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Increasing the Entrapment of Protein-Loaded Liposomes with a Modified Freeze–Thaw Technique: A Preliminary Experimental Study

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The objective of this study was to investigate the possibility of increasing the entrapment of protein-loaded liposomes with a modified freeze–thaw technique. Blank liposomes were prepared by ethanol injection method. Then recombinant human growth hormone (rhGH) was added to blank liposome suspension. rhGH encapsulation efficiency was enhanced by modified freeze–thaw technique. We separated each step and studied the effect of each parameter on encapsulation: incubation temperature (water bath from 0 to 15°C), incubation duration (from 5 to 90 min), number of freeze–thaw (from 0 to 4 cycle), and the ratio between rhGH and phospholipids (from 1:5 to 1:10). The effect of cryoprotectants on the encapsulation and particle size distribution was finally examined. rhGH encapsulation efficiency was determined by Bradford's dye-binding assay. Morphology and size distribution of rhGH liposomes were also observed. The optimum parameters for rhGH encapsulation were incubation temperature of 5°C, incubation duration of 40 min, and three to four cycles of freeze–thaw. The ratio between rhGH and phospholipids did not affect the encapsulation percentage. Trehalose exhibited the highest integrated value among the cryoprotectants investigated. From the results, this study demonstrates the suitability of the modified freeze–thaw technique for obtaining rhGH liposomes with high entrapment.

Keywords rhGH; liposome; freeze–thaw; encapsulation efficiency; protein

INTRODUCTION

Liposomes were first studied in England in 1961 by Bangham (1961), and since then, they become the most popularly used protein delivery carriers for a great variety of molecules such as small drug molecules, proteins, nucleotides, and even plasmids (Banerjee, 2001; Giannantoni, Di Stasi, Chancellor, Costantini, & Porena, 2006; Gregoriadis, 1994; Langner & Kral, 1999; Pal et al., 2005; Patil, Rhodes, & Burgess, 2004, 2005).

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In recent years, investigation on increasing the liposome entrapment for gene and protein drugs attracts considerable interest (Chaize, Colletier, Winterhalter, & Fournier, 2004; Colletier, Chaize, Winterhalter, & Fournier, 2002; Kawaura, Hasegawa, Hirashima, & Nakanishi, 2000; Khalil, Kogure, Akita, & Harashima, 2006; Nakanishi, 2003; Noguchi, Hirashima, & Nakanishi, 2002). The distinct advantages associated with the use of liposomes include their (i) robust manufacture, (ii) ease in handling and preparation techniques, (iii) ability to deliver large protein molecules, and (iv) low immunogenic response. Liposomes can be classified as (1) multilamellar vesicles (MLVs; 0.2–10 μm), (2) small unilamellar vesicles (SUVs; 25–50 nm), or (3) large unilamellar vesicles (LUVs; 100 nm to 1 μm).

However, the volume of the inner phase for these liposomes is small and the loading volume of water-soluble drugs is low. For chemical drugs, many methods such as the film hydration method and reverse-phase evaporation method can be used for producing LUV with a large inner phase. However, high temperature, strong shear stress, long procedure, and organic solvent used in the microencapsulation process negatively affect the stability of unstable proteins (Van de Weert, Hoechstetter, Hennink, & Crommelin, 2000). To maintain the maximum activity of protein, more liposome preparation methods with mild manufacturing procedure are expected.

Freeze–thaw technique is one of the reliable methods to prepare LUV with large internal aqueous space and high capture capacity. After several cycles of freeze–thaw hydration of empty liposomes in aqueous protein solution, many kinds of protein drugs can be encapsulated highly efficiently, and particle size can be controlled well. However, there are major limitations associated with this approach: many cycles of freeze–thaw, long incubation in each freeze–thaw process, and additional manufacturing procedure of nitrogen addition.

In our unpublished experiments, some disadvantages of freeze–thaw technique seemed to be overcome by combining incubation under vacuum rotation in low temperature. Therefore, the objective of this study was to establish a mild manufacturing process for protein-loaded liposomes with high entrapment. In this study, recombinant human growth hormone (rhGH) was

used as the target protein. rhGH (22 kDa) is used to treat short stature in children due to growth hormone deficiency (GHD), Turner's syndrome, or chronic renal failure, and has also been approved for treatment of adults with GHD (Vance & Mauras, 1999). Liposomes may be particularly suitable devices for the administration of rhGH because of their facility of use and the improved rhGH stability. To achieve the goal, modified freeze–thaw technique was used and the influences of formulation variables in freeze–thaw process were examined, such as incubation temperature, incubation duration, number of freeze–thaw, and the ratio between rhGH and phospholipids.

MATERIALS AND METHODS

Blank Liposome Preparation

Blank liposomes were prepared by ethanol injection method. Briefly, 10 mg hydrogenated phosphatidylcholine (HPC) (HPC >99%, Doosan Corporation Biotech BU, Kyonggi Do, Korea) and 5 mg cholesterol (Beijing Chemical Reagent Corp., Beijing, China) were accurately weighed and dissolved in 10 mL dehydrated alcohol. The solution was poured into 10 mL 0.02 mol/L phosphate buffer (pH 7.4). Then sonication was carried out at 30°C (JY 92-II ultrasonic processor, KunShan Ultra-Sound Instrument Inc., Kunshan, China) at a frequency of 40 kHz and power of 160 W for 30 s to obtain a liposome suspension. With a vacuum pump, alcohol in the liposome suspension was removed by evaporation in a 25°C water bath with constant rotation. The final volume of liposome suspension was 10 mL.

rhGH Liposome Preparation

rhGH liposomes were prepared by a modified freeze–thaw technique. rhGH (Hengtong Bioengineering Company, Zhuhai, China) solution (1.5 mg/mL, 4.0 IU/mL) was added to 10 mL blank liposome suspension. Then the mixture underwent different numbers of freeze (liquid nitrogen)–thaw (designed water bath temperature) cycles. The process in this study was modified by combining incubation under vacuum rotation in low temperature. The freezed mixture in the thaw process was poured into a flask and agitated using a rotary evaporator at 1×10^{-3} Pa pressure with stirring speed at 120 rpm under low temperature. As each freeze–thaw cycle can cause change of encapsulation efficiency, the effects of incubation duration (from 5 to 90 min), incubation temperature (water bath from 0 to 15°C), number of freeze–thaw (from 0 to 4 cycle), and the ratio between rhGH and phospholipids (from 1:5 to 1:10) on the encapsulation efficiency were investigated. Finally, rhGH liposome suspension underwent lyophilization to obtain a dry sample. The effect of cryoprotectants on the encapsulation and particle size distribution was examined. After added with different cryoprotectants (20 mg), the liposome suspension was frozen and lyophilized at 5×10^{-4} Pa pressure for 20 h. All liposome formulations were prepared in triplicate. The preparation process of rhGH liposomes is shown in Figure 1.

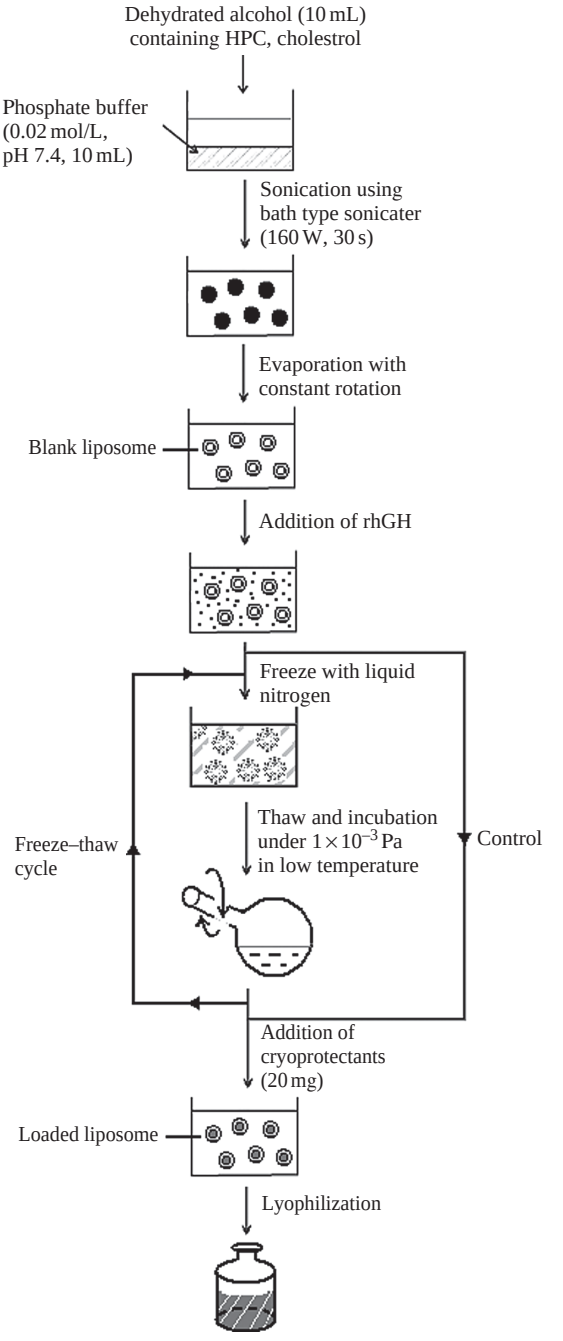

FIGURE 1. Schematic illustration of the preparation process of rhGH liposomes.

As a control for experiments on the factors in freeze–thaw cycle, rhGH liposomes (rhGH : phospholipids = 1:5) were prepared by incubating rhGH and blank liposomes at 5°C for 40 min before lyophilization. Then after added with 20 mg trehalose, the mixture solution underwent lyophilization to obtain a dry sample.

Morphology and Size Distribution of rhGH Liposomes

The morphology of rhGH liposomes was observed using an electron microscopy (XSZ-4G, COIC Company, Chongqing, China). Size distribution of rhGH liposomes was examined by laser particle measuring instrument (LS800, OMEC Company, Zhuhai, China). The mean size was reported from at least three samples.

rhGH Encapsulation Efficiency

Sephadex G-75 column was used to separate free rhGH from rhGH encapsulated in the liposomes. rhGH liposome suspension (0.5 mL) was added in a Sephadex G-75 (1.0 × 25 cm) column. The effluent was 0.02 mol/L PBS (pH 7.4). The effluent liquid containing free rhGH was collected, and the content of rhGH was determined by Bradford's dye-binding assay (Bradford, 1976). Coomassie Brilliant Blue G-250 (CBB G-250, Fluka Company, Buchs, Switzerland) 100 mg was dissolved in 50 mL 95% ethanol. To this solution, 100 mL 85% (wt/vol) phosphoric acid was added. The resulting solution was diluted to a final volume of 1 L. Final concentration in the reagent was 0.01% (wt/vol) CBB G-250, 4.7% (wt/vol) ethanol, and 8.5% (wt/vol) phosphoric acid. Bovine serum albumin (BSA, 300,000 U/g, SINO-American Biotechnology Company, Shanghai, China) standard solution was 0.1% (wt/vol). To BSA, deionized water was added based on proportion variously. Final concentrations in the protein solutions were 0, 0.01, 0.02, 0.04, 0.06, 0.08, and 0.1% (wt/vol) BSA, respectively. BSA solutions 1 mL and CBB G-250 solution 5 mL, respectively, were added to the test tubes. The absorbance at 595 nm was measured after 5 min. Based on the result, the standard curve of Bradford's dye-binding assay was described. The effluent liquid containing free rhGH 0.1 mL and CBB G-250 solution 5 mL were added to the test tube. And the content of rhGH was determined based on BSA standard curve.

$$\text{Encapsulation efficiency} = \left(1 - \frac{C_r}{C_0}\right) \times 100\%$$

C_r: free rhGH amount
C_0: total rhGH amount

Recovery experiment samples were prepared by physically mixing blank liposomes with rhGH in different concentration before Sephadex column separation. The mean percent recovery of rhGH was 95.4%, which verified the separation method and encapsulation assay for rhGH liposomes.

Statistical Analysis

Student's *t* test and the one-way ANOVA were adopted for statistical comparison using the SAS 8.01 (1999–2000, SAS Institute Inc., Cary, NC, USA). The data difference was considered to be statistically significant when the *p*-value was less than .05.

RESULTS AND DISCUSSION

Morphology and Size Distribution of rhGH Liposomes

Ethanol injection method is one of the simplest methods of obtaining liposomes. It is a mild procedure that affords a reasonably homogeneous vesicle population. As shown in Figure 2, rhGH liposomes showed a majority of spherical vesicles and no aggregation or fusion was observed. Narrow size distributions were obtained for all rhGH liposome suspension, with the mean size 1.7–2.5 μm.

Different Factors Affecting the rhGH Encapsulation Efficiency

Effect of Incubation Temperature

In a first series of experiments, we investigated the influence of incubation temperature on the encapsulation. The rhGH liposome suspension was freezed in liquid nitrogen and incubated in water bath at 0, 5, 10, and 15°C for 30 min, respectively, and repeated with three freeze–thaw cycles. As shown in Figure 3, encapsulation efficiency showed no significant difference between the incubation temperature at 0°C (71.5 ± 2.5%) and 5°C (75.2 ± 2.9%). We observed a decrease tend of encapsulation efficiency according to increasing incubation temperature exceed 5°C. The encapsulation efficiency in control experiment (without freeze–thaw process) was 37.1 ± 2.2%, which showed great difference with that of the experiments under freeze–thaw process ($p < .05$).

In many studies on liposomes, protein encapsulation efficiency was increased with high incubation temperature. With

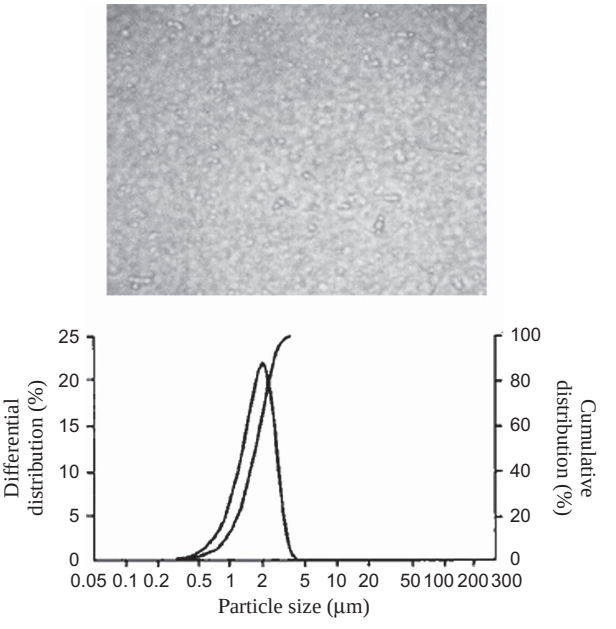

FIGURE 2. Morphology (×400) and size distribution of rhGH liposomes.

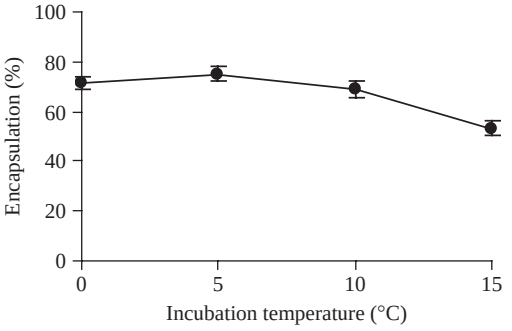

FIGURE 3. Effect of incubation temperature on rhGH encapsulation percentage.

high temperature above the gel–fluid phase transition temperatures of these lipids, the affinity for protein and liposome bilayer would be enhanced and adsorption would be progressively increased. However, high temperature causes deamidation, oxidation, denaturation, or aggregation of protein, which can turn protein biologically inactive and even immunogenic. In this study, incubation temperature at 5°C resulted in the highest encapsulation percentage. This suggests that gel–fluid phase transition temperature can be decreased to rather low temperature under vacuum condition. Therefore, liposomes containing unstable proteins can be obtained in low incubation temperature by modified freeze–thaw technique. In the following experiments, we fix the incubation temperature at 5°C.

Effect of Incubation Duration

In the following series of experiments, we investigated the effect of incubation duration on the encapsulation efficiency. The rhGH liposome suspension was freezed in liquid nitrogen and incubated in water bath at 5°C for 5, 20, 40, 60, and 90 min, respectively, and repeated with three freeze–thaw cycles. As shown in Figure 4, incubation duration had a strong effect on the encapsulation percentage of rhGH liposomes. From Figure 4, incubation duration showed a linear increase in encapsulation efficiency when incubation duration was below 40 min. The encapsulation percentage reached a maximum (79.7 ± 3.2%) when incubation time was 40 min. Then the encapsulation efficiency declined gradually to 72.6 ± 2.2% at 90 min incubation. All the experiment results under freeze–thaw process showed significant differences with that of control (37.1 ± 2.2%) ($p < .05$). This result suggests that the adsorption for protein and liposome bilayer can be promoted efficiently under vacuum condition.

Effect of Freeze–Thaw Number

In many studies, high encapsulation percentage was obtained by 10–30 freeze–thaw cycles (Colletier et al., 2002). With optimum conditions as mentioned above (incubation temperature at 5°C and incubation duration at 40 min), rhGH liposomes underwent different numbers of modified freeze–thaw cycles from 0 (control experiment) to 4. As shown in Figure 5, an increase of encapsulation efficiency according to the number of freeze–thaw cycles was observed. Highest encapsulation percentage was obtained at freeze–thaw cycle 4 (82.5 ± 3.4%). However, there was no significant difference between the freeze–thaw cycles 3 and 4 ($p > .05$).

It has been reported that repetitive freeze–thaw cycles of aqueous suspensions of liposome suspension improve the adsorption of protein into liposome bilayer (Colletier et al., 2002). However, repetitive freeze–thaw cycles reduce vesicles diameter and therefore decline the inner phase for loading protein which offsets the promotion of adsorption. In this study, the optimum parameters for rhGH encapsulation were found at freeze–thaw cycles 3–4. In the following experiments, we fix the freeze–thaw cycles to 4.

Effect of the Ratio Between rhGH and Phospholipids

The influences of different ratio between rhGH and phospholipids on rhGH encapsulation percentage are shown in Figure 6. From the results, the ratio between rhGH and phospholipids did not affect the encapsulation percentage. It means that the ratio between protein and phospholipids could be

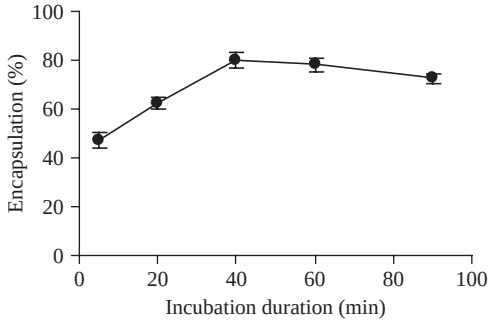

FIGURE 4. Effect of incubation duration on rhGH encapsulation percentage.

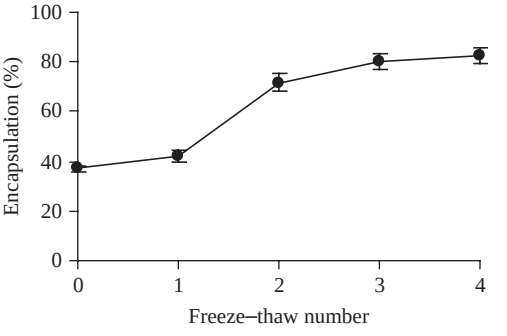

FIGURE 5. Effect of freeze–thaw number on rhGH encapsulation percentage.

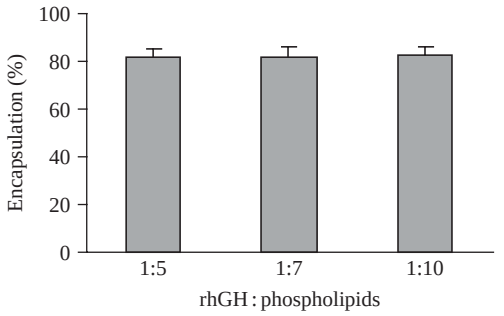

FIGURE 6. Effect of the ratio between rhGH and phospholipids on rhGH encapsulation percentage.

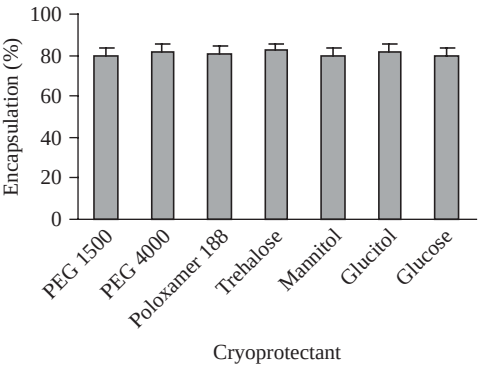

FIGURE 7. Effect of the cryoprotectants on rhGH encapsulation percentage.

adjusted in a large range without loss of encapsulation percentage. This result will be favorable in clinic use according to different patient situations.

Effect of Cryoprotectants

Most of protein capsulated liposomes will be agglomerated after lyophilization. Therefore, this step of the lyophilization process is a key step for the particle size distribution and it cannot be skipped. Addition of stabilizing additives as cryoprotectants in formulations is the most common tool to solve the problem. These compounds are often chosen on an empirical basis because the protective effect of solutes is variable, depending on chemical characteristics. We therefore evaluated a series of cryoprotectants (sugars or polymer) with different molecules through integrated evaluation of the encapsulation efficiency, appearance, and particle size distribution after redissolution of the lyophilized rhGH liposomes.

As shown in Figure 7 and Table 1, liposome suspension added with different cryoprotectants had nearly the same encapsulation ($p > .05$) but different morphology and particle size. The results suggested that liposome structure does depend on the stabilization and protection of the cryoprotectants. As shown in Table 1, trehalose exhibited the highest integrated value among the cryoprotectants investigated.

This result was similar to those resulted from previous studies (Chandrasekhar & Gaber, 1988; Madden et al., 1985). Under same concentration, the glass-transition temperature of liposomes containing trehalose is the highest among the ordinary cryoprotectants such as mannitol, glucitol, and glucose. Therefore, the devitrification action and the agglomeration of liposomes with trehalose as cryoprotectant are less than those for liposomes with mannitol, glucitol, and glucose as cryoprotectants.

From the experiment, efficient encapsulation could not be weakened by modified freeze–thaw method. Combined with vacuum rotation, this modification not only decreased the number of freeze–thaw cycle and incubation temperature, but shortened incubation duration as well. In addition, the addition of nitrogen was simplified during the preparation procedure.

TABLE 1
Morphology and Particle Size of Liposomes with Different Cryoprotectants

Cryoprotectants	Evaluation Appearance of Freeze Drying Sample[a]	Number of Debris after Redissolution[b]	Liposome Morphology[c]	Particle Size[d] (Mean Value)	Integrated Value
PEG 1500	2	1	1	1 (3.21 μm)	5
PEG 4000	2	1	1	1 (4.67 μm)	5
Poloxamer 188	1	3	1	2 (2.98 μm)	7
Trehalose	3	3	3	3 (1.69 μm)	12
Mannitol	1	1	1	1 (3.60 μm)	4
Glucitol	3	3	2	3 (1.89 μm)	11
Glucose	3	3	2	2 (2.56 μm)	10

[a]Very sticky = 1; porous but a little sticky = 2; porous and fragile = 3.
[b]Many = 1; some = 2; few = 3.
[c]Agglomerated and hard to disperse = 3; agglomerated but can be dispersed = 2; disintegrated = 3.
[d]Particle size: 1–2 μm = 1; 2–3 μm = 2; >3 μm = 3.

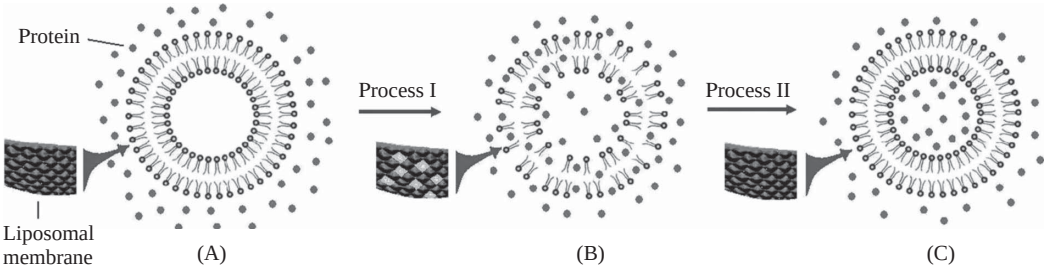

FIGURE 8. Effect of the environmental temperature or atmosphere on the liposomal membrane. (A) Below T_c under normal atmosphere, the membrane structure of liposome was formed orderly and compactly. (B) As temperature increasing near T_c or atmosphere declining to certain vacuity, the lipid material reached in liquid-crystal state, which resulted in structural flaw and fragility. With the osmotic pressure and lipid affinity, hydrophilic proteins outside of the liposomal shell were brought into liposomal interior. (C) When environmental temperature or atmosphere restored to normal value as phase "a," a great amount of protein could be sealed in liposome. Process I: increasing temperature or declining atmosphere. Process II: declining temperature or increasing atmosphere. T_c: phase transition temperature.

Though the reasons for these findings were unclear, it seemed that the reasons were related to the gel–liquid crystalline phase transition temperature (T_c). Effect of T_c on the liposomal membrane was shown in Figure 8. Usually, the membrane structure of liposomes was formed orderly and compactly below T_c (Figure 8A). When environmental temperature increased beyond T_c, liposomal shell was liquefied and membrane structure was lost. With the osmotic pressure and lipid affinity, hydrophilic proteins outside of the liposomal shell were brought into liposomal interior (Figure 8B). Therefore, under suitable thaw temperature near T_c, high encapsulation for protein-loaded liposomes could be obtained (Figure 8C).

The lipid membrane of rhGH liposomes in this study was formed by HPC, whose T_c was 42–45°C under normal atmosphere. As a rule, atmosphere condition had a positive relationship with the colligative characters such as boiling point and freezing point. T_c of the liposomal shell seemed to be greatly declined under vacuum condition than that under normal atmosphere. Therefore, the effect of declining atmosphere had a same effect as increasing temperature for liposomal shell. Therefore, thaw and incubation process under suitable vacuum condition could give a high entrapment for rhGH-loaded liposomes.

CONCLUSIONS

This study demonstrated the possibility of increasing the entrapment of rhGH liposomes with a modified freeze–thaw technique. From the results, efficient encapsulation could be obtained by manipulation of the incubation temperature, incubation duration, and number of freeze–thaw during the modified freeze–thaw cycles. However, the release profile of rhGH from liposomes was not detected in this study. In addition, more experiments are needed to detect the real T_c under different vacuity. It will be interesting to continue investigation in this direction to improve the freeze–thaw technique for protein-loaded liposome preparation.

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