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Optimisation of preparation conditions and properties of phytosterol liposome-encapsulating nattokinase

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Phytosterol liposomes were prepared using the thin film method and used to encapsulate nattokinase (NK). In order to obtain a high encapsulation efficiency within the liposome, an orthogonal experiment (L9 $(3)^4$) was applied to optimise the preparation conditions. The molar ratio of lecithin to phytosterols, NK activity and mass ratio of mannite to lecithin were the main factors that influenced the encapsulation efficiency of the liposomes. Based on the results of a single-factor test, these three factors were chosen for this study. We determined the optimum extraction conditions to be as follows: a molar ratio of lecithin to phytosterol of 2 : 1, NK activity of 2500 U mL^{-1} and a mass ratio of mannite to lecithin of 3 : 1. Under these optimised conditions, an encapsulation efficiency of 65.25% was achieved, which agreed closely with the predicted result. Moreover, the zeta potential, size distribution and microstructure of the liposomes prepared were measured, and we found that the zeta potential was -51 ± 3 mV and the mean diameter was 194.1 nm. From the results of the scanning electron microscopy, we observed that the phytosterol liposomes were round and regular in shape and showed no aggregation.

Keywords: liposome; nattokinase; phytosterol preparation; encapsulating properties

1. Introduction

Natto is a traditional high-protein food commonly consumed in Asia and is produced using the bacterium *Bacillus natto* to ferment a protein source over a short period of time. In the recent years, this hypothesis has been confirmed by several clinical trials using animals and humans. Nattokinase (NK) is a potent fibrinolytic enzyme produced by *B. natto*, and has been isolated from natto by Sumi et al. (1990), Sumi (1987) and Fujita, Nomura, and Hong (1993). NK can break down fibrin directly, while enhancing the body's production of both plasmin and other

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clot-dissolving agents (Tetsumei et al., 2001; Yamashita, Oda, Giddings, & Yamamoto, 2003). NK has been demonstrated to be stable to pH and temperature, and can therefore exist in the gastrointestinal tract in a stable condition (Chaize & Fournier, 2004). In some ways, NK is actually superior to conventional clot-dissolving drugs. While traditional drugs have to be administered intravenously and within 12 h of a stroke or heart attack, this natural dietary supplement taken orally on a regular basis at a daily dose may help prevent the formation of thrombi (Sumi, Hamada, & Nakanishi, 1990).

Although early clinical studies utilised intravascular administration of thrombolytic agents, it is now clear that some agents may be successfully administered orally when encapsulated for delivery into the small intestine (Fujita, Ito, Hong, & Nishimuro, 1995; Suzuki, Kondo, Ichise et al., 2003). For instance, clinical and animal studies have demonstrated that the intestinal absorption of urokinase produces a prolonged level of activity in plasma together with a significant lytic effect on thrombi (Suzuki, Kondo, Matsumotoa et al., 2003). As an effective and efficient system of drug delivery, liposomes are being developed to increase the use and effectiveness of active components (Derycke & Witte, 2004). Encapsulating NK may lead to the development of a new method of exploiting its activity and delivery as a drug. Hsieh et al. (2009) employed Na-γ-PGA (polyglutamic acid) as a coating material for preserving NK, and found that the stability to temperature and pH of the microencapsulated NK was higher than that of the free form (Chang et al., 2008). Moreover, immobilisation of NK on polyhydroxybutyrate (PHB) nanoparticles resulted in a 20% increase in the enzymatic activity (Deepak, Pandian, Kalishwaralal, & Gurunathan, 2009). A liposome is composed of a double bilayer membrane similar to the cell membrane. With regard to the characteristics of biodegradability and compatibility with the organism, the toxicity and immunogenicity of liposomes were found to be low (Jia, Joly, & Omri, 2008). Recently, liposomes have been used in the food and pharmaceutical industries for encapsulating bioactive substances. Normally, the cholesterol in a liposome serves as a component providing rigidity to the membranes and improving the properties of the bilayer membranes (Frans, Sanders, & Thompson, 2005). Compared with dietary levels in healthy subjects, the cholesterol present in liposomes is negligible. Patients with hypercholesterolemia should severely restrict their intake of cholesterol even at low concentrations (Jogchum & Ronald, 2005; Laura, Joan, & Francisco, 2009). Phytosterols have been reported to show a similar function and chemical structure as that of cholesterol in membranes. Chan, Chen, Chiu, and Lu (2004) suggested that replacing cholesterol with phytosterols in the preparation of liposomes was feasible and hence desirable. Phytosterols have been found to be effective in lowering the concentration of cholesterol in plasma by inhibiting its absorption from the small intestine (Konan, Gurny, & Allemann, 2002; Pass, 1993).

Liposome-encapsulated NK would be beneficial for disrupting thrombi, especially those caused by hyperlipidemia. Moreover, NK can be more efficiently used by the organism when it is encapsulated in a liposome. The preparation of liposome-encapsulated NK is described in this article. An orthogonal design was adopted in order to optimise the preparation conditions of the liposomes on the basis of the encapsulation efficiency (EE). The properties of the liposomes were also determined.

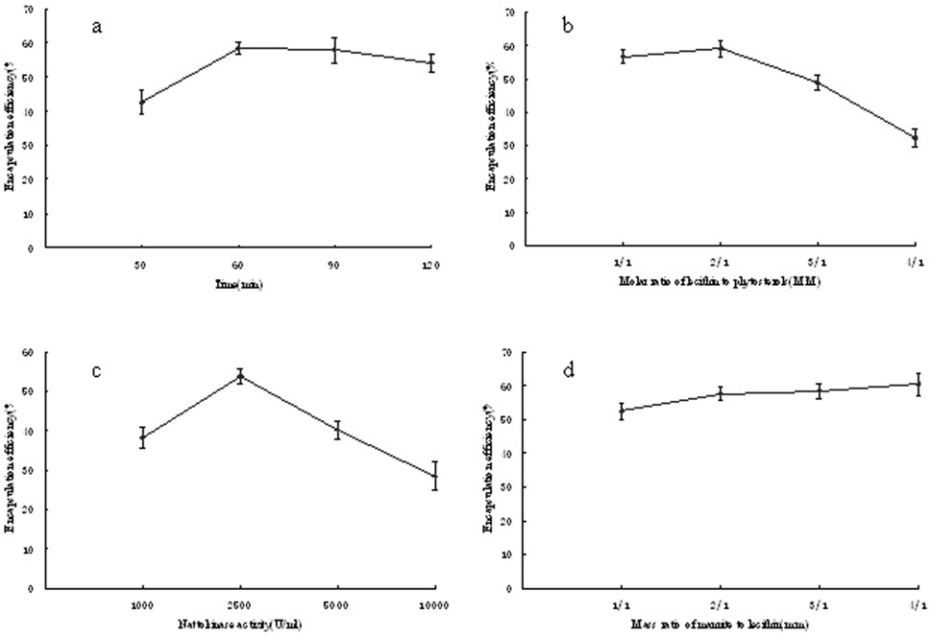

Figure 1. The effect of rotary evaporation time, the molar ratio (M : M) of lecithin to phytosterols, NK activity and the mass ratio (m : m) of mannite to lecithin on the EE of liposomes.

2. Results and discussion

2.1. *The effect of rotary evaporation time, the molar ratio of lecithin to phytosterols, NK activity and the mass ratio of mannite to lecithin on the EE of liposomes*

The effect of the length of time required for the rotary evaporation step on the EE of the liposomes was evaluated when the molar ratio of lecithin to phytosterols (M : M) was set at 2 : 1, NK activity was 2500 U mL^{-1} and the mass ratio of mannite to lecithin (m : m) was set at 2 : 1. Figure 1(a) shows the effect of rotary evaporation time on the EE of liposome. When the rotary evaporation time increased from 30 to 60 min, the EE of liposome increased due to the increase in film formation of lecithin. There was no change in the level of film formation with increasing evaporation time. There was no noticeable enhancement and very little decline in film formation over the period of evaporation from 60 to 120 min. Thus, 60 min was selected as the optimum period for rotary evaporation.

Using the optimal result for the rotary evaporation time as described above, the liposomes were prepared with different molar ratios of lecithin to phytosterols, using NK activity of 2500 U mL^{-1} and a 2 : 1 mass ratio of mannite to lecithin. Figure 1(b) shows the effect of the molar ratio of lecithin to phytosterols on the EE of liposomes. When the molar ratio of lecithin to phytosterols was below 1 : 1, comparative difficulties were observed in film formation process. Therefore, the EE resulting from a molar ratio of lecithin to phytosterol of below 1 : 1 was unacceptable. As shown in

the figure, the EE decreased when the molar ratio of lecithin to phytosterols was increased to 3 : 1. An even more dramatic decline was observed when the ratio was increased to above 4 : 1. The EE was highest when the molar ratio of lecithin to phytosterols was 2 : 1.

The effect of different NK activities was evaluated when liposomes were prepared with the rotary evaporation time set at 60 min, the molar ratio of lecithin to phytosterols set at 2 : 1 and the mass ratio of mannite to lecithin set at 2 : 1. As shown in Figure 1(c), the EE increased with the increase in NK activity. However, when the NK activity was set above 2500 U mL^{-1}, a significant decline in the EE was observed, which can be ascribed to the attainment of the maximum EE at the 2500 U mL^{-1} level of NK. Further increase in NK activity resulted in the decline of EE.

The effect of the mass ratio of mannite to lecithin on the EE of liposomes was evaluated when the rotary evaporation time was set at 60 min, the ratio between lecithin and phytosterols at 2 : 1 and an NK activity of 2500 U mL^{-1}. It can be seen from Figure 1(d) that with the increase in the mass ratio of mannite to lecithin, EE experienced a significant upward trend. However, no significant increase was seen when the mass ratio of mannite to lecithin was above 2 : 1. Mannite, as lyophilised protective solute, may raise the EE of liposomes.

2.2. *Orthogonal experiment*

From our experiment, various parameters were found to play critical roles in the experimental conditions required for the optimisation of the EE of liposomes, such as the molar ratio of lecithin to phytosterols (A), NK activity (B) and the mass ratio of mannite to lecithin (C), which are generally considered to be the most important factors. The levels of each factor investigated were selected, according to the previously mentioned results of the single-factor experiments. The independent variables, the molar ratio of lecithin to phytosterols (1 : 1, 2 : 1, 3 : 1), the NK activity (1000, 2500, 5000 U mL^{-1}),the mass ratio of mannite to lecithin (1 : 1, 2 : 1, 3 : 1) and the three levels of variation tested were tested using an orthogonal L9 $(3)^4$ design. The total evaluation index was used for statistical analysis. Analysis of the orthogonal tests is given in Table 1. The liposomes obtained from each test were pretreated and quantitatively analysed according to the method discussed above. Although the maximum EE of the liposomes was 64.4%, the corresponding preparation conditions may not be the optimum. According to orthogonal analysis, the values of K and R were calculated using statistical software. The factors influencing the EE are listed in decreasing order as follows: $B > A > C$ with respect to the R value. The maximum EE was therefore obtained when the molar ratio of lecithin to phytosterols, NK activity and the mass ratio of mannite to lecithin were $B_2A_2C_3$ (2500 U mL^{-1}, 2 : 1, 3 : 1), respectively. According to the R value and the results of the analysis of variance (ANOVA) table, the NK activity and molar ratio of lecithin to phytosterols were the major factors affecting the EE. We propose that the optimum conditions are as follows: $B_2A_2C_3$ (2500 U mL^{-1}, 2 : 1, 3 : 1). In a confirmatory test, a high EE of 65.25% was obtained.

Table 1. Orthogonal experiment (L9 $(3)^4$) to determine the EE of liposomes.

Treat	Variable levels			EE (%) ($n=3$)
	A	*B*	*C*	
1	1	1	1	22.9
2	1	2	2	52.2
3	1	3	3	29.2
4	2	1	2	44.1
5	2	2	3	64.4
6	2	3	1	48.3
7	3	1	3	38.4
8	3	2	1	55.2
9	3	3	2	32.2
K1	104.3	105.4	126.4	
K2	156.8	171.8	128.5	
K3	125.8	109.7	132.0	
R	52.5	66.4	5.6	

2.3. *Size distribution and microstructure of liposomes*

Freeze-dried liposomes were hydrated for the mean diameters and size distribution measurements at 25°C. The zeta potential was $-(51 \pm 3)$ mV, the mean diameter was 194.1 ± 4.98 nm and the coefficient of variation was 2.57%. Liposomes are nano-single-room-liposome capsules.

The liposomes were diluted to form a suspension and one drop of the liposome suspension was observed at an objective magnification of ×10,000 and an accelerating voltage of 15,000 V. The result showed that the liposomes prepared in this manner were round and regular in shape and did not adhere to one another.

2.4. *Stability of liposomes during storage*

The experimental liposome particle suspension was stored for 14 days at 4°C. After 2, 4, 6, 8, 10, 12 and 14 days, the residual percentage was determined. The residual percentage of NK is shown in Figure 2. From this figure, it can be seen that the residual percentage of NK in the liposome suspension decreased with storage time at 4°C. After 14 days of storage, the residual percentage declined from 100% to 81.9%. From 2 to 6 days, the residual percentage fell rapidly from 6 to 14 days, the residual percentage showed a relatively downward trend. The results suggest that the liposomes containing NK were stable at 4°C. Some studies have demonstrated that the residual percentage of BSA in a liposome suspension stored at 4°C was the highest of all the three temperatures tested (4°C, −20°C and 25°C) (Chan et al., 2004). Lecithin and phytosterols were tightly packed in a regular formation which resulted in membrane rigidity and decreasing membrane permeability. Storage of liposomes at 4°C may reduce susceptibility to oxidation, lower the residual percentage of encapsulated materials and increase the stability of liposomes during storage. Storing liposomes made with phytosterols as a freeze-dried powder may be

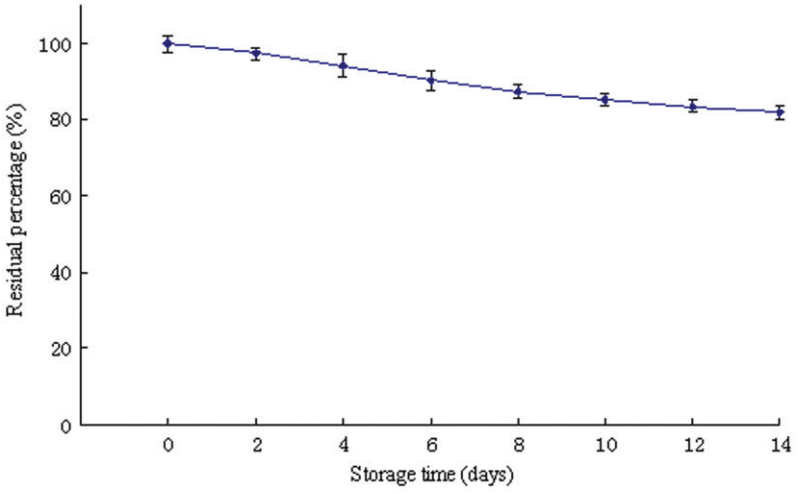

Figure 2. Residual percentage of NK from liposomes stored at 4°C. All values are the mean ± SD of triplicate experiments.

a good storage option, particularly, when they are stored at 4°C under an atmosphere of nitrogen.

The encapsulation of protein drugs (e.g. enzyme) is a viable and promising approach for the delivery of proteins. The most commonly used methods for protein drug encapsulation in polymeric microparticles include coacervation, spray cooling/chilling, extrusion, alginated beads, liposomes and spray drying (Tamber, Johansen, Merkle, & Gander, 2005). Previously, researchers have encapsulated NK in a Na-γ-PGA coating material and PHB nanoparticles. These methods enabled efficient encapsulation of NK and increased its stability against varying conditions of pH and temperature (Chang et al., 2008; Deepak et al., 2009). Phytosterol liposomes encapsulating NK have dual functional components and belong to the nano-single-room-liposome group of capsules. Thus, liposomes may be used to prolong NK activity in multiple-unit, site-specific drug-delivery systems which should be further investigated in future studies.

3. Experimental

3.1. *Chemicals and instruments*

Lecithin was extracted from soyabean, and biochemical agents were purchased from Beijing Aoboxing Biotech Company Ltd. NK extraction was from natto in our laboratory and 50,000 U mL^{-1} solution of NK was prepared with barbitone buffer (pH 7.8). Phytosterols were obtained from Sigma. Fibrinolytic protease, urokinase and thrombase were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). All other reagents were of analytical grade. The mean diameters and size distribution of the liposomes were determined by dynamic light scattering (Mastersizer, Malvern instruments Ltd.,

Nano ZS90 (Red badge), UK). The microstructure of liposomes was observed using a S-3000N scanning electron microscope (Hitachi Science Systems, Ltd. Japan).

3.2. *Liposome preparation*

The thin film method was used to prepare liposomes (Chaize & Fournier, 2004; New, 1990), but with minor modifications. Briefly, a lecithin/phytosterol mixture in ethyl ether was dried under reduced pressure in a rotary evaporator to leave a thin film on the wall of a 100-mL volume round-bottomed flask. Of the mannite buffer, 5 mL was added to the flask, and the mixture was agitated with glass beads at 60°C for 5 min. A total of 5 mL of the encapsulating material (NK in barbitone buffer, pH 7.8) was added to the suspension and thoroughly mixed. The suspension was treated with ultrasound at room temperature for 20 min, and then freeze-dried. The freeze-dried material was exposed to humidified nitrogen and then rehydrated by adding 5 mL of water with subsequent incubation at 37°C for 10 min, thus producing the liposomes.

3.3. *Encapsulation efficiency*

The liposomes prepared were precipitated by centrifugation at $1{,}80{,}000 \times g$ for 20 min at 4°C. The activity of NK in the supernatant was determined. The NK EE was calculated using the following equation:

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Activity of added NK} - \text{Activity of uncapsulated NK}}{\text{Activity of added NK}} \times 100.$$

3.4. *NK activity analysis*

Fibrinolytic activity was determined by comparison with urokinase which was used as a standard of fibrinolytic protease activity. The fibrinogen plates were synthesised using the Astrup method (Liu, Xing, Shen, Yang, & Liu, 2004). Wells were made in the fibrin plate and 10 μL of each sample was placed into the wells. The plate was then incubated for 18 h at 37°C, after which the activity of the samples was determined by measuring the dimensions of the clear zone around the fibrin clots and comparing the data to a standard curve.

3.5. *Optimisation of EE of the liposomes*

An orthogonal L9 $(3)^4$ test design was used to investigate the optimal encapsulation conditions of the liposomes. The extraction experiment was carried out by three factors and three levels based on the results of single-factor experiments. The EE (%) of the liposomes was the dependent variable. The EE of the liposomes was obtained from the above nine tests.

3.6. *Statistical analysis*

The data were analysed using ANOVA. Experiments were repeated at least three times.

4. Conclusion

This article reports a novel method of encapsulating NK and phytosterols, which has proved to be feasible. Phytosterols showed a similar function and chemical structure to cholesterol which is present in membranes. In this study, the preparation conditions were optimised. Under optimised conditions, the highest EE achieved was 65.25%. After appropriate preparation, the liposomes were found to be round and regular in shape with no adherence. In addition, the liposomes containing NK were stable at 4°C. NK encapsulated in liposomes can be more efficiently utilised in the small intestine. These results lay a solid foundation for further utilisation of liposomes. We have also carried out animal model experiments on thrombi *in vitro* and *in vivo* which indicate that liposomes may be a potential clot-dissolving agent that is superior to urokinase. Product development will be the core of our future studies.

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