Determining the critical parameters that influence drug loading and release from a thermosensitive liposomal formulation of vinorelbine

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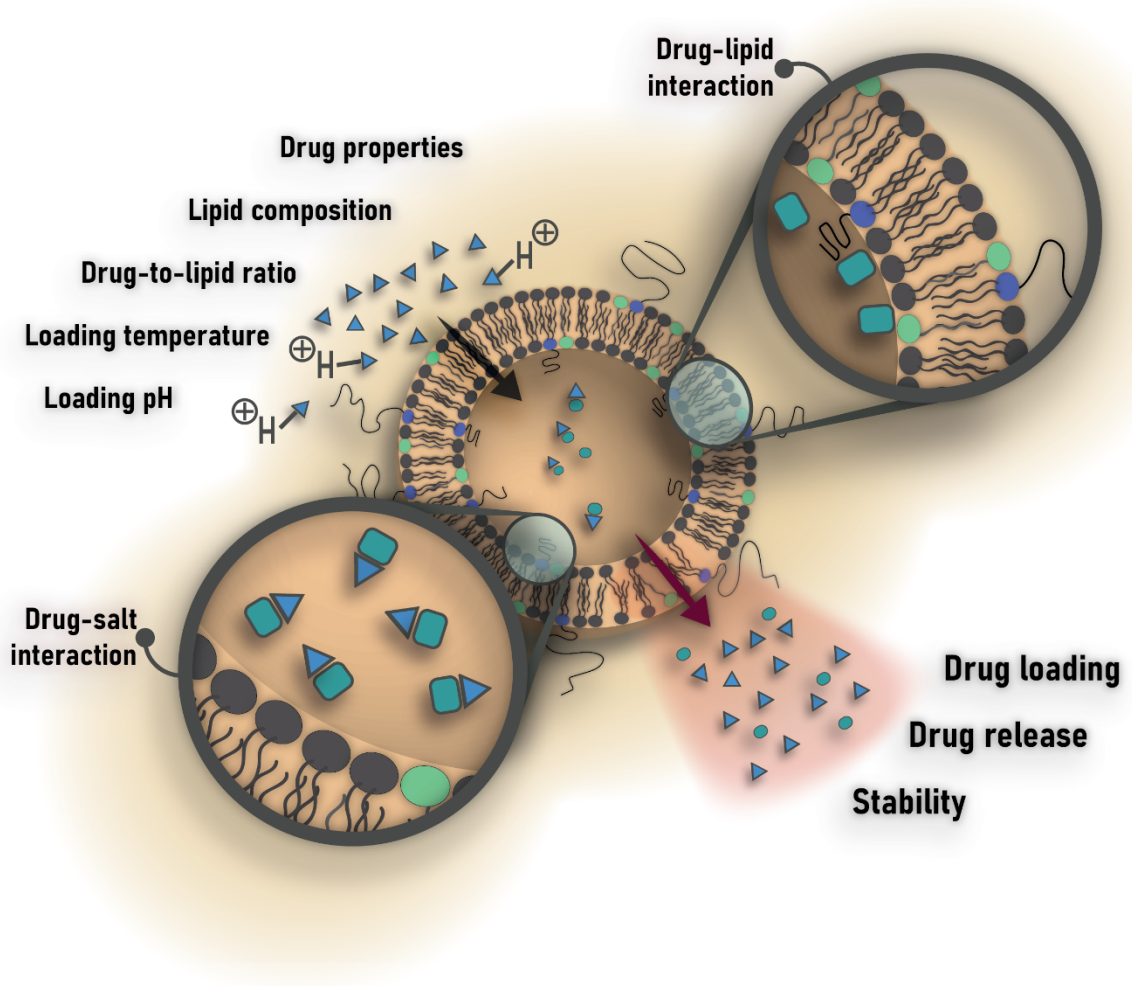
Abstract

Currently many studies have demonstrated the advantages associated with heat-triggered drug delivery via thermosensitive liposomes for the treatment of localized cancer. Challenges that traditional liposomal systems face such as limited drug release and homogeneous distribution throughout the region of interest can potentially be overcome when triggering intravascular drug release. The most prominent example is a thermosensitive liposome formulation of doxorubicin commercially known as ThermoDox®. Many other drugs may benefit from the same targeted and localized delivery approach using thermosensitive liposomes as it can result in a significant improvement in the therapeutic index. Vinorelbine is a semi-synthetic vinca alkaloid which has shown to be active in a broad range of cancers. Several liposome formulations encapsulating vinorelbine have been developed as a means to reduce systemic drug exposure. The present study takes a systematic approach in exploring formulation and drug loading parameters and their influence on performance characteristics of a rapidly releasing thermosensitive liposome formulation of vinorelbine. More broadly, this study shows that trends observed for non-thermosensitive liposome formulations of specific drugs can not be easily translated to their thermosensitive counterparts. The deep impact of the presence of albumin on stability and *in vitro* release is also highlighted. This is of significance given that a number of recent reports examine drug release in the absence of biologically relevant components. As a result, a strong recommendation emanating from this is a thorough challenge of the liposome formulation *in vitro* in order to gain a better understanding of its likely behaviour *in vivo* as well as potential for future clinical translation.

Keywords:

Thermosensitive liposome; drug delivery; hyperthermia; vinorelbine; drug release; nanomedicine.

Graphical abstract



1. Introduction

Liposomes are recognized as the most advanced delivery technology for cancer chemotherapy. Major milestones in their development, such as active drug loading techniques and significant prolongation of their *in vivo* circulation lifetime through the addition of PEGylated lipids, have led to several clinically approved formulations [1]. Liposomes encapsulation of commonly used chemotherapy drugs has been shown to improve their therapeutic index, a measure of associated efficacy and toxicity [2]. This is largely attributable to an improvement in toxicity profile, which results from a reduction in systemic drug exposure, relative to administration of free drug. Improvements in therapeutic efficacy of liposome formulated drugs, relative to conventional chemotherapy, have been variable among patient populations [3]. This may in part be attributed to the design of the liposomes or nanocarriers. In order to ensure retention of drug within the carriers while in transit to the diseased sites, the liposomes are designed to exhibit stable drug entrapment. Once at the target site, this can lead to poor or limited drug release and tumor penetration [3,4].

Triggered drug release from nanocarriers has been proposed as a potential strategy to overcome these challenges [5]. The most clinically advanced approach relies on heating the target tissue within the range of ablative temperatures in combination with administration of chemotherapy in thermosensitive liposomes. ThermoDox® is a low temperature sensitive liposome (LTSL) formulation of doxorubicin which is currently under Phase III clinical investigation for the treatment of hepatocellular carcinoma in combination with radiofrequency ablation (NCT02112656). Other clinical trials (e.g. NCT02536183, NCT03749850) are evaluating ThermoDox® in combination with localized heating of tumors in the range of mild hyperthermia (HT). ThermoDox® demonstrates rapid and complete drug release within the tumor blood vasculature upon reaching the heated tissue [6]. As a consequence, tumor accumulation and penetration of the drug is no longer reliant on extravasation of the whole carrier and subsequent drug release, but rather on the physico-chemical properties of the drug itself.

The majority of papers studying drug release via thermosensitive liposomes encapsulate the drug doxorubicin. Doxorubicin is the drug of choice in these efforts in part because it was encapsulated in the first liposome formulation which received clinical approval (i.e. Doxil®). It is also well established that a lack of drug release at the tumor site limits the efficacy of Doxil®. There are common fluorescence-based analytical techniques developed that enable real-time quantification of the time dependent release of doxorubicin from thermosensitive liposomes. However, there are many other chemotherapy drugs that may benefit from this delivery approach. The parameters affecting the formulation of drugs in non-thermosensitive liposomes have been extensively researched and are well understood, but there are still many questions to be answered regarding thermosensitive liposomes. In particular, how do the formulation properties (e.g. lipid composition, internal and external buffer) and loading parameters (e.g. loading pH, loading temperature, and loading process) influence the performance characteristics (i.e. drug loading, stability, drug release) of rapidly releasing, lyso-lipid containing, thermosensitive liposomes? Developing a LTSL formulation of the vinca alkaloid, vinorelbine (VRL) aimed to answer some of these unexplored questions.

The anti-mitotic chemotherapy agent VRL is commonly used for the treatment of locally advanced or metastatic non-small cell lung cancer and metastatic breast cancer and has recently been granted orphan designation for the treatment of soft tissue sarcoma [7,8]. Several non-thermosensitive liposome formulations encapsulating VRL have been developed [9–16]. TLC178 is a non-thermosensitive liposome formulation of VRL that is currently in clinical (Phase I/II) development (Taiwan Liposome Company). To build upon the success of TLC178, in this study, the development of a thermosensitive liposome formulation of VRL is pursued as a means to further enhance tumor accumulation of VRL when administered in combination with localized heating at the tumor site. The influence of the aforementioned

formulation properties and loading parameters on the performance characteristics of a rapid releasing LTSL formulation are examined.

In brief, this study results in a stable LTSL formulation of VRL with a relatively high drug loading that provides triggered burst release in the temperature range of mild HT. More generally, this study shows that the relationships established between composition and performance for non-thermosensitive liposome formulations of a specific drug are not necessarily applicable to their thermosensitive counterparts. As a result, there is a need for additional studies which examine the relationships between composition and performance of thermosensitive liposome formulations. Moreover, this study highlights the need to challenge liposome formulations *in vitro* with biologically relevant components in order to gain a more accurate assessment of stability and drug release.

2. Materials and Methods

2.1. Materials

Vinorelbine tartrate was purchased from Selleck Chemicals (Houston, TX, USA). Sodium sucrose octasulfate (Na_8SOS) was purchased from Toronto Research Chemicals (North York, ON, Canada). 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), N-(carbonyl-methoxypolyethylenglycol 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (PEG_{2k}-DSPE), 1-stearoyl-2-lyso-sn-glycero-3-phosphocholine (lyso-SPC, MSPC) were obtained from Corden Pharma (Plankstadt, Germany). Triethylamine (TEA), Dowex® 50WX8-200 and 50WX4 200-400, bovine serum albumin (BSA, heat shock fraction, pH 7, ≥98 %) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Sepharose CL-4B was purchased from GE Healthcare Bio-Sciences (Mississauga, ON, Canada).

2.2. Liposome preparation

Liposomes were prepared following a modified protocol by Viglianti et al [17]. Briefly, DPPC, MSPC, and PEG_{2k}-DSPE were dissolved in chloroform at a molar ratio of 86/10/4, respectively and the solvent was removed using a rotary evaporator. The lipid film was then dried under vacuum overnight to remove any residual solvent. The lipids were hydrated for 30 minutes at 55 °C in triethylammonium sucrose octasulfate (TEA_8SOS) containing buffer to a lipid concentration of 125 mM. The TEA_8SOS buffer was prepared from the respective sodium salt described by Drummond et al [18]. In brief, the sodium ions were exchanged using a Dowex 50WX8-200 resin in its hydrogen form and the eluted free acid of sucrose octasulfate was immediately titrated with neat triethylamine to a pH of 5.7. The concentration of sulfate groups was calculated from the amount of TEA added and adjusted to 0.22-0.75 M. Sulfate group concentrations in TEA_8SOS buffers are reported throughout this paper. The rehydrated lipids were then extruded three times through two stacked track-etch 200 nm pore size polycarbonate membranes (Whatman Inc., Clifton, NJ, USA) and 10 times through two stacked 100 nm pore size membranes at 55 °C and 400 psi nitrogen pressure using a 10 mL Lipex Extruder from Northern Lipids (Vancouver, BC, Canada). Unencapsulated TEA_8SOS was removed by dialysis at 4 °C overnight (50 kDa MWCO) against a 1000-fold volume excess of HEPES-buffered dextrose (HBD; 5 mM HEPES, 5 % dextrose, pH 6.5) or HEPES-buffered saline (HBS; 20 mM HEPES, 150 mM sodium chloride, pH 6.5) solution. The liposomes were stored at 4 °C until loading with VRL.

2.3. Liposome loading with VRL

VRL was loaded following a modified protocol by Drummond et al [13]. In brief, a 10 mg/mL solution of VRL tartrate in water was prepared and added to the liposomes in order to achieve a specific drug-to-lipid ratio (D/L ratio). The pH was adjusted to the desired values using 1 N NaOH and the dispersion was incubated in a water bath at various temperatures while being gently stirred. The liposomes were subsequently chilled on ice for 10 min and unencapsulated drug was removed by size exclusion chromatography (SEC) on Sephadex CL-4B gel columns. For the *in vitro* release studies, in order to minimize sample dilution during removal of the unencapsulated drug, an alternative method was used as described under section 2.7.

2.4. Liposome characterization

Liposomes were diluted 100 times in HEPES-buffered saline (0.02 M HEPES, 0.15 M NaCl, pH 7.4) and the size was determined using an intensity-based analysis and the dynamic light scattering (DLS) system Zetasizer Nano ZS from Malvern Instruments (Malvern, WOR, UK). The zeta potential of liposomes was measured using the same system, after diluting the liposomes in Milli-Q water.

2.5. VRL quantification

VRL was quantified using an Agilent Technologies 1260 Infinity LC system (Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was achieved using an Agilent EC-C18 column (2.1 x 50 mm, 1.9 µm) at 40 °C and a mobile phase composed of 5 mM ammonium formate (A) and acetonitrile (B) both

with 0.1 % (v/v) formic acid. The initial mobile phase was 80 % A with a flow rate of 0.3 mL/min, which was gradually decreased to 10 % A over a time course of 3 min. Following a one-minute equilibration at this ratio, the composition was changed back to 80 % A accompanied by an increase in the flow rate to 0.5 mL/min.

Detection of vinorelbine was achieved using a ThermoScientific TSQ Endura Triple Quadrupole Mass Spectrometer (Mississauga, ON, Canada) with an H-ESI in positive mode. The optimal ion source settings consisted of a spray voltage of 3700 V, sheath gas 1 arbitrary unit (a.u.), auxiliary gas 8.5 a.u., sweep gas 3 a.u. and an ion transfer tube temperature of 275 °C. Selected reaction monitoring of m/z 799.05 → 122.2, 323.1, 457.2, 626.2, 658.3, 696.2 was used to quantify vinorelbine. The collision energy ranged between 21 V and 35 V and the scan time per transition was 0.13 seconds.

2.6. Lipid quantification

Phospholipids, lyso-lipids, as well as free fatty acids, were quantified using an Agilent 1260 Infinity II HPLC system (Agilent Technologies, Santa Clara, CA, USA) coupled with an evaporative light-scattering detector (ELSD) and a protocol previously published by Shibata et al. [19]. Briefly, chromatographic separation of a 20 µL injected sample was achieved at 50 °C using an Eclipse XDB-C18 column (150 x 4.6 mm, 5 µm) and a gradient elution starting with 80 % A (i.e. methanol with 0.1 % trifluoroacetic acid (TFA)) and 20 % B (i.e. water with 0.1 % TFA) which was increased to 100 % A after 10 min at a flow rate of 1 mL/min. This was continued for another 10 min, followed by a change to the initial ratio and a short equilibration before continuing with the next injection. The ELSD was set to an evaporator temperature of 70 °C, a nebulizer temperature of 50 °C, the nitrogen gas flow was 1.6 SLM and the gain was set to 4. The assay was calibrated using DPPC, lyso-SPC, PEG_{2k}-DSPE, palmitic acid (PA) and stearic acid (SA) diluted in methanol over a broad range of concentrations.

2.7. Removal of unencapsulated VRL

In order to avoid liposome dilution when using gel columns to remove unencapsulated VRL, a modified protocol previously described by Amselem et al. for the removal of unencapsulated doxorubicin was used to prepare purified liposomes for subsequent evaluation of drug release, differential scanning calorimetry (DSC) analysis or cryogenic transmission electron microscopy (cryo-TEM) studies [20]. Briefly, Dowex 50WX4 200-400 mesh in its hydrogen form was converted to the sodium form by washing with 2 M NaOH in a ratio of 400 mL NaOH per 100 g of dry resin using a Buchner funnel. The pH was subsequently neutralized by several washes with 1 M NaCl solution. The resin was then dried at 70 °C and stored at room temperature for further use. Following the manufacturer's declaration of resin exchange capacity plus a sufficient excess, the resin was prepared and pre-cooled on ice in a glass vial. The loaded liposomes were then added and incubated for 20 min at 4 °C while gently mixed on a shaker. Finally, the unencapsulated VRL bound to the resin was removed using a Buchner funnel and the collected liposomes were stored at 4 °C.

2.8. VRL release from thermosensitive liposomes

Temperature dependent release of VRL was tested by adding 200 µL liposomes to 5 mL of pre-heated phosphate buffered saline (PBS, pH 7.4) solution with or without the addition of 45 g/L BSA. A water bath was used to control the temperature while the solution was stirred with a magnetic stir bar. Release was tested at 37 °C, 38 °C, 39 °C, 40 °C, 41 °C and 42 °C. 200 µL samples were withdrawn at 15 seconds, 0.5 min, 1 min, 1.5 min, 2 min, 3 min, 4 min, 5 min and 10 min and immediately placed onto Sephadex CL-4B gel columns. The columns were pre-equilibrated with chilled HBS. Samples heated at 37 °C were also collected at 30 min and 60 min. The eluent fraction containing the liposomes, as well as the fraction containing the released drug, were collected, diluted greater than 2.5 times with methanol, and the VRL concentration was quantified using the LC/MS assay described above. The percentage of VRL released was calculated by comparing the amount of VRL remaining in the liposomes at a certain temperature and time point relative to the initial amount of VRL present in the liposomes prior to heating.

2.9. Phase transition temperature measurement using differential scanning calorimetry

Thermal analysis was performed using a TA Q100 DSC (TA Instruments, New Castle, DE, USA). A 10 µL aliquot of each liposome formulation was pipetted into a hermetic aluminum pan and analyzed with the relevant external buffer as the reference. Each sample was subjected to three heating cycles from 25 °C to 60 °C and back to 25 °C at a rate of 1 °C/min. The obtained data was analyzed using the TA Universal Analysis Software. Extracted data included the melting phase transition temperature (T_m), the temperature of peak onset (T_{on}) and the peak width at half peak height ($T_{1/2}$).

2.10. Cryogenic transmission electron microscopy

Liposomes were diluted with buffer to a lipid concentration of 6-7 mM. A 10 µL aliquot of each sample was then added onto a Quantifoil Multi A holey carbon film (Quantifoil Micro Tools GmbH, Großlöbichau, Germany) supported by a 300 mesh copper grid at room temperature and under controlled humidity. Excess sample was removed using filter paper and immediately after, the samples were flash frozen by plunging them into liquid ethane at a temperature of -183 °C. The samples were kept below -170 °C throughout the imaging process using liquid nitrogen. Images were obtained using a FEI Tecnai G2 F20 microscope (FEI company, Hillsboro, OR, USA) equipped with a bottom mount Gatan 4k CCD camera (Gatan Inc., Warrendale, PA, USA) in bright field mode with a 200 kV acceleration voltage.

A method adapted from Semple et al. was used to assess the state of the drug in the liposomes [9]. In brief, the exterior and interior of the liposomes were compared by evaluating the mean gray value using ImageJ (Version 1.52a, NIH, Bethesda, MD, USA). Forty independent regions inside and outside the liposomes of various different images and a minimum of two separate liposome batches imaged on different days were analyzed. On 8-bit grayscale images, the area inside the liposome was compared to its immediate surroundings using a linear scale from a minimum of 0 to a maximum of 255. In order to compare liposomes loaded at different drug-to-lipid ratios the mean gray value of the vesicles inside was subtracted from the respective outside.

2.11. Calculation of entrapped VRL molecules per vesicle

The number of VRL molecules loaded into the vesicles was calculated as described previously by Van Raath et al. [21]. In brief, the number of lipids per vesicle was calculated from the number of lipids in the outer (L_{OL}) and inner (L_{IL}) membrane leaflet (3.93 nm subtracted from vesicle radius (r) to account for membrane thickness [22]):

$$Number\ lipids\ in\ outer\ leaflet\ (L_{OL}) = \frac{4\pi r^2}{A_{(weighed)}}$$

$$Number\ of\ lipids\ in\ inner\ leaflet\ (L_{IL}) = \frac{4\pi(r - 3.93\ nm)^2}{A_{(weighed)}}$$

The area was weighed according to the mole fraction (χ) of each lipid in the lipid composition (DPPC, lyso-SPC, PEG_{2k}-DSPE 86/10/4) and the following previously reported areas per lipid molecule were used: 49.4 Å² for DPPC [23], 48.0 Å² for lyso-SPC [24] (assuming a comparable surface area of lyso-SPC and lyso-PPC) and 40.0 Å² for PEG_{2k}-DSPE [25]:

$$A_{(weighed)} = \chi_{DPPC} \times A_{DPPC} + \chi_{lyso-SPC} \times A_{lysoSPC} + \chi_{PEG_{2k}-DSPE} \times A_{PEG_{2k}-DSPE}$$

The number of VRL molecules per vesicle was obtained from the number of lipids per vesicle (L_{OL} + L_{IL}) and the drug-to-lipid ratio.

2.12. Statistical analysis

All experiments were performed in independent triplicates and all statistical analysis was performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego, CA, USA) or SPSS Statistics 22.0 (IBM, Armonk, NY, USA). Formulation characteristics between liposomes loaded with different amounts of VRL were compared by unpaired t-tests. Significant differences in mean gray values of either the interior of formulations loaded with different amounts of drug, or differences between the exterior and interior of one specific formulation were analyzed by one-way ANOVA with Bonferroni post hoc testing or unpaired t-test, respectively.

3. Results

3.1. Systematic evaluation of parameters affecting VRL loading into thermosensitive liposomes

Studies were conducted to determine the influence of various factors on the loading of VRL into thermosensitive liposomes with a goal towards obtaining a stable formulation with a high drug-to-lipid ratio. In order to achieve this, the loading pH, loading temperature, initial drug-to-lipid (D/L) ratio, and the intraliposomal TEA_8SOS sulfate group concentration were varied and their subsequent influence on drug entrapment investigated.

As shown in Figure 1 A, VRL loading into thermosensitive liposomes was generally found to be fast and reached a maximum loading level between 15 and 30 minutes. The amount of drug loaded did not significantly ($p > 0.12$) increase with longer incubation.

The influence of the external pH of the loading buffer (i.e. pH = 6.0, 6.5, 7.0 and 7.5) was examined (Supplementary Information, Figure S1) with an initial D/L ratio of 150 g VRL/mol lipid and incubation for one hour at a loading temperature of 35 °C. Overall, over the pH range tested, it was found that the pH did not significantly impact the drug loading efficiency with an average of 96 % under all conditions examined ($p > 0.809$).

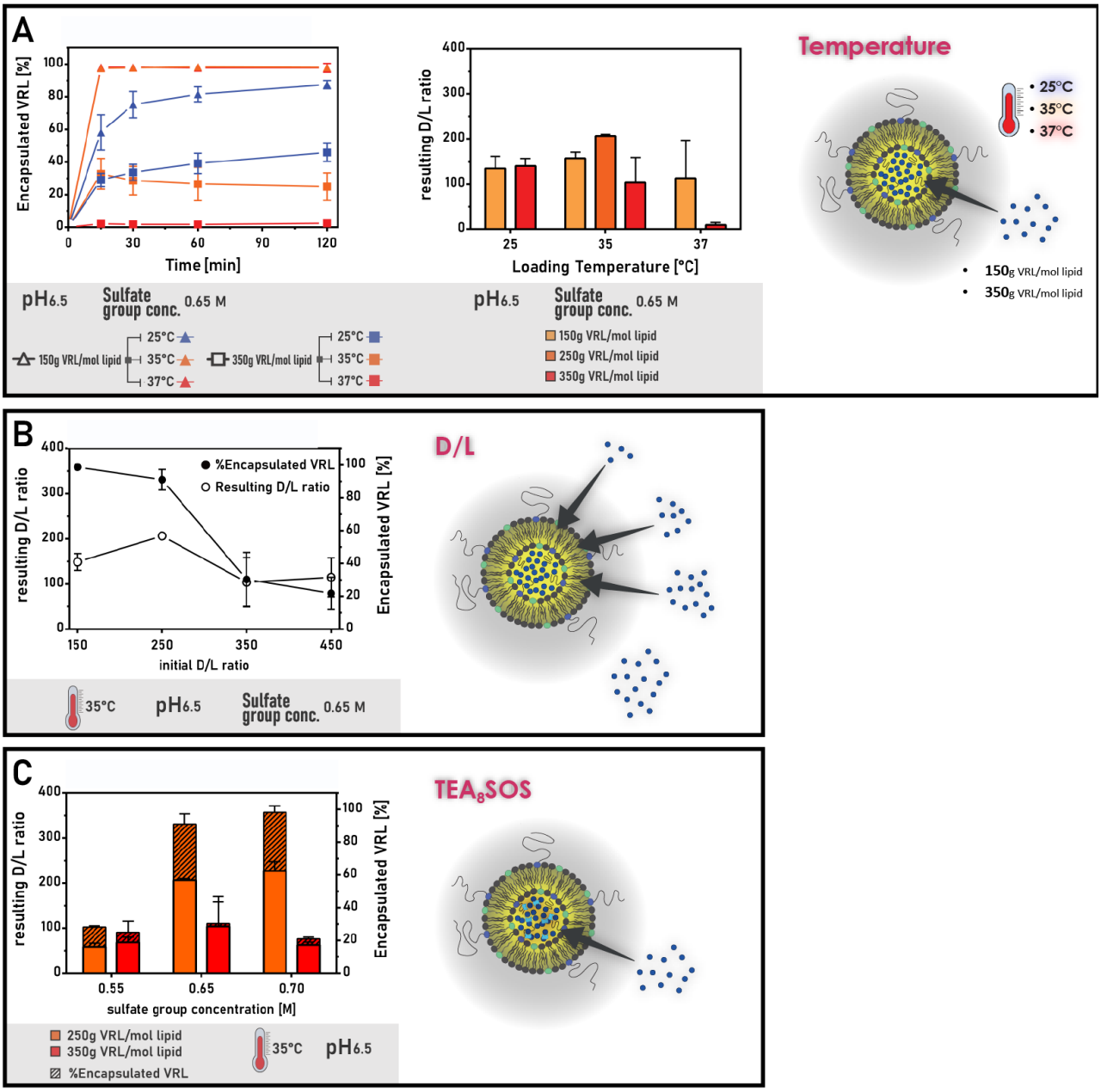
As shown in Figure 1 A, the temperature used during drug loading and the initial D/L ratio were found to influence the drug loading. At the lower D/L ratio of 150 g VRL/mol lipid, the loading rate as well as the resulting loading efficiency increased with an increase in temperature, whereas for higher D/L ratios the opposite was found. For example, less than 10 % VRL was encapsulated when loading at 37 °C with a D/L ratio of 350 g VRL/mol lipid.

In order to further investigate these findings, additional D/L ratios were examined (Figure 1 B). Increasing the initial D/L ratio from 150 g VRL/mol lipid to 250 g VRL/mol lipid resulted in a similarly high drug loading efficiency and anticipated increase in the amount of encapsulated VRL. However, a further increase in the initial D/L ratio to 350 g VRL/mol lipid led to a significant decrease in the loading efficiency as well as the resulting encapsulated amount of VRL per mol lipid ($p = 0.031$). This trend continued when increasing the initial D/L ratio up to 450 g VRL/mol lipid resulting in significantly decreased amounts of VRL loaded compared to an initial D/L ratio of 250 g VRL/mol lipid ($p = 0.021$).

A significant increase of ~50-100 nm in the hydrodynamic diameter (measured via DLS) was observed when the drug was not efficiently loaded (data not shown). Unencapsulated drug molecules may interact with the negatively charged liposomes facilitating liposome aggregation. Similar behaviour between positively charged drug and negatively charged lipid bilayers has previously been reported for doxorubicin as well as in general for divalent cations [26,27].

The influence of the internal concentration of TEA_8SOS sulfate groups on drug loading was also evaluated. As shown in Figure 1 C, an increase in the TEA_8SOS sulfate group concentration from 0.55 M to 0.65 M with a D/L ratio of 250 g VRL/mol lipid, resulted in an improvement in the VRL loading ($p < 0.001$). However, raising the internal TEA_8SOS sulfate group concentration from 0.65 M to 0.70 M did not result in a further increase ($p = 0.168$). A different trend was found for formulations with an initial D/L ratio of 350 g VRL/mol lipid where raising the internal TEA_8SOS sulfate group concentration did not improve the amount of VRL loaded.

Based on the results of these studies, the optimal conditions for drug loading were found to include an external pH of 6.5, a loading temperature of 35 °C, a maximum initial D/L ratio of 250 g VRL/mol lipid and an internal TEA_8SOS sulfate group concentration of 0.65 M. These conditions, yield formulations with a drug-to-lipid ratio of approximately 1/4 (mol/mol) (i.e. which corresponds to approximately 206 g VRL/mol lipid).



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296 **Figure 1:** Lyso-lipid containing thermosensitive liposomes were loaded with vinorelbine (VRL) under
297 different drug loading conditions. Parameters highlighted in grey were held constant throughout the
298 drug loading experiment with the pH referring to that of the external buffer, the sulfate group
299 concentration is due to presence of TEA_8SOS levels within the internal compartment of liposomes, and
300 the D/L ratio (in units of g VRL/mol lipid) refers to the initial ratio of VRL and lipid. The graphs show the
301 drug loading following one hour of incubation. Error bars represent the SD of three independent
302 experiments (n=3).

303

3.2. Temperature triggered release of VRL from thermosensitive liposomes

As shown in Figure 2 A, preliminary assessment of *in vitro* drug release from the 250 g VRL/mol lipid formulation in buffer alone (i.e. absence of protein) confirmed the temperature sensitivity of LTSL loaded with a relatively high level of VRL. Less than 10 % VRL was released at 37 °C over a time course of 30 min (data not shown in graph). Similar results were found for the release at 38 °C. When heated to 39 °C, after an initial lag time, VRL was released from the liposomes. Heating of the liposomes to temperatures of 40 °C or more, resulted in rapid and efficient release of the drug within one minute.

In vitro release from the 250 g VRL/mol lipid was also examined under more physiologically relevant conditions. Under these conditions, the influence of protein was found to have a profound impact on the drug release profile of the formulation. At 37 °C, approximately 60 % of drug loaded was released within 10 min (Figure 2 B). The 250 g VRL/mol lipid formulation includes approximately 26,000 molecules per liposome (Table 1). Liposome formulations including lower levels of drug were prepared and evaluated in terms of stability and drug release. A reduction in the number of molecules loaded to 80 g VRL/mol lipid significantly improved the stability of the liposomes (Figure 2 C). Only 3 % of VRL was released following 10 min at 37 °C. Lowering the D/L ratio to 30 g VRL/mol lipid (i.e. equivalent to 2,700 molecules per liposome) retained the stability at 37 °C, but further improved the stability at 38 °C and 39 °C (Figure 2 D). The improved stability at temperatures below 39 °C is best highlighted in Figure 3 which shows the difference in drug released between the different formulations at specific time points. The significant impact of the number of VRL molecules loaded was especially apparent when evaluating the stability at 37 °C over a time course of one hour (Figure 3 D). Notably, the increased stability at temperatures below 40 °C did not affect the burst release behavior once heated to higher temperatures.

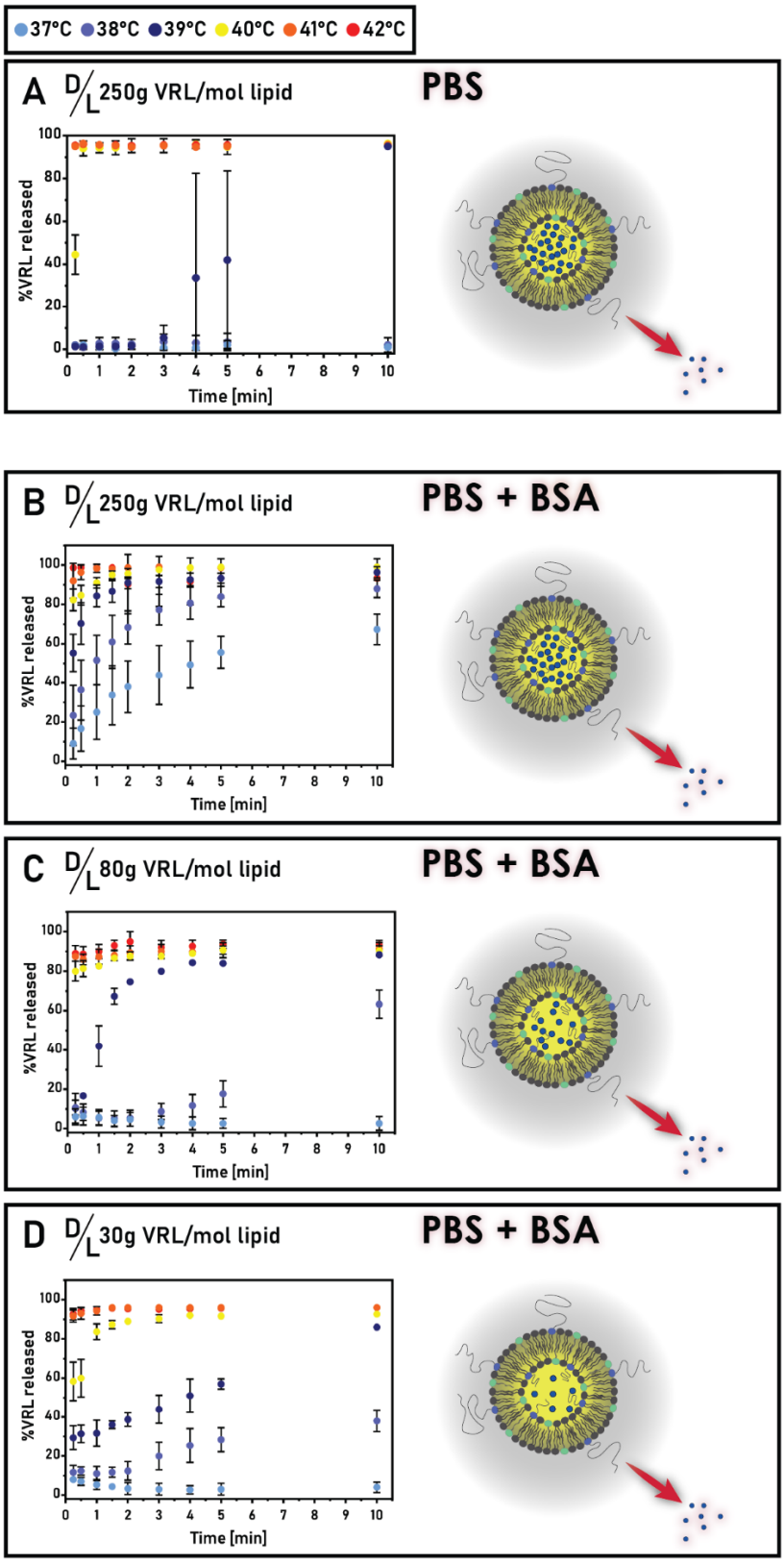
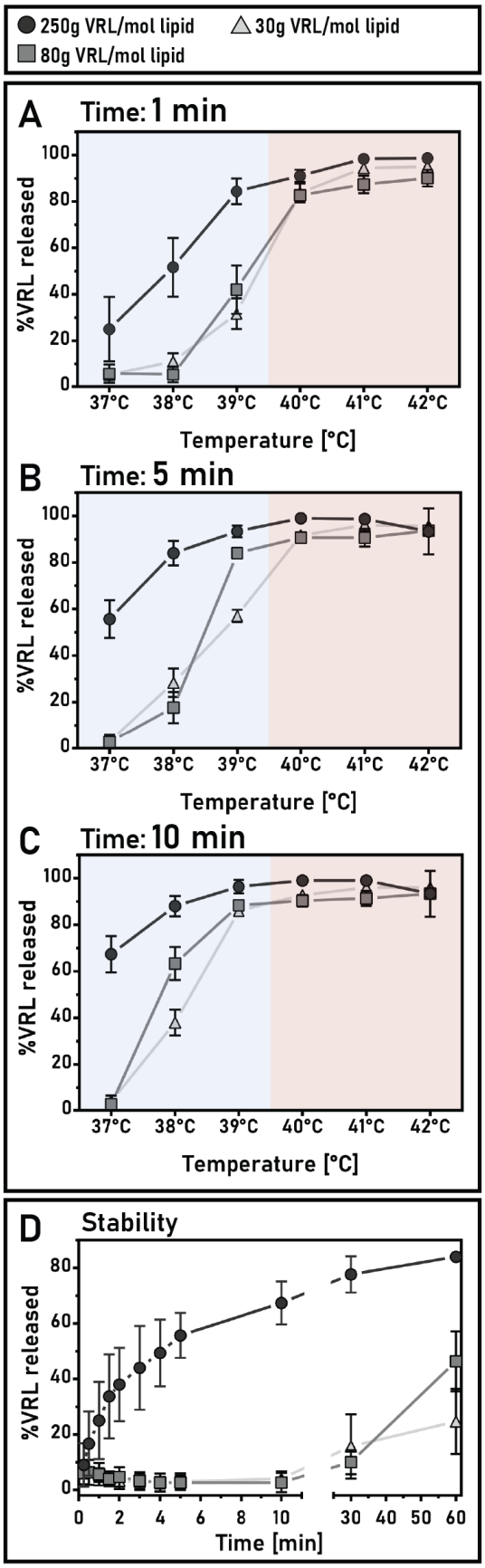


Figure 2: VRL release from thermosensitive liposomes loaded with different D/L ratios at various temperatures. Liposomes were added to pre-heated release media and released drug was immediately separated from liposomes using size exclusion chromatography at various time points. PBS without (A) or with protein added (B-D) was used as release media. Liposomes loaded with the same amount of drug were significantly less stable in the presence of protein (i.e. BSA 45 g/L). Reducing the amount of drug loaded led to an improvement in stability at temperatures below 40 °C while maintaining burst release properties when heated to higher temperatures. Error bars represent SD of three independent experiments (n=3).



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336 **Figure 3:** Drug release comparison of the formulations loaded at different D/L ratios at (A) 1, (B) 5, and
337 (C) 10 min. Blue and red indicate the temperature ranges that had desired negligible or little drug release
338 and burst release, respectively. (D) Drug release at 37 °C over one hour demonstrates the significant
339 differences in stability between the three formulations loaded with different amounts of VRL. Error bars
340 represent SD of three independent experiments (n=3).

3.3. Physico-chemical characteristics of drug-loaded thermosensitive liposomes

The thermal properties of the liposome formulations with different drug-to-lipid ratios were examined to better understand the influence of drug loading on formulation stability and release. As shown in Table 1, the unloaded LTSL formulations have an average phase transition temperature (T_m) of 41.1 ± 0.1 °C. Loading of the LTSL formulations with VRL affected their T_m. A decrease in the amount of drug loaded from 250 g VRL/mol lipid to 80 g VRL/mol lipid and 30 g VRL/mol lipid resulted in an increase in T_m from 39.3 ± 0.2 °C to 39.9 ± 0.1 °C and 40.6 ± 0.1 °C, respectively (see thermograms in Supplementary, Figure S2).

Table 1: Physico-chemical properties of LTSL composed of the same lipid mixture loaded with different amounts of VRL. TEA_8SOS was used to actively load VRL into liposomes and unloaded drug was removed using a cation exchange resin. The average hydrodynamic diameter (i.e. size) of liposomes was determined by DLS.

Initial D/L ratio [g VRL/mol lipid]	VRL molecules/ liposome	Size [nm]	PDI	Zeta potential [mV]	T_m unloaded [°C]	T_m loaded [°C]
250	≈26000 ± 5000	104 ± 2	0.107 ± 0.014	-26 ± 3	41.1 ± 0.1	39.3 ± 0.2
80	≈7600 ± 300	100 ± 1	0.067 ± 0.010	-26 ± 1	41.1 ± 0.02	39.9 ± 0.1
30	≈2700 ± 200	92 ± 3	0.055 ± 0.010	-26 ± 1	41.0 ± 0.04	40.6 ± 0.1

Cryo-TEM images of formulations were obtained to evaluate morphology and to gain insight on the state of the encapsulated drug. The unloaded LTSL (Figure 4 A) appeared spherical in morphology, whereas loading VRL at a D/L ratio of 250 g VRL/mol lipid (Figure 4 B) resulted in liposomes with a polygonal structure with darkened regions inside the liposomes which are typically indicative of drug precipitate. The majority of liposomes loaded with a medium level of VRL (Figure 4 C, 80 g VRL/mol lipid) appeared spherical in morphology, with only smaller liposomes exhibiting similar features to those observed for LTSL with the higher drug loading level. A further reduction in the D/L ratio to 30 g VRL/mol lipid resulted in polygonal and bean-like structures without visible signs of drug precipitation (Figure 4 D). The apparent differences in electron density between the interior and exterior of drug loaded and unloaded liposomes were analyzed by quantifying the mean gray value inside and outside of the liposomes. The differences between the intensities in the interior and exterior of the liposomes were found to vary depending on the amount of drug loaded. Due to the obvious differences in appearance of the liposomes loaded with 80 g VRL/mol lipid depending on their size, the analysis was separated into liposomes larger and smaller than 70 nm in diameter. The intensity value for the formulation with the 250 g VRL/mol lipid (i.e. 12.4 ± 5.6; Mean ± SD) was significantly higher than the value obtained for the larger 80 g VRL/mol lipid (7.2 ± 3.7; $p < 0.001$), the 30 g VRL/mol lipid (8.4 ± 3.3; $p < 0.001$) and the unloaded liposomes (8.1 ± 4.7; $p < 0.001$). However, no significant difference between the 250 g VRL/mol lipid loaded liposomes and the smaller 80 g VRL/mol lipid liposomes was detected ($p = 1.0$). Additionally, there was no significant difference between the larger 80 g VRL/mol lipid loaded liposomes or the 30 g VRL/mol lipid ones, as well as the unloaded liposomes, suggesting that drug precipitation within the vesicles mainly appears at the higher D/L ratios in combination with the vesicle size and not at the lower drug loading levels.

Comparison of the internal and external mean gray values of each liposome formulation revealed significant differences for the liposomes loaded at 250 g VRL/mol lipid ($p = .02$) and 80 g VRL/mol lipid ($p = .032$), but not for liposomes loaded at 30 g VRL/mol lipid ($p = .113$) or unloaded liposomes ($p = .082$).

The electron density differences in combination with the morphology observations suggest drug precipitation inside the liposomes loaded at a high D/L ratio (250 g VRL/mol lipid). Whether the precipitate

is of amorphous or crystalline structure remains to be identified and will be investigated in future studies. The resulting nanoparticles no longer exhibit a spherical morphology, with areas of increased lipid bilayer curvature. As previously discussed by Hossann et al. high curvature results in a more loosely packed lipid bilayer and increased number of membrane defects causing an overall increase in membrane permeability [28].

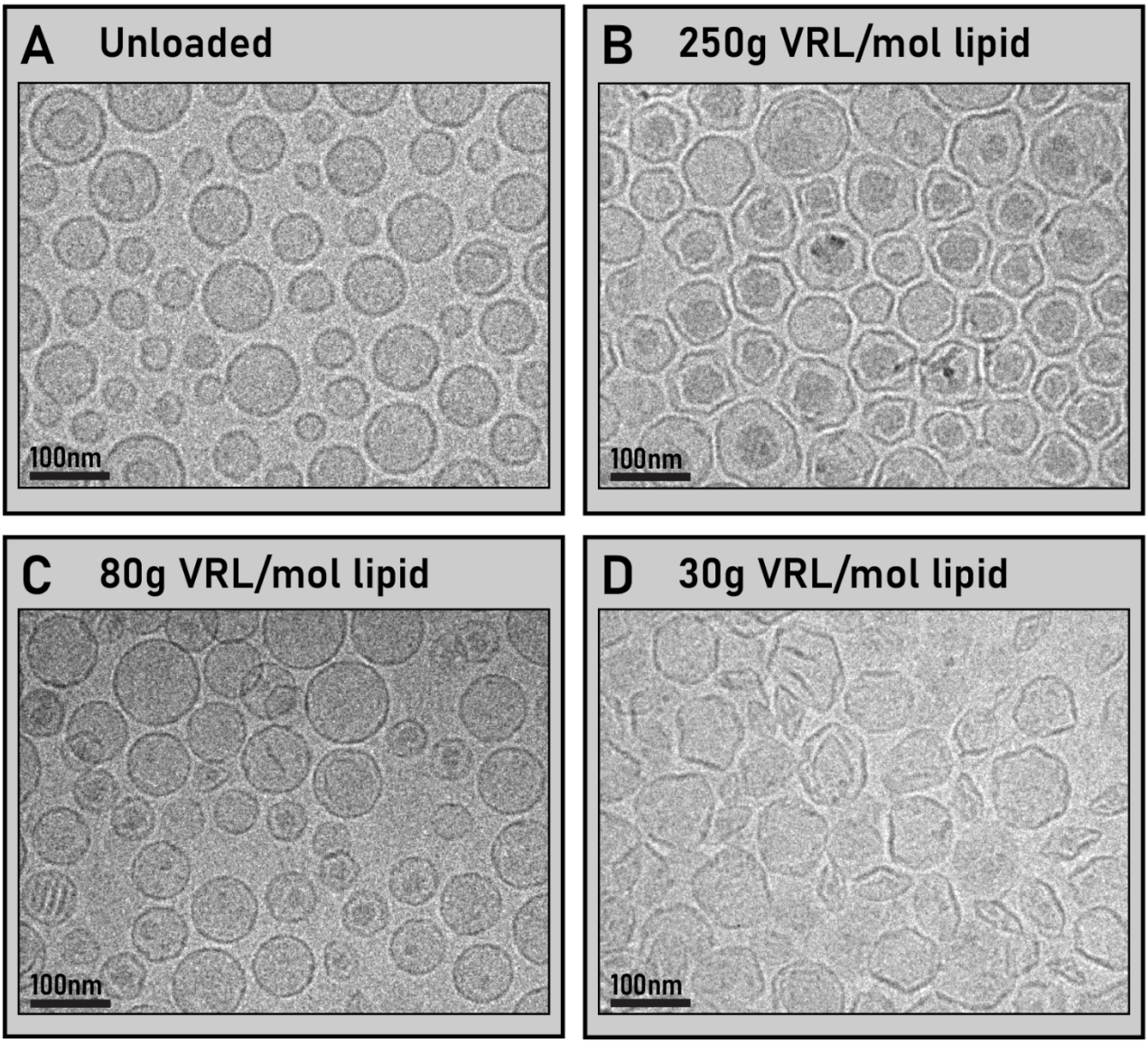

Figure 4: Representative cryo-TEM images of LTSL loaded with different amounts of VRL. Unloaded liposomes underwent the same protocol as loaded liposomes. A) Unloaded LTSL appear spherical, whereas loaded LTSL (B-D) exhibit a polygonal morphology and areas of increased darkness within the interior of the vesicles.

4. Discussion

VRL is a semi-synthetic vinca alkaloid with a higher degree of lipophilicity, membrane permeability, and therapeutic index relative to other compounds in the vinca alkaloid family such as vinblastine or vincristine [9,29]. It has been shown to be active in a broad range of cancers including non-small cell lung carcinoma, breast cancer and soft tissue sarcomas, as well as rhabdomyosarcoma [29–31]. Hematological and neurological toxicities have been reported as the dose limiting adverse effects of VRL [30,32]. Thus, several liposome formulations encapsulating VRL have been developed as a means to reduce systemic drug exposure [9,13–15,33]. TLC178 is a non-thermosensitive liposome formulation of VRL from the Taiwan Liposome Company which is currently undergoing Phase I/II clinical evaluation for the treatment of advanced malignancies (NCT02925000). The EMA and FDA recently granted TLC178 orphan drug designation for the treatment of soft tissue sarcoma.

The advantages associated with heat-triggered drug delivery as an approach for cancer treatment have been highlighted through pre-clinical and clinical studies examining the thermosensitive liposome formulation of doxorubicin, known as ThermoDox® [6,17,34]. In comparison to administration of doxorubicin in non-thermosensitive liposomes, the thermosensitive liposome formulation has been shown to significantly increase the amount of drug delivered to the tumor [35]. However, as with other dual modality therapeutic approaches, the combination of thermosensitive liposomes and heat delivery, to achieve drug release, has been met with challenges [36]. To date, doxorubicin is the drug that has been most commonly incorporated and subsequently evaluated in thermosensitive liposomes. Review of the scientific literature on thermosensitive liposomes that has been published over the past decades revealed that roughly 50 % of all studies either aimed to develop formulations encapsulating doxorubicin or studied heat-triggered drug delivery aspects using doxorubicin as the compound of interest. The use of doxorubicin is likely, among other considerations, due to its fluorescence properties which facilitates straightforward, real-time assessment of drug release. However, there are many other drugs that may benefit from delivery using thermosensitive liposomes. Our group has been interested in evaluating thermosensitive liposome formulations of other anti-cancer agents including cisplatin, alvespimycin, and in the current study vinorelbine [2,37]. Many studies have evaluated the influence of lipid composition and conditions during formulation preparation on drug loading and release from non-thermosensitive liposomes with clear composition-property-performance relationships established [1,38–41]. However, in terms of thermosensitive liposomes, these relationships remain an interesting area of research as there are many aspects that have not yet been evaluated. It cannot be assumed that the established relationships between composition and performance for non-thermosensitive liposomes are directly applicable to their thermosensitive counterparts. For this study, in addition to the D/L ratio employed for drug loading, the influence of several other liposome preparation parameters on the performance of a thermosensitive vinorelbine liposome formulation were evaluated.

It is desirable to achieve high D/L ratios when developing a liposomal drug formulation. This ensures delivery of a therapeutic drug dose without administering large amounts of lipid. In addition, as reported by Drummond et al, increasing the D/L ratio actually improved the *in vivo* stability of a non-thermosensitive liposome formulation containing VRL [13,42]. Thus, the initial experiments aimed to understand and optimize the loading behaviour of VRL into LTSL.

The lipophilicity of VRL complicates its stable entrapment within the liposome core [9,11,13]. Several techniques have been developed to actively load VRL into non-thermosensitive liposomes. These methods are based on the A23187 ionophore technique coupled with magnesium sulfate [9,10] or calcium hydroxybenzenesulfonate [11], resulting in high D/L ratios for non-PEGylated liposomes. In addition, traditional approaches based on pH gradients and sodium citrate have also been utilized [10,12,33]. However, most of these techniques have been used in combination with sphingomyelin and cholesterol based liposomes since it was shown that DSPC containing formulations exhibited poor *in vitro* stability as

well as increased *in vivo* drug leakage (as was observed with another vinca alkaloid, vincristine) [43]. Using a triethylammonium sucrose octasulfate (TEA_8SOS) gradient, Drummond et al. were able to develop a DSPC-based formulation that was capable of stably entrapping VRL at high D/L ratios [13].

Several thermosensitive liposome formulations of varied lipid composition have been reported [5]. This study builds upon the success of the LTSL formulation ThermoDox®, with the goal of rapid drug release within the tumor blood vasculature once the liposomes are heated in the range of mild HT. The LTSL lipid bilayer of ThermoDox® is composed of DPPC, lyso-SPC and PEG_{2k}-DSPE in a molar ratio of 86.5/9.7/3.8 [44]. However, in a separate study it had been shown that incorporating PEG_{2k}-DSPE at molar ratios higher than 3 mol% significantly impedes the loading of VRL [13]. PEG_{2k}-DSPE is generally included in liposome formulations to allow for longer *in vivo* circulation times but has also been shown, in the case of LTSL, to play an important role in ensuring rapid drug release [13,44].

Interaction between a drug and the lipid bilayer can have a significant effect on the loading and stability of the formulation. Several findings from this study point to a possible interaction between VRL and the lipid bilayer. First, the loading of VRL appeared to be highly dependent on the ratio of drug added to the liposomes. And secondly, the stability was found to be significantly decreased with an increase in the level of drug loading.

Drummond et al. have reported low loading efficiencies when actively loading VRL into various PEG_{2k}-DSPE containing liposome formulations [13,43]. In these studies, the authors suggest that the loading is limited in the presence of PEG_{2k}-DSPE due to ionic interaction between PEG_{2k}-DSPE and the drug. They also found that replacing PEG_{2k}-DSPE with a non-ionic PEGylated lipid resulted in efficient drug loading even at relatively high D/L ratios [13]. Similarly, Li et al. found that post-insertion of PEG_{2k}-DSPE, following the drug loading process, maintained a high level of drug loading [15]. In the current study, there was found to be a threshold level for the number of VRL molecules per liposome beyond which the loading was impaired in a similar manner to that described for PEG_{2k}-DSPE (i.e. between 250 and 350 g VRL/mol PL). The limitations in loading of VRL into the LTSL formulation may be attributed to an ionic interaction between VRL and constituents of the liposome membrane bilayer. Based on the findings from Drummond et al. and the current study, this interaction appears to depend on the ratio between VRL and ionic PEGylated lipid (i.e. PEG_{2k}-DSPE) and can thus be avoided through fine-tuning of the D/L ratio and/or temperature during the loading process as well as the lipid composition.

Another key piece of data which alludes to an interaction between VRL and the lipid bilayer is the decrease in liposome stability that is observed with an increase in drug loading. It is important to note, unlike the trend observed in the study performed by Drummond et al. where higher amounts of drug loaded improved *in vivo* stability, results from this study show an increase in drug loading led to *in vitro* destabilization of the LTSL [13].

Previous studies have shown that given its physico-chemical properties, VRL can become partially interdigitated within the liposome bilayer. This interdigitation of the drug in the lipid bilayer can increase the number of membrane packing defects, which in turn can result in an increase in membrane permeability [45,46]. Cryo-TEM images of the 250 g VRL/mol lipid formulation reveal a dramatic change in morphology, with a polygonal shape and clear presence of drug precipitated within the internal aqueous volume of the vesicles. In other studies, the precipitation of vinca alkaloids within non-thermosensitive liposomes has been shown to result in an increase in formulation stability [47]. This highlights that relationships and trends observed for specific drug/non-thermosensitive liposome combinations cannot be easily translated to thermosensitive liposome formulations. The specific properties of the lipid composition or the encapsulated drug require a customized optimization approach tailored around the scope of application.

In the design of thermosensitive liposomes, analysis of T_m provides insight into the temperature dependent stability and drug release properties. A sharp T_m in the range of mild HT has shown to be crucial

for rapid and complete drug release from LTSL formulations in vivo [48]. The reduction in T_m and T_{on} following loading of the liposomes with increasing amounts of VRL, indicates an interaction between the drug and lipid. Loading high amounts of VRL resulted in an approximate 1.85 °C decrease in the T_m, suggesting a fluidization of the lipid membrane due to drug-lipid interactions [49,50].

Studies in our field continue to examine the heat-triggered release of drugs from carriers in the absence of biologically relevant components. However, plasma proteins are known to affect cargo release from thermosensitive liposomes [51]. Interactions between the lipid bilayer and plasma components resulting in increased release have been observed for a multitude of drugs and thermosensitive liposome formulations [52–57]. Thus, in order to successfully predict *in vivo* performance, evaluating *in vitro* release in biorelevant media is crucial. The presence of proteins such as albumin within the release media can facilitate lipid extraction from the lipid-bilayer, and thus result in increased membrane defects and an increased membrane permeability [51,58]. As demonstrated in these studies, the release of VRL from lyso-lipid containing thermosensitive liposomes is dramatically increased in BSA containing media (Figure 2). Recent papers publish drug release from thermosensitive and non-thermosensitive liposomes in the absence of biologically relevant components. Given the findings of the current study and the community's interest in translation of formulations to the clinic, we recommend challenging liposome formulations *in vitro* in the early stages of research in order to gain a true sense of their potential.

5. Acknowledgements

These studies were supported by a CHIR project grant to C.A (GrantNo.). C.A. acknowledges GlaxoSmithKline for an endowed chair in Pharmaceutic and Drug Delivery. MR holds a Centre for Pharmaceutical Oncology scholarship. The authors thank Linyu Fan for her assistance with the lipid analysis and acknowledge the use of equipment provided by the Centre of Pharmaceutical Oncology (CPO) at the University of Toronto.

6. Abbreviations

BSA: bovine serum albumin; Cryo-TEM: cryogenic transmission electron microscopy; D/L ratio: drug-to-lipid ratio; DPPC: 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DSC: differential scanning calorimetry; HBD: HEPES buffered dextrose; HBS: HEPES buffered saline; HT: hyperthermia; L_{IL}: lipids in inner membrane leaflet; L_{OL}: lipids in outer membrane leaflet; LTSL: low temperature sensitive liposome; lyso-SPC: 1-stearoyl-2-lyso-sn-glycero-3-phosphocholine; PA: palmitic acid; PBS: phosphate buffered saline; PDI: polydispersity index; PEG_{2k}-DSPE: N-(carbonyl-methoxypolyethylenglycol 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine; SA: stearic acid; SD: standard deviation; SEC: size exclusion chromatography; $T_{1/2}$: peak width at half peak height; TEA: triethylamine; TEA_8SOS: triethylamine sucrose octasulfate; TFA: trifluoroacetic acid, T_m: melting phase transition temperature; T_{on}: temperature of peak onset; VRL: vinorelbine

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Supplementary Information

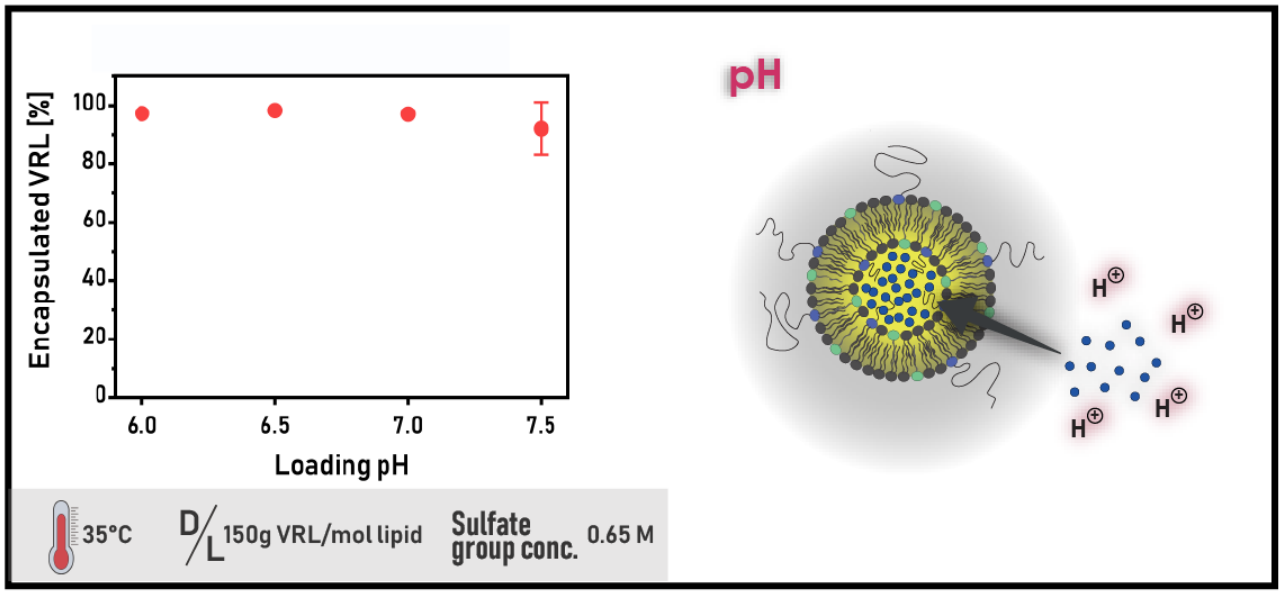

Figure S1: Lyso-lipid containing thermosensitive liposomes were loaded with vinorelbine (VRL) under various pH. Parameters highlighted in grey were held constant throughout the drug loading experiment with the pH referring to that of the external buffer, the sulfate group concentration is due to presence of TEA_8SOS levels within the internal compartment of liposomes, and the D/L ratio refers to the initial ratio of VRL added to liposomes. The graphs show the drug loading following one hour of incubation. Error bars represent SD of three independent experiments (n=3).

Thermal properties of the thermosensitive liposomes

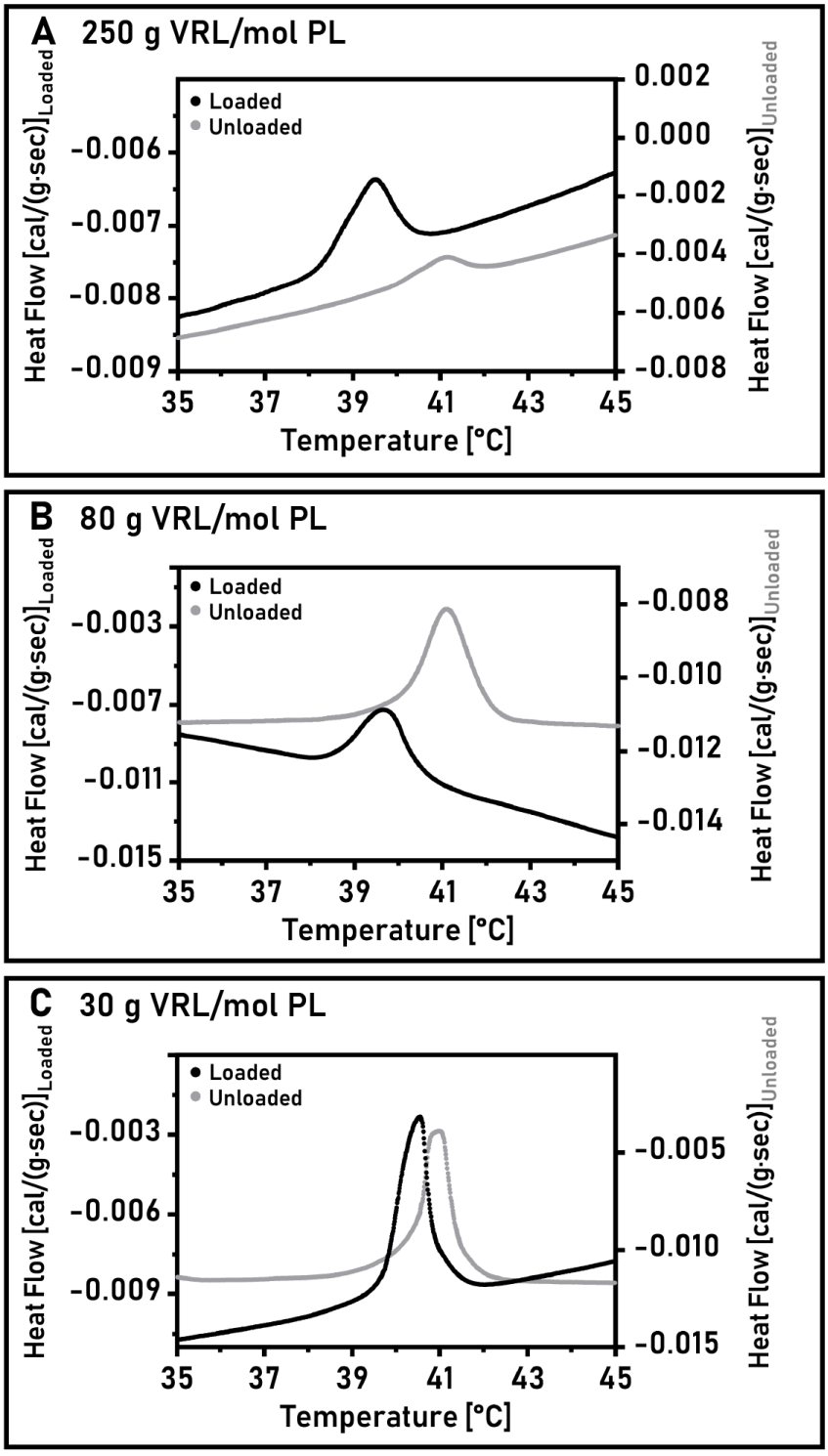

Figure S2: A-C Thermograms of unloaded LTSL and LTSL loaded with different amounts of VRL. Liposomes in solutions were heated at a rate of 1 °C/min from 25 °C to 60 °C. For clarity, heat flows of unloaded/ loaded samples are plotted on separate axes.