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Preparation and pH Stability of Ferrous Glycinate Liposomes

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Ferrous glycinate liposomes were prepared by reverse phase evaporation method. The effects of cholesterol, Tween 80, ferrous glycinate concentration, hydrating medium, pH of hydrating medium, and sonication strength on the encapsulation efficiency of liposomes were investigated. Encapsulation efficiency was significantly influenced by the different technique parameters. Ferrous glycinate liposomes might be obtained with high encapsulation efficiency of 84.80% under the conditions of optimized technique parameters. The zeta potential and average particle size of liposomes in the hydrating medium of pH 7.0 were 9.6 mV and 559.2 nm, respectively. The release property of ferrous glycinate liposomes in vitro was investigated in simulated gastrointestinal juice. A small amount of ferrous glycinate was released from liposomes in the first 4 h in simulated gastrointestinal juice. The mean diameters of liposomes increased from 559.2 to 692.9, 677.8, and 599.3 nm after incubation in simulated gastrointestinal juice of pH 1.3, 7.5, and 7.5 in the presence of bile salts, respectively. Results showed that the stability of ferrous glycinate in strong acid environment was greatly improved by encapsulation in liposomes, which protected ferrous glycinate from disrupting the extracapsular environment by lipid bilayer. The bioavailability of ferrous glycinate, as the iron source for biological activity including hemoglobin formation, may be increased. The ferrous glycinate liposomes may be a kind of promising iron fortifier.

KEYWORDS: Ferrous glycinate; liposomes; encapsulation efficiency; stability; gastrointestinal juice

INTRODUCTION

Iron deficiency is considered to be one of the most common worldwide nutritional deficiencies and affects people of all ages in both developing and developed countries (*1*). 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 (*2*). Although technical challenges limit the amount of bioavailable iron compounds that can be used in food fortification, studies show that iron fortification can be an effective strategy against nutritional iron deficiency (*3*−*5*).

Compared with the most commonly used iron salts, ferrous glycinate is more bioavailable, more stable, and safer (*6*−*8*). In acute doses, ferrous glycinate was less toxic than ferrous sulfate, one of the most popular iron supplements. In previous work, bioavailability of ferrous glycinate was improved and gastric irritability was reduced (*8*). Ferrous glycinate had proven to be compatible as a dietary fortificant with foods that were high in fiber and to be protected from inhibition by dietary phytate (*9*). However, the glycine complex was not so stable within the gastrointestinal tract because of the low pH, and it was fully or partially dissociated from the glycine complex (*10, 11*). It is therefore proposed that ferrous glycinate may be microencapsulated, like another iron salt (*12*), to prevent it from being dissociated by protecting the core ingredients from the environment of the gastrointestinal tract during the absorption process.

Liposome technology is a special kind of microencapsulation technique. Liposomes, microscopic lipid vesicles, usually formed from phospholipids, have been extensively investigated, developed, and used in many fields, such as drug, cosmetic, nutrition, and food, as the delivery system (*13*−*16*). One important aspect of this application is the protection afforded by encapsulation against potentially damaging conditions in the extracapsular environment. Liposomes are particularly well suited for use in the food industry as delivery systems because they are well characterized, easily made, highly versatile in their carrier properties, highly biocompatible, and "generally recognized as safe" materials (*17*). Another important application of liposomes is site-specific targeting. Passive targeting exploits the natural tendency of some cells (i.e., Kupffer cell in the liver and the macrophages of the reticule endothelial system) to phagocytize foreign microparticles, such as liposomes. The effect of this phagocytosis is the passive incorporation of the entrapped drug into a desirable organ such as the liver, spleen, or kidney (*18*). The common iron salt ferrous sulfate has been microencapsulated with liposomes (*12, 19, 20*). Because iron compounds were encapsulated in the interior phase of liposomes, the iron was kept from contacting the other components of food by the

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phospholipid bilayer membrane, so the interaction between iron compounds in liposomes and the other components of food was restricted. The iron compounds in liposomes have lower prooxidant properties, and the foods supplemented with iron compound liposomes have longer shelf life and better sensorial properties than foods directly supplemented with iron compounds (*12*). Furthermore, the bioavailability of core material in liposomes may be improved (*15*, *21*, *22*).

The synthesis of ferrous glycinate has been investigated in our previous study (CN Patent ZL200410065260.3). The ferrous glycinate produced was soluble and stable in aqueous solution, even in the boiled aqueous solution. However, the application was restricted by the acid sensitivity. The main objective of the present study was to prepare ferrous glycinate liposomes with high encapsulation efficiency to provide physicochemical stability in the simulated gastrointestinal tract. During the preparation of liposomes, the influence of several factors on their encapsulation efficiency was examined; the zeta potential and size distribution of ferrous glycinate liposomes were determined. In addition, the controlled release of ferrous glycinate liposomes in the simulated gastrointestinal juice was investigated to evaluate the effect of liposomes.

MATERIALS AND METHODS

Materials. Egg phosphatidylcholine (EPC) or lecithin was purchased from the Chemical Reagent Plant of East China Normal University (Shanghai, China). Sodium citrate, citric acid, ascorbic acid, diethyl ether, cholesterol, Tween 80, and pig bile salts were obtained from Shanghai Chemical Reagent Co. (Shanghai, China). All chemicals were of reagent grade and used without further purification. Ferrous glycinate was synthesized according to the method given in CN Patent ZL200410065260.3.

Preparation of Ferrous Glycinate Liposomes. The ferrous glycinate liposomes were prepared by reverse-phase evaporation method (REV) (*23*). The mixture, containing EPC (200 mg) and various amounts of cholesterol (from 0 to 100 mg), was dissolved in an organic solvent (diethyl ether, 10 mL). Aqueous solutions (3 mL) with various quantities of ferrous glycinate and buffer solution of citric acid and Na_2HPO_4 were 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 with a sequence of 1 s of sonication and 1 s rest resulted in a homogeneous w/o emulsion. After the removal of 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 aqueous phase containing various amounts of Tween 80 surfactants (from 50 to 200 mg) was added with gentle vortexing, and the final volume was kept at 10 mL. The remaining ether was evacuated by nitrogen gas. The liposomes were incubated at the room temperature for 12 h and then stored at 4 °C in a refrigerator.

Measurement of Ferrous Glycinate Concentration. Because all iron occurred in chelated form in ferrous glycinate, the concentration of ferrous glycinate was determined by measuring the corresponding iron concentration in the solution. The ferrous glycinate was dissolved in a 37% hydrochloric acid solution. Then the bathophenanthrolin method (*24*) was used to measure the iron concentration in this study. One milliliter of 10% hydrochloric acid, 2 mL of 10% hydroxylamine hydrochloride, and 5 mL of 10% sodium acetate buffer were added to 25-mL flasks in turn. One milliliter of 0.12% bathophenanthrolin (4,7-diphenyl-1,10-phenanthrolin) aqueous solution was added to each flask. The total volume for each flask was then increased to 25 mL with deionized water. Reagent blanks were analyzed simultaneously with the same procedure. The absorbance of each sample was measured in a 1-cm glass cuvette, at 510 nm, using a model 722 spectrophotometer (Exact Science Apparatus Ltd., Shanghai, China).

Analysis of Encapsulation Efficiency. Liposome samples (1 mL) were placed in a dialytic membrane bag (molecular weight cutoff 5000, Shanghai Huamei Biochemistry Ltd., China) and dialyzed against 100 mL of deionized water for 24 h under churning by a magnetic force stirrer to remove the nonencapsulated ferrous glycinate remaining in the dispersion medium. The liposome samples were disrupted by ashing for 5 h at 550 °C to remove organic compounds (e.g., EPC, cholesterol, Tween 80, glycine) and to release elemental iron thoroughly, so that the amount of iron in liposomes can be determined. The ash was dissolved with 37% hydrochloric acid and then diluted in a volumetric flask with deionized water. The concentrations of iron in the dispersion medium and in the ash solution were determined according to the method of bathophenanthrolin colorimetry. The amount of ferrous glycinate in the dispersion medium and in liposomes was calculated on the basis of the absorbency. The encapsulation efficiency (EE) was calculated using the following equation:

$$\mathrm{EE}\ (\%) = \frac{\text{total ferrous glycinate in liposome (mg)} - \text{unencapsulated ferrous glycinate (mg)}}{\text{total ferrous glycinate in liposome (mg)}} \times 100$$

Measurement of Liposomal Zeta Potential. The zeta potential of liposome samples was determined according to laser Doppler electrophoretic mobility theory using the Zetasizer 2000 (Malvern Instruments Ltd., U.K.) at 25 °C. The concentration of liposome was diluted to 0.1% by citric acid−Na_2HPO_4 buffer solution. Each sample was measured and repeated three times.

Determination of Liposomal Sizes. The sizes of liposomes were determined by dynamic laser light scattering technology using a size measurer (Nano-ZS90, Malvern Instruments Ltd.) at 25 °C. The concentration of liposome was diluted to 0.1% by citric acid−Na_2HPO_4 buffer solution. Each sample was measured and repeated three times.

pH Stability of Ferrous Glycinate. The ferrous glycinate (0.05 g) was dissolved in water solution (5 mL) with different pH values (pH 1.3, 2.5, 3.5, 4.5, and 5.5, respectively; adjusted with 2 M HCl) for 30 min at 25 °C. Ethanol (50 mL) as a kind of precipitator for ferrous glycinate was added into the solution to separate ferrous glycinate from its bulk phase. The deposit was separated by centrifugation (TG16-WS centrifuge, Changsha Xiangyi Centrifuge Instruments Ltd., China), and then the content of ferrous glycinate of the deposit was determined. The retention ratio was calculated using the following equation:

$$\text{retention ratio}\ (\%) = \frac{\text{amount of ferrous glycinate in the deposit (g)}}{\text{total amount of dissolved ferrous glycinate (g)}} \times 100$$

In Vitro Release of Ferrous Glycinate from Liposomes. Because ferrous glycinate was not so stable in the gastrointestinal tract and it was fully or partially dissociated (*10*, *11*), the controlled release was examined in simulated gastric juice pH of 1.3 and intestinal juice pH of 7.5 with and without bile salts (10 mM). The solution of pH 1.3 consisted of NaCl, HCl, and deionized water, whereas the solution of pH 7.5 was made up of KH_2PO_4, NaOH, and deionized water.

The in vitro release of ferrous glycinate from liposomes was measured by a dialysis method using a dialytic membrane bag (molecular weight cutoff = 5000, Shanghai Huamei Biochemistry Ltd., China). This membrane assures the permeation of nonencapsulated ferrous glycinate.

One milliliter of ferrous glycinate liposomal dispersion was transferred to a dialytic membrane bag (donor compartment). It was placed in a glass cylinder containing 100 mL of simulated gastrointestinal juice (receiver compartment). The whole setup was placed on a magnetic stirrer adjusted to a constant speed of 150 rpm at 37 °C. Aliquots of 15 mL were sampled from the receiver compartment at 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 8.0, 10.0, and 20.0 h followed by immediate replenishment of the same volume of simulated gastrointestinal juice. The samples were determined by the method of bathophenanthrolin colorimetry. The release of ferrous glycinate from the inner aqueous phase of liposomes was evaluated by cumulative release ratio. The cumulative release ratio was calculated using the equation

$$\mathrm{CRR}_t\,(\%) = \frac{\sum_{i=1}^{i=t-1} c_i V_i + c_t V_t}{\text{encapsulated ferrous glycinate (mg)}} \times 100$$

where CRRt_t is the cumulation release ratio of ferrous glycinate in liposomes at time t. c_i and c_t are the concentrations of ferrous glycinate in the release medium at times i and t, respectively. V_i is the withdrawing volume (15 mL) from release medium at time i. V_t is the release medium volume (100 mL) at time t.

RESULTS AND DISCUSSION

Effect of Cholesterol Content on the Encapsulation Efficiency of Ferrous Glycinate Liposomes. Cholesterol is beneficial in increasing the stability of liposomes in an environment having low pH (*15*). Studies of ferrous glycinate liposomes incorporated with different ratios of cholesterol indicated the effect of its variables on the degree of encapsulation efficiency. The effect of cholesterol content on encapsulation efficiency of liposomes is shown in **Figure 1**. The encapsulaltion efficiency of cholesterol-free liposomes was lower than that of cholesterol-containing liposomes. This may be attributed to the saturation of lipid domains. However, the encapsulation efficiency could be enhanced up to 66.07% by increasing the cholesterol content. The increase of the encapsulation efficiency was attributed to the ability of cholesterol to cement the leaking space in the bilayer membranes, which in turn allowed enhanced core material levels inside the liposomes. With a further increase in this level of cholesterol the encapsulation efficiency decreased. Agarwal et al. (*25*) reported that decreasing entrapment efficiency with increasing cholesterol ratio above a certain limit for dithranol liposomes may be due to the fact that increasing the cholesterol level beyond a certain level can disrupt the regular bilayer structure, leading to the loss of core material entrapment levels.

The amount of encapsulated substance in liposomes was dependent on the capacity of bilayers to accommodate it and on its concentration in the inner aqueous phase of the vesicles (*26*). For aqueous soluble compounds, improvement of the inner volume of the vesicle can increase encapsulation efficiency accordingly (*27*). At the first stage, cholesterol can be incorporated into incompact bilayer structure of liposomes, resulting in the increase of liposomes' size. Inner aqueous phase volume was expanded with increasing liposomal size, so that the encapsulation efficiency of ferrous glycinate liposomes was enhanced. When the ratio between cholesterol and EPC was excessive, liposomal membrane rigidity decreased, making it more fragile and allowing ferrous glycinate to leak more easily from liposomes, so that the encapsulation efficiency of ferrous glycinate decreased. It was concluded that appropriate proportions of cholesterol to EPC in the liposomes were important for increasing the encapsulation efficiency.

Effect of Tween 80 Content on Encapsulation Efficiency of Ferrous Glycinate Liposomes. As a kind of nonionic surfactant, Tween 80 was added in the lipid system, and the liposome bilayer was covered with polyoxyethylene chains from Tween 80 and a hydrophilic layer was formed on the surface of liposome microvesicles (*28*). The relationship between Tween 80 level and encapsulation efficiency of ferrous glycinate liposomes is shown in **Figure 2**. An increase in the Tween 80 concentration led to an increase in the entrapment of ferrous glycinate from 36.69 to 63.04%. However, the addition of excess Tween 80 resulted in significant liposome destruction (*29*), so that the encapsulated ferrous glycinate was leaked from liposomes and the encapsulation efficiency decreased. This case may indicate that the membrane rigidity can be improved when the concentration of Tween 80 was in an appropriate range.

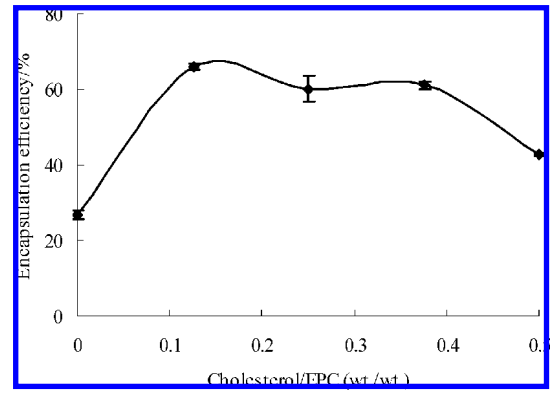

Figure 1. Relationship between encapsulation efficiency and weight ratio of cholesterol to EPC. Data reported are the mean values ± standard variation of three replications. EPC/Tween 80/ferrous glycinate, 10:10:1; sonication strength, 400 W; hydrating medium pH, 6.8.

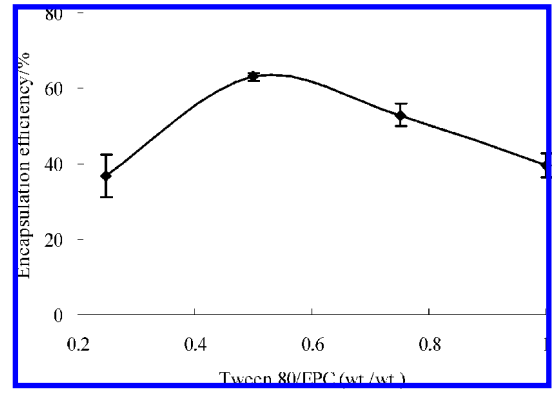

Figure 2. Relationship between encapsulation efficiency and weight ratio of Tween 80 to EPC. Data reported are the mean values ± standard variation of three replications. EPC/cholesterol/ferrous glycinate, 10:2.5:1; sonication strength, 400 W; hydrating medium pH, 6.8.

Sterically stabilized liposomes, that is, Stealth liposomes, have recently been improved by using glycolipids or polymers such as polyethylene glycol and polyacrylamide. Because hydrophilic macromolecules were directly linked to liposomes or through a space arm, the steric stability of liposomes was strengthened and the interaction among microvesicles was weakened (*30*). Kronberg et al. proved that sterically stabilized liposomes with Tween 80 surfactants incorporated into the lipid bilayer in serum increased the stability of systems compared with the corresponding liposomes without Tween 80 (*31*).

Effect of Ferrous Glycinate Load Capacity on Encapsulation Efficiency of Ferrous Glycinate Liposomes. Changes in encapsulation efficiency with the increase of ferrous glycinate load capacity are shown in **Figure 3**. The encapsulation efficiency of liposomes increased from 59.75 to 84.80% with increasing weight ratio of ferrous glycinate to EPC from 0.1 to 0.3. The increase of entrapment was observed up to the weight ratio of ferrous glycinate to EPC of 0.3. For the water-soluble compounds, encapsulation efficiency depended on the inner volume of vesicle, whereas the encapsulation capacity of a certain amount of liposomes was finite (*27*). Further increase in the weight ratio of ferrous glycinate to EPC did not produce any enhancement in ferrous glycinate entrapment; rather, it indicated saturation, which led to the decrease of encapsulation efficiency when the ferrous glycinate concentration was too high.

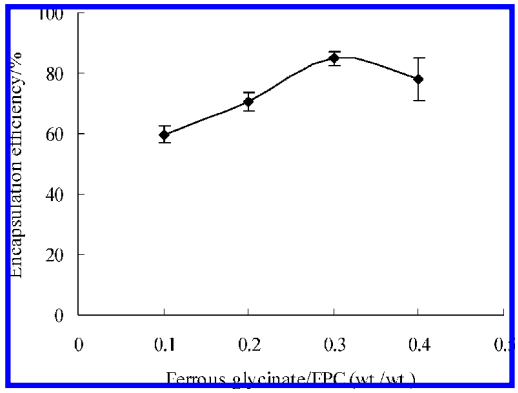

Figure 3. Relationship between encapsulation efficiency and weight ratio of ferrous glycinate to EPC. Data reported are the mean values ± standard variation of three replications. EPC/cholesterol/Tween 80, 10:2.5:10; sonication strength, 400 W; hydrating medium pH, 6.8.

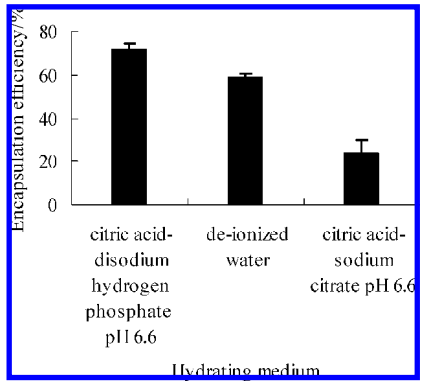

Figure 4. Effect of different hydrating media on encapsulation efficiency. Data reported are the mean values ± standard variation of three replications. EPC/cholesterol/Tween 80/ferrous glycinate, 10:2.5:10:2; sonication strength, 400 W.

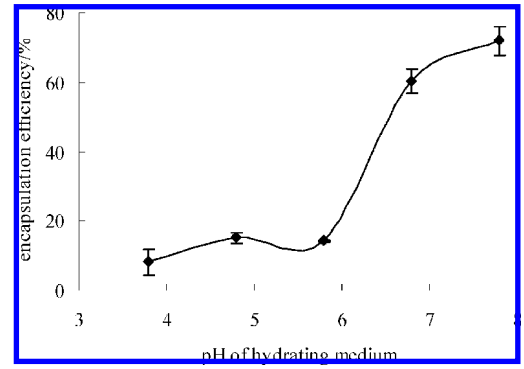

Figure 5. Effect of hydrating media with different pH values on encapsulation efficiency. Data reported are the mean values ± standard variation of three replications. EPC/cholesterol/Tween 80/ferrous glycinate, 10:2.5:5:1; sonication strength, 400 W.

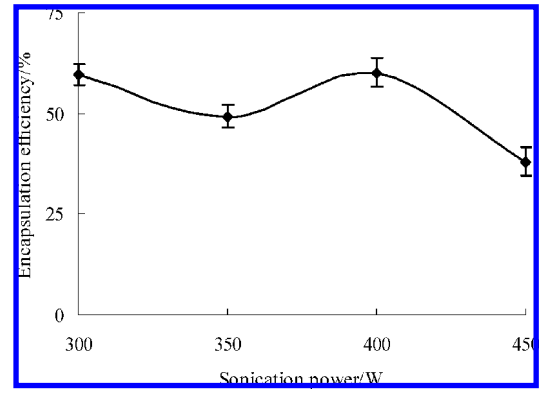

Figure 6. Effect of sonication power on encapsulation efficiency. Data reported are the mean values ± standard variation of three replications. EPC/cholesterol/Tween 80/ferrous glycinate, 10:2.5:5:1; hydrating medium pH, 6.8.

The optimal weight ratio of ferrous glycinate to EPC for the maximal entrapment efficiency, under the defined conditions, was 0.3 (w/w).

Effect of Different Hydrating Media on Encapsulation Efficiency of Ferrous Glycinate Liposomes. The encapsulation efficiency of ferrous glycinate liposomes in different hydrating media is presented in **Figure 4**. Different hydrating media had significant effects on the encapsulation efficiency of ferrous glycinate. According to the encapsulation efficiency, citric acid−Na_2HPO_4 buffer solution of pH 6.6 with citric acid (0.0227 mol/L) and Na_2HPO_4 (0.1545 mol/L) was found to be the optimal hydrating medium. However, the encapsulation efficiency in the hydrating medium of citric acid−sodium citrate buffer solution was the lowest. This may be due to the fact that the stability of ferrous glycinate was improved by an appropriate level of citric acid, whereas it may be damaged by excess levels of citric acid. Ferrous glycinate was dissociated and the total concentration reduced, which caused the encapsulation efficiency to be decreased. Furthermore, monovalent cations were found to fluidize the bilayer (*32*). The encapsulation efficiency may be influenced by the fluidization of the bilayer. However, the precise nature of the interaction between ions and phospholipids was still open to debate (*32*).

Effect of Buffer Solution pH on Encapsulation Efficiency of Ferrous Glycinate Liposomes. The encapsulation efficiency of ferrous glycinate liposomes in solution with different pH values is shown in **Figure 5**. The results demonstrated that the encapsulation efficiency was significantly decreased with decreasing pH of buffer solution from 7.8 to 3.8. The decrease of encapsulation efficiency may be due to the dissociation of ferrous glycinate and hydrolyzation of EPC. The actual concentration of core material in the liposomal system decreased with dissociating ferrous glycinate, leading to the decrease of ferrous glycinate level in the inner aqueous phase. The fixed amount of lipid produced the constant amount of liposomes in the system (*27*). The amount of lipid decreased with the hydrolyzation of EPC, and fewer microvesicles were formed in the system, so that less ferrous glycinate was encapsulated into the inner volume of microvesicles.

Effect of Sonication Strength on Encapsulation Efficiency of Ferrous Glycinate Liposomes. The encapsulation efficiency of ferrous glycinate liposomes with different sonication strengths is shown in **Figure 6**. The encapsulation efficiency was decreased with increasing ultrasonic power from 300 to 350 W and from 400 to 450 W. The w/o emulsion can be formed under a certain sonication strength, and the encapsulation efficiency can be increased (*33*). With the increase of sonication strength, the decrease of encapsulation efficiency may be attributed to the high input of energy so that the liposomes from multilamellar vesicles turned to unilamellar vesicles and some amount of ferrous glycinate was leaked from microvesicles. Also, the dissipation of energy into the system resulted in overheating, so that EPC may be de-esterified and liposomes were not stable and fused (*23*).

Zeta Potential and Size of Ferrous Glycinate Liposomes. The zeta potential was an important and useful indicator of particle surface charge, which can be used to predict the storage stability of emulsions and colloidal suspensions. The zeta

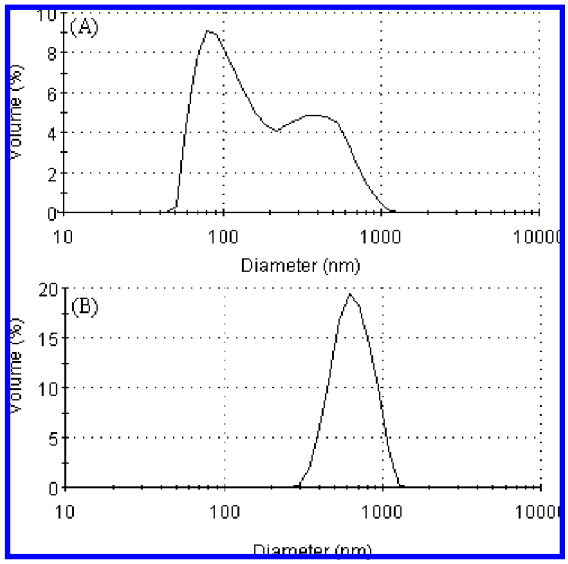

Figure 7. Size distribution of (**A**) empty liposomes and (**B**) ferrous glycinate-containing liposomes. EPC/cholesterol/Tween 80/ferrous glycinate, 10:2.5:10:1; sonication strength, 400 W; hydrating medium pH, 6.8.

potential of the empty liposomes was +9.1 mV. The mean zeta potentials of ferrous glycinate liposome were +9.2 and +9.6 mV in the hydrating media of pH 6.0 and 7.0, respectively. The results showed that ferrous glycinate liposomes had positive charges, indicating that electrostatic repulsive force occurred between the particles. However, there was no obvious difference between the empty liposomes and ferrous glycinate liposomes and between ferrous glycinate liposomes with different hydrating media. The zeta potential of ferrous glycinate liposomes could not be affected by the interaction between ferrous glycinate in the inner aqueous phase and the liposomal bilayer.

The measurement of size was generally used as a characterization tool to evaluate the stability of liposomes. Polydispersity is usually expressed as an index of particle diameter distribution in colloidal systems. The smaller the value of polydispersity index (PDI), the more likely the particle diameter distribution is narrower, and thus particles show better uniformity in diameter (*27*).

The effect of ferrous glycinate on the size distribution of liposomes is shown in **Figure 7**. It seemed likely that entrapment of ferrous glycinate resulted in an apparent increase in liposome size. For liposome-containing ferrous glycinate, the mean diameter was 559.2 nm and the PDI was 0.313, compared with the mean diameter of 207.2 nm and the PDI of 0.240 for empty vesicles. The increase of size distribution for liposome-containing ferrous glycinate may be attributed to the neutralization between the zeta potential of ferrous glycinate (−15.2 mV) and the zeta potential of empty liposomes (+9.1 mV) and led to microvesicle aggregation. Fan et al. (*27*) investigated the effect of the water-soluble core material loading capacity on the mean diameter of liposomes and also found that the average diameters of liposomes containing salidroside greatly increased.

pH Stability of Ferrous Glycinate and in Vitro Release of Ferrous Glycinate from Liposomes in Simulated Gastrointestinal Juice Conditions. The pH dependence of ferrous glycinate is shown in **Figure 8**. Ferrous chloride can be dissolved in ethanol, and ferrous glycinate is insoluble, so they can be separated from each other. The retention ratio of ferrous glycinate was dramatically reduced with the change of solution pH from 5.5 to 1.3. It was found that around 95% of ferrous

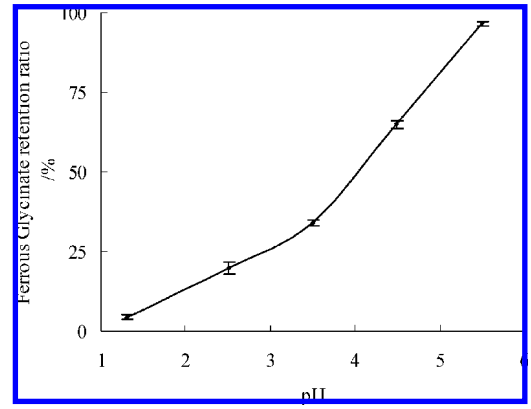

Figure 8. Ferrous glycinate retention ratio in solutions with different pH values. Ferrous glycinate (0.05 g) was dissolved in water solution (5 mL) with different pH values (adjusted with HCl) and incubated at room temperature for 30 min. Data reported are the mean values ± standard variation of three replications.

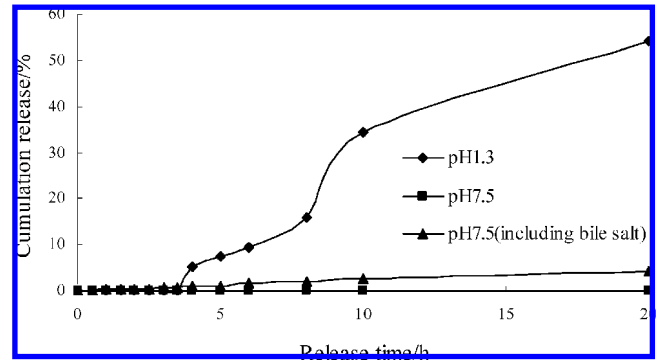

Figure 9. Cumulative release of ferrous glycinate from liposomes in media of pH 1.3, 7.5, and 7.5 in the presence of bile salts. EPC/cholesterol/Tween 80/ferrous glycinate, 10:2.5:10:1; sonication strength, 400 W; hydrating medium pH, 6.8.

glycinate was dissociated after incubation in the simulated gastric juice of pH 1.3 for 30 min. This may be due to the increase of H^+ concentration, which results in significant ferrous glycinate destruction as for the combination of H^+ and $H_2NCH_2COO^-$. However, ferrous glycinate was stable in the simulated intestinal juice of pH 5.5 and <5% was dissociated.

The cumulative release of ferrous glycinate from liposomes in the three media of pH 1.3, 7.5, and 7.5 in the presence of bile salts is shown in **Figure 9**. Almost no ferrous glycinate was released from liposomes in the medium of pH 7.5. The release of ferrous glycinate from liposomes was slightly affected by the bile salts in pH 7.5. Bile salts are a special class of surfactants that seem to break down the bilayer membranes. According to Walde et al. (*34*), the general mechanism of bile salt insertion in the membrane is a two-step process. First, the molecules of bile salts are located only in the external leaflet of the vesicle bilayer and do not modify its permeability. Second, bile salts molecules flip to the internal monolayer as the bile salt concentration increases. This second step might be accompanied by the formation of a transient pore followed by the redistribution of the bile salt within the membranes, finally leading to destabilization of the vesicles (*35*, *36*). This increased the release of ferrous glycinate liposomes. Furthermore, a small amount of ferrous glycinate was also released from liposomes in the first 4 h in the medium of pH 1.3. After 4 h, the ferrous glycinate was dramatically released from liposomes. The leakage of ferrous glycinate liposomes may be attributed to the low pH. The instability of the liposomes would be related to the

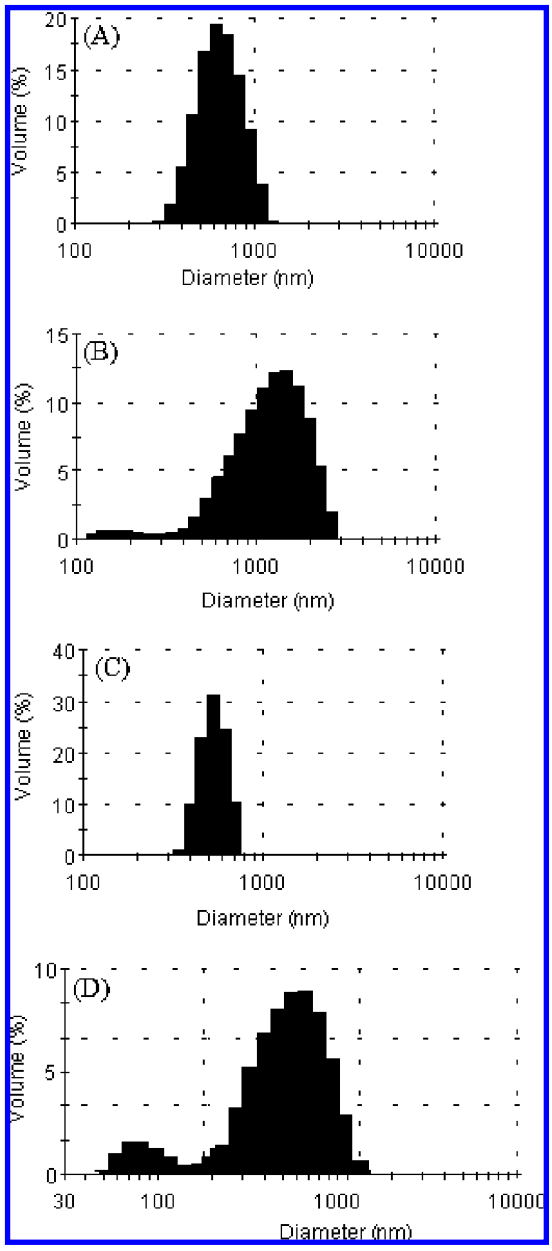

Figure 10. Size distribution of (**A**) ferrous glycinate-loaded liposomes, (**B**) ferrous glycinate-loaded liposomes after incubation in a medium of pH 1.3, (**C**) ferrous glycinate-loaded liposomes after incubation in a medium of pH 7.5, and (**D**) ferrous glycinate-loaded liposomes after incubation in a medium of pH 7.5 in the presence of bile salts. EPC/cholesterol/Tween 80/ferrous glycinate, 10:2.5:10:1; sonication strength, 400 W; hydrating medium pH, 6.8; incubation time, 20 h.

permeation of protons (*37*). Because the permeability properties of bilayer membranes increased, ferrous glycinate more easily permeated the bilayer membranes. However, because food usually remains in the stomach for more or less 4−5 h, ferrous glycinate from the liposomal inner aqueous phase can be effectively protected in the simulated gastric juice.

The size distribution of liposomes after incubation of different media is shown in **Figure 10**. For ferrous glycinate liposomes after release in the medium of pH 1.3, the mean diameter was 692.9 nm and the PDI was 0.406; for ferrous glycinate liposomes after release in the medium of pH 7.5, the mean diameter was 677.8 nm and the PDI was 0.316 nm; for ferrous glycinate liposomes after release in the medium of pH 7.5 in the presence of bile salts, the mean diameter was 599.3 nm and the PDI was 0.410, compared with the mean diameter was 559.2 nm and PDI value 0.313 for liposome-containing ferrous glycinate. Therefore, the observed leakage at low pH among these liposomes entrapping ferrous glycinate might primarily be due to membrane destabilization. The liposome particle aggregated and fused, so that the diameter was increased after incubation in simulated gastrointestinal juice. The mean diameter of ferrous glycinate liposomes after release in the medium of pH 7.5 in the presence of bile salts was less than that of ferrous glycinate liposomes after release in the medium of pH 7.5 without bile salts. The decrease of mean diameter may be due to nature's own nanoparticle delivery system of bile salt micelles and vesicles (*38*). Bile salt micelles and vesicles can solubilize phospholipid bilayer membranes. The difference of PDI between ferrous glycinate liposomes after release in the medium of pH 7.5 in the presence of bile salts and ferrous glycinate liposomes after release in the medium of pH 7.5 without bile salts may be due to the same reason. Even though the mean diameter of liposome after incubation was enhanced more than 100 nm, the PDI indicated that the change in liposome size was smaller after incubation in simulated intestinal juice than after incubation in simulated gastric juice.

In conclusion, liposomes containing ferrous glycinate in the inner aqueous phase were prepared successfully by the method of reverse phase evaporation with high encapsulation efficiency. Leakage characteristics of ferrous glycinate liposomes in different conditions showed that the stability of ferrous glycinate in liposomal delivery system was increased in simulated gastrointestinal juice of pH 1.3, 7.5, and 7.5 in the presence of bile salts, especially in a strong acid environment. According to the above results, the bioavailability of ferrous glycinate may be improved. It is well-known and documented that the biodistribution of liposomes was significantly affected by liposome size, surface properties, and stability. Therefore, the possibility of targeting the encapsulated ferrous glycinate to specific tissues will be highly influenced. Further work is needed to verify in vivo and in human bioavailability of ferrous glycinate liposomes for oral administration.

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