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Use of a passive equilibration methodology to encapsulate cisplatin into preformed thermosensitive liposomes

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Abstract

A conventional, cholesterol-containing liposome formulation of cisplatin has demonstrated insignificant activity in clinical trials, due in part, to insufficient release of encapsulated content following localization within solid tumors. For this reason, the development of a triggered release liposome formulation is desirable. In this report, cisplatin was encapsulated into lysolipid-containing thermosensitive liposomes (LTSL) using a novel technique, which relies on the equilibration of cisplatin across the liposomal membrane at temperatures above the gel-to-liquid crystalline phase transition temperature (T_C) of the bulk phospholipid. Mild heating and drug loading into LTSL did not induce morphological changes of the liposomes. In vitro data demonstrated that >95% of encapsulated cisplatin was released from LTSL within 5 min following mild heating at 42 °C, while <5% was released at 37 °C. Under similar conditions, lysolipid-free thermosensitive liposomes exhibited 70% release of cisplatin at 42 °C, and cholesterol-containing liposomes exhibited negligible drug release at 42 °C. The pharmacokinetic profiles of LTSL- and TSL-cisplatin indicated that these formulations were rapidly eliminated from circulation (terminal $t_{1/2}$ of 1.09 and 2.83 h, respectively). The therapeutic utility of LTSL-cisplatin formulation will be based on strategies where hyperthermia is applied prior to the administration of the liposomal drug—a strategy similar to that used in the clinical assessment of LTSL-doxorubicin formulation.

Keywords: Thermosensitive liposomes; Cisplatin; Passive equilibration; Drug release

1. Introduction

Liposomes have proven to be a versatile and reasonably successful systemic drug delivery system (Allen and Cullis, 2004), and this is exemplified by the regulatory approval and advanced clinical studies in patients of several liposomal formulations of anticancer drugs (Allen and Cullis, 2004). Liposomes are often designed to extend the circulation time of encapsulated drug and to improve in vivo drug retention, so as to result in the greatest improvements in drug accumulation within tumor tissues (Lim et al., 1997, 2000). However, enhanced tumor drug delivery does not directly translate into optimal therapeutic activity, because the therapeutic effects are dependent on the rate at which the

Abbreviations: AAS, atomic absorption spectroscopy; cryo-TEM, cryo-transmission electron microscopy; CHE, cholesterylhexadecyl ether; chol, cholesterol; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; D:L, drug-to-lipid ratio (w/w); DSPE-PEG 2000, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (ammonium salt); i.v., intravenous; LTSL, lysolipid-containing thermosensitive liposomes; mPEG-PE, methoxypolyethylene glycol-distearoyl phosphatidylethanolamine; MSPC, 1-stearoryl-2-hydroxy-*sn*-glycero-3-phosphocholine; PC, phosphatidylcholine; QELS, quasi-elastic light scattering; T_C, gel-to-liquid crystalline phase transition temperature; TSL, thermosensitive liposomes (non-lysolipid containing).

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encapsulated drug is released from the liposomes (Drummond et al., 1999; Lim et al., 1997, 2000; Maeda et al., 2000). This is best exemplified by SPI-077—the non-temperature sensitive, long circulating liposome formulation of cisplatin (Newman et al., 1999). SPI-077 exhibited poor drug dissociation from liposomes following administration and negligible therapeutic activity in patients (Bandak et al., 1999; Harrington et al., 2001; Meerum Terwogt et al., 2002). For this reason, effective triggering mechanisms to allow release of liposomal contents following tissue accumulation of long circulating, serum stable liposomes have been intensively pursued, and the use of mild hyperthermia has received considerable interest, both from a therapeutic (Dewhirst, 1994; van der Zee, 2002) and a mechanistic/delivery perspective (Song et al., 1980; Kong and Dewhirst, 1999; Kong et al., 2000; Needham and Dewhirst, 2001). Numerous studies have demonstrated that the use of hyperthermia in combination with liposomal delivery has resulted in increased intra-tumoral liposome concentrations, which in turn, is associated with increased drug delivery compared to that achieved with delivery of free drug or liposomal drug in the absence of heating (reviewed in Kong and Dewhirst, 1999).

Since thermosensitive liposome formulations were first described (Yatvin et al., 1978; Weinstein et al., 1979), continued research has resulted in the engineering of a thermosensitive liposome formulation that exhibits maximal drug release upon heating at temperatures (40–42 °C) and time frames (30–60 min) achievable with clinical hyperthermia treatment protocols. This liposome formulation, described by Anyarambhata and Needham as LTSL (Anyarambhatla and Needham, 1999), is composed of DPPC/MSPC/DSPE-PEG$_{2000}$ (90:10:4 mole ratio), in which the lysolipid (MSPC) facilitates rapid release (within tens of seconds) of doxorubicin at 40–42 °C, and the PEG-lipid enhances liposomal circulation lifetimes and promotes liposome accumulation within tumors (Needham et al., 2000). This LTSL-doxorubicin formulation has entered Phase I clinical trials for liver and prostate cancers. Based on the drug release profile of the LTSL formulation, the objective of the current study is to encapsulate cisplatin into LTSL in hopes to trigger cisplatin release upon mild heating. A novel method of drug encapsulation, termed "passive equilibration," is described here for the loading of cisplatin into preformed LTSL.

This method was developed to overcome the technical challenge of producing liposomal cisplatin by the conventional method which involves the passage of concentrated cisplatin solutions with the lipid mixture through the extruder apparatus that could lead to a permanent, black deposit in the extruder. The passive equilibration loading method is based on the findings from biophysical characterization that membrane permeability of PC-based vesicles increases as temperature increases due to expansion in vesicle area (Xiang and Anderson, 1998). Thus, equilibration of drug across the liposomal membrane occurs at temperatures above T_C and trapping of drug occurs when the liposomes are cooled below T_C because of the differences in the relative membrane permeability at the different temperatures. We reported here drug encapsulation capacity, liposome size and morphology, in vitro drug release and pharmacokinetic behavior of the LTSL-cisplatin formulation.

2. Methods and materials

2.1. Materials

All lipid products were obtained from Avanti Polar Lipids (Alabaster, USA). Sephadex G-50 size exclusion gel, *cis*-diamminedichloroplatinumII (cisplatin), platinum standard and other chemicals (reagent grade) were purchased from Sigma–Aldrich (St. Louis, USA). [^{3}H]-cholesterylhexadecyl ether ([^{3}H]-CHE) and [^{14}C]-lactose were purchased from NEN Life Sciences Products (Oakville, Canada). [^{14}C]-methylamine hydrochloride was purchased from Amersham Pharmacia Biotech (Oakville, Canada). Pico-Fluor 15 scintillation fluid was purchased from Packard Bioscience (Groningen, The Netherlands).

2.2. Preparation of liposomes

The thermosensitive liposome formulations evaluated here include lysolipid-containing thermosensitive liposomes (LTSL) composed of DPPC/MSPC/DSPE-PEG$_{2000}$ (90:10:4 mole ratio), lysolipid-free thermosensitive liposomes (TSL) composed of DPPC/DSPE-PEG$_{2000}$ (100:4 mole ratio) and a cholesterol-containing formulation composed of DPPC/chol/DSPE-PEG$_{2000}$ (55:45:4 mole ratio) which served as a non-temperature sensitive liposome control. All liposome samples were prepared by the extrusion method (Olson et al., 1979; Hope et al., 1985). Briefly, lipids at the appropriate molar ratios were dissolved in chloroform. [^{3}H]-CHE (0.03 and 0.1 μCi/μmol for in vitro and in vivo studies, respectively) was added to the dissolved lipid mixture as a non-exchangeable and non-metabolizable lipid marker (Derksen et al., 1987). The preparation was subsequently dried under a stream of nitrogen gas, and the resulting lipid film was placed under high vacuum overnight to remove organic solvent. The dried lipid films were hydrated with gentle mixing in 150 mM NaCl adjusted to pH 7.4 at 55 °C for 1 h to form multilamellar vesicles. The resulting preparation was extruded 10 times at 55 °C through two stacked 0.1 μm pore size polycarbonate filters (Northern Lipids Inc., Vancouver, BC, Canada) with an extruder apparatus (Northern Lipids Inc., Vancouver, BC, Canada). The resulting mean diameter of the liposomes was determined by quasi-elastic light scattering (QELS) using the Nicomp submicron particle sizer model 370/270 (Pacific Scientific, Santa Barbara, CA) operating at 632.8 nm. Liposomal lipid was quantitated by liquid scintillation counting of samples labeled with [^{3}H]-CHE using the Packard 1900TR Liquid Scintillation Analyzer (Meriden, CT).

2.3. Cisplatin solubility determination

Briefly, 12 mg/mL cisplatin was added to 150 mM NaCl with gentle mixing for 30 min and incubated at room temperature, 37, 55 and 70 °C. The sample remained at the incubation temperature for another 30 min without mixing to allow sedimentation of undissolved cisplatin. An aliquot of the cisplatin solution was then transferred to 1 mL of NaCl at room temperature,

and measured against a standard curve by atomic absorption spectroscopy (AAS) using a SpectrAA 300Z spectrometer (Varian Inc., Palo Alto, CA) with a graphite tube atomizer. The instrument was operated at a wavelength of 265.9 nm and with the following temperatures at 90 °C for 30 s, 120 °C for 10 s, 1100 °C for 15 s and 2800 °C for 8 s sequentially. A commercially available platinum standard was used to obtain the standard curve. All measurements were made in duplicates.

2.4. Drug loading methods

The passive equilibration loading method is described as follows. Briefly, preformed liposomes were prepared as described in Section 2.2, and were warmed at indicated temperatures for 5 min before adding to pre-warmed cisplatin powder. An initial D:L ratio of 0.15 (w/w) was used. The mixture was incubated at indicated temperatures for 2 h in a water bath with gentle mixing. The final, external cisplatin concentration used in the studies was either 1.5 mg/mL (the maximum solubility achievable at room temperature as determined in Section 2.3) or 10 mg/mL (the concentration achievable at 70 °C without any visible precipitation as determined in Section 2.3). For time course studies, aliquots from the liposome–cisplatin mixture were taken at indicated time points, equilibrated to room temperature and followed by centrifugation (1000 × *g*, 3 min) to pellet insoluble cisplatin. All subsequent processing and sample handling were performed at room temperature. The supernatant was then passed down a 1-mL Sephadex G-50 size exclusion spin column pre-equilibrated with saline. The spin column was prepared by adding glass wool to a 1-mL syringe, followed by packing of Sephadex G-50 beads by centrifugation (680 × *g*, 2 min). Liposomes were eluted by centrifugation (680 × *g*, 3 min) and collected in the void volume. Liposomal lipid and cisplatin concentrations were then analyzed as described above.

To prepare cisplatin-loaded DPPC/Chol/DSPE-PEG_{2000} liposomes, the method of passive encapsulation was used, where a 10 mg/mL cisplatin solution prepared by dissolving the drug at 70 °C was used to hydrate the dried lipid film. In this case, drug encapsulation occurred during the formation of liposomes, and preformed DPPC/Chol/DSPE-PEG_{2000} liposomes could not be used. Following a 1-h hydration period at 70 °C, the sample was extruded at 70 °C through stacked polycarbonate filters. Liposomal lipid and drug concentrations were analyzed as described above. The initial D:L ratio used in the preparation was 0.15 (w/w). Unencapsulated cisplatin was removed by passing the liposomes down a Sephadex G-50 column pre-equilibrated with saline.

2.5. Trapped volume determination

Trapped volume was estimated using [^{14}C]-lactose as the aqueous marker. To determine the trapped volume of liposomes prepared by the passive encapsulation method, [^{3}H]-CHE labeled lipid films were prepared as described above and subsequently hydrated in saline containing [^{14}C]-lactose. The liposomes were hydrated and extruded at 70 °C. The resultant liposome sample was passed down the Sephadex G-50 column pre-equilibrated with saline to remove unencapsulated [^{14}C]-lactose. Trapped volume (μL/μmole lipid) was calculated using the following equation:

$$\text{trapped volume} = \frac{M}{L} \quad (1)$$

where M = [^{14}C]-lactose dpm per μL eluted from the column divided by [^{14}C]-lactose dpm per μL of the initial liposome suspension prior to separation on the column, and L = [^{3}H]-CHE dpm per μL eluted from the column divided by the specific activity of the liposomal preparation in dpm per μmole total lipid.

To determine the trapped volume of liposomes prepared by the passive equilibration method, [^{14}C]-lactose was added to the exterior of preformed liposomes at 70 °C, and the levels of [^{14}C]-lactose accumulated in liposomes were monitored over time. When the level of liposome-associated [^{14}C]-lactose reached a plateau ($t > 8$ h), the value was used to compute M and to subsequently estimate the trapped volume using Eq. (1) presented above.

2.6. Cryo-transmission electron microscopy (cryo-TEM)

The method and interpretation of liposome images have been previously described (Almgren et al., 1996; Ickenstein et al., 2003). Briefly, in a climate controlled chamber, a 1–2 μL diluted liposomal sample was placed onto a copper grid coated with a cellulose acetate butyrate polymer film and blotted, forming a thin aqueous layer on the membrane. The sample was flash frozen in liquid ethane, allowing the film to vitrify, and was immediately transferred to liquid nitrogen so as to maintain the temperature below −165 °C. This step was to minimize formation of ice crystals and sample perturbation. The grid containing the sample was transferred to a Zeiss EM902 transmission electron microscope (Carl Zeiss Inc., Oberkochen, Germany) for analysis in a zero-loss bright-field mode and an accelerating voltage of 80 kV.

2.7. In vitro cisplatin release assay

Cisplatin-loaded liposomes were divided into two fractions and adjusted to a final liposomal lipid concentration of 2 mg/mL, with one fraction incubated at 37 °C and the other at 42 °C. Changes in D:L ratio were monitored over time, and decreases in D:L ratio reflected cisplatin release from the liposomes. At various time points, aliquots were taken and passed down the spin columns to separate the released cisplatin from the liposomes. The eluted liposomes were analyzed for liposomal lipid and cisplatin concentrations as described above.

2.8. Plasma elimination of cisplatin-loaded liposomes

Plasma elimination of cisplatin encapsulated in LTSL, TSL and cholesterol-containing liposomes were evaluated in female, 20–22 g, Rag-2M mice. All animal studies were conducted according to procedures approved by the University of British

Columbia's Animal Care Committee and in accordance with the current guidelines of the Canadian Council of Animal Care. To control the animal body temperature, mice were anesthetized i.m. with 6 mg/kg acepromazine prior to injection of the liposomal formulations, and were subsequently kept in a custom designed temperature controlled chamber (BC Cancer Agency workshop). The temperature of the animals kept in the chamber was maintained at 37° ± 0.5 °C throughout the 8-h time course of the study. The temperatures of the chamber and of the tranquillized mice were monitored at regular time intervals on a multi-channel Model 46 TUC Tele-Thermometer using YSI 400-thermistor probes (Yellow Springs Inc., Yellow Springs, USA). The probe was inserted into the rectum of the animal for body temperature monitoring. Free cisplatin and liposomal cisplatin (labeled with [^{3}H]-CHE) were injected i.v. as a single bolus via the lateral tail vein at 2 mg/kg cisplatin (injection volume = 200 μL). The lateral tail vein was dilated by submerging the tail in a 37 °C water bath for 1–2 min. At various time points post-injection, mice were terminated by CO_2 asphyxiation, and blood was collected by cardiac puncture, placed into EDTA-coated microtainers (Becton Dickinson, Oakville, Ont., Canada), and kept at room temperature until ready for centrifugation (1000 × *g*, 15 min). Plasma liposomal lipid and cisplatin concentrations were determined by radioactivity counting and AAS, respectively, as described above.

2.9. Pharmacokinetic modeling and statistical analysis

Pharmacokinetic parameters were estimated from the plasma elimination data by noncompartmental analysis using WinNonlin software (Version 1.5; Pharsight Corp., Mountain View, CA). First-order elimination was assumed, and the standard trapezoidal rule was used to calculate the mean area under the curve (AUC). A standard one-way analysis of variance (ANOVA) was used for comparisons. The Newman–Keuls test for the post hoc comparison of means was employed.

3. Results and discussion

3.1. Cisplatin encapsulation via passive equilibration method

Encapsulation of cisplatin into LTSL via passive equilibration method was characterized as a function of time at 37, 45, 55 and 70 °C. Cisplatin was added at a final concentration of 2 mg/mL to the exterior of preformed LTSL. As shown in Fig. 1, cisplatin accumulation in LTSL was negligible when the incubation temperature was 37 °C (Fig. 1, ▲). Rapid equilibration of cisplatin across the liposomal membrane was achieved within 30 min when the sample was incubated at temperatures of 45, 55 and 70 °C. The rates of drug uptake were comparable at these temperatures, and loading was stable over the 2-h time course. The final D:L ratio was approximately 0.0028 (w/w) regardless of the incubation temperature (45, 55 and 70 °C), as the D:L ratios for the different incubation temperatures were not statistically different ($p > 0.05$). The loading efficiency was approximately 10% under the conditions used.

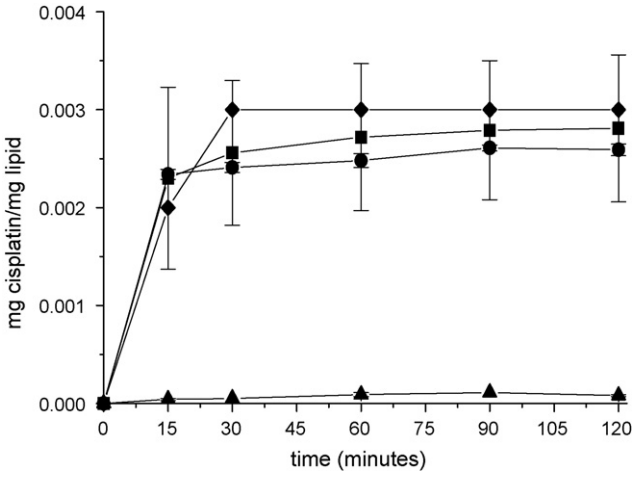

Fig. 1. Time-dependent uptake of cisplatin (2 mg/mL final drug concentration) via passive equilibration into LTSL at various temperatures. Cisplatin encapsulation was determined at 37 °C (▲), 45 °C (♦), 55 °C (■) and 70 °C (●). At the indicated time points during the incubation, aliquots of sample were taken and lipid and drug content were analyzed as described in Section 2. Each point represents mean ± S.D. ($n = 3$).

To increase the final D:L ratio of LTSL achievable by passive equilibration, one approach is to increase the external cisplatin concentration for loading. This was achieved by dissolving cisplatin in saline at higher temperatures to obtain the maximum cisplatin solubility at the various temperatures for drug loading (i.e., 37, 55 and 70 °C). As shown in the inset of Fig. 2, the maximum solubility of cisplatin in saline at room temperature was found to be 1.49 ± 0.21 mg/mL, and was increased

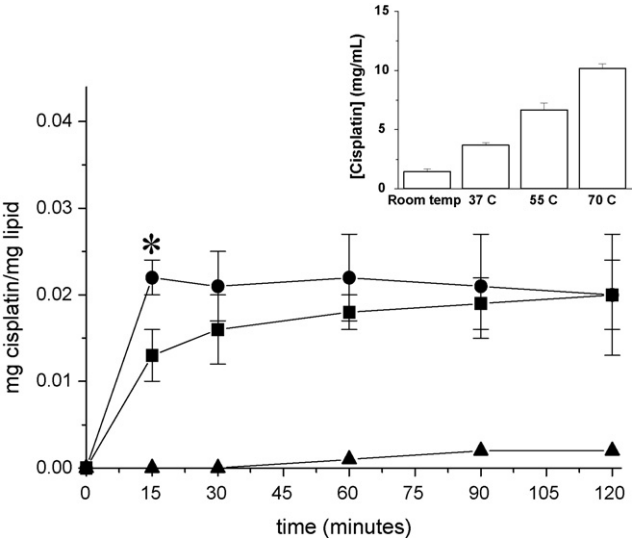

Fig. 2. Time-dependent uptake of cisplatin via passive equilibration into LTSL (●), TSL (■) and DPPC/chol/DSPE-PEG$_{2000}$ liposomes (▲) at 70 °C as a function of time. The initial cisplatin drug concentration for encapsulation was 10 mg/mL. At the indicated time points during incubation, aliquots of sample were taken for analyses of liposomal lipid and drug content as described in Section 2. Each point represents mean ± S.D. ($n = 3$). (Inset) Cisplatin solubility in 150 mM NaCl at different temperatures. Cisplatin was mixed with 150 mM NaCl at the indicated temperature with gentle vortexing ($n = 3$). The asterisk (*) indicates a statistical significant difference of LTSL from TSL and DPPC/chol/DSPE-PEG$_{2000}$ liposomes with $p < 0.05$.

to 10.20 ± 0.36 mg/mL at 70 °C. Thus, subsequent drug loading studies via the passive equilibration method were done at 70 °C and with a cisplatin concentration of 10 mg/mL to ensure complete dissolution of drug. As expected, a fivefold increase in the final D:L ratio was observed when the cisplatin concentration was increased to 10 mg/mL (Fig. 2), which was found to be 0.018 ± 0.002 (w/w). This D:L ratio was comparable to that achieved in the Stealth liposome formulation of cisplatin (SPI-077) (Newman et al., 1999; Meerum Terwogt et al., 2002). Consistent with results from Fig. 1, maximum cisplatin loading into LTSL was achieved within 15 min at 70 °C. Under these cisplatin loading conditions (incubation at 70 °C for 2 h with a pH value of 7.4), the extent of lipid hydrolysis should be minimal (Grit and Crommelin, 1993). Incubation temperatures greater than 70 °C were not considered to avoid liposome instability as well as safety concerns about working with very hot solutions of cisplatin. Passive equilibration loading method was also assessed in preformed TSL and DPPC/Chol/DSPE-PEG$_{2000}$ liposomes. After 15 min of incubation, the D:L ratios achieved in TSL and DPPC/Chol/DSPE-PEG$_{2000}$ liposomes were significantly lower than that in LTSL ($p < 0.05$). This indicates that the incorporation of lysolipid in LTSL formulation enhanced the permeability of cisplatin across the liposome membrane, and this is consistent with the postulation that lysolipid plays an important role in stabilizing the nanopores at the grain boundaries of the melting LTSL membrane (**). The time to achieve maximum cisplatin loading was increased to 2 h for TSL (Fig. 2, ■), and negligible loading was observed in the 2-h time course when DPPC/Chol/DSPE-PEG liposomes were used (Fig. 2, ▲). This is consistent with the ability of cholesterol incorporated at >40 mol% to reduce liposome membrane permeability by at least fivefold (Bittman et al., 1986).

Table 1
Trapped volume of LTSL and TSL as determined by passive encapsulation and passive equilibration methods

Liposome formulation	Trapped volume (μL/μmole lipid)		Liposome size (nm)[c]
	Passive encapsulation[a]	Passive equilibration[b]	
LTSL	1.88 ± 0.15	1.92 ± 0.22[d]	88 ± 5
TSL	2.4 ± 0.15	1.79 ± 0.17[e]	86 ± 5

[a] Measurements determined by trapped [^{14}C]-lactose added during sample rehydration.
[b] Measurements determined by trapped [^{14}C]-lactose into preformed liposomes over time.
[c] Liposome size determined by quasi-elastic light scattering following extrusion.
[d] Data represents mean ± S.D. ($n = 3$).
[e] Significantly different from data obtained for passive encapsulation ($p < 0.05$).

The trapped volumes of LTSL and TSL were estimated using radiolabeled [^{14}C]-lactose as the aqueous marker. Two approaches were followed: one where [^{14}C]-lactose was added to the buffer used to hydrate the lipid film, and a second approach that used the passive equilibration method (see Section 2). The results, summarized in Table 1, indicate that LTSL and TSL exhibited mean trapped volumes of 1.88 ± 0.15 and 2.4 ± 0.15 μl/μmole lipid, respectively, when estimated using the first approach. The second approach resulted in measured trapped volumes of 1.92 ± 0.22 and 1.79 ± 0.17 μL/μmole lipid for LTSL and TSL, respectively. It is important to point out that the time required to achieve [^{14}C]-lactose equilibration was significantly greater than the time required for cisplatin equilibration (>8 h at 70 °C for LTSL membrane). This finding was

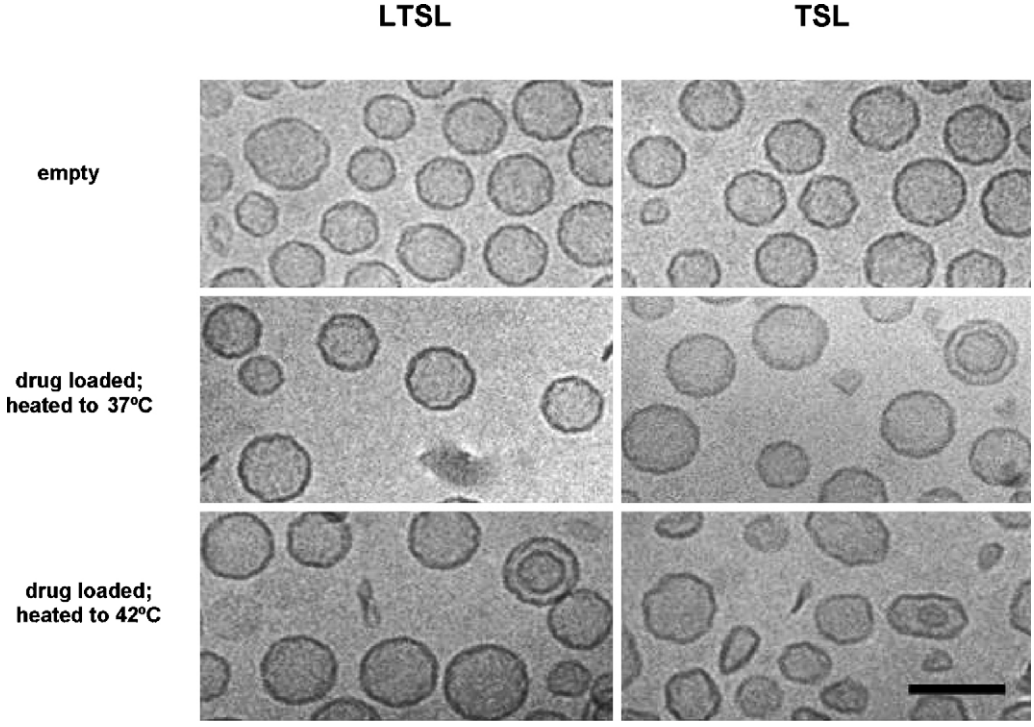

Fig. 3. Cryo-TEM electron micrographs of empty and cisplatin-loaded LTSL and TSL prepared using the passive equilibration technique. Bar represents 100 nm.

not surprising given that lactose is not very membrane permeable and it demonstrates that the nature of the equilibrating solute would affect the time required to reach equilibrium at a given temperature in addition to the liposomal lipid composition. In general terms, these data suggest that the physicochemical properties (including lipophilicity and molecular cross-sectional area) of the therapeutic agent to be encapsulated in thermosensitive liposomes by passive equilibration will influence the rate of drug loading, which in turn, may relate to the rate of in vivo drug release from the liposomes.

3.2. Cryo-TEM analyses of LTSL- and TSL-cisplatin formulations

Since cisplatin was encapsulated at a concentration of 10 mg/mL and the liposomes were cooled to room temperature after preparation, it was anticipated that cisplatin may precipitate within the liposome core. Using cryo-TEM to analyze the LTSL- and TSL-cisplatin formulations, substantial differences between empty or cisplatin-loaded LTSL and TSL were not observed (Fig. 3). It is possible that cisplatin precipitates are not detectable by cryo-TEM analyses; however, this possibility is not likely considering that cisplatin is an electron dense compound. Alternatively, cisplatin did not form a precipitate in liposome core. A previous study using nuclear magnetic resonance method demonstrated that encapsulated cisplatin, prepared in cholesterol-containing liposomes stabilized with PEG-modified lipids, exists in a soluble form within the aqueous liposome core and not as a drug precipitate (Peleg-Shulman et al., 2001). It is likely that the encapsulated cisplatin in LTSL and TSL is in a soluble form based on this previous finding. In terms of morphology, substantial changes were not observed in cisplatin-loaded LTSL and TSL that were imaged following a 30-min incubation at 37 or 42 °C. LTSL and TSL surfaces appeared angular regardless of the temperature and of the encapsulated content, a feature which is consistent with that obtained with the LTSL- and TSL-doxorubicin formulations (Ickenstein et al., 2003).

3.3. In vitro and in vivo cisplatin release from LTSL and TSL formulations

It is important to assess cisplatin release from the thermosensitive liposomes (LTSL and TSL) upon mild heating (42 °C), and the results from in vitro drug release assay are shown in Fig. 4. As expected, cisplatin was well retained in all formulations incubated at 37 °C—a temperature that is below T_C. Although TSL showed a small decrease in D:L ratios over time at 37 °C (Fig. 4B), the change in cisplatin-to-liposomal lipid ratios observed over the 1-h time course was not statistically different ($p > 0.05$). When mild heating (incubation at 42 °C) was applied, ~95% cisplatin was released from LTSL within 5 min, with 100% release achieved by 30 min (Fig. 4A, filled symbols). In comparison, TSL released only 70% of the encapsulated cisplatin at the 5-min time point at 42 °C, and 95% release of drug was not achieved until the formulation was heated for 60 min (Fig. 4B, filled symbols). Negligible cisplatin release

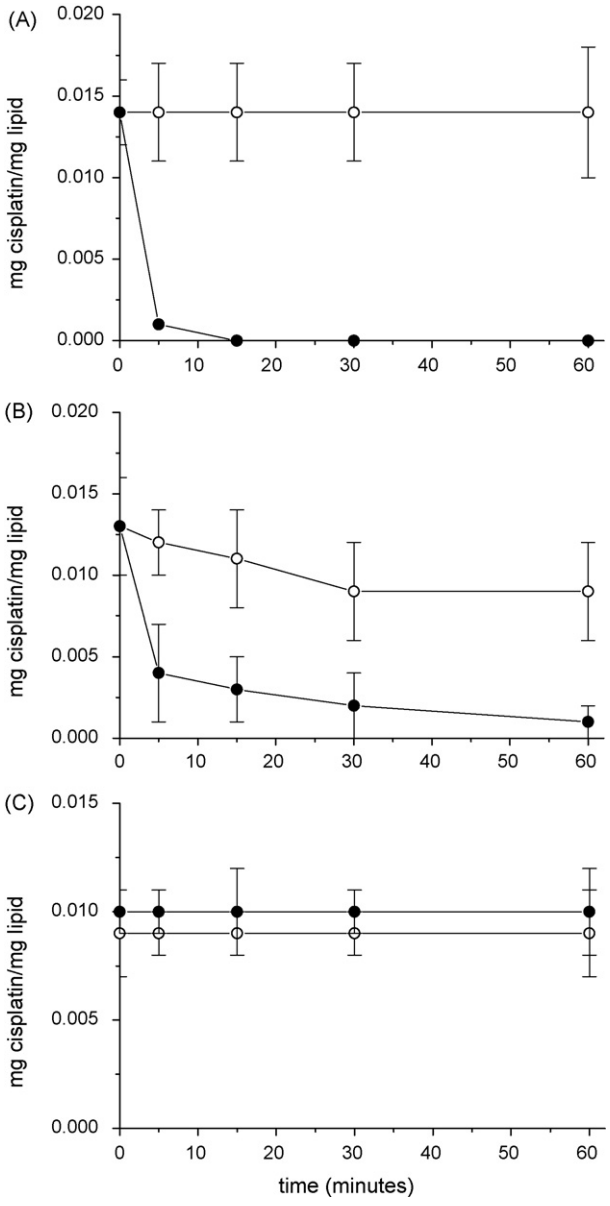

Fig. 4. Time-dependent release of cisplatin from (A) LTSL, (B) TSL and (C) DPPC/chol/DSPE-PEG$_{2000}$ liposomes at 37 °C (open symbols) and 42 °C (filled symbols). At the indicated time points during the incubation, aliquots of sample were taken and lipid and drug content were analyzed as described in the methods. Each point represents mean ± S.D. ($n = 3$).

was observed for DPPC/Chol/DSPE-PEG$_{2000}$ liposomes when incubated at 37 or 42 °C. Taken together, these in vitro data demonstrate that the passive equilibration loading method did not compromise the thermosensitivity of the LTSL- and TSL-cisplatin formulations, and that lysolipid is important for the enhancement of membrane permeability based on its role in stabilizing the nanopores in grain boundaries of LTSL (Sandstrom et al., 2005).

It is established that in vitro drug release profiles may not correlate with in vivo drug release (Shabbits et al., 2002). For this reason, pharmacokinetic studies of cisplatin encapsulated in LTSL, TSL and DPPC/Chol/DSPE-PEG$_{2000}$ liposomes were performed in mice. The objectives of these studies were

to determine: (1) whether the cisplatin-loaded thermosensitive liposomes were long circulating, and (2) whether cisplatin was well retained within circulating liposomes. The studies were completed under conditions where the core body temperature of the mouse was carefully controlled at 37 °C because handling during injections and following anesthesia cause wide fluctuations in body temperature. Results were presented and summarized in Fig. 5 and Table 2.

As expected, free, unencapsulated cisplatin was rapidly eliminated from plasma, with <1% of the injected dose remaining after 15 min (Fig. 5A, ♦), whereas the plasma cisplatin concentrations were significantly higher when the drug was administered in the liposomal formulations. This is reflected by the $AUC_{0–8h}$ values of 0.27, 0.16 and 0.06 mg h mL^{-1} for DPPC/Chol/DSPE-PEG_{2000} liposomes, TSL and LTSL, respectively (Table 2). The LTSL carrier was less stable than TSL and DPPC/Chol//DSPE-PEG_{2000} liposomes, as reflected by the % injected lipid dose remaining in plasma (Fig. 5B). The plasma cisplatin-to-liposomal lipid ratios (Fig. 5C) suggest, however, that 50% of the encapsulated cisplatin was released from LTSL and TSL formulations within 1–1.5 h following injection. The TSL-cisplatin formulation exhibited improved in vivo drug retention properties compared to the LTSL formulation, as reflected by higher D:L ratios remaining in plasma at 4 and 8 h. The DPPC/Chol/DSPE-PEG_{2000} liposomal formulation of cisplatin, however, showed a significantly ($p < 0.05$) higher plasma D:L ratio at 8 h compared to those observed following injection of LTSL and TSL (Fig. 5C).

When in vitro and in vivo drug release profiles at 37 °C were compared (Figs. 4 and 5C), two important points can be made. First, the presence of plasma proteins and cellular components (such as red blood cells and macrophages) in circulation may act as potential liposome destabilizing agents, resulting in cisplatin release from the LTSL- and TSL-cisplatin formulations at body temperature (37 °C). Second, the LTSL formulation appears to be the most susceptible to cisplatin leakage when injected into

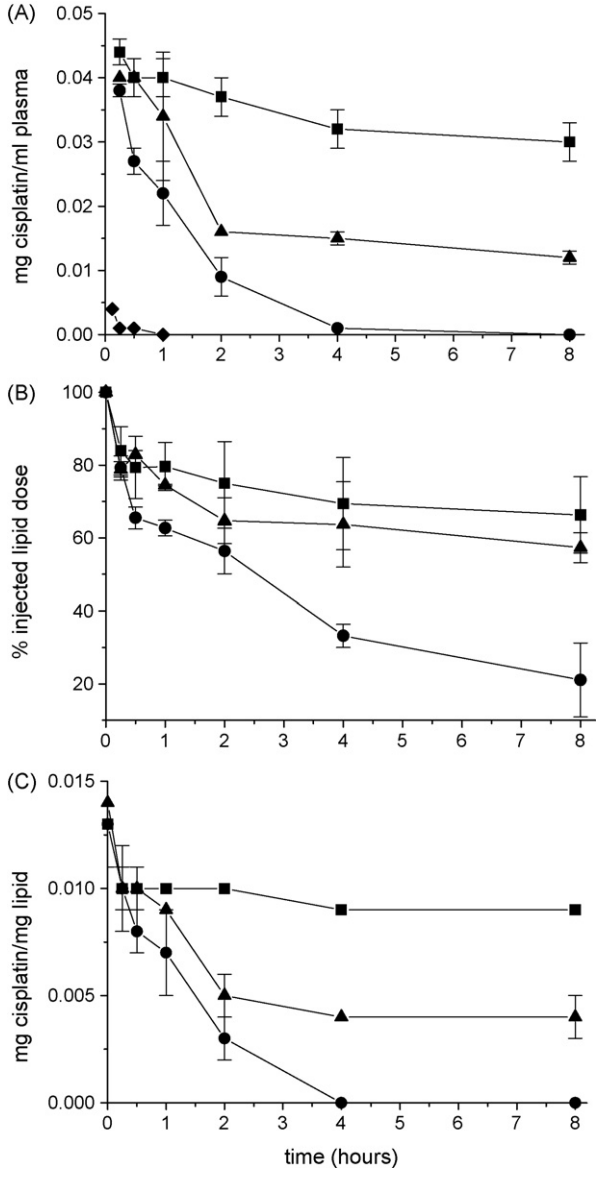

Fig. 5. Plasma elimination and in vivo drug release of free cisplatin (♦), cisplatin-loaded LTSL (●), TSL (▲) and DPPC/chol/DSPE-PEG_{2000} liposomes (■) where body temperature was maintained at 37 ± 0.5 °C throughout the entire time course of the study. (A) Plasma drug levels, (B) plasma liposomal lipid levels, and (C) plasma D:L ratio (w/w) were measured as described in the methods. Each point represents mean ± S.D. ($n = 3$, except TSL $n = 6$).

Table 2
Summary of pharmacokinetic parameters of liposomal cisplatin formulations injected i.v. in Rag2-M mice

	DPPC/MSPC/ DSPE-PEG_{2000} (90:10:4)	DPPC/DSPE-PEG_{2000} (100:4)	DPPC/chol/ DSPE-PEG_{2000} (55:45:4)
$AUC_{0–8h}$[a,b] (mg h mL^{-1})	0.06	0.16	0.27
$AUMC_{0–8\,h}$ (mg h^2 mL^{-1})	0.06	0.42	1.01
MRT_{last} (h)	1.09	2.69	3.71
CL (mL h^{-1})	0.67	0.22	0.04
V_{ss} (mL)	0.76	0.88	0.97
Terminal $T_{1/2}$ (h)	1.09	2.83	16.42
R^2	0.89	0.92	0.89
Corr (*x*:*y*)	−0.94	−0.96	−0.94

Rag2-M mice (20–22 g) were injected with 2 mg/kg cisplatin as a single IV bolus dose via the lateral tail vein ($n = 3$). Blood was collected and processed as described in Section 2.9.

[a] Noncompartmental analysis based on the linear trapezoidal rule was performed using the software WinNonlin Version 1.5 to estimate the values of various pharmacokinetic parameters. Five to six time points were used for the estimation of the terminal elimination phase. The dose was entered as an IV bolus with no lag time and first-order elimination was assumed. Uniform weighting was used.

[b] $AUC_{0–8\,h}$, area under curve from time of dosing ($t = 0$ h) up to the last measured concentration ($t = 8$ h); $AUMC_{0–8\,h}$, area under moment curve from time of dosing ($t = 0$ h) up to the last measured concentration ($t = 8$ h); MRT_{last}, mean residence time which is calculated based on values up to the last measured concentration ($t = 8$ h) and is not extrapolated to infinity; CL, total body clearance (predicted); V_{ss}, volume of distribution at steady state (predicted); terminal $T_{1/2}$, half-life of the terminal elimination phase; R^2, coefficient of determination for the points used in the estimation of the first-order rate constant associated with the terminal portion of the plasma concentration–time curve via linear regression; Corr (*x*:*y*), correlation coefficient for the points used in the estimation of the first-order rate constant associated with the terminal portion of the plasma concentration–time curve via linear regression.

circulation, suggesting that plasma proteins and cellular components may have greater destabilizing effects on the LTSL formulation than on TSL and cholesterol-containing liposomes. Currently, the interaction of LTSL carrier with plasma proteins and cellular components are being investigated in our laboratory, with emphasis on the role of lysolipid in LTSL in vivo stability.

From the in vivo drug release profiles (Fig. 5C), ~50% of cisplatin was released from LTSL and TSL within 1–1.5 h, and based on these biological performances of the formulations, the results suggest that the thermosensitive cisplatin formulations developed here could be efficacious under conditions where mild hyperthermia is applied prior to or concurrent with the injection of the formulations (Ponce et al., 2007). Indeed, this is the current administration protocol for the LTSL-doxorubicin formulation in combination with mild hyperthermia in clinical trials. Recently, the therapeutic effect of LTSL-doxorubicin has been suggested to be due to release of liposomal contents within the microvessels of the heated tumor, and this specific delivery of drug due to rapid, localized release within the tumor vasculature has led to apoptosis of tumor endothelial cells and subsequent shutdown of tumor blood supply (Needham and Dewhirst, 2001; Chen et al., 2004). Interestingly, cisplatin-based combination chemotherapy has recently been shown to sensitize or trigger apoptosis of endothelial cells (Goto et al., 2004; Yap et al., 2005). Thus, it would be of value to evaluate the molecular effects of LTSL-cisplatin in combination with mild hyperthermia on tumor endothelial cells in light of the potential as a vascular targeting approach for cancer treatment.

4. Conclusion

The studies presented here focused on the development and characterization of a versatile method to prepare cisplatin-loaded thermosensitive liposomes. The passive equilibration loading method will likely be most useful for liposomes prepared without cholesterol or those that contain cholesterol incorporated at levels which would not significantly decrease membrane permeability, and it will be applicable to other small molecule drug compounds. More importantly, this loading method did not compromise the thermosensitivity of LTSL and TSL formulations. Our data suggest that the physicochemical properties of the therapeutic agent to be encapsulated in thermosensitive liposomes by passive equilibration method will affect the rate of drug loading, which in turn, may influence the rate of drug release from the liposomes following administration. The LTSL-cisplatin formulation could be further developed for use in combination with clinical hyperthermia, and we have a particular interest in pursuing this formulation for the treatment of head and neck cancers where cisplatin is active as a single agent (Einhorn and Williams, 1979) and hyperthermia is commonly used (Valdagni et al., 1988; Valdagni and Amichetti, 1994). The formulation is likely to be administered in conjunction with or after mild hyperthermia to achieve therapeutic effects—an approach that is currently undertaken for the administration of the LTSL-doxorubicin formulation. What remains to be addressed in pre-clinical models is whether this approach could reduce cisplatin-induced nephrotoxicity while enhancing the therapeutic activity of cisplatin.

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