Stable emulsion produced from casein and soy polysaccharide compacted complex for protection and oral delivery of curcumin

Guangrui Xu, Chaonan Wang, Ping Yao*

State Key Laboratory of Molecular Engineering of Polymers, Collaborative Innovation Center of Polymers and Polymer Composite Materials, Department of Macromolecular Science, Fudan University, Shanghai 200433, China

A R T I C L E   I N F O
Article history:
Received 10 March 2017
Received in revised form 29 April 2017
Accepted 11 May 2017
Available online 12 May 2017

Keywords:
Curcumin
Emulsion
Protein
Polysaccharide
Oral delivery
Stability

A B S T R A C T
Casein and soy soluble polysaccharide formed compacted complex aggregates in pH 3–4.5 aqueous solutions by electrostatic and hydrophobic interactions. The diameters of the complex aggregates were about 133 nm. The oil in water emulsion produced from the complex at pH 4 was stable after 500 days of storage in pH 2–6.8 media at 4 °C due to the compacted complex interfacial film. The curcumin loading efficiency of the emulsion was 99.9% and diameter of the droplets was about 324 nm. Only 3% of the loaded curcumin degraded when the emulsion was stored at 4 °C for 40 days. About 73% of the curcumin was released after 2 h digestion of the emulsion in simulated gastric fluid followed by 2 h digestion in simulated intestinal fluid. The curcumin pharmacokinetics in mice was analyzed after oral administration. The curcumin absorption of the emulsion treatment group was more rapid and effective than the absorption of curcumin/Tween 20 suspension treatment group. The curcumin oral bioavailability of the emulsion group was 11-fold higher than the bioavailability of curcumin/Tween 20 suspension group. This study demonstrated that casein and soy polysaccharide complex emulsion is an applicable system for oral delivery of lipophilic nutrients and drugs.

© 2017 Elsevier Ltd. All rights reserved.

1. Introduction

Many lipid soluble nutrients, such as β-carotene and curcumin, are beneficial to health including disease prevention; however, their low solubility and poor stability in aqueous solution result in their low bioavailability (Li, Jiang, Xu, & Gu, 2015). In order to enhance oral bioavailability of lipophilic nutrients and also lipophilic drugs, many delivery systems have been reported (Date, Hanes, & Ensign, 2016; Porter, Trevaskis, & Charman, 2007; Yao, McClements, & Xiao, 2015). Oil in water emulsion is one of the oral delivery systems, which can effectively load lipophilic drugs and nutrients in oil droplets, increase their dispersibility and stability in aqueous solution as well as improve their oral bioavailability (Kotta et al., 2012; McClements, 2013). So far, the reported emulsions for drug and nutrient delivery are mainly produced from synthetic surfactants (Wadhwa, Nair, & Kumria, 2012). Due to the potential toxicity of synthetic surfactants (Chassaing et al., 2015), the emulsions produced from protein and polysaccharide are of great interest (Bouyer, Mekhloufi, Rosilio, Grossiord, & Agnely, 2012).

Food proteins and polysaccharides have advantages of low cost, high nutrition and safety. Many food proteins, such as soy protein, whey protein and casein are widely used as emulsifiers in food industry (Chen, Wu, McClements, Li, & Li, 2017; Dickinson, 2006; Nishimari, Fang, Guo, & Phillips, 2014). Generally, the emulsions stabilized by proteins are sensitive to temperature, pH, salt etc. environmental stresses, which result in coalescence, flocculation, creaming and phase separation (Dickinson, 2010; Lam & Nickerson, 2013; McClements, 2004). Polysaccharides can improve the stability of protein emulsions on storage (Dickinson, 2009; Evans, Ratcliffe, & Williams, 2013) and also on simulated gastrointestinal conditions, therefore can change the digestion behavior of the emulsions as well as the absorption efficiency of the loaded drugs, nutrients and lipids in gastrointestinal tract (Chang & McClements, 2016; Wang, Liu, Xu, Yin, & Yao, 2016; Xu et al., 2014; Yang et al., 2015).

Soy soluble polysaccharide (SSPS), extracted from the byproduct of the isolation of soy protein, is used as dietary fiber in food (Chen, Duizer, Corredig, & Goff, 2010). SSPS is a negatively charged polysaccharide which contains galacturonic acid (about...
Casein (CN) is a predominant protein in milk, and is consisted of αs1-, αs2-, β- and κ-casein approximately in proportions of 4:1:4:1 by weight in cow milk (Suh, Decker, & McClements, 2006). CN is soluble in neutral and alkaline aqueous solutions in the form of sodium caseinate; its solubility is poor at pH around its isoelectric point, pH 4.6 (Li, Fang, Phillips, & Al-Assaf, 2013). All four CN components are linear amphiphatic macromolecules and have high surface activity (Horne, 2002). The emulsion produced using CN as emulsifier could increase postprandial bioavailability of interesterified-lipids after oral gastric feeding to SD rats (Farfan, Villalon, Ortiz, Nieto, & Bouchon, 2015). Unlike globular protein, CN does not have heat-induced gelation property (Suh et al., 2006) and therefore cannot form irreversible oil-water interfacial films by heat treatment. The emulsions produced from CN are not stable, especially at the pH around its isoelectric point (Liu, Verespej, Corredig, & Alexander, 2008). Polysaccharides, for example, xanthan gum (Liu, Zhao, Kong, & Zhao, 2012), pectin (Liu, Verespej, Alexander, & Corredig, 2007; Liu et al., 2008; Suh et al., 2006), SSPS (Liu et al., 2007; Liu et al., 2008) and dextran sulfate (Jourdain, Leser, Schmitt, Michel, & Dickinson, 2008) were introduced to adsorb on the droplet surfaces after emulsification to enhance the stability of CN emulsions. The complexes of CN with polysaccharides, such as dextran sulfate (Jourdain et al., 2008), pectin (Li et al., 2013) and flaxseed gum (Zhuo et al., 2015), formed before emulsification, were also reported as complex emulsifiers to produce more stable emulsions. CN is insoluble in the pH range of 3.5–5.5; negatively charged dextran sulfate could inhibit the precipitation of CN by binding with CN via electrostatic interaction, and soluble CN/dextran sulfate complex could produce fine emulsions at pH 2, 4 and 6 (Jourdain et al., 2008). Sugar beet pectin (SBP) could bind with CN via electrostatic and hydrophobic interactions, increased proportions of SBP might suppress self-aggregation of CN at pH < 5.6, and the emulsions containing 1.2 wt% SBP and 0.3 wt% CN were stable at pH 4.5 (Li et al., 2013). However, to the best of our knowledge, the CN emulsion with long-term stability in a broader pH range has not been reported.

Our previous study proved that apo cytchrome c, a linear protein, could bind with alternating copolymers of maleic acid and alkene via electrostatic and hydrophobic interactions; the binding could destroy the protein aggregates as well as the polymer aggregates, forming more compacted complex aggregates (Liang, Yao, Jiang, Zhang, & Yan, 2005). Similarly, CN is a linear protein and forms micelles (Li et al., 2013), and SSPS is a negatively charged amphiphatic macromolecule. Therefore, we speculated that CN and SSPS could form compacted complex aggregates via electrostatic and hydrophobic interactions. Furthermore, we speculated that CN and SSPS could form compacted complex interfacial films at optimal condition and the emulsions with compacted complex interfacial films were stable in a broad pH range without heat treatment. Herein, we investigated the complexion of CN with SSPS and the stability of the complex emulsion in the pH range of 2–7 to prove our speculations. In addition, curcumin (CUR), a natural polyphenol isolated from the herb Curcuma longa, was used as a model of lipophilic drugs and nutrients. The CUR loading and protection effect of the complex emulsion were investigated, the release of the loaded CUR on simulated gastrointestinal conditions was studied, and the enhancement effect of CUR oral bioavailability of the emulsion in mice was evaluated.

2. Materials and methods

2.1. Materials

Casein (CN, technical grade) was purchased from Sigma-Aldrich (Shanghai, China). Soybean soluble polysaccharide (SSPS, Soyabife-S-CA100, crude protein 6.3%, moisture 5.6%, ash 7.8%) was from Fuji Oil Co., Ltd. (Osaka, Japan). Medium chain triglyceride (MCT) for injection was from Avic (Tieling) Pharmaceutical Co., Ltd. (Tieling, Liaoning, China). Pancreatin-8.0 (from porcine pancreas; protease 214 USP/j, amylase 214 USP/j and lipase 24.2 USP/j), pepsin (from porcine stomach; 3000–3500 NFU/j, curcumin (CUR, 95%), vitamin E (VE) and bile salts (from bovine) were purchased from Sangon Biotech Shanghai Co., Ltd. (Shanghai, China). DiR (1,10-dioctadecyl-3,3,30,30-tetramethyl indotricarbocyanine iodide) was supplied by Rufai Bio Beijing Co., Ltd. (Beijing, China). All other reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Preparation of CN and SSPS stock solutions and CN/SSPS complex solutions

CN was added in deionized water and the solution was adjusted to pH 7.5 with 1 M NaOH. After overnight stirring, the pH value was checked and adjusted to pH 7.5 again. The final CN concentration was 20 mg/mL in CN stock solution. SSPS stock solution with a concentration of 20 mg/mL was prepared by dissolving SSPS in deionized water and then adjusting the solution pH to 7.5. NaN3 with a final concentration of 0.02% was added to inhibit microbial growth. The CN and SSPS stock solutions were mixed at a volume ratio of 1:1, and then the mixed solution was adjusted to predetermined pH value using 1 M HCl or NaOH to obtain CN/SSPS complex solution.

2.3. Preparation of CN/SSPS emulsion (@CN/SSPS) and curcumin-loaded CN/SSPS emulsion (CUR@CN/SSPS)

For @CN/SSPS emulsion, the aqueous phase was prepared as follows. The CN and SSPS stock solutions were mixed at a volume ratio of 1:1; the mixed solution was stirred at room temperature for 3 h, subsequently, the solution was adjusted to pH 3.0, 3.5, 4.0 or 4.5 and then stirred for another 3 h. MCT and VE mixture with a volume ratio of 1:1 was used as the oil phase. The oil phase with 16.7% volume fraction was added into the aqueous phase. The mixture was pre-emulsified using a homogenizer (FJ200-S, Shanghai Specimen Model Co.) at 10,000 rpm for 1 min and was immediately emulsified using a high-pressure homogenizer (AH100D, ATS Engineering Inc.) at 800 bar for 4 min. The resultant @CN/SSPS
emulsion was adjusted to predetermined pH values using 1 M HCl or NaOH solution and then was stored at 4 °C. Similarly, individual CN emulsion (@CN) and individual SSPS emulsion (@SSPS) were prepared using the MCT and VE mixture as the oil phase. For @CN and @SSPS emulsions, the CN and SSPS concentrations in the aqueous phases were 10 and 20 mg/mL, and the emulsification pH values were 7.0 and 5.5, respectively.

For CUR@CN/SSPS emulsion, the aqueous phase was the CN/SSPS complex solution with pH 4.0 prepared as described above. The oil phase contained 10 mg/mL CUR, 10% (v/v) ethanol and 90% (v/v) MCT. The other emulsification conditions were the same as the conditions of @CN/SSPS emulsion. Similarly, hydrophobic fluorescence probe DiR was dissolved in MCT with DiR concentration of 100 μg/mL to prepare DiR-loaded CN/SSPS emulsion (DiR@CN/SSPS) for fluorescence imaging.

2.4. Characterization

2.4.1. Dynamic light scattering (DLS) and ζ-potential measurements

Z-average hydrodynamic diameter (Dh), polydispersity index (PDI) and ζ-potential measurements were performed on a laser light scattering instrument (Zetasizer Nano ZS90, Malvern Instruments). For Dh and PDI measurements, the CN and SSPS aqueous solution samples and CN/SSPS complex solution samples contained 10 mg/mL CN and/or 10 mg/mL SSPS. The emulsion samples were prepared by 1000-fold dilution of the emulsions with deionized water freshly before the measurement. The measurements were carried out at 25 °C and 90° scattering angle. The Dh and PDI values were obtained by Automatic analysis mode. At least 2 batches of the emulsions produced at the same condition were measured to assess reproducibility.

For ζ-potential measurements, the samples were prepared as follows. Firstly, the CN solution, SSPS solution, CN/SSPS complex solution and the emulsions were adjusted to desired pH values. Subsequently, the aqueous solutions were diluted to 1 mg/mL CN and/or 1 mg/mL SSPS concentrations, and the emulsions were diluted by 1000-fold with the same pH aqueous solutions containing 5 mM NaCl. The ζ-potentials were calculated by the Dispersion Technology Software provided by Malvern according to the Henry equation and Smoluchowski approximation (Pan, Yu, Yao, & Shao, 2007).

2.4.2. Transmission electron microscopy (TEM) measurements

TEM images of the emulsion droplets were acquired on an electron microscope (FEI Tecnai G2 TWIN, FEI Company). The TEM sample was prepared by depositing diluted emulsion onto a carbon-coated copper grid and drying at room temperature in a desiccator containing anhydrous silica gel.

2.4.3. CUR loading efficiency of CUR@CN/SSPS emulsion

Unloaded CUR in CUR@CN/SSPS emulsion was extracted by addition of 2 mL chloroform into 1 mL CUR@CN/SSPS emulsion for three times. The collected chloroform solutions were mixed together and dried by rotational evaporation under reduced pressure, and then the extracted CUR was redissolved in acetonitrile. The absorbance of the CUR acetonitrile solution at 420 nm was measured to determine the CUR concentration. Similarly, hydrophobic fluorescence probe DiR was dissolved in MCT with DiR concentration of 100 μg/mL to prepare DiR-loaded CN/SSPS emulsion (DiR@CN/SSPS) for fluorescence imaging.

2.4.4. CUR chemical stability of CUR@CN/SSPS emulsion

Freshly prepared CUR@CN/SSPS emulsion was stored at 4, 25 or 37 °C in the dark or without shading light for predetermined period. During the storage, the appearance of the emulsion was observed and the CUR concentration in the emulsion was analyzed as follows. The emulsion was diluted by 1000-fold with pH 4.0 aqueous solution and the absorbance of the diluted emulsion at 420 nm was measured using the same diluted @CN/SSPS emulsion as the reference solution. Integrating sphere was used in the measurement to eliminate the influence of scattering light. The work curve was obtained by analysis of freshly prepared CUR@CN/SSPS emulsion after diluting to desired CUR concentrations.

Free CUR solution with CUR concentration of 1.67 mg/mL was prepared by dilution of 10 mg/mL CUR ethanol solution with pH 4.0 aqueous solution. After storage at 37 °C in the dark, the absorbance of the free CUR solution at 420 nm was measured to determine the CUR degradation ratio.

2.5. Ex vivo fluorescence imaging of mice gastrointestinal tracts

All animal experiments of this study were carried out at Experimental Animal Center of School of Pharmacy of Fudan University in full compliance with the guidelines approved by Shanghai Administration of Experimental Animals. Male ICR mice (18–22 g) were from Sino-British SIPPR/BK Lab. Animal Ltd. (Shanghai, China). The mice were fasting overnight with free access to water prior to administration. DiR@CN/SSPS emulsion of 0.5 mL was administered by oral gavage. The mice were free access to water and standard chow after oral administration. At 0.5, 1, 2, 4, 6, 8 and 24 h post-administration, the mice were sacrificed and the organs of stomach and intestine were excised. After washing the organ surfaces with deionized water, fluorescence images of the organs were acquired and the fluorescence intensities were measured on a small animal imaging system (In Vivo Xtreme, Bruker) at excitation wavelength of 720 nm and emission wavelength of 790 nm.

2.6. CUR release from CUR@CN/SSPS emulsion at simulated gastrointestinal conditions

In vitro sequential digestions of the emulsion at simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were carried out according to the method reported by Gumus, Davidov-Pardo, and McClements (2016) with a bit of modification. SGF was prepared by dissolving 32 mg pepsin in 10 mL pH 1.2 HCl solution. SIF was prepared by dissolving pancreatin and bile salts in PBS (10 mM pH 7.4 phosphate buffer containing 150 mM NaCl) together at the concentrations of 3.2 mg/mL pancreatin and 25 mg/mL bile salts. After adjusting the pH to 1.2, CUR@CN/SSPS emulsion was mixed with SGF at 1:1 volume ratio to simulate gastric digestion process. After 2 h of the digestion in SGF, the digestion solution was adjusted to pH 7.4 with 1 M NaOH and then equal volume of SIF was added to simulate intestinal digestion process; the volume ratios of CUR@CN/SSPS:SGF:SIF were 1:1:2. The digestions were performed at 37 °C with shaking. At predetermined time intervals, 1 mL of the digestion solution was taken out and released CUR in the solution was extracted using chloroform as described above. The absorbance of the chloroform solution at 420 nm was measured to determine the CUR concentration. The work curve was obtained by analysis of a series of standard CUR chloroform solutions.

2.7. CUR plasma concentration analysis

Male ICR mice (about 25 g) were randomly assigned to two treatment groups with 30 in each group. CUR suspension was...
freshly prepared before administration by dispersing CUR in 1% Tween 20 aqueous solution and used as a control as reported in the literature (Mohanty & Sahoo, 2010; Wang et al., 2016). The mice were fasting overnight with free access to water before administration. CUR@CN/SSPS emulsion and CUR/Tween 20 suspension were separately administered by oral gavage at a single CUR dose of 50 mg/kg. After oral administration, the mice were free access to water and standard chow. At each predetermined interval (0.5, 1, 2, 4, and 24 h post-administration), five mice in each group were sampled. About 500 μL blood was collected from eye ground vein, placed into microtube preprocessed with EDTA, and centrifuged at 1810 g and 4 °C for 10 min immediately. The plasma was stored at −80 °C before analysis. The plasma sample of 150 μL was mixed with 350 μL acetonitrile. After vortex for 2 min, the mixture was centrifuged at 7260 g and 4 °C for 30 min. The supernatant was analyzed on a HPLC system (Agilent 1100, Agilent Technologies) and the CUR was detected by 420 nm absorbance (Wang et al., 2016). A series of standard CUR acetone solutions were separately mixed with blank plasmas without CUR, after the same vortex, centrifugation and HPLC analysis described above, the CUR work curve was obtained.

2.8. Statistical analysis

The data were expressed as mean ± s.d. (standard deviation). Statistical analysis was performed using Microsoft Excel 2010 software. A P value < 0.05 was considered to be statistically significant.

3. Results and discussion

3.1. CN/SSPS complex

In this study, we used CN and SSPS electrostatic complex as emulsifier and stabilizer to produce oil in water emulsion to encapsulate hydrophobic CUR. As mentioned above, CN forms micelles in aqueous solution due to its amphiphilic property and CN precipitates at the pH around its isoelectric point where its net charge is about zero. The commercial CN we used in this study was precipitated. About 500 μL blood was collected from eye ground vein, placed into microtube preprocessed with EDTA, and centrifuged at 1810 g and 4 °C for 10 min immediately. The plasma was stored at −80 °C before analysis. The plasma sample of 150 μL was mixed with 350 μL acetonitrile. After vortex for 2 min, the mixture was centrifuged at 7260 g and 4 °C for 30 min. The supernatant was analyzed on a HPLC system (Agilent 1100, Agilent Technologies) and the CUR was detected by 420 nm absorbance (Wang et al., 2016). A series of standard CUR acetone solutions were separately mixed with blank plasmas without CUR, after the same vortex, centrifugation and HPLC analysis described above, the CUR work curve was obtained.

negative charges in the pH range of 3–8 and the negative charges increased gradually from pH 3 to 6. The ζ-potential result indicates that CN and SSPS carry opposite charges and thus they can form electrostatic complex in the pH range of 3–4.5.

The CN and SSPS solutions with pH 7.5 were mixed together. After mixing, the CN concentration was 10 mg/mL and SSPS concentration was also 10 mg/mL. The mixed solution was adjusted to different pH values using 1 M HCl and aggregation behaviors of the acidized solutions were investigated. The CN/SSPS solutions with pH 3–5 did not present precipitates, indicating that CN and SSPS formed complex in this pH range and the complex could inhibit the CN from precipitation completely. The Dh values of the aggregates in the pH 3–5 CN/SSPS solutions were 132–140 nm (Table 1), which were smaller than the Dh values of the aggregates formed in the individual CN and SSPS solutions, and also smaller than the Dh values of the particles formed in the CN/SSPS solutions with pH 2, 6, 7 and 8. The scattering intensities of the pH 3–5 CN/SSPS solutions were much stronger than the intensities of the individual CN and SSPS solutions, and also much stronger than the intensities of the CN/SSPS solutions with pH 2, 6, 7 and 8. As we know, scattering intensity is proportional to both the number of scatterers and the square of the scattering’s mass (Li, He, Ng, Wu, & Ng, 2000). The smaller Dh values and stronger scattering intensities of the pH 3–5 CN/SSPS solutions mean that there were more aggregates in these solutions and the aggregates had more compacted structure. As mentioned above, both CN and SSPS are amphiphilic macromolecules and they carry opposite charges in the pH range of 3–4.5. Therefore, CN and SSPS can form complex via electrostatic and

![Fig. 1. ζ-Potentials of CN, SSPS and CN/SSPS solutions at various pH conditions. CN concentration was 1 mg/mL and/or SSPS concentration was 1 mg/mL in the solutions.](image)

Table 1

<table>
<thead>
<tr>
<th>pH</th>
<th>CN</th>
<th>Intensity (kcps)</th>
<th>SSPS</th>
<th>Intensity (kcps)</th>
<th>CN/SSPS</th>
<th>Intensity (kcps)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>206 ± 12</td>
<td>1097 ± 22</td>
<td>206 ± 12</td>
<td>1097 ± 22</td>
<td>206 ± 12</td>
<td>1097 ± 22</td>
</tr>
<tr>
<td>3.0</td>
<td>306 ± 10</td>
<td>833 ± 18</td>
<td>306 ± 10</td>
<td>833 ± 18</td>
<td>306 ± 10</td>
<td>833 ± 18</td>
</tr>
<tr>
<td>4.0</td>
<td>306 ± 10</td>
<td>833 ± 18</td>
<td>306 ± 10</td>
<td>833 ± 18</td>
<td>306 ± 10</td>
<td>833 ± 18</td>
</tr>
<tr>
<td>5.0</td>
<td>206 ± 12</td>
<td>1097 ± 22</td>
<td>206 ± 12</td>
<td>1097 ± 22</td>
<td>206 ± 12</td>
<td>1097 ± 22</td>
</tr>
<tr>
<td>6.0</td>
<td>206 ± 12</td>
<td>1097 ± 22</td>
<td>206 ± 12</td>
<td>1097 ± 22</td>
<td>206 ± 12</td>
<td>1097 ± 22</td>
</tr>
<tr>
<td>7.0</td>
<td>206 ± 12</td>
<td>1097 ± 22</td>
<td>206 ± 12</td>
<td>1097 ± 22</td>
<td>206 ± 12</td>
<td>1097 ± 22</td>
</tr>
<tr>
<td>8.0</td>
<td>206 ± 12</td>
<td>1097 ± 22</td>
<td>206 ± 12</td>
<td>1097 ± 22</td>
<td>206 ± 12</td>
<td>1097 ± 22</td>
</tr>
</tbody>
</table>

* CN concentration was 10 mg/mL and/or SSPS concentration was 10 mg/mL.

* CN precipitated.
hydrophobic interactions. At pH 5, both CN and SSPS carry negative charges as shown in Fig. 1. Possibly, the formation of CN/SSPS complex at pH 5 is similar to the formation of CN/pectin complex at pH 5.6 reported by Li et al. (2013). Protein molecules have heterogeneous charge distribution; the electrostatic and hydrophobic interactions between protein and the macromolecule with amphipathy and negative charges at the pH higher than the isoelectric point of protein account for the formation of the complex (Liang et al., 2005). In addition, the $D_h$ values of the CN/SSPS complex aggregates formed at pH 3–5 were significantly smaller than those of individual CN and SSPS aggregates formed in the pH range of 2–8, suggesting that the complexation of CN with SSPS via electrostatic and hydrophobic interactions at pH 3–5 destroyed the original aggregates of CN and SSPS. This result is consistent with our previous study that the binding of linear protein with linear polymer via electrostatic and hydrophobic interactions can destroy the protein aggregates and polymer aggregates forming more compacted complex aggregates (Liang et al., 2005).

When the CN/SSPS solution was acidized to pH 2, the $D_h$ value increased and the intensity decreased compared with the $D_h$ values and intensities of the pH 3–5 CN/SSPS solutions (Table 1). This result can be explained by the fact that SSPS has no charges at pH 2 (Fig. 1). The interactions between CN and SSPS decreased, and thus the number of the complex aggregates formed at pH 2 decreased and also the aggregates had less compacted structure compared with the aggregates formed at pH 3–5. At pH 6, the $D_h$ of the CN/SSPS solution was 624 nm, which was much larger than that of the CN solution. However, the scattering intensity of the CN/SSPS solution was much weaker than the intensity of the CN solution, indicating that the number of the aggregates in the CN/SSPS solution was much smaller than the number of the aggregates in the CN solution. This result suggests that there were interactions between CN and SSPS at pH 6, which inhibited the aggregation of CN, on the other hand, the interactions were not strong enough and thus CN and SSPS could not form compacted complex aggregates.

### 3.2. @CN/SSPS emulsion

Both VE and MCT are good solvents for some hydrophobic drugs (Wang, Maitani, & Takayama, 2002; Xu, Yin, Li, & Yao, 2015). In this study, we used mixture of MCT and VE as the oil phase to investigate the emulsion stabilities. The emulsification pH was chosen at pH 3–5 at which CN and SSPS can form compacted complex aggregates as discussed above. The emulsion produced at pH 5 was not stable and therefore was not studied further. For the emulsions produced at pH 3, 3.5, 4 and 4.5, the freshly prepared emulsions were adjusted to pH 2, 5 and 7, and then the emulsions were stored at 4°C to investigate their long-term stability in pH 2–7 media. During the storage, the pH 7 samples changed their pH values to 6.8, the other samples did not change their pH values significantly, and all the samples were homogeneous in appearance. Fig. 2 shows that the droplet sizes of the emulsions produced at pH 3–4.5 were 300–350 nm. The droplet sizes decreased a bit after adjusting the emulsions to pH 7, whereas the sizes increased somewhat after adjusting the emulsions to pH 2. The droplet sizes did not change significantly after adjusting the emulsions to pH 5, indicating that the droplets had SSPS surface which prevented the CN from self-aggregation. All the samples did not change their droplet sizes much after 210 days of storage, demonstrating that the complex emulsions produced at pH 3–4.5 were long-term stable in the media of pH 2–6.8. Particularly, the emulsion produced at pH 4 was stable after 500 days of storage in the media of pH 2–6.8 (Figs. S1 and S2 of Supplementary data). In the following study, pH 4 was chosen to produce CN/SSPS complex emulsion. Individual CN and SSPS emulsions were also produced for comparison. The droplet
size was 145 nm for CN emulsion and 896 nm for SSPS emulsion. For CN emulsion, when the emulsion produced at pH 7 was adjusted to pH 5, flocculation and creaming appeared. For SSPS emulsion, creaming appeared after short-term storage.

Fig. 3 shows the ζ-potentials of the emulsions. For CN emulsion samples with pH 3–5, the ζ-potentials are for reference only because flocculation and creaming appeared in these samples. In pH 2–8 range, the ζ-potentials of @CN/SSPS emulsion were close to the ζ-potentials of @SSPS emulsion, confirming that @CN/SSPS emulsion droplets were covered by SSPS. It is the SSPS surface that enables the droplets to be dispersible in the pH range of 3–4.5 as discussed above, and therefore it is reasonable that CN and SSPS can form compacted complex interfacial films by use of the same electrostatic and hydrophobic interactions.

As we know, three factors account for the stability of the emulsions: droplet sizes, stability of the interfacial films and dispersity of the droplets in the aqueous solutions (Yin et al., 2012; Yin et al., 2015). The surface structure of CN/SSPS complex emulsions may be similar to the structures of soy protein/SSPS and pea protein/SSPS complex emulsions with crosslinked interfacial films (Yin et al., 2012; Yin et al., 2015). Due to the compacted CN/SSPS complex interfacial films, the SSPS chains were fixed on the droplet surfaces. As mentioned above, only about 18% of the total sugar units of SSPS are negatively charged which can bind with the positively charged CN. The uncharged sugar units of the SSPS tend to stretch in the aqueous solutions that make the nano-sized droplets dispersible.

3.3. CUR@CN/SSPS emulsion

CUR was dissolved in MCT containing 10% ethanol (v/v) to increase CUR solubility in the oil phase. Ethanol is a cosolvent/cosurfactant, which can penetrate into surfactant layer to improve emulsifying property (Date & Nagarsenker, 2008; Kuentz, 2012). CUR@CN/SSPS emulsion was produced at pH 4 with the same emulsification condition as @CN/SSPS emulsion. The CUR concentration in the emulsion was 1.7 mg/mL. Table 2 shows that about 99.9% of the CUR was encapsulated in the droplets. The CUR encapsulation did not influence the droplet properties. The Dh, PDI and ζ-potential values of CUR@CN/SSPS emulsion at pH 4 were 324 nm, 0.07 and −3.2 mV, respectively, which were close to the values of the @CN/SSPS emulsion. The TEM images shown in Fig. 4 confirm that there is no significant difference between @CN/SSPS and CUR@CN/SSPS droplets in droplet size and morphology. During
the storage at pH 4 and 4 °C for 380 days, the CUR@CN/SSPS emulsion was homogeneous in appearance. The droplet size distributions before and after the storage were almost the same (Fig. S3 of Supplementary data). These results verify that CUR@CN/SSPS emulsion has perfect physical stability.

3.4. CUR chemical stability of CUR@CN/SSPS emulsion

Fig. 5 shows that about 94% of the free CUR in pH 4 aqueous solution degraded after 2 days of storage at 37 °C in the dark. For CUR@CN/SSPS emulsion with pH 4, almost no CUR degraded after 2 days of storage and about 25% of the CUR degraded after 40 days of the storage at 37 °C in the dark. The CUR decomposition rate decreased with the decrease of the storage temperature. When the emulsion was stored at 4 °C in the dark for 40 days, only 3% of the CUR degraded. Natural light facilitated the CUR decomposition. When the emulsion was stored at 4 °C without shading light for 40 days, the CUR decomposition rate increased to 20%. Recently, we studied CUR loaded BSA-dextran conjugate emulsion (CUR@BSA-dextran) (Wang et al., 2016). The CUR@BSA-dextran emulsion had long-term physical stability and CUR chemical stability only when the emulsion had integrated and crosslinked interfacial film. In this study, the very good physical stability and CUR chemical stability of CUR@CN/SSPS emulsion at acidic condition confirm that the CN/SSPS complex interfacial film formed at pH 4 is integrated and compacted because the interfacial film can act as an efficient physical barrier to protect the loaded component (Wang et al., 2016; Xiao, Lo, & Huang, 2015).

3.5. Ex vivo fluorescence imaging of mice gastrointestinal tracts

The retention time and distribution of the loaded compound in mice gastrointestinal tracts were investigated after oral administration of DiR@CN/SSPS emulsion. Fig. 6 shows that at 0.5 h post-administration, the stomach presented strong fluorescence and the intestine also presented fluorescence, suggesting that the loaded compound could quickly enter into intestinal tract. The fluorescence intensity in gastrointestinal tracts decreased gradually and disappeared at 24 h post-administration, indicating that the emulsion digestion was completed.

3.6. In vitro sequential digestions of CUR@CN/SSPS emulsion

It was reported that the absorption of the loaded compound in the emulsion depends on the emulsion digestion in gastrointestinal tract (McClements & Li, 2010). The sequential digestions of CUR@CN/SSPS emulsion in simulated digestive juices were investigated. The SGF was pH 1.2 HCl solution containing pepsin; SIF was

<table>
<thead>
<tr>
<th>Sample</th>
<th>Loading efficiency (%)</th>
<th>(\zeta)-Potential (mV)</th>
<th>(D_0) (nm)</th>
<th>PDI</th>
<th>(D_0) (nm)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>CUR@CN/SSPS</td>
<td>99.9 ± 0.1</td>
<td>3.2 ± 0.1</td>
<td>324 ± 7</td>
<td>0.07 ± 0.04</td>
<td>330 ± 7</td>
<td>0.07 ± 0.04</td>
</tr>
</tbody>
</table>

* CUR concentration in the emulsion was 1.7 mg/mL and the emulsion was homogeneous in appearance during the storage.
pH 7.4 phosphate buffer solution containing trypsin, amylase, lipase and bile salts. Fig. 7 shows that the emulsion remained homogeneous in appearance and about 7.3% of the loaded CUR was extractable after 2 h digestion in SGF. When the SGF digestion solution was mixed with SIF, the mixture divided into two layers. The lower layer was clear and the upper layer was cloudy with oil and chyme. After 2 h digestion in SIF, the oil and chyme layer disappeared and flocules appeared in the bottom. About 73% of the loaded CUR was extractable after 2 h digestion in SGF followed by 2 h digestion in SIF, indicating that the emulsion is digestible and the loaded CUR is releasable in gastrointestinal tract.

For single sodium caseinate-stabilized emulsion, it was reported that no intact casein remained on the interface after 10 min digestion in SGF, and flocculation and coalescence occurred immediately (Li, Ye, Lee, & Singh, 2012). SSPS is a non-starch polysaccharide and cannot be digested by amylase (Choct, Dersjant-Li, McLeish, & Peisker, 2010). In this study, the release of CUR from CN/SSPS emulsion was only 7.3% and the emulsion was homogenous in appearance after 2 h digestion in SGF, indicating that the SSPS surface as well as the compacted interfacial films protected the CN from pepsin digestion. On the other hand, the concentration of SSPS (10 mg/mL in water phase) was not high enough to prevent the digestion completely. During the incubation in SIF, about 65.7% of the CUR was released from CN/SSPS emulsion, which was much higher than that in SGF. The isoelectric point of porcine pepsin is pH 2.8 and the isoelectric point of porcine trypsin is pH 10.7 (Patrickios & Yamasaki, 1995). In SIF condition (pH 7.4), porcine trypsin carried positive charges while CN and SSPS carried negative charges. Trypsin could adsorb on the droplet surface via electrostatic attraction, which might enhance the hydrolysis efficiency of trypsin because of the enrichment of trypsin on the surface. As reported in the literature, the released CUR was transferred into water-soluble mixed micelles formed by bile salts, free fatty acids and monoaoylglycerol in digestive juices that can increase the bioaccessibility of CUR in digestive tract (Yu & Huang, 2012).

3.7. CUR pharmacokinetics analysis of CUR@CN/SSPS emulsion in mice

CUR pharmacokinetics in mice delivered by CUR@CN/SSPS emulsion and also by CUR/Tween 20 suspension was studied after oral gavage at CUR dose of 50 mg/kg. The CUR plasma concentration changes are shown in Fig. 8 and the CUR pharmacokinetic parameters are shown in Table 3. For the group treated with CUR/Tween 20 suspension, the maximum CUR plasma concentration (C_{max}) was 29 ng/mL and appeared at 4 h post-administration, and the area under the curve in the period of 0–24 h (AUC_{0-24}) was 250 h ng/mL. For the emulsion group, the C_{max} was 330 ng/mL and appeared at 0.5 h post-administration, and the AUC_{0-24} was 2747 h ng/mL. These results reveal that the CUR delivered by the emulsion was absorbed more rapidly and effectively than the CUR delivered by CUR/Tween 20 suspension. The CUR bioavailability of
CUR@CN/SSPS emulsion group increased 11-fold compared to the bioavailability of CUR@Twee 20 suspension group. Recently, we reported that the CUR bioavailability of CUR@BSA-dextran conjugate emulsion was 4.8-fold higher than that of CUR@Twee 20 suspension (Wang et al., 2016). That is, the CUR bioavailability of CUR@CN/SSPS complex emulsion is 2.3-fold higher than that of CUR@BSA-dextran conjugate emulsion. This difference indicates that the structure of protein/polysaccharide interfacial film has an impact on the digestion and/or absorption of the loaded CUR. Fig. 7 shows that CUR@CN/SSPS emulsion is digestible and the loaded CUR is releaseable. It was reported that emulsions are able to generate mixed micelles that increase the bioaccessibility of the loaded compounds after oral administration and digestion (Porter et al., 2007; Yu & Huang, 2012). At this stage, we do not have direct evidence that the CUR was absorbed in intestinal tract only when the CUR was released from the emulsion, which needs further study. In this study, we used CN and SSPS, which are low cost, high nutrition and safety, to produce stable CUR@CN/SSPS complex emulsion by means of convenient and green emulsification method. The 11-fold increase of the CUR bioavailability clearly demonstrates that CN/SSPS complex emulsion is an applicable system for oral delivery of lipophilic nutrients and drugs.

4. Conclusion

Linear protein CN can form compact complex aggregates with SSPS at the pH range of 3–4.5 via electrostatic and hydrophobic interactions. CUR@CN/SSPS emulsion produced at pH 4 has very good physical stability and CUR chemical stability due to compacted CN/SSPS complex interfacial film. CUR@CN/SSPS emulsion is digestible and the loaded CUR is releaseable in simulated digestive juices. The CUR oral bioavailability of CUR@CN/SSPS emulsion in mice is 11-fold higher than that of CUR@Twee 20 suspension. This study demonstrates that food grade protein and polysaccharide can fabricate long-term stable emulsion without heat treatment and CN/SSPS complex emulsion is an applicable system for oral delivery of lipophilic nutrients and drugs.

Acknowledgment

Financial support of National Natural Science Foundation of China (NSFC Project 21274026) is acknowledged.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.foodhyd.2017.05.010.

Table 3

<table>
<thead>
<tr>
<th>Group</th>
<th>Tmax (h)</th>
<th>Cmax (ng/mL)</th>
<th>AUC0-24 (ng/mL h)</th>
<th>Relative bioavailability</th>
</tr>
</thead>
<tbody>
<tr>
<td>CUR@Twee 20 suspension</td>
<td>4.0</td>
<td>29</td>
<td>250</td>
<td>1</td>
</tr>
<tr>
<td>CUR@CN/SSPS emulsion</td>
<td>0.5</td>
<td>330</td>
<td>2747</td>
<td>11.0</td>
</tr>
</tbody>
</table>

References


