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Stability and Release Performance of Curcumin-Loaded Liposomes with Varying Content of Hydrogenated Phospholipids

Kedong Tai [a], Michael Rappolt [b], Like Mao [a], Yanxiang Gao [a], Fang Yuan [a,]*

[a] *Beijing Advanced Innovation Center for Food Nutrition and Human Health, Beijing Laboratory for Food Quality and Safety, College of Food Science & Nutritional Engineering, China Agricultural University, Beijing 100083, P.R. China*

[b] *School of Food Science and Nutrition, University of Leeds, Leeds LS2 9JT, U.K.*

**Corresponding author: Fang Yuan.*

Tel: +86-10-62737034

Address: No.17 Qinghua East Road, Haidian District, Beijing 100083, China

E-mail: yuanfang0220@cau.edu.cn

ABSTRACT

The aim of this study was to substitute part of soybean phospholipid (SPC) with hydrogenated soybean phospholipid (HSPC) in curcumin-loaded liposomes (Cur-LP), in order to further enhance stability and release performances of curcumin. When the SPC/HSPC mass ratio changed from 10:0 to 5:5, vesicle size, encapsulation efficiency and alkali resistance of curcumin increased, although a small decrease in centrifugal stability was observed. Salt stability became worse as more HSPC was used (3:7 and 0:10). Owing storage at 4 °C and 25 °C, Cur-LP at a SPC/HSPC mass ratio of 5:5 performed well considering vesicle size, lipid oxidation and curcumin retention. These vesicles displayed also the best sustained-release performance in simulated digestion, attributed to the tighter lipid packing in membranes as indicated by fluorescence probes, DSC and FTIR. This study can guide the development of a Cur-LP product with improved shelf-life stability by using HSPC.

KEYWORDS: Liposomes; Curcumin; Hydrogenated Phospholipid; Stability

1. Introduction

Poor stability is the major drawback for liposomes formed by natural unsaturated phospholipids, especially when encapsulating nutraceuticals. This instability is mainly attributed to lipid oxidation and vesicles aggregation (Huang, Chung, & Wu, 1998). The double bonds in fatty acid chains of unsaturated phospholipids disturb the molecular packing and cause interspaces in the hydrophobic region of liposomal membranes, which increases the membrane fluidity (Grit & Crommelin, 1993). To solve this problem, cholesterol and phytosterol are employed in the preparation of traditional liposomes to stabilize the liposomal vesicles and improve their encapsulation efficiency by reducing the bilayers fluidity in the liquid crystalline phase (Sulkowski, Pentak, Nowak, & Sulkowska, 2005; K. D. Tai, Liu, He, Ma, Mao, Gao, et al., 2018). Nonetheless, this does not solve the oxidation problem of unsaturated phospholipids, which limits the shelf-life stability of liposomes. In recent years, antioxidants such as α-tocopherol (Sahari, Moghimi, Hadian, Barzegar, & Mohammadi, 2017), quercetin (Frenzel & Steffen-Heins, 2015) and vitamin E (Amiri, et al., 2018) were tried to be embedded into liposomes. Although a considerable antioxidant effect was achieved in liposomes, this protocol might induce some molecular interaction between the antioxidant and the main embedded bioactive compounds. For example, promising bioactive nutrients and auxiliary agents such as protein (Wang & Wang, 2015) and polysaccharide (Caddeo, Diez-Sales, Pons, Carbone, Ennas, Puglisi, et al., 2016) strongly interact with polyphenols, which decreases their antioxidant effect in liposomes and renders the preparation process more complicated as well.

Compared with unsaturated phospholipids, saturated or hydrogenated ones exhibit higher phase transition temperatures, and gel-phases with a lower membrane fluidity can be formed at or below human body temperature. It contributes to the stability of the bioactive compound encapsulated in liposomes. Huang et al. found that adding HSPC effectively reduced lipid oxidation in haemoglobin-loaded liposomes (Huang, Chung, & Wu, 1998). Apart from a few

studies about liposomes prepared by hydrogenated phospholipids alone in field of medicine, little research has been carried out from a food perspective. Sebaaly et al. prepared eugenol (Sebaaly, Greige-Gerges, Stainmesse, Fessi, & Charcosset, 2016) and essential oil (Sebaaly, Jraij, Fessi, Charcosset, & Greige-Gerges, 2015) liposomes by non-hydrogenated and hydrogenated phospholipids, respectively. Of these, non-hydrogenated phospholipids liposomes displayed improved loading capacity. However, a better encapsulation efficiency (34.6%) of nisin Z in hydrogenated phospholipid liposomes was obtained, compared with liposomes contained a high percentage of unsaturated phospholipids (Laridi, Kheadr, Benech, Vuillemard, Lacroix, & Fliss, 2003). Chen et al. confirmed that a greater HSPC content leads to the higher phase transition temperature of liposomes, due to the longer alkyl chain and stronger chain-chain van der Waals interactions (Chen, Liang, Wang, Yokoyama, Chen, & Zhong, 2018). It is worth noting that the cost of hydrogenated phospholipids is much higher than that of unsaturated ones, which make it unrealistic to prepare liposomes using hydrogenated phospholipids alone in food processing.

In our previous study, curcumin-loaded liposomes (Cur-LP) have been successfully prepared by unsaturated phospholipids naturally extracted (Tai, Rappolt, He, Wei, Zhu, Zhang, et al., 2019). The physicochemical stability, encapsulation efficiency and bioavailability were significantly improved as more β-sitosterol was incorporated into the liposomes. Nevertheless, an unsatisfying phospholipid oxidation in these liposomes still displayed soon afterwards. Thus, the objective of this study was to investigate the usage of hydrogenated phospholipids in the manufacturing of more stable Cur-LP, which could prolong the availability of encapsulated curcumin. At present, most reported studies about Cur-LP only concentrate on usage of hydrogenated phospholipids alone, e.g., an ultrasound-assisted supercritical antisolvent method was established (Jia, Song, Gai, Zhang, & Zhao, 2016), which proves the feasibility of preparing Cur-LP with HSPC. But it is not suitable for application in the current food industry

due to the special high costs involved. Further, Chaves et al. studied the curcumin proliposomes with different phospholipids compositions, who found that curcumin loading in proliposomes prepared by HSPC mixed with some non-hydrogenated phospholipids was higher than that in samples formed by HSPC only (Chaves & Pinho, 2019).

In contrast to Chaves' research about powdery Cur-LP, our study tries to reveal the influence of HSPC content on stability and digestive release property of liquid-crystalline phase based Cur-LP. In our study, vesicle characteristics were determined by vesicle size, zeta-potential, polydispersity index (PDI) and morphology. A series of stability tests was conducted, such as centrifugal, salt and pH stability. Storage stability under refrigerated (4 °C) and room temperature (25 °C) conditions was closely monitored in terms of vesicle size, lipid oxidation and curcumin retention. Fluorescence probe, differential scanning calorimeter (DSC) and Fourier transform infrared spectroscopy (FTIR) were applied to measure the molecular ordering in liposomal membranes, which is closely related with liposomal stability. The *in vitr*o release performance during simulated digestion was finally evaluated to verify the application potential of Cur-LP containing some HSPC.

2. Materials and methods

2.1 Materials and chemicals

Non-hydrogenated soybean phospholipid (SPC) Lipoid S100 (94% soybean phosphatidylcholine, 3% lysophosphatidylcholine, 0.1% phosphatidylethanolamine, 0.5% N-acyl-phosphatidylethanolamine, 0.1% phosphatidylinositol, 2% water, 0.2% ethanol) was purchased from Lipoid GmbH (Ludwigshafen, Germany). Hydrogenated soybean phospholipid (HSPC, 97% 1,2-distearoyl-*sn*-glycero-3-phosphocholine) was purchased from Advanced Vehicle Technology Pharmaceutical Co., Ltd (Shanghai, China). Curcumin (> 95%) was obtained from Hebei Food Additive Co., Ltd (Hebei, China). Cholesterol was purchased

from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). The fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene (DPH, 98%), mucin from porcine stomach (M2378), pepsin from porcine gastric mucosa (P7125), pancreatin from porcine pancreas (P1750) and bile salts were purchased from Sigma-Aldrich (St. Louis MO, USA). Dimethyl sulfoxide (DMSO, 99%) was purchased from Xilong Scientific Co., Ltd (Guangzhou, China). All other reagents used were analytical grade without further purification.

2.2 Preparation of curcumin-loaded liposomes

Curcumin-loaded liposomes (Cur-LP) was prepared by the thin film hydration method illustrated in our previous study with some modifications (K. Tai, et al., 2019). Briefly, soybean phospholipid, cholesterol and curcumin (40:8:1, w/w/w) were adequately dissolved into anhydrous methanol. The soybean phospholipid contained SPC and/or HSPC, the concentration of total soybean phospholipids was fixed at 10 mg/mL, of which the investigative mass ratios of SPC/HSPC are 10:0, 7:3, 5:5, 3:7 and 0:10, respectively. Whereafter, the solution was transferred into a round-bottom flask and methanol was vacuum-removed under rotary evaporating at 50 °C. The thin lipid film inside the surface of the flask was hydrated with phosphate buffered saline (PBS, 0.05M, pH 7.0) to form a crude curcumin-loaded liposomes suspension. A micro-fluidizer (M-110EH30, Microfluidic Corp, Newton, MA, USA) operated for 3 cycles at 150 MPa was used to decrease the liposomal vesicle size. All freshly prepared liposomes were stored in a refrigerator at 4 °C for further analysis.

2.3 Liposomes characterization

The mean vesicle size, zeta potential, polydispersity index (PDI) and size distribution were determined by dynamic light scattering (DLS) using a Malvern Zetasizer Nano-ZS90 (Malvern Instruments Ltd., Malvern, UK) equipped with a 633 nm He/Ne laser at a detector angle of 90°. The prepared Cur-LP were diluted (10-fold) with 0.05 M PBS in order to avoid multiple scattering that could affect the accuracy of the DLS-measurements. For size

measurement, the diluted suspension was transferred into disposable polystyrene cuvettes with a path length of 10 mm. Refractive index was set to 1.490. The zeta potential was obtained from the electrophoretic mobility of the vesicles. Folded capillary cell were used for the measurements. PDI represents the width of vesicle size distribution ranging from 0 to 1, of which PDI $\leq$ 0.1 is considered to be highly monodispersed, values of 0.1-0.4 and > 0.4 are considered to be moderately and highly poly-dispersed, respectively (Bhattacharjee, 2016). Each sample was carried out in triplicate at 25 °C and each measurement needs 2 min of equilibration before starting.

The encapsulation efficiency (EE) and loading capacity (LC) of curcumin was measured by UV-1800 spectrophotometer (Shimadzu, Japan). Liposomes were centrifuged at 15000 × *g* for 1 h (3K15 refrigerated versatile centrifuge, Sigma Laborzentrifugen GmbH, Germany) to remove the free or absorbed curcumin on the surface of the vesicles. The sediment was re-dispersed in buffer and centrifuged three times to remove the unembedded curcumin as much as possible. The sediment was adequately disrupted by methanol to release the encapsulated curcumin, which was detected by absorbance at 428 nm based on full wavelength scanning. The content of curcumin was then calculated from a calibration curve. Cur-LP was used to measure the gross amount of curcumin in the samples. EE and LC of curcumin were calculated using the following equations:

$$\mathrm{EE}(\%)=\frac{\text{Amount of encapsulated curcumin}}{\text{Total amount of curcumin}}\times 100 \quad (1)$$

$$\mathrm{LC}(\%)=\frac{\text{Amount of encapsulated curcumin}}{\text{Total amount of curcumin, phospholipids and cholesterol}}\times 100 \quad (2)$$

2.4 Field emission scanning electron microscope (FE-SEM)

The microstructure of liquid and lyophilized liposomes was observed by FE-SEM (SU8020, Hitachi Ltd, Japan). For liquid samples, SEM samples were prepared by dropping diluted liposomes onto the silicon wafer followed by air-drying and gold sputtering. The

lyophilized samples were directly adhered to the silicon wafer before gold sputtering, which avoids the influence of charging under electron beam. The thickness of gold layer is about 10 nm. SEM images were captured at an accelerating voltage of 3 kV.

2.5 The fluidity of liposomal bilayer membranes

The fluidity of liposomal membranes was measured by fluorescence spectrometer (F-7000, Hitachi, Japan) according to the protocol applied in a previous study (K. Tai, et al., 2019). The inclination degree of DPH penetrated into hydrophobic region of liposomal membranes is readily affected by adjacent phospholipid molecules moving laterally. Thus, the fluidity of liposomal membranes is reflected by polarization of DPH, which is also independent of probe concentration. Liposomes diluted (10-fold) with 0.05 M PBS were mixed with DPH solution (2 μM in dimethyl sulfoxide) at a volume ratio of 5:1. The mixture was incubated in the dark for 60 min at room temperature. For investigating the change of membrane fluidity with temperature, the samples were thermally equilibrated in a water bath at different temperatures ranging from 25-80 °C for 10 min before measurement. Samples were excited with vertically polarized light (360 nm), and the emission intensities were recorded at 430 nm. The bandwidth was set to 5 nm for both the exciting and emitting light beam. The steady state polarization (P) of DPH was calculated using the following equations:

$$P=\left(I_{0,0}-G\times I_{0,90}\right)/\left(I_{0,0}+G\times I_{0,90}\right) \quad (3)$$

$$G=I_{90,0}/I_{90,90} \quad (4)$$

where $I_{0,0}$, $I_{0,90}$, $I_{90,0}$, $I_{90,90}$ are fluorescence intensities of emitted light (exciting light) polarized to exciting light (emitted light) in parallel (0) and vertical (90), respectively. G is the grating correction coefficient. The fluorescence intensity of DPH in aqueous is basically ignored.

2.6 Differential scanning calorimetry (DSC)

The phase transition temperature (T_m) and enthalpy (ΔH) of Cur-LP were obtained from

DSC thermograms. Briefly, transferring accurately-weighted mass of freeze-dried samples into aluminium pans, which was subsequently sealed with a lid. Heating sample was operated by DSC Q200 (TA Instruments Inc., New Castle, Delaware, USA) at rate of 10 °C/min from 25 to 80 °C under nitrogen gas flow (50 mL/min). An empty aluminum crucible was used as a reference. All DSC curves were base-line corrected and fitted with a sum of Gaussian distributions using the software Origin 9.0 (Origin Lab Inc., Northampton, MA, USA).

2.7 Stability study

2.7.1 Accelerated stability analysis

The accelerated stability of liposomes was evaluated by multi-sample analytical centrifuge LUMiSizer® (L.U.M GmbH, Berlin, Germany), which traces instability phenomena by recording the evolution of transmission profiles for test tubes with the dispersions in terms of time and position. The continuous instability process characterized by vesicle migration and sedimentation would be accelerated during centrifugation. All liposome formulations were subjected to centrifugation at 2300 × *g* for 1 h. A total of 360 profiles were recorded in intervals of 10 s.

2.7.2 Salt stability

In view of the common usage of salt in food products, the stability of liposomes in salt solutions with diverse concentrations is important to know. Undiluted Cur-LP formulation was mixed with NaCl solutions at a volume ratio of 1:9. The final concentration of NaCl in the mixed solution varied in the range of 100-1000 mM. Vesicle size of treated liposomes was measured after 1 h of incubation at room temperature. Extent of change in vesicle size (ΔD_z) was calculated using the following equation:

$$\Delta D_z(\%) = \frac{\text{Vesicle size after incubation-vesicle size initially prepared}}{\text{Vesicle size initially prepared}} \times 100 \qquad (5)$$

2.7.3 pH stability

To determine the pH sensitivity of curcumin and pH-driven deformation of liposomal membranes, Cur-LP was subjected to an alkaline environment (pH= 8.0, 9.0, 10.0, 11.0 and 12.0). The pH of PBS was respectively adjusted to pre-set values using NaOH solution. Then, undiluted Cur-LP samples were mixed with PBS at a volume ratio of 1:9. All mixtures were incubated at room temperature for 1 h followed by measuring the amount of curcumin retained in the liposomes by absorbance. The retention degree of curcumin (%) was calculated using following equation:

$$\text{Retention}\ (\%) = \frac{C_t}{C_0} \times 100 \qquad (6)$$

where C_t and C_0 are the concentration of curcumin remained in samples after treatment and in freshly prepared samples, respectively.

2.7.4 Storage stability

15 mL of freshly prepared liquid Cur-LP were transferred and sealed into 20 mL brown glass bottles. The storage temperatures were controlled at 4 °C and 25 °C, respectively. Vesicle size, lipid oxidation and retention of curcumin were monitored at scheduled time intervals during three weeks storage. The method of vesicle size measurement is described in Section 2.3.

The thiobarbituric acid reactive substances (TBARS) method was applied to determine the lipid oxidation of liposomes. Briefly, 1 mL of undiluted Cur-LP was mixed with 5 mL of thiobarbituric acid (TBA) solvent (15% TBA + 0.6% trichloroacetic acid + 2% hydrochloric acid, w/v) and heated in a boiling water bath for 30 min. Whereafter, the mixture was cooled immediately in an ice bath to terminate the reaction, followed by centrifugation at 825 × *g* for 15 min. The absorbance of the supernatant was measured at 535 nm against a blank containing all solvents except the liposomes. The concentration of malondialdehyde (MDA) formed from oxidative degradation of lipids was calculated using the extinction coefficient (1.56×10^5 M^{-1}

cm^{-1}) as 'TBARS value'.

The retention degree of curcumin in liposomal suspensions was also monitored. 500 μL of suspension was taken at a pre-set interval and the residual curcumin was measured by absorbance. The percentage of curcumin retained was calculated, using Eq. 6.

2.8 Fourier transform infrared (FTIR) spectroscopy

FTIR was applied to investigate the possible molecular interactions of curcumin with the membrane matrix. The analysis was performed using a Spectrum™ 100 FTIR spectrometer (PerkinElmer, Waltham, MA, USA) in the range of 400-4000 cm^{-1} with a resolution of 4 cm^{-1}. All Cur-LP formulations were freeze-dried before analysis, which were mixed with anhydrous potassium bromide (KBr) and pressed into a tablet. A pure KBr tablet was measured as background.

2.9 In vitro release study

The *in vitro* release of curcumin from liposomes with different phospholipid compositions was carried out during simulated digestion. This method was preferred over the dialysis method, since it more closely reflects the human digestive environment. *In vitro* simulated gastrointestinal tract (GIT) model established in this study could reveal the release performance of Cur-LP during simulated digestion, which might be affected by saturated level of phospholipids in liposomes. The basic GIT model includes three digestion steps: (i) mouth phase (simulated saliva fluid, SSF, pH=6.8), (ii) gastric phase (simulated gastric fluid, SGF, pH=1.5), and (iii) small intestine phase (simulated intestinal fluid, SIF, pH=7.0).

In detail, (i) 1 L of SSF contained 1.594 g of NaCl, 0.202 g of KCl and 0.6 g of mucin, (ii) 1 L of SGF contained 2 g of NaCl, 3.2 g of pepsin and 7 mL of 12 M HCl, and (iii) 1 L of SIF contained 6.8 g of K_2HPO_4, 8.775 g of NaCl, 5 g of bile salts and 3.2 g of pancreatin. All simulated digestive juices and liposomes were preheated at 37 °C. Firstly, liposomes and SSF were mixed 1:1 by volume for simulating mouth digestion. When the simulated mouth phase

ended, the digestive mixture was mixed with SGF in the same volume ratio (1:1) for simulated gastric digestion. At the end of the gastric phase, the gastric effluent was continuously mixed with an equal volume of SIF. These three digestive phases took 10 min, 2 h and 2 h, respectively. The pH of the gastric effluent was adjusted to 6.8-7.0 before simulated intestine digestion. The whole simulated digestion process was carried out in a water bath shaker at 37 °C, to simulate gastrointestinal peristalsis.

The release of curcumin from liposomes constructed with mixed phospholipids was investigated by monitoring its cumulative release during simulated digestion. 500 μL of digestive mixture was collected at defined time intervals and cooled in an ice bath. Each control sample was centrifuged at 4 °C for 30 min (15000 × *g*). The supernatant was collected and the absorbance measured for quantification the amount of curcumin released from the liposomes into the digestive mixture. For calculation of the released amount of curcumin, the unembedded fraction of curcumin in primary sample needs to be subtracted. The cumulative release rate (%) of curcumin was calculated and plotted as a function of time:

$$\text{Cumulative release}(\%) = \sum_{0}^{t} \left(\frac{M_t}{M_0}\right) \times 100 \qquad (7)$$

where M_0 and M_t are the initial amount of curcumin encapsulated in liposomes and the cumulative amount of released curcumin in digestive medium at different sampling times.

2.10 Statistical analysis

All experiments were carried out in triplicate and data are expressed as mean ± standard deviation. One-way ANOVA and Duncan's significant difference test at 5% level of significance by IBM SPSS software version 25 (IBM Corp., NY) were performed. Origin 9.0 (OriginLab Inc., Northampton, MA, USA) was used to draw graphs.

3. Results and discussion

3.1 Liposomal vesicle characteristics

In our previous studies, sonication and high-pressure homogenization were commonly applied to decrease liposomal vesicle size and prevent vesicles sedimentation (K. Tai, et al., 2019; K. D. Tai, et al., 2018). Although the stability was improved in these liposome suspensions, we note that by applying greater homogeneous pressures (>100 MPa) with the microfluidizer technique, vesicle sizes below 100 nm can be achieved. Microfluidisation at 117 MPa was applied as a preparation method of stable liposomes formed with sunflower lecithin (W. Liu, Ye, & Singh, 2015; Peng, Zou, Liu, Liu, & McClements, 2018). Hence, using a microfluidizer, Cur-LPs with different SPC/HSPC mass ratios were prepared (each containing 33 mol% cholesterol).

Vesicle sizes of Cur-LPs from 84 nm (SPC:HSPC=0:10) to 220 nm (SPC:HSPC=3:7) were obtained (Table 1), by increasing the ratio of HSPC in the total phospholipids gradually, but the vesicle size decreased to 146 nm for Cur-LP with 100% HSPC. The same trend is observed for the PDI (Table 1) and SEM results (Fig. 1, air-dried samples). From the vesicle size distribution results (Fig. 1), unimodal distributions for 10:0 and 7:3 SPC/HSPC ratios and a bimodal distribution above 50% HSPC are evident. Similarly, Sebaaly et al. showed that eugenol-loaded liposomes using only hydrogenated phospholipids were larger than those formed by non-hydrogenated ones (Sebaaly, Greige-Gerges, Agusti, Fessi, & Charcosset, 2016). The same phenomenon was also found for quercetin-loaded liposomes (Azzi, Jraij, Auezova, Fourmentin, & Greige-Gerges, 2018). The larger vesicle sizes were attributed to the diverse saturation of the hydrocarbon chains in phospholipids molecules. Increasing the degree of chain saturation increases van der Waals interactions, leading to reduced membrane fluidity (increased membrane rigidity) (Zhang, Han, Ye, Liu, Tian, Lu, et al., 2019), and hence to larger vesicle diameters with lower membrane curvature. Generally, increasing membrane rigidities

enhances the “curvature frustration”, when membranes are bent. That is, following the widely applied concept of the molecular “packing parameter” (Israelachvili, 2011), the ideal molecular shape in planar structures such bilayers is the cylinder. However, when forming liposomes, the curvature of the membranes is slightly deviating from zero, i.e. the embedded molecules adopt slightly truncated cone shapes, deviating from their spontaneous intrinsic curvature. That is, the greater the fluidity of a membrane (e.g., displayed in the liquid disordered phase (L_d)), the easier it can be bent (low curvature frustration). On the other hand, stiffer membranes such as in the liquid ordered (L_o) and lamellar gel (L_β) phase cannot be bent as easily, i.e. the curvature frustration is higher and consequently liposomes with bigger diameters are expected to form. Thus, the observation of increasing vesicle size with increasing HSPC content makes good sense, since based on the thermotropic phase behaviour of hydrogenated soybean PC in the presence of cholesterol (Kitayama, Takechi, Tamai, Matsuki, Yomota, & Saito, 2014), the following phase transition from the L_o phase (100% SPC) to L_o/L_d phase coexistence (7:3 SPC/HSPC) to the L_o/L_β phase coexistence (HSPC > 50%) is expected. Note, the bimodal vesicle distribution might be related to L_o-rich and L_β-rich vesicles, respectively.

The reason for the shrinking of the liposomes when using HSPC alone remains speculative. The most rigid membranes should actually lead to the largest vesicle sizes. A possible explanation could be that the overall membrane undulations reduce drastically, once there is no SPC present in the liposomes, and hence, the Helfrich undulation repulsion force (Helfrich, 1973) reduces. Consequently, the internal water layer within the liposomes is expected to reduce as well. In contrast, the liposomes are well hydrated when the SPC/HSPC mass ratio varied from 10:0 to 3:7, while at ratio of 0:10 the liposomes might expel interlamellar water and therefore might overall shrink in size. Alternatively, it might be that the curvature frustration at 100% HSPC becomes so large, that stacking disorder effects lead to the formation of liposomes with significantly less lamellae, and hence leading to smaller vesicle sizes.

3.2 Thermal behavior

The thermal property of the liposomal membrane was investigated, using DSC (Fig. 2A and 2B). As previously described, the phase transition temperature (T_m) of Lipoid S100 is below 0 °C (Li, Wang, Zhang, Wang, Huang, Luo, et al., 2015), which explains why no endothermic peak is apparent in thermogram of Cur-LP with SPC alone. The same result was also obtained in another study (Li, Liu, Zhang, Fang, Xu, Zhang, et al., 2016). Further, it is important to note, that the SPC was mixed with 33 mol% cholesterol, which decreases the membrane fluidity of the SPC membranes in the liquid crystalline phase. We note, that DPH polarization of curcumin-loaded liposomes prepared by SPC alone (~0.44) is clearly lower than SPC liposomal membranes containing 33 mol% cholesterol (~0.55; cp. also Figure 2C). However, cholesterol in this case, does not induce the formation of a purely liquid ordered state (L_o) (Hodzic, Rappolt, Amenitsch, Laggner, & Pabst, 2008; Rappolt, Vidal, Kriechbaum, Steinhart, Amenitsch, Bernstorff, et al., 2003; Singer & Finegold, 1990). Thus, SPC/cholesterol (33 mol%) is clearly a liquid phase (free lateral movement of the lipids take place), but it can neither be described satisfactorily as a liquid disordered phase (L_d) nor as a fully liquid ordered (L_o) phase, it is somewhat in between. For the sake of simplicity though, we call this the "L_o phase" all the same. When HSPC is added to the system, the phase behavior changes entirely. Indeed, for 7:3, 5:5, 3:7 and 0:10 SPC/HSPC, two endothermic peaks are observed (Fig. 2B). In accordance to Kitayama et al. on the phase behavior of hydrogenated soybean PC (Kitayama, Takechi, Tamai, Matsuki, Yomota, & Saito, 2014), we observe two transitions: (i) L_o+L_β to L_o+L_d phase transition (melting point) at 46 to 50 °C and (ii) the L_o+L_d to L_d phase transition at 54 to 64 °C (Fig. 2A and 2B). For increasing amounts of HSPC in liposomes, increased transition temperatures and total enthalpies were synchronously observed.

Phospholipid membranes are highly fluid above melting point when the amount of unsaturated lipid is high, and 80% of the fatty acids in SPC used in this study are unsaturated.

Incorporating cholesterol in the membrane reduces the membrane fluidity above T_m due to the effective limitation of mobility of acyl chains and the induction of the liquid ordered phase, while it increases the membrane fluidity below T_m (Sułkowski, Pentak, Nowak, & Sułkowska, 2005). Fig. 2C shows the fluorescence micropolarity of DPH in different Cur-LPs as a function of temperature. The micropolarity of all Cur-LP gradually reduced with increasing temperature. This is attributed to the higher mobility of phospholipids molecules at elevated temperatures, resulting from an increased lipid chain disorder (increased number of *trans-gauche* conformations per chain) (Balanc, Ota, Djordjevic, Sentjurc, Nedovic, Bugarski, et al., 2015; Seelig & Seelig, 1974). Cur-LP containing HSPC had better resistance on the heat-induced fluidity increase of membranes, especially evident for Cur-LP composed of HSPC alone. This is understood, since the saturated fatty acid chains of HSPC decrease the steric repulsion between phospholipids in membranes, which in turn, restricts the rotational mobility of phospholipids owing to an increased lateral lipid packing density (Neves, Nunes, Amenitsch, & Reis, 2016; Takechi-Haraya, Sakai-Kato, Abe, Kawanishi, Okuda, & Goda, 2016).

3.3 Stability assessment under environmental stress

3.3.1 Centrifugal stability

Given the thermodynamic instability of liposomes, the LUMiSizer® was used to monitor dispersion properties of liposome suspensions by the evolution of the space- and time-related transmission profile. As shown in Fig. 1 (A1-E1 at 25 °C and A2-E2 at 60 °C), the red and green profiles represent transmissions detected in earlier and later time, respectively. 130 mm and 108 mm shown in abscissa represent the physical bottom and top position of sample, respectively. Under the action of centrifugal force, migration of the vesicles gradually caused density variations at different positions in the sample cells, which is reflected by transmission variations. The density of transmission profiles reflects the time required for sample phase separation. More dense profile mean that more time is needed for the phase separation. Results

in Fig. 1 demonstrates that increasing the ratio of HSPC decreased the centrifugal stability of Cur-LP. This change is best reflected in transmission changes in the middle of sample cells. Compared with the low transmission changes observed for Cur-LPs with no or low content of HSPC (Fig. A1 and B1), obvious transmission changes were observed in Cur-LP at SPC/HSPC of 3:7 (Fig. D1) and 0:10 (Fig. E1) at 25 °C. The trends are similar at 60 °C (Fig. A2-E2), but display faster changes in transmissions due to more intense Brownian motions of liposomal vesicles at higher temperature. In accordance with vesicle size results, only the 10:0 and 7:3 SPC/HSPC ratio liposomes deliver satisfyingly low sedimentation results, whereas bigger vesicles and in particular bimodal vesicle size distributions lead to faster migration rates under the influence of centrifugation.

3.3.2 Salt stability

Salt is commonly used in food products like energy drinks for electrolyte supplementation. Thus, it is necessary to test salt tolerance of Cur-LP in the case of HSPC addition. In this study, all Cur-LPs were incubated in NaCl solutions of different ion strength at room temperature. The change in vesicle size (ΔD_z, %) as a function of NaCl concentration is shown in Fig. 3A. Cur-LP exhibited a good salt stability for vesicles with SPC/HSPC mass ratios ranging from 10:0 to 5:5. However, vesicle size increased significantly at high HSPC concentrations (SPC/HSPC=3:7 and 0:10). This observation is somewhat counterintuitive, since at high salt concentrations osmosis is induced. Thus, it is expected that liposomes increasingly dehydrate with an increase in the sodium concentration and therefore, the vesicle size should reduce. On the other hand, dehydration of the membranes leads to denser lipid packings within the membranes, and hence to more rigid membranes within the liposomes. In turn, this induced curvature frustration might lead to stacking defects and subsequent annealing of the HSPC-rich liposomes to form actually vesicle of bigger size with lower curvature frustration (Mouritsen, 2011).

3.3.3 pH stability

Curcumin is very sensitive to the pH of the environment. It was confirmed that more than 90% of curcumin decomposes rapidly in buffer systems at neutral-basic pH condition (Wang, Pan, Cheng, Lin, Ho, Hsieh, et al., 1997). Nevertheless, a previous study reported that stabilizing the SPC membranes with cholesterol and Tween 80 would improve the pH stability of encapsulated curcumin in liposomes under alkaline condition (~~X.~~ Chen, Zou, Niu, Liu, Peng, & Liu, 2015). As for our study, results shown in Fig. 3B illustrates that Cur-LPs containing HSPC display a better pH-stability than the SPC-liposomes (10:0). The curcumin retention rates of the former were significantly higher than that of the latter, when exceeding pH 9.0. For instance, at moderate alkaline conditions (pH=10.0), the pH stability ranking for Cur-LP is 5:5 > 0:10 > 3:7 > 7:3 > 10:0 (SPC/HSPC mass ratio), and almost the same curcumin retention rates (about 87%) were determined at pH=12.0 for Cur-LP at SPC/HSPC mass ratios of 5:5, 7:3 and 10:0. It manifests that the increasing HSPC concentration, leading to lower membrane fluidity, also helps to resist the leakage of curcumin from membranes, when the pH increased. Similar observations were made for large unilamellar vesicles composed of PC/cholesterol/phosphatidylglycerol. Here, the employment of HSPC offered both, stronger membrane rigidity and lesser propensity for peroxidation, leading to an optimized liposomal L-cysteine-stability (El Kateb, Cynober, Chaumeil, & Dumortier, 2008). It is worth noting that all HSPC concentrations lead to a significant pH-stabilization of the liposomes lying usually in a retention interval of 5%, with the ratios 0:10, 3:7 and 5:5 being slightly more promising than the 7:3 ratio of SPC to HSPC.

3.4 Storage stability

The thermodynamic instability of liposomes may lead to vesicle fusion and leakage of embedded curcumin, which is reflected in the shelf-life stability of Cur-LP in terms of size, lipid oxidation and curcumin retention.

3.4.1 Vesicle size

As presented in Fig. 4A and 4B, all Cur-LPs guarantee good vesicle size stability for 30 day storage period, whether at 4 °C or 25 °C, apart from Cur-LP with SPC/HSPC at mass ratio of 3:7. Here a significant increase in vesicle size (from 220 nm to 382 nm (25 °C) and 447 nm (4 °C)) was observed during the 30 day storage period. We note that the formulation with a SPC/HSPC ratio of 3:7 displays also the strongest bimodal size distribution (Fig. 1). This either reflects an exceptional tendency of vesicle aggregation in this formulation, or it might be explained by L_o-rich and L_β-rich vesicle formulation, in which the latter vesicle contribution grows in size over time, especially in the first week. Nonetheless, the high-pressure microfluidics technique applied in this study produced Cur-LP with smaller vesicle sizes (less than 150 nm) with a greater size stability, when compared to results of high-pressure homogenization in our previous study (K. Tai, et al., 2019). The same preparation method used in another Cur-LP study achieved stable vesicle sizes at 4 °C and a 90 day storage period, but displayed poor stability at 25 °C (X. Chen, Zou, Niu, Liu, Peng, & Liu, 2015). In contrast, in the present investigation a higher amount of cholesterol and greater homogenization pressure helped to obtain a better stability also at 25 °C. Importantly, liposomes at 25 °C do remain in the L_o+L_β phase (Fig. 2B), and hence guarantee more rigid membranes with enhanced curcumin retention, but at the same time lead to liposomes with a very good size stability over a longer storage time.

3.4.2 Lipid peroxidation

In many previous studies on liposomes for food applications, unsaturated phospholipids were widely used to improve the absorption or bio-distribution of bioactive compounds. However, with respect to long-term storage in a commercial environment, these studies commonly paid no attention to the lipid oxidation of liposomes, which plays an important role in their stability (Niki, Yamamoto, Komuro, & Sato, 1991). Although some groups did achieve

antioxidant properties for liposomes by embedding antioxidants like zein hydrolysates (Y. Y. Li, Liu, Han, Kong, & Liu, 2017), the simple strategy of replacing a certain amount of SPC with HSPC, performed well in restraining the oxidation process in liposomes during storage, as shown in Fig. 4C (4 °C) and 4D (25 °C). Cur-LP composed of HSPC alone (0:10) showed little change in TBARS values during storage at the two different temperatures owing to the lack of unsaturated double bonds in the fatty acid chains. On the contrary, liposomal membranes formed with SPC alone (10:0) showed the most lipid oxidation. For mixed phospholipids samples, adding more HSPC significantly slowed down the lipid oxidation of Cur-LP during storage. Of these, Cur-LP at a SPC/HSPC ratio of 5:5 exhibited the least lipid oxidation. One reason is that greater concentrations of saturated fatty acid chains in the membranes reduces obviously the possibility of phospholipid oxidation. However, another reason is that reduced vesicle fusion and aggregation under storage decreased also the likelihood of unsaturated phospholipids being exposed to oxygen (Gast, Zirwer, Ladhoff, Schreiber, Koelsch, Kretschmer, et al., 1982). An interesting phenomenon is that the TBARS values of Cur-LPs containing SPC at 4 °C were just a little bit lower than that at 25 °C. Actually, we expected a greater difference owing to the acceleration of the oxidation rate at higher temperature. One possible explanation is that antioxidant of curcumin itself was also involved during storage (Ak, & Gülçin, 2008; Huang, Chen, Liu, Wang, Shen, Chen, et al., 2017), which deserves to be further studied in the future.

3.4.3 Curcumin retention

Leaked curcumin can be gradually degrade during storage. Hence, the retention of curcumin in Cur-LP suspensions were monitored, as shown in Fig. 4E (4 °C) and 4F (25 °C). The slowest curcumin degradation curve was obtained for Cur-LP at a SPC/HSPC ratio of 5:5, while curcumin degradation was fasted in Cur-LP at a SPC/HSPC ratio of 10:0 (lowest oxidation stability) and 3:7 (lowest vesicle size stability), regardless of temperature. Thus, in

our opinion, the stable vesicle size and lower phospholipids oxidation during storage synergistically guaranteed the stability of mixed phospholipids membranes on embedded curcumin. As to the relatively lower curcumin retention of Cur-LP with HSPC alone, it is speculated that the stiffness of this liposomal vesicles failed to make uniform distribution of curcumin molecules in the membranes owning to the lower temperature used in preparation and storage than phase transition of HSPC. Thus, curcumin leaked and degraded after two days storage. That is consistent with a slightly lower encapsulation efficiency of curcumin in liposomes with HSPC alone. As for the temperature effect, the degradation of curcumin was expectedly faster at 25 °C than at 4 °C. The main reason for this finding, is the higher permeability of liposomal membranes at higher temperature, which led to a stronger leakage of curcumin from the internal hydrophobic area of bilayers (Jin, Lu, & Jiang, 2016). Furthermore, the rapid degradation period in the first week indicates that a process of gradual stabilization and annealing takes place in Cur-LP after preparation. This is also consistent with the rapid growth stages in vesicle size and TBARS values in the same period.

3.5 Intermolecular interaction

There has been little research being reported on molecular interactions between SPC and HSPC in liposomal membranes, while these interactions indeed contribute to the stability of membranes. In accordance to our previous FTIR study on liposomes (K. D. Tai, et al., 2018), the pair of peaks located at 2800-3000 cm^{-1} relate to symmetric (~2850 cm^{-1}) and asymmetric (~2920 cm^{-1}) stretching vibration of C-H bonds in CH_2, with the peak near 1160 cm^{-1} displaying the asymmetric stretching vibration of the C-O-C aliphatic ester. Concerning the polar head group of phospholipids, representative peaks are the symmetric (~1070 cm^{-1}) and asymmetric stretching vibrations (~1240 cm^{-1}) of PO_2^- groups and the asymmetric stretching vibration (~950 cm^{-1}) of the N^+-CH_3 group. All wavenumbers of different stretching vibration peaks are labelled in Fig. 5A. As the HSPC content of the membranes increased, the two stretching vibration peaks of C-H simultaneously shifted to the lower wavenumber (shift of 2926 to 2917 and 2854 to 2850, respectively), which is mainly attributed to the diverse saturation of fatty acid chains in phospholipids. Denser phospholipid chain packings due to increased van der Waals attractions greatly limit the bond vibrations deep in the hydrophobic region of membranes. A similar observation was made for the stretching vibration in the C-O-C group (shift from 1162 to 1160), that is located between the polar head groups and hydrophobic fatty acid tail chains. Likely, the C-O-C group is involved in the formation of hydrogen (H) bonds with other molecules, such as cholesterol and curcumin containing hydroxyl groups. The PO_2^- groups are also involved in the formation of H-bonds, which led its symmetric and asymmetric stretching vibrations peaks shifting to lower wavenumbers (shift of 1240 to 1236 and 1073.3 to 1072.7, respectively) as more HSPC was added. The miniscule wavenumber shift of the N^+-CH_3 stretching vibration illustrates that the hydrophilic choline in the head group did not participate in intermolecular interactions. In general, the formation of more H-bonds and denser lipid chain packing in the hydrophobic core synergistically made liposomal vesicles more

stable, to allow increase in the HSPC content.

3.6 In vitro release performance in simulated digestion

In order to clarify the possible application potential of this binary liposomes, *in vitro* simulated digestion experiments were carried out that includes three stages: mouth, stomach and small intestinal digestions. The digestion fate of curcumin can be expressed by its cumulative release from the liposomal matrix as shown in Fig. 5B. In the simulated mouth stage, only about 5% of curcumin was released from all Cur-LPs. The absence of specific enzymes for disrupting phospholipids bilayers and short processing time are responsible for the low curcumin release during this stage (Wickham, Faulks, & Mills, 2009). The cumulative release of curcumin was still less than 20% at the end of simulated gastric digestion. We attribute this mainly to the retained integrity of liposomal structures after simulated gastric digestion, which has been ascertained in our previous study (K. D. Tai, He, Yuan, Meng, Gao, & Yuan, 2017) and by others (W. L. Liu, Ye, Liu, Liu, & Singh, 2012). Notably, Cur-LPs with SPC/HSPC ratios of 10:0 and 7:3 displayed evidently higher curcumin release during this stage. Their relatively higher membrane fluidity (the fluid L_d phase is dominant, cp. Fig. 2B) causes more curcumin to be released. Additionally, the simulated stomach peristalsis (shaking) might also have a stronger influence on the integrity of the high-SPC liposomes. In simulated small intestinal digestion, a sharp increase in curcumin release was observed for all Cur-LP formulations, which we mainly attribute to the hydrolysis of phospholipids by lipolytic enzymes in pancreatin and the emulsifying effects of bile salts on liposomal membranes (W. L. Liu, Ye, Liu, Liu, & Singh, 2012; Sadeghpour, Rappolt, Misra, & Kulkarni, 2018). The former disrupts the phospholipids assembly by chemical hydrolysis, while the latter leads to solubilizing liposomal vesicles into micelles (Maherani, Arab-Tehrany, Kheirolomoom, Geny, & Linder, 2013). The fastest release was obtained for Cur-LP composed of SPC alone (10:0). Here more than 80% of curcumin was released after digestion. On the contrary, Cur-LP

composed of HSPC alone (0:10) successfully sustained the curcumin release when compared with the SPC-formed one. Noteworthy, the Cur-LP at SPC/HSPC ratio of 5:5 achieved the slowest curcumin release during simulated digestion, even slightly better than pure HSPC vesicles. The Cur-LP with SPC/HSPC ratio of 3:7 displays a slightly worse release rate in agreement with its poor vesicle size stability (Fig. 4A and 4B). In view of the complicated digestive environment, including warm temperature (37 °C), longstanding mechanical shaking and salt ions (NaCl), the best physicochemical stability obtained above guaranteed a digestive stability for the high-content HSPC liposomes, especially for Cur-LP at SPC/HSPC ratio of 5:5.

4. Conclusions

In this study, we have systematically analyzed the effect of increasing addition of HSPC with concomitant lowing of the SPC content on the stability and *in vitro* release of Cur-LP. It turned out that liposomes prepared by HSPC alone is not the best choice for curcumin encapsulation, although better encapsulation efficiency and loading capacity, stability and sustained release were achieved when compared to pure SPC liposomes. Combining advantages of SPC liposomes displaying better physical stability (during centrifugation and at high NaCl concentrations) with the more rigid membranes structures of HSPC liposomes, the mixed phospholipids liposomes with a SPC/HSPC ratio of 5:5 exhibited overall improved performances in encapsulating curcumin. Its good physicochemical stability is mainly attributed to (□) the denser lipid chain packing of the membranes (L_o+L_β phase coexistence) and (□) the good thermal stability of its liposomes up to 50 °C as probed by fluorescence and DSC. The intermolecular interaction results from FTIR measurements also confirm the denser lipid packing and increased H-bond formation, when HSPC-rich vesicles are employed. Finally, the enhanced liposomal stability (pH, centrifugal and shelf-life stability) of particularly the Cur-LP with a SPC/HSPC ratio of 5:5 is reflected also in the slowest *in vitro* simulated

digestion release of encapsulated curcumin. This formulation has the greatest potential for developing liposomes with enhanced health benefits for future functional food development. Future work will concentrate on improving annealing procedures for high HSPC-content liposomes formulations in order to enhance their stability even further.

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Figure captions & Figures

Figure 1. A, B, C, D and E represent Cur-LP with SPC/HSPC mass ratios of 10:0, 3:7, 5:5, 3:7 and 0:10, respectively. LUMiSizer transmission profiles at 25 °C (A1-E1) and at 60 °C (A2-E2). SEM images of air-dried samples are shown in (A3-E3). Here superimposed corresponding vesicle size distributions measured by DLS are also presented. SEM images of freeze-dried samples are shown in A4-E4. Appearance photographs of curcumin-loaded liposomes are shown in the last column.

Figure 2. DSC heating thermograms (A), SPC/HSPC ratio-temperature phase diagram (B), and temperature-dependent fluorescence polarization of DPH (C) embedded in the Cur-LP membrane at different SPC/HSPC mass ratios of 10:0, 3:7, 5:5, 3:7 and 0:10. Note, the DSC data (black dots) have been fitted by a sum of two turnovers, i.e., one referring to the (L_β + L_o) to (L_d + L_o) (blue) and the other to the (L_d + L_o) to L_d (red) phase transition. The fitted DSC curves are plotted in green.

Figure 3. Environmental stress stability assessment: (A) vesicle size changes of Cur-LP with SPC/HSPC mass ratios of 10:0, 3:7, 5:5, 3:7 and 0:10, incubated in sodium chloride solution with a gradient of concentrations (100-1000 mM); (B) curcumin retention of Cur-LP after 1 h incubation in PBS at pH ranging from 7 to 12.

Figure 4. Variations of vesicle sizes (A, B), thiobarbituric acid reactive substances (TBARS) (C, D) and curcumin retention (E, F) of Cur-LP with SPC/HSPC mass ratios of 10:0, 3:7, 5:5, 3:7 and 0:10 during 30 days storage at 4 °C (dashed lines) and 25 °C (solid lines), respectively. Each data point represents the mean value ± standard deviation (n = 3).

Figure 5. (A) FTIR spectrogram of Cur-LP at different SPC/HSPC mass ratios and curcumin, respectively. V_s and V_{as} represent symmetric and asymmetric stretching vibrations of molecular bonds in functional groups, respectively. The hydrophobic core region (CH_2 vibrational modes) and molecular vibrations in the head group region are highlighted in grey and yellow, respectively, as well as zoomed out for clarity on the right; (B) Cumulative release curves of curcumin from liposomes at different SPC/HSPC mass ratios during *in vitro* simulated digestion at 37 °C.

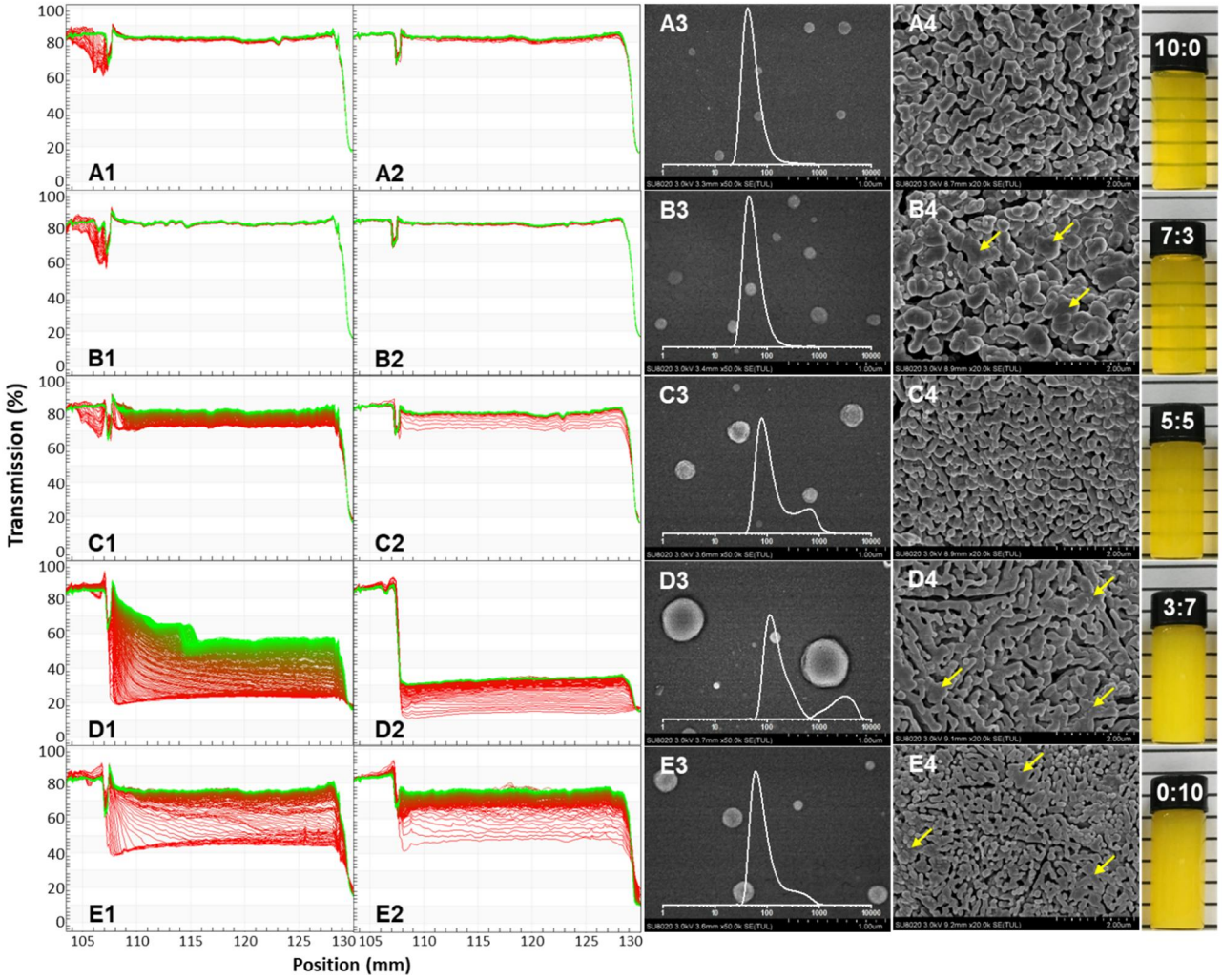

Transmission (%)
Position (mm)
A1
A2
A3
A4
B1
B2
B3
B4
C1
C2
C3
C4
D1
D2
D3
D4
E1
E2
E3
E4
10:0
7:3
5:5
3:7
0:10

Figure 1

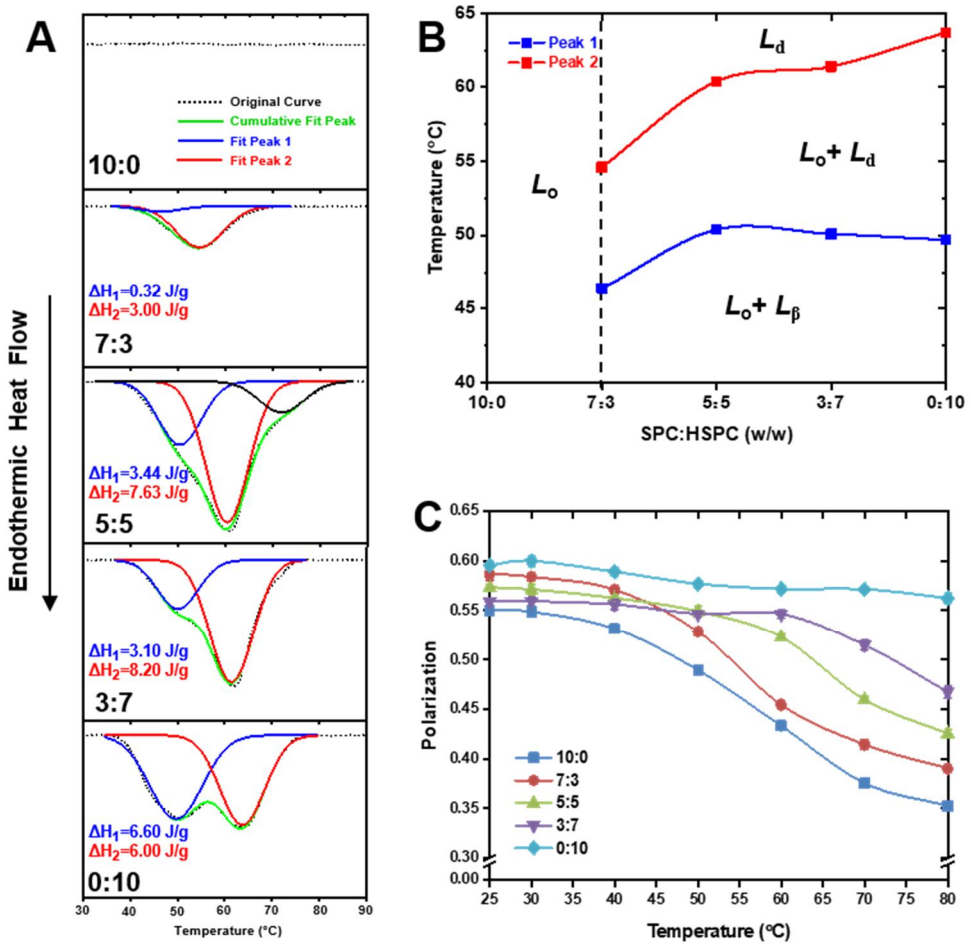

A
Original Curve
Cumulative Fit Peak
Fit Peak 1
Fit Peak 2
10:0
ΔH1=0.32 J/g
ΔH2=3.00 J/g
7:3
ΔH1=3.44 J/g
ΔH2=7.63 J/g
5:5
ΔH1=3.10 J/g
ΔH2=8.20 J/g
3:7
ΔH1=6.60 J/g
ΔH2=6.00 J/g
0:10
Endothermic Heat Flow
Temperature (°C)
B
Peak 1
Peak 2
Ld
Lo+ Ld
Lo
Lo+ Lβ
Temperature (°C)
SPC:HSPC (w/w)
C
10:0
7:3
5:5
3:7
0:10
Polarization
Temperature (°C)

Figure 2

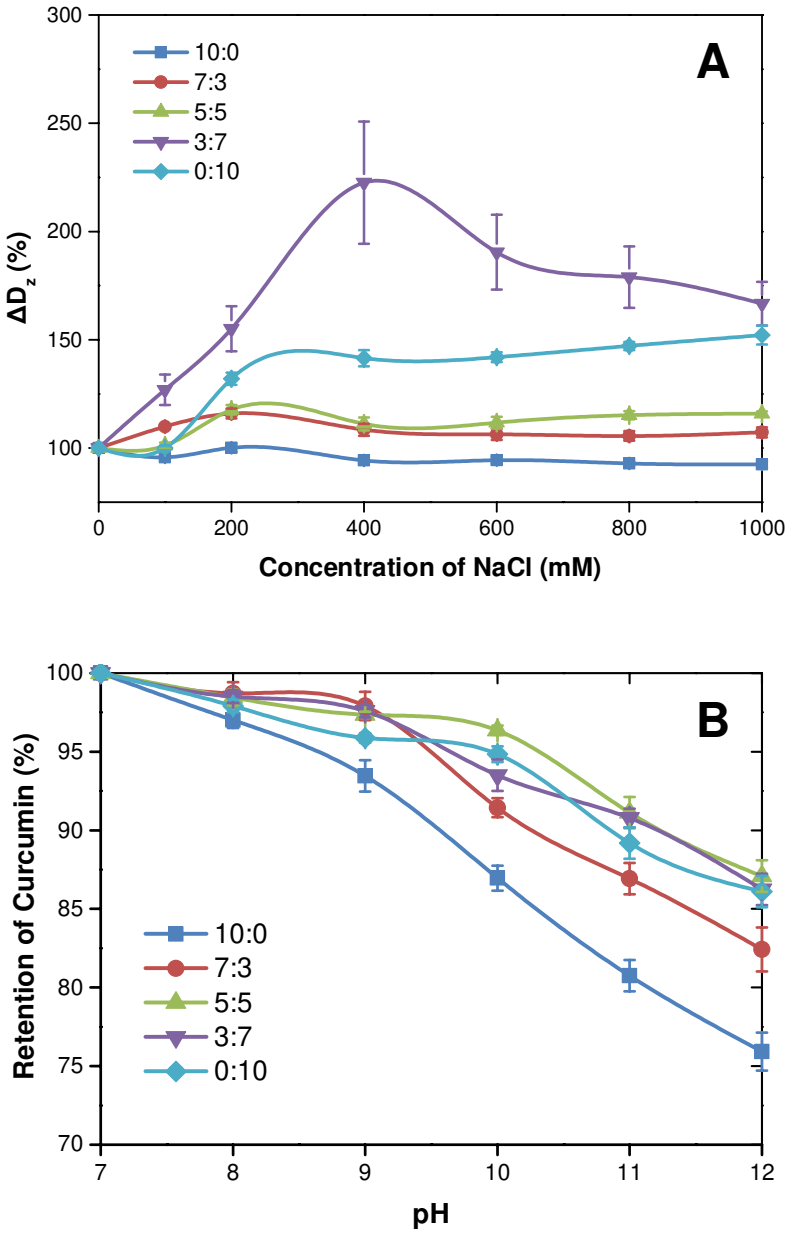

A
10:0
7:3
5:5
3:7
0:10
ΔDz (%)
Concentration of NaCl (mM)
B
Retention of Curcumin (%)
pH

Figure 3

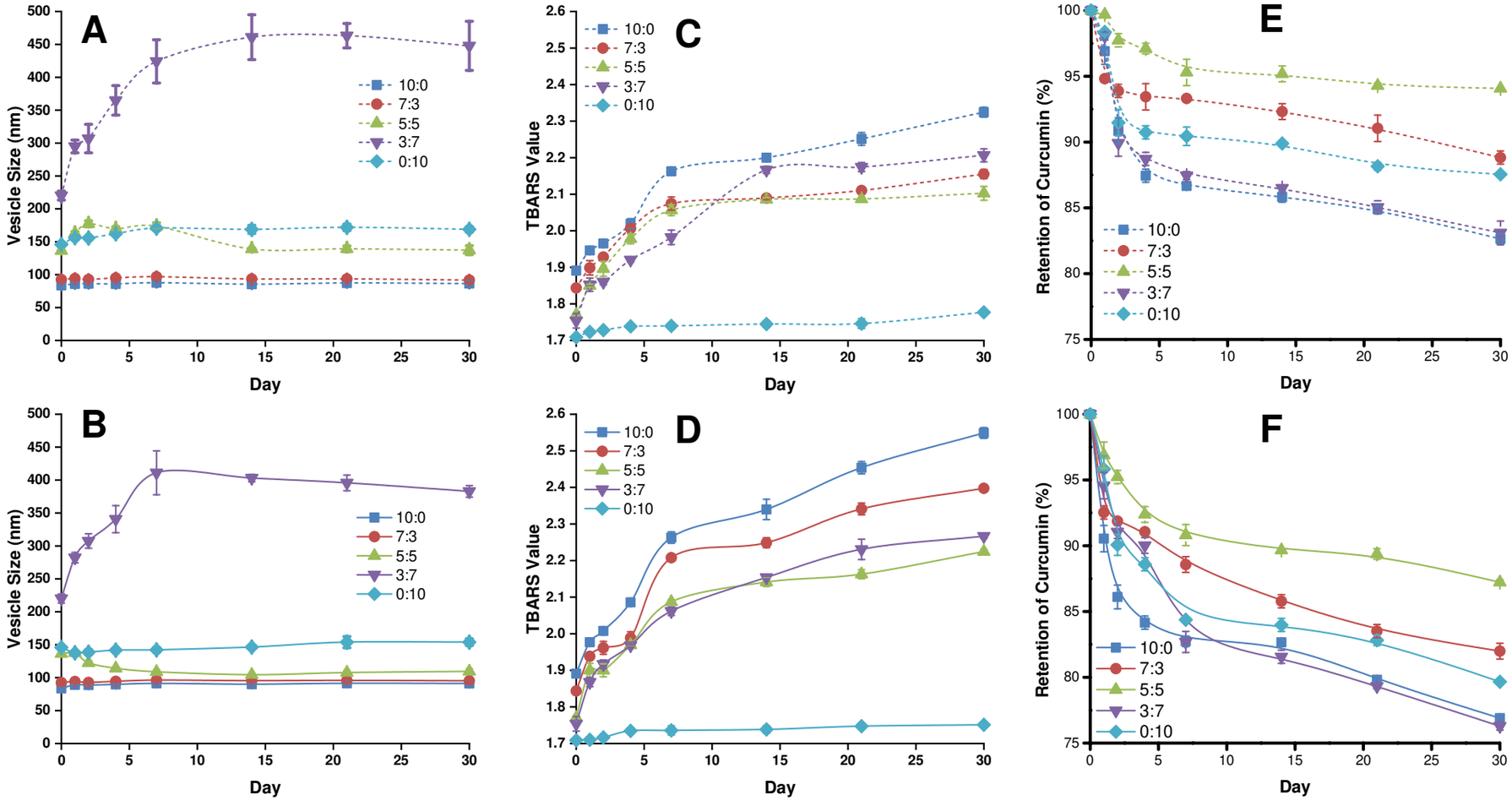
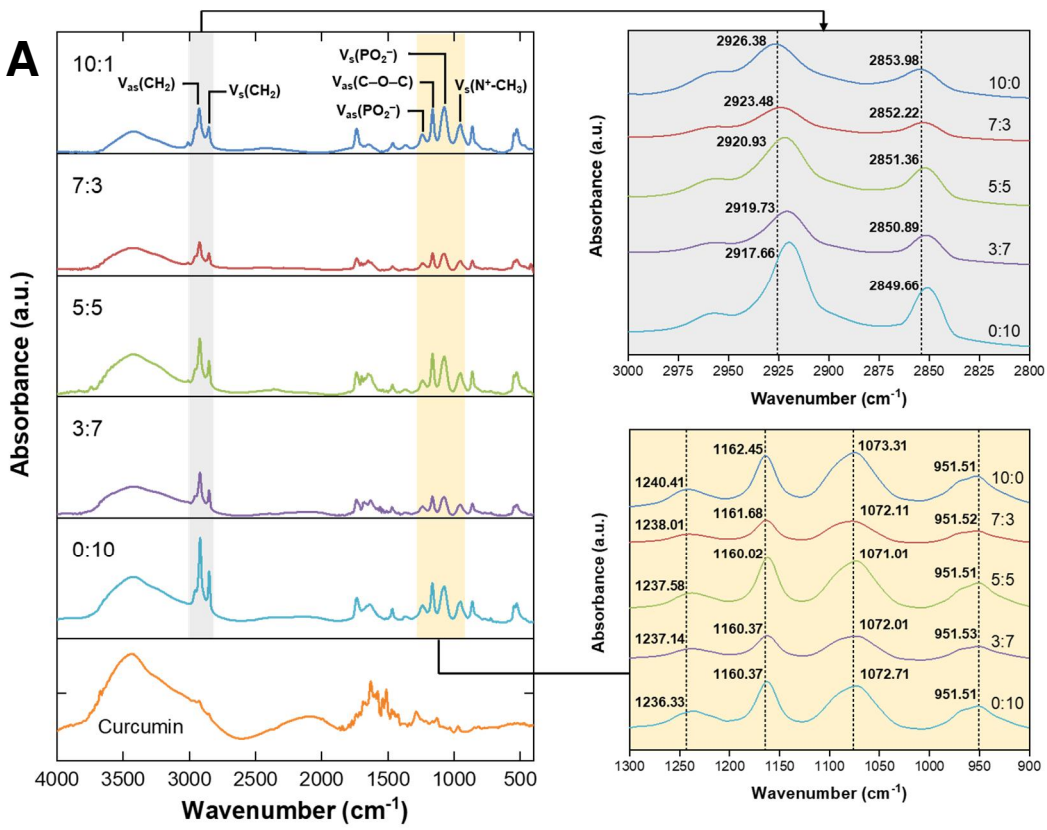

A
B
C
D
E
F
Vesicle Size (nm)
TBARS Value
Retention of Curcumin (%)
Day
10:0
7:3
5:5
3:7
0:10

Figure 4


A
10:1
7:3
5:5
3:7
0:10
Curcumin
Absorbance (a.u.)
Wavenumber (cm-1)
Vas(CH2)
Vs(CH2)
Vs(PO2-)
Vas(C–O–C)
Vas(PO2-)
Vs(N+-CH3)
2926.38
2853.98
2923.48
2852.22
2920.93
2851.36
2919.73
2850.89
2917.66
2849.66
1162.45
1073.31
951.51
1240.41
1161.68
1072.11
951.52
1238.01
1160.02
1071.01
951.51
1237.58
1160.37
1072.01
951.53
1237.14
1160.37
1072.71
951.51
1236.33

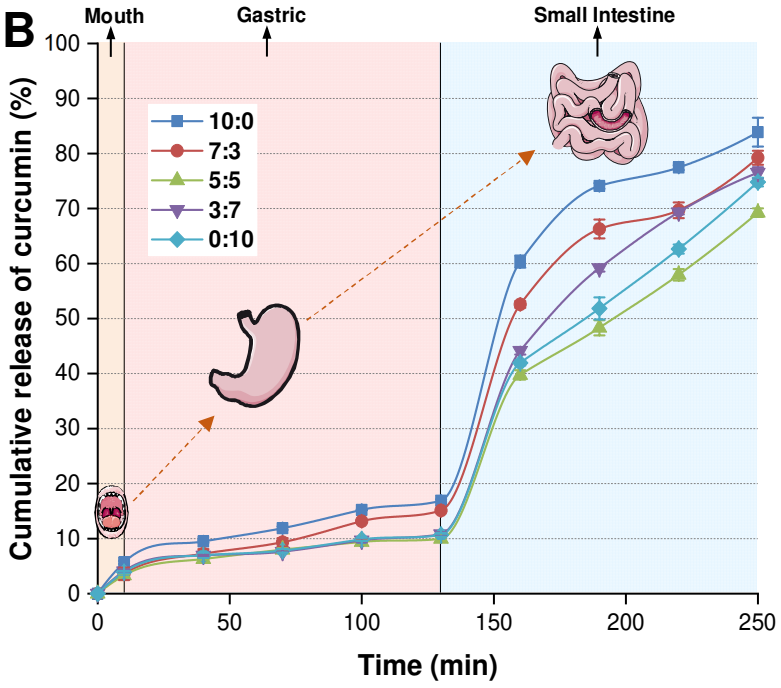

B
Mouth
Gastric
Small Intestine
10:0
7:3
5:5
3:7
0:10
Cumulative release of curcumin (%)
Time (min)

Figure 5