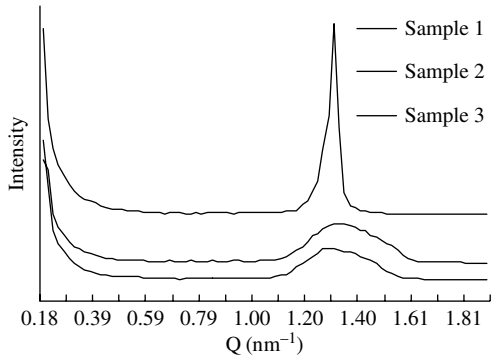

FIGURE 2. Small angle X-ray scattering curves of lyophilized MLVs (sample 1), lyophilized LUVs (sample 2), and lyophilized FDE liposomes (sample 3). All the lyophilized products were composed of HSPC/CHO/PEG2000-PE (3:1:1 mass ratio) with the inner phase containing 300 mM Na_2SO_4 formulation (pH 5). Sucrose was used as a lyoprotectant.

unilamellar vesicles and from regularly stacked bilayers of multilamellar vesicles (Bouwstra, Gooris, Bras, & Talsma, 1993). For the unilamellar vesicles, where the bilayers have no fixed geometrical relationship to each other, the scattering curve displays a very broad peak. On the other hand, the regularly stacked bilayers will display a relatively sharp peak (Bouwstra et al., 1993; Skalko et al., 1998). As seen from Figure 3, the lyophilized MLVs (sample 1) showed a lamellar repeat spacing of approximately 5.16 nm (λ = 0.1542 nm, $2\theta = 1.713$; Bouwstra et al., 1993; Wang et al., 2006). The lyophilized LUVs (sample 2) displayed a relatively broad scattering curve. Sample 3 had a similar scattering curve to that of sample 2, which means that the FDE liposomes are not multilamellar vesicles.

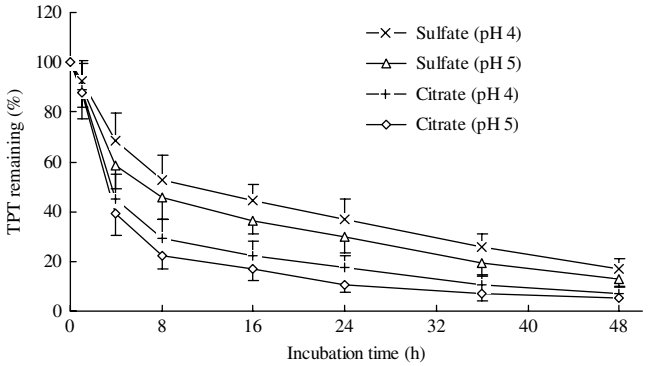

FIGURE 3. Release of topotecan from the FDE liposomes composed of HSPC/CHO/PEG2000-PE (3:1:1 mass ratio) with sucrose as a lyoprotectant. Release was measured in the presence of 90% human blood plasma at an incubation temperature of 37°C (n = 3).

In Vitro Topotecan Release Assay

To develop methods to control drug release from the FDE liposomes, different compositions of W1 were used to manipulate the physicochemical state of the drug in the liposome interior. Also, in vitro release experiments were performed, and the results are shown in Figure 3. Changes in the percent of topotecan remaining associated with liposomes, measured at various time points, indicated that different liposome formulations released drug at different rates, in the following order: formulation of Na_2SO_4 (pH 4) < Na_2SO_4 (pH 5) < citrate (pH 4) < citrate (pH 5).

Solvent Residue

No organic solvent residue was found in FDE powder tested by gas chromatograph (GC). This is an advantage compared with other methods in which organic solvents are usually removed by evaporation (New, 1990b).

Storage Stability

Liposomes were formed by rehydration of lyophilized products which were stored and protected from light at 4°C for 6 months without encapsulation or size change. Only the formulation of Na_2SO_4 (pH of 5) was shown in Figure 4.

Influencing Factors

A number of variables may be responsible for determining the final products in terms of the FDE vesicle size, structure, and encapsulation efficiency (Wang et al., 2006). These mainly include the type of phospholipid and its solubility in the organic solvents, lyoprotectants, and the loaded agents.

PEG-PE or charged lipids are necessary for preparation of double emulsions with phospholipids (Wang et al., 2006). Without PEG-PE, the double emulsions are rather unstable, and

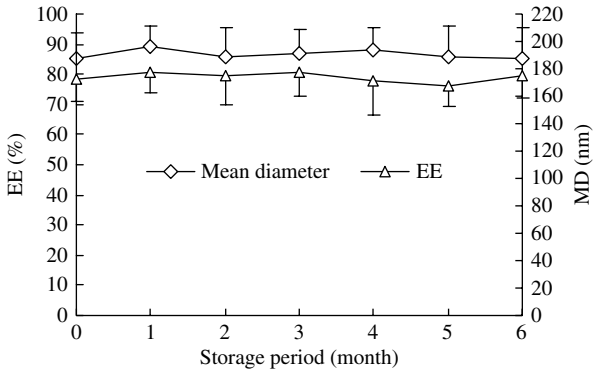

FIGURE 4. The storage stability of the lyophilized products composed of HSPC/CHO/PEG2000-PE (3:1:1 mass ratio) with the inner phase containing 300 mM Na_2SO_4 (pH of 5). The stability was assessed by determination of the changes in encapsulation efficiency and mean diameters of the liposomes reconstituted from lyophilized products protected from light at 4°C for different periods of time ($n = 3$).

aggregate and delaminate shortly after preparation, especially in the case of the formulation of HSPC which possesses a high phase transition temperature, T_m, of 52°C (Garbuzenko, Barenholz, & Priev, 2005). PEG can increase the hydrophilicity of the emulsion surface and sterically prevent aggregation of the double emulsions (Klibanov, Maruyama, Torchilin, & Huang, 1990). This might also be explained by the high mobility of PEG chains associated with conformational flexibility and water-binding ability (Needham, McIntosh, & Lasic, 1992; Blume & Cevc, 1993).

The solubility of HSPC in cyclohexane decreases markedly as the temperature decreases. Thus, the temperature should be controlled at 35°C to prevent lipids from precipitating or crystallizing from the oil phase (O) during the preparation procedure (New, 1990b; Cevc, 1993). For the same reason, the process of freezing must be rapidly completed and performed in liquid nitrogen. It was found that the FDE liposome size and size distribution increased markedly and the EE for topotecan was below 10% when the double emulsions of HSPC/CHO/PEG-PE were not frozen in liquid nitrogen, but in a refrigerator container with the temperature of −70°C. The reason may be that precipitation or crystallization of HSPC occurred, or not, when frozen under different conditions.

Even using soy phosphatidylcholine (SPC), which does not precipitate or crystallize from O as temperature decreases, the protocol of slow cooling (0.5°C/minute), often used for lyophilization of liposomes (Zuidam, van Winden, de Vrueh, & Crommelin, 2003), will lead to delamination and destruction of the double emulsions formed, and so it cannot be used to prepare the FDE liposomes.

Disaccharides, such as trehalose, sucrose, and lactose, are lyoprotectants known to protect membranes from damage during freeze drying (Wolfe & Bryant, 1999; Wolkersa, Oldenhof, Tablina, & Crowe, 2004; van Winden, 2003). In the FDE procedure, carbohydrates act as lyoprotectants as well as physical support for the phospholipid bilayer or monolayer of the submicron solid spheres, which might have similar structure to that of freeze-dried liposomes (Wang et al., 2006). Therefore, the best possible lyoprotectants, such as trehalose, sucrose, and lactose, are also necessary in the whole process.

Advantages of the FDE Liposomes Encapsulation of Topotecan

Long-Term Stability

Although there has been great interest in the development of liposomal formulations of topotecan for years (Burke et al., 1993; Burke & Gao, 1994; Tardi et al., 2000; Abraham et al., 2004), up to now, liposomal topotecan has not been used in clinical trials, and there have been no reports of the long-term stability of aqueous suspensions of topotecan liposomes. The leakage of topotecan from liposomes in aqueous suspensions could be a serious barrier to the development of liposomal topotecan for clinical applications (Sharma & Sharma, 1997). An initial examination of the stability of an aqueous suspension of topotecan-entrapping liposomes, with a composition of HSPC/CHO/PEG2000-PE (3:1:1 mass ratio) and a drug encapsulated by active loading of an ammonium gradient has been made, and it was found that more than 30% of the entrapped topotecan had been lost after being in light-protected storage at 4°C for 1 month (unpublished data). The FDE products containing topotecan are stable freeze-dried powders that can be stored for a long time and form liposomes by rehydration just before use. Thus, many of the problems, such as leakage and aggregation, associated with aqueous suspensions of topotecan liposomes can be avoided.

Controlled Release

It is worth noting that the FDE liposomes having different inner phases exhibit different drug release properties. When the inner phase has a similar pH value, the sulfate formulation exhibits a reduced topotecan release rate compared with the citrate formulation. The explanation for this may be that sulfate might help topotecan form a more ordered crystal structure while citrate might encourage amorphous precipitates that are labile and liable to dissolve (Abraham et al., 2004).

The in vitro drug release experiments also demonstrated that, for the identical formulations, the one with a lower pH value also exhibits a reduced topotecan release rate. The reason for this might be that the more acidic the liposome interior, the more topotecan molecules tend to be in the protonated (charged) form and, thus, in a membrane-impermeable state (Maurer-Spurej, Wong, Maurer, Fenske, & Cullis, 1999).

It should also be pointed out that the formulations presented here are not liposomes containing a saturated suspension in equilibrium with a precipitate, as in the case of others previously described using active loading (Lasic et al., 1995; Cheung et al., 1998; Abraham et al., 2002; Abraham et al., 2004; Johnston et al., 2006; Taggar et al., 2006; Zhigaltsev et al., 2006). Instead, they are liposomes containing an unsaturated

agent solution arising from a precipitate, or a solid state, which has been formed during the lyophilization process. The actual pattern of release, however, will also be dependent on the dissolution rate of the precipitate (Zhigaltsev et al., 2006). The dissolution rate of the precipitate should be slower than the membrane diffusion rate of the neutral species; thus, the release is biphasic, starting with a rapid loss of the drug and, after establishing an equilibrium, followed by a slower linear phase (Zhigaltsev et al., 2006).

It is well established that the physical state of a drug dictates its pharmaceutical behavior. Other researchers have shown that on increasing drug-to-lipid ratio, there was also an associated reduction in drug release (Abraham et al., 2004; Johnston et al., 2006). Based on active loading, the formation of transition metal complex and/or precipitation was also investigated to regulate drug release from LUVs, and improved drug retention was observed (Cheung et al., 1998; Abraham et al., 2002; Taggar et al., 2006; Zhigaltsev et al., 2006). These findings all suggest that drug release can be controlled by manipulation of the chemical and/or physical state of a drug, which might be achieved through regulation of the inner phase of the liposomes. This can easily be performed in the FDE procedure, in which the inner phase is prepared and regulated prior to, and independently of, the process (Wang et al., 2006). This was proved by the preparation of the FDE liposomes entrapping an anticancer drug, topotecan, which tends to rapidly leak out of liposomes (Abraham et al., 2004; Taggar et al., 2006). This drug release was successfully controlled.

Although no quantitation of the lactone and carboxylate forms of topotecan was carried out, the topotecan-entrapping FDE liposomes had an internal pH of 5, and the lactone ring of topotecan can be made very stable (Burke & Gao, 1994).

CONCLUSION

In this investigation, using saturated phospholipids, a novel procedure of freeze drying double emulsions was successfully used to prepare submicron topotecan-entrapping liposmes with a high encapsulation efficiency of up to 80%. Since the FDE liposome encapsulation of topotecan produces a dry product, many of the problems, such as leakage and aggregation, associated with aqueous suspensions of topotecan-entrapping liposomes have been solved. Specifically, the drug release can be controlled for the topotecan-entrapping FDE liposomes through regulation of the composition of the inner phase by manipulation of the physicochemical properties and states, such as protonation and precipitation of the incorporated drugs. Also, this may define a viable methodology to prepare liposomal drugs with controlled release. Due to these advantages, the topotecan-entrapping FDE liposomes are expected to be suitable for therapeutic applications.

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