Highlights

- The 1:1 mass ratio of WPI to CN was chosen as the external phase to prepare the emulsions.
- The α-tocopherol-loaded emulsions was stable against environmental stresses.
- The WPI/CN complex could protect α-tocopherol from degrading in gastric condition.
- The complex could be used to achieve controlled release of α-tocopherol to the intestinal tract.
Encapsulation of \(\alpha\)-tocopherol in whey protein isolate/chitosan particles using oil-in-water emulsion with optimal stability and bioaccessibility

Weili Xu\(^1\)*, Kangxing Lv\(^1\), Wei Mu\(^2\), Shaobo Zhou\(^3\), Yang Yang\(^1\)

\(^1\)Department of Food Science and Engineering, School of Chemistry and Chemical Engineering, Harbin Institute of Technology, 150001 Harbin, China

\(^2\)Department of Biomolecular and chemical engineering, School of Chemistry and Chemical Engineering, Harbin Institute of Technology, 150001 Harbin, China

\(^3\)School of Life Sciences, Institute of Biomedical and Environmental Science and Technology, University of Bedfordshire, Luton, LU1 3JU, UK

Funding: This work was supported by the National Natural Science Foundation of China (31501481).
Abstract

The aim of this study was to develop an O/W emulsion using whey protein isolate (WPI)-chitosan (CN) complex to encapsulate α-tocopherol and to characterize their stability and bioaccessibility in vitro. The O/W emulsions prepared under the optimal conditions (mass ratio of WPI:CN=0.2%:0.2%, 5% corn oil containing 5% of α-tocopherol) exhibited a monomodal distribution (d = 803.30 ± 6.89 nm) with encapsulation rate of 86.30±2.31%. Morphological observations showed that the emulsion particles were spherical and the oil phase was embedded inside. The emulsions were stable under NaCl (0 mM-150 mM), sugar (0%~5%), 55°C for 30 min, pH 5-6.5, even storage for 20 d at 4°C and 25°C. During gastric digestion, WPI situated at the surface of emulsion particles can be digested into small molecular peptides by pepsin, but the structure of the core-shell particles remained due to the cross-linking with CN. During intestinal digestion, the structure of the particles disintegrated over the digestion time, and the inner-oil phase was released. Release profiles of the α-tocopherol and free fatty acids showed a burst effect followed by slow release. These results suggest that the WPI-CN complex could be used to achieve a controlled and sustainable release of liposoluble bioactive compounds from O/W emulsions.

Keywords: Whey protein isolate, Chitosan, Encapsulation, α-tocopherol, in vitro digestion
1. Introduction

Vitamin E has been widely used in medicine, cosmetics and food industry as a nutritional supplement and antioxidant. Among the subtypes of vitamin E, i.e., tocopherols (α, β, γ, δ) and tocotrienols (α, β, γ, δ), α-tocopherol is the one with the highest biological activity. But its hydrophobicity and sensitivity to light, oxygen, metal ions limit its applications, and the free α-tocopherol decomposes rapidly in simulated gastrointestinal conditions (R. Brigelius-Flohé, 1999; Wongsasulak, Pathumban, & Yoovidhya, 2014). Microparticle (MP) technology used to embed α-tocopherol is an effective method to help improve stability, bioavailability and targeted release to specific sites (Liang, Line, Remondetto, & Subirade, 2010; Raza, Abid, Azam, & Rehman, 2020; Relkin, Yung, Kalnin, & Ollivon, 2008; Wongsasulak, Pathumban, & Yoovidhya, 2014; Zhiyang, Jingbo, Liu, Hui, Zhang, Xinling, et al., 2019). Currently, zein (Wongsasulak, Pathumban, & Yoovidhya, 2014), β-lactoglobulin (Liang, Line, Remondetto, & Subirade, 2010), chitosan (CN) (Raza, Abid, Azam, & Rehman, 2020), sodium caseinate (Relkin, Yung, Kalnin, & Ollivon, 2008), and whey protein isolate (WPI) (Chen & Subirade, 2005; Hwang, Ha, Lee, Kim, Kim, & Lee, 2017; Zhiyang, et al., 2019; Zimet & Livney, 2009) are frequently used as wall materials to entrap bioactive compounds in micro or nanoparticle preparations.

WPI is a mixture of globular proteins that are isolated from milk whey, a by-product of cheese production (Fang, Xu, Cheng, Li, Guang, & Liang, 2018). WPI has been used as an efficient emulsifier because of its charged hydrophilic and hydrophobic groups, and its good ability to bind hydrophobic substances. It is therefore a suitable carrier for the delivery of fat-soluble drugs or other bioactive ingredients, e.g., β-LG-epigallocatechin-3-gallate, curcumin, DHA (Chen & Subirade, 2005; Hwang, Ha, Lee, Kim, Kim, & Lee, 2017; Zhiyang, et al., 2019; Zimet & Livney, 2009). Bioactive...
compounds encapsulated by WPI individually or jointly with others showed enhanced bioavailability and increased physical and chemical stability during food storage and digestion (Avi, Shpigelman, and, Gal, Israeli, and, et al., 2010; Fang, Xu, Cheng, Li, Guang, & Liang, 2018; Ron, Zimet, Bargarum, & Livney, 2010; Zimet & Livney, 2009). WPI-based delivery systems have been shown to reduce odor and off-flavor development. Previous studies have shown that the stability of WPI-encapsulated α-tocopherol was markedly improved during storage and under gastric or intestinal conditions (Fang, Xu, Cheng, Li, Guang, & Liang, 2018; Ozturk, 2015; Wang L., 2016). However, WPI are poor in solubility at pH higher than its isoelectric point (≈pH 5.0) such as that in intestinal conditions, prone to denaturation at high temperature and to degradation in digestive fluids by enzymes in the digestive tract. These properties limit its applications as a common emulsifier.

CN is a unique cationic polysaccharide that is positively charged due to the protonation of its amino residues when dissolved in an aqueous solution with pH≤6.5. This feature has been used for film formation, flocculation process and the immobilization of proteins, drugs and other biologically active substances, or as a wall material for delivery systems (Liu & Park, 2009; Naghibzadeh, Amani, Amini, Esmaeilzadeh, Mottaghi-Dastjerdi, & Faramarzi, 2010; Raza, Abid, Azam, & Rehman, 2020; Sahoo, Sahoo, Mohanty, Sasmal, & Nayak, 2012). CN has been exploited in formulations encapsulating vitamin E (Naghibzadeh, Amani, Amini, Esmaeilzadeh, Mottaghi-Dastjerdi, & Faramarzi, 2010). It has also been used to coat vitamin E-loaded nano-size liposomes to improve the stability (Liu & Park, 2009). Furthermore, CN is an excellent biomaterial (biocompatible, biodegradable and nontoxic) with mucoadhesive, antifungal and bactericide properties (Kean & Thanou, 2010; Naghibzadeh, Amini, Amini, Esmaeilzadeh, Mottaghi-Dastjerdi, & Faramarzi, 2010). The α-tocopherol-
encapsulated CN nanospheres have been incorporated in skin care products that
demonstrated good antibacterial and antioxidant properties (Raza, Abid, Azam, &
Rehman, 2020). The DL-α-tocopheryl-modified chitosan nanoparticles can gradually
release α-tocopherol as an antioxidant along with the microbicidal properties of CN
(Quiñones, Gothelf, Kjems, Yang, Caballero, Schmidt, et al., 2013). A formulation
consisted of trans-cinnamaldehyde-loaded poly (DL-lactide-co-glycolide (PLGA) and
CN nanoparticles with an encapsulation rate of 33% showed faster release rates at low
pH (Pola, Moraes, Medeiros, Teófilo, Soares, & Gomes, 2019). However, α-tocopherol-
loaded particles stabilized with CN are highly soluble in acidic aqueous solution, and
the fat-soluble active substances embedded in the nanoparticles can be rapidly released
in the gastric phase thus are not protected for targeted release in the intestinal
environment (Yoksan, Jirawutthiwongchai, & Arpo, 2010). While many recent studies
have focused on the preparation, characterization and application of WPI and CN
complexes (Daniele S. Bastos, et al., 2010; Laplante, Turgeon, & Paquin, 2005, 2006;
Souza, Bai, Gonçalves, & Bastos, 2009; Xu, Tang, Yang, Wang, & Zhou, 2020),
developing WPI-CN particles for encapsulation with controlled release of bioactives
has not been reported. Therefore, using WPI/CN complex to encapsulate α-tocopherol
may present an opportunity to overcome the disadvantages of emulsions stabilized by
WPI or CN alone.

In present study, α-tocopherol-loaded oil-in-water (O/W) emulsions were prepared
using the WPI-CN complex as water phase (W) and corn oil as the oil phase. The
optimum mass ratios of WPI/CN, and corn oil content for the formation of a stable and
efficient α-tocopherol-loaded emulsions were studied by evaluating the particle size,
zeta potential, and α-tocopherol encapsulation rate. The morphology was investigated
by laser confocal microscope (LCM) and transmission electron microscope (TEM). The
stability of $\alpha$-tocopherol-loaded emulsions under NaCl, sugar, temperature, thermal processing, storage duration was examined. The release of free fatty acid and $\alpha$-tocopherol from the WPI-CN emulsions under simulated gastrointestinal conditions was also assessed.

2 Material and methods

2.1 Materials and reagents

WPI (97.5% w/w protein) were purchased from Davisco Foods International, Inc. (Le Sueur, MN, USA). CN (Medium molecular weight) and $\alpha$-tocopherol (biochemical grade) were both from Sigma-Aldrich Chemical Company, St. Louis, MO, USA. Other chemicals are of analytical grade from certified companies.

2.2 Preparation of emulsions using WPI-CN to encapsulate $\alpha$-tocopherol

The microparticles were prepared by oil-in-water emulsion method. Stock solutions of 1wt% of WPI and 0.25wt% of CN were prepared as described in our previous report (Xu, Tang, Yang, Wang, & Zhou, 2020). WPI and CN were mixed in different mass ratios (4:1, 2:1, 1:1, and 1:2), using a magnetic stirrer bar at room temperature for 1 h. A stock solution of corn oil containing 5% (wt/wt) of $\alpha$-tocopherol was prepared by mixing 5 g of $\alpha$-tocopherol into 95 g corn oil, thoroughly vortexed until the compound was completely dissolved, and sealed and stored at -20°C in the dark for further uses. The emulsions were prepared by mixing 2.5%, 5.0% and 7.5% (w/w) of $\alpha$-tocopherol stock solutions with 97.5%, 95.0% and 92.5% (w/w) of WPI/CN mixed aqueous solutions, respectively, and dispersed at a high speed at 12000 rpm for 10 min to form a coarse emulsion at room temperature. The coarse emulsions were immediately homogenized at 500 bar for 2 min to obtain $\alpha$-tocopherol-loaded emulsions which were stored at 4°C before use.

2.3 Particle size and zeta potential
Different samples were diluted 100 fold and their Zeta potential, particle size and distribution were measured based on the method previously reported (Xu, Tang, Yang, Wang, & Zhou, 2020) using Zetasizer Nano-ZS90 (Malvern Panalytical, Malvern, UK).

2.4 Assessment of the encapsulation rate of α-tocopherol-loaded emulsions

Emulsions-encapsulated α-tocopherol (0.4 mL) was added into 3.6 mL of n-hexane, vortexed for 2 min, and sonicated at 6000 rpm for 10 min to completely release α-tocopherol from the emulsion particles, and then centrifuged at 4000 rpm for 10 min at 4°C. The supernatant containing total α-tocopherol was collected and its absorbance was measured at 290 nm with a UV spectrophotometer (FP5600, Hitachi, Japan). For determination of free α-tocopherol in emulsions, the same procedure was followed except vortex was used instead of sonication to remove the α-tocopherol entrapped inside the WPI/CN complex (Minekus, Alminger, Alvito, Ballance, & Brodkorb, 2014). The total and free α-tocopherol contents (μg/mL) were calculated according to the standard curve (50-300 μg/mL, R²=0.9977). The embedding rate was calculated as:

\[ \text{embedding rate (\%)} = \frac{(C_a-C_b)}{C_a} \times 100\% \]

where \( C_a \) is the total α-tocopherol content of the sample (μg/mL), \( C_b \) is the total free α-tocopherol content of the sample (μg/mL).

2.5 Morphological examination of α-tocopherol-loaded emulsions

The morphology of α-tocopherol-loaded emulsions was examined using LCM and TEM. Briefly, 10 μL of Nile Red (dissolved in 0.1% acetone solution, excitation wavelength 488 nm) and 5 μL of Fast green (dissolved in 1% acetone solution, excitation wavelength 633 nm) were added into 200 μL of emulsion and stained for 5 min, then 20 μL of the above solution was loaded onto a glass slide for observation. After dyeing with Nile red and Fast green, the oil phase of α-tocopherol-loaded emulsion particles and the proteoglycan complex appeared red and green, respectively.
under the laser confocal microscope (Olympus FV3000). A drop of the freshly prepared emulsions was placed on a 400-mesh copper grids for 3 min to dry, then a drop of 2 wt% uranium, bis (acetato-kO) dixo-hydrate was added to its surface and treated for 2 min, and then observed with TEM.

2.6 The stability of α-tocopherol-loaded emulsions under different stresses

The stability of the α-tocopherol-loaded emulsions was assessed in the following environmental conditions. 1) NaCl or sugar was added into 20 g emulsions to obtain the final concentrations of NaCl at 0, 25, 50, 75, 100, 150, 200, 250 and 300 mM, and of sugar (% wt/wt) at 0%, 1%, 2%, 3%, 4% and 5%. Then the complex solutions were stored in 50 mL tube at 4°C before analysis. 2) The emulsions were incubated for 15 min at 25°C, 35°C, 45°C, 55°C, 65°C, 75°C, 85°C and 95°C, then immediately cooled to 4°C before further analysis; 3) The emulsions was heated in a water bath at 65°C for 0, 15, 30, 45 and 60 min, then immediately cooled to 4°C before further analysis. 4) The emulsions were adjusted to the pH (3, 4, 5, 5.7, 6.5 and 7) with NaOH or 1% citric acid in a 50 mL centrifuge tube and stored at 4°C for 24 h before analysis. 5) The emulsions were stored at 4°C and 25°C for 0, 5, 10, 15, 20 and 30 d. The particle size and zeta potential and distribution of all the above samples were measured.

2.7 In vitro simulation of gastric and intestinal digestion of α-tocopherol-loaded emulsions

A simulated digestion model of a stomach and intestine was constructed respectively as described in previous report with slight modifications (He, Gu, Wang, Xu, & Ma, 2019). The simulated digestive solutions included a simulated gastric fluid (SGF, pH=2.5) and a simulated intestinal fluid (SIF, pH=7.5). After configuration, the pH was adjusted to desired value with 1M HCl and 1M NaOH and store at 4°C (Raza, Abid,
Azam, & Rehman, 2020). SGFB solutions containing pepsin (4.8 mg/mL) and CaCl₂ (0.15 mmol/L) were prepared with distilled water. Meanwhile, 100 g WPI/CN-stabilized emulsion was placed in an erlenmeyer flask, and incubated at 37°C in a water bath shaker at 100 rpm/min. Two peristaltic pumps were used by adding SGF and SGFB with flow rate of 2.0 mL/min and 0.5 mL/min, respectively. The gastric digestion stage was maintained at 37 °C for 120 min, and 2 mL samples were taken at 0, 5, 10, 15, 20, 60, 90 and 120 min for analysis.

In the intestinal digestion stage, SIFB solutions containing 2.5 mg/mL bile extract were prepared using SIF solution. Then, 50 mL SIFB was added into 50 mL of the above gastrically digested solution, and the mixture was adjusted to pH 7.5 with 1M NaOH, followed by addition of pancreatin (0.14 mg/mL) and CaCl₂ (0.40 mg/mL). The digestion stage was continued at 37°C for 120 min. Then, 2 mL samples were taken at 0, 1, 10, 30, 60, 90 and 120 min for analysis. The mixtures were titrated with 0.25 M NaOH during digestion period to keep the pH at 7.5. The amount of NaOH consumed during digestion was recorded at 5, 10, 15, 20, 25, 30, 60, 90 and 120 min, and the amount of free fatty acid (FFA) was calculated according to the following formula:

\[
FFA = \frac{V_{NaOH(t)} \times C_{NaOH} \times M_{lipid}}{2m_{lipid}}
\]

where, FFA, the released rate of free fatty acids (%); \(V_{NaOH}\), the volume of NaOH solution consumed at digestion time \(t\) (mL); \(C_{NaOH}\), the concentration of NaOH solution (mol/L); \(m_{lipid}\), the quantity of oil in the sample; \(M_{lipid}\), the average molecular weight of oil (g/mol); The molecular weight of corn oil is about 880 g/mol.

2.8 SDS-PAGE of α-tocopherol-loaded nanoparticles

SDS-PAGE was performed as described in a previous report with some modifications (Xu, Mi, & He, 2017). The sample was added with the loading buffer (sample:
buffer=4:2, v/v) and heated at 90°C for 5 min, then 15 μL of each treated sample solution was loaded per well and run on 5% stacking gel 80 V for 30 min. The SDS-PAGE was carried out in 12% separating gel with 120 V for 1.5 h. The gels were stained with Coomassie Brilliant Blue-250, and decolorized with a decolorizing solution (glacial acetic acid: methanol: water = 10: 30: 60). Images of the gel were taken by a digital camera for analysis.

2.9 Rheological analysis
Rheological measurements followed the same method as described in previous report (He, Gu, Wang, Xu, & Ma, 2019).

2.10 Statistical analysis
All measurement was obtained in triplicate and experiments were repeated three times using freshly prepared samples. Data are expressed as mean ± standard deviation. Analyses were performed using one-way ANOVA followed by Tukey’s post-hoc analysis with the GraphPad Prism 7 (San Diego, CA, USA). A p value of less than 0.05 was considered statistically significant.

3. Results and discussion
3.1 The optimum mass ratio of WPI to CN and corn oil content for preparing O/W emulsions
In our previous study, the WPI / CN complex prepared at mass ratio of 4:1 [0.2% WPI (wt/wt):0.05% CN (wt/wt)], without loading α-tocopherol, was the most stable under a variety of environmental stresses (Xu, Tang, Yang, Wang, & Zhou, 2020). In this study, the effects of WPI:CN at different mass ratios (4:1, 2:1, 1:1, and 1:2) on the characteristics and stability of the emulsion particles loaded with α-tocopherol were investigated. A precipitation was observed at WPI:CN = 4:1 and 2:1, but not at 1:1 and 1:2. Larger portion of CN in the latter two conditions might have enhanced the
electrostatic repulsion between the particles, making the systems more stable. Zeta potential was higher and the average particle obviously lower in the WPI:CN=1:1 group compared to the WPI:CN=1:2 group (Figure 1A, a). The emulsions also exhibited a monomodal particle size distribution for the WPI:CN=1:1 group (Figure 1A, b), suggesting this emulsion to be the most homogeneous and stable. Thus, WPI:CN=1:1 was chosen as the wall material to prepare emulsions with 2.5%, 5%, and 7.5% of corn oil containing 5% α-tocopherol for further exploration.

Figure 1B shows the zeta potential, particle size and distribution, as well as the encapsulation rate of the emulsions stabilized by WPI/CN-encapsulated 2.5%, 5%, 7.5% corn oil containing 5% α-tocopherol. Among the three groups, the 2.5% group had the lowest zeta potential and smallest particle size, but the greatest α-tocopherol encapsulation rate. The zeta potential was used to characterize the stability of the emulsions. Higher absolute value of zeta potential leads to greater repulsive force between colloidal particles, making the emulsion more stable. The zeta potential value was increased as a function of oil content, reaching its maximum at 7.5% oil. Our data suggests that the emulsion system is stable when the oil content is at 5% and 7.5%. This is also explained by increased repulsive effect among the particles brought by the more positive charges carried by the WPI-CN complex. Although the 2.5% group had the highest encapsulated rate, it produced more precipitates and was least stability. The 5% oil group had little precipitation, while the 7.5% group had no precipitation (results not shown). This result is consistent with the measurement of the zeta potential. Considering the stability of the emulsion system and the encapsulation rate, the formulation with 5% corn oil was selected for the following studies.

The O/W emulsions prepared under the optimal conditions (WPI:CN=1:1, 5% corn oil) exhibited a monomodal distribution (d = 803.30 ± 6.89 nm), with a relatively high
Zeta potential (39.20 ± 1.20 mV) and α-tocopherol encapsulation rate (86.30 ± 2.31) 
(Figure 1B), which were much higher than previous reported (Raza, Abid, Azam, & Rehman, 2020).

The results of laser confocal microscopy are shown in Figure 2A. The WPI-CN was 
distributed on the surface of the particles and the corn oil containing α-tocopherol was 
well embedded inside them. The distribution of oil droplets in the particles was 
relatively uniform, which basically formed a spherical structure, and no aggregation of 
oil droplets occurred. Micromorphology of WPI-CN particles loaded with α-tocopherol 
was further observed by TEM (Figure 3A). The center of the particles had bright color 
compared to the gray and black surroundings, indicating that the particles were core-
shell structures, and the bright part located in the center of the particles is the corn oil 
containing α-tocopherol, and the surrounding gray area was wall material (WPI-CN 
complex). The result of morphologic observation indicated that the oil phase was 
successfully embedded in the WPI-CN complex.

3.2 Stability of α-tocopherol-loaded emulsion under variety environmental stresses
The zeta potential, particle size and α-tocopherol encapsulation rate of WPI-CN 
emulsions depended on NaCl concentration, and reached the peak values of 39.7±0.44 
mV, 936.47±9.84 nm, 89.90±2.2%, respectively at 75 mM NaCl (Figure 4A). 
Increasing NaCl concentration promoted the stability of α-tocopherol-loaded particles 
when the concentration was less than 75 mM, but between 75 and 300 mM NaCl, the 
zeta potential, particle size and encapsulation rate of the emulsions decreased with the 
increase of NaCl concentration, leading to instability of the emulsions. In general, the 
WPI-CN emulsions had good stability when NaCl concentrations were between 0 to 
150 mM (Figure 4A).

The zeta potential, particle size, and distribution of the α-tocopherol-loaded
emulsions were consistent at different sugar concentrations (0%, 1%, 2%, 3%, 4% and 5%), so was the α-tocopherol encapsulation rate (around 87%) (Figure 4B), suggesting the emulsions are not affected by sugar content.

In terms of the effect of temperature, both the zeta potential and the α-tocopherol retention rate decreased, but the particle size increased gradually with increasing temperature (Figure 4C). The particle size was significantly higher at temperatures over 35 °C (p<0.05 or p<0.01) and α-tocopherol retention rate were lower significantly at >55 °C (p<0.05 or p<0.01). While the emulsions were more stable at temperatures <35 °C. Degradation of α-tocopherol occurred in both the unembedded group and embedded group at different temperatures after 15 min incubation, but the loss in the former was obviously greater than that in the latter (Figure 4C). So, it is reasonable to conclude that the WPI-CN emulsions have a protective effect on α-tocopherol due to their good stability at 25 -55 °C.

Prolonged heating (at 65 °C) of the WPI-CN emulsions led to decreased Zeta potential and α-tocopherol retention rate, but increased particle size. Compared with unheated group, α-tocopherol retention rate and particle size were significantly different at heating time 30 min (p < 0.05), and highly significantly at ≥45 min (p < 0.01) (Fig. 4D). This may be due to the aggregation of particles induced by heating, resulting a decreased stability (Dickinson & Parkinson, 2004). In general, our results showed that the α-tocopherol emulsion stabilized by WPI-CN had good stability when treated at 65 °C for 0 min – 30 min. Similarly, degradation of α-tocopherol occurred in both the unembedded group and embedded group, but the loss was more significant in unembedded formulations, suggesting the emulsion can reduce the loss of α-tocopherol during heat treatment.

pH can affect the stability of the emulsions by altering the charges on the surface of
particles. Increasing pH from 3 to 7 resulted in continuously decreased zeta potential, but for the particle size, it decreased first and then increased. The opposite effect was observed for embedding rate, i.e., it increased first and then decreased with increasing pH value (Figure 4E).

At pH≤5, both WPI and CN are positively charged, and the electrostatic attraction between them is weakened. WPI carries net positive charge at low pH, but may still have negatively charged patches on the surface that interacts with chitosan which possesses positive charges (Elmer, Karaca, Low, & Nickerson, 2011; Mounsey, O’Kennedy, Fenelon, & Brodkorb, 2008). However, the interaction is not tight, making the particle size relatively large. At pH ≥ 6.5, WPI possesses a large number of negative charges, and CN possesses positive charge (Xu, Tang, Yang, Wang, & Zhou, 2020). However, at higher pH, the solubility of CN is decreased to a point it may even be precipitated, making it difficult for WPI and CN to form stable complexes through electrostatic adsorption. This ultimately results in significantly increased particle size and unstable emulsion systems (Kulmyrzaev, Chanamai, & McClements, 2000). We found that at pH 5.7, the electrostatic interaction between WPI and CN was strongest because of their high opposite charges, which resulted in the most compact complexation. At this pH, the emulsion was the most stable, with the smallest particle size and the highest encapsulation rate of α-tocopherol (88.80%) (Figure 4E).

Both zeta potential and α-tocopherol encapsulation rate were gradually decreased but the particle size was increased over the 30 d storage time at both 4ºC and 25ºC, but no significant difference was found in zeta potential and α-tocopherol encapsulation rate compared to day 0 (p>0.05) (Fig. 5). However, the particle size increased significantly when stored for ≥20 days compared with the control group (0 d). At the same storage interval, the zeta potential and encapsulation rate of the emulsions were higher at 4 ºC,
but the particle size was smaller compared to that at 25 °C (Figure 5). Higher
temperature usually increases the frequency of particle collisions and promotes particle
aggregation thus affect the stability of particles in the emulsion (Favé et al., 2004). Our
results suggest that α-tocopherol emulsions should be stored as 4 °C for longer storage
stability.

The above results showed that the α-tocopherol-loaded emulsion particles composed
of WPI and CN with mass ratio of 1:1 as the wall materials and 5% (w/w) corn oil as
the core material were stable against the abovementioned environment stresses.

3.3 *In vitro* gastrointestinal digestion of WPI-CN emulsions loaded with α-
tocopherol

Physical-chemical properties of the oil and water phases in O/W particles not only
affect α-tocopherol encapsulation rate and stability of the emulsions, but also impact
greatly on the release of bioactive components during gastrointestinal digestion (Furr
& Clark, 1997). The morphological changes of the emulsions were evaluated visually
using images (Figure 6), and the microstructure changes of α-tocopherol-loaded
particles at different stages of in vitro digestion were evaluated using LCM, TEM and
dynamic light scattering technique (Figures 2, 3 and 7). Due to the short residence time
of liquid emulsion in the oral cavity, this digestion experiment mainly includes
simulated gastric and intestinal digestion phases. After gastric digestion for 60 min, the
zeta potential of the emulsions increased from 37.67 ± 3.78 mV to 45.70 ± 3.32 mV,
and then remained stable (Figure 7A). The particle size increased significantly from
766.33 ± 42.06 nm to 1114.3 ± 3.15 nm after gastric digestion for 10 min, but dropped
back to 787.53 ± 33.53 nm at 20 min (Figure 7A). The higher zeta potential might be
due to the lower pH that increased the electrostatic repulsive force between the particles.

During gastric digestion, the particles in the emulsions remained evenly distributed
without aggregation, and their micromorphology did not have any apparent changes (Figure 2B, 10 min; Figure 3B) compared with freshly prepared emulsion (Figure 2A; Figure 3A). A small amount of oil was released from the kernels likely due to the action of pepsin which hydrolyzed the WPI adsorbed on the surface of particles into small molecular peptides (Figure 8A), consequently leading to damaged structure of the WPI-CN complex. However, the structure of the core-shell particles did not appear to be significantly disrupted and the integrity remained due to the cross-linking with CN (Figure 3B, b). Nevertheless, our results indicated that the particles exhibited good stability during the gastric digestion.

Transition from the simulated gastric to the intestinal conditions and contact with intestine digestive juice, caused delamination of the emulsion initially which then gradually disappeared (Figure 6B). This is mainly because the pH value of intestinal digestive juice is 7.5, at which the Zeta potential of the emulsions is low (-2.17 ± 5.23 mV) and the electrostatic repulsion between particles decreases, allowing particles to aggregate (Figure 2C, 0 min; Figure 3C, a) to form larger particle sizes (d=1924.67 ± 138.73 nm) (Figure 7B). The zeta potential decreased from -2.17 ± 5.23 mV at 0 min to -70.50 ± 2.43 mV at 30 min intestinal digestion, then gradually increased to -53.33 ± 5.28 mV at 120 min. There was a negative correlation between particle size and digestion time; the size was 697.07 ± 31.31 nm at 120 min (Figure 7B). This might be caused by a combined action of the bile salts, pancreatic lipase and ions in the small intestine that further broke-down the structure of the particles, thus as the digestion proceeded, and inner-oil phase was released. These small oil droplets were evenly dispersed in the digestive juice due to the presence of a large amount of salt ions, free fatty acids, monoacylglycerols and amphiphilic peptides on the oil droplet surface (Figure 2C, 10 min and 120 min; Figure 3C, b; Figure 6B).
3.6 Protein breakdown in O/W emulsions during *in vitro* digestion

The protein bands during gastric and intestinal digestions are shown in Figure 8A and Figure 8B. The original bands started to disappear and new small molecular diffuse bands emerged 10 min after gastric digestion; the diffuse bands gradually narrowed down as the digestion continued (Figure 8A). The results indicated that the WPI on the particle surface was digested by pepsin into smaller molecular peptides. This, combined with the results from the morphological observation, suggest that the addition of CN helped to maintain the structure of the particles and prevented the leakage of α-tocopherol. The pepsin band appeared between 22.0 kDa and 31.0 kDa, and its intensity increased over digestion time. There were no protein bands in the CN lane. The WPI adsorbed on the surface of the particles disappeared completely at the start of the intestinal digestion, and the bands that appeared during the intestinal digestion were of pancreatin (Figure 8B).

3.7 Release of free fatty acid and α-tocopherol from the emulsion particles during *in vitro* intestinal digestion

Usually the hydrolysis of oil mainly occurs in the small intestine (Furr & Clark, 1997). The high intestinal concentration of lipase is adsorbed at the oil-in-water interface of the emulsion, leading to immediate hydrolysis of lipids (Troncoso, Aguilera, & McClements, 2012). Lipase enzymatically hydrolyzes oils during digestion to form products such as surface active lipids, including monoacylglycerol and free fatty acids that accompanied the release of α-tocopherol (Mun, Decker, & McClements, 2007). Release profile showed that both free fatty acids and α-tocopherol were released sharply at 10 min of the intestinal digestion (Figure 8C). This may be a result of decreased solubility of CN at pH 7.5 and dissociated crosslinking of WPI-CN that led to rapid release of the oil phase, exposing the oil to lipase for burst release of fatty acids and α-
tocopherol in the initial stage of intestinal digestion. The release rate of free fatty acids became slower and steady after 10 min, which may be related to the release of free fatty acid molecules with interfacial activity, because they will compete oil molecules for lipase that are adsorbed at the oil droplet interface thus reducing the lipase activity (Troncoso, Aguilera, & Mcclements, 2012). Approximately 80% of α-tocopherol was released from the emulsion in 2 h of the intestinal digestion. A similar observation was also reported by Yangchao et al. (Luo, Zhang, Whent, Yu, & Wang, 2011) and Somchue et al. (Somchue, Sermsri, Shiowatana, & Siripinyanond, 2009), who showed that polysaccharides, as a wall material to coat α-tocopherol-loaded protein particles, can result in significant slower release rate of α-tocopherol and free fatty acids from the emulsion particles in the gastrointestinal tract.

3.8 Rheological properties of O/W emulsions during in vitro simulated gastrointestinal digestion

The viscosity of the systems containing microparticles in different stages of in vitro digestion was measured using a rheometer (Figure 8D). The viscosity of the α-tocopherol-encapsulated emulsions before and after in vitro gastric and intestinal digestion was decreased with the increasing shear rate. A shear thinning phenomenon was observed, indicating these emulsions had non-Newtonian fluid behavior at shear rates of 0.1–1000 1/s.

The zeta potential of the emulsions increased obviously, and the electrostatic repulsion between the particles also increased, so the viscosity was decreased 2 h into the gastric digestion when compared with the control group. After this, the emulsions were well mixed with bile salts, and the pH was adjusted to 7.5. At this time, the zeta potential of the emulsion tended to be 0, the electrostatic repulsion force between particles was reduced, and a large amount of the particles were gathered together
resulting in maximum viscosity (Figure 8D). Under the collective action of bile salts and lipase in simulated intestinal digestive juice for 2 h, the oil phase was released from the emulsion particles, and hydrolyzed by the lipase to release fatty acids. Meanwhile, these small oil droplets can be further emulsified by a large number of salt ions, free fatty acids, monoacylglycerols, and amphiphilic peptides. Therefore, the viscosity of the digestive fluid was lower compared with that at the beginning of the intestinal digestion (Figure 8D).

Conclusion

In this study, 0.2% WPI and 0.2% CN solutions were mixed under acidic conditions (pH=5.7) in equal volumes to form WPI-CN complexes, which were successfully used to prepare corn oil containing α-tocopherol emulsions. Our results showed that the emulsions with high α-tocopherol encapsulation rate had good water solubility, and excellent stability. The α-tocopherol-loaded emulsion particle structure remained unchanged after gastric digestion for 120 min. The α-tocopherol and free fatty acids was released from the emulsion particles with initial burst effect within the first 10 min followed by sustained release during simulated intestinal digestion. The results indicated that the WPI-CN complex is a good carrier system for supplementation of hydrophobic bioactive compounds such as α-tocopherol to achieve targeted delivery and controlled release to the intestinal tract. Our future research will be focused on in vivo evaluation of α-tocopherol-loaded emulsion particles in animal models.

Declaration of competing interest

The authors declared no conflict of interest.

Acknowledgement
This work was supported by the National Natural Science Foundation of China (NSFC Projects (31501481). The authors wish to thank Dr. Rong Tsao at Guelph Research and Development Centre, Agriculture and Agri-Food Canada (AAFC) for revising and improving the manuscript.

References


Figure 1 Effect of WPI/CN ratio on the particle size, zeta potential (A, a) and particle size distribution (A, b) in O/W emulsions; Effect of corn oil content on particle size and zeta potential (B, a), particle size distribution (B, b), and encapsulation rate of α-tocopherol (B, c) in O/W emulsions. The concentration ratio of WPI/CN was 4:1 [0.2% (wt/wt): 0.05% (wt/wt)], 2:1 [0.2% (wt/wt):0.1% (wt/wt)], 1:1 [0.2% (wt/wt): 0.2% (wt/wt)], and 1:2 [0.1% (wt/wt):0.2% (wt/wt)], respectively.
**Figure 2** The images of α-tocopherol-loaded emulsions stabilized by WPI/CN under laser confocal microscopy (60×) from in vitro digestion model. (A) before digestion; (B) simulated gastric digestion for 10 min; (C) simulated intestinal digestion for 0 min, 10 min, and 120 min, respectively.
Figure 3 TEM image of α-tocopherol-loaded emulsion particles prior to and following in vitro gastric and intestinal digestion. (A) control, fresh preparation; (B) gastric digestion for 0 min (a) and 120 min (b); (C) intestinal digestion for 0 min (a) and 120 min (b).
Figure 4 Effect of NaCl (A) and sugar concentration (B), temperature (C), thermal treatment (D), and pH (E) on particle size and zeta potential (a), distribution (b) and encapsulation rate of α-tocopherol (c) in the emulsion particles stabilized by WPI 0.2(wt%)-CN (0.2wt%) encapsulated with 5% of corn oil containing 5% of α-tocopherol, pH 5.7 for A-D. Values are mean ± SD, n = 3. * p < 0.05 versus the control group, ** p < 0.01 versus the control group. The control group is 0 mM NaCl, or 25°C, or 0 min, or pH=5.7, respectively.
Figure 5 Effect of storage time and temperature on the zeta potential, particle size and distribution, and encapsulation rate of α-tocopherol in the particles. Values are mean ± SD, n = 3. * p < 0.05, ** p < 0.01 versus the control group (0 day).
Figure 6 Photographs of α-tocopherol-loaded emulsion after in vitro digestion. (A) Gastric digestion for 0, 5, 10, 15, 20, 60, 90 and 120 min, respectively. (B) intestinal digestion for 0, 1, 10, 30, 60, 90 and 120 min, respectively.
Figure 7 Changes of the zeta-potential, particle size and distribution of α-tocopherol-loaded emulsion particles after in vitro gastric (A) and intestinal (B) digestion at the desired time.
Figure 8 SDS-PAGE analysis of the α-tocopherol-loaded emulsions stabilized using WPI/CN during gastric (A) and intestinal (B) digestion; Release profile of free fatty acids and α-tocopherol from the emulsions during intestinal digestion (C); Rheological characterization of the emulsions during in vitro digestion (D).
Declarations of interest: none

The authors declared no conflict of interest.