Liposomes containing glycocholate as potential oral insulin delivery systems: preparation, in vitro characterization, and improved protection against enzymatic degradation

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Background: Oral delivery of insulin is challenging and must overcome the barriers of gastric and enzymatic degradation as well as low permeation across the intestinal epithelium. The present study aimed to develop a liposomal delivery system containing glycocholate as an enzyme inhibitor and permeation enhancer for oral insulin delivery.

Methods: Liposomes containing sodium glycocholate were prepared by a reversed-phase evaporation method followed by homogenization. The particle size and entrapment efficiency of recombinant human insulin (rhINS)-loaded sodium glycocholate liposomes can be easily adjusted by tuning the homogenization parameters, phospholipid:sodium glycocholate ratio, insulin:phospholipid ratio, water:ether volume ratio, interior water phase pH, and the hydration buffer pH.

Results: The optimal formulation showed an insulin entrapment efficiency of 30% ± 2% and a particle size of 154 ± 18 nm. A conformational study by circular dichroism spectroscopy and a bioactivity study confirmed the preserved integrity of rhINS against preparative stress. Transmission electron micrographs revealed a nearly spherical and deformed structure with discernable lamella for sodium glycocholate liposomes. Sodium glycocholate liposomes showed better protection of insulin against enzymatic degradation by pepsin, trypsin, and α-chymotrypsin than liposomes containing the bile salt counterparts of sodium taurocholate and sodium deoxycholate.

Conclusion: Sodium glycocholate liposomes showed promising in vitro characteristics and have the potential to be able to deliver insulin orally.

Keywords: liposomes, glycocholate, insulin, enzymatic degradation, oral

Introduction
Nonparenteral delivery is by far the most convenient route for drug delivery, especially when repeated or routine administration is necessary. However, the oral route, as a whole, presents a major barrier to macromolecular drugs, the oral bioavailability of which is invariably well below the therapeutic level. This limitation is especially pronounced for proteins and peptides because of their gastric instability, susceptibility to enzymatic degradation in the gastrointestinal tract, and low permeability across the intestinal epithelium. In order to improve the oral bioavailability of peptides, researchers have employed a variety of strategies, including enhancing permeation by absorption enhancers and cell-penetrating peptides, or by opening the tight junction of the epithelia, protection from gastric and enzymatic destabilization, and...
enhancing lymphatic absorption through uptake by M cells in the Peyer's patches. Multiple mechanisms are usually employed to enhance the oral bioavailability of these drugs, for example, in the design of particulate or colon-specific delivery systems for protein and peptide drugs.

Liposomes, being among the most studied particulate carrier systems, have shown appealing potential in enhancing oral bioavailability of proteins and peptides. However, the efficacy of conventional liposomes, mainly composed of phospholipid and cholesterol, has been compromised due to the instability of liposomes in the gastrointestinal tract and poor permeability across the epithelial membrane. Modification of the composition and properties of liposomes is effective in improving the stability and uptake of protein and peptide drugs by the gastrointestinal tract. Recently, liposomes containing bile salts such as sodium deoxycholate, also called “bilosomes,” have shown promising potential in oral immunization. Oral administration of bilosomes incorporating vaccines such as the influenza subunit vaccine and cholera toxin B subunit was found to create as potent an antibody response as an equivalent dose administered parenterally. Hepatitis B surface antigen-loaded bilosomes produced both a systemic as well as a mucosal antibody response upon oral administration. It is reported that although bile salts act exteriorly to destabilize liposomal membranes, bile salts embedded in the lipid bilayers act to stabilize lipid vesicles against further destabilization by physiological bile salts in the gastrointestinal tract. On the other hand, bile salts presenting in the lipid bilayers of the bilosomes are assumed to increase the fluidity of epithelial membranes and contribute to enhanced permeation of the vesicles. These ideas sparked our interest in the oral delivery of protein and peptide drugs using liposomes containing bile salts.

In this study, we investigated the feasibility of using liposomes containing bile salts to enhance the oral bioavailability of insulin, a model protein drug. More specifically, sodium glycocholate was used as a new bile salt in the liposomal formulation to strengthen the enzyme-inhibiting and permeation-enhancing effect. It is reported that sodium glycocholate has good permeation-enhancing properties, with relatively low toxicity, as well as a protease-inhibiting effect. We hypothesized that liposomes containing sodium glycocholate would provide increased protection of insulin from enzymatic degradation and enhanced permeation across the gastrointestinal epithelium. In this first report, liposomes containing sodium glycocholate were prepared by a reversed-phase evaporation method and characterized in vitro.

Methods and materials

Materials

Recombinant human insulin (rhINS, 27 units/mg) was provided by Novo Nordisk (Copenhagen, Denmark). Sodium glycocholate was purchased from Amresco (Solon, OH). Soybean phosphotidylcholine and cholesterol was supplied by Lipoid (Ludwigshafen, Germany). Sodium taurocholate and sodium deoxycholate were obtained from Sigma (Shanghai, China). Pepsin (Ph Eur) and trypsin (more than 6000 BAEE units/mg) were purchased from Sigma (Shanghai, China). α-chymotrypsin (more than 1500 USP units/mg) were obtained from BD Falcon (New Jersey, USA). A Sephadex G50 column was purchased from Pharmacia (Shanghai, China). Deionized water was prepared by a Milli-Q purification system (Molsheim, France). High-pressure liquid chromatography (HPLC) grade acetonitrile was supplied by Merck (Darmstadt, Germany). All other chemicals were of analytical grade and used as received.

Four-week-old male Kunming mice weighing 18–20 g were purchased from the Animal Center at the Shanghai University of Traditional Chinese Medicine (Shanghai, China) and were raised in rooms controlled at 23 ± 1°C and 55% ± 5% relative humidity with 12-hour light/12-hour dark time cycles. The mice received a standard laboratory chow diet and tap water during acclimatization. All experimental protocols concerning the handling of mice were in accordance with the requirements of the Institutional Animal Care and Use Committee at Fudan University.

Preparation of insulin-loaded liposomes

rhINS-loaded liposomes containing sodium glycocholate were prepared by a reversed-phase evaporation method. Briefly, soybean phosphotidylcholine and sodium glycocholate were dissolved in absolute ether, into which rhINS citric-Na$_2$HPO$_4$ buffer solution (pH 3.0, if not specified otherwise) was added drop by drop. The mixture was sonicated for 5 minutes in a water bath until a w/o emulsion was formed. The emulsion was rota-evaporated under 0.07–0.08 mPa pressure with a rotating speed of 50 rpm at 30°C for 20 minutes to remove the organic solvent. A citric acid-Na$_2$HPO$_4$ buffer (pH 5.6) was then added to hydrate the dry lipids until a homogeneous dispersion was formed. Finally, this dispersion was extruded through a high-pressure homogenizer (AH 100 D; ATS Engineering Inc, Brampton, ON) to obtain the sodium glycocholate liposomes. Before assaying, free rhINS was removed by eluting the liposome dispersion through the Sephadex G50 column, following similar procedures as described below for measurement of entrapment efficiency.
To prepare rhINS-loaded liposomes containing sodium taurocholate or sodium deoxycholate, similar procedures were followed, but substituting sodium glycocholate for sodium taurocholate or sodium deoxycholate, and likewise for conventional liposomes composed of soybean phosphotidylcholine and cholesterol as a control. Blank liposomes were prepared following the same procedures without adding rhINS.

Particle size analysis
Sizes of the sodium glycocholate liposomes were examined using a dynamic light scattering instrument (NICOMP 380 ZLS Particle Sizing Systems; PSS, Inc, Santa Barbara, CA). Liposomes were placed in a disposable cuvette, and photon counts were collected over 5 minutes using an angle of 90° at 25°C. Particle size was analyzed by the ZPW388 software program (PSS, Inc) and expressed with intensity-based Gaussian distribution. The result was expressed as mean ± standard deviation (n = 3).

Entrapment efficiency
Entrapment efficiency of the rhINS in sodium glycocholate liposomes was determined after separating free rhINS from the liposomes using molecular exclusion chromatography with a 30 cm long Sephadex G50 column. Briefly, liposome samples were introduced into the column and subsequently eluted with citric acid-Na2HPO4 buffer (pH 5.6). The eluted fraction containing the entrapped liposomal rhINS was analyzed by HPLC as described below. The entrapment efficiency was defined as the ratio of liposome-associated rhINS (rhINS_{liposomal}) to total rhINS (rhINS_{total}), and calculated as shown in Eq (1):

\[ EE(\%) = \frac{rhINS_{liposomal}}{rhINS_{total}} \times 100\% \] (1)

Determination of insulin
rhINS was assayed by a reversed-phase HPLC/ultraviolet method. The Agilent 1100 series HPLC system (Agilent Technologies, Inc, Santa Clara, CA) was composed of a quaternary pump, a degasser, an autosampler, a column heater, and a tunable ultraviolet detector. rhINS was separated at 25°C using a C18 column (Zorbax®, 5 µm, 4.6 mm × 150 mm, Agilent) with a C18 precolumn (2 mm × 20 mm), and detected by measuring the 220 nm absorbance of the eluate. The mobile phase was a mixture of acetonitrile and 0.57% H3PO4 (adjusted to pH 2.25 with triethylamine) in a volume ratio of 26/74, pumped at a flow rate of 1.0 mL/min.

Conformational stability
The conformational stability of rhINS was studied by circular dichroism spectroscopy. To prepare samples for the circular dichroism test, rhINS was released by disrupting the liposomes using 1% Triton X-100. rhINS was then separated from soybean phosphotidylcholine and sodium glycocholate using an ENVI-18 solid-phase extraction column (Supelco, Bellefonte, PA) to avoid interference. The separation details were as follows. A 1 mL sample of destabilized liposomal mixture was applied onto an ENVI-18 column which had been activated by methanol. Elution was then performed with 1 mL aqueous methanol (40%) to wash out sodium glycocholate and soybean phosphotidylcholine. After that, 60% aqueous methanol was added to elute the rhINS, which was collected and adjusted to a concentration of approximately 20 µg/mL for the circular dichroism test. A rhINS solution of equal concentration, dissolved in 60% aqueous methanol, was used as the control. The conformational characteristics of the insulin released and the control were examined by circular dichroism on a Jasco-715 circular dichroism spectrophotometer (Jasco Co Ltd, Hachioji, Japan). Five scans were performed and averaged for each sample.

Bioactivity
rhINS solution in 0.9% NaCl solution was used as a positive control and 0.9% NaCl solution as a negative control. The control and rhINS solutions retrieved from the different formulations as described earlier were injected subcutaneously into normal mice at a dose of 0.5 IU/kg. Blood samples were collected from the retro-orbital plexus before dosing and 1 hour after dosing, and then centrifuged (without addition of anticoagulants) at 4000 g for 5 minutes. Serum was collected and blood glucose levels were determined using a glucose GOD-PAD kit (Shanghai Rongsheng Biotech Co Ltd, Shanghai, China). Bioactivity was expressed as the reduction ratio of postdosing to predosing blood glucose level.

Transmission electron microscopy
The morphology of the liposomes was studied by negative transmission electron microscopy following a standard procedure. Briefly, a drop of liposome dispersion was placed on 300-mesh and carbon-coated copper grids, and allowed to adsorb. The surplus was removed using blotting paper. A drop of 1% phosphotungstic acid was added, and the liposomes were stained for 30–60 seconds. The stained...
liposomes were allowed to dry in ambient conditions and inspected with transmission electron microscopy (JEM-1230; JEOL, Tokyo, Japan) at an acceleration voltage of 120 kV. The micrographs were recorded at a final magnification of 60,000x.

**Leakage of insulin**
The leakage of rhINS from the sodium glycocholate liposomes was measured by detecting the change in entrapment efficiency change at different time intervals. Briefly, rhINS-loaded liposomes were suspended in pH 2.0, 5.6, and 6.8 citric acid-Na₂HPO₄ buffers. The suspensions were placed in a 37°C water bath and shaken at 60 rpm in a reciprocal shaker (SHZ-C, Pudong Physical Optical Co Ltd, Shanghai, China) over 6 hours. At specific time intervals, the entrapment efficiency of the liposomes was detected using the analytical method described earlier. Sink conditions were maintained through the study.

**Protection of insulin from enzymatic digestion**
The protective effect on rhINS-loaded sodium glycocholate liposomes was studied using a dissolution tester (ZRS-8G; Tianda Technology Co Ltd, Tianjin, China) following procedures similar to those described in the Chinese Pharmacopoeia (2010) for dissolution testing by the small beaker method. Briefly, 0.5 mL of liposome suspension was diluted in 50 mL of digestive media and subjected to enzymatic degradation. The digestive media comprised either simulated gastric fluid (containing 1% pepsin, pH 1.2) or simulated intestinal medium (containing 1% trypsin, pH 6.8), or α-chymotrypsin solution (100 µg/mL, in phosphate buffer, pH 7.8). The temperature was maintained at 37 ± 1.0°C and stirred with a paddle at 100 rpm. At appropriate time intervals, 200 µL of the suspension was withdrawn and diluted with an equal volume of 0.1 M NaOH for simulated gastric fluid, 0.1 M HCl for simulated intestinal medium, and α-chymotrypsin solution to terminate degradation. Samples were subsequently treated with Triton X-100 to release rhINS from the liposomes, prior to HPLC assay.

**Statistical analysis**
The results were expressed as means ± standard deviations. One-way analysis of variance was performed to assess the significance of the differences between the data. Results with *P* < 0.05 were considered to be statistically significant.

**Results**

**Preparation and characterization of rhINS sodium glycocholate liposomes**
Sodium glycocholate liposomes were obtained easily following the preparative procedures similar to our previous report using sodium deoxycholate. Replacing sodium deoxycholate with sodium glycocholate did not lead to significant variation in the preparative process. The primary objective of the formulation study was to optimize the experimental conditions to achieve high rhINS loading and smaller particle size, as well as robust rhINS stability during preparative stress.

Figure 1 shows the effect of the homogenization parameters, including homogenizing pressure and number of cycles, on entrapment efficiency and particle size. Increasing the homogenization pressure from 100 to 500 bar led to a significant decrease in particle size (from 430 nm to 100 nm), and a slight decrease in entrapment efficiency from 18% to 11%.
was enough to achieve high entrapment efficiency because a further increase in hydration time did not lead to a further increase in entrapment efficiency.

Based on the results of the formulation study, the optimal preparative conditions were chosen as follows: 334 mg soybean phosphotidylcholine and 66 mg sodium glycocholate (4:1, mol/mol) were dissolved in 10 mL absolute ether, into which 2 mL rhINS buffered solution (4 mg/mL) was added. The mixture was sonicated and hydrated for 30 minutes to obtain a sodium glycocholate liposome dispersion. Finally, this suspension was homogenized at 300 bar for four cycles. The resulting rhINS-loaded sodium glycocholate liposomes showed an entrapment efficiency of 30.2 ± 2.2%, drug loading of 120.8 ± 2.7 µg/mL, and a particle size of 154 ± 18 nm with a polydispersity index of 0.340 ± 0.024 (Figure 3).

Conformational and bioactive stability

Far-ultraviolet circular dichroism spectroscopy is usually used as a fingerprint for the identification of various secondary structural elements of peptides. Therefore, it was employed as an easy tool to study the conformational stability of the rhINS. Preparative variables, such as homogenization pressure and number of cycles, hydration time, amount of soybean phosphotidylcholine and insulin, and volume of organic solvents volume, at their extreme levels, may have an impact on the conformational stability of rhINS. Figure 4 shows the circular dichroism spectra of rhINS in sodium glycocholate liposomes prepared under extreme conditions, including the maximum amount of sodium glycocholate (soybean phosphotidylcholine:sodium glycocholate ratio of 2:1), maximum amount of rhINS (rhINS:soybean phosphotidylcholine ratio of 0.0025:1), maximum ether volume (water:ether ratio of 1:7), the strongest ionic strength (1.2), and the extreme preparation conditions (hydration for 90 minutes; homogenization at 500 bar for six cycles), as well as a combination of the extreme conditions. The circular dichroism spectrum of rhINS released from the optimal sodium glycocholate liposome formulation was also recorded and compared with rhINS solution (Figure 4, insert).

The circular dichroism spectra for free rhINS in solution show a valley at 208 nm and a shoulder at around 223 nm, which is characteristic of the α-helix conformation and in close agreement with the spectra obtained by other researchers. The ratio of \( \theta_{208}/\theta_{223} \) can be used as a qualitative measure of rhINS association. This is due to the antiparallel β structure which forms when two monomers dimerize. This results in an increase of \( \theta_{223} \) without an
Figure 2 Effects of soybean phosphotidylcholine:sodium glycocholate ratio (A), recombinant human insulin:soybean phosphotidylcholine ratio (B), water:ether volume ratio (C), pH of the inner water phase (D), ionic strength of the hydration buffer (E), and hydration time (F) on recombinant human insulin entrapment efficiency.

Note: Data expressed as means ± standard deviations (n = 3).

Abbreviations: SPC, soybean phosphatidylcholine; SGC, sodium glycocholate; rhINS, recombinant human insulin.

increase of $\theta_{208}$. The $\theta_{208}/\theta_{223}$ ratios of rhINS released from the six sodium glycocholate liposomes were within the range of 1.638–1.852, which was close to that of free rhINS (1.734, $P > 0.05$). Moreover, the helix ratio was 21.6%–24.5%, which was in good agreement with that of natural rhINS (23.3%, $P > 0.05$). Such spectral features indicated that there was only a minor difference between the circular dichroism spectra of the sodium glycocholate liposome samples.
prepared under different preparative conditions and that of the free rhINS solution.

The six sodium glycocholate liposome formulations prepared under extreme stress were disrupted by Triton X-100 to release rhINS, which was then administered to the mice subcutaneously at a dose of 0.5 IU/kg. Blood glucose levels were then determined and are shown in Table 1. No significant difference \( P > 0.05 \) was observed as compared with free rhINS solution, ie, the positive control, where a 53.1 ± 12.6% reduction of the initial blood glucose level was obtained. However, a significant difference \( P < 0.01 \) was observed for both the released rhINS and the positive control as compared with the negative control (blank sodium glycocholate liposomes). Therefore, it was concluded that the

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**Figure 3** Particle size and distribution of recombinant human insulin-loaded sodium glycocholate liposomes prepared under optimal conditions.

**Figure 4** Circular dichroism spectra of free insulin solution and insulin released from sodium glycocholate liposomes prepared under various extreme stress conditions at a recombinant human insulin concentration of 20 \( \mu \)g/mL. The insert indicates circular dichroism spectrum of liposomal recombinant human insulin for the optimal formulation.

**Abbreviations:** SPC, soybean phosphatidylcholine; SGC, sodium glycocholate; rhINS, recombinant human insulin; I.S., ionic strength.
Table 1 Blood glucose level after subcutaneous administration of 0.5 IU/kg recombinant human insulin released from different sodium glycocholate liposomal formulations in mice. Data expressed as means ± standard deviations (n = 6)

<table>
<thead>
<tr>
<th>SG liposome formulation</th>
<th>Blood glucose level</th>
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<tbody>
<tr>
<td></td>
<td>0 hours (mmol/L)</td>
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<tr>
<td>Free rhINS solution</td>
<td>10.70 ± 2.71</td>
</tr>
<tr>
<td>Blank SGC liposomes</td>
<td>9.93 ± 1.59</td>
</tr>
<tr>
<td>At maximum SGC amount (SPC:SGC ratio 2:1)</td>
<td>10.98 ± 2.74</td>
</tr>
<tr>
<td>At maximum rhINS:SPC ratio of 0.0025:1</td>
<td>10.21 ± 1.59</td>
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<tr>
<td>At maximum ether:water ratio of 7:1</td>
<td>10.09 ± 1.84</td>
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<tr>
<td>At the strongest ionic strength (1.2)</td>
<td>8.48 ± 1.17</td>
</tr>
<tr>
<td>At extreme homogenization conditions (500 bar, 6 cycles)</td>
<td>7.94 ± 1.35</td>
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<tr>
<td>At combination of extreme stress conditions</td>
<td>8.83 ± 2.2</td>
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Note: *P < 0.05, compared with free rhINS solution.

Abbreviations: rhINS, recombinant human insulin; SGC, sodium glycocholate; SPC, soybean phosphotidylcholine.

bioactivity of insulin was well preserved after drug loading, confirming the results of the conformational study.

The optimal formulation, which was prepared at less stress, showed good conformational stability (Figure 4, insert) and well preserved bioactivity, with a hypoglycemic percentage of 45.2% ± 6.5%, which is similar to that of the free rhINS solution.

Transmission electron microscopy
Figure 5 shows the transmission electron micrographs of the blank and rhINS-loaded sodium glycocholate liposomes, as well as the conventional liposomes. An almost spheroidal morphology was observed for the conventional liposomes, whereas a less spheroidal and deformed morphology was observed for both blank and rhINS-loaded sodium glycocholate liposomes. This was possibly due to the deformability of the sodium glycocholate liposomes. The vesicular structure was discernable, and the inner lamella could be weakly observed for sodium glycocholate liposomes. No obvious changes in sodium glycocholate liposome morphology were observed as a result of drug loading.

Leakage of insulin
Figure 6 shows the variation in entrapment efficiency in pH 2.0, 5.6, and 6.8 buffers over 6 hours. There was no significant difference (P > 0.05) in entrapment efficiencies obtained at different time points and different pH values. The results indicate that leakage of rhINS from sodium glycocholate liposomes was slow within the observed time duration. Therefore, the effect of potential variations in entrapment efficiency can be ignored when interpreting the in vitro characterization results.

Protection of insulin against digestion by pepsin, trypsin, and α-chymotrypsin
After treatment with pepsin, rhINS content in the conventional cholesterol liposomes was remarkably decreased from 100% to less than 5% of the initial value after 4 hours (Figure 7A). Liposomes containing bile salts all showed some resistance to pepsin because less degradation of rhINS was observed. Sodium glycocholate provided the best protection of liposomal rhINS, preserving 50% of rhINS at 4 hours. This may be attributable to the enzyme-inhibiting ability of sodium glycocholate. However, surprisingly, as the amount of sodium glycocholate increased further, less protection of...
liposomal rhINS was observed (Figure 7B). As the sodium glycocholate:soybean phosphotidylcholine ratio in the liposome formulation increased from 1:8 to 1:2, the amount of intact insulin decreased from 70% to 28% at 4 hours. This can possibly be explained by the increased flexibility of the lipid bilayers due to the presence of excessive amounts of sodium glycocholate, which may lead to the leakage of rhINS. The protective effect of bile salts on liposomal rhINS against trypsin and α-chymotrypsin was different from that against pepsin. Conventional liposomes showed better resistance to trypsin than liposomes containing sodium taurocholate or sodium deoxycholate (Figure 8A). However, sodium glycocholate liposomes still exhibited the best protection against these two enzymes, preserving 67% and 81% of rhINS content at 4 hours for trypsin and α-chymotrypsin, respectively (Figures 8A and 9A). Again, an increase in the amount of sodium glycocholate in the liposome membrane leads to increased enzymatic degradation (Figures 8B and 9B).

Discussion

The application of liposomes containing bile salts in the field of oral immunization and oral delivery of poorly water-soluble drugs has triggered our interest in using this vehicle to deliver rhINS by the oral route. To achieve better protection against the detrimental gastrointestinal environment, we employed sodium glycocholate, a special bile salt that has been reported to have both enzyme-inhibiting and permeation-enhancing effects. Therefore, the purpose...
of this series of studies was to provide proof of concept of this new glycocholate-containing vehicle and to elucidate the underlying mechanisms. In this first report, we focus on issues of encapsulation of rhINS in sodium glycocholate liposomes and the protective effect of this vehicle against enzymatic degradation in simulated in vitro models. The hypoglycemic as well as mechanistic studies will be reported in upcoming publications.

The aim of the formulation study was to obtain liposomes with particle sizes as small as a few hundred nanometers, which, as previously reported, are very important in achieving good permeation across biomembranes. By increasing the homogenization pressure, the particle size can be easily reduced to as low as 100 nm. However, reduction in particle size also leads to reduced entrapment efficiency of rhINS. Therefore, the homogenization pressure was finally settled at 300 bar, resulting in a particle size of about 150 nm. Because the particle size can be adjusted easily using this method, we did not investigate the effect of other preparative variables on particle size.

The second goal of this formulation study was to achieve high rhINS loading. Through screening the critical process and formulation variables, an entrapment efficiency of rhINS in sodium glycocholate liposomes as high as 30% can be achieved for the optimal formulation. The objective of incorporating as much sodium glycocholate in the liposomes as possible was compromised by the fact that an excess of sodium glycocholate would lead to significant reduction in entrapment efficiency. This can be explained by the fluidizing effect of sodium glycocholate on the lipid bilayers, which probably results in leakage of rhINS. An increase in the rhINS:soybean phosphotidylcholine ratio was helpful to increase drug loading, but was at the expense of entrapment efficiency.

rhINS may be sensitive to interfacial stress during the ultrasonication or homogenization process. Therefore, it is very important to ensure preservation of rhINS stability in the final product. HPLC assay gives the chemical stability of rhINS content, whereas, conformational studies and bioactivity assays are usually utilized to ensure the integrity of rhINS. In this study, we investigated the conformational and bioactive stability of rhINS in liposomes prepared under extreme stress conditions. Both conformation, as measured by circular dichroism, and bioactivity was preserved. Therefore, we conclude that preparative stress does not cause destabilization of rhINS.

The clinical potential of liposomes containing bile salts would depend on their integrity in the gastrointestinal tract in order to achieve absorption-enhancing activity and protection of the rhINS. Thus, it is desirable not to release rhINS into the gastrointestinal tract. Therefore, we evaluated in vitro release for a duration of 6 hours by measuring the change in entrapment efficiency of sodium glycocholate liposomes. The pH of the release medium used in this section was 2, 5.6, and 6.8, which are the pH of the gastric environment, isoelectric point of rhINS, and intestinal environment, respectively. It is designed to evaluate the stability of the liposome in the gastrointestinal environment compared with that on the shelf; however, for protection from the enzyme degradation component, the pH values were settled at 1.2, 6.8, and 7.8, which were the most appropriate pH values for pepsin, trypsin, and α-chymotrypsin. Stability of entrapment
efficiency confirmed little leakage of rhINS. However, the integrity of sodium glycocholate liposomes needs to be studied in vivo.

The bile salts used could be either sodium glycocholate, sodium taurocholate,20 or sodium deoxycholate.40,41 These three bile salts were compared to highlight the effect of sodium glycocholate as an enzyme inhibitor. The in vitro protective effect against enzymatic degradation by pepsin, trypsin and α-chymotrypsin, the three main proteases in the gastrointestinal tract, confirmed the advantage of glycocholate. Moreover, the safety of sodium glycocholate was better. As bile salts are naturally secreted by the gall bladder, the sodium glycocholate concentration in our formulation (6.77 mM) was far lower than the toxic concentration of bile salts (30 mM) for destroying the integrity/viability of Caco-2 monolayer cells.42

**Conclusion**

rhINS can be loaded into sodium glycocholate liposomes with high efficiency by a reversed-phase evaporation method followed by homogenization. The sodium glycocholate liposome particle size can be easily adjusted by tuning the homogenization pressure. The stability of rhINS was well preserved, even at extreme stress conditions, as confirmed by a conformational and bioactivity study. The formulation prepared under optimal conditions showed an entrapment efficiency of 30 ± 2% and a particle size of 154 ± 18 nm. In vitro study of the protective effect of bile salts on liposomal rhINS against enzymatic degradation by pepsin, trypsin, and α-chymotrypsin suggest superiority of sodium glycocholate. It is concluded that sodium glycocholate liposomes have potential for use in the oral delivery of protein and peptide drugs.

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**Disclosure**

The authors report no conflicts of interest in this work.

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