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Chitosan-coated nano-liposomes for the oral delivery of berberine hydrochloride

Thanh Xuan Nguyen,[a,b,c] Lin Huang,[a,b,d] Li Liu,[a,b] Ahmed Mohammed Elamin Abdalla,[a,b] Mario Gauthier[e] and Guang Yang*[a,b]

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Berberine hydrochloride (BH) possesses various pharmacological properties including anticancer; unfortunately, it has a low oral bioavailability and potential side effects for its parenteral administration. Nanoscale delivery carriers can increase the oral bioavailability of BH. Chitosan has interesting biopharmaceutical properties such as nontoxicity, biocompatibility, biodegradability, mucoadhesiveness, and the ability to open epithelial tight junctions. This study aims to engineer a chitosan-coated nano-liposomal carrier for the oral delivery of BH. The engineered formulation had a size ranging nanoscale. Chitosan-coated nano-liposomes displayed better stability and slower BH release in simulated gastrointestinal (GI) environment as compared to the uncoated ones. All values of pharmacokinetic analysis for chitosan-coated nano-liposomes were higher than for uncoated ones. These findings demonstrate that chitosan-coated nano-liposomes are more efficient than uncoated ones for the oral delivery of BH. It can be concluded that the stability and delayed BH release in simulated GI environment were improved with engineered chitosan-coated nano-liposomes. Moreover, since desirable *in vitro* and *in vivo* characteristics were achieved, they are promising release devices for the oral delivery of BH increasing the bioavailability of the drug.

1. Introduction

Berberine hydrochloride (BH, $C_{20}H_{18}ClNO_4$, M = 371.82) is a well-known plant alkaloid with a long history, that has been used in both Vietnamese and Chinese traditional medicine (Fig. 1). It is used to treat diarrhea because of its antimicrobial, antimotility and antisecretory properties. Recently, studies have also demonstrated that BH can possess other pharmacological properties that include anticancer, anti-HIV, anti-diabetic, anti-obesity, antirheumatic, muscle-relaxing, anti-inflammatory, cardioprotective, hepatoprotective, GI protective, anticonvulsant, anti-skin-aging, anti-uveitis, immunoregulative, antimalarial, antioxidantive, neuroprotective, vasorelaxing, anxiolytic and analgesic effects.[1, 2]

Unfortunately BH has a low bioavailability, which has hampered its therapeutic applications for a long time. The poor water solubility of BH results in decreased absorption in the GI tract and subtherapeutic plasma concentrations. These disadvantages have limited the development and applications of BH as a pharmaceutical formulation. On the other hand, BH also has potential side effects associated with its intramuscular and intravenous administration, such as anaphylactic shock and drug rash. It is therefore necessary to design an oral drug delivery carrier to increase the solubility and bioavailability of BH.[3, 4]

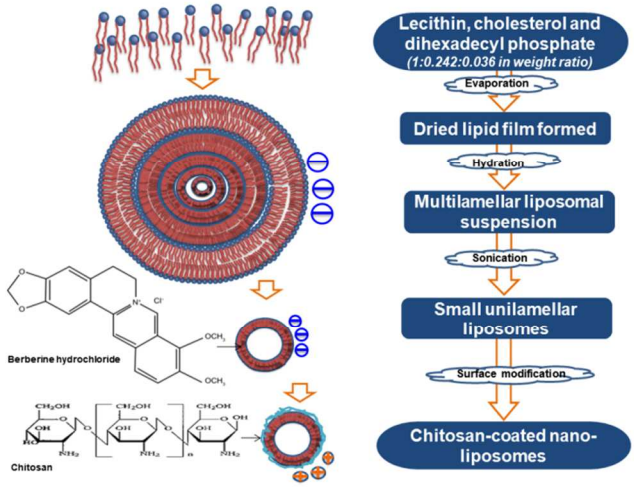

Fig. 1 Schematic illustration of preparation of uncoated and chitosan-coated nano-liposomes incorporating berberine hydrochloride.

Oral administration is one of the most preferred and traditional routes for drug delivery. As compared with parenteral drug delivery carriers, its main advantages include greatest safety, simplicity, convenience and patient compliance, which increase

the therapeutic efficacy of the drugs.[5, 6] Oral drug administration also suppresses the risk of disease transmission, reduces expenses, and allows more flexible or controlled dosing frequency.[6, 7]

Recent reports on oral nanoscale delivery carriers for BH utilized liposomes, solid lipid nanoparticles and nanoemulsions, all of which are common delivery carriers to enhance in vivo oral bioavailability.[4] Unfortunately conventional liposomes, structured as concentric bilayers of phospholipids, are sensitive to damage caused by the harsh chemical and enzymatic GI environment, resulting in reduced oral material bioavailability.[5]

In recent years, various attempts have been made to modify the liposomal surface not only to improve their stability, but also to functionalize them.[8, 9] Coating with chitosan can increase the stability of liposomes in various biological fluids including simulated gastric fluid (SGF) and intestinal fluid (SIF), as well as their mucoadhesive properties, cellular uptake, and the solubility of drugs.[10]

Chitosan, a linear copolymer of β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine (Fig. 1), is a commercially available and abundant natural polysaccharide obtained by alkaline deacetylation of the natural polysaccharide chitin,[11] which is the structural material of the shells of crustaceans and insects, and the second most abundant natural polymer on earth after cellulose.[12, 13]. Chitosan possesses interesting biopharmaceutical properties such as nontoxicity, biocompatibility, biodegradability, mucoadhesiveness, the ability to open epithelial tight junctions, and it has FDA-GRAS (Food and Drug Administration-Generally Recognized as Safe) status which has allowed it to be widely applied in the biomedical and biotechnological fields.[14, 15] It has been recently reported that it is capable of enhancing berberine absorption via the paracellular route, due to its ability to improve the berberine paracellular pathway in the intestinal tract.[14]

Up to now chitosan has been mainly utilized to coat liposomes as a protective and mucoadhesive polymer, and as a permeation enhancer for controlled drug delivery due to its ability to prolong the residence time in GI tract, and to open epithelial tight junctions to allow for an increase in paracellular transport for drugs such as cyclosporine A,[16] indomethacin,[10] alendronate,[17] furosemide,[18] atenolol,[19] DNA vaccines,[20] elcatonin,[21] calcitonin,[22, 23] insulin,[24] superoxide dismutase,[25] and so on.

However, to the best of our knowledge, studies on chitosan-coated nano-liposomes as BH delivery carriers for oral administration have not yet been reported in the literature. Therefore, there is an urgency to provide evidence for the in vitro and in vivo efficiency of chitosan-coated nano-liposomes incorporating BH for oral delivery.

2. Experimental section

2.1 Materials

Lecithin from egg yolk (EPC) and cholesterol were obtained from Sinopharm Chemical Reagent Co., Ltd in China. Dihexadecyl phosphate (DHP) and bile salts were purchased from Sigma-Aldrich in the USA. Chitosan with a degree of deacetylation of ≥ 90 % was obtained from Shanghai Ruji Biology Technology Co., Ltd in China. Berberine hydrochloride with purity > 98 % and palmatine (IS) with purity > 98 % were obtained from Shanghai Yuanye Biotechnology Co., Ltd in China. Pancreatin was purchased from Tokyo Chemical Industry Co., Ltd in Japan. Pepsin (high purity) and dialysis bags (molecular weight cut-off, MW: 8000-14000) were purchased from BioSharp, in the USA. All other reagents used in this study were reagent grade.

2.2 Preparation of buffers and other solutions

Phosphate buffered saline (PBS)[24] prepared from 137 mM NaCl, 2.6 mM KCl, 6.4 mM $Na_2HPO_4.12H_2O$, 1.4 mM $NaH_2PO_4.2H_2O$ and pH = 7.4 in water. BH solution (1 mg/mL)[26] was composed of 100 mg of BH in 100 mL of PBS, pH 7.4. An acetic acid solution (0.1 % : v/v, pH = 3.5) was prepared by adding 1.0 mL of acetic acid solution to 1 L of ultrapure water, and the pH was adjusted with 1.0 M NaOH. Chitosan solutions were prepared by dissolving 0.1 or 0.3 % (w/v) of chitosan in 0.1 % acetic acid solution with stirring overnight. The SGF was prepared by mixing 2.0 g of NaCl, 7.0 mL of HCl 36 - 38 % and 3.2 g of pepsin in 1 L of water, and adjusting the pH to 1.2 with 1.0 M HCl. For the preparation of SIF, 6.8 g of KH_2PO_4, 10 g of pancreatin, and 5.0 g of bile salts were added to 1 L of water, and the pH was adjusted to 7.4 with 1.0 M NaOH.

2.3 Preparation of nano-liposome-encapsulated berberine hydrochloride

Uncoated nano-liposomes were prepared by thin film hydration followed by sonication (Fig. 1). This was described in detail in the Supplementary information.

2.4 Preparation of chitosan-coated nano-liposomes incorporating berberine hydrochloride

The uncoated liposome suspension was added dropwise to an equal volume of chitosan solution and the mixture was incubated at 10 °C for 1 hour with continuous stirring (Fig. 1). The final lipids and chitosan concentrations were half of the original solutions, and the final BH concentration in the chitosan-coated nano-liposomes was 0.5 mg/mL.

2.5 Characterization of the uncoated and coated nano-liposomes

To assess the quality of the nano-liposomes and to obtain quantitative measurements allowing comparisons between different nano-liposomes batches, various parameters were measured. These include the average mean size, zeta potential, microscopic analysis, encapsulation efficiency (*EE*) and drug release measurements.

2.5.1 Measurements of particle size, size distribution and zeta potential

The mean particle size, polydispersion index (*PDI*) and zeta potential of the nano-liposomes prepared were measured using dynamic light scattering with a Zetasizer Nano ZS90 (Malvern Instruments Ltd.) at 25 °C. Prior to the measurements, the nano-liposome dispersions were diluted 20-fold with ultrapure water to avoid multiple scattering phenomena arising from interparticle interactions. Each sample was measured at least three times.

2.5.2 Morphological assessment

The shape and surface morphology of the nano-liposomes was examined by transmission electron microscopy (TEM, FEI

Company, Netherlands) for different uncoated and chitosan-coated samples. Briefly, the dispersion of prepared nano-liposomes was diluted with ultrapure water to obtain a very dilute suspension for TEM imaging. After dilution, 8 µL of the liposome suspension was carefully dropped onto a clean copper grid and air-dried for 2 minutes at room temperature after removing excess solution with a filter paper. Negative contrast staining was carried out with 8 µL of 2 % aqueous phosphotungstic acid solution (pH = 6.0) and air-drying for 2 minutes at room temperature after removing excess solution with a filter paper. The copper grids were dried at room temperature before imaging by TEM.

2.5.3 Determination of encapsulation efficiency

The *EE* was determined by an indirect method using dialysis bags. The dialysis membranes were stored overnight in the dissolution medium before dialysis, to ensure thorough wetting of the membrane. To remove unencapsulated BH from the suspension of uncoated nano-liposomes or chitosan-coated nano-liposomes, each suspension (5 ml) was filled into a dialysis bag (MW: 8,000-14,000) and dialyzed against 250 mL of deionized water at room temperature for 24 h. The dialysate was collected and the absorbance (A) of BH in the dialysate was measured on a UV–vis spectrophotometer (TU-1810, Beijing Purkinje General Instrument Co., Ltd, China) at 345 nm, which is the absorption maximum for BH. The BH concentration (µg/mL) in the dialysate was determined from a standard curve between 3 and 10 µg/mL, corresponding to the regression equation y = 0.00143 + 0.06127x (where y is the absorbance of the drug solution and x is the concentration of the drug; $R^2 = 0.9996$). The *EE* was calculated according to Equation 1.

$$EE\ (\%) = \frac{(Q_t - Q_d)}{Q_t} \times 100\ \%\ (1)$$

where *EE* is the encapsulation efficiency, Q_t the theoretical amount of added BH and Q_d is the amount of the dialyzed BH. Each experiment was repeated in triplicate.

2.5.4 Assay of drug leakage

The uncoated and chitosan-coated nano-liposomes incorporating BH were stored at 4 °C and 25 °C for 30 days in a sealed container. The leakage ratio of BH was determined after 15 days and 30 days, by measuring the *EE* after the set storage time (EE_2) as compared to the *EE* before storage (EE_1) (as described in Section 2.5.3), and calculated according to Equation 2. It should be mentioned that each experiment was repeated in triplicate.

$$\text{Leakage ratio}\ (\%) = \left(1 - \frac{EE_2}{EE_1}\right) \times 100\ \%\ (2)$$

2.5.5 *In vitro* drug release study

The *in vitro* release of BH from solution and from liposome formulations was analysed by membrane dialysis at 37 °C. The dialysis membranes (MW: 8,000-14,000) were stored overnight in the dissolution medium before dialysis, to ensure thorough wetting of the membrane. The *in vitro* release studies were carried out in GI environment (SGF, SIF). Briefly, 2 mL of each liposome formulation mixed with 2 mL of each aqueous receptor medium was placed in the dialysis bag, which was hermetically sealed and dropped into 200 mL of the aqueous receptor medium. Perfect sink conditions prevailed during the drug release studies, and the entire system was kept at 37 °C under continuous stirring with a heat collection-constant temperature type magnetic stirrer (DF-101S) at 100 rpm. The receptor compartment was closed to avoid evaporation of the dissolution medium. Samples (10 mL) of the dialysate were removed at different time intervals (0.5, 1, 2, 4, 6, 8, 12, and 24 h) and assayed for BH concentration by spectrophotometry at 345 nm. The same volume was replaced with fresh dissolution medium, so that the volume of the receptor compartment remained constant. All the kinetic experiments were conducted in triplicate and the mean values were calculated. The BH concentration (µg/mL) in the dialysate was determined as described in Section 2.5.3. The percent release of BH from the nano-liposomes was calculated as shown in Equation 3.

$$RR\ (\%) = \frac{C_t \times V_1 + \sum_{i=1}^{i=n-1} C_i \times V_2}{m} \times 100\ \%\ (3)$$

where *RR* is release ratio, C_t is the concentration of BH in the buffer solutions at t time, V_1 is the volume of buffer solution at different pH values (200 mL), *n* is the number of samples removed from the release medium, V_2 = 10 mL, and *m* is the initial total amount of BH in the dialysis bag.

2.5.6 Physicochemical stability study

The uncoated and chitosan-coated nano-liposomes encapsulating the BH were sealed in glass bottles and stored at 4 °C or 25 °C for a period of up to 30 days. At predetermined time intervals, samples were withdrawn and analyzed for their size, size distribution, and zeta potential using a Zetasizer Nano ZS as described in Section 2.5.1. Each experiment was repeated in triplicate.

To investigate the stability of nano-liposomes in simulated GI fluids, the liposome formulations were incubated in SGF and SIF as described in Section 2.2. For each liposome formulation, 3 mL of liposome suspension was added to 3 mL of simulated fluid (SGF or SIF), followed by incubation for up to 24 h in a water bath at 37 °C. At different time intervals (0, 0.5, 1, 2, 4, 6, 8, 12, and 24 h) the particle size, size distribution and zeta potential were analyzed as described in Section 2.5.1. Each experiment was repeated in triplicate.

2.6 Animal experiments

Twelve New Zealand white rabbits (female, weighing 2.0–2.5 kg) were purchased from the Hubei research centre for laboratory animals in China. The rabbits were fasted for 24 hours before the experiment and had free access to water. On the day of experiment, the rabbits were randomly divided into four groups (n = 3 for each group). Each drug solution in the different formulations was given to the rabbits via oral gavage at a single dose equivalent to 5 mg/kg of BH: group 1 (non-liposome drug), group 2 (uncoated liposomes), group 3 (0.1 % chitosan-coated liposomes) and group 4 (0.3 % chitosan-coated liposomes).

Blood samples (1.5 mL) were obtained from the marginal ear vein before dosing (to serve as a blank) and subsequently at 0.5, 1, 2, 4, 6, 8, 12, and 24 h after drug administration. The blood samples were collected in tubes containing heparin as

anticoagulant. Immediately after collection, each blood sample was gently inverted several times to ensure complete mixing with the anticoagulant and immediately centrifuged at 10,000 rpm for 10 minutes at 4 °C to separate the plasma. The supernatant plasma was transferred to a clean tube and stored at -20 °C until analysis.

2.7 HPLC assay

2.7.1 Chromatographic apparatus and conditions

HPLC analysis was performed using a system (Prominence LC-20A, Shimadzu Corporation, Japan) consisting of a LC-20AD pump and a SPD-20A UV detector. Chromatographic separation was achieved on an Agilent TC-C18 analytical column (4.6 mm × 150 mm, 5 µm, Agilent, USA) maintained at 30 °C. The mobile phase consisted of 30 % acetonitrile and 70 % buffer (mixture of 10 mM KH_2PO_4, pH value adjusted to 3.03 ± 0.01 with phosphoric acid); the flow rate was set at 1 mL/min. The UV absorbance was determined at 345 nm, at which BH has the highest absorption.

2.7.2 Preparation of standard and working solutions

Individual standard stock solutions of BH (100 µg/mL) and an internal standard (IS: palmatine, 100 µg/mL) were prepared by accurately weighing of the required amounts into volumetric flasks and dissolution in methanol. The working solutions containing BH at concentrations of 0.01, 0.025, 0.05, 0.1, 0.5, 1.0, and 5.0 µg/mL were prepared in methanol from the stock solutions to plot the calibration curve. Quality control (QC) samples were prepared by adding the stock solution of BH into blank plasma to obtain final concentrations of 0.05, 0.1 and 1.0 µg/mL, representing low, medium, and high concentration QC samples, respectively. The QC samples were used to access the accuracy and precision of the assay methods. All the calibration and QC samples were extracted by the method described in the subsequent section and then analyzed. The QC samples were stored along with the test samples at −20 °C until analysis.

2.7.3 Preparation of sample solution

The plasma samples containing BH for HPLC analysis were prepared by liquid-liquid extraction method. Briefly, plasma samples were removed from -20 °C storage and immersed in a 37 °C water bath for 5 minutes to thaw. Following vortexing, 1 mL of plasma was extracted with 5 mL of methanol (1:5, v/v) after addition of 10 µL of IS (5.0 µg/mL) and 100 µL of 1.5 M NaOH solution. The mixture was vigorously vortexed for 3 minutes and subsequently centrifuged at 12,000 rpm for 15 minutes at 4 °C. The upper organic layer was transferred to a glass tube and filtered once with a 0.22 µm pore size membrane. The filtered sample (1.5 mL) was transferred to a microtube and evaporated at 40 °C until a completely dry residue was formed. The residue was then reconstituted in 100 µL of methanol, vortexed for 30 minutes, and centrifuged at 12,000 rpm for 15 minutes. Finally, 25 µL of the supernatant layer was injected into the HPLC system for analysis.

2.7.4 Validation of analytical method

The HPLC method was validated for selectivity, linearity, limit of quantitation, precision, and accuracy according to current guidelines (described in detail in the Supplementary information).

2.8 Pharmacokinetic parameters

The area under the plasma concentration-time curve (*AUC*) was calculated by using the linear trapezoidal method.[27] The peak concentration (C_{max}) and the time to reach the peak concentration (T_{max}) of drug in the plasma were obtained by visual inspection of the data from the concentration–time curve. The area under the plasma concentration-time curve (AUC_{0-t}) from time zero to the time of the last measured concentration (C_{last}) was calculated by the linear trapezoidal rule. The elimination rate constant (K_{el}) was estimated from the slope of the terminal phase of the log plasma concentration-time points fitted by the method of least-squares. The *AUC* from time zero to infinite ($AUC_{0-\infty}$) was obtained by the addition of AUC_{0-t} and the extrapolated area determined by C_{last}/K_{el}. The mean residence time (*MRT*) was derived from the ratio *AUMC:AUC*, where *AUMC* was the area under the curve for the plot of the product of concentration and time versus the time from zero to infinity.

2.9 Statistical analysis

Statistical analysis of the differences in the measured properties of the groups was performed using Excel with one-way analysis of variance and the determination of confidence intervals. All the data are presented as means with standard deviations, indicated as "mean ± SD". The differences are considered to be statistically significant when the p values are less than 0.05. The pharmacokinetic parameters were calculated by the software DAS 2.0 (issued by the State FDA of China for pharmacokinetic study).[28]

3. Results and discussion

3. 1 Characterization of the prepared nano-liposomes

3.1.1 Evaluation of size, *PDI* and zeta potential

The results in Table 1 show that the size of the nano-liposomes in the suspension was highly dependent on the concentration of chitosan solution added to the system, increasing from 143 nm in the absence of chitosan to above 264 nm in the presence of chitosan. The formation of the coating layer on the surface of the nano-liposomes, and an increase in the thickness of the coated layer with the polymer concentration is observed as a change in size. The *PDI* of all the preparations were between 0.26 and 0.53, suggesting a narrow size distribution. However the size of the nano-liposomes did not increase too much before and after coating, only 1.84-fold as compared to the original size.

The relatively small change in size of the nano-liposomes upon coating is preferable for GI absorption and systemic circulation. Firstly, drug carriers or free drugs must adhere to the mucus and cross the mucus layer to be absorbed into the systemic circulation. Drug carriers or drugs delivered to the mucosal surface are usually efficiently removed by mucus clearance mechanisms and systemic absorption.[29] The mucus acts to entrap and remove pathogens and foreign particles, in order to protect the epithelial surface. Drug nanocarriers are nevertheless a good alternative to diffuse into the mucus layer and minimize

elimination by this clearance mechanism. In addition, there is a size limit to cross the intestinal mucosal barrier since the mesh-pore spacing of the mucus layer varies from 50 – 1800 nm.[30] Many studies have shown that nanoparticles with a size under 200 nm effectively diffuse through the mucus layer.[31-34] Secondly, liposomes smaller than 70 nm are removed from the systemic circulation by liver parenchymal cells, while those larger than 300 nm accumulate in the spleen. An optimum size range of 70 - 200 nm has been identified to give the highest blood concentration of liposomes.[35-37]

Table 1 Characteristics of uncoated and chitosan-coated nano-liposomes (n = 3).

Formulations	Diameter (nm)	*PDI*	Zeta potential (mV)	*EE* (%)	pH (25.4 °C)
Uncoated nano-liposomes	143 ± 5	0.26 ± 0.01	-39.5 ± 1.2	/	5.5 ± 0.01
Uncoated nano-liposomes in acetic acid 0.1 %	142 ± 5	0.27 ± 0.01	-26.8 ± 0.9	83.2 ± 0.4	4.3 ± 0.01
0.1 % Chitosan-coated nano-liposomes	194 ± 3	0.34 ± 0.03	24.1 ± 0.5	81.6 ± 0.4	4.8 ± 0.01
0.3 % Chitosan-coated nano-liposomes	264 ± 8	0.53 ± 0.01	29.3 ± 0.5	78.4 ± 0.5	5.3 ± 0.02

The presence of strong intra-molecular electrostatic interactions is an explanation for the relatively thin layer formed around the nano-liposomes prepared.[38] After adsorption, the attraction between segments of the polyelectrolyte chains of chitosan and the oppositely charged groups on the surface of the liposomes forces them to come into close proximity.[39]

The zeta potential has often been utilized to characterize colloidal drug delivery systems. It is a measure of the surface electrical charge of the particles. The magnitude of the zeta potential gives an indication of the stability of colloidal systems: As the potential increases, the repulsion between particles is greater, thus leading to more stable colloidal dispersions. If all the particles in suspension have a large negative or positive zeta potential they will repel each other, and there will be no tendency for the particles to aggregate.[40] It is clear from Table 1 that coating the nano-liposomes with chitosan shifted the zeta potential from negative (-39.5 ± 1.2) to positive values (29.3 ± 0.5). The zeta potential of the nano-liposomes became increasingly positive as the chitosan concentration was increased from 0.1 % (24.1 ± 0.5) to 0.3 % (29.3 ± 0.5). The increased particle size and zeta potential of chitosan-coated nano-liposomes reflect several changes in the surface properties of nano-liposomes due to the polymer-liposome interactions.

3.1.2 Encapsulation efficiency

The *EE* was determined by dialysis, which has been shown to have better accuracy than ultracentrifugation techniques.[41]

As shown in Table 1, the *EE* for BH was 83.2 ± 0.4 % for uncoated nano-liposomes. The addition of an incremental concentration of chitosan decreased *EE* slightly from 83.2 ± 0.4 % to 78.4 ± 0.5 %, possibly due to the interactions of chitosan with the apolar head groups on the surface of the phospholipid bilayers. Moreover, previous studies demonstrated that when the concentration of chitosan was increased to 0.3 %, no more leakage of BH was observed because the adsorption of chitosan onto the surface of the nano-liposomes was saturated.[10, 22]

3.1.3 Morphological observation

TEM analysis served to examine the structure of the nano-liposomes. The morphological appearance of uncoated and coated nano-liposomes is presented in Fig. 2. It can be seen that both uncoated and coated nano-liposomes have a spherical morphology. Although the previous results indicated that the particle size increased slightly after coating and increasing the concentration of chitosan, no large differences among these were observed by TEM. This is probably due to strong adsorption between the polymer and the liposomal bilayer, resulting in a flat configuration and making it difficult to observe the polymer at the surface of the liposomes.[42]

3.1.4 Physicochemical stability

Physical instability results in drug leakage and the aggregation or fusion of liposomes, while chemical instability is caused by oxidation of the unsaturated fatty acids and hydrolysis of the ester bonds.[43] The storage stability of uncoated, 0.1 % and 0.3 % chitosan-coated nano-liposomes was evaluated in the present study. The liposomes were stored at 4 °C and 25 °C in an aqueous environment for up to 30 days while monitoring their size, zeta potential and leakage ratio as shown in Fig. 3, 4 and 5.

At 4 °C (Fig. 3 and 4), chitosan-coated nano-liposomes exhibited superior stability as compared with the uncoated liposomes: They showed little change in size and zeta potential over 5 days during storage, while the uncoated nano-liposomes started to show significant changes just after 2 days. After this relative stability period, remarkable changes in size were observed. This may be due to to swelling of the nanosized carriers and changes in their surface properties under the conditions designed for the stability study.[44, 45] Swelling of the nano-liposomes could cause the release of the encapsulated materials. After 15 days, the size of the uncoated nano-liposomes increased significantly (1.27-fold, from 142 to 181 nm) and the leakage ratio was enhanced to 3.6 %, whereas the size of chitosan-coated nano-liposomes increased approximately 1.15-fold (from 194 to 223 nm) and 1.0-fold (from 264 to 271 nm) for 0.1 and 0.3 % chitosan, respectively. The leakage ratio of coated samples increased to 2.1 and 1.3 % for 0.1 and 0.3 % chitosan, respectively.

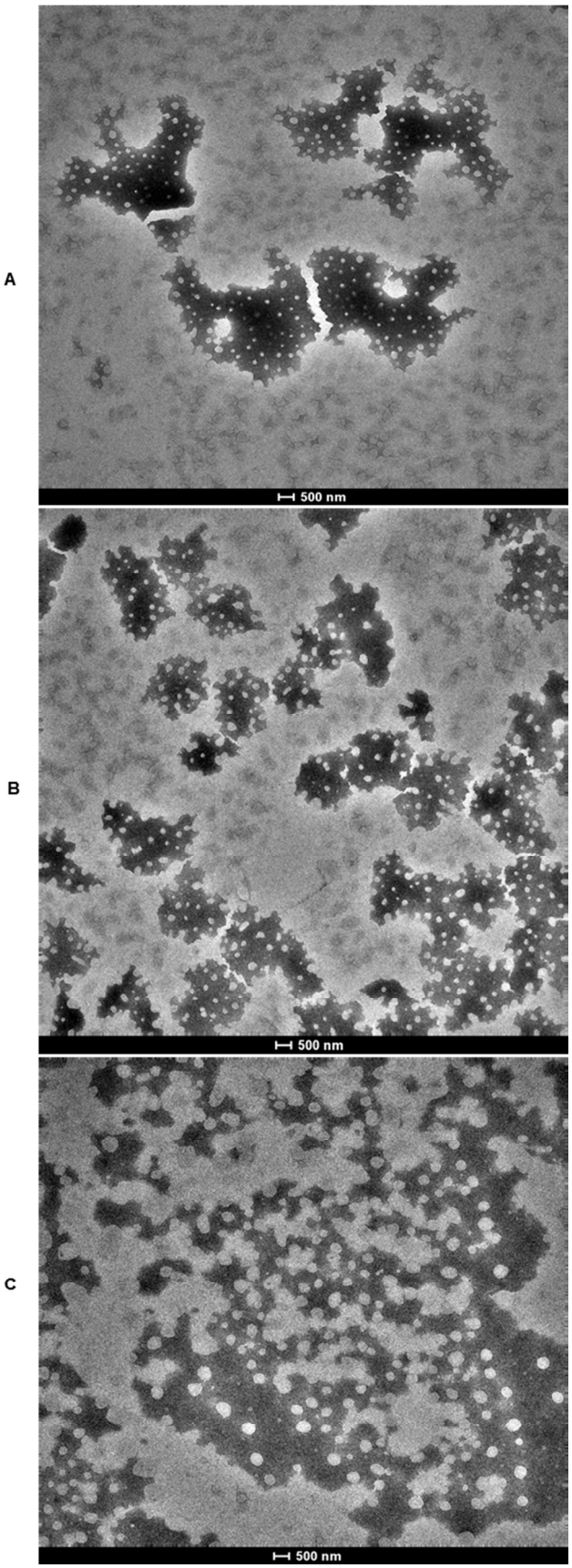

Fig. 2 TEM of uncoated nano-liposomes (A), 0.1 % chitosan-coated nano-liposomes (B), 0.3 % chitosan-coated nano-liposomes (C). bar is 500 nm.

After 30 days, the size of uncoated nano-liposomes increased 1.37–fold and the leakage ratio to 9.4 %, whereas the size of chitosan-coated nano-liposomes increased 1.2-fold and 1.07-fold for 0.1 and 0.3 % chitosan, respectively. The leakage ratio of the coated samples increased to 6.2 and 2.1 % for 0.1 and 0.3 % chitosan, respectively. Therefore the 0.3 % chitosan-coated nano-liposomes showed the highest stability, while the 0.1 % chitosan-coated nano-liposomes were still more stable than uncoated nano-liposomes during storage for 30 days at 4 °C.

These differences in stability are ascribed to the presence of the chitosan coating layer forming a wall that hindering swelling and the release of the encapsulated materials.

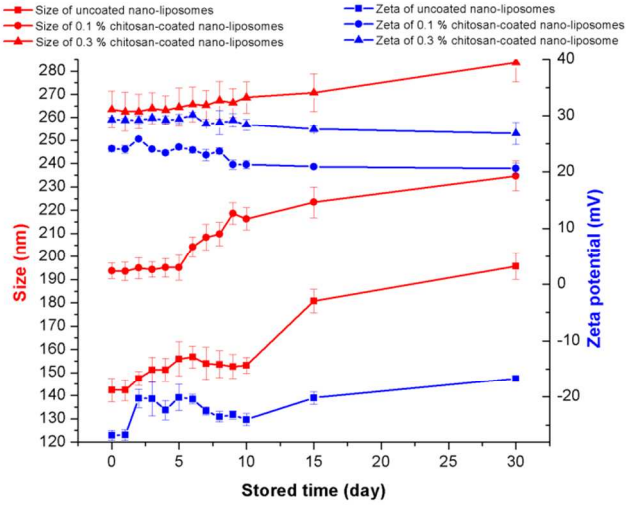

Fig. 3 Size and zeta potential of nano-liposomes stored at 4 °C for 30 days.

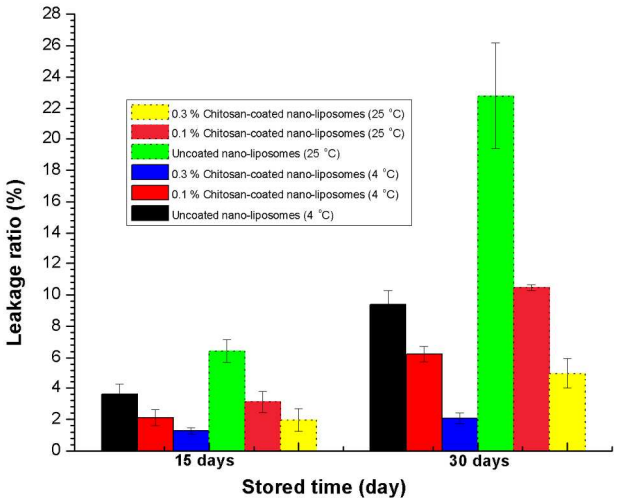

Fig. 4 Leakage ratio of nano-liposomes stored at 4 °C and 25 °C after 15 and 30 days.

At 25 °C (Fig. 4 and 5), both coated and uncoated nano-liposomes showed significantly decreased stability as compared to 4 °C. When stored at 25 °C for 30 days, the size and zeta potential of the uncoated samples decreased significantly (1.42- and 1.43-fold, respectively), while their leakage ratio increased to 22.8 %, whereas the coated samples were still stable with a slow increase in size (1.39- and 1.17-fold for 0.1 and 0.3 % chitosan, respectively), zeta potential, and leakage ratio. The changes in

size, zeta potential and leakage ratio were nevertheless higher than at 4 °C. This could be explained by hydrolysis and oxidation of the lipids at room temperature, which induces decomposition and aggregation of the liposome vesicles.[46] This also resulted in drug leakage and a slight decrease in zeta potential. In contrast, the decomposition of liposomes was inhibited at low temperature (at 4 °C).[47, 48] The stability of uncoated nano-liposomes was therefore much lower than for chitosan-coated liposomes at 25 °C in terms of aggregation, fusion, leakage, and oxidation. The chitosan layer may provide shielding for the surface of the liposomes, because it has a higher stability in water. The instability of lipids cannot be avoided, which may explain why the size of the uncoated nano-liposomes decreased 1.42-fold after 30 days at 25 °C.

The results for storage stability in the present study are in agreement with previous studies.[44-46] Li et al.[47] thus demonstrated that polymers forming a layer around liposomes reduced the oxidation of the lipids and prevent the leakage of drugs. Therefore, the chitosan-coated nano-liposomes displayed better storage stability than uncoated nano-liposomes at both 4 °C and 25 °C over 30 days likely because the chitosan successfully coated their surface. Moreover, the 0.3 % chitosan-coated sample was more stable than the 0.1 % chitosan sample, which might be due to reaching a thicker coating layer.[42, 49]

To further evaluate the effect of surface modification on the stability of liposomes, they were incubated in SGF and SIF. The particle size and zeta potential determined under these conditions are represented in Fig. 6 and 7.

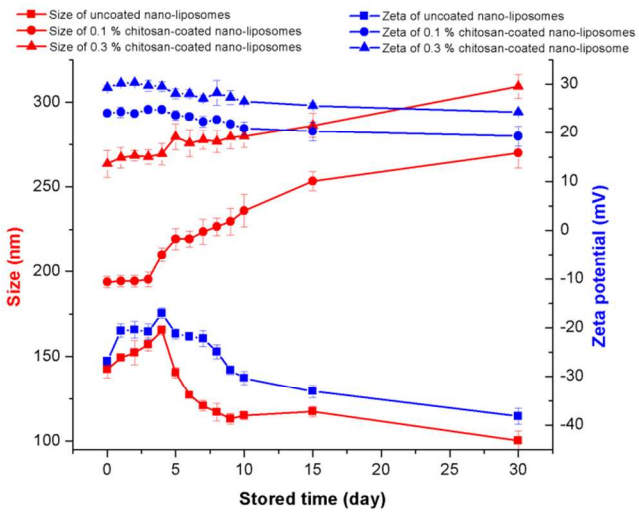

Fig. 5 Size and zeta potential of nano-liposomes stored at 25 °C for 30 days.

The results in Fig. 6 show that the size of uncoated nano-liposomes increased 1.38-fold from 0 h to 1 h when incubated in SGF, and then gradually decreased. This is due to the negative charge on their surface that may have led to the adsorption of positively charged ions from the surrounding medium, resulting in aggregation and destabilization of the liposomes.[50] In contrast, the size of chitosan-coated nano-liposomes gradually decreased significantly from 0 h. This can be mainly explained by enhanced interactions between chitosan and the surface of liposomes under low pH conditions in SGF (pH 1.2), because the ionization of amino groups in chitosan is increased by protonation. The molecular configuration of chitosan also became more expanded, leading to stronger affinity for the liposome surface.[51] These results are also in agreement with the gradual increase in zeta potential of the coated nano-liposomes. The changes in the size and zeta potential of the coated samples were less than for the uncoated samples in SGF. The coating layer can therefore improve the stability of liposomes in SGF.

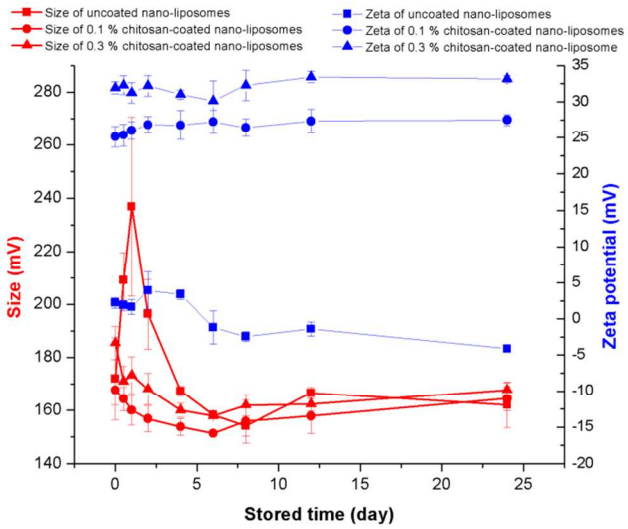

Fig. 6 Size and zeta potential of nano-liposomes incubated in SGF at 37 °C for 24 hours.

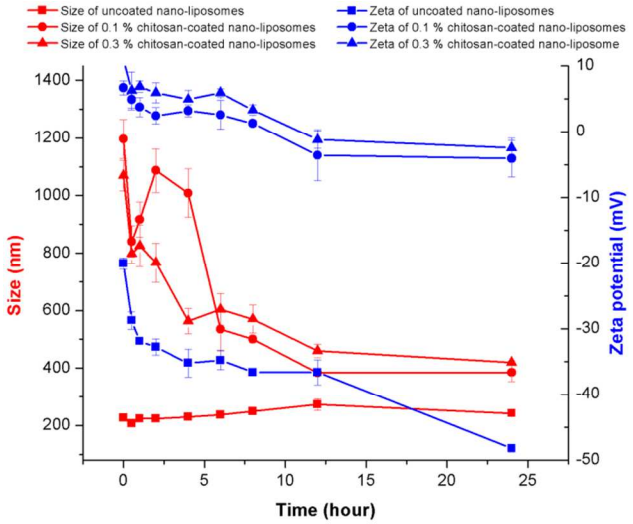

Fig. 7 Size and zeta potential of nano-liposomes incubated in SIF at 37 °C for 24 hours.

The results in Fig. 7 show that the size of all the liposomes dramatically increased after mixing in SIF, with a gradual decrease observed thereafter. The size of the uncoated samples increased 1.6-fold, while the coated samples increased 6.2- and 4.2-fold for 0.1 and 0.3 % chitosan, respectively. A potential explanation for this observation is reduced interactions between chitosan and the surface of liposomes in SIF (pH 7.4), due to a decrease in the number of charged cationic groups on chitosan. Since the electrostatic interactions were weaker, the medium

gradually entered into the particles and their average diameter increased.[52] Moreover, constituents of the SIF such as bile salts act as surfactants promoting lipid solubilization. Pancreatic lipases have a digestive action on phospholipids, which also contributes to destabilization of the liposomal system.[53] The zeta potential of all the samples decreased significantly during incubation in SIF, but this reduction for the coated samples (1.6- and 1.2-fold for 0.1 and 0.3 %, respectively) was less than for the uncoated samples (2.4-fold). The decrease in potential of uncoated samples may be attributed to the hydrolysis of phospholipids by pancreatin enzymes, the smaller changes observed for the coated samples being probably due to the protection of the chitosan layer.[52]

Despite the fact that the stability of nano-liposomes was similar in SIF and in SGF, significant differences still existed. It was thus found that changes in size and zeta potential for all the samples in SGF were lower than in SIF. Similar results were also obtained in previous studies.[17, 20, 52, 54, 55] Rowland et al.[54] demonstrated that most liposomes were little affected by the low pH during passage through the stomach. The well-organized assembly of phospholipids can protect liposomes from gastric environment disintegration. Liu et al.[52] showed that the deposition of polymers on the surface of liposomes may further stabilize their structure under low-pH conditions.

3.1.5 *In vitro* sustained release

The *in vitro* release profiles of BH from uncoated nano-liposomes and chitosan-coated nano-liposomes in SGF and SIF are presented in Fig. 8. The profiles of all the liposomes show a biphasic pattern of drug release. An initial burst effect, exhibited during the first 2 hours, which may be related to the immediate release of unencapsulated and surface-associated drug, followed by a slow release phase of the encapsulated drug.

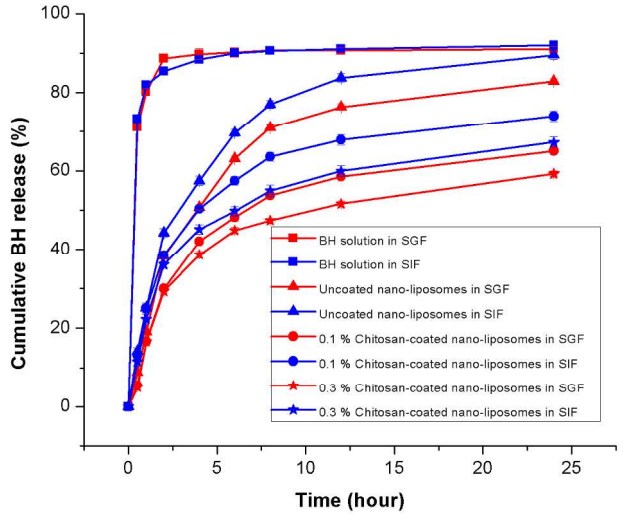

Fig. 8 Cumulative BH release of nano-liposomes in SGF and SIF at 37 ºC for 24 hours.

The results reveal that the BH release rate for both uncoated and coated liposomes was significantly lower than for the BH solution in both SGF and SIF. As expected, the chitosan concentration in the coating process had a strong influence on the drug release behaviour of the liposomes. Increasing the chitosan concentration from 0.1 % to 0.3 % resulted in a decrease in the percentage of drug released in both SGF and SIF as compared to uncoated liposomes. Slow release of the contents in SGF is desirable for an oral delivery system, because there would be more BH available for absorption in the intestine. The BH release rate from all the liposomes was enhanced in SIF relatively to SGF.

These findings are also in agreement with previous studies.[52, 55-57] Liu et al.[55] argued that liposomes released more entrapped ingredients in SIF than in SGF because of the disruption of the liposomal membrane by the pancreatic enzyme in SIF conditions. Moreover, it was clear that delayed and reduced release rates were obtained for the polyelectrolyte delivery system as compared with bare liposomes.[52] Other studies also found that uncoated liposomes lost their structural integrity easily and thus released the entrapped materials under SIF conditions.[56, 57] In addition, the cumulative release rates in our study show that the steady release percentages of BH solution reached above 90 % over 24 hours, while the BH loaded in liposomes attained almost 80 %. The delayed release observed for BH loaded in nano-liposomes shows that these have great potential as carriers to provide sustained BH delivery during a treatment.[58] Moreover, our previous study showed that the delayed release observed for BH loaded in bacterial cellulose in both SGF and SIF.[59]

Release kinetics analysis of samples was described in detail in the Supplementary information.

3. 2 HPLC assay

3. 2.1 Validation of the analytical method

Assay selectivity was evaluated by analysing blank plasma samples obtained from six rabbits. The results in the present study show that all samples were free of interference with BH or IS. Good separation was achieved with retention times of 7.15 and 7.9 min for palmatine (IS) and BH, respectively (Detailed in Fig. S1 in the Supplementary information). This method yielded good peaks without any significant drift in the baseline over the entire runtime of 10 min. Linearity was verified by least squares linear regression analysis of the calibration curve. Good linearity between the peak area and concentration of BH was obtained throughout the concentration range, with a regression equation $y = 2.1631x - 0.0834$ for BH and a correlation coefficient (R^2) of 0.9993, where y represents the peak area ratio of BH to IS and x represents the concentration of BH in the plasma (μg/mL). These results demonstrate an excellent correlation between the peak area and the BH concentration in the range studied. The limit of detection for BH was found to be 0.01 μg/mL (Detailed in Fig. S2 in the Supplementary information), indicating good sensitivity for the analytical method selected. The intra- and inter-day RSD for all the samples were below 6 %, and the relative errors (RE) ranged from −12.3 % to -5.1 % (Detailed in Table S2 in the Supplementary information). All these values are within an acceptable range, so the analytical method was considered accurate and sufficiently precise for our purpose. The recovery values at BH concentrations of 0.05, 0.1 and 1.0 μg/mL from blood plasma were 90.8 %, 96.1 % and 94.3 %, respectively (Detailed in Table S3 in the Supplementary information). The extraction recovery of IS was also calculated in the same manner

as for the QC samples at a concentration of 0.5 µg/mL. The mean recovery of IS was 95.3 ± 2.5 %.

3.2.2 Pharmacokinetic analysis

Pharmacokinetic analysis was carried out using a two-compartmental method, and pharmacokinetic parameters were obtained at the same time. The plasma concentration–time curves for BH after a single oral administration of liposomes containing 5 mg/kg of BH are illustrated in Fig. 9. The results of the pharmacokinetic analysis of the plasma concentration-time data are summarized in Table 2.

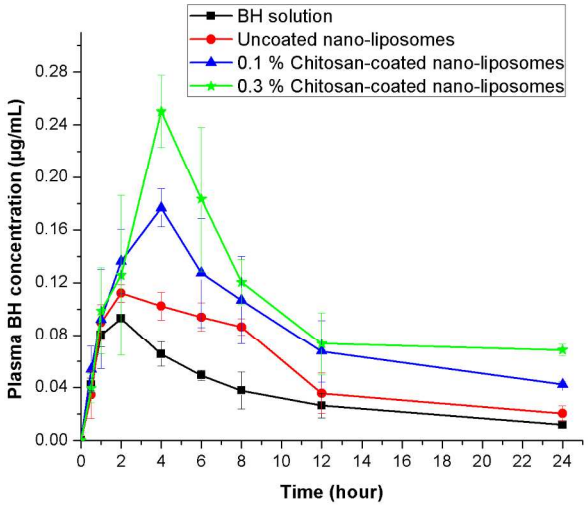

Fig. 9 Mean plasma concentration–time profiles for BH after oral administration of BH solution and nano-liposomes at 5 mg/kg in rabbits; each point and bar represents the mean ± SD (n = 3).

It is worth noting that the oral administration of BH solution and uncoated nano-liposomes led to a very low peak plasma concentration (C_{max} = 0.09 and 0.11 for BH solution and uncoated nano-liposomes, respectively), while significantly higher ($p < 0.05$) C_{max} values were achieved for chitosan-coated liposomes (C_{max} = 0.18 and 0.25 for 0.1 and 0.3 % chitosan, respectively). Similarly, T_{max} for 0.1 and 0.3 % chitosan-coated liposomes was 4 h, while it was 2 h for the BH solution and the uncoated liposomes. Moreover, all values of $AUC_{(0-t)}$, $AUC_{(0-\infty)}$, $AUMC_{(0-t)}$, $AUMC_{(0-\infty)}$, $MRT_{(0-t)}$ and $MRT_{(0-\infty)}$ for the coated liposomes were higher than for the uncoated samples. The AUC is inversely proportional to the clearance of the drug From the AUC and $AUMC$ values we can calculate the mean residence time, MRT. This is the average time that the drug stays in the body. Therefore coated samples could prolong time of the drug in the systemic circulation. These findings for our *in vivo* studies are in agreement with the *in vitro* results: This can be rationalized in terms of the previous observation that chitosan-coated liposomes were more stable and delayed the release in simulated GI fluids. Moreover, the mucoadhesive properties of chitosan should result in better oral bioavailability for coated than uncoated nano-liposomes. These results demonstrate that chitosan-coated nano-liposomes are more efficient than uncoated nano-liposomes in the oral delivery of BH.

Table 2 Pharmacokinetic parameters for BH after oral administration of solution and liposomes to rabbits at a dose of 5 mg/kg (n = 3).

Parameter	Unit	BH solution	Uncoated nano-liposomes	0.1 % Chitosan-coated nano-liposomes	0.3 % Chitosan-coated nano-liposomes
T_{max}	h	2	2	4	4
C_{max}	mg/L	0.09 ± 0.001	0.11 ± 0.01	0.18 ± 0.01	0.25 ± 0.03
$AUC_{(0-t)}$	mg/L.h	0.84 ± 0.10	1.31 ± 0.12	2.02 ± 0.36	2.41 ± 0.60
$AUC_{(0-\infty)}$	mg/L.h	1.09 ± 0.20	1.57 ± 0.22	2.66 ± 0.63	2.93 ± 1.06
$AUMC_{(0-t)}$	-	6.59 ± 1.36	10.79 ± 2.01	18.56 ± 2.88	24.43 ± 3.75
$AUMC_{(0-\infty)}$	-	17.13 ± 6.99	20.51 ± 6.92	50.52 ± 38.49	43.95 ± 22.26
$MRT_{(0-t)}$	h	7.77 ± 0.72	8.19 ± 0.93	9.21 ± 0.40	10.34 ± 1.27
$MRT_{(0-\infty)}$	h	15.40 ± 3.82	12.81 ± 2.99	17.70 ± 10.87	14.43 ± 2.54

4. Conclusions

This study has successfully demonstrated the engineering of a nano-sized BH delivery carrier for oral administration. Chitosan-coated nano-liposomes were prepared by deposition of positively charged chitosan on the surface of anionic nano-liposomes. The optimized formulation exhibited a nanoscale size ranging from 143 ± 5 nm to 194 ± 3 nm and 264 ± 8 nm for uncoated, 0.1 and 0.3 % chitosan-coated liposomes, respectively.

Chitosan-coated nano-liposomes had a better storage stability than uncoated nano-liposomes at both 4 °C and 25 °C over 30 days. Moreover, the 0.3 % chitosan-coated sample was more stable than the 0.1 % chitosan-coated one. The coated samples were also more stable in simulated GI fluid, but changes in size and zeta potential in SGF were less than in SIF. Increasing the chitosan concentration from 0.1 % to 0.3 % led to a decrease in the percentage of drug released in both SGF and SIF as compared to uncoated liposomes. Moreover, the BH release rate from all the liposomes was higher in SIF than in SGF.

All values of pharmacokinetic analysis for chitosan-coated nano-liposomes were higher than for the uncoated species. These results demonstrate that the chitosan-coated samples are more efficient than uncoated samples in the oral delivery of BH.

It can be concluded that the stability and delayed BH release in simulated GI environment were improved by using engineered chitosan-coated nano-liposomes. Moreover, since desirable *in vitro* and *in vivo* characteristics were achieved, they are promising release devices for the oral delivery of BH increasing the bioavailability of the drug.

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[a] *Department of Biomedical Engineering, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan 430074, China*
[b] *National Engineering Research Center for Nano-Medicine, Huazhong University of Science and Technology, Wuhan 430074, China*
[c] *Department of Human and Animal Physiology, Faculty of Biology-Agricultural Technology, Hanoi Pedagogical University No.2, Vietnam*
[d] *Wuhan East Lake High-tech Zone Administrative Committee, Wuhan 430079, China*
[e] *Department of Chemistry, University of Waterloo, 200 University Ave West, Waterloo, Ontario, Canada N2L 3G1*

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