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RESEARCH PAPER

Strategies to Maximize Liposomal Drug Loading for a Poorly Water-soluble Anticancer Drug

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ABSTRACT

Purpose To develop a liposomal system with high drug loading (DL) for intravenous (i.v.) delivery of a poorly water-soluble basic drug, asulacrine (ASL).

Methods A thin-film hydration and extrusion method was used to fabricate the PEGylated liposomal membranes followed by a freeze and thaw process. A novel active drug loading method was developed using ammonium sulphate gradient as an influx driving force of ASL solubilized with sulfobutyl ether-β-cyclodextrin (SBE-β-CD). DL was maximized by optimizing liposomal preparation and loading conditions. Pharmacokinetics was evaluated following i.v. infusion in rabbits.

Results Freeze-thaw resulted in unilamellar liposome formation (180 nm) free of micelles. Higher DL was obtained when dialysis was used to remove the untrapped ammonium sulphate compared to ultracentrifuge. The pH and SBE-β-CD level in the loading solution played key roles in enhancing DL. High DL ASL-liposomes (8.9%*w/w*, drug-to-lipid mole ratio 26%) were obtained with some drug "bundles" in the liposomal cores and were stable in a 5% glucose solution for >80 days with minimal leakage (<2%). Surprisingly, following administration of ASL-liposomes prepared with or without SBE-β-CD, the half-lives were similar to the drug solution despite an increased area under the curve, indicating drug leakage from the carriers.

Conclusions High liposomal DL was achieved with multiple strategies for a poorly-water soluble weak base. However, the liposomal permeability needed to be tailored to improve drug retention.

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KEY WORDS active loading · cyclodextrin · liposomes · pharmacokinetics · supersaturated

ABBREVIATIONS

ASL	Asulacrine
ASL-L	Asulacrine liposomes
Cryo-TEM	Cryo-Transmission electron microscopy
DL	Drug loading
DLS	Dynamic light scattering
EE	Entrapment efficiency
EPR	Enhanced permeability and retention
PDI	Polydispersity index
PEG	Polyethylene glycol
PIP	Post-injection precipitation
RES	Reticuloendothelial system
SBE-β-CD	Sulfobutyl ether-β-cyclodextrin
TFH	Thin-film hydration

INTRODUCTION

Liposomes are established carriers for anticancer drugs due to their potential of targeting solid tumors and reducing systemic toxicity by exploiting the enhanced permeability and retention (EPR) effect [1, 2]. Controlled drug release from the liposomes during circulation is essential to ensure enough drug to reach the tumor site; and a sufficient drug content in the carriers could lead to efficient drug uptake by the cancer cells [3, 4]. Therefore, controlled drug release and high drug loading are vital in liposomal preparation. However, both have often posed a number of technical challenges to the formulation scientists.

Drugs can be incorporated into liposomes by either a passive or active (remote) loading method. With the former approach, drugs are loaded during liposome formation [5]; whereas in remote loading, drugs are loaded in the preformed liposomes with an additional trans-membrane potential as the

driving force(s). Compared to passive loading, the remote loading method has been proven to be more effective in achieving a higher drug-to-lipid ratio due to its active pumping mechanism and more controlled drug release by "locking" the drug inside liposomes [6]. Various chemical gradients can be used as an influx driving force depending on the drug properties. Ammonium sulphate [7] and calcium acetate [8] are employed for weak bases and acids, respectively. EDTA [9], cyclodextrin [10] and transition metals [11] are used for drugs with which a less soluble complex can be formed in the vesicles.

Although remote loading methods are superior to passive loading, a poorly water-soluble drug seems to defy ready encapsulation via the trans-membrane transport due to its low concentration gradient. Zucker *et al.* [12] reported a working model to predict loading efficiency based on a study of nine model drugs with different physicochemical properties. As described in this model, the poor aqueous solubility of drug candidates is usually a major limitation for efficient active loading. Boman *et al.* [13] have constructed an equation describing that the drug uptake rate into liposomes was proportional to the extra-liposomal concentration of the neutral species of the drug. In practice, a high trans-membrane concentration gradient is difficult to achieve given the poor water solubility of compounds especially in neutral species. To address this problem, supersaturated drug solutions with or without the use of cyclodextrin (CD) have been recently explored by Anderson's group [14] and have achieved a high drug-to-lipid ratio (17% mole ratio). This novel strategy has not been widely applied to different poorly water-soluble drugs. Also, the effects of CD on the liposome physicochemical stability and particularly the *in vivo* retention properties have not yet been reported.

Asulacrine (ASL), 9-[2-methoxy-4-methylsulphonylamino) phenylamino]-N, 5-dimethyl-4-acridinecarboxamide, also known as SN 21407 or CI-921 (Fig. 1), is an analogue of amsacrine synthesized by The University of Auckland, New Zealand [15]. ASL is an inhibitor of topoisomerase II [16] and has shown greater activity than amsacrine against breast and lung cancer in clinical trials Phase I and II [17, 18]. However, phlebitis resulting from intravenous (i.v.) infusion hampered its further development. ASL is a weakly basic drug with a reported pK_a value of 6.7 [19] and was administrated as a 1 mg/ml solution of isethionate salt (pH ~ 4). Our preformulation data [19] showed that the water solubility of ASL was high in acidic solutions, however, it was remarkably reduced at physiological pH (843 μg/l at pH 7.4), possibly resulting in post-injection precipitation (PIP) in the vein [20, 21]. Therefore, it was envisioned that the irritancy of drug as well as PIP [22] were the probable main causes of phlebitis for ASL. Based on their ability to separate the encapsulated drugs from the surrounding tissues and to prevent PIP, reducing the degree of tissue damage [23, 24], liposomes are proposed as a new formulation for parenteral administration to improve the venous tolerance with favorable pharmacokinetic behavior and tumor targeting.

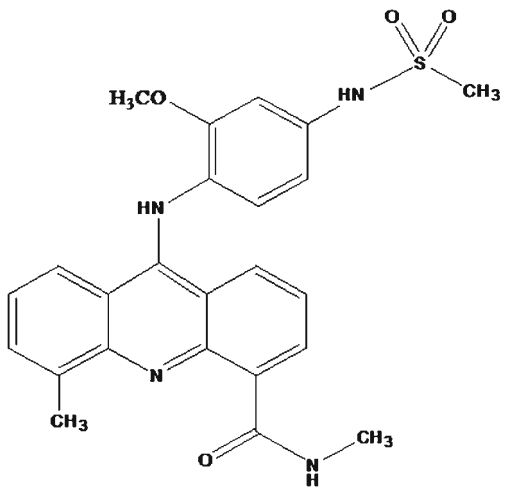

Fig. 1 Chemical structure of ASL free base.

The aim of this paper was to develop a stable long-circulating liposome system containing a high content of ASL for i.v. administration. ASL also served as a "challenging" model drug with low water solubility, high log P and a pK_a (6.7) close to intra-liposomal pH (pH = 5.6 with 250 mM ammonium sulphate for developing strategies to improve drug loading in liposomes for active reagents of its kind. To obtain a high drug-to-lipid ratio (drug loading, DL) and entrapment efficiency (EE), the remote loading using an ammonium sulphate gradient was employed, and a negatively charged cyclodextrin, sulfobutyl ether-β-cyclodextrin (SBE-β-CD) was utilized to create a highly concentrated supersaturated solution for drug loading [14] in addition to acidification by using its isethionate salt. The preparation process was optimized to maximize the volume of aqueous cores in the liposomes and trans-membrane ammonium sulphate gradient prior to drug loading. DL and EE were maximized through manipulation of the key factors affecting extra-liposomal drug concentration and pH, SBE-β-CD levels and loading duration. Finally, the effects of SBE-β-CD on the liposome stability, particularly the *in vivo* drug retention, were investigated following a one-hour i.v. infusion in New Zealand white rabbits.

MATERIALS AND METHODS

Materials

The phospholipids, 1,2-dipalmitoyl-sn-glycero-3-phospocholinemonohydrate (DPPC), N-(carbonyl-methoxy-polyethyleneglycol 2000) -1,2- distearoyl-sn-glycero-3-phosphoethanolamine (DSPE-mPEG 2000) were purchased

from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol was obtained from Sigma-Aldrich Co., Ltd. Asulacrine isethionate salt (99% pure) was synthesized and kindly provided by Auckland Cancer Society Research Centre, The University of Auckland. Sulfobutyl ether-β-cyclodextrin (Captisol®) (SBE-β-CD) was a gift sample from Captisol Technology (La Jolla, USA). All other reagents used in this study were of analytical grade except methanol and acetonitrile of chromatographic grade.

Animals

New Zealand white rabbits were housed in individual cages in an animal room maintained at 23 ± 3°C and 55 ± 10% relative humidity with ventilation 13-16 times per hour and a 12-h light–dark cycle. The rabbits were allowed free access to diet and water. This animal study was approved by the Committee on Animal Experiments of The University of Auckland (Ethics Approval No. C881).

Preparation of Liposomes with and without Freeze-thaw

The preformed liposomes (empty) were prepared according to a thin-film hydration (TFH) method followed by extrusion. Briefly, DPPC, DSPE-mPEG 2000 and cholesterol (mole ratios 6:1:3, total mass 20 mg) were dissolved in 1 ml of chloroform: methanol (3:1, *v*/*v*) and dried in an eggplant-shaped flask using a rotary evaporator under vacuum condition (R-215, Büchi, Switzerland). The thin film obtained was hydrated with 1 ml of 250 mM ammonium sulphate solution at 45°C for 10 min, followed by ultrasonication for 180 s (70 amplitude) using a probe sonicator (UP200S Ultrasonic processor, Germany) or subjected the hydrated suspension to 3, 5, 7 or 10 freeze-thaw cycles to optimize the process for maximized formation of unilamellar liposomes [25] and absorbing any potential micelles formed by the PEG-lipid during hydration [26]. Each cycle consisted of freezing in liquid nitrogen at−180°C for 3 min and thawing in water bath at 45°C for 7 min. Thereafter, the liposome suspension was extruded through 0.2 μm and then 0.1 μm pore sized polycarbonate membrane filters (Whatman, UK) with a stainless steel Avanti extruder fitted with two gas-tight 1 ml syringes (Hamilton, USA) in formulation development, or a 10 ml LIPEX™ Extruder (Northern Lipids Inc, Burnaby, Canada) for animal studies. After extrusion, the size, polydispersity index (PDI) of the liposomes, and the mass of the liposome pellet (after ultracentrifugation) with different number of cycles of freeze-thaw were compared.

The optimum process to prepare liposomes to load the drug was chosen after confirmation of the absence of micelles with cryo-transmission electron microscopy (cryo-TEM).

Optimization of Remote Drug Loading Conditions

To maximize DL and EE, various factors were investigated including methods to remove free ammonium sulphate, concentrations of drug loading solutions and the presence or absence of SBE-β-CD, loading duration and extra-liposomal pH, as well as SBE-β-CD concentration, as follows:

(1) A trans-membrane ion gradient of ammonium sulphate was generated by removing the untrapped ammonium sulphate using either dialysis against an iso-osmotic NaCl solution (100 ml for 1 ml liposomes each time) 4 times for a total of 20 h at 37°C or ultracentrifuge at 188, 272×*g* (4°C) for 1 h in a F50L-24×1.5 rotor within Sorvall® WX 80 Ultra centrifuge (Thermo Scientific, Auckland, New Zealand).
(2) The above blank liposomes with higher trans-membrane ion gradient were incubated at 37°C with the same volume of saturated (with excess drug in solid) or supersaturated drug solution established by 5% *w*/*w* SBE-β-CD. The final drug-to-lipid weight ratio (including the excess drug in solid) was kept at 1:10 in both cases and the loading solutions were adjusted to different pHs. After incubation for 1.5 h, excess drug precipitate was removed firstly by centrifuge at 700×*g* for 10 min. Then the supernatant containing free drug, ASL-CD complex and SBE-β-CD was removed immediately by ultracentrifuge at 188, 272×*g* (at 4°C) for 1 h. EE and DL were compared with different loading solutions.
(3) After the loading solution conditions were selected, the maximum concentration of SBE-β-CD in supersaturated drug loading solution was determined at which the integrity of liposomes was not damaged. The above blank liposomes were incubated at 37°C with the same volume of supersaturated drug solution established by 4%, 5%, 6%, 7% *w*/*w* SBE-β-CD with drug-to-lipid weight ratio kept at 1:10. After incubation for 1.5 h, the EE was determined as described below.
(4) To optimize the loading pH and duration, various concentrated supersaturated drug solutions in the presence of SBE-β-CD were used for drug loading, and dynamic drug uptake process was monitored over 3 h. Blank liposomes with a trans-membrane ammonium sulphate gradient were incubated with drug solutions containing the optimal ratio of SBE-β-CD with pH adjusted to 4.25 (the initial pH of drug solution as salt), 5, 5.4 and 5.8, respectively. At each pre-determined time interval, 100 μl aliquots of ASL-L suspension were taken to determine the EE.

The finished ASL-liposomes (ASL-L) were centrifuged and the pellets were re-suspended with a glucose solution (5%, *w*/*v*) for further studies.

Size, Zeta Potential, Entrapment Efficiency and Drug-loading of ASL-L

Particle sizes and PDI of ASL-L were measured by dynamic light scattering (DLS) using a Malvern Nano ZS (Malvern Instruments, UK). Samples were diluted with distilled water to obtain liposome suspensions with lipid concentration below 10 mg/ml. Zeta potentials of liposomes were measured in glucose solution using the same instrument. All measurements were conducted at 25°C in triplicate.

To determine EE and DL, unencapsulated drug existed in the form of solute and/or crystal was separated from liposomes by 2 steps. Firstly, the drug precipitate was removed from the nano-liposome suspension by centrifuge at 700×*g* for 10 min. Then the supernatant containing ASL-L (C_1) was ultra-centrifuged at 188, 272×*g* (4°C) for 1 h allowing liposomes to be separated from the drug solution (C_f) including free soluble ASL and ASL-CD complex. The concentration of drug was determined by HPLC after extracting the drug with acetonitrile. EE and DL were calculated using the following formulae:

$$EE(\%) = \frac{\left(C_1 - C_f\right) \times V_{total}}{M_{drug}} \times 100 \quad (1)$$

$$DL(\%) = \frac{\left(C_1 - C_f\right) \times V_{total}}{M_{lipids} + M_{drug}} \times 100 \quad (2)$$

where V_{total} is the total volume of original liposomes, M_{drug} and M_{lipid} is the mass of the drug and total lipids used in the liposome preparation, respectively.

The concentration of ASL was analyzed by a validated stability-indicating HPLC method. The HPLC method employed an Aglient 1200 instrument, a Phenocifcifmenex RP18 column (150×4.6 mm, 5 μm), and a 20 mM monopotassium phosphate (pH 2.5) buffer-acetonitrile (72:28, *v*/*v*) mobile phase with a flow rate of 1 ml/min. The detection wavelength was set at 254 nm and the injection volume was 20 μl.

Cryo-Transmission Electron Microscopy

Morphology of empty and drug-loaded liposomes prepared with different freeze-thaw cycles, were analyzed by cryo-TEM. A drop of the sample (theoretical lipid concentration 10 mg/ml) was placed on the copper grid in the climate chamber and blotted, leaving a thin film stretched over the holes. The samples were shock-frozen by dipping into liquid ethane and cooled to 90 K by liquid nitrogen. The samples were transferred to the Tecnai 12 electron microscope (FEI, Hillsboro, USA) operating at 120 KV. Liposomal membrane lamellae, presence of micelles and the drug form in the aqueous cores were observed.

ASL-liposomes Long-term Stability

The optimized ASL-L in 5% glucose solution were stored at 4°C in the dark. The size, zeta potential and drug leakage were monitored over 80 days. The drug leakage ratio was calculated as the increased percentage of free drug over storage.

Pharmacokinetics Following Intravenous Infusion

The pharmacokinetics of ASL-L prepared with SBE-β-CD (DL =8.9% *w*/*w*) was compared with an ASL solution in 5% glucose (pH =4), simulating that used in the clinical trial [17, 18]. To investigate the effect of SBE-β-CD on membranes, liposomes actively loaded using a saturated drug solution at pH 4.25 (1 mg/ml) without SBE-β-CD (DL =4.9% *w*/*w*) was also tested. All the formulations were adjusted with 5% glucose solution to the same drug concentration of 0.5 mg/ml and sterilized by filtration. Twelve rabbits (body weight 3.0–3.5 kg) were randomly divided into 3 treatment groups (n =4). The formulations were administrated by a 1 h-infusion via the ear vein at ASL dose of 6.67 mg/kg through a 23 gauge plastic catheter with outer diameter of 0.65 mm (Terumo, Tokeyo, Japan). The rate of administration was set and maintained at 20 mg/h by a syringe pump (Model KDS200, KD Scientific Inc., USA). At the end of infusion (0 h) and 0.17 (10 min), 0.5, 1, 1.5, 2, 3, 5, 8, 12 and 24 h post infusion, 1 ml blood samples were collected from the contralateral ear vein.

The blood samples in heparinized tube were immediately centrifuged (700×*g*, 10 min, 25°C) to obtain the plasma. Then 100 μl of plasma was mixed with 700 μl acetonitrile and vortex mixed for 2 min. After centrifuging the mixture, all the supernatant was taken and dried in a SpeedVac (SVC 100 H; Savant Instruments Inc.) at 25°C. HPLC mobile phase was added to re-dissolve the residue. The concentration of ASL was analyzed by HPLC as described for EE determination. The drug concentration was linear ranging from 0.1 to 5 μg/ml with absolute recoveries >90%.

The non-compartmental pharmacokinetic parameters were calculated using DAS software program (version1.0, Pharmacometrics Professional Committee of China, Shanghai, China).

Statistical Analysis

Student *T*-test for data comparison was performed using GraphPad Prism 6, version 6.01 (GraphPad Software, Inc.). The *p* value for significance was set at 0.05.

RESULTS

Effect of Freeze-thaw on Liposome Formation

The results (Table 1 and Fig. 2) showed that with the increase of freeze and thaw cycles the size of liposomes became more uniform (PDI values reduced). Increased volume and mass of liposome pellets were obtained as the cycles number increased, reaching a maximum at 7 cycles with no significant increase at 10 cycles ($p > 0.05$). Therefore, 7 cycles of the freeze and thaw process was used in the rest of the studies. In addition, no significant changes to zeta potential occurred between samples subjected to all the cycles (Table 1).

The Cryo-TEM micrographs (Fig. 3) revealed that most liposomes had unilamellar structures. The blank liposomes without freeze-thaw treatment had fewer vesicles and exhibited a 'sun' like image: each of the liposomes was surrounded with tiny disks. These disks were reported to be micelles formed by PEG lipids [26]. However, there were no disks found in liposomes prepared with 7 or 10 cycles of freeze-thaw (Fig. 3b).

The DLS (Nano ZS) gave a larger average size than TEM as it measured the hydrodynamic diameter of the particles [27], which could be dehydrated and therefore shrunk (~150 nm) during TEM sample preparation.

Effect of Method of Removing Ammonium Sulphate on EE and DL

The dialysis method appeared to be more efficient in removing the free ammonium sulphate and resulted in higher EE and DL (98.20 ± 0.10 and 8.93 ± 0.08% respectively) compared to the ultracentrifuge method (80.43 ± 1.25 and 7.07 ± 0.08% respectively). Therefore, the dialysis method was employed to remove ammonium sulphate for later drug loading.

Table I The effect of Freeze-thaw Cycles on the Formation of Liposomes as the Mass Collected from Tubes (mean ± SD, $n = 3$). Liposomes Contained 10% PEGylated Lipid and no Drug

Cycles	Size	PDI	Zeta potential	Recovery as pellet mass (mg)
0[a]	184.7 ± 1.53	0.089 ± 0.003	-47.3 ± 1.5	2.4 ± 0.6
3	183.9 ± 1.37	0.060 ± 0.002	-48.8 ± 2.1	2.8 ± 0.4
5	183.5 ± 1.70	0.058 ± 0.001	-47.4 ± 1.9	3.7 ± 0.1
7	185.2 ± 1.27	0.047 ± 0.001*	-48.6 ± 2.0	7.0 ± 0.2*
10	189.2 ± 0.36	0.044 ± 0.001*	-49.3 ± 2.4	7.2 ± 0.2*

[a] by ultrasonication without freeze-thaw treatment

*represent significant differences from 0, 3 or 5 cycles ($p < 0.05$)

Determination of the ASL Concentration for EE and DL

Both the pH and presence of SBE-β-CD in the loading solution affected ASL concentration in the loading solution directly, resulting in different EE and DL (Table 2). When saturated solutions (with excess solid drug) were used for drug loading, low EE (49%) and DL (4.5%) were obtained at pH 4.25 and decreased dramatically as concentration dropped when pH increased to 5.8. In contrast, only when SBE-β-CD was used in drug loading, supersaturated solutions at higher drug concentrations at different pH were created. Drug loading was maximized by 5.6 times with the aid of SBE-β-CD, 8.9% *w/w* at pH 4.25 with no change when pH increased to 5.4. However, EE and DL decreased as pH increased to 5.8. Therefore the supersaturated drug solution with SBE-β-CD as loading solution at low pH was better for drug loading.

Optimization of SBE-β-CD Concentration

Maximized EE was achieved when 5% SBE-β-CD were employed. Although 4% SBE-β-CD could keep ASL solubilized at a supersaturated state initially, precipitation was observed after loading for 1.5 h. As SBE-β-CD concentration rose to 6 and 7%, EE dropped to 72.59 ± 0.23 and 23.23 ± 0.12% respectively without drug precipitation and in a concentration-dependent manner along with a reduced mass of liposome pellets.

Loading Kinetics at Different Extra-liposomal pH

As seen from Fig. 4, the maximal uptake of ASL by liposomes was accomplished at 1 h with EE above 90% regardless of the pH used, and the initial drug influx was faster with elevated pH. After 1 h, however, there was a drug efflux observed at pH 5.8 resulting in a low EE with drug precipitation observed at this pH (where more than 10% drug was unionized).

Therefore a loading duration of 1 h with pH at 4.25 (the initial pH of drug solution as salt) with addition of 5% SBE-β-CD was selected for the preparation of final formulation for cryo-TEM observation, stability and pharmacokinetic study. The physicochemical properties were shown in Table 3. Figure 3 C showed that drug-loaded liposomes appeared more electron-dense under Cryo-TEM compared with empty liposomes and a large proportion of trapped drug in liposomes was globular. Some formed bundled or fibre-like structures (precipitates) which induced a slight change in liposomal shape into a 'coffee bean' appearance, similar to doxorubicin-liposomes loaded via an ammonium sulphate gradient method [28].

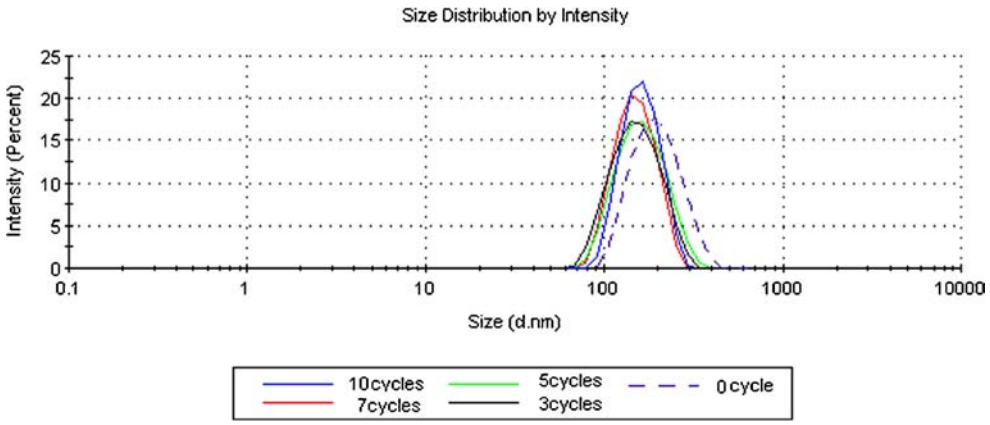

Fig. 2 Size and size distribution of the liposomes subjected to different cycles of freeze-thaw, 0 cycle means samples were treated by ultrasonication without freeze-thaw.

Long-term Stability of ASL-L

The optimized ASL-L were prepared as following: the empty liposomes were prepared with 7 cycles of freeze-thaw and dialysis, then incubated with supersaturated drug solution with 5% SBE-β-CD at 37°C for 1 h with extra-liposomal pH at 4.25. ASL-L were centrifuged immediately and resuspended in 5% glucose solution (pH ~6), an i.v. infusion fluid frequently used in clinic. Table 4 shows that the size and zeta potential did not change significantly after storage for 80 days ($p>0.05$). The total drug concentration in the samples was maintained above 95% of the original, and the drug leakage ratio was minimal, suggesting satisfactory physical and chemical stability of ASL-L.

Pharmacokinetics in Rabbits

Figure 5 depicts the pharmacokinetic profiles of ASL-liposomes prepared with (ASL-L + CD) and without (ASL-L-CD) SBE-β-CD in comparison with the ASL solution following 1 h i.v. infusion in the rabbits. The drug concentration-time profile of the ASL solution followed a one-compartment model with logarithms of all the concentration data *vs* time linear ($R^2>0.99$). Although the onset concentrations of both ASL-L + CD and ASL-L-CD were higher than those of ASL-solution, showing a bigger area under the curve (AUC) ($p>0.05$), a smaller volume of distribution (V_d) ($p>0.05$) and a slower distribution process from the central bloodstream, interestingly, the clearance phases of both ASL-Ls, prepared with CD and without CD, conincided with that of ASL-solution with a similar short half-life ($p>0.05$). Also, no significant differences ($p>0.05$) were found on all the phamacokinetic parameters between ASL-L prepared with or without SBE-β-CD despite the different DL in the two formulations.

DISCUSSION

In this study combination strategies were used to improve drug loading into the PEGylated liposomal system for a poorly water-soluble weak base. With the aid of a cyclodextrin as a solubilization enhancer and the manipulation of the conditions of active drug loading, a maximal DL of 8.9% w/w (drug-to-lipid mole ratio 26%) with high EE (>98%) were achieved. The liposomes showed little leakage over 80 days. These results may also be extended to other drugs with similar

Table 2 Effects of ASL Concentration on EE and DL in Preparation of ASL-liposomes. The Drug Loading Time and Temperature was 1.5 h and 37°C Respectively (Data are means ± SD, $n=3$)

External drug loading medium		Extra-liposomal pH		
		4.25	5.4	5.8
Saturated solution	Initial soluble drug concentration (μg/ml)	934.25 ± 0.47	223.95 ± 0.50	26.50 ± 0.18
	DL (%)	4.53 ± 0.21	1.60 ± 0.04	1.57 ± 0.06
	EE (%)	49.83 ± 0.16	17.60 ± 0.15	17.27 ± 0.12
Supersaturated solution with SBE-β-CD	Initial soluble drug concentration (μg /ml)	2,000.24 ± 0.41	2,000.15 ± 0.28	2,000.19 ± 0.36
	DL (%)	8.93 ± 0.08	8.95 ± 0.09	6.44 ± 0.07
	EE (%)	98.20 ± 0.10	98.47 ± 0.14	70.84 ± 0.18

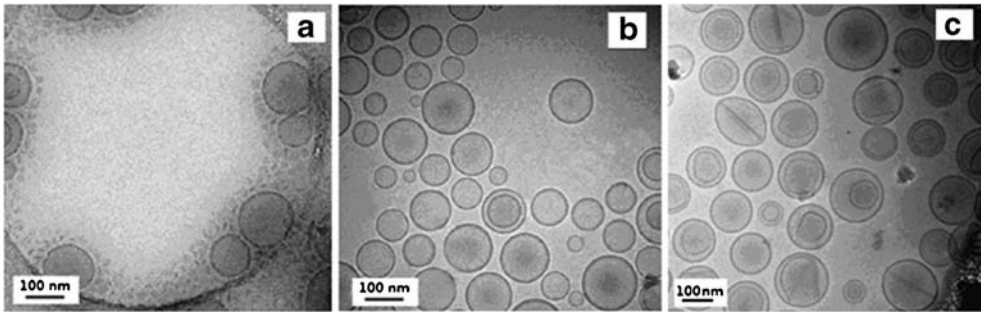

Fig. 3 Cryo-TEM micrographs of blank liposomes (**a** and **b**), and liposomes containing 8.9% of ASL (**c**). **a** is sample prepared with ultrasonication without freeze and thaw, showing the presence of micelles with less number of liposomes; **b** and **c** were typical liposomes prepared with 7 or 10 freeze-thaw cycles.

physicochemical properties. However, the short half-life *in vivo* indicated poor drug retention in the carriers despite the formation of drug 'bundles' (precipitates) in the liposomal cores. The possible mechanisms for efficient drug loading and the absence of long circulation are discussed below.

In a liposomal active drug loading model with an acidic interior, the quantity of loaded amino-containing drugs was reported to be proportional to 1) the volume of aqueous phase of liposomes, 2) the extra-liposomal concentration of neutral drug and 3) the trans-membrane proton gradient with a favorable interior concentration of H^+ [29]. In the present study of maximization of the drug loading, various factors were investigated and optimized (Fig. 6).

Firstly, the maximum entrapment volume of aqueous cores which accommodate the drug, was achieved by reducing the number of lamellae of the liposomes [25]. A freeze-thaw process was applied and unilamellar nano-sized liposomes were obtained (Fig. 3). It was also found that sufficient freeze and thaw was effective in preventing formation of micelles by the PEGylated lipid, increasing the amount of liposomes. It is well known that modification of liposomes with polyethylene glycol (PEG) can protect liposomes from elimination by the reticulo-endothelial system (RES) and achieve prolongation of blood circulation lifetime. The *in vivo* circulation time of PEGylated liposomes was prolonged with increased PEG content when the ratio of PEG is within 10% (mole) [30]. However, the use of >5% PEG lipid could result in formation of micelles [26], which were also shown in cryo-TEM photos in this study where disk-like images were revealed, similar to those reported [26], in samples without freeze-thaw treatment. These disks disappeared when more than 7 cycles of freeze-thaw were applied, leading to an increase in the mass of resulting liposomes.

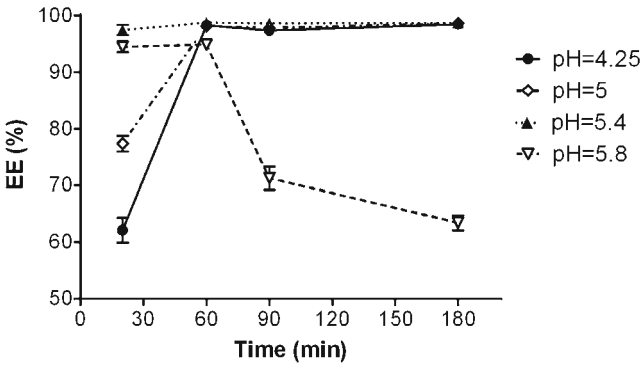

Fig. 4 Kinetic profiles of ASL uptake by liposomes at different extra-liposomal pH in the presence of 5% SBE-β-CD (means ± SD, $n = 3$).

Of the two methods to remove the untrapped ammonium sulphate, dialysis appeared to be more efficient than ultracentrifuge resulting in a higher EE and DL. With the ammonium sulphate gradient method to load the drug, after removing the extra-liposomal ammonium sulphate, the higher concentration of ammonium inside the liposomes caused efflux of the neutral ammonia molecules. For every ammonium molecule that leaves the liposome, one proton is left inside. Thus, an imbalanced pH gradient is formed. The free uncharged ASL base passing through the membrane would be protonated intra-liposomally. Then the new extra-liposomal, non-protonated ASL can diffuse into the liposome due to the concentration gradient. Therefore a 'proton pool' inside the liposomes is necessary as a driving force for ASL loading and accumulation. Apart from the protonation and charging effect, precipitation of ASL (Fig. 3) in the hydrophilic interior of the vesicle also served as a driving force. Unlike ultracentrifugation, dialysis does not only remove the external ammonium sulphate, but it also allows an efflux of ammonium from liposomes leaving more protons in the liposomal cores prior to the active drug loading, which highly affects the rate of drug uptake by liposomes. For most soluble drugs, the rate of uptake was not so important because a similar high EE can be achieved by extending the loading time [12]. However, it would be crucial when a supersaturated solution was used, as

Table 3 Physicochemical Stability of ASL-liposomes Stored in 5% Glucose and Kept in the Dark at 4°C (means ± SD, $n = 3$)

Storage time (days)	Size (nm)	PDI	Zeta potential (mV)	Leakage ratio (%)
0	182.3 ± 0.6	0.065 ± 0.020	-49.3 ± 0.1	–
10	180.0 ± 1.2	0.092 ± 0.015	-40.8 ± 0.2	0.93 ± 0.01
20	179.9 ± 1.2	0.104 ± 0.008	-45.6 ± 5.1	1.01 ± 0.05
30	179.6 ± 0.7	0.110 ± 0.011	-49.2 ± 0.6	1.45 ± 0.03
80	180.2 ± 0.5	0.107 ± 0.006	-40.2 ± 0.5	1.80 ± 0.03

Table 4 Pharmacokinetic Parameters of ASL Formulations Following i.v. Infusion to Rabbits at a Dose of 6.67 mg/kg (Means ± SD; $n = 4$)

	ASL-S	ASL-L + CD	ASL-L-CD
$T_{1/2}$ (min)	84.28 ± 18.41	81.70 ± 16.77	83.81 ± 14.58
V_d (L/kg)	0.71 ± 0.20	0.56 ± 0.22	0.46 ± 0.20
$AUC_{0\text{-}t}$ (mg·h/L)	19.12 ± 2.27	27.29 ± 10.20	28.81 ± 4.03

a slow transport of the less soluble neutral species would result in drug precipitation in the extra-liposomal medium.

To overcome the limitations of the low aqueous solubility of ASL, a supersaturated drug solution at a high concentration of 2 mg/ml was created by addition of SEB-β-CD. The negatively charged SEB-β-CD (as sodium salt) with a hydrophilic exterior and large molecular weight (2163 Da) could not cross the lipid bilayers but acts as a 'bridge' to deliver drug to liposomes. The most important mechanism by which SBE-β-CD increased DL and EE is its well-known solubilization effect through the inclusion of the neutral species in the hydrophobic cavity, allowing a rich drug solution reservoir for drug loading. Furthermore, SEB-β-CD was able to stabilize the supersaturated ASL solutions, similar to other findings [14] which allowed time for drug to be taken by liposomes. Secondly, ASL was a weak base (pK_a =6.7) and the majority was ionized in the drug loading settings (pH <5.8); one SBE-β-CD molecule (3° substitution) could provide three anions which may bond with a maximum of three positively charged ASL^+, reducing the ratio of neutral ASL and further increasing the solubility. Drug loading could complete very well when pH was lower than 5.8. However, an extra-liposomal pH of 5.8 resulted in incomplete drug loading due to drug precipitate in the supersaturated solution. This may be explained by the fact that SBE-β-CD could not maintain the high drug concentration with less ASL in charged form at higher pH. In the contrast, when the liposomes were loaded with saturated solutions, EE and DL were much lower at all pHs and decreased as pH increased, this is probably due to the lower solubility of ASL at high pH, leading to a lower concentration gradient.

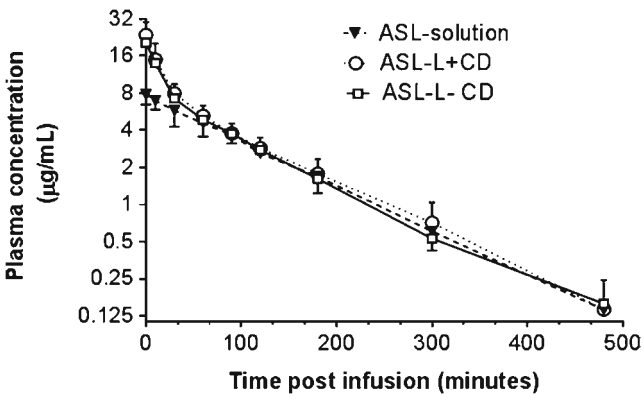

Fig. 5 Pharmacokinetics profiles in rabbits after 1-h i.v. infusion of ASL formulations at a rate of 20 mg/h (the dose was approximatley 6.67 mg/kg) (means ± SD, $n = 4$).

The drug uptake kinetic study allowed optimization of pH-dependent loading duration. For poorly water-soluble drugs, the concentration of neutral species was extremely limited by their solubility. Since ionization is a dynamic process, a suitable extra-liposomal pH can be utilized at which a proportion of the drug to be loaded is ionized to warrant a high solubility acting as a reservoir providing unionized species for liposomal uptake. However, the drug uptake rate slowed at low pH when less drug molecules existed as neutral form. The higher liposomal uptake rate of drug was found with higher pH. The results also revealed that supersaturated drug solution could provide sufficient driving force for loading, even with less than 1% of neutral drug species with pH at 4.25 within 1 h.

Despite the long-circulating property of the PEGylated carriers as designed, the half-life of ASL-L in rabbits was far shorter than that of Doxil [31]. Unfortunately, a half-life less than 6 h [32] is generally considered not long enough to exploit the EPR effect for tumor-targeted drug delivery. One-hour post i.v. infusion, the elimination phase half-lives of both ASL-L prepared with or without SBE-β-CD (DL was 8.9 and 4.9% respectively) was almost identical with the free drug solution, suggesting rapid drug leakage from liposomes, although ASL-L in 5% glucose solution had a leakage ratio less than 5% even after 80 days. Complement activation by first injection of PEGylated liposomes has been reported in human [33] which could result in opsonization with C3b and iC3b and a subsequent more rapid clearance than even conventional liposomes. However, if this were the case, the elimination phase of the two liposome formulations (containing different amount of total PEG-lipid) was unlikely to be identical to the free drug solution.

The use of cyclodextrins in liposomal drug loading has significant potential to increase drug to lipid ratio and encapsulation efficiency. However, care should be taken as high concentration of CDs may destabilize the liposomal membrane by forming inclusion complexes with phospholipids or cholesterol in the membrane [34, 35]. It has also been reported that (α-, β-, γ-) cyclodextrins can be incorporated into phospholipid bilayers of the liposomes and lowered the molecular packing in the DPPC-membranes [35]. Therefore in this study, the concentration of SBE-β-CD was carefully tailored and SBE-β-CD was removed immediately after loading. A concentration of 5% (DPPC/SBE-β-CD mole ratio = 3:5) was found to preserve the integrity of liposomes while stabilizing the drug solution for efficient loading. The pharmacokinetic profile of this formulation showed no statistical difference from that prepared without SBE-β-CD, strongly supporting the view that the leakage was predominately due to the liposome membrane, rather than the damage by SBE-β-CD.

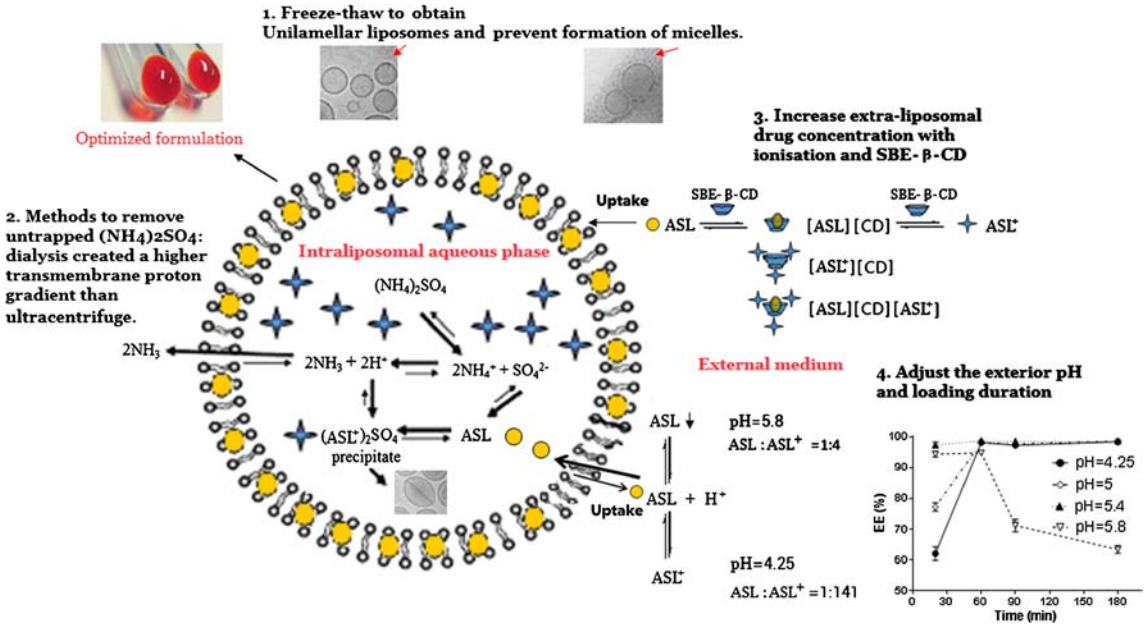

Fig. 6 Mechanisms of active loading using $(NH_4)_2SO_4$ gradient coupled with SBE-β-CD and various strategies used to maximize DL.

Drug release from liposomes can be influenced by both the membrane composition and the choice of drug [36], and could be drug-dependent for each membrane [38]. For instance, vincristine (log P=2.82) was found to leak out much faster than doxorubicin (log P=1.26) even using the same remote loading approach and lipid composition, despite the larger molecular weight of vincristine [13]. Liposome leakage has been reported widely for lipophilic drugs, such as vinca alkaloids [37], ciprofloxacin [38], and idarubicin [9] even using an active loading method. *In vitro* drug leakage was found, in hindsight, when pH 7.4 PBS was used as release medium especially in the presence of serum (data were not shown). ASL is a lipophilic molecule with logD >1 at pH 3.6 [19], which is the minimum pH within the liposomes containing 250 mM ammonium sulphate in theory [7]. Therefore, the drug concentration in the bilayer membrane is more than 10 times higher than that in aqueous cores (not including the precipitate as shown in TEM). Drug partitioned into the membrane could diffuse out quickly following the large concentration gradient in the medium and precipitate in a pH 7.4

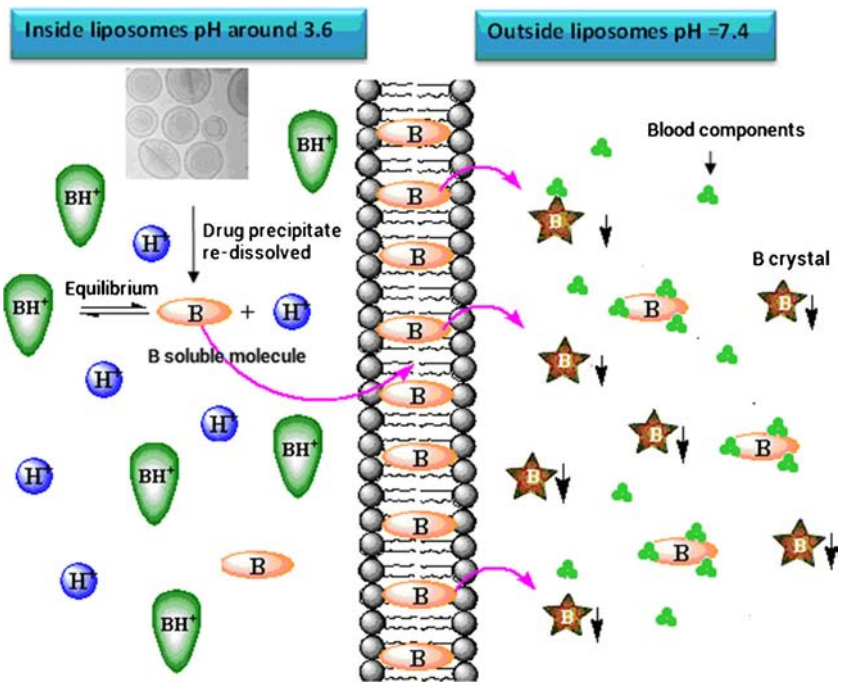

Fig. 7 Possible mechanisms of ASL leakage from liposomes in the blood. B is ASL base in neutral form, BH^+ is ASL in protonated form.

medium. The rapid drug precipitation may accelerate drug leakage. The dynamic intra-liposomal drug dissociation equilibrium (BH^+ and B in Fig. 7) would shift to supply more neutral species to the membrane, eventually making drug precipitate in the cores re-dissolve. A close pK_a of ASL (6.7) to intra-liposomal pH (5.5) facilitated ionization of drug (become soluble) and accelerated leakage [39]. In addition, lipophilic drugs repartitioned within the liposomal membrane are most likely susceptible to interaction with blood components [19, 38], resulting in a short circulation time. Therefore, the drug retention should be optimized in further study. Attempts to improve drug retention in liposomes have been made in the literature, including optimization of lipid component(s) [38], coating [40] or cross-linking membrane and forming a less-soluble drug complex inside liposomes [9, 11].

CONCLUSIONS

By optimizing the method of preparation and manipulating the loading conditions including the use of SBE-β-CD, a high drug-to-lipid ratio (26% by mole) was achieved with drug precipitate being observed in the liposomal cores. The multiple strategies to achieve high DL and EE could be applied to other poorly-water soluble weak bases. However, the *in vivo* stability of the liposomes was compromised. The half-life of ASL-L was identical with that of free drug, suggesting drug leakage from liposomes. Further study to improve drug retention by reducing liposomal permeability is under way.

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The authors declare that they have no conflicts of interest to disclose.

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