Ferrous sulfate liposomes: preparation, stability and application in fluid milk

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Abstract

The effects of cholesterol and Tween 80 on the physical stability of empty liposomes were investigated. Results showed that the physical stability of liposomes, including electrostatic and steric stability, was improved by addition of cholesterol and Tween 80. Liposomes prepared by different methods, thin-film hydration, thin-film sonication, reverse-phase evaporation and freeze-thawing, were tested for their capacity to encapsulate ferrous sulfate. Technology parameters to microencapsulate ferrous sulfate, concentration of ferrous sulfate, hydrating media and sonication strength, were optimized. The concentration of ferrous sulfate and the hydrating medium had a significant effect on the amount of encapsulated ferrous ions. The encapsulation efficiency (EE) of 67% was obtained by using the reverse-phase evaporation method. In milk fortified with ferrous sulfate liposomes, the concentration of iron increased to 15 mg L\(^{-1}\), and it was stable to heat sterilization (100 °C, 30 min) and storage at 4 °C during one week.

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1. Introduction

Iron deficiency has earned distinction as the most common nutritional deficiency in the world today (Horton & Ross, 2003; Navarrete, Camacho, Lahuerta, Monzo, & Fito, 2002). Because iron is important for blood formation, iron deficiency often leads to anemia, defined as having a blood hemoglobin level below standard. Iron deficiency is usually the result of insufficient dietary intake of iron, poor utilization of iron from ingested food, or a combination of the two (Gaucheron, 2000). Milk is relatively poor in iron (0.2–0.4 mg L\(^{-1}\)). Direct addition of iron to milk or dairy products might be an effective means of increasing the dietary intake of iron to the general population.

Soluble iron salts such as ferrous sulfate are cheap and have higher availability, yet may reduce vehicle-food quality and shelf-life due to potential oxidized off-flavors, color changes, and metallic flavors (Jackson & Lee, 1991). It is therefore proposed that these salts may be microencapsulated to prevent these negative effects, by protecting the core ingredients from the environment or from interaction with other ingredient during processing (Augustin, Sanguansri, Margetts, & Young, 2001). So microencapsulation technology may be an effective way to solve the problem of iron fortification in food.

Liposome technology is a special kind of microencapsulation technique, which has been extensively investigated and developed in the biomedical field as a drug delivery system. An important aspect of this application is the protection afforded by encapsulation, against potentially damaging conditions in the extracapsular environment. In comparison with other encapsulation systems used within the food industry, they are particularly well suited since they are well characterized, easily
made, highly versatile in their carrier properties, and composed of food-acceptable ingredients (AFRC, 1988; Kibry, 1991; Kibry, Whittle, Rigby, Coxon, & Law, 1991). Several researchers have investigated the bioavailability of ferrous sulfate liposomes. Results revealed that microencapsulated FeSO₄ with lecithin (Boccio et al., 1996, 1997; Lysionek, Zubillaga, Sarabia, Caro, & Weill, 2000, 2002; Uicich et al., 1999; Zubillaga et al., 1996) has the same bioavailability as FeSO₄, but has the advantage of being coated with a phospholipid membrane which keeps the iron from contacting with the other components of food, thus preventing the undesirable interactions that happened when conventional FeSO₄ was used.

However, liposomes are thermodynamically unstable. The liposome particles will aggregate, fuse, flocculate and precipitate during storage. Increasing interparticle repulsion, either electrostatic or steric, can enhance their efficiency and physical stability. The influences of cholesterol and Tween 80 on the physical stability of liposomes were examined by determining ζ-potential and turbidity of empty liposomes; ferrous sulfate liposomes prepared from different kinds of methods were assessed through encapsulation efficiency. In addition ferrous sulfate liposomes were added to fluid milk to evaluate the possibility of application.

2. Materials and methods

2.1. Raw materials

Egg PC (phosphatidylcholine) or lecithin was purchased from the Chemical Reagent Plant of East China Normal University (Shanghai, China). Ferrous sulfate, sodium citrate, citric acid, ascorbic acid, diethyl ether, cholesterol, and Tween 80 were obtained from Shanghai Chemical Reagent Co. (Shanghai, China). Macroporous cation exchange resin was bought from Jiangyin Organic Chemical Plant (Jiangsu Province, China). The fluid milk (fat content = 3.1%) samples were bought from supermarket, manufactured by Bright Dairy Co. (Shanghai, China), which were already pasteurized, and had a shelf life of 7 days at 4 °C. All other chemicals were of reagent grade and used without further purification.

2.2. Preparation of empty liposomes

Preparation of empty PC-cholesterol liposomes: The empty PC-cholesterol liposomes were prepared by reverse-phase evaporation method (REV) (Lasic, 1993). The lipid mixture, containing egg PC (20 mmol L⁻¹) and various quantities of cholesterol (from 0 to 50 mol%), was dissolved in 30 mL diethyl ether. One-third volume (10 mL) of buffer solution composed of 10 mmol citric acid and 10 mmol Na₂HPO₄ (pH being adjusted to 6.8) was added to the organic phase. Ultrasonication with a probe sonicator (VCX400, Sonics & Material Vibra Cell, 400 W, 20 kHz) in an ice bath for 5 min resulted in a homogeneous w/o emulsion. After the removal of the organic solvent at controlled reduced pressure with a rotary evaporator, a gel was formed. Upon continued rotary evaporation the gel was broken, and then the remaining aqueous phase (20 mL) was added with gentle vortexing. The remaining ether was evaporated at 0.01 MPa. The liposomes were stored at 4 °C in a refrigerator.

Preparation of empty Tween-grafted liposomes: The empty Tween-grafted liposomes were also prepared by REV method. The lipid mixture contained egg PC (20 mmol L⁻¹) and cholesterol (2 mmol L⁻¹). When the gel was broken, the remaining aqueous phase containing various quantities of Tween 80 surfactants (molar ratio from 0 to 1.0) was added. The other procedures were the same as the above.

2.3. Preparation of ferrous sulfate liposomes

Based on the study of empty liposomes, the basic composition of the membrane fraction of the liposomes was fixed at 80% (mol/mol) egg lecithin, 10% (mol/mol) cholesterol and 10% (mol/mol) Tween 80. Ascorbic acid was used as an anti-oxidant to protect the ferrous ion against oxidation. Four kinds of methods were used to prepare ferrous sulfate liposomes in order to compare their encapsulation efficiency (EE). The most suitable method was selected according to the EE. Then parameters such as weight ratio of iron to lipids, hydrating media and sonication strength were optimized. The effect of weight ratio of iron to lipids (0.04, 0.06 and 0.1) on the EE was investigated. The effect of hydrating media on the EE was studied by encapsulating the ferrous sulfate solution (weight ratio of iron to lipids was 0.04) with different hydrating media (deionized water, 0.01 mol L⁻¹ citric acid–Na₂HPO₄ buffer solution and 0.01 mol L⁻¹ citric acid–sodium citrate buffer solution). In addition, the effect of sonication strength (60%, 70%, 80% and 90%) on the EE was studied.

Reverse-phase evaporation (REV) (Lasic, 1993): Ferrous sulfate liposomes were prepared slightly differently from empty liposomes. In this system, one-third volume of aqueous solution containing ferrous sulfate and ascorbic acid (15:1 weight ratio of ferrous sulfate to ascorbic acid) was added to the organic phase. Final li-
The liposome samples were disrupted by ashing to release the encapsulated iron, solubilized with 3 mol L\(^{-1}\) hydrochloric acid, and then diluted in a volumetric flask with de-ionized water to a ferrous ion concentration of about 30 mg mL\(^{-1}\). The amount of iron in the solution was determined by the method of bathophenanthrolin colorimetry.

### 2.7. Encapsulated iron in liposomes

In the iron trapping efficiency experiments, 1 mL of the liposome samples were placed in a cellulose membrane bag (molecular weight cut-off 5000, Shanghai Huamei Biochemistry Ltd., China) and dialyzed against 250 mL of de-ionized water for 24 h at 4 °C to remove the non-encapsulated iron remaining in the dispersion medium. The liposomes inside the dialyzer were then destroyed by ashing and solubilized with 3 mol L\(^{-1}\) hydrochloric acid, and diluted in a volumetric flask with de-ionized water to a ferrous ion concentration of about 30 mg mL\(^{-1}\). The amount of iron in the solution was also determined by the method of bathophenanthrolin colorimetry.

### 2.8. \(\zeta\)-Potential measurement of liposomes

The electrophoretic mobility and the \(\zeta\)-potential of the liposomes sample were determined by laser Doppler electrophoretic mobility measurements using the Zetasizer 2000 (Malvern Instruments Ltd, Malvern UK). All measurements were done at 25 °C, which was controlled with a precision of 0.1 °C. The measurements were repeated three times.

### 2.9. Determination of liposomal sizes

According to Rayleigh–Gans–Debye (RGD) theory, turbidity (\(T\)) determination technique may be used to estimate relative sizes of liposomes via spectrophotometry. Furthermore, absorbance (\(A\)) is proportional to turbidity (\(T = 2.303A/\ell, \ell\) is the length of light path) (Fennema, 1996), so the absorbance values of liposomes increase with increasing liposomal size. Herein the turbidity was determined by measuring the absorbance of
the liposome samples at 500 nm, at 25 °C, in a spectrophotometer, Model 722 (Exact Science Apparatus Ltd., Shanghai, China).

2.10. Removal of free ferrous ion via ion-exchange column

Macroporous cation exchange resin was used to prepare plenty of ferrous sulfate liposomes without free ferrous ion, because the encapsulated ferrous ions are positively charged. Ferrous sulfate liposomes were passed through a 2.6 ID × 30 cm column packed with macroporous cation exchange resin to remove free ferrous ions. The column was washed with excess de-ionized water to rinse off the liposomes until the absorption at 500 nm was zero. Then the resin was regenerated by washing with 2 mol L\(^{-1}\) NaCl.

2.11. Preparation of fluid milk samples fortified with ferrous sulfate liposomes

Fluid whole milk samples (250 mL), at room temperature, were enriched with about 10 mL ferrous sulfate liposomes to produce final iron concentrations to about 15 mg L\(^{-1}\). Ferrous sulfate liposomes were added with gentle stirring to ensure rapid and complete mixing. Preparation of iron-fortified milk samples and subsequent treatments were carried out in triplicate.

2.12. Heat treatment of fluid milk samples fortified with ferrous sulfate liposomes

The fluid milk samples, which were bought from the supermarket, manufactured by Bright Dairy Co., had been already pasteurized. In order to evaluate the effect of heat treatment on the iron-fortified milk samples, 250 mL of milk samples in sealed bottles were submerged at 100 °C for 30 min. A control sample without fortification was tested under the same experimental conditions.

2.13. Sensory evaluation of milk fortified with ferrous sulfate liposomes

The stability indexes (precipitation and coagulation) of fluid milk fortified with ferrous sulfate liposomes were evaluated by visual observation at 4 °C for one week. The sample that had no coagulation or precipitation after one week was considered stable.

Sensory analyses (color and off-flavor) of the milk samples after holding at 4 °C for one week were performed by 8 trained members of a sensory panel. Panelists evaluated the difference in color and off-flavor between fortified milk and control milk. Unsalted water was provided to cleanse the palate.

2.14. Statistical analysis

All experiments were performed in triplicate; the means and standard deviation were calculated.

3. Results and discussion

3.1. Effect of cholesterol on electrostatic stability of empty liposomes

It has long been known that cholesterol can improve liposome membrane stability in biological fluids such as blood plasma (Kirby, Clarke, & Gregoriadis, 1980) and milk (Kirby, Brooker, & Law, 1987), and reduce the fluidity and permeability of the liposomal bilayer (Lasic, 1993). Herein the \(\zeta\)-potential of the empty PC liposomes prepared by REV method was measured by incorporating different quantities of cholesterol into the PC bilayer. Experimental results showed that the PC liposomes possessed negative charges, indicating that a strong electrostatic repulsive force occurred among the PC liposomes. Fig. 1 describes the effects of cholesterol on the \(\zeta\)-potential of the PC liposomes. The incorporation of different amounts of cholesterol into the PC liposomal bilayer significantly affected the \(\zeta\)-potential at 25 °C. The \(\zeta\)-potential value of the liposomes increased as the concentration of the cholesterol increased from 0 to 10 mol%. This is because the polar head of cholesterol contain hydroxyl groups, which are easily combined with choline in the polar region of PC to produce a kind of dipole tropism that increases the liposomal surface negative charges. However, the \(\zeta\)-potential dramatically dropped when the concentration of the cholesterol was increased further, which destabilized the liposomes. The mechanism of this phenomenon has to be elucidated.

A significant change in the \(\zeta\)-potential was found in lipid bilayer with negative charges, which was affected by cholesterol. This illustrated that cholesterol affected...
the electrostatic interaction among PC liposome membranes. It is concluded that appropriate cholesterol content in the liposomes may improve the encapsulation efficiency and physical stability of ferrous sulfate liposomes.

3.2. Effect of Tween 80 on steric stability of empty liposomes

Sterically stabilized liposomes have recently been designed by altering their surface by using glycolipids or polymers such as polyethylene glycol (PEG), polyvinyl alcohol or polyacrylamide. The attachment of the hydrophilic macromolecule to the biologically stabilized liposomes, also referred to stealth liposomes, makes them heavily hydrated, thus reducing protein adsorption (Liu, Hsieh, Chang, & Chen, 2003). Kronberg et al. proved that sterically stabilized liposomes increased the stability of systems that incorporated Tween 80 surfactants into the lipid bilayer in serum, compared with the corresponding liposomes without Tween 80 (Tasi, Liu, & Chen, 2003). The interaction between PC liposomes incorporated with varying quantities of Tween 80 surfactant was examined from the aspect of liposomal size in order to elucidate the steric stability properties of empty liposomes.

Turbidity is not only an effective indicator of particle size, but also an estimator of relative size (Lasic, 1993). For example, turbidity increases as the liposomal size increases. Herein turbidity was expressed by the value of $A_{500\text{ nm}}$. Experimental results showed that the liposomal size decreased dramatically when the amount of Tween 80 surfactant was increased from 0 to 0.2 ( Tween 80:lipids, molar ratio) (Fig. 2). This observation might be due to a steric repulsion among the Tween 80 surfactants, which are exposed from the outer and inner leaflets of the liposomal bilayer membrane. The Tween 80 surfactants exposed from the outer leaflet of the bilayer membrane increased the liposome particle curvature, whereas the Tween 80 exposed to the inner leaflet did the opposite. Therefore, adding Tween 80 surfactants reduced the liposomal size since there was more Tween 80 existing in the outer leaflet than that in the inner leaflet of the liposomal bilayer membranes (Tasi et al., 2003). When the ratio was increased from 0.2 to 0.8, the turbidity almost kept constant, but when it was further increased to 1.0, the turbidity dropped sharply again. The interaction between surfactant and liposomal membrane may be explained via Lichtenberg’s three-step model (Alino, Iruarrizaga, Alfaro, Pajean, & Herbage, 1991): when surfactant concentration is increased further (molar ratio from 0.2 to 0.8), liposomes can be gradually dissolved into mixed micelles; however once surfactant concentration is above a limitative value (maybe above 0.8), liposomes can be dissolved entirely and the liposomal bilayer structure is destroyed completely, hence the entrapped core materials leak out. Therefore, an appropriate addition amount of Tween 80 had to be further optimized.

3.3. Encapsulation efficiency of ferrous sulfate liposomes prepared under different conditions

Nowadays, many methods are used for the preparation of liposomes. Due to the difference in the preparation procedures, the same type of liposomes prepared by different methods may have different properties, such as encapsulation efficiency, bilayer permeability and stability. Herein four kinds of methods, thin-film (TF), thin film and sonication (TFS), reverse-phase evaporation (REV) and freeze-thawing (FT), were used to prepare ferrous sulfate liposomes in which de-ionized water was used as the hydrating medium. Based on the stability of empty liposomes, the basic composition of the membrane fraction of the liposomes was fixed at 80% (mol/mol) egg lecithin, 10% (mol/mol) cholesterol and 10% (mol/mol) Tween 80. Comparing the different preparation methods, the ferrous sulfate liposomes prepared by REV had highest EE (Fig. 3). This may be due to the fact that all the water soluble molecules are contained in the small water droplets of the w/o emulsions which may coalesce into liposomes without being open when the remaining aqueous phase is added later during the REV process (Lasic, 1993). Because REV had highest EE, it was used in the rest of this study.

3.3.1. Effect of ferrous ion concentration on EE of ferrous sulfate liposomes

The effect of ferrous ion concentration on the encapsulation efficiency of liposomes prepared by the REV method is shown in Fig. 4. Increased ferrous ion concentration correlated with a decreased amount of encapsulated ferrous ion when de-ionized water was used as the hydrating medium. This is related with core material
properties because ferrous sulfate is a type of strong electrolyte and has higher ionic strength that may influence electric charge properties of liposomes. Also the entrapped water phase volume may be reduced.

3.3.2. Effect of different hydrating media on EE and \( \zeta \)-potential of liposomes

The relationship between the encapsulation efficiency of ferrous sulfate liposomes prepared by the REV method and different hydrating media is shown in Fig. 5. Different hydrating media had great effects on the encapsulation efficiency of liposomes. EE could be increased evidently by using citric acid–\( \text{Na}_2\text{HPO}_4 \) buffer solution or citric acid–sodium citrate buffer solution as the hydrating medium, because citrate can chelate the ferrous ions.

The addition of a chelating agent did not only increase the encapsulation efficiency of iron in liposomes, but also improved their electrostatic stability. The effect of different hydrating media on the \( \zeta \)-potential of liposomes is shown in Table 1. When de-ionized water was used as the hydrating medium, core material Fe\( ^{2+} \) produced a reversion of the negative \( \zeta \)-potential of PC liposomes due to an electrostatic interaction between the ferrous ion and the liposomes. However, when citric acid–\( \text{Na}_2\text{HPO}_4 \) buffer solution was used as the hydrating medium, the \( \zeta \)-potential of either empty liposomes or ferrous sulfate liposomes were higher. This phenomenon can be explained by using Gouy-Chapman diffusion double layer theory. Polyvalent anions (\( \text{PO}_4^{3-} \) or \( \text{HPO}_4^{2-} \)) penetrate through the stern layer and diffuse layer of double layer, and these polyvalent counter ions may lead to \( \zeta \)-potential values of liposomes with or without ferrous ion that are larger those that of liposomes prepared using water as hydrating medium or of opposite sign. Therefore the \( \zeta \)-potential of liposomes increased and their electrostatic stability was improved.

3.3.3. Effect of sonication strength on EE of ferrous sulfate liposomes

Ultrasoundication with a probe sonicator in an ice bath for 5 min resulted in a homogeneous w/o emulsion. The water droplets in the emulsion were covered by a lipid monolayer while the rest of the system contained the organic solvent. The effect of different sonication strengths on the encapsulation efficiency is shown in Fig. 6. The results demonstrated that the encapsulation efficiency...
decreased with increasing sonication strength and the optimal sonication strength was 70%. This phenomenon could be explained by the higher sonication strength destroying the emulsion.

3.4. Stability of fluid milk fortified with ferrous sulfate liposomes

After the removal of free ferrous ions via macroporous cation exchange column, ferrous sulfate liposomes were added to fluid whole milk and their stability was determined by visual observation of the milk emulsion under the conditions of the technological processes of elaboration and manufacturing. These procedures, which are those to which milk is usually submitted, are the thermic and long-term storage processes. In order to evaluate the thermic effect on milk samples fortified with ferrous sulfate liposomes, milk with ferrous sulfate liposomes added was heated at 100 °C for 30 min. Heat treatments of iron-fortified fluid milk induced no visible heat instability: no coagulation or aggregation was observed. During 1-week storage in which the milk samples were held at 4 °C, fluid whole milk samples were very stable without any signs of precipitation and coagulation, and a sensory evaluation proved that fluid milk fortified with ferrous sulfate liposomes did not differ greatly from control milk in color and off-flavor. The results showed that the ferrous sulfate liposomes made in our lab can be applied in liquid milk. But more research work on ferrous sulfate liposomes in application of liquid milk in pilot plant scale will need to be done in the next study.

4. Conclusion

In general, the electrostatic and steric stability of empty liposomes can be improved by the addition of certain amounts of cholesterol and Tween 80. And the reverse phase evaporation method is effective for microencapsulating ferrous sulfate to get ferrous sulfate liposomes with a high EE of 67%. Furthermore the concentration of ferrous sulfate and the hydrating medium, as well as the sonication strength, have a significant effect on the amount of encapsulated iron. Preliminary results suggest that ferrous sulfate liposomes may provide a powerful iron fortifier and can improve ferrous ion stability in food vehicles.

References


Fig. 6. Relationship between the encapsulation efficiency and the sonication strength.


