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Improving drug retention in liposomes by aging with the aid of glucose

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ABSTRACT

This paper describes a novel method to improve drug retention in liposomes for the poorly water-soluble (lipophilic) model drug asulacrine (ASL). ASL was loaded in the aqueous phase of liposomes and the effects of aging conditions and drug loading levels on drug retention were investigated using an in vitro bio-relevant drug release test established in this study. The status of intra-liposomal drug was investigated using differential scanning calorimetry (DSC) and cryo-transmission electron microscopy (cryo-TEM). Pharmacokinetics and venous tolerance of the formulations were simultaneously studied in rabbits following one-hour intravenous infusion via the ear vein. The presence of glucose
during aging was found to be crucial to accelerate drug precipitation and to stabilize the liposomal membrane with high drug loading (8.9% over 4.5% w/w) as a prerequisite. Although no drug crystals were detected, DSC showed a lower phase-transition peak in the glucose-assisted aged ASL-liposomes, indicating interaction of phospholipids with the sugar. Cryo-TEM revealed more ‘coffee bean’ like drug precipitate in the ASL-liposomes aged in the glucose solution. In rabbits, these liposomes gave rise to a 1.9 times longer half-life than the fresh liposomes, with no venous irritation observed. Inducing and stabilizing drug precipitation in the liposome cores by aging in the presence of sugar provided an easy approach to improve drug retention in liposomes. The study also highlighted the importance of bio-relevance of \textit{in vitro} release methods to predict \textit{in vivo} drug release.

\textit{Keywords:} liposomes; active loading; drug retention; aging; glucose

1. Introduction

Asulacrine (ASL), a topoisomerase II poison and an analogue of the clinical drug amsacrine, was developed at The University of Auckland (Baguley et al., 1984). ASL was 2-4 times more potent than amsacrine against solid tumors in experimental models (Baguley and Wilson, 1987), possibly due to its enhanced lipophilicity (Paxton et al., 1986). However, during the clinical trials of ASL (Fyfe et al., 2001), severe phlebitis in patients was observed after intravenous (i.v.) infusion, hampering further development. Our recent studies (See et al., 2014, Zhang et al., 2015 a and b) showed that post-injection drug precipitation at the wall of veins was probably the main reason for the cause of phlebitis
with ASL. And ASL-loaded PEGylated liposomes were developed to prevent these adverse events.

Furthermore, PEGylated liposomes with a size ranging from 100 to 200 nm (Li and Huang, 2008) were found to offer tumor-targeted drug delivery, controlled drug release (Charrois and Allen, 2004) and long circulation, as in the case of Doxil® (doxorubicin-loaded PEGylation liposomes). Surprisingly, although the area under the plasma concentration-time curve (AUC) significantly increased with the use of PEGylated ASL-liposomes, a similar elimination half-life to ASL solution was observed following i.v. infusion to rabbits, indicating that undesirable drug leakage from the carriers had occurred. This drug leakage would compromise the tumor targeting capacity of liposomes as well as their venous protection effects.

There are many literature reports of drug leakage from liposomes, even if they were actively loaded in the cores (Table 1). Drug release behaviors are in part related to their physicochemical properties (Lindner and Hossann, 2010). Drugs with high log P values (i.e. lipophilic) tend to diffuse through liposomal membranes easily, resulting in quick drug leakage (Table 1). In this case, permeability of the liposomal membrane is a determining factor for drug retention. However, the leakage does not apparently correlate to the drug pKa, possibly due to the different intra-liposomal pH and solubility. For instance, liposomes containing an ammonium sulphate gradient for the loading of weakly basic drugs could have an intra-liposomal pH of 5.5 (Bolotin et al., 1994). A lower pH than the pKa value is required to keep drug molecules ionized within the liposomes, reducing the drug efflux through lipid membranes (Maurer-Spurej et al., 1999).
Another important factor influencing drug stability in liposomes is the physical state of the drug inside the liposomes. A slow dissolution of intra-liposomal drug, when an insoluble complex or precipitate is formed, would give rise to better drug retention (Gubernator et al., 2010; Lasic et al., 1995; Zhigaltsev et al., 2006). For example, doxorubicin formed sulphate-based or citrate-based aggregates after being actively loaded and showed longer retention than that in liposomes without forming aggregates (Lasic et al., 1995; Li et al., 2000). The importance of the physical status of drug inside liposomes was also demonstrated with ciprofloxacin-loaded liposomes which leaked due to the lack of compact precipitate even if the intra-liposomal drug concentration exceeded its solubility by two orders of magnitude (Maurer et al., 1998). If the drug is in a solution state or forms amorphous precipitate, the neutral species will flow (or dissolve out) and be replenished according to the pKₐ and intra-liposomal pH, in a similar manner to active drug loading. ASL, with a pKₐ of 6.7, actively loaded with ammonium sulphate into liposomes, presumably falls into this category. This is supported by the fact that during drug loading (See et al., 2014), ASL with <10% unionized form could be rapidly loaded into the liposomes.

Forming a less-soluble drug complex inside liposomes (Gubernator et al., 2010; Taggar et al., 2006) has been a popular method to load the drug as well as to reduce drug leakage. However, it is a challenging task to find a suitable chemical to form a less soluble complex and effectively release at the tumor target. In addition, reducing permeability of the liposomal membrane is a widely-used strategy. This can be achieved by the use of cholesterol-free liposomes (Dos Santos et al., 2002), saturated phospholipids (Lindner and
Hossann, 2010) and cross-linked lipid structures in the liposomal membrane (Liu and O’Brien, 2002). Coating liposomes with chitosan (Mady and Darwish, 2010) or forming nanoshells by calcium phosphate (Thakkar et al., 2012) have also been employed to strengthen the liposomal membrane.

In the present work, we attempted to develop a simple approach to improve ASL retention in our previously developed liposome formulations (Zhang et al., 2015 a). A novel method, called sugar assisted ‘aging’, was investigated with the aim of improving drug retention by changing the physical state of intra-liposomal drug and stabilizing the liposomal membrane via interaction between phospholipids and glucose. The optimal aging conditions and factors influencing the physical state of drug inside liposomes during aging were also studied using a bio-relevant in vitro release study. Pharmacokinetics and venous tolerance were simultaneously evaluated following a one-hour i.v. infusion in New Zealand White rabbits.

2. Materials and methods

2.1 Materials

Asulacrine isethionate salt (CI-921, 99% pure) was synthesized and kindly gifted by Auckland Cancer Society Research Centre. The phospholipids, 1, 2-dipalmitoyl-sn-glycero-3-phosphocholinemonohydrate (DPPC), N-(carbonyl-methoxy-polyethyleneglycol2000)-1 and 2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE-mPEG 2000) were obtained from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol was purchased from Sigma-Aldrich Co., Ltd.
Sulfobutyl ether β-cyclodextrin (Captisol®) was a gift sample from Captisol Technology (La Jolla, US). All other reagents used in this study were of analytical grade except methanol and acetonitrile which were of chromatographic grade.

2.2 Animals

New Zealand White rabbits weighing between 3.0 and 3.5 kg were obtained from the Vernon Jansen Unit (VJU) of The University of Auckland. Animals were maintained according to the standards relating to the care and management of experimental animals in New Zealand. The experiments were carried out in accordance with the guidelines for animal experimentation and approved by the Committee on Animal Experiments of The University of Auckland (Ethics Approval No. C00881).

2.3 Preparation and characterization of liposomes

The liposomes (empty) were prepared using a thin film hydration (TFH) method as described previously (Zhang et al., 2015 a). Briefly, DPPC, DSPE-mPEG 2000 and cholesterol (6:1:3 mole ratios) were dissolved in organic solvent and dried under vacuum conditions. Then the thin film obtained was hydrated with 250 mM ammonium sulphate solution at 45 °C for 10 min, followed by a 7-cycle freeze and thaw. Size was controlled with extrusion through 0.1 μm-pore sized polycarbonate membrane filters (Whatman, UK) with a LIPEX™ Extruder (Northern Lipids Inc, Burnaby, Canada). A trans-membrane ion (H⁺ and SO₄²⁻) gradient was generated by removing extra-vesicular ammonium sulphate using dialysis method. The liposomes were then incubated with supersaturated drug solution in the presence of 5% sulfobutyl ether β-cyclodextrin (SBE-β-CD) at 37 °C for 1.5
h. The SBE-β-CD was employed to solubilize the drug by inclusion of the neutral species in its hydrophobic cavity, acting as a rich drug solution reservoir for drug loading. When drug loading was completed, free drug, ASL-CD complex and SBE-β-CD were removed by ultracentrifuge at 188,272×g (4 °C) for 1 h (Zhang et al., 2015 a). The obtained ASL-liposome (ASL-L) pellets were re-suspended in a glucose solution (5% w/v) and kept at 4 °C for further aging.

The particle sizes, polydispersity index (PDI) and zeta potentials of different ASL-Ls were determined using a Malvern Nano ZS (Malvern Instruments, UK). Samples were diluted to the required concentration with 5% glucose solution. All measurements were conducted at 25 °C in triplicate.

The encapsulated drug was separated using two-step centrifugation as described previously (Zhang et al., 2015 a). Drug entrapment efficiency (EE) was calculated as the percentage (%) of the mass of the drug encapsulated versus the total drug used for loading. The drug loading (DL) was expressed as the percentage (%) of the mass of encapsulated drug versus the mass of the obtained drug-loaded liposomes.

2.4 Factors affecting in vitro drug release

To achieve satisfactory drug retention, the following factors were investigated. Drug retention was evaluated after a bio-related in vitro release study method was established.

2.4.1 Establishment of a bio-relevant release study method
To determine the most bio-relevant release condition, different release methods were compared using the fresh liposomes proven leaky in vivo (Zhang et al., 2015a). Briefly, the drug release from liposomes was performed using a dialysis method against an isotonic release medium, PBS (0.12 M, 280mOsm) at pH 7.4 or pH 6. Liposome samples of the same volume (1 ml) were condensed into pellets by ultracentrifuge, and then the liposome pellet was re-suspended with pH 7.4 PBS (0.12 M) or a 5% glucose solution and transferred into the dialysis bag (MWCO of 12000-14000 Da). Then the bags were immersed in 500 ml of release medium in different beakers on a shaking water bath (GLS aqua 18 plus, Grant Instruments, UK) preset at 37 °C. At each pre-determined time interval, samples (0.1 ml of ASL-L suspension) were withdrawn from the dialysis bags. ASL in the liposomes was extracted by dissolving the liposomes with acetonitrile and the drug concentration determined by a validated stability-indicating HPLC method (See et al., 2014). The release method capable of identifying the ‘leaky’ liposomes was considered to be bio-relevant, and used in the sequential release studies.

### 2.4.2 Effect of drug loading level on drug release

To study the effect of drug loading level on drug retention, ASL-L with different drug loadings (4.5% and 8.9%) were suspended in 5% glucose solution and stored at 4 °C for 30 days before being subjected to a release study.

### 2.4.3 Effects of aging conditions on drug release

Liposome aging conditions including aging medium and aging period were optimized using a drug release study. All liposomes used had a drug loading of 8.9% (w/w). The freshly-prepared liposomes were suspended in a 5% glucose solution and were aged at 4 °C.
for 10, 20 and 30 days (aged ASL-L suspensions), respectively, before release study. The freshly-prepared liposomes centrifuged into pellets (without adding aging medium) were also stored at 4 °C for 30 days and used for comparison.

2.5 Aging simulation study

ASL (~10 mg/ml) was dissolved in a 250 mM (NH₄)₂SO₄ solution acidified by HCl to pH 3.6. The excess ASL was removed and the solution was neutralized with NaOH to induce drug precipitation (pH 5.5). The precipitates were isolated and transferred in 250 mM (NH₄)₂SO₄ or 5% glucose. They were stored at 4 °C for 30 days to simulate the physical change of drug within liposomes. After drying, these drug precipitates were observed by eye and polarized light microscope before analyzed by DSC.

2.6 Thermal analysis by DSC

In order to reveal the physical state of drug within the vesicles, calorimetric investigations of ASL powder, freshly-prepared ASL-L (within 24 h), 30-day aged ASL-L as pellets or colloidal suspensions, simulated drug precipitate aged in 250 mM (NH₄)₂SO₄ or 5% glucose, were carried out using differential scanning calorimetry (DSC), using DSC Q2000 series with Refrigerated Cooling System 90 and TA Instrument Explorer software (TA Instruments-Waters LLC, US). The liposome samples and simulated drug precipitates were condensed into pellets by ultracentrifuge and dried for 2 days in a thermostat at 25 °C. The reference and sample pans were prepared using the aluminum hermetic pans. For liposomes, a scan rate of 5 °C/min was applied in the range of 5 to 70 °C for several heat/cool cycles (Kastantin et al., 2009) to ensure that data represented equilibrium
measurements. Then the graphs were recorded for the last cycle of liposomes using a scan rate of 10 °C/min in the temperature range of 10 to 300 °C. For the drug powder and simulated drug precipitate, only one cycle with a scan rate of 10 °C/min between 10 to 300 °C was applied.

2.7 Cryo-transmission electron microscopy

The physical state of drug in freshly-prepared (within 24 h) and aged liposome suspensions (lipid concentration approximately 10 mg/ml) were analyzed by cryo-transmission electron microscopy (cryo-TEM): a drop of each sample was placed on the copper grid in the climate chamber and blotted, forming a thin aqueous layer on the membrane. The samples were then shock-frozen by dipping into liquid ethane and cooled to 90 K by liquid nitrogen. The copper grid containing the sample was transferred to the Tecnai 12 electron microscope (FEI, Hillsboro, OR) operating at 120 KV where it was analyzed.
2.8 Pharmacokinetics and venous tolerance in rabbits

2.8.1 Drug administration

The pharmacokinetic studies were conducted in comparison with a 0.5 mg/ml ASL solution (ASL dissolved in 5% glucose, pH 4.25), the same formulation used in clinical trials (Fyfe et al., 2001; Sklarin et al., 1992). Twelve rabbits were divided into three formulation groups randomly: ASL solution, freshly-prepared ASL-L, well-aged ASL-L with all the drug concentrations kept at 0.5 mg/ml. All formulations were sterilized by filtration (0.22 µm). Formulations were infused to the animal through a plastic catheter (23 gauge, outer diameter of 0.65 mm, Terumo, Tokyo, Japan) implanted into the marginal ear vein. The ASL dose was 6.67 mg/kg and the rate of infusion was controlled at 40 ml/h by a syringe pump (Model KDS200, KD Scientific Inc., USA).

2.8.2 Pharmacokinetics in rabbits

At the end of the 1-hour i.v. infusion (time set as 0), and at 10 min, 30 min, 1, 1.5, 2, 3, 5, 8, 12, 24 h after infusion, blood samples (1 ml) were collected from the contralateral ear vein. The blood samples in heparinized tubes were immediately centrifuged (700×g, 10 min, 25 °C) to obtain the plasma. Then an aliquot (100 µl) of plasma was mixed with 700 µl acetonitrile by vortexing for 2 min to allow the proteins precipitate. After centrifuging the mixture, all the supernatant was taken and dried in a SpeedVac (SVC 100H; Savant Instruments Inc.) at 25°C. HPLC mobile phase was added to re-dissolve the residue and the concentration of ASL was analyzed by HPLC as described previously (See et al., 2014).
2.8.3 Venous irritancy using rabbit ear vein

Following i.v. infusion, the injection site of the ear vein was visually observed over the first 24 hours. The animals were then killed and skin specimens ~10 mm away from the injection site along with the ear vein were excised and fixed in phosphate-buffered saline containing 10% formalin. Each sample was embedded in paraffin and stained with hematoxylin and eosin (HE) for histopathological examination.

3. Results

3.1 Characteristics of liposomes before and after aging

The size of ASL-L did not show any differences with a narrow size distribution after aging. All of their zeta potentials were negative, showing good physical stability (Table 2). No drug leakage occurred during aging inferred from unchanged EE.

3.2 Effects of drug loading level and aging conditions on drug retention

Fig. 1 A shows that a burst release from the fresh ASL-L was only observed when pellet ASL-L was re-suspended in pH 7.4 PBS in the dialysis bag and released in the same medium (method ‘a’), which reflected the in vivo leakage (Zhang et al., 2015a and b). In contrast, when either the suspension medium or the release medium was changed to 5% glucose, the drug would exhibit a false ‘sustained release’. Therefore, the method ‘a’ was considered to be bio-relevant and employed for the later release studies to identify optimal ‘aging’ conditions.
The release study showed that only high drug loaded liposomes (8.9% w/w) aged for 30 days in the 5% glucose solution exhibited a ‘healing effect’ of aging. There was < 35% drug released after 24 h from this well-aged ASL-L in contrast to the liposomes with 4.5% DL aged under the same condition with up to 92% drug released, and this ‘healing effect’ was in a time-dependent manner. Interestingly, the burst release was only slightly controlled for ASL-L aged in pellet form without an aging medium for 30 days.

3.3 Aging simulation study

As shown in Fig.2, after 10 days of storage for oversaturated ASL suspensions, drug in 5% glucose and 250 mM (NH₄)₂SO₄ precipitated and aggregated forming a porous gel-like bulk (A and B). With the extension of the storage period, drug aged in 5% glucose solution started to convert to solid cake and the supernatant became clearer (D), suggesting little drug in the solution. However, the drug aged in 250 mM (NH₄)₂SO₄ was still gel-like although becoming a little more compact (C). There were no observable drug crystals in either sample by eye or polarized light microscope.

3.4 Thermal analysis

Fig.3 shows the DSC curves of drug powder and dried liposome samples: freshly-prepared ASL-L, 30 days aged ASL-L pellet and suspensions. A phase transition peak of lipids was seen in all liposomes. However, no drug peak around the melting point of the drug powder (271.43 °C) was found in either the fresh ASL-L or the aged ASL-L,
indicating the drug inside liposomes was still amorphous after aging. The endothermic peaks at 110°C and 120°C for both the fresh and aged ASL-L were due to dehydration. Compared to the fresh ASL-L (51.12 °C) or aged ASL-L pellet (51.03 °C), a slightly lower phase transition temperature of the lipids in aged ASL-L suspensions (49.96 °C) occurred. As for the simulation samples (Fig. 3b), no melting peaks of drug were observed in either of the drug precipitates, consistent with those observed with both ASL-Ls. Rather, there was a significant exothermic broad peak at 150 °C in the precipitate aged in the glucose, corresponding to the melting points of the additives. The endothermic peak around 110 °C in the precipitates aged in ammonium sulphate (ammonium sulphate melting peak 230-280°C) was most possibly due to the dehydration over heating.

[Insert Fig.3 here]

3.5 Cryo-transmission electron microscopy

The representative cryo-TEM micrographs are shown in Fig.4. Most of the liposomes were unilamellar and the samples were free of micelles, due to the freeze and thaw process (Zhang et al., 2015 a). Compared to the empty liposomes (A), drug-loaded liposomes displayed more electron-dense cores under cryo-TEM, due to the highly concentrated drug. In the freshly-prepared liposomes, a large proportion of drug entrapped in the cores was globular. Some formed bundled or fiber-like structures which induced a slight change in liposomal shape into a “coffee bean”, similarly to doxorubicin-liposomes (Ohvo and Slotte, 1996). In the ASL-L suspensions aged for 30 days, most of the drug inside the vesicles
rearranged to bundled precipitates. Moreover, some square or needle stack-shaped drug precipitates were observed.

[Insert Fig.4 here]

3.6 Pharmacokinetics in rabbits

Fig.5 depicts the pharmacokinetic profiles of optimized aged ASL-L (aged in 5% glucose for 30 days at 4 °C) in comparison with the freshly-prepared liposomes (DL were both 8.9% w/w) and ASL solution following 1 hour i.v. infusion in the rabbits. Both liposomes showed a biphasal distribution process. Although the fresh ASL-L had much higher onset concentrations than ASL-solution and aged ASL-L, increasing the AUC and slowing the distribution from the central bloodstream, the clearance phase of fresh ASL-L coincided with that of ASL-solution with a similar short half-life (81.7 v.s 84.3 min; p>0.05). In contrast, ASL was retained better in aged liposomes, giving rise to a 1.8 times longer half-life (152 min).

[Insert Fig.5 here]

3.7 Venous irritancy using rabbit ear vein

Our previous study (Zhang et al., 2015 b) showed that infusion of ASL solution caused inflammatory cell infiltration near the venous wall, loss of endothelial cells as well as oedema, thrombus and hyperaemia. These pathological changes were reported to be typical symptoms of phlebitis (Worker and Dodd, 1960). Surprisingly, the animals which received fresh ASL-L showed more severe inflammatory responses than ASL solution as reported
previously (Zhang et al., 2015 b). In contrast, the ear veins and tissues exposed to aged ASL-L were similar to the negative controls.

[Insert Fig.6 here]

4. Discussion

To achieve effective tumor-targeting, drug retention in the long circulating liposomes is crucial. Although long-circulating PEGylated carriers have been designed, the fresh ASL-L failed to achieve this with an elimination half-life similar to drug solution, suggesting a drug leakage occurred during blood circulation. To deal with leakage, in a hindsight study, a more acidic core (pH below 4) was used to load ASL, and alternative lipids such as DSPC, DPPG, DSPG, SPC were used to prepare liposome membrane (data are not reported), however, there was no success in controlling the leakage. In addition, drug loading failed with a phosphate buffer at pH 7.4 as the inner aqueous phase for liposomes, suggesting a weak driving force of precipitation within vesicles.

The current study investigated the factors affecting drug precipitation in the liposome core by an established bio-relevant release method, and provided strategies to improve drug retention properties. It was postulated that aging ASL-L in the presence of glucose could accelerate drug precipitation within liposomes and decrease drug leakage. This was evidenced by the changed microstructures and increased drug precipitates in the simulation study, a better-controlled drug release profile \textit{in vitro}, prolonged half-life in rabbits, and less venous irritancy than fresh ASL-L. Once the drug precipitate increased inside the liposomes, the drug concentration in solution (both ionized and unionized) within these
Liposomes would considerably decrease, reducing the trans-membrane drug concentration gradient for efflux. DSC was carried out to investigate the aging mechanism by the change of the drug state and liposomal membrane during aging.

Liposome with a favorable size of 100~200 nm can be employed to exploit the EPR effect for tumor-targeted drug delivery (Li and Huang, 2008). In the present study, liposomes with a size of 180 nm were produced after extrusion, which are much larger than the pores size (~100 nm) of the polycarbonate membranes. It was reported that liposome vesicles could be deformed, and undergo a decrease in volume during extrusion either by an efflux of water or through rupture (Hunter and Frisken, 1998). The pressure and temperature for extrusion, and the lipid properties such as transition temperature (Tc) determine the size and distribution of the resulting liposomes (Hunter and Frisken, 1998). In this study, a pressure of ~500 psi and a temperature (42 °C, above the Tc of DPPC) were used in extrusion, which favored the deformation of liposomes, allowing liposomes larger than the membrane pore size (100 nm) pass through.

Nanoparticles with zeta potentials > 30 mV or < −30 mV are normally considered to be stable due to the static repulsion (Lyklema and Fleer, 1987; Hunter et al., 2001). The zeta potentials are presented due to ionization of surface groups of the nanoparticles and adsorption of charged species from the medium. A highly negative zeta potential (< -40 mV) of ASL-L was observed in 5% glucose solution. When PBS (pH 7.4) was used as medium, the zeta potential was close to zero (data are not presented). Indeed, the ASL-L were more stable in 5% glucose than in PBS (pH 7.4) with less tendency for aggregation. This negative zeta potential value may be due to absorption of anions such as the residual
SBE-β-CD which carries triple negative charges. In general, the hydrated PEG layers of PEGylated liposomes have a charge-shielding effect for the liposome surface (Nakamura et al., 2012). However, at higher degree of PEGylation, the negative zeta-potential value may increase, for example, Nakamura and co-workers (Nakamura et al., 2012) reported that liposomes with 0.75–4% PEG-lipid gave rise to a increasing zeta potential from -12.5 to -27.8 mV in purified water.

Most of the drug inside the fresh liposomes was still in condensed solution based on the cryo-TEM photo. Although the interior concentration of ASL, estimated from intra-liposomal volume based on the particle size according to the method reported by Xu et al. (2012), is approximately 28 times higher than its solubility at pH 5.5 (>1 mg/ml), the intra-liposomal pH using (NH₄)₂SO₄ reported (Bolotin et al., 1994), as most of the drug encapsulated did not precipitate in the freshly prepared ASL-L. This finding is similar to a previous study with ciprofloxacin liposomes using H-NMR (Maurer et al., 1998). In contrast, after aging, more drug precipitated within the vesicles which distorted into bundles compared with fresh ASL-L as observed from cryo-TEM (Fig. 4B).

To obtain greater drug retention through precipitation, a high drug loading level is a prerequisite (Johnston et al., 2008), therefore it is also a limitation. It was reported that intra-liposomal doxorubicin crystals were observed as the drug-to-lipid ratio was raised from 0.05 to 0.46 (w/w, e.g. 5% to 46%) and the drug retention increased with drug-to-lipid ratio. This is also evidenced in the current study that the ASL-L where lower DL (4.5% w/w) failed to produce a controlled release profile (Fig. 1 B). However, even the ASL-L with higher DL of 8.6% w/w did not achieve a comparable long-circulation as doxorubicin
liposomes. Further improvement in DL may increase the ASL retention due to the increase in the amount of precipitate. In addition, not all the drug retention would be improved as drug loading increased, possibly due to the different solubility of the drug in the liposomal aqueous cores. For instance, the retention properties of ciprofloxacin which did not precipitate following accumulation into liposomes were not affected by the drug-to-lipid ratio in the range that was investigated (Johnston et al., 2006, 2008).

Other than drug loading, equally crucial were aging conditions. A relatively low temperature (4 °C) was employed to maximize drug precipitation, which was also of benefit for the stability of liposomes. Since ASL-L were quite stable when stored in 5% glucose solution or pellets for at least 80 days, liposomes aging in both forms were utilized to optimize the aging conditions. Glucose medium was also employed to simulate clinical practice where ASL was diluted with it before infusion for patient use. Surprisingly, ASL-L aged in glucose medium (aged ASL-L suspensions) showed more drug precipitate and improved drug retention.

The presence of glucose may have two roles in the aging process of ASL-L: accelerating drug precipitation and stabilizing the phospholipid bilayer. It was speculated that during aging of liposomes in glucose solution, neutral glucose molecules diffused into the liposomal cores due to a hypertonic effect. Moreover, the intra-liposomal ions (H⁺, SO₄²⁻) also diffused out slowly (Deamer and Bramhall, 1986), which altered the pH, counter-ion types or osmotic pressure of the intra-liposomal medium and facilitated the drug to precipitate. Although aged ASL-L suspensions showed a one-off small endothermic peak at 271 °C (the graph was not shown) which may hint that crystalized drug existed,
most of the results suggested that the aging only increased drug precipitate instead of
forming crystals within liposomal vesicles, and the retention behavior was greatly
improved.

The improved drug retention may result from the interaction of glucose with the
liposomal bilayer membrane during storage, judging by the decreased phase transition
temperature ($T_c$) of phospholipids (endothermic peak $49.96 ^\circ C$ vs $51.03 ^\circ C$ or $51.12 ^\circ C$). Glucose, commonly used as a lyoprotectant, could replace the water for hydration of phospholipids through the hydrogen bonds between its OH groups and the polar head groups of DPPC, strengthens the DPPC chain packing (Nagase and Ueda, 1997). This direct interaction between the sugar and lipid in the presence of water was reported to lower the $T_c$ of the lipid in a lipid/sugar mixture (Koster et al., 2000), and also during liposomal lyophilization with sugar acts as lyoprotectant (Chen et al., 2010). Therefore, in this study the packing order of DPPC molecules should be increased during the aging period due to the rigid matrix containing hydrogen bonds of DPPC with glucose, inducing a more stable liposomal membrane (Harrigan et al, 1990) which in turn facilitates drug precipitation inside liposomes by preventing drug leakage. However, the effect of glucose on liposomal membrane and on drug retention should be limited and became effective only when drug can precipitate at high drug loading levels.

For the dehydration endothermic peak around $110 ^\circ C$, the aged ASL-L pellet was
similar to the simulated drug precipitate aged in ammonium sulphate. This may be
attributed to the hydrated phospholipids during aging, however, for the aged ASL-L
suspensions and fresh ASL-L, these peaks were smaller. The speculated reason was that the
hydrated water in phospholipids for the aged ASL-L suspensions was replaced by glucose, the phospholipids in fresh ASL-L have not been hydrated and the water has been removed before DSC analysis. From the DSC graph for the simulation study, it was shown that the drug precipitates in both media were amorphous after aging. Therefore, the drug can still release easily from the drug bundles inside the liposomes. The exothermic peak for sample 2 (drug precipitate aged in 5% glucose) appeared at the melting point of glucose and this provides an explanation for the enhanced drug retention for ASL-L aged in glucose solution. However, the present results could not explain this phenomenon.

The in vitro release media played an important role in the ASL release from liposomes. To establish a bio-relevant in vitro release method, the in vivo proven leaky liposomes were used in a different release setup. The leakage was only able to be identified when pH 7.4 PBS was used as the re-suspending and release medium. ASL could precipitate immediately in pH 7.4 PBS, which increased the drug concentration gradient from the bilayer membrane to release medium and speeded up drug leakage. It was also worth noting that no burst release was observed if the liposomes in the dialysis bag were re-suspended in the glucose solution, further confirming the stabilizing effect of glucose on the liposomal membrane.

Although the elimination half-lives of the two liposomes, measured with ASL concentrations, were different. Their AUC$_{0-8h}$ were not significantly different (Table 3). The initial drug concentration for the aged liposomes declined more rapidly than that for the fresh liposomes. This is possibly due to the uptake of the liposomes by the liver, despite the PEGylation effect, with more unreleased drug in the aged ASL-L than that in the fresh
ASL-L during circulation. Despite the improved drug retention for the aged ASL-L, unfortunately, the half-life of less than 6 h is not ideal to exploit the tumor targeting effect (Fenske and Cullis, 2008). Future studies will be conducted with combination of more effective method to load drug or impede liposomal membrane permeability including the use of alternative phospholipids with higher Tc.

5. Conclusions

These studies defined a method to improve drug retention by aging drug precipitate inside vesicles and stabilizing liposomal membranes with the aid of glucose and in conjugation with high drug loading, which resulted in controlled drug release in vitro and in vivo and less drug irritancy in rabbits following i.v. infusion. For ASL, good drug retention in the liposomes is crucial for preventing phlebitis. The present study also highlighted the importance of bio-relevance of an in vitro release method in liposomal formulation screening.

Acknowledgements and disclosures

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

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Fig. 1. Percentage of cumulative drug release from ASL-L at 37 °C (mean ±SD, n=3). (A) Effect of release method: ‘a’: ASL-L suspended in pH 7.4 PBS and released in pH 7.4 PBS; ‘b’ and ‘c’: ASL-L suspended in 5% glucose, and released in pH 6 PBS and pH 7.4 PBS, respectively; (B) Effect of drug loading levels; (C) Effect of aging conditions. ASL-L were freshly prepared for A and B. All the drug loadings were 8.9% for A and C.
Fig. 2. Oversaturated drug suspensions in 250 mM (NH₄)₂SO₄ at 10 (A) and 30 (C) days or in 5% glucose for 10 (B) and 30 (D) days, showing more drug turned into precipitate in the presence of glucose over time.
Fig. 3. DSC curves of (a) ASL powder, freshly-prepared ASL-L and ASL-L aged for 30 days at 4 °C as pellets or colloidal suspensions; (b) drug precipitate aged in 250 mM (NH₄)₂SO₄ (sample 1) and 5% glucose (sample 2) for 30 days.
**Fig. 4.** Cryo-TEM micrographs of empty liposomes (A), freshly-prepared ASL-L (B) and ASL-L aged in suspensions in the presence of glucose for 30 days (C). The drug loading for B and C were both around 8.9%.

**Fig. 5.** Pharmacokinetic profiles in rabbits after i.v. infusion of ASL formulations at a rate of 20 mg/h for 1 hour (mean ± SD, n=4).
Fig. 6. Typical histological photomicrographs of cross section of rabbit ear specimens after infusion of fresh ASL-L (A) and 30-day aged ASL-L suspensions (B).
Table 1 Physicochemical properties (pKₐ and log P) of different drugs that are reported to have ‘leakage’ from liposomes. All these drugs are anticancer agents apart from ciprofloxacin (an antibiotic).

<table>
<thead>
<tr>
<th>Drug</th>
<th>pKₐ</th>
<th>Reference</th>
<th>LogP</th>
<th>Reference</th>
<th>Leakage</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vinorelbine</td>
<td>5, 7.4</td>
<td>(Owellen et al., 1977)</td>
<td>2.82</td>
<td>a</td>
<td>In vitro and in vivo (mice)</td>
<td>(Zhigaltsev et al., 2005)</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>7.4</td>
<td>(Gaertner et al., 1998)</td>
<td>3.4</td>
<td>(Etievant et al., 1998)</td>
<td>In vitro and in vivo (mice)</td>
<td>(Zhigaltsev et al., 2005)</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>10.36, -1</td>
<td>c</td>
<td>3.2, 3.54</td>
<td>b and c</td>
<td>In vivo (Human)</td>
<td>(Sharma et al., 1997; Soepenberg et al., 2004)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>5.61-6.18</td>
<td>(Pisal et al., 2004)</td>
<td>2.3</td>
<td>(Botté et al., 2011)</td>
<td>In vitro</td>
<td>(Maurer et al., 1998)</td>
</tr>
<tr>
<td>Idarubicin</td>
<td>8.2</td>
<td>(Robert, 2005)</td>
<td>1.69, 1.9</td>
<td>b and c</td>
<td>In vitro and in vivo (mice)</td>
<td>(Dos Santos et al., 2002; Gubernator et al., 2010)</td>
</tr>
<tr>
<td>Asulacrine</td>
<td>6.7</td>
<td>(See et al., 2014)</td>
<td>3.0 (pH 7)</td>
<td>(See et al., 2014)</td>
<td>In vivo (rabbit)</td>
<td>(Zhang et al., 2015 a)</td>
</tr>
</tbody>
</table>

aMERCKINDEX (1996); bALOGPS; cChemAxon.
Table 2  Size, PDI, zeta potential (measured in a 5% glucose solution), EE and DL of freshly-prepared and aged ASL liposomes aged in different conditions (mean ± SD, n=3).

<table>
<thead>
<tr>
<th>Storage</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>Zeta potential (mV)</th>
<th>DL (%)</th>
<th>EE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freshly-prepared*</td>
<td>182.3 ± 0.6</td>
<td>0.065 ± 0.020</td>
<td>-49.3 ± 0.1</td>
<td>8.93 ± 0.08</td>
<td>98.2 ± 0.1</td>
</tr>
<tr>
<td>4 °C for 10 days*</td>
<td>180.0 ± 1.2</td>
<td>0.092 ± 0.015</td>
<td>-40.8 ± 0.2</td>
<td>8.85 ± 0.05</td>
<td>97.3 ± 0.0</td>
</tr>
<tr>
<td>4 °C for 20 days*</td>
<td>179.9 ± 1.2</td>
<td>0.104 ± 0.008</td>
<td>-45.6 ± 5.1</td>
<td>8.84 ± 0.03</td>
<td>97.2 ± 0.0</td>
</tr>
<tr>
<td>4 °C for 30 days*</td>
<td>179.6 ± 0.7</td>
<td>0.110 ± 0.011</td>
<td>-49.2 ± 0.6</td>
<td>8.80 ± 0.04</td>
<td>96.8 ± 0.0</td>
</tr>
<tr>
<td>4 °C for 30 days*</td>
<td>181.4 ± 0.6</td>
<td>0.084 ± 0.010</td>
<td>-46.1 ± 0.4</td>
<td>8.83 ± 0.06</td>
<td>97.1 ± 0.1</td>
</tr>
</tbody>
</table>

* As liposome colloidal suspensions in 5% glucose solution; p As liposome pellet.
Table 3 Pharmacokinetic parameters of ASL formulations following i.v. infusion to rabbits at a dose of 6.67 mg/kg (mean ±SD, n=4).

<table>
<thead>
<tr>
<th></th>
<th>ASL-S</th>
<th>Freshly-prepared ASL-L</th>
<th>Aged ASL-L</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₀ (μg/ml) *</td>
<td>7.81 ± 1.33</td>
<td>23.73* ± 6.18</td>
<td>9.66 ± 1.54</td>
</tr>
<tr>
<td>T₁/₂ (min)</td>
<td>84.28 ± 18.41</td>
<td>81.70 ± 16.77</td>
<td>151.64* ± 19.39</td>
</tr>
<tr>
<td>MRT (min)</td>
<td>125.16 ± 20.70</td>
<td>94.73 ± 10.15*</td>
<td>172.32 ± 22.04 *</td>
</tr>
<tr>
<td>AUC₀ₜ (mg·h/l)</td>
<td>19.12 ± 2.27</td>
<td>27.29 ± 10.20</td>
<td>19.44 ± 3.27</td>
</tr>
</tbody>
</table>

* C₀: The drug concentration immediately after infusion.

* Significant differences with other formulations (p<0.05).