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Effect of Different Preparation Methods on Physicochemical Properties of Salidroside Liposomes

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Salidroside liposomes were prepared by using five different methods: thin film evaporation, sonication, reverse phase evaporation, melting, and freezing−thawing. The effect of different preparation methods and salidroside loading capacity on the formation of liposomes and their physicochemical properties were evaluated by means of encapsulating efficiency, particle size, morphology, and ζ potential. Results showed that the encapsulating efficiency of liposomes was highest when prepared by freezing−thawing, followed by thin film evaporation, then reverse phase evaporation and the lowest with melting and sonication. Loading capacity of salidroside had a significant effect on encapsulating efficiency, average diameter, and ζ potential of liposomes. Liposomal systems prepared by sonication, melting, and reverse phase evaporation displayed better dispersivity. Determination of leakage of salidroside from different liposomal systems revealed that the melting method had the lowest leakage of 10% and 15%, at 4 and 30 °C after 1 month of storage, respectively. In all cases, a straight-line leakage behavior of salidroside was found. This revealed that the leakage of salidroside was a diffusion process from the membrane of liposomes. Furthermore, the storage stability of different liposomal systems showed that salidroside liposomes prepared by melting had a better physicochemical stability. Instability in the systems was exacerbated when temperature increased. Salidroside liposomes showed the slower increase in particle size than liposomes without salidroside. This could indicate that salidroside played an important role in preventing the aggregation and fusion of liposomes.

KEYWORDS: Liposome; salidroside; loading capacity; encapsulating efficiency; ζ potential; stability

INTRODUCTION

Liposomes are formed from polar lipids, having a unique closed structure and special physical and chemical properties, and can incorporate a wide variety of functional components in their interior (*1*, *2*). Researchers recognized that liposomes can be an effective drug delivery vesicle (*2*). This is due to many distinct advantages of liposomes, including biocompatibility, targetability, controlled release, and the possibility of producing them in different sizes (*3*). In developing targeting dosage control strategies for traditional Chinese medicine, particular emphasis has recently been placed on liposome due to the above-mentioned advantages. For example, notoginseng noside liposomes, ginseng saponin liposome, and tanshinone proliposome have been prepared using various techniques (*4*−*6*).

Rhodiola sachalinensis A. Bor, a popular traditional herb with health function in China, Russia, and other countries, has been used as an adaptogen by Russian researchers for its activities to increase body resistance to a variety of chemical, biological, and physical agents (*7*). The major bioactive components in *Rhodiola* species are salidroside and *ρ*-tyrosol (**Figure 1**), which have functions such as resisting anoxia, fatigue, and microwave radiation. In the late 20th century, salidroside had been reported for special purposes such as for astronauts, pilots, mountaineers, and divers to enhance the body's survival in adverse environment (*8*, *9*). Unfortunately, functional foods containing salidroside presently developed have some drawbacks, such as fluctuation of the concentration in blood, low bioavailability, and too high intake frequency. To solve these problems, some researchers attempted to encapsulate salidroside in alginate-chitin microcapsulate and to prepare phytosome containing salidroside (*10*, *11*). However, for alginate-chitin microcapsulate, salidroside showed a poor controlled release because of the too larger pore in the surface of the microcapsulate. Furthermore, only salidroside extract from materials by microwave assisted extraction was used. There were still impurities in the salidroside extract, such as polysaccharides, proteins, tannins, gum, and polar pigments. Phytosome containing salidroside was only developed for the application in pharmaceutical injection, and it suffered from a requirement for the unacceptable Poloxamer 188, an emulsifier used medically, which could give an undesirable safety problem in food (*12*).

In the present study, the first aim was to prepare salidroside containing liposomes by five different methods: thin film evaporation, sonication, reverse phase evaporation, melting, and freezing−thawing. Second, the effect of different preparation methods and salidroside loading capacity on the formation of liposomes and their physicochemical properties was evaluated

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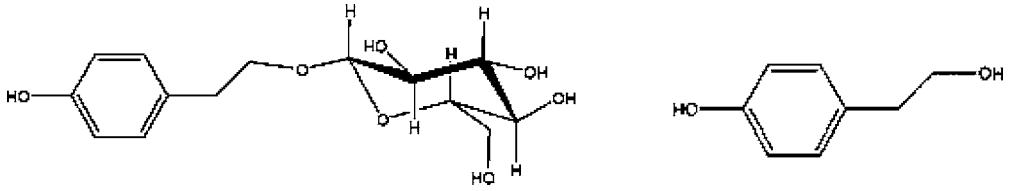

Figure 1. Structures of the major bioactive components in *Rhodiola* species: salidroside (left) and *p*-tyrosol (right).

by means of encapsulating efficiency, particle size, morphology, and ζ potential. We also determined the encapsulating efficiency of the prepared liposomes. Finally, the storage stability of liposomal systems was determined at 4 and 30 °C for more than 1 month. Leakage characteristics of salidroside from liposomes were studied.

MATERIALS AND METHODS

Materials. Lecithin [egg yolk phosphatidylcholine (PC), with 85% purity], cholesterol, and Tween 80 were purchased from Sinopharm Chemical Reagent Co, Ltd. (Shanghai, China). Salidroside standard was purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). *Rhodiola sachalinensis A. Bor* was purchased from a Ginseng Factory in Jilin Province, China. DA 201-C nonionic polymeric absorbent was obtained from Nankai University Chemical Co. (Tianjin, China). Polyamide powder was purchased from Dongtai Chemical Co. (Zhejiang, China). All reagents were of analytical grade.

Extraction and Isolation of Salidroside. The raw material was soaked with ethanol (ethanol−water 20/80, v/v) according to the ratio of solid and liquid 1:25 (g/mL). The mixture was irradiated for 120 s in a microwave oven modified with Fiso microwave workstation (MWS-8, FISO Company, Canada). The microwave radiation power was set on 130 W. The mixture was filtered by vacuum. The residual ethanol in the extract was removed under reduced pressure. The procedure was repeated twice. The salidroside extract was diluted by deionized water (1:5, v/ v) and centrifuged (8000 rpm, 10 min) (Sigma Co., German), and the clear supernatant was collected.

Salidroside was isolated with DA 201-C (diameter 2.6 cm, length 30 cm). Polysaccharides, proteins, tannins, gum, and polar pigments were eluted from salidroside extract with deionized water and 30% ethanol solution (ethanol−water 30/70, v/v) in DA 201-C column. Salidroside remaining in column was eluted with 50% ethanol (ethanol−water 50/50, v/v). The solvent was then evaporated and salidroside extract was dissolved in deionized water. After that, the salidroside extract was added to polyamide column (diameter 2 cm, length 50 cm) and eluted with deionized water. Salidroside-containing fraction was concentrated. The purity of salidroside was 30% and the total phenol content was beyond 95% (*13*).

Preparation of Liposomes. Liposomes consisting lecithin and cholesterol (5:1, w/w) were prepared by different methods. Salidroside of different concentration in phosphate buffer solution (0.05 M, pH 7.0) was added to the aqueous phase used to hydrate liposomes.

Thin Film Evaporation. Thin film evaporation was operated according to Bangham's technique (*1*). Lipid phase consisting of a mixture of 200 mg of lecithin and 40 mg of cholesterol was dissolved in diethyl ether that was then removed under reduced pressure in a rotary evaporator at 40 °C, thus to form a thin film of dry lipid on the wall of the flask. Evaporation was continued for half an hour to completely remove all organic solvent residues. For the preparation of salidroside solution, the partially purified salidroside (10 mg or 20 mg) and the appropriate amount of Tween 80 (100 mg) were dissolved in 20 mL of phosphate buffer solution (0.05 M, pH 7.0). The film was hydrated by adding salidroside solution under vigorous stirring at 40 °C until the liposomal system was formed.

Freezing−Thawing Method. Thin film evaporated liposomes were rapidly frozen at −80 °C and then placed in a water bath at 50 °C. The procedure was repeated five times.

Sonication Method. Liposomal system obtained by thin film evaporation was used for sonication treatment (Sonics & Material Vibra Cell, 400 W, 20 kHz, U.K.) at 50% amplifying strength for 5 min with 1 s pulse-on and 1 s pulse-off.

Reverse Phase Evaporation. The lipid phase consisting of a mixture of 200 mg of lecithin and 40 mg of cholesterol was dissolved in diethyl ether and mixed with the aqueous solution containing salidroside (organic phase:aqueous phase 3:1, v/v). Sonication treatment for 5 min at 50% amplifying strength resulted in a water-in-oil emulsion. After removal of the organic solvent with a rotary evaporator under vacuum, a gel was formed. Upon vigorous rotary evaporation, the dispersion of liposomes was formed.

Melting Method. The lipid phase consisting of a mixture of 200 mg of lecithin and 40 mg of cholesterol was dissolved in 3 mL of ethanol, dried by evaporation, followed by adding the mixture of Tween 80 and oleic acid at 70 °C, and then heated until melting. The salidroside solution was added and stirred at 70 °C for 30 min. Liposomal system was applied to the sonication treatment at 50% amplifying strength for 1 min with 1 s pulse-on and 1 s pulse-off.

Salidroside Content and Encapsulating Efficiency Measurement. The content of salidroside was analyzed by the chemical colorimetric technique (*10*). Maximum absorbance was at 480 nm, detectable by the UV/visible spectrophotometer. The linear equation of salidroside standard curve was obtained:

$$y = 11.834x + 0.0217 \ (r^2 = 0.9923)$$

Liposome encapsulating efficiency was determined using the dialysis technique for separating the non-entrapped salidroside from liposomes. One milliliter of salidroside liposomal dispersion was placed into a dialysis bag of cellulose membrane (MW cutoff 12000, Shanghai Huamei Biochemistry Ltd., Shanghai, China) and immersed in 100 mL of phosphate buffer (pH 7.0, 0.05 M) for 8 h, with mild stirring. The dialyzed liposomes were disrupted with 5 mL of 10% Triton X-100 to obtain a clear solution.

The percentage of encapsulating efficiency (EE%) was calculated according to the following equation:

$$\text{EE\%} = (\text{salidroside encapsulated in liposomes/total salidroside added}) \times 100$$

ζ Potential Measurement. The ζ potential of liposomes was measured with the laser Doppler electrophoretic mobility measurements using the Zetasizer 2000 (Malvern Instruments Ltd., Malvern, U.K.), at 25 ± 0.1 °C. All analysis was done in triplicate.

Liposomal Size Measurement. The size distribution of liposomes prepared using different methods was measured using a dynamic light scattering technique (DLS), at 25 °C. The intensity of the laser scattered by the samples was detected at an angle of 90°. The liposomal dispersion was diluted with phosphate buffer (0.05 M, pH 7.0) to avoid multiple scattering phenomena due to interparticle interaction. At least 10 runs were performed for each sample. The viscosity value (0.8872 mPa s) and the refractive index (1.33) of water were used for all the measurements. The *z*-average diameter and the polydispersity index of the liposomes were automatically generated by the instrument using cumulants analysis.

Confocal Laser Scanning Mmicroscope. According to Lopez-Pinto et al., lamellarity of liposomes was observed by confocal laser scanning microscope (CLSM) (*14*). CLSM has the ability to penetrate deep beneath the surface of a tissue and to produce much higher resolution images than with conventional fluorescence microscopy. With the conventional fluorescence microscope, images can be obscured by lack of focus and excess fluorescent light from the specimen both above

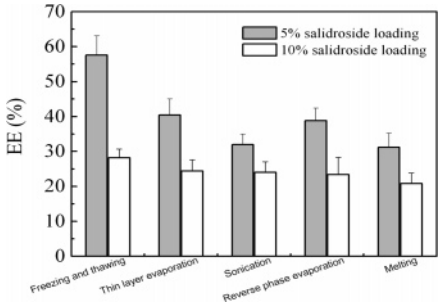

Figure 2. Relationship between encapsulating efficiency of salidroside liposomes and different preparation methods and loading capacity. Data reported are the mean values ± standard variation of three samples. Values with 10% salidroside loading were not significantly different ($p > 0.05$).

and below the plane of focus. The CLSM has advanced optics which eliminates all the information outside of a narrow plane of focus, thus yielding a well-defined image.

The equipment used in this investigation was a Zeiss LSM510 confocal laser scanning microscope (Heidelberg, Germany) using Zeiss Fluar 100*/1.3 with magnifications of 10×, 20× (dry), and 100× (immersion objective) on its oil position. To view with the CLSM, salidroside liposomes were stained with the fluorescent dye, rhodamine. The following parameters were used for the confocal microscope starts: magnification, 10×, 20×, and 40×; laser power, 5%; scan modus, slow; gain values, 400−600; $x-y-z$ mode; 488 nm emission line at 31% maximum power; transmission light channel activated; and bidirectional scanning pattern.

Leakage Study. Liposomal systems prepared using different methods were stored at 30 and 40 °C for more than 1 month. Samples were withdrawn at different time intervals, and the residual amount of the salidroside in liposomes (i.e., encapsulating efficiency) was determined as described earlier.

$$\text{leakage ratio \%} = (\text{EE during the storage period}/\text{EE of the beginning storage}) \times 100$$

Statistical Analysis. The data were compared by one-way analysis of variance (ANOVA), and differences between sample means were analyzed by a least-squares difference method at $\alpha = 0.05$.

RESULTS AND DISCUSSION

Effect of Different Preparation Methods and Loading Capacity. As shown in **Figure 2**, the encapsulating efficiency of liposomes was highest when prepared by freezing−thawing, followed by thin film evaporation, then reverse phase evaporation and the lowest with melting and sonication ($p < 0.05$). It was obvious that encapsulating efficiency was related with liposome type. As it was known, multilamellar vesicles (MLV) can be obtained by thin film evaporation, larger unilamellar vesicles (LUV) by reverse phase evaporation. and small unilamellar vesicles (SUV) by sonication or extrusion technique (*1*, *2*). MLV can be reduced lamellars by freezing−thawing treatment to produce special freezing and thawing MLV (FTMLV) (*15*). For the water-soluble compounds, encapsulating efficiency depended on the inner volume of vesicle. The improvement of inner volume of vesicle could increase encapsulating efficiency accordingly. The freezing−thawing circles caused an amount of salidroside to permeate the bilayers, which led to the higher encapsulating efficiency.

The effect of loading capacity on encapsulating efficiency of salidroside liposomes was examined by varying the amount of salidroside added while keeping the level of lipid phase. As shown in **Figure 2**, in all cases there was an obvious decrease in encapsulating efficiency of liposomes when salidroside loading capacity increased from 5% to 10%. The encapsulating efficiency of liposomes for 10% of salidroside loading capacity was not statistically significant ($p > 0.05$), suggesting that different preparation methods slightly affected the encapsulating efficiency of liposomes. This indicates that the fixed amount lipid produced the constant amount of liposomes in the system. A certain given amount of liposomes had a limited encapsulating capacity, which led to the decrease of encapsulating efficiency when salidroside concentration was too high. Theoretically, encapsulating efficiency should increase with increasing lipid concentration when the drugs were lipid-soluble. But this does not fit for the water-soluble drugs (*16*). A fact should be emphasized that salidroside has a hydroxybenzene function in its structure (*7*−*9*). The compatibility of the lipid bilayer and salidroside was enhanced because the strong polar group was helpful for the interaction between salidroside molecule and the polar head of lipid. This could result in salidroside molecules being encapsulated easily into the inner aqueous phase.

Morphology Study of Liposomes. It was reported that the outer morphology and the lamellarity of liposomes can be demonstrated and observed by means of CLSM (*14*). CLSM images of liposomes prepared with different methods were shown in **Figure 3**. MLV liposomes (**Figure 3B**) presented a concentric lamellar structure, although irregular to some extent, whereas FTMLV (**Figure 3A**) showed a less regular morphology due to the "traumatic" preparation method, as reported also by other authors (*17*). LUV liposomes (**Figure 3C**) were unilamellar and had a smaller particle size than MLV and FTMLV ones (*18*).

Particle Diameter Distribution and Polydispersity of Liposomes. Different preparation methods resulted in different mean particle size of liposomes. The experimental data from **Table 1** showed that the average diameter of salidroside liposomes prepared using freezing-thawing method was much larger than that with other methods. The mean diameter of salidroside liposomes prepared using thin film evaporation and reverse phase evaporation was greatly affected by salidroside loading capacity. As salidroside loading capacity increased from 5% to 10%, the average diameters of liposomes prepared using thin film evaporation and reverse phase evaporation increased 95% and 80%, respectively. In contrast, salidroside loading capacity influenced the mean diameter of liposomes slightly for sonication and melting method. Corresponding to encapsulating efficiency, the larger particle size resulted in a higher encapsulating efficiency. Salidroside structure probably played an important role to get it packed in the inner.

Polydispersity is usually expressed as an index of particles diameter distribution in colloidal systems. The smaller the value of polydispersity index, the more likely the particle diameter distribution is narrower, and thus particles show better uniformity in diameter (*19*). The fluctuating in polydispersity index was irregular as shown in **Table 1**. However, the liposomal system treated with sonication always displayed better dispersivity (lower polydispersity index).

ζ Potential of Liposomes. The ζ potential of the empty liposomes prepared using different methods almost reached zero (data not shown here), and the ζ potential of salidroside liposomes gradually increased with the concentration increase of salidroside (**Table 1**). The results showed that salidroside liposomes had positive charges, indicating the electrostatic repulsive force occurred between the particles. The ζ potential of salidroside liposomes could be attributed to the interaction between salidroside and lecithin. The hydroxyl group of salidroside interacted with choline in the polar region of lecithin

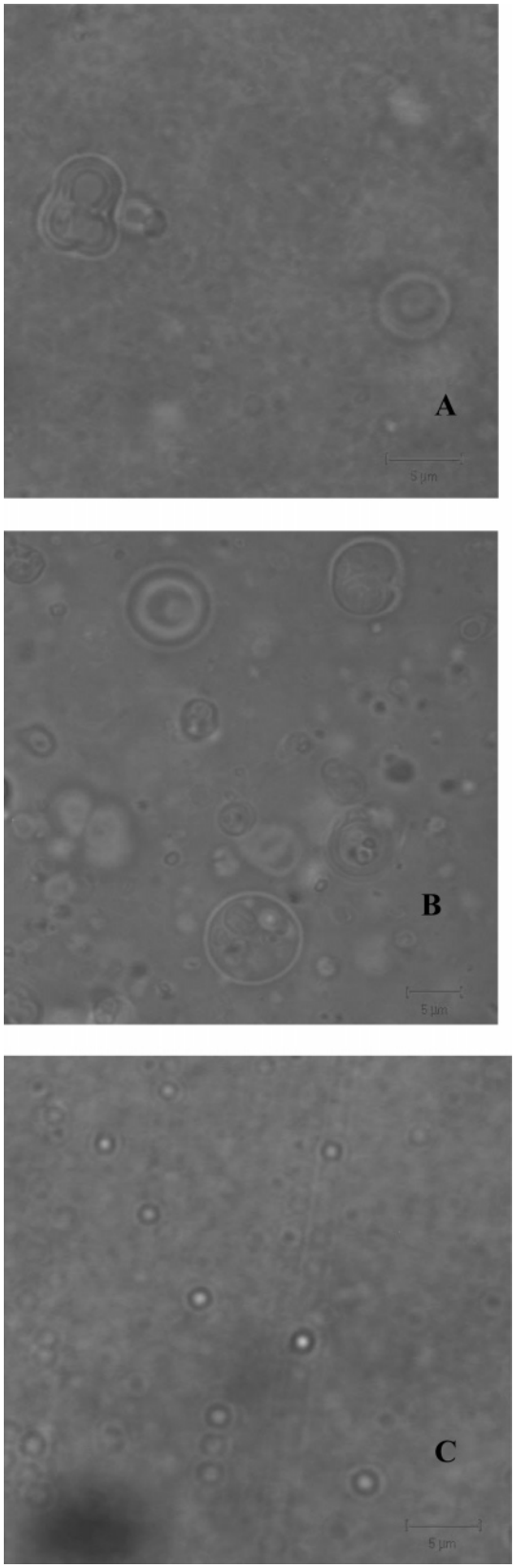

Figure 3. CLSM microphotographs corresponding to liposomes by different preparation methods: (**A**) freezing–thawing method; (**B**) thin layer evaporation; (**C**) reverse phase evaporation.

to produce the dipole tropism and increased the particle surface charges.

Table 1 also shows that different preparation methods led to the variation on ζ potential of salidroside liposomes. The

Table 1. Effect of Different Preparation Methods and Loading Capacity on Particle Diameter Distribution of Salidroside Liposomes

preparation methods	salidroside loading (%)	mean diameter (nm)	polydispersity index	ζ potential
thin layer evaporation	5	604 ± 4.4[a]	0.360 ± 0.002	7.5 ± 1.1
	10	1179 ± 7.6	0.319 ± 0.002	14.9 ± 0.8
sonication	5	114 ± 2.5	0.095 ± 0.005	–1.5 ± 0.8
	10	114 ± 2.9	0.093 ± 0.003	–1.3 ± 0.5
freezing–thawing	5	1157 ± 1.5	0.361 ± 0.003	16.8 ± 0.3
	10	1342 ± 3.5	0.300 ± 0.02	19.7 ± 2.1
reverse phase evaporation	5	250 ± 2.6	0.237 ± 0.001	–0.4 ± 1.0
	10	449 ± 3.0	0.224 ± 0.002	–2.5 ± 0.5
melting	5	99 ± 1.0	0.219 ± 0.02	–8.7 ± 0.4
	10	105 ± 2.1	0.205 ± 0.06	–10.8 ± 0.9

[a] Mean value ± standard deviation of three samples.

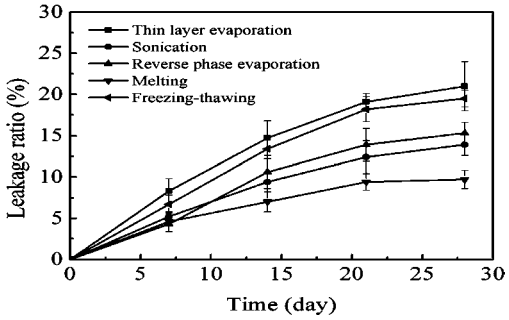

Figure 4. Salidroside leakage from liposomes with the various preparation methods at 4 °C versus time.

absolute values of the ζ potential of salidroside liposomes prepared by sonication and reverse phase evaporation were extremely lower than those by thin film evaporation and freezing−thawing. This obviously depended on the type of liposomes prepared. Compared with SUV and LUV, MLV or FTMLV could entrap more salidroside and other polyphenol molecules, which further enhanced the interaction of salidroside and lecithin.

It is currently regarded that the absolute value of ζ potential under 30 mV is required for full electrostatic stabilization; ζ potential between 5 and 15 mV is in the region of limited flocculation (*20*). Thus particle aggregation is less likely to occur for charged particles due to electric repulsion. However, this rule could not be applied to our liposomal systems. In our study, the absolute values of ζ potentials were under 30 mV for all liposomal systems. It was reported that Tween 80 would adsorb or incorporate into the liposomal bilayer and could decrease the ζ potential due to the shift in the shear plane of the particle (*21*). According to Schwarz et al., solid lipid nanoparticles (SLNs) constituted of trilaurin of less low ζ potential (*21*). Generally, it was considered not high enough to stabilize the dispersion solely by electrostatic repulsion. But Poloxamer 188 was used as a steric stabilizer which can easily compensate for the missing electrostatic repulsion. This was corresponded with our results. Tasi et al. investigated the effect of Tween surfactant on ζ potential of liposomes and concluded that the incorporation of different amounts of Tween surfactant into liposomal bilayer does not significantly impact the ζ potential at 25 °C (*22*). So, it should be noted that ζ potential is not considered as the most important indicator of liposomal stability. Tween 80 used in this work improved and stabilized the dimensional structure of liposomes.

Leakage Characteristics of Salidroside from Liposomes. As shown in **Figures 4** and **5**, there was more leakage of

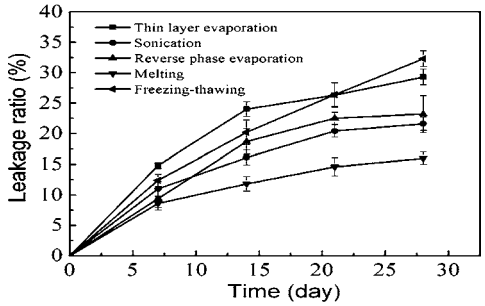

Figure 5. Salidroside leakage from liposomes with the various preparation methods at 30 °C versus time.

Table 2. Nonlinear Fits of Salidroside Leakage Cumulation from Liposomes during Different Storage Time and Condition

preparation methods	Q vs $\sqrt{t}$ [a]		r	
	4 °C	30 °C	4 °C	30 °C
thin layer evaporation	$y = 3.9938x - 0.5693$	$y = 5.697x + 0.3512$	0.9901	0.9934
sonication	$y = 2.3275x - 0.2499$	$y = 4.2079x + 0.6541$	0.9941	0.9942
freezing–thawing	$y = 3.7713x - 0.9859$	$y = 6.141x - 0.1931$	0.9801	0.9789
reverse phase evaporation	$y = 3.0542x - 1.1134$	$y = 4.7147x - 0.5742$	0.9714	0.9837
melting	$y = 1.9235x - 0.116$	$y = 2.7825x + 0.8103$	0.9939	0.9850

[a] Q: the leakage cumulation during storage. t: time in days.

salidroside from liposomes prepared with thin film evaporation and freezing−thawing method than that with sonication, reverse phase evaporation, and melting. At the temperature of 4 °C or 30 °C, the leakage behavior of salidroside liposomes by thin film evaporation and freezing−thawing method was similar. In like manner, the leakage of salidroside liposomes by sonication and reverse phase evaporation was similar. Determination of leakage of salidroside from different liposomes revealed that melting method had the lowest leakage after 1 month of storage at 4 and 30 °C (respectively 10% and 15%). Furthermore, instability increased drastically when the system temperature increased. Considering the mechanical properties of the liposomal structure being heavily dependent on temperature, the rise of temperature accelerated the speed of liposomes fusion because the enhancement of temperature increased the frequency of collision between liposomes and promoted the thermal motion of the liposomal bilayer, and it resulted in a decreased order of the bilayer structure.

According to the equations in **Table 2**, the leakage cumulation was plotted against the square root of time at 4 and 30 °C, respectively. In all cases, a straight-line leakage pattern was found ($0.9714 \leq r \leq 0.9942$). This corresponds well with the conclusion that the leakage of salidroside is a process of diffusion from the membrane of liposomes (*23*).

Obviously, the leakage pattern was related to the characteristics of liposomes. At 30 °C, similar leakage trends of salidroside from liposomes by thin film evaporation and freezing and thawing method could attribute to the same MLV type. However, the similar leakage trends of salidroside from liposomes by sonication and reverse phase evaporation could attribute to producing SUV and LUV, respectively. MLV showed more leakage of salidroside, and the reason was probably that the multilamellar bilayers fused to form unilamellar vesicles.

Stability−Temperature Characteristics of Liposomes. Drug leakage from liposomes may be related to the hydrolysis and oxidation of lipids in the bilayers, which consequently result in defects in membrane packing (*24*, *25*). For phosphatidylcholine liposomes, the oxidation and hydrolysis of ester bonds depended on pH. **Table 3** shows the pH change of salidroside liposomes during different storage conditions. The decreasing trend of pH values of all prepared liposomal systems was found. The lowest pH was found at 6.10 for LUV prepared by reverse phase evaporation after 180 days of storage. The pH value (from pH 6.98 to pH 6.73) of salidroside liposomes prepared by the melting method showed the slowest decline. In aqueous dispersion, phosphatidylcholine was hydrolyzed to form free fatty acids and lysophosphatidylcholine as the main degradation products. The higher the temperature was, the lower pH value was. This indicated that the increase of the temperature stimulated the oxidation and hydrolysis in the systems. The decrease in pH resulted in an increased electrolyte concentration. The higher electrolyte concentration can lead to the compression of the diffuse layer resulting in aggregation of the particles at the low pH (*26*).

The stability of liposomes over time is an important concern in applications because the liposomes have to retain their vesicle integrity while in the circulation. The study on the physicochemical stability of salidroside liposomes showed variations among different preparation methods (**Figure 6**). The particle size of salidroside liposomes, prepared by thin film evaporation, freezing−thawing, reverse phase evaporation, and sonication, increased gradually during the storage. For salidroside liposomes by melting method, stored at either 4 or 30 °C, the particle size was maintained in the same range over 180 days. Salidroside liposomes (MLV and FTMLV) prepared by thin layer evaporation and freezing−thawing respectively showed distinctive particle size growth or aggregation. Furthermore, liposome

Table 3. ζ Potential of Salidroside Liposomes by Different Preparation Methods during Different Storage Times and Conditions

preparation methods	storage time and conditions					
	0 days		90 days		180 days	
	4 °C	30 °C	4 °C	30 °C	4 °C	30 °C
thin layer evaporation	14.9 ± 0.8[a] (pH 6.95)	14.9 ± 0.8 (pH 6.95)	14.2 ± 1.0 (pH 6.78)	11.9 ± 1.2 (pH 6.75)	10.2 ± 2.7 (pH 6.60)	17.8 ± 2.3 (pH 6.57)
sonication	–1.3 ± 0.5 (pH 6.96)	–1.3 ± 0.5 (pH 6.96)	–7.3 ± 1.5 (pH 6.80)	–9.3 ± 2.3 (pH 6.68)	–8.4 ± 1.5 (pH 6.63)	–9.9 ± 3.8 (pH 6.43)
freezing–thawing	19.7 ± 2.1 (pH 6.96)	19.7 ± 2.1 (pH 6.96)	15.7 ± 2.9 (pH 6.72)	17.7 ± 3.1 (pH 6.52)	13.3 ± 1.1 (pH 6.49)	10.6 ± 0.8 (pH 6.19)
reverse phase evaporation	–2.5 ± 0.5 (pH 6.99)	–2.5 ± 0.5 (pH 6.99)	–7.4 ± 1.5 (pH 6.79)	–7.5 ± 1.0 (pH 6.45)	–8.9 ± 1.1 (pH 6.53)	–8.2 ± 1.5 (pH 6.10)
melting	–10.8 ± 0.9 (pH 6.98)	–10.8 ± 0.9 (pH 6.98)	–14.8 ± 0.7 (pH 6.86)	–15.6 ± 1.3 (pH 6.84)	–19.9 ± 2.3 (pH 6.77)	–20.8 ± 3.6 (pH 6.73)

[a] Mean value ± standard deviation of three samples.

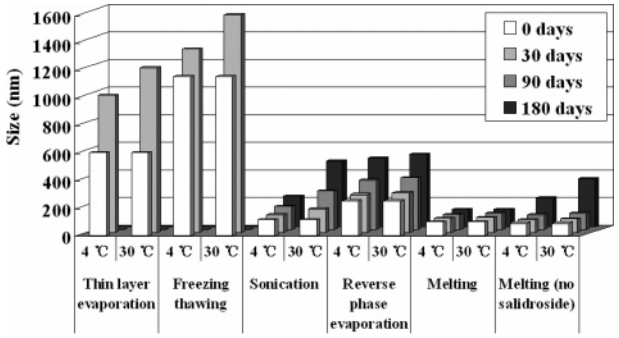

Figure 6. Mean particle size variation as a function of time measured by DLS analysis of salidroside liposomes stored at 4 and 30 °C. Each value was the average of three experiments ± standard deviation.

suspensions of MLV and FTMLV exhibited significant phase separation at 30 days due to aggregation and fusion of liposomes. Heavy aggregation resulted in the formation of few but large particles that could no longer be subjected to particle size analysis by DLS. Therefore, the particle size of MLV and FTMLV storage after 30 days was not determined. The instability of particle size in all systems was worsened when stored at 30 °C. Thus, liposomal systems became unstable when storage temperature rose.

An interesting finding was that the encapsulated salidroside had an important influence on liposomes stability. The particle size of liposomes prepared by melting method was stable for 180 days of storage at 4 or 30 °C when salidroside was encapsulated into liposomes (**Figure 6**). However, the particles became unstable for empty liposomes with an increase of particle size from 110 to 210 nm and 119 to 335 nm stored at 4 °C and 30 °C, respectively. The difference of the particle size change can be explained by the protection of salidroside molecule and other phenolic compounds. Salidroside can afford a better antioxidant activity by the greater affinity of the polar hydroxyl group toward the polar surface of the lecithin bilayers, and it prevents phospholipids from oxidation and minimizes the hydrolysis reaction.

The surfactant Tween 80 provided a better stability of liposomes which did not show high ζ potential. Low ζ potential was caused by the coverage of the diffusion layer by the uncharged polymer layer. The aggregation of the lipid particles was prevented efficiently by steric stabilization. Comparing liposomes consisting of the same lipid and surfactant Tween 80 but prepared by different methods, we conclude that salidroside liposomes by using melting method showed a better stability stored at 4 or 30 °C. The instability of salidroside liposomes prepared by sonication and reverse phase evaporation was due to very low ζ potential (only -1.3 mV and -2.5 mV, respectively). The steric stabilization of Tween 80 could not compensate for the decrease of electrostatic repulsion to prevent aggregation. The relation between particle size and ζ potentials showed that the steric stabilization alone was not sufficient for salidroside liposomes. It needed to be combined with a minimum of electrostatic stabilization (ζ potential approximately 10 mV). The results showed that ζ potentials below approximately 10 mV led to physical instable liposomes in any case.

In conclusion, liposomes containing salidroside in the inner aqueous phase were obtained successfully. Salidroside liposomes prepared by the melting method showed better encapsulating efficiency and physicochemical stability. Loading and leakage characteristics of salidroside liposomes showed that salidroside loading efficiency highly influenced encapsulating efficiency, and preparation method had a great effect on the leakage of salidroside from liposomes. It is well-known and documented that liposome size, surface properties, and stability have a profound effect on their biodistribution and thereby will highly influence the possibility of targeting the encapsulated salidroside to specific tissues. Additional work is needed to verify in vitro release and in vivo bioavailability of salidroside liposomes under different conditions, followed by a subsequent aim of liposomal formulation for oral administration.

ABBREVIATIONS

TCM, traditional Chinese medicine; DLS, dynamic light scattering technique; CLSM, confocal laser scanning microscope, MLF, multilamellar vesicles; LUV, larger unilamellar vesicles; SUV, small unilamellar vesicles; FTMLV, freezing and thawing MLV.

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