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A Supersaturating Delivery System of Silibinin Exhibiting High Payload Achieved by Amorphous Nano-Complexation with Chitosan

Minh Hiep Nguyen, Hong Yu, Kunn Hadinoto*

School of Chemical and Biomedical Engineering, Nanyang Technological University, Singapore 637459

*To whom correspondence should be addressed:

Tel.: (65) 6514 8381, Fax: (65) 6794 7553, E-mail: kunnong@ntu.edu.sg

Abstract

The therapeutic potentials of silibinin - a **phytochemical** isolated from milk thistle plants - have not been fully realized due to its poor oral bioavailability caused by the low aqueous solubility. Existing solubility enhancement strategies of silibinin by nanonization were limited by their low payload. Herein we developed a supersaturating delivery system of silibinin exhibiting a high payload ($\approx 76\%$) in the form of amorphous silibinin-chitosan nanoparticle complex (or silibinin nanoplex in short) prepared by self-assembly drug-polysaccharide complexation. The effects of (1) pH and (2) charge ratio of chitosan to silibinin on the nanoplex's physical characteristics (i.e. size, zeta potential, and payload) and preparation efficiency (i.e. silibinin utilization, overall yield) were investigated. The formation of nanoplex (≈ 240 nm) was feasible only in a narrow pH range (5.1-5.8) and favored charge ratio below unity. At the optimal condition (pH 5.8 and charge ratio of 0.30), the nanoplex preparation exhibited 87% silibinin utilization rate and 63% yield signifying its high efficiency. The amorphous state and colloidal stabilities of the nanoplex during storage, and prolonged supersaturation generation (3 h) at more than $10\times$ of the saturation solubility were successfully demonstrated.

Reviewer #1:

The manuscript entitled "A Supersaturating Delivery System of Silibinin Exhibiting High Payload Achieved by Amorphous Nano-Complexation with Chitosan" is attempt to develop the amorphous Silibinin as a complex with the Cationic polymer Chitosan. In my view, a major revision is required on following points before being considered.

1. Abstract

Author said "silibinin - a medicinal herb isolated from milk thistle plants. Actually it's a phytochemical, not an herb (herb indicate a plant). It's is the major active constituent of silymarin (SLB).

Authors' response:

The authors would like to thank the reviewer for highlighting this mistake. In the revised submission, the above sentence has been corrected accordingly (line 31)

In Methods

2. In the XRD and FTIR study, got get the better presentation of the data it is important to write about the sample size (amount) used in the study. Therefore author should give the information about how much silibinin (alone), nanoplexes were used in the study.

Authors' response:

In the revised submission, the amounts of silibinin (alone) and silibinin nanoplex used in the PXRD and FTIR analysis have been provided (line 173 for FTIR and line 175 for PXRD). The same information has also been provided for the DSC analysis (lines 181-182).

3. The supportive study to corroborate the XRD, author should perform the DSC (Differential Scanning Calorimetry) analysis.

Authors' response:

The DSC analysis has been performed with the methods presented in lines 179-185 of the revised submission. The TGA/DSC results were presented in Fig. 7 and discussed in lines 277-282.

4. The analysis of SLB; author need to provide the brief about the UV-Vis method used. For example- what was the λ_{max} for the analysis? Moreover, what was the Conc. range for which the method is able to quantify (LOQ and the Max limit)?

Authors' response:

In the revised submission, the λ_{max} , LOQ, and the maximum limit for the UV-Vis spectrophotometry have been provided (lines 152-154)

Study need to perform:

5. To exemplify the significance of developing the amorphous nanoplexes, author need to perform the release study of SLB or in another word, the dissolution. After all, the improved solubility of amorphous

drug-polymer complex must improve the dissolution in simulated GIT fluid that will ultimately will indicate the possible improvement in oral bio-availability. So, the dissolution need to be performed in gastric simulated condition (pH1.2) As well as in the simulated intestinal condition (pH6.8).

Authors' response:

The dissolution time-profile of the silibinin nanoplex in SIJ and SGJ have been provided in the revised submission in Fig. 8. The results of the dissolution time-profile were discussed in lines 283-291.

6. For this study, in my view UV-Vis method for drug analysis will not be effective. So, it is advice here that author should reproduce any available HPLC method in the literatures or can develop their own in-house method for the quantification of drug from dissolution media.

Authors' response:

The authors agree with the reviewer that UV-Vis will not be effective enough to capture the dissolution time-profile of the nanoplex. Thus, the authors have decided to use HPLC instead of UV-Vis to carry out the dissolution time-profile study. The details of the HPLC protocols were presented in lines 192-195 of the revised submission.

7. Moreover, Modulated DSC STUDY OR XRD analysis OF THE SAMPLE FORMAUCTION IS REQUIRED TO CHECK THE STABILITY OF silymarin AS AMORPHOUS FORM. Author can perform the simple Modulated DSC STUDY. OR CAN CARRIED OUT THE XRD by keeping the sample on elevated temperature and humidity (best condition in my view is: accelerated stability testing as per ICH/us-FDA guideline that used for the solid dosage from).

Authors' response:

The authors agree with the reviewer that the long-term stability of the amorphous nanoplex ought to be demonstrated. Fortunately, we have kept the nanoplex sample prepared on 13 April 2015 in a dry cabinet set at 25°C and 55% relative humidity exactly for this purpose.

The PXRD analysis of the nanoplex sample after 11-month storage (i.e. 13 April 2015 to 11 March 2016) was presented in Fig. 4B in the revised submission. The appearance of amorphous halos showed the amorphous state stability of the nanoplex after the 11-month storage. The results were discussed in the text in lines 274-276.

Reviewer #2:

1. List of abbreviations (line 57): change curcumin with silibinin

Authors' response:

The authors would like to thank the reviewer for highlighting this mistake on our part. The abovementioned text has been corrected in the revised submission (line 59)

2. What is the supersaturation of silibinin obtained? Can the author provide any justification of choosing only one storage condition and the storage period of 3 months (not more than 3 months)?

Authors' response:

The supersaturation of silibinin was obtained at 12 and 22 times of its saturation solubility as discussed in lines 296-299 of the revised submission.

The authors agree with the reviewer that 3-month storage may be too short to obtain any conclusive data. Fortunately, we have been keeping the sample prepared on 13 April 2015 in a dry cabinet at 25°C and 55% relative humidity. Thus, we have replaced the results from 3-month storage with the results from 11-month storage in the revised submission (lines 177-178).

3. Fig. 4 (B): Some changes in the PXRD peak of SLB-CHI nanoplex after 3-month-storage can be observed (especially at 2θ of 5-15) as compared with freshly prepared sample. Please provide any reason for these changes.

Authors' response:

As addressed in Query #2, the authors have replaced the PXRD pattern from 3-month storage with that from 11-month storage. Unlike the PXRD pattern from 3-month storage, the new PXRD pattern presented in Fig. 4B of the revised submission did not show noticeable differences across all theta range from the PXRD pattern of the freshly prepared sample.

4. There could be higher chances for the amorphous samples to undergo chemical changes and the halo pattern of samples after 3 months storage might be due to the presence of degradant. It would be better if the author can provide chemical stability data of SLB-CHI nanoplex after storage.

Authors' response:

The authors agree with the reviewer that other stability data of the silibinin nanoplex was needed. For this purpose, we have carried out DSC analysis for the nanoplex stored for 11 months to show that the amorphous state has not recrystallized. The DSC thermogram was presented in Fig. 7 of the revised submission and the results were discussed in lines 277-282.

5. Is that possible to maintain the enhanced supersaturation of the SLB-CHI nanoplex after 3 months? If possible please provide the supersaturation data after 3 months storage.

Authors' response:

In the revised submission, the supersaturation level generated by the nanoplex after 11-month storage was presented in Fig. 9A at feed concentration equal to 15 times of the saturation solubility of silibinin. The results showed there was minimal variation in the supersaturation generation between the freshly prepared nanoplex sample and the stored nanoplex sample. This discussion has been added in the revised submission (lines 302-304)

1 **A Supersaturating Delivery System of Silibinin Exhibiting High Payload**
2 **Achieved by Amorphous Nano-Complexation with Chitosan**

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4 Minh-Hiep Nguyen, Hong Yu, **Bingxue Dong**, Kunn Hadinoto*
5 School of Chemical and Biomedical Engineering, Nanyang Technological University, Singapore 637459

6 *To whom correspondence should be addressed:
7 Tel.: (65) 6514 8381, Fax: (65) 6794 7553, E-mail: kunnong@ntu.edu.sg

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Abstract

The therapeutic potentials of silibinin - a phytochemical isolated from milk thistle plants - have not been fully realized due to its poor oral bioavailability caused by the low aqueous solubility. Existing solubility enhancement strategies of silibinin by nanonization were limited by their low payload. Herein we developed a supersaturating delivery system of silibinin exhibiting a high payload ($\approx 76\%$) in the form of amorphous silibinin-chitosan nanoparticle complex (or silibinin nanoplex in short) prepared by self-assembly drug-polysaccharide complexation. The effects of (1) pH and (2) charge ratio of chitosan to silibinin on the nanoplex's physical characteristics (i.e. size, zeta potential, and payload) and preparation efficiency (i.e. silibinin utilization, overall yield) were investigated. The formation of nanoplex (≈ 240 nm) was feasible only in a narrow pH range (5.1-5.8) and favored charge ratio below unity. At the optimal condition (pH 5.8 and charge ratio of 0.30), the nanoplex preparation exhibited 87% silibinin utilization rate and 63% yield signifying its high efficiency. The amorphous state and colloidal stabilities of the nanoplex during storage, and prolonged supersaturation generation (3 h) at more than $10\times$ of the saturation solubility were successfully demonstrated.

Keywords: silybin; silymarin; milk thistle; amorphous drug; nanopharmaceuticals

45 **List of abbreviations**

46	AA	acetic acid
47	C	supersaturated concentration
48	C _{Sat}	saturated solubility
49	CE	complexation efficiency
50	CHI	chitosan
51	DSC	differential scanning calorimetry
52	FESEM	field emission scanning electron microscopy
53	FTIR	Fourier transform infrared spectroscopy
54	HPLC	high performance liquid chromatography
55	HPMC	hydroxypropyl methylcellulose
56	PBS	phosphate buffered saline
57	PCS	photon correlation spectroscopy
58	PXRD	powder x-ray diffraction
59	R _{CHI/SLB}	charge ratio of chitosan to silibinin
60	SGJ	simulated gastric juice
61	SIJ	simulated intestinal juice
62	SLB	silibinin
63	TGA	thermogravimetric analysis
64	UV-Vis	ultraviolet visible spectroscopy
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66		

1. Introduction

The hepatoprotective effect of silibinin - a natural biologically active flavonolignans isolated from milk thistle plants - has been well established (Fraschini et al., 2002). Silibinin has also been demonstrated to possess antioxidant (Naso et al., 2011), anti-inflammatory (Gupta et al., 2000), and anticancer properties (Deep and Agarwal, 2010). The oral bioavailability of silibinin, however, is extremely low ($< 1\%$) caused primarily by its poor gut absorption and phase II metabolism in the liver (Theodosiou et al., 2014; Wu et al., 2007). Complexation of silibinin with phospholipids was shown to improve the gut absorption and in turn the bioavailability of silibinin attributed to the higher lipophilicity of the silibinin-phospholipid complex, resulting in better gut permeability (Kidd and Head, 2005; Xiao et al., 2006). A separate study, however, determined that the gut permeability was not the rate-limiting step in the gut absorption of silibinin, instead it was the slow dissolution rate of silibinin due to its poor solubility in the gastrointestinal fluid (Wang et al., 2010).

Hence, not coincidentally, a majority of studies on bioavailability enhancement of silibinin set their aims at improving the dissolution rate by means of nanonization to take advantage of the large specific surface areas afforded by nanoparticles. Various nanoformulation platforms ranging from liposomes (El-Samaligy et al., 2006), solid lipid nanoparticles (Zhang et al., 2007), polymer nanoparticles (Pooja et al., 2014) to porous silica nanoparticles (Cao et al., 2013) and nano-emulsions (Wu et al., 2006) have been employed as delivery vehicles for silibinin. Recently, a combined approach in which silibinin-phospholipid complex was encapsulated in liposomes was also pursued (Angelico et al., 2014).

These nanoformulation strategies, however, possess a major drawback in their low silibinin payloads ($< 15\%$ wt%). The low payload leads to a high dosing requirement to achieve the therapeutic effect in which a large fraction of the administered dose is made up of carrier materials that not only end up wasted, but also possibly have adverse health effects due to their large amount. Moreover, the high dosing requirement would make therapy regimen of silibinin too costly for most patients, hence limiting its potential for widespread clinical applications. To address this drawback, Wang et al. (2010) avoided the use of carriers altogether and developed carrier-free silibinin crystalline nanoparticles exhibiting high payload (75%). However, their nano-silibinin preparation was lengthy and energy-intensive involving multiple cycles of high-pressure homogenizations.

Furthermore, even though the abovementioned nanoformulation strategies could enhance the dissolution rate of silibinin, the amount of silibinin available for absorption remained limited due to its low thermodynamic saturation solubility (< 0.1 mg/mL) (Bai et al., 2006). A supersaturating drug delivery system that can generate a

highly supersaturated drug concentration upon dissolution is therefore ideal for silibinin as the said system can produce an apparent solubility that is multifold higher than the thermodynamic saturation solubility (Brouwers et al., 2009). Enhanced bioavailability would then ensue provided that the high apparent solubility is maintained for duration sufficient for gut absorption. This is achieved by incorporating crystallization inhibitors, such as hydroxypropyl methylcellulose (HPMC), in the dosage formulations (Tajarobi et al., 2011).

For this purpose, several studies have developed supersaturating delivery systems of silibinin in the form of microscale amorphous solid dispersions (Li and Hu, 2004; Qiu et al., 2005; Sun et al., 2008). These amorphous silibinin formulations, however, also exhibited low payloads due to the large amount of polymer excipient required in solid dispersions to stabilize the metastable amorphous form (Laitinen et al., 2013). Herein we developed a high-payload supersaturating delivery system of silibinin in the form of amorphous silibinin-chitosan nanoparticle complex (or nanoplex in short).

The amorphous silibinin nanoplex was prepared by the self-assembly electrostatically-driven drug-polysaccharide complexation developed previously by our group (Cheow et al., 2014; Nguyen et al., 2015). The method was simple, rapid, and having a low energy requirement involving only ambient mixing of the drug and polysaccharide solutions. Chitosan was used as the polysaccharide because (1) it could be readily ionized in base to produce charge opposite to silibinin, and (2) its inclusion in dosage formulation has been shown to improve intestinal absorption of the drug attributed to the chitosan-mediated epithelial tight junction opening (Sonaje et al., 2012; Yeh et al., 2011).

In this method, negatively charged silibinin (after its deprotonation in base) were mixed with oppositely charged chitosan, resulting in the self-assembly formation of soluble silibinin-chitosan complex as illustrated in Fig. 1. The soluble complex subsequently aggregated due to hydrophobic interactions among the bound silibinin molecules. Upon reaching a critical aggregate concentration, whose value was dictated by the hydrophobicity of silibinin, the complex aggregates precipitated out to form the silibinin nanoplex. The amorphous form was realized because the strong electrostatic interactions between silibinin and chitosan inhibited the former from assembling into ordered crystalline structures during precipitation.

The objectives of the present work were to investigate the effects of the two governing process variables in the drug-polysaccharide complexation (i.e. pH and charge ratio of chitosan to silibinin) on the (1) physical characteristics of the nanoplex produced (i.e. size, colloidal stability, and payload) and (2) preparation efficiency (i.e. silibinin utilization, overall yield). Subsequently, the silibinin nanoplex prepared at the optimal pH and

charge ratio was characterized for its (i) colloidal stability during storage at 25°C, (ii) supersaturation generation, and (iii) amorphous state stability during prolonged storage after drying.

2. Materials and Methods

2.1. Materials

Silibinin (SLB) (98% purity), low molecular weight chitosan (CHI) (50-190 kDa), potassium hydroxide (KOH), glacial acetic acid (AA), hydroxypropyl methylcellulose (HPMC), phosphate buffered saline (PBS, pH 7.4), sodium chloride (NaCl), hydrogen chloride (HCl), potassium phosphate monobasic (KH_2PO_4), potassium bromide (KBr), and ethanol were purchased from Sigma-Aldrich (USA).

2.2. Methods

2.2.1. Preparation of SLB-CHI nanoplex

SLB having pK_a of 6.6, 8.0, and 11.0 was deprotonated at pH 13 to form anionic SLB⁽³⁻⁾ with a charge density of 6.22×10^{-6} mol-charge/mg (Biedermann et al., 2014). CHI having pK_a of 6.5 was fully protonated at pH 2.8-3.1 with a charge density of 5.58×10^{-6} mol-charge/mg (Rinaudo et al., 1999). Briefly, SLB was dissolved in 0.1 M aqueous KOH solution (pH 13) at a fixed concentration of 5 mg/mL and CHI was dissolved in 0.2 to 1.0% (v/v) aqueous AA solution (pH 2.8-3.1). The amount of CHI dissolved in the AA solution was varied according to the charge ratio of CHI to SLB ($R_{\text{CHI/SLB}}$) investigated, which ranged from 0.30 to 1.20.

Next, the SLB solution was mixed with an equal volume of the CHI solution under gentle stirring after which the resultant SLB-CHI nanoplex suspension was vortexed for 10 s and ultrasonicated for 25 s using a probe sonicator (CV24, Sonics, USA). The suspension then underwent two cycles of centrifugation (14,000×g for 25 min) and washing, followed by its re-suspension in deionized water for characterizations. A control run in which the SLB solution was mixed with an equal volume of 0.5% (v/v) acetic acid solution (i.e. without CHI) was prepared. The SLB-CHI nanoplex preparation was carried out in five replicates on five different days.

The efficiency of the SLB-CHI nanoplex preparation was characterized by the complexation efficiency (CE) and the overall yield. The CE, which characterized the utilization rate of SLB, was defined in Eq. (1) and determined by taking the difference between the initial mass of SLB added and the mass of SLB recovered in the supernatant after the first centrifugation. The mass of SLB recovered in the supernatant was quantified by first diluting the supernatant in 80% (w/v) aqueous ethanol solution after which the SLB concentration was measured by ultraviolet visible (UV-Vis) spectrophotometry (UV Mini-1240, Shimadzu, Japan) at 287 nm at which the maximum absorbance was observed. At this wavelength, the minimum and maximum SLB

concentrations detectable were 4.5 and 36.7 µg/mL, respectively. The yield, which characterized the overall utilization of SLB and CHI, was defined in Eq. (2) and determined from the freeze-dried mass of the SLB-CHI nanoplex recovered after the two cycles of centrifugation and washing.

$$CE (\%) = \frac{\text{Mass of SLB that formed nanoplex}}{\text{Initial mass of SLB added}} \times 100 \quad (1)$$

$$\text{Yield } (\%) = \frac{\text{Mass of SLB-CHI nanoplex produced}}{\text{Initial mass of SLB and CHI added}} \times 100 \quad (2)$$

2.2.2. Physical characterizations of SLB-CHI nanoplex

The zeta potential and size of the SLB-CHI nanoplex suspension were determined by photon correlation spectroscopy (PCS) using Brookhaven 90 Plus Nanoparticle Size Analyzer (Brookhaven Instruments Corporation, USA). The nanoplex suspension was diluted 100 times with deionized water prior to the PCS measurement at 25°C. The morphology of the nanoplex was examined by Field Emission Scanning Electron Microscope (FESEM) (JSM-6700F, JEOL, USA) in which freeze-dried nanoplex powders were used after sputter coating them with platinum.

$$\text{Payload } (\%) = \frac{\text{Mass of SLB in SLB-CHI nanoplex}}{\text{Mass of SLB-CHI nanoplex}} \times 100 \quad (3)$$

The payload defined in Eq. (3) was determined by dissolving a known mass of the freeze-dried SLB-CHI nanoplex powders in 80% (v/v) ethanol after which the mass of SLB in the ethanol solution was quantified by UV-Vis spectrophotometry at 287 nm. The presence of SLB in the SLB-CHI nanoplex was verified by Fourier transform infrared spectroscopy (FTIR) (Spectrum One, Perkin-Elmer, USA) performed between 450 and 4000 cm⁻¹ at 1 cm⁻¹ spectral resolution. The FTIR was performed for the native SLB, native CHI, SLB-CHI nanoplex, and a physical mixture of the native SLB and CHI at an equal mass ratio. The FTIR pellets were prepared by die pressing of 2 mg of the nanoplex (containing 1.52 mg of SLB) and 100 mg KBr at 10 tons for 1 min.

The amorphous state of the SLB-CHI nanoplex was evaluated by powder x-ray diffraction (PXRD) using 10 mg of the nanoplex (containing 7.6 mg of SLB). The PXRD analysis was performed from 5° to 70° (2θ) with a step size of 0.02°/s using D8 Advance X-ray diffractometer equipped with Cu Kα radiation (Bruker, Germany). The PXRD analysis was performed for the native SLB and the SLB-CHI nanoplex immediately after its preparation and after long-term storage (i.e. eleven months) at 25°C and 55% relative humidity.

The amorphous state stability of the SLB-CHI nanoplex after long-term storage was examined by differential scanning calorimetry (DSC) using DSC 822e (Mettler Toledo, USA). The DSC analysis was

performed for the native SLB, native CHI, and SLB-CHI nanoplex. Briefly, 4 mg of the nanoplex (containing 3.04 mg of SLB) were filled into a sealed aluminium crucible and heated from 25°C to 400°C at 10°C/min. Simultaneously, thermogravimetric analysis (TGA) was performed using SDT Q600 (TA Instruments, USA), where 5 mg of powder (containing 3.8 mg of SLB) was filled into an alumina pan and heated from 25°C to 400°C at 10°C/min.

2.2.3. Supersaturation generation of the optimal SLB-CHI nanoplex

For the SLB-CHI nanoplex prepared at the optimal pH and $R_{\text{CHI/SLB}}$, its supersaturation generation was characterized in triplicates by the ratio of the supersaturated concentration generated (C) to the thermodynamic saturation solubility of SLB (C_{Sat}). C_{Sat} was first determined by incubating native SLB in excess in 20 mL PBS for 48 h under gentle stirring at 37°C. Afterwards, the SLB solution in PBS was centrifuged to remove excess SLB. Subsequently, the SLB concentration in the supernatant was quantified by UV-Vis spectrophotometry at 287 nm after its dilution in 80% (v/v) ethanol. C_{Sat} was verified with high performance liquid chromatography (HPLC) (Agilent 1100, Agilent Technologies, Singapore) using ZORBAX Eclipse Plus C18 column (250×4.6 mm and 5-μm particle size). The detection wavelength was set at 287 nm using 80% (v/v) aqueous ethanol solution as the mobile phase at 1 mL/min, resulting in SLB's retention time of 2.5 min.

The supersaturated SLB solution was generated by adding the SLB-CHI nanoplex in excess (i.e. 15 and $30 \times C_{\text{Sat}}$) to 8.5 mL PBS containing 2 mg/mL HPMC in a shaking incubator at 37°C. At a fixed time interval over three hours, 0.2 mL aliquot was withdrawn, filtered, and then diluted tenfold with fresh PBS to prevent precipitation of SLB from the supersaturated solution. The SLB concentration in the aliquot (C) was subsequently determined by UV-Vis spectrophotometry at 306 nm that represented the wavelength of maximum absorbance for SLB in PBS. The supersaturation generation at $15 \times C_{\text{Sat}}$ was also performed for the optimal SLB-CHI nanoplex after its long-term storage.

2.2.4. Dissolution time-profile of the optimal SLB-CHI nanoplex

The dissolution time-profile of the SLB-CHI nanoplex prepared at the optimal pH and $R_{\text{CHI/SLB}}$ was investigated under sink condition in simulated gastric juice (SGJ) and simulated intestinal juice (SIJ). Following Cook et al. (2011), the SGJ was prepared by adjusting the pH of 0.2% (w/v) NaCl solution to pH 1.2 using 1.0 M HCl, whereas the SIJ was prepared by dissolving 0.68 g of KH_2PO_4 in 75 mL of deionized water, followed by adjusting the pH to ≈ 6.8 using 0.2 M KOH and topping up the volume to 100 mL. Briefly, 0.1 mL of the nanoplex suspension was placed inside a dialysis bag filled with 1.9 mL PBS. The dialysis bag was then

immersed in 18 mL PBS held at 37°C in a shaking incubator. At fixed time intervals over a 2 h-period, 0.5 mL aliquots were withdrawn and the dissolution medium was replenished with fresh PBS of the same volume. The SLB concentration in the aliquot was subsequently quantified by the aforementioned HPLC protocols.

2.2.5. Colloidal stability of the optimal SLB-CHI nanoplex during storage

The colloidal stability of the SLB-CHI nanoplex prepared at the optimal pH and $R_{\text{CHI/SLB}}$ during a prolonged storage after its preparation was examined by incubating the nanoplex in deionized water at 25°C and neutral pH for 24 h. The colloidal stability was characterized in triplicates using PCS by monitoring the changes in the size and zeta potential of the nanoplex at fixed time intervals.

3. Results

3.1. Effect of pH on the SLB-CHI complexation

The effect of pH on the feasibility of preparing the SLB-CHI nanoplex was investigated at a fixed value of $R_{\text{CHI/SLB}}$ (≈ 1.0) at which there were equal amounts of charges available for complexation between SLB and CHI. The size of the particulate products obtained upon the mixing of SLB and CHI solutions was found to be highly dependent on the resultant pH of the mixture, which was dictated by the AA concentration used to dissolve CHI. At 0.2% and 0.4% (v/v) AA, which resulted in mixtures having alkaline pH of 12.2 and 11.6, respectively, microscale particles in the size range of 1.10 ± 0.08 and 1.51 ± 0.10 μm were produced, respectively. Larger particles in the size range of ≈ 3.4 to 7.7 μm were produced upon increasing the AA concentrations to 0.6%, 0.8%, and 1.0% (v/v), which resulted in pH of 5.1, 4.9, and 4.7, respectively (Fig. 2A).

Significantly, particles in the nanosize range (298 ± 24 nm) were produced only at 0.5% (v/v) AA in which the pH of the mixed solution was equal to 5.8. The nanoscale size reported from the PCS measurement was confirmed by the FESEM image showing individual nanoparticles around ≈ 100 nm in size (Fig. 2B). The nanoparticles exhibited a positive zeta potential at 25.6 ± 0.9 mV denoting the presence of CHI on their surface (Fig. 2A). This observation signified a successful preparation of the SLB-CHI nanoplex at pH 5.8. For comparison, the control run in which the SLB solution was mixed with 0.5% (v/v) aqueous AA solution without CHI produced particles with a strongly negative zeta potential at -36.9 ± 3.6 mV in the size range of 548 ± 11 nm (Fig. 2A). The particles in the control run were produced by pH-shift precipitation of SLB in acidic pH.

Significantly, all the particles produced upon mixing of the SLB and CHI solutions exhibited positive zeta potentials suggesting that the SLB-CHI complexation took place in the range of pH investigated, albeit a majority of the runs led to the production of microparticles, instead of nanoparticles. FESEM images of the

microparticles produced at 0.4%, 0.6%, and 1.0% (v/v) AA in Fig. 3 showed various morphologies suggesting that different phenomenon of particle formation occurred at the different pH.

At 0.6% (v/v) AA, the FESEM image in Fig. 3A showed that the microparticles detected by PCS were in fact attributed to the coalescence of several nanoparticles, where the size of the individual nanoparticles involved in the coalescence was comparable to the size of the dispersed nanoparticles produced at 0.5% (v/v) AA. The coalescence of the nanoplex prepared at 0.6% (AA) was likely attributed to its lower zeta potential compared to the nanoplex prepared at 0.5% (AA). In contrast, at 0.4% and 1.0% (v/v) AA, the FESEM images in Fig. 3B and 3C, respectively, indicated the absence of nanoparticle formation.

The presence of SLB in the SLB-CHI nanoplex prepared at 0.5% (v/v) AA was verified by the FTIR analysis. The peaks at 1639, 1509, and 1084 cm^{-1} , which corresponded to the stretching of the ketone group, aromatic ring vibrations, and benzopyran ring vibrations (Das et al., 2011), respectively, were used as the SLB identifiers as these functional groups were not present in the native CHI. The three peaks, which appeared in the FTIR spectra of the native SLB and the physical mixture of SLB and CHI, were also observed in the spectrum of the SLB-CHI nanoplex, hence confirming the presence of SLB in the nanoplex (Fig. 4A). The amorphous state of the SLB-CHI nanoplex was evident from its PXRD pattern in which broad amorphous halos were observed as opposed to the sharp crystalline peaks of the native SLB (Fig. 4B).

3.2. Optimal $R_{\text{CHI/SLB}}$ for SLB-CHI nanoplex preparation

Next, the effect of $R_{\text{CHI/SLB}}$ on the physical characteristics of the SLB-CHI nanoplex produced was investigated at the optimal pH of 5.8 obtained at 0.5% (v/v) AA. The size of the SLB-CHI nanoplex was found to be minimally affected by the $R_{\text{CHI/SLB}}$ variation for $0.30 \leq R_{\text{CHI/SLB}} \leq 1.05$, where the size fell in the narrow range of $\approx 240\text{-}340$ nm (Fig. 5A). At $R_{\text{CHI/SLB}} = 1.20$, however, significantly larger particles around 1.19 ± 0.29 μm were produced. The zeta potential, on the other hand, was found to increase from 20.8 ± 2.7 mV at $R_{\text{CHI/SLB}} = 0.30$ to reach the maximum of 28.8 ± 0.4 mV at $R_{\text{CHI/SLB}} = 0.75$, before it gradually decreased to 18.3 ± 1.2 mV at $R_{\text{CHI/SLB}} = 1.20$ (Fig. 5A).

The payload was found to gradually decrease with increasing $R_{\text{CHI/SLB}}$ from $76.3 \pm 2.9\%$ at $R_{\text{CHI/SLB}} = 0.30$ to $39.8 \pm 1.7\%$ at $R_{\text{CHI/SLB}} = 1.05$ (Fig. 5B). Upon increasing $R_{\text{CHI/SLB}}$ further to 1.20, the payload was drastically reduced to $4.0 \pm 1.3\%$ suggesting that the abovementioned large particles were made up of mostly CHI, hence their formation was not attributed to the coalescence of the SLB-CHI nanoplex.

In terms of the preparation efficiency, the CE and yield were found to be minimally affected by the change in $R_{\text{CHI/SLB}}$ for $0.30 \leq R_{\text{CHI/SLB}} \leq 1.05$, where their variations were still within the experimental uncertainties (Fig. 6). Specifically, the CE and yield were in the range of ≈ 83 -87% and 55-63%, respectively. At $R_{\text{CHI/SLB}} = 1.20$, the CE and yield were significantly lower at 21 ± 3 and $38 \pm 4\%$, respectively, which was not unexpected as the particles produced had a very low payload. Thus, the optimal $R_{\text{CHI/SLB}}$ was determined to be at 0.30 at which the SLB-CHI nanoplex exhibited the highest payload ($\approx 76\%$), while possessed relatively similar size (≈ 243 nm), zeta potential (≈ 21 mV), CE ($\approx 87\%$), and yield ($\approx 63\%$) as those obtained at the other $R_{\text{CHI/SLB}}$.

The amorphous state stability of the nanoplex prepared at the optimal $R_{\text{CHI/SLB}}$ after its long-term storage was successfully demonstrated by the appearance of broad amorphous halos in the PXRD pattern, denoting the absence of recrystallization of the amorphous form (Fig. 4B), which was further reaffirmed by the DSC analysis. The TGA thermograph showed that the native SLB and the native CHI started to degrade at 245 and 230°C, respectively, whereas the SLB-CHI nanoplex degraded at a slightly lower temperature of 215°C (Fig. 7A). Below the degradation temperature, the DSC thermograph of the native SLB showed an endothermic peak at 130-160°C corresponding to the melting point of the crystalline form, where the wide melting peak was likely caused by the presence of impurities in the native SLB (Fig. 7B). The DSC thermograph of the nanoplex did not show the melting point peak signifying that the nanoplex remained amorphous after the long-term storage. .

3.3. Dissolution time-profile of the optimal SLB-CHI nanoplex

The SLB-CHI nanoplex dissolved rapidly in the SIJ in which approximately 90% of the SLB was released in 20 min as the presence of salt ions in the SIJ provided the charge screening effect that weakened the electrostatic interaction between SLB and CHI, resulting in their de-complexation (Fig. 8A). For comparison, the native SLB powders of the same concentration took more than 24 h to fully dissolve in the SIJ due to its low solubility (data not shown). The SLB-CHI nanoplex dissolved at a slower rate in the SGJ caused by the lower solubility of SLB in acidic condition, where only about 60% of the SLB was released in 20 min (Fig. 8A). A closed-up look of the dissolution time-profile in the first 3 min (0.05 h) showed comparable dissolution rates of the nanoplex in SIJ and SGJ (Fig. 8B).

3.4 Supersaturation generation of the optimal SLB-CHI nanoplex

The supersaturation generation of the SLB-CHI nanoplex prepared at the optimal $R_{\text{CHI/SLB}}$ was successfully demonstrated in the presence of HPMC (Fig. 9A). The roles of HPMC here were twofold, i.e. (1) to suppress the solution-mediated crystallization of the dissolved SLB and (2) to inhibit the Ostwald-ripening crystallization of

the remaining SLB-CHI nanoplex undergoing dissolution. The addition of the SLB-CHI nanoplex at $15\times C_{\text{Sat}}$ to the dissolution medium containing 2 mg/mL HPMC produced a supersaturation level at $\approx 12\times C_{\text{Sat}}$ that was maintained for 3 h. A similarly effective supersaturation generation was observed upon adding the SLB-CHI nanoplex at $30\times C_{\text{Sat}}$, where a prolonged supersaturation level at $\approx 22\times C_{\text{Sat}}$ was produced (Fig. 9A).

The rate of the supersaturation generation was very rapid in which the maximum level was reached in just five minutes after dissolution attributed to the amorphous state and small size of the SLB-CHI nanoplex. Moreover, the effect of the long-term storage on the supersaturation generation upon the addition of the nanoplex at $15\times C_{\text{Sat}}$ was found to be minimal (Fig. 8A), which was not unexpected as the amorphous state stability of the nanoplex had been established earlier from the PXRD and DSC analysis.

3.5. Colloidal stability of the optimal SLB-CHI nanoplex

The colloidal stability of the SLB-CHI nanoplex prepared at the optimal $R_{\text{CHI/SLB}}$ was successfully demonstrated during ambient 24-h storage (Fig. 9B). While the nanoplex size showed some fluctuations during storage, the variation was limited to the narrow range of ≈ 195 to 255 nm. The zeta potential, on the other hand, was found to gradually decrease from 20.8 ± 2.7 mV immediately after preparation to 14.4 ± 1.8 mV and 9.3 ± 2.7 mV after 1 and 24 h, respectively, denoting the decreased presence of CHI on the nanoplex surface over time. Despite the lower zeta potential at 24 h, the SLB-CHI nanoplex did not agglomerate suggesting sufficient steric stabilization afforded by the CHI chains.

4. Discussion

4.1. pH dependence of SLB-CHI nanoplex formation at $R_{\text{CHI/SLB}} = 1.0$

The results showed that the formation of well-dispersed SLB-CHI nanoplex was highly sensitive to the resultant pH of the mixed SLB and CHI solution. This was despite both SLB and CHI were primed for electrostatic complexation as they were fully ionized in their alkaline (KOH) and acidic (AA) solutions, respectively. As the electrostatic binding between oppositely charged drug and polysaccharide molecules in drug-polysaccharide complexation takes place instantaneously (CaramLelham et al., 1997), the unsuccessful formation of the SLB-CHI nanoplex at certain pH was likely attributed to the disengagement of the SLB-CHI complex after the electrostatic binding took place.

In the highly alkaline pH region of the mixed solution (i.e. pH 11.6 and 12.2), the oppositely charged SLB and CHI molecules immediately formed electrostatic binding upon mixing, resulting in the formation of the soluble SLB-CHI complex. As the pH of the mixed solution equilibrated to the alkaline region, the high alkaline

solubility of SLB overcame the electrostatic binding causing the SLB-CHI complex to disengage. At the same time, as the pH rose above the pK_a of CHI, the charge of the bound CHI molecules gradually diminished, hence making the complex more prone to disengagement.

This phenomenon was evident from the photograph of the mixed solutions immediately after their preparation shown in Fig. S1 of the Supplementary Materials, where light yellowish color was observed for the mixed solutions prepared at the alkaline pH. This color was identical to the color of the SLB solution in KOH prior to mixing, thus indicating the disengagement of the complex. In contrast, the mixed solutions at pH 5.8 and 5.1 at which the SLB-CHI nanoplex was successfully prepared were cloudy due to the turbidity generated by the nanoparticles. As CHI precipitated out of the mixed solution due to its poor alkaline solubility, the large particles recovered after washing shown earlier in Fig. 3B were likely made up of mostly CHI.

Likewise, the unsuccessful formation of the SLB-CHI nanoplex at the two lowest pHs investigated (i.e. pH 4.7 and 4.9) was also believed to be caused by the disengagement of the electrostatically-bound SLB-CHI complex. As the pH of the mixed solution dropped further below the pK_a of SLB, the reduced charge of the bound SLB molecules was significant enough to cause the bound molecules to disengage from the complex. The SLB molecules then precipitated out of the mixed solution due to their poor solubility at acidic pH, which was evident from the cloudy appearance of the mixed solution denoting the presence of particulates (Fig. S1 of the Supplementary Materials). In this regard, the angular morphology of the particles recovered after washing shown earlier in Fig. 3C indicated that they were likely crystalline SLB, which was later confirmed by PXRD analysis (data not shown). The positive zeta potentials of the SLB precipitates indicated that the disengaged CHI molecules accumulated on the surface of the SLB precipitates.

4.2. $R_{CHI/SLB}$ dependence of SLB-CHI nanoplex prepared at the optimal pH

First, the high CE ($\approx 85\%$) obtained at $R_{CHI/SLB} < 1.05$ suggested that the SLB-CHI nanoplex formation did not require equal amounts of available charges between SLB and CHI to be effective. In drug-polysaccharide complexation, the electrostatic binding of drug molecules to the oppositely charged sites in the polysaccharide chains encourages the binding of other drug molecules at the same sites via inter-drug hydrophobic interactions, which is commonly referred as complexation by cooperative binding (CaramLelham et al., 1997). The high CE at $R_{CHI/SLB} < 1.05$ signified the importance of the cooperative binding in the SLB-CHI nanoplex formation, where hydrophobic interactions between the free SLB molecules and those that were already electrostatically

bound to the CHI chains resulted in the consistently high CE, regardless of the amount of oppositely charge available for electrostatic complexation from CHI.

Second, as the effect of $R_{\text{CHI/SLB}}$ was studied at a fixed SLB concentration, the constant CE values at $R_{\text{CHI/SLB}} \leq 1.05$ meant that the amount of the SLB molecules that formed the nanoplex was the same for all the $R_{\text{CHI/SLB}}$ investigated. The reduction in the payload with increasing $R_{\text{CHI/SLB}}$ thus indicated that a larger amount of CHI was present in the nanoplex at higher $R_{\text{CHI/SLB}}$. In other words, the more CHI molecules available for complexation, the more CHI molecules participated in the nanoplex formation, resulting in the lower payload. The increased participation of the CHI molecules at higher $R_{\text{CHI/SLB}}$ was also reflected in the constant yield observed at different $R_{\text{CHI/SLB}}$ for $R_{\text{CHI/SLB}} \leq 1.05$. As the size, CE, and yield were minimally affected by the $R_{\text{CHI/SLB}}$ variation, a lower $R_{\text{CHI/SLB}}$ was preferred owed to the higher payload obtained.

Third, the positive zeta potentials of the nanoplex prepared at different $R_{\text{CHI/SLB}}$ suggested that not all of the positive charges of the CHI molecules in the nanoplex participated in one-to-one electrostatic binding with the negative charges of the SLB molecules. In fact, the excess charges of the bound CHI molecules served as the charge stabilizer of the SLB-CHI nanoplex, in addition to their steric stabilization role, resulting in the high colloidal stability of the nanoplex. On this note, the increasing zeta potential with $R_{\text{CHI/SLB}}$ for $R_{\text{CHI/SLB}} \leq 0.75$ indicated that there were more excess charges of CHI in the nanoplex with increasing $R_{\text{CHI/SLB}}$. This was not unexpected as there was an increased presence of CHI in the nanoplex at higher $R_{\text{CHI/SLB}}$ as reflected by the correspondingly lower payload.

Upon increasing $R_{\text{CHI/SLB}}$ above 0.75, however, the zeta potential started to decrease denoting the reduction in the excess charge despite the ever increased presence of CHI in the nanoplex. The reduced excess charge implied that there was an increase in the one-to-one electrostatic binding between SLB and CHI for $0.75 < R_{\text{CHI/SLB}} \leq 1.05$. The increased in the electrostatic binding, however, did not lead to higher CE, which suggested that the extent of the cooperative binding was reduced in this $R_{\text{CHI/SLB}}$ range. The increased presence of CHI molecules at higher $R_{\text{CHI/SLB}}$ was believed to hinder the hydrophobic interactions among the SLB molecules.

On the same note, the unsuccessful formation of the SLB-CHI nanoplex at $R_{\text{CHI/SLB}} = 1.20$ signified that the excessive presence of the CHI molecules could hinder the complexation process. The electrostatic binding between SLB and CHI still occurred at $R_{\text{CHI/SLB}} = 1.20$ as both molecules were fully charged, resulting in the formation of soluble SLB-CHI complex. The low CE ($\approx 20\%$), which was determined from the supernatant of the mixed solution after the first centrifuge, indicated that a large amount of SLB was detected in the

supernatant. Thereby, it hinted that a majority of the SLB-CHI complex did not precipitate out of the mixed solution. The inhibited precipitation of the complex occurred when the complex had not reached the critical concentration due to the lack of aggregation among the complex.

As the aggregation of the complex was driven by the cooperative binding as illustrated earlier in Fig. 1, it was thus postulated that the cooperative binding was hindered by the excessive presence of CHI at $R_{\text{CHI/SLB}} = 1.20$. Moreover, the low payload ($\approx 4\%$) of the recovered particles indicated that the small amount of the SLB-CHI complex that had precipitated out of the mixed solution was disengaged during the washing step. The complex disengagement was likely caused by its loose structure due to the lack of cooperative binding, resulting in mostly CHI recovered as the particulate products.

5. Conclusion

A supersaturating delivery system of SLB exhibiting a high payload ($76 \pm 3\%$) was successfully developed in the form of amorphous SLB-CHI nanoplex prepared by a simple method based on drug-polysaccharide complexation. The nanoplex formation was found to be only feasible in a narrow pH range of around 5.1-5.8, which was due to the disengagement of the soluble SLB-CHI complex outside this pH range as a result of the weakened electrostatic binding between SLB and CHI. Subsequently, the optimal pH was determined to be at pH 5.8 attributed to the absence of nanoplex coalescence at this pH.

At the optimal pH, the impacts of $R_{\text{CHI/SLB}}$ for $R_{\text{CHI/SLB}}$ below unity on the size, zeta potential, CE, and yield of the nanoplex produced were found to be relatively insignificant. The minimal influence of $R_{\text{CHI/SLB}}$ on the CE signified the important role of the cooperative binding in the SLB-CHI nanoplex formation. The optimal $R_{\text{CHI/SLB}}$ was thus determined to be at the lowest $R_{\text{CHI/SLB}}$ investigated (i.e. 0.30) owed to the highest payload produced. The increasing presence of CHI at higher $R_{\text{CHI/SLB}}$, however, gradually hindered the cooperative binding, which eventually resulted in the failed nanoplex formation at $R_{\text{CHI/SLB}} = 1.20$.

At the optimal pH and $R_{\text{CHI/SLB}}$, the SLB-CHI nanoplex exhibited size and zeta potential of 243 ± 16 nm and 21 ± 3 mV, respectively, with CE and yield of $87 \pm 0.6\%$ and $63 \pm 2\%$, respectively. The amorphous state stability after long-term storage (i.e. eleven months), colloidal stability after 24 h, and prolonged supersaturation generation (3h) in the presence of HPMC at a level $>10\times$ of the saturation solubility were successfully established. These positive results bode well for the clinical potential of the amorphous SLB-CHI nanoplex as a new bioavailability enhancement strategy of SLB.

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References

- Angelico, R., Ceglie, A., Sacco, P., Colafemmina, G., Ripoli, M., Mangia, A., 2014. Phyto-liposomes as nanoshuttles for water-insoluble silybin-phospholipid complex. *Int. J. Pharm.* 471, 173-181.
- Bai, T.C., Yan, G.B., Hu, J., Zhang, H.L., Huang, C.G., 2006. Solubility of silybin in aqueous poly(ethylene glycol) solution. *Int. J. Pharm.* 308, 100-106.
- Biedermann, D., Vavrikova, E., Cvak, L., Kren, V., 2014. Chemistry of silybin. *Nat. Prod. Rep.* 31, 1138-1157.
- Brouwers, J., Brewster, M.E., Augustijns, P., 2009. Supersaturating Drug Delivery Systems: The Answer to Solubility-Limited Oral Bioavailability? *J. Pharm. Sci.* 98, 2549-2572.
- Cao, X., Deng, W.W., Fu, M., Zhu, Y., Liu, H.F., Wang, L., Zeng, J., Wei, Y.W., Xu, X.M., Yu, J.N., 2013. Seventy-two-hour release formulation of the poorly soluble drug silybin based on porous silica nanoparticles: In vitro release kinetics and in vitro/in vivo correlations in beagle dogs. *Eur. J. Pharm. Sci.* 48, 64-71.
- CaramLelham, N., Hed, F., Sundelof, L.O., 1997. Adsorption of charged amphiphiles to oppositely charged polysaccharides - A study of the influence of polysaccharide structure and hydrophobicity of the amphiphile molecule. *Biopolymers* 41, 765-772.
- Cheow, W.S., Kiew, T.Y., Yang, Y., Hadinoto, K., 2014. Amorphization Strategy Affects the Stability and Supersaturation Profile of Amorphous Drug Nanoparticles. *Mol. Pharmaceutics* 11, 1611-1620.
- Cook, M.T., Tzortzis, G., Charalampopoulos, D., Khutoryanskiy, V.V., 2011. Production and Evaluation of Dry Alginate-Chitosan Microcapsules as an Enteric Delivery Vehicle for Probiotic Bacteria. *Biomacromolecules* 12, 2834-2840.
- Das, S., Roy, P., Auddy, R.G., Mukherjee, A., 2011. Silymarin nanoparticle prevents paracetamol-induced hepatotoxicity. *Int. J. Nanomed* 6, 1291-1301.
- Deep, G., Agarwal, R., 2010. Antimetastatic efficacy of silibinin: molecular mechanisms and therapeutic potential against cancer. *Cancer Metastasis Rev.* 29, 447-463.
- El-Samaligy, M.S., Afifi, N.N., Mahmoud, E.A., 2006. Evaluation of hybrid liposomes-encapsulated silymarin regarding physical stability and in vivo performance. *Int. J. Pharm.* 319, 121-129.
- Fraschini, F., Demartini, G., Esposti, D., 2002. Pharmacology of silymarin. *Clin. Drug Invest.* 22, 51-65.
- Gupta, O.P., Sing, S., Bani, S., Sharma, N., Malhotra, S., Gupta, B.D., Banerjee, S.K., Handa, S.S., 2000. Anti-inflammatory and anti-arthritic activities of silymarin acting through inhibition of 5-lipoxygenase. *Phytomedicine* 7, 21-24.
- Kidd, P., Head, K., 2005. A review of the bioavailability and clinical efficacy of milk thistle phytosome: A silybin-phosphatidylcholine complex (siliphos (R)). *Altern. Med. Rev.* 10, 193-203.
- Laitinen, R., Lobmann, K., Strachan, C.J., Grohgan, H., Rades, T., 2013. Emerging trends in the stabilization of amorphous drugs. *Int. J. Pharm.* 453, 65-79.
- Li, F.Q., Hu, J.F., 2004. Improvement of the dissolution rate of silymarin by means of solid dispersions. *Chem. Pharm. Bull. (Tokyo)*. 52, 972-973.
- Naso, L.G., Ferrer, E.G., Butenko, N., Cavaco, I., Lezama, L., Rojo, T., Etcheverry, S.B., Williams, P.A.M., 2011. Antioxidant, DNA cleavage, and cellular effects of silibinin and a new oxovanadium(IV)/silibinin complex. *J. Biol. Inorg. Chem.* 16, 653-668.
- Nguyen, M.H., Yu, H., Kiew, T.Y., Hadinoto, K., 2015. Cost-effective alternative to nano-encapsulation: Amorphous curcumin-chitosan nanoparticle complex exhibiting high payload and supersaturation generation. *Eur. J. Pharm. Biopharm.* 96, 1-10.

457 Pooja, D., Bikkina, D.J.B., Kulhari, H., Nikhila, N., Chinde, S., Raghavendra, Y.M., Sreedhar, B., Tiwari, A.K.,
458 2014. Fabrication, characterization and bioevaluation of silibinin loaded chitosan nanoparticles. *Int. J.Biol.*
459 *Macromol.* 69, 267-273.

460 Qiu, M.F., Jia, W., Li, S.S., Xu, Z.H., Sun, X., Wang, X.R., Zhang, Y.Y., Xie, G.X., 2005. A new silymarin
461 preparation based on solid dispersion technique. *Adv. Ther.* 22, 595-600.

462 Rinaudo, M., Pavlov, G., Desbrieres, J., 1999. Influence of acetic acid concentration on the solubilization of
463 chitosan. *Polymer* 40, 7029-7032.

464 Sonaje, K., Chuang, E.Y., Lin, K.J., Yen, T.C., Su, F.Y., Tseng, M.T., Sung, H.W., 2012. Opening of Epithelial
465 Tight Junctions and Enhancement of Paracellular Permeation by Chitosan: Microscopic, Ultrastructural, and
466 Computed-Tomographic Observations. *Mol. Pharmaceutics* 9, 1271-1279.

467 Sun, N., Wei, X., Wu, B., Chen, J., Lu, Y., Wu, W., 2008. Enhanced dissolution of
468 silymarin/polyvinylpyrrolidone solid dispersion pellets prepared by a one-step fluid-bed coating technique.
469 *Powder Technol.* 182, 72-80.

470 Tajarobi, F., Larsson, A., Matic, H., Abrahmsen-Alami, S., 2011. The influence of crystallization inhibition of
471 HPMC and HPMCAS on model substance dissolution and release in swellable matrix tablets. *Eur. J. Pharm.*
472 *Biopharm.* 78, 125-133.

473 Theodosiou, E., Purchartova, K., Stamatis, H., Kolisis, F., Kren, V., 2014. Bioavailability of silymarin
474 flavonolignans: drug formulations and biotransformation. *Phytochem. Rev.* 13, 1-18.

475 Wang, Y., Zhang, D., Liu, Z., Liu, G., Duan, C., Jia, L., Feng, F., Zhang, X., Shi, Y., Zhang, Q., 2010. In vitro
476 and in vivo evaluation of silybin nanosuspensions for oral and intravenous delivery. *Nanotechnology* 21.

477 Wu, J.-W., Lin, L.-C., Hung, S.-C., Chi, C.-W., Tsai, T.-H., 2007. Analysis of silibinin in rat plasma and bile for
478 hepatobiliary excretion and oral bioavailability application. *J. Pharm. Biomed. Anal.* 45, 635-641.

479 Wu, W., Wang, Y., Que, L., 2006. Enhanced bioavailability of silymarin by self-microemulsifying drug delivery
480 system. *Eur. J. Pharm. Biopharm.* 63, 288-294.

481 Xiao, Y.Y., Song, Y.M., Chen, Z.P., Ping, Q.N., 2006. The preparation of silybin-phospholipid complex and the
482 study on its pharmacokinetics in rats. *Int. J. Pharm.* 307, 77-82.

483 Yeh, T.H., Hsu, L.W., Tseng, M.T., Lee, P.L., Sonjae, K., Ho, Y.C., Sung, H.W., 2011. Mechanism and
484 consequence of chitosan-mediated reversible epithelial tight junction opening. *Biomaterials* 32, 6164-6173.

485 Zhang, J.Q., Liu, J., Li, Y.L., Jasti, B.R., 2007. Preparation and characterization of solid lipid nanoparticles
486 containing Silibinin. *Drug Deliv.* 14, 381-387.

Figure captions

- Fig. 1 Complexation of silibinin (SLB) with oppositely charged chitosan (CHI) to form amorphous silibinin-chitosan nanoparticle complex (or SLB-CHI nanoplex in short)
- Fig. 2 A) The effects of preparation pH as dictated by the acetic acid concentration used to dissolve CHI on the size and zeta potential of the particulate products from the SLB-CHI complexation; (B) FESEM image of the SLB-CHI nanoplex successfully produced at 0.5% (v/v) acetic acid (pH 5.8)
- Fig. 3 FESEM images of the particles produced at acetic acid concentrations (v/v) of (A) 0.6% (pH 5.1); (B) 0.4% (pH 11.6); (C) 1.0% (pH 4.7)
- Fig. 4 (A) FTIR spectra of the native SLB, native CHI, their physical mixture (Phys. mixture), and the SLB-CHI nanoplex confirmed the presence of SLB in the nanoplex; (B) broad amorphous halos in the PXRD pattern of the SLB-CHI nanoplex confirmed its amorphous state and its solid-state stability after prolonged storage
- Fig. 5 The effects of the charge ratio of chitosan to silibinin ($R_{\text{CHI/SLB}}$) on the (A) size and zeta potential; (B) payload of the SLB-CHI nanoplex produced
- Fig. 6 The effects of the charge ratio of chitosan to silibinin ($R_{\text{CHI/SLB}}$) on the complexation efficiency (CE) and yield of the nanoplex preparation
- Fig. 7 (A) TGA and (B) DSC thermographs of the SLB-CHI nanoplex, native SLB, and native CHI
- Fig. 8 Dissolution time-profile of the SLB-CHI nanoplex in SIJ (pH 6.8) and SGJ (pH 1.2) during (A) 1-h period and (B) first 0.05 h
- Fig. 9 (A) Prolonged supersaturation generation of the SLB-CHI nanoplex in the presence of crystallization inhibitor (i.e. 2 mg/mL HPMC) upon initial nanoplex addition at 15× and 30× of the saturation solubility, where the minimal effect of long-term storage on the supersaturation generation was established; (B) colloidal stability of the SLB-CHI nanoplex characterized by the size and zeta potential variations during 24-h ambient storage after its preparation

Fig. 1

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Fig. 2

Fig. 2 (A) The effects of preparation pH as dictated by the acetic acid concentration used to dissolve CHI on the size and zeta potential of the particulate products from the SLB-CHI complexation; **(B)** FESEM image of the SLB-CHI nanoplex successfully produced at 0.5% (v/v) acetic acid (pH 5.8)

Fig. 3

Fig. 3 FESEM images of the particles produced at acetic acid concentrations (v/v) of (A) 0.6% (pH 5.1); (B) 0.4% (pH 11.6); (C) 1.0% (pH 4.7)

Fig. 4

Fig. 4 (A) FTIR spectra of the native SLB, native CHI, their physical mixture (Phys. mixture), and the SLB-CHI nanoplex confirmed the presence of SLB in the nanoplex; **(B)** broad amorphous halos in the PXRD pattern of the SLB-CHI nanoplex confirmed its amorphous state and its solid-state stability **after long-term storage of eleven months**

Fig. 5

Fig. 5 The effects of the charge ratio of chitosan to silibinin ($R_{CHI/SLB}$) on the (A) size and zeta potential; (B) payload of the SLB-CHI nanoplex produced

Fig. 6

Fig. 6 The effects of the charge ratio of chitosan to silibinin ($R_{\text{CHI/SLB}}$) on the complexation efficiency (CE) and yield of the nanoplex preparation

Fig. 7

Fig. 7 (A) TGA and (B) DSC thermographs of the SLB-CHI nanoplex, native SLB, and native CHI

Fig. 8

Fig. 8 Dissolution time-profile of the SLB-CHI nanoplex in SIJ (pH 6.8) and SGJ (pH 1.2) during (A) 1-h period and (B) first 0.05 h

Fig. 9

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Supplementary Material

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