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Fabrication of bilayer emulsion by ultrasonic emulsification: Effects of chitosan on the interfacial stability of emulsion

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ABSTRACT

In this study, the stable system of bilayer emulsion was fabricated by ultrasonic emulsification. The effect of chitosan (CS) addition (0.05 %-0.4 %, w/v) at pH 5.0 on the stability of rice bran protein hydrolysate-ferulic acid (RBPH-FA) monolayer emulsion was investigated. It was found that the addition of CS (0.3 %) could form a stable bilayer emulsion. The droplet size was 3.38 μm and the absolute ζ-potential value was 31.52 mV. The bilayer emulsion had better storage stability, oxidation stability and environmental stabilities than the monolayer emulsion. The results of *in vitro* simulations revealed the bilayer emulsion was able to deliver the β-carotene to the small intestine digestive stage stably and the bioaccessibility was increased from 22.34 % to 61.36 % compared with the monolayer emulsion. The research confirmed that the bilayer emulsion prepared by ultrasonic emulsification can be used for the delivery of hydrophobic functional component β-carotene.

1. Introduction

Emulsions are thermodynamically unstable systems and widely applied in the processing of beverages, milk, coffee, ice cream and other foods [1]. Emulsions can also be served as a medium for the delivery of bioactive substances (e.g., vitamins, carotenoids and curcumin) in the human body [2]. However, due to the change of external environmental conditions and their own properties, emulsions are prone to destabilization phenomena such as flocculation and coalescence. It has certain limitations on the application of emulsions in food processing production, for instance, poor functional properties, storage stability and environmental stress stability. Therefore, there are deep demands of fabricating stable emulsion systems to resist the negative environmental effects.

The researches on the applications of multilayer emulsions have generated the interest in the food field and the biological field recently. The layer-by-layer electrostatic deposition technique is usually used to prepare multilayer emulsions [3]. Compared with the monolayer emulsions, bilayer emulsions have superior physical properties, such as the storage stability and the pH stability [4]. Lipid droplets coated with multilayer biopolymers can improve the stability of emulsion under environmental stress [5,6]. Thicker interfacial layers can provide good chemical stability for the encapsulated lipophilic compounds and reduce the rate of lipid oxidation. Multilayer emulsion can be formed by many kinds of proteins and polysaccharides. Generally speaking, the first layer is formed by proteins because they have better surface activity. Then, the second layer can be formed by the polysaccharides [7]. The selection of polysaccharides depends on the electrical properties of the first interfacial layer during the fabrication of multilayer emulsions by electrostatic adsorption. Chitosan (CS) is the only natural cationic polysaccharide and has been paid much attention to its non-toxicity, biocompatibility and antimicrobial activity [8]. It has been demonstrated that CS and whey isolate protein were used for the preparation of bilayer emulsions, which were more stable than the monolayer emulsions [9]. Yan et al. found that the bilayer emulsion formed after CS loading at the soybean isolate protein-EGCG emulsion interface improved the physicochemical stability of the emulsion [10]. Liu et al. prepared bilayer emulsions using succinylated soybean isolate and CS

Abbreviations: RBP, rice bran protein; RBPH, rice bran protein hydrolysate; HHP, high hydrostatic pressure; FA, ferulic acid; CS, chitosan; POV, peroxide value; TBARS, thiobarbituric acid reactive substance; G′, storage modulus; G″, loss modulus; GIT, gastrointestinal tract.

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and revealed that the CS could improve the storage and oxidative stability of the emulsions [11]. At present, the researches on plant-based protein bilayer emulsions mainly focused on soy protein, whereas there are few researches on rice bran protein bilayer emulsions.

It is well known that ultrasonic emulsification is a green and efficient way to fabricate emulsions [12]. The emulsifier can be dispersed rapidly on the interface effectively by the acoustic cavitation and shearing effect generated by ultrasound wave, which can form the emulsion with small and uniform droplets [13–15]. In our previous study, limited enzymatic hydrolysis of rice bran protein (RBP) by trypsin was conducted after high hydrostatic pressure (HHP) pretreatment, then the rice bran protein hydrolysate (RBPH) was obtained [16]. RBPH and ferulic acid (FA) were covalently interacted under the alkaline condition to prepare a novel plant-based conjugate emulsifier (RBPH-FA conjugate) [17]. RBPH-FA covalent conjugate emulsion was fabricated by ultrasonic emulsification under neutral conditions. The emulsion had better emulsifying property and environmental stress stability [18]. Nevertheless, the emulsion was not stable under the acidic conditions (pH 5.0). Emulsions are used in food applications, especially in beverage processing which is often under acidic conditions, and it is expected that emulsions could maintain a stable state. Therefore, the fabrication of a bilayer emulsion stabilization system might be a valid way.

In the research, bilayer emulsion was fabricated by ultrasonic emulsification, the CS was used to form the second interface layer. The aim was to solve the issue destabilization of monolayer emulsion at pH 5.0. The effects of CS on the droplet size, ζ-potential and rheological properties of bilayer emulsions were investigated. Bilayer emulsion formed by ultrasonic emulsification gained better environmental stresses stability under acidic conditions by the addition of CS. This study can provide a theoretical basis for the application of ultrasound as an emulsification method of fabricating bilayer emulsion in food industry.

2. Materials and methods

2.1. Materials

Fresh rice bran was purchased from Heilongjiang Great Northern Wilderness Agribusiness Group Co., Ltd. (Harbin, Heilongjiang, China). RBP was self-extracted via alkali extraction and acid precipitation in laboratory [16]. The protein content of RBP was 91.67 % (w/w), which was determined by the Kjeldahl method (N% × 5.95). Trypsin (3×10^4 U/g) and FA were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Soybean oil was purchased from Harbin Huikang Food Co., Ltd. (Harbin, Heilongjiang, China). Chitosan (deacetylation degree ≥ 90 %) was purchased from Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Chemicals and other reagents were of analytical grade.

2.2. Preparation of conjugate

RBPH-FA conjugate was prepared by our previous research [17]. The detail steps were as follows: firstly, RBP was pretreated by HHP instrument (Ren-He Electromechanical Engineering CO., Shenyang, China) at 25 °C with 200 MPa for 30 min; secondly, the pretreated RBP was hydrolyzed by trypsin (pH 8.0, 37 °C) at normal pressure for 60 min. then, the RBPH was obtained. Thirdly, the RBPH (1 %, w/v) and FA (0.15 %, w/v) interacted for 24 h (pH 9.0, 25℃). The mixture was dialyzed with the 3 kDa dialysis bag for 48 h, and then it was freeze-dried till to analysis.

2.3. Preparation of monolayer emulsion

RBPH-FA monolayer emulsion was prepared according to our previous research [18]. The RBPH-FA solution was mixed with soybean oil (9:1, v/v) at 20000 rpm for 3 min by homogenizer (Ultra-Turrax T18, Angni Co., Shanghai, China). Then, the coarse emulsion was emulsified by ultrasound generator (Scientz-II D, Scientz Biotechnology Co., ltd., Ningbo, China) at 300 W for 3 min.

2.4. Preparation of bilayer emulsion

The monolayer emulsion was adjusted to pH 5.0. The CS was dissolved with acetic acid (1 %, w/v) and the pH value was adjusted to 5.0. The CS solution (10 mL) was then dropped into the monolayer emulsion (10 mL) and the mixture was emulsified for 3 min at 300 W (pulse duration, 4 s; turn-off time, 2 s) by ultrasound generator. Finally, the bilayer emulsions with different CS concentrations (0, 0.05 %, 0.1 %, 0.2 %, 0.3 % and 0.4 %, w/v) were obtained. The β-carotene was dissolved in soybean oil and then the β-carotene emulsion (1 wt% β-carotene content) was formed by the procedure of the bilayer emulsion.

2.5. Measurement of the droplet size and ζ-potential of emulsion

The droplet size and ζ-potential of emulsion were determined according to our research [18]. The droplet size was determined with a Particle Size Analyzer (Microtrac S3500, Microtrac Inc., Krefeld, Germany). The ζ-potential of emulsion was measured using Zetasizer Nano ZS (Malvern Instrument ltd., Malvern, Worcestershire, UK).

2.6. Rheology

A HAAKE Mars 40 rheometer (Thermo Scientific, Bremen, Germany) was used to determine the rheological properties. The viscosity was determined with the shear rate from 0.1 to 100 s^{-1}. The storage modulus (*G'*) and loss modulus (G″) changes were measured with the Frequency scanning from 0.1 to 100 rad/s [10].

2.7. Microstructure of emulsion

2.7.1. Inverted fluorescent microscopy

The emulsion was stained by Nile red for 30 min. Then the emulsion was observed by the inverted fluorescent microscope (LEICA DMi8, Leica Microsystems, Germany) [18].

2.7.2. Cryo-scanning electron microscopy (Cryo-SEM)

The emulsion droplet was observed by the Cryo- scanning electron microscope (S-3400 N, Hitachi, Japan) [18]. The acceleration voltage was 5 kV. The magnification was adjusted from 1.00 k to 10.00 k.

2.7.3. Optical microscopy

The microstructure of β-carotene emulsion was observed by optical microscope (DP27, Olympus, USA) in each vitro digestion phase. The magnification was 40×.

2.8. Emulsion storage stability

The droplet sizes of monolayer and bilayer emulsion were measured after storage experiment for 14 days at 25℃.

2.9. Environmental stresses

2.9.1. Temperature stability

The bilayer emulsions (with 0.3 % CS) were incubated in water bath (30 °C to 90 °C) for 30 min. The samples were cooled down and stored for 24 h. The changes of emulsion droplet size and ζ-potential were recorded.

2.9.2. Ionic strength stability

NaCl solution (5 mol/L) was added to the bilayer emulsion (with 0.3 % CS), and the bilayer emulsion with NaCl (0–300 mmol/L) was obtained separately. Then the emulsions were placed for 24 h with 25 °C. The droplet size and ζ-potential were measured.

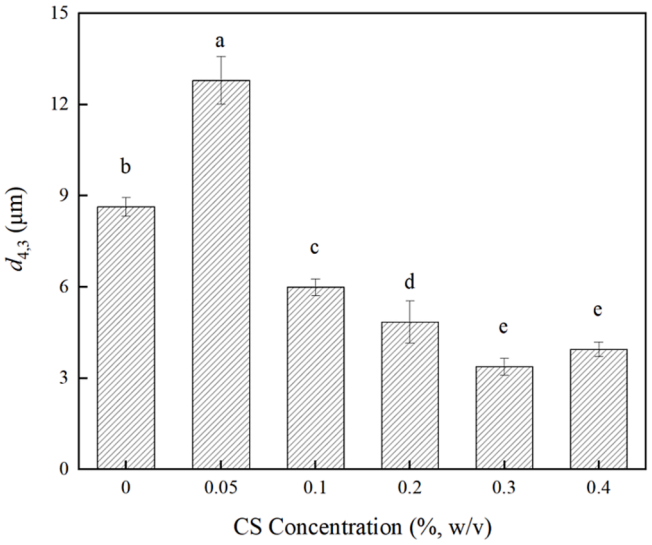

Fig. 1. Effect of the CS on the average droplet size ($d_{4,3}$) of RBPH-FA emulsion The error bars indicate the standard deviation obtained from triplicate determinations. The letters a–e represent the significant differences ($p < 0.05$).

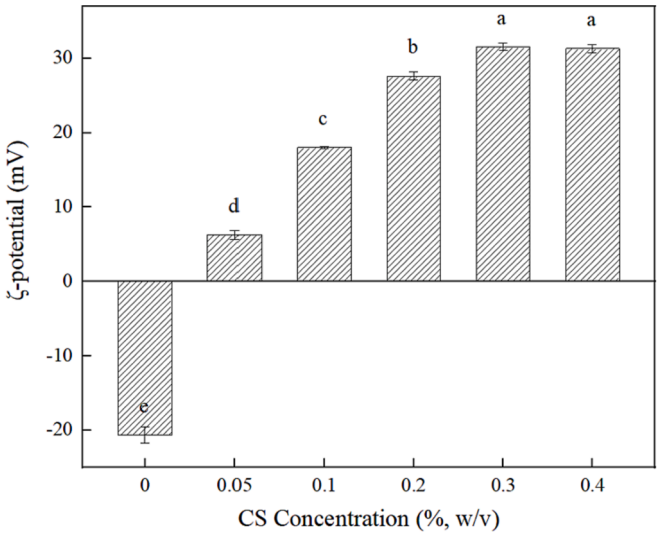

Fig. 2. Effect of the CS on the ζ-Potential of RBPH-FA emulsion The error bars indicate the standard deviation obtained from triplicate determinations. The letters a–e represent the significant differences ($p < 0.05$).

2.9.3. pH stability

The bilayer emulsions (with 0.3 % CS) were adjusted to pH 3.0–9.0. The droplet size and ζ-potential were detected.

2.10. Oxidative stability

The fresh emulsion was stored in sealed glass tubes at 40℃ for 14 days to evaluate the oxidative stability. The peroxide value (POV) [19] and thiobarbituric acid reactive substance (TBARS) [20] values of the emulsion were measured after 1, 4, 7, 10 and 14 days of storage.

2.11. In vitro digestion analysis

2.11.1. Simulated gastrointestinal tract (GIT) digestion

A simulated gastrointestinal tract model for conducting *in vitro* digestion studies was referred to the methods of Wei et al. [21].

Oral stage: 20 mL of simulated saliva was mixed with the initial emulsion; adjusted the pH value to 6.8. Digestion temperature was 37℃, the shaker speed was 100 rpm and the digestion time was 10 min.

Gastric stage: the sample digested from oral stage was mixed with the same volume of simulated gastric solution, and then the mixture was adjusted to pH 2.5. Digestion temperature was 37℃, the shaker speed was 100 rpm and the digestion time was 2 h.

Small intestinal stage: the sample after gastric digestion was adjusted to pH 7.0 for inactivating the pepsin. 10 mL of this sample was mixed with the same volume of simulated intestinal solution, adjusting to pH 7.0 again. The sample was digested continuously for 2 h in a water bath at 37℃ to simulate intestinal digestion.

In the whole process of the experiment, the samples after gastrointestinal digestion were measured by optical microscope and droplet size distribution.

2.11.2. Bioaccessibility of β-carotene

Bioaccessibility of β-carotene was determined by the method of Liu et al. [11]. Digestive solution was treated with a centrifuge at a speed of 8000 *g*, and the micellar phase with soluble β-carotene was collected at 4℃. The β-carotene was extracted by the mixture of *n*-hexane, acetone and ethanol (2:1:1, v/v). The content of β-carotene from the initial emulsion and micelle was determined at 450 nm. The bioaccessibility of β-carotene (%) was calculated by the formula:

$$Bioaccessibility\% = \frac{C_{micelle}}{C_{initial}} \times 100$$

where C_{micelle} and C_{initial} are the concentrations of β-carotene in the micelle and the initial emulsion, respectively.

2.12. Statistical analysis

Three replicate measurements were performed for each experiment, the experimental results were reported in terms of mean value ± standard deviation by SPSS 22.0. Origin 9.0 was used for drawing. Duncan's test was used to determine significant differences ($p < 0.05$).

3. Results and discussion

3.1. Effect of the CS on droplet size of emulsion

In Fig. 1, the droplet size of the conjugate emulsion changed significantly with the addition of CS ($p < 0.05$). The droplet size of the sample without CS was large (8.64 μm). The isoelectric point of the RBPH-FA was about 3.5 (unpublished). The monolayer conjugate emulsion was unstable at pH 5.0 (which was close to the isoelectric point of the conjugate), resulting in the larger droplet size [18]. Generally, small droplets can be produced after emulsification. In order to prevent emulsion droplets aggregation and flocculation or emulsion instability caused by other factors, the emulsion can be stabilized by adding stabilizers. CS is a common stabilizer [22]. When the concentration of CS was 0.05 %, the droplet size was the largest (12.8 μm). That was as the amount of CS with positive charge was not enough to form a complete covering film on the surface of the RBPH-FA conjugate monolayer emulsion. This made the aggregation and bridging flocculation occur between the emulsion droplets. When the CS concentration was above 0.1 %, the CS could form the second layer completely and densely on the interface of conjugate monolayer emulsion by electrostatic adsorption, fabricating the bilayer emulsion. The sufficient electrostatic and spatial repulsion could be provided by CS effectively. In addition, CS can also give a certain viscosity to the emulsion for preventing the emulsion from aggregating. Thirdly, the droplets were dispersed to be small and uniform ones forcibly by ultrasonic emulsification. Hence, the droplet size was the smallest (3.38 μm) with the CS concentration 0.3 %. Interestingly, when the CS concentration was 0.4 %, the emulsion droplet size increased slightly but not significantly. As the concentration of CS

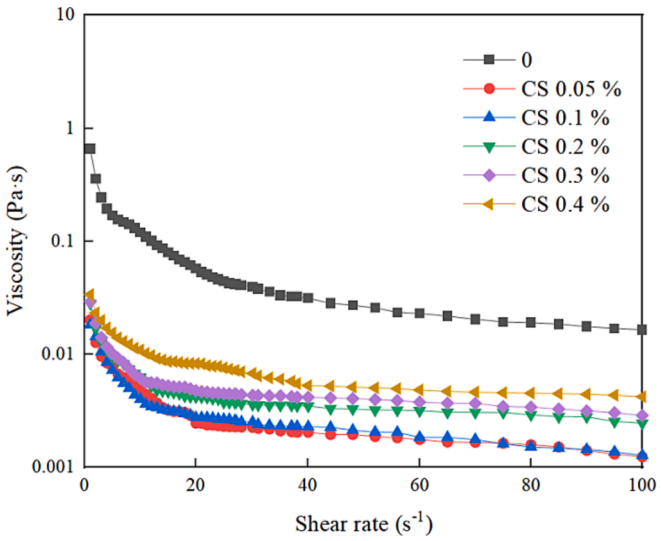

Fig. 3. Effect of the CS on the viscosity of RBPH-FA emulsion.

increased to 0.4 %, the gravitational force for flocculation was greater than the repulsive force between droplets, which caused flocculation of the emulsion. Studies have shown that the biopolymers in O/W emulsion could affect the stability of the emulsion through the depletion flocculation and the bridge flocculation [23]. This was consistent with the study of Chen et al. [24].

3.2. Effect of the CS on ζ-potential of emulsion

As the only cationic polyelectrolyte in nature, CS can form polyelectrolyte complexes with negatively charged polymers by electrostatic adsorption [25]. As Fig. 2 shown, the ζ-potential of the conjugate emulsion without CS was −20.63 mV. The ζ-potential of CS 0.05 % sample was 6.25 mV, and the absolute ζ-potential was the lowest. Then, the ζ-potential increased significantly ($p < 0.05$) with the increase of CS concentration. The ζ-potential value reached the maximum (31.52 mV) with the CS concentration 0.3 %.

The electrical properties of the emulsion formed by emulsifiers can be affected by the type of emulsifier, the pH value of emulsion and the ionic composition of emulsion [26]. The ζ-potential of the sample without CS was negative because the pH value of the emulsion was higher than the isoelectric point of the emulsifier. The polyelectrolytes or emulsifiers with different charges can form multilayer emulsions by electrostatic attraction [5]. It was found that the ζ-potential of CS 0.05 % sample was positive, but the absolute ζ-potential value was the smallest. The reason was that the CS had formed the interface film on the surface of the monolayer emulsion, making the emulsion positively charged. This proved the bilayer emulsion was prepared successfully. However, because the concentration of CS was not enough to form a dense interfacial film, the absolute ζ-potential value was small. With the increasing of CS addition, the bilayer interface film became thicker and denser. Therefore, the absolute ζ-potential value increased significantly ($p <$ 0.05). This could provide strong repulsive force between emulsion droplets, which can make the emulsion stable [27]. This was consistent with the droplet size results in the research. When the CS concentration was 0.4 %, the ζ-potential value did not increase. It was because the CS had been already adsorbed sufficiently on the surface of the emulsion droplets.

In summary, ultrasonic emulsification could make the CS disperse on the interface of the monolayer emulsion efficiently in the period of emulsion formation. The bilayer emulsion formed by CS could provide much more electrostatic repulsion and space repulsion, which made the emulsion more stable.

3.3. Effect of the CS on rheology of emulsion

The rheological behavior can not only characterize the mechanical behavior of different components of the emulsion system, but also be used to study the relationship between molecular structure and material mechanics of the polymer composed of the emulsion interface layer. The changes of the apparent viscosity, the G' and the G'' of the bilayer emulsion prepared by ultrasonic emulsification with the addition of CS are shown in Fig. 3 and Fig. 4.

In Fig. 3, the apparent viscosities of all emulsions decreased with the shear rate increasing. This indicated these emulsions were non-Newtonian fluids. The emulsion droplets turned form disordered to orderly, and the flow resistance turned to be smaller [28]. The apparent viscosity of the monolayer emulsion was the highest. This was because the monolayer emulsion flocculated under acidic conditions. After the bilayer emulsion forming by ultrasonic emulsification, the positive charge was carried by CS provided electrostatic repulsion and spatial repulsion to the emulsion, and the aggregation and flocculation phenomena of emulsion droplets were improved. From the Fig. 3, it was also found that the apparent viscosity of the bilayer emulsion increased with the increase of CS concentration, and the apparent viscosity of the CS 0.4 % sample was the highest. This is because the CS increases the repulsion between emulsion droplets, which made the entangled molecular chains of CS unfold adequately.

The G' and G'' values of the emulsions increased with the scanning frequency increasing in Fig. 4. The G' and G'' of the conjugate emulsion without CS intersected at high shear frequency, this demonstrated that the emulsion droplets aggregated seriously, cross-linking occurred and the viscosity increased. The G' of the emulsion sample with CS was larger than that of G'' in the scanning range, this indicated the rheological behavior of the emulsion was predominantly elastic. Therefore, it proved that there was a well-developed network between the emulsion droplets [29]. The G' decreased with the increase of CS concentration gradually, indicating the elasticity of the emulsion samples decreased. While the G'' gradually increased, indicating the viscosity increased. This was CS enhanced the repulsion between emulsion droplets by electrostatic adsorption on the RBPH-FA interfacial layer, this was mainly as that the CS enhanced the repulsion between emulsion droplets; on the other side, the network formed by CS was resilient [30]. The formation of the network between emulsion droplets was meaningful to maintain the emulsion stable, it also could resist the lipid oxidation occurring [31].

3.4. Effect of the CS on the microscopic morphology of RBPH-FA emulsion

3.4.1. Inverted fluorescent microscope

The inverted fluorescent microscope can directly show the apparent morphology of the emulsion, reflecting the distribution of the emulsion droplets. Fig. 5 shows the droplet distribution of the emulsions with different CS concentrations (0–0.4 %, w/v). The emulsion droplets of the sample without CS were large, and the droplets aggregated and flocculated. This was because the stability of RBPH-FA conjugate emulsion was unstable under acidic condition. In order to improve this situation, the CS was added as the stabilizer to form the bilayer emulsion by electrostatic adsorption under acidic condition. It was worth noting that the CS could be rapidly dispersed on the interface by ultrasonic emulsification, which was helpful to form stable bilayer emulsion. However, the CS 0.05 % sample showed obvious aggregation and flocculation, and the droplet size was the largest. The distribution of droplets became more uniform and the droplet size became smaller from the CS addition concentration above 0.1 %. Whereas, when the CS concentration was 0.4 %, the droplets were slightly larger than those of the CS 0.3 %. The result was the same with the finding of this study on droplet size. The bilayer emulsion formed by suitable concentration of CS had small and uniform droplet size and uniformity. The droplets of bilayer emulsion

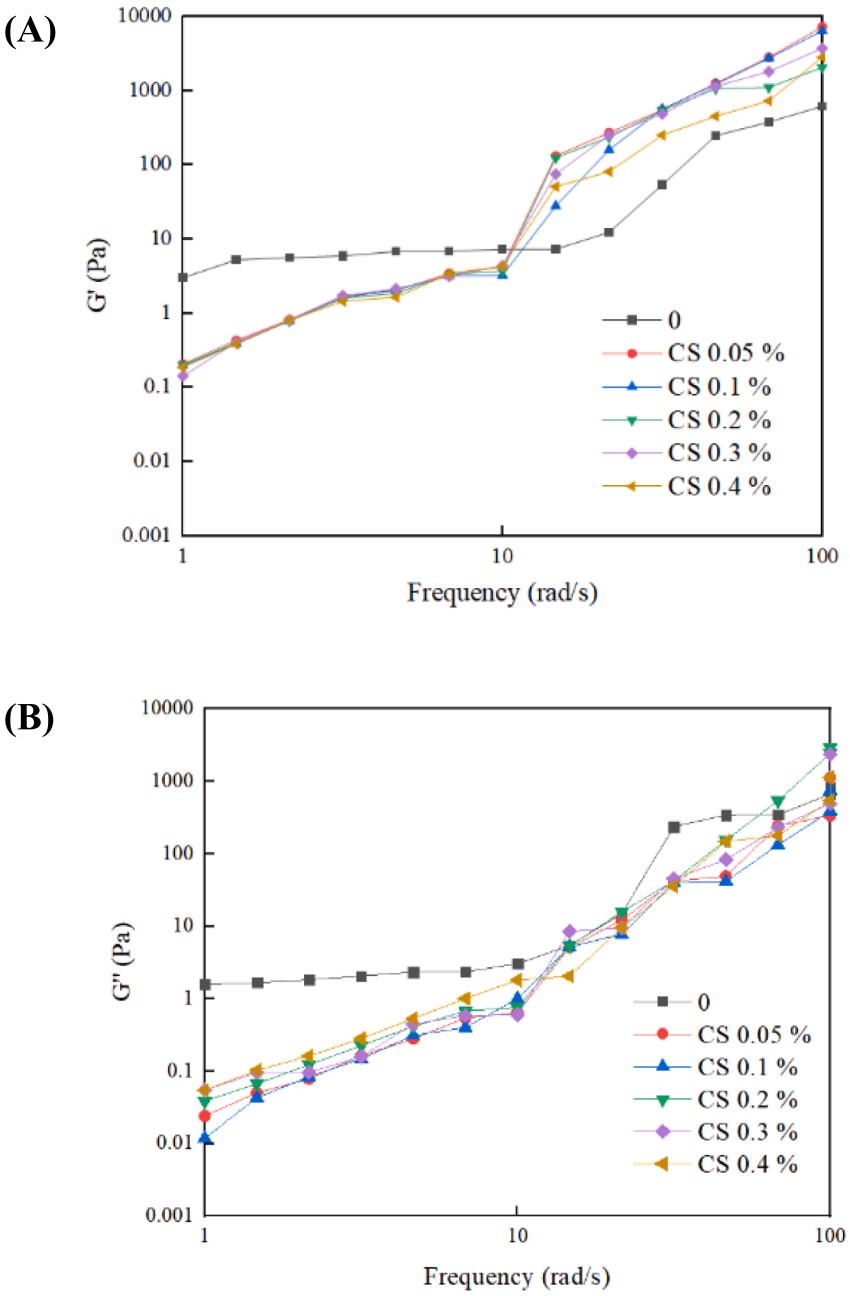

Fig. 4. Effect of the CS on the rheological properties (G' and G'') of RBPH-FA emulsion.

fabricated by ultrasonic emulsification were small and uniform.

3.4.2. *Cryo-SEM analysis*

The information of emulsion droplet was furtherly gained by Cryo-SEM. The result is exhibited in Fig. 6 (A). With the increase of CS concentration, the surface of the droplet changed from rough (0.05 %) to relatively rough (0.1 %, 0.2 %), until smooth (0.3 %, 0.4 %). This could show the process directly that CS formed a dense coating on the interface of monolayer emulsion gradually. The emulsion droplets with CS addition (above 0.1 %) were decreased significantly ($p < 0.05$), comparing with that without CS. Otherwise, in Fig. 6 (B), the overall picture of the CS 0.3 % sample was observed at the magnification of 1000×. The network could be found between the droplets. Due to the existence of the network microstructure, it could maintain "safe distance" between the droplets, which made the emulsion stable. The rheological study of the emulsion also confirmed the existence of this phenomenon. Therefore, CS could form the second layer on the surface of RBPH-FA emulsion and maintained the emulsion steadily by the network structure.

3.5. *Effect of the CS on storage stability of emulsion*

The storage stability of the emulsion can be reflected by the change of emulsion droplet size during the storage period. The change of droplet size and the appearance of the emulsion stabilized by CS (0–0.4 %) after 14 days of storage are shown in Fig. 7. With storage time passing, the droplet size of all emulsions increased significantly ($p < 0.05$). The emulsion with CS 0.05 % was unstable, the droplet size reached 19.91 μm which was the largest of all. The droplet size of the CS 0.3 % sample was the smallest (3.95 μm) of all the samples. The appearances of emulsions were shown in Fig. 7. The emulsions with CS (0.3–0.4 %)

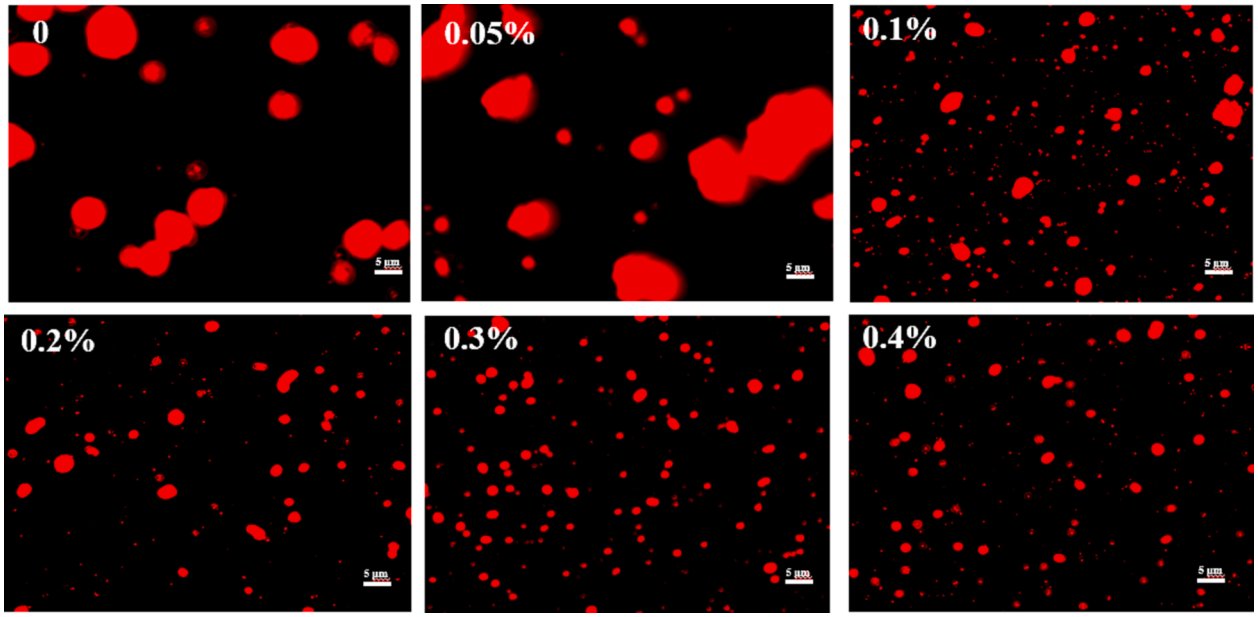

Fig. 5. Effect of the CS on the microstructure of RBPH-FA emulsion: Inverted fluorescent microscope.

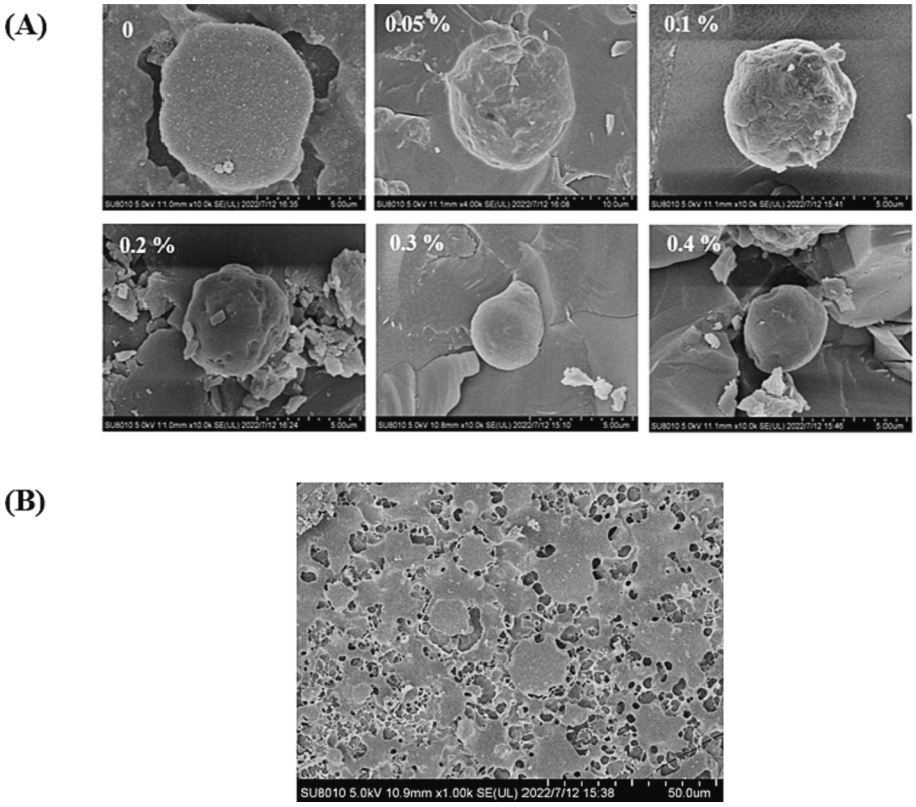

Fig. 6. Effect of the CS on the microstructure of RBPH-FA emulsion: Cryo-SEM (A) CS concentration (0–0.4%); (B) CS concentration (0.3%).

showed a uniform appearance and there was no cream separation. The bilayer emulsion was able to be stabilized by the physical barrier provide by CS. This was as the reason that there were enough electrostatic repulsion and spatial repulsion, these were positive to prevent the droplets from aggregating.

3.6. *Effect of the CS on stability of emulsion against environmental stress*

3.6.1. *Temperature stability*

The effects of heating temperature (30℃, 50℃, 70℃ and 90℃) on the stability of bilayer emulsions were evaluated (Fig. 8A). The data obtained in the experiment revealed the droplet of bilayer emulsion could still maintain a small droplet size (3.93 μm) at 50℃. The effect of heating temperature was not significant at the conditions below 50 °C,

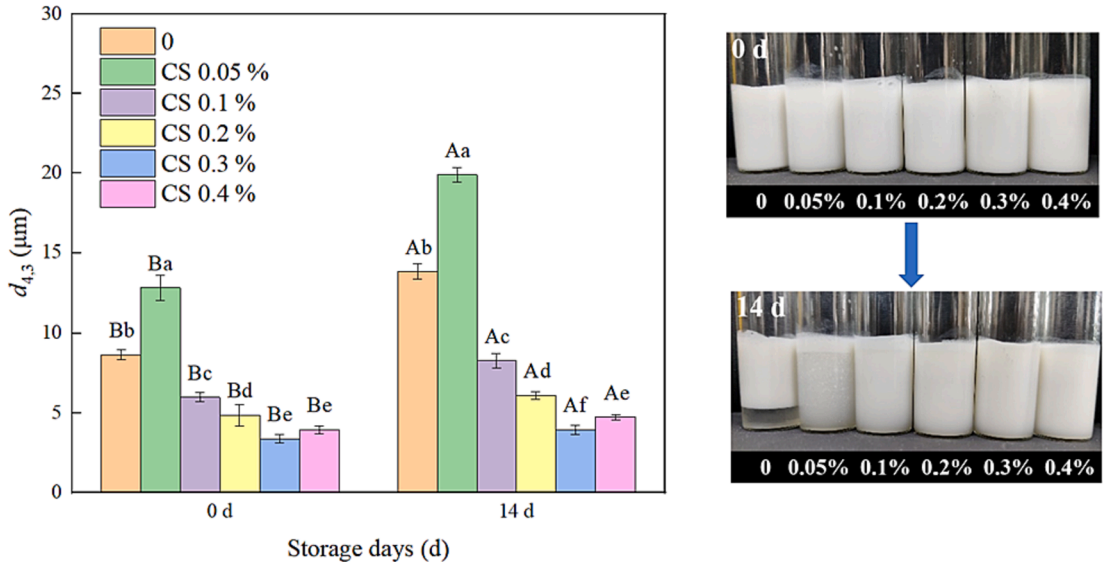

Fig. 7. Effect of the CS on the storage stability of RBPH-FA emulsion The error bars indicate the standard deviation obtained from triplicate determinations. The letters A–B and a–f represent the significant differences ($p < 0.05$) in the $d_{4,3}$.

the differences of droplet size between 30 °C and 50 °C were not significant ($p > 0.05$). From the emulsion appearance picture, it also can be seen that the appearance of the emulsion below 50℃ remained uniform and stable, there was no obvious creaming separation. The droplet size increased significantly at 70℃. When the heating temperature was 90℃, the droplet size was 6.29 μm, which was not significantly different from that at 70℃. The emulsion appearance picture clearly showed that the emulsion exhibited obvious creaming at 70-90℃. This reason was that the solubility of CS increased with the temperature increasing, whereas the non-adsorbed CS in the continuous phase would result in the depletion flocculation of bilayer emulsion. On the other hand, too many protons attack the acetal bond of CS, resulting in the degradation of CS, the disruption of interfacial layer, the aggregation and flocculation of emulsion, and then the destabilization of the emulsion [32,33]. The ζ-potential did not change significantly during the whole heat treatment interval. This indicated that the structures of the components in the continuous and discontinuous phases were not disrupted significantly [34].

3.6.2. *Ionic strength stability*

The emulsion stability can be affected as the existence of salt ions in the emulsion. The effects of NaCl on the droplet size and the ζ-potential of emulsion were evaluated in this study. As the results showed in Fig. 8 (B), the droplet size increased significantly with the ionic strength increasing. The droplet size of the sample with 300 mmol/L of NaCl was the largest (5.77 μm). The ζ-potential of the emulsion decreased gradually with the increase of ionic strength, and that of the initial emulsion was 32.68 mV. When the ionic strength reached the maximum (300 mmol/L), the ζ-potential decreased to the minimum value of 20.53 mV.

With the 50 mmol/L of NaCl, the emulsion was able to remain stable although the droplet size increased and the absolute ζ-potential value decreased slightly. The CS on the interface can also reduce the van der Waals gravitation between the droplets, which can maintain the emulsion stable well [35,36]. The appearance picture shows no obvious creaming occurred at<50 mmol/L of NaCl. But the phenomena of creaming separation were observed in the emulsions at above 100 mmol/L of NaCl. There even generated flocculent complexes in the emulsions with 300 mmol/L of NaCl.

The electrostatic shield occurred as the counter-ions accumulated around the interface of the bilayer emulsion. This would affect the electrostatic interactions between all the charged surfactants and biopolymers [37]. The dense CS layer interface was destroyed, which led to bridge flocculation [38].

3.6.3. *pH stability*

The emulsion interfacial properties depend on the electrical properties of the weak electrolyte and the properties of the interfacial film. In this study, the effect of pH on the stability of bilayer emulsion was investigated. The droplet size and ζ-potential were used to characterize the emulsion stability change trend with pH (3.0–9.0). The results are shown in Fig. 8 (C). The droplet size of the bilayer emulsion was small and the change was not significant ($p > 0.05$) with the pH 3.0–5.0. At pH 6.0, comparing with the emulsion sample with lower pH, the droplet size increased significantly ($p < 0.05$), the droplet size was 4.88 μm. The CS could form fine interfacial layer as the proper solubility under acidic condition. Therefore, the emulsion droplet size was smaller, the absolute ζ-potential value was larger and the emulsion could keep stable. It can be seen furtherly in the Fig. 8 (C). The bilayer emulsion droplet size began to increase significantly from pH 7.0 ($p < 0.05$), and reached the maximum of 17.76 μm at pH 9.0. The absolute ζ-potential value of bilayer emulsion decreased with the increase of pH. Under alkaline conditions (pH 8.0 and 9.0), the ζ-potential was negative and the absolute value reached the minimum value of 5.05 mV. The CS molecule was deprotonated with the higher pH (pH ≥ 7.0), as the pH was greater than or equal to pKa of CS (pKa 7.0) [39], which led to the CS solubility decreased. This caused the CS layer (the second layer of emulsion) to change from dense to loose, resulting in the bridging flocculation of the bilayer emulsion under alkaline conditions. The picture showed the appearance of emulsions with different pH, it also proved the bilayer emulsion was stable under acidic condition.

3.7. *Effect of the CS on oxidative stability of emulsion*

Lipids, especially plant lipids, contain unsaturated bonds, they are prone to redox reactions in the process of storage. This has negative effects on the edible and nutritional value of the lipids. Lipids can also dissolve fat-soluble nutrients; therefore, it can be used as medium in the delivery of nutrients. The change trends of lipid oxidation primary (peroxides) and secondary (TBARS) products were used to reflect the protection of bilayer emulsion on lipids during storage period. The results are shown in Fig. 9.

The POV of all emulsions increased gradually with the extension of storage time. It was found that the POV of the CS 0.05 % sample (17.54 mmol/kg oil) was even higher than that of the sample without CS (15.17

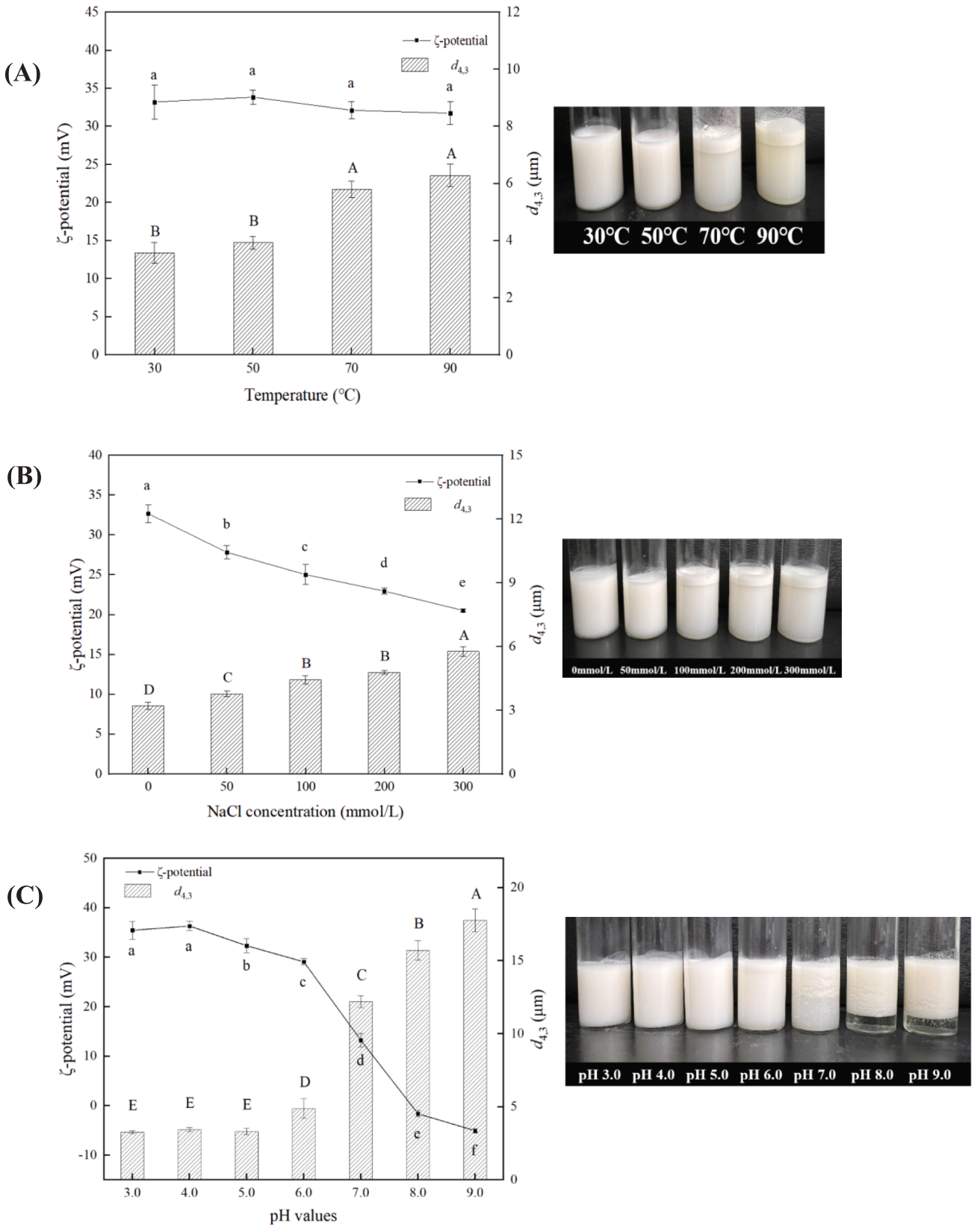

Fig. 8. Effect of the CS on the stability of emulsion against environmental stress (A) temperature stability; (B) ionic strength stability; (C) pH stability The error bars indicate the standard deviation obtained from triplicate determinations. The letters A–E and a–f represent the significant differences ($p < 0.05$) in the $d_{4,3}$ and ζ-potential.

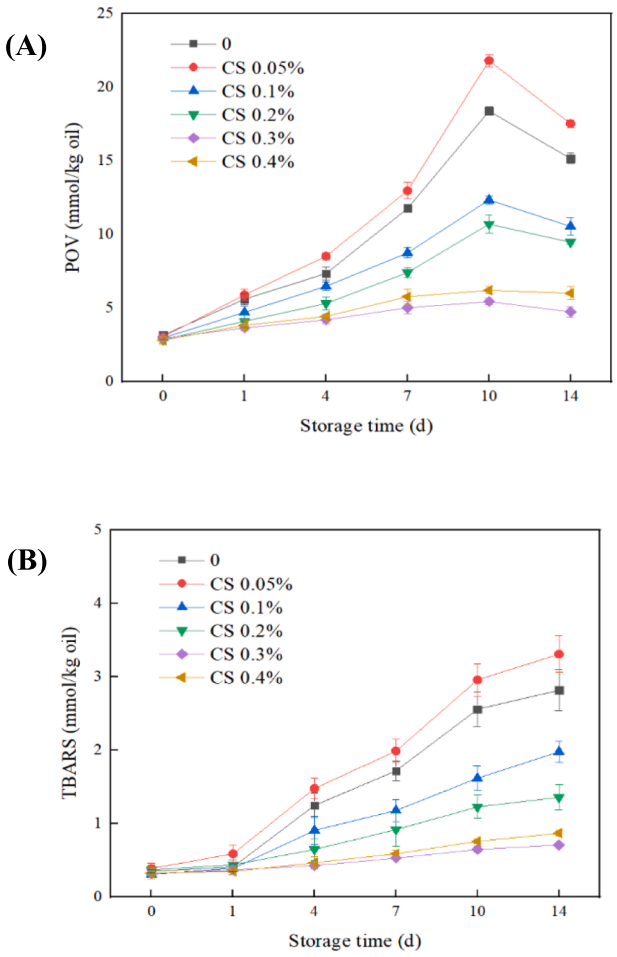

Fig. 9. Effect of the CS on POV (A) and TBARS value (B) of RBPH-FA emulsion The error bars indicate the standard deviation obtained from triplicate determinations.

mmol/kg oil) in the 14 days' storage experiment. The reason was that the CS addition was not enough to form a bilayer emulsion completely. The emulsion was unstable, the droplets were tended to aggregate together and flocculate. The bilayer interface was disrupted as the aggregation of emulsion droplets. The lipids were explored outside to the oxidative environment, contacted with oxygen and metal ions (especially iron ions) in the system, leading to rapid lipid oxidation. The POVs of the samples with 0.3 % and 0.4 % CS concentration were smaller (4.75 mmol/kg oil and 6.01 mmol/kg oil, respectively). It has been demonstrated that multilayer emulsion had better oxidation resistance and stability than monolayer emulsion [40]. With the increase of CS concentration, the amount of lipid oxides in the bilayer emulsion gradually decreased. This indicated that the bilayer emulsion with suitable CS concentration had lipids antioxidant capacity. The changing trend of TBARS value was basically consistent with that of POV.

3.8. *In vitro digestion*

The β-carotene is a lipid-soluble nutrient, which is widely applied in food processing. As the affecting of the external condition, it is susceptible to oxidation for conjugated unsaturated bonds, resulting in the loss of nutritional value. In this study, β-carotene was delivered by bilayer emulsion system, and the changes of encapsulated β-carotene emulsion at different stages of simulated human digestion were investigated by the complete GIT model.

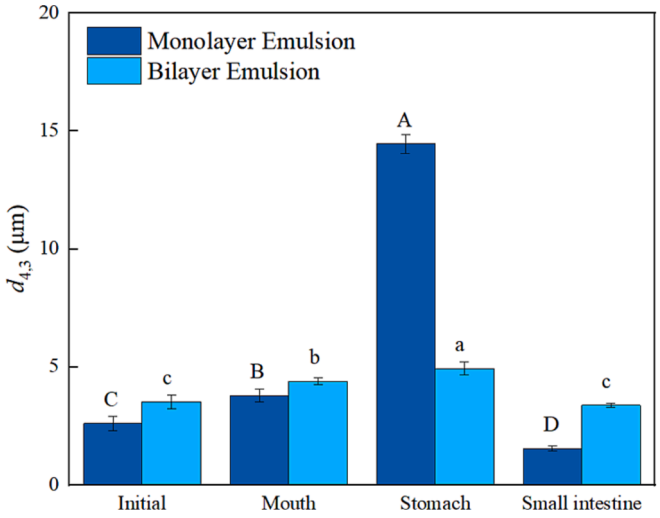

Fig. 10. Droplet sizes of β-carotene emulsion *in vitro* simulated digestion The error bars indicate the standard deviation obtained from triplicate determinations. The letters A–D and a–c represent the significant differences ($p < 0.05$) in the monolayer emulsion and bilayer emulsion.

3.8.1. *Droplet size of β-carotene emulsion in vitro simulated digestion*

The droplet sizes of the monolayer and bilayer emulsions at different stages of simulated digestion conditions are shown in Fig. 10. In the initial stage, the droplet sizes of monolayer emulsion and bilayer emulsion were 2.61 μm and 3.53 μm, respectively. As the bilayer emulsion had thicker interfacial layer formed by RBPH-FA and CS, hence, the droplet size was larger than that of monolayer emulsion. The two kinds of emulsions were evenly distributed, and there was no creaming in the emulsion. This was because the fresh emulsion had a good distribution and stayed a stable state after ultrasonic emulsification, and there is no obvious difference between the two emulsions from the appearance.

After simulated oral digestion, it was found that the droplet sizes of the two kinds of emulsions increased. This was due to the aggregation and flocculation of emulsion droplets. The simulated oral fluid contained the mucin, a glycoprotein composed of mucopolysaccharide, which increased the osmotic attraction between droplets, leading to the bridging flocculation or the depletion flocculation of the emulsions [41].

After simulated stomach digestion, the droplet size of the two emulsions continued increasing. The droplet size of monolayer emulsion increased significantly ($p < 0.05$). This was due to the effects of the electrostatic shielding, the pepsin and the oral mucin on the interfacial properties of the monolayer emulsion [42–43]. While the bilayer emulsion was stable under acidic conditions, the droplet size change was not obvious. In addition, the second interface formed by CS could prevent pepsin from hydrolyzing the conjugate on the interface layer.

After simulated small intestinal digestion, the droplet size of the two emulsions decreased significantly compared with the stage of gastric digestion. The main place of emulsion digestion is the small intestine stage [21]. The CS cannot be digested in the small intestine. Compared with the monolayer emulsion, the bilayer emulsion in the simulated intestinal fluid remained relatively stable. This forecasted that the second layer formed by CS could resist the external environment and prevent bile salt and lipase from contacting with lipids, having a slow release effect on β-carotene [44]. From the results of 3.6.3 (the effect of chitosan on the pH stability of the emulsion), it can be concluded that the bilayer emulsion was less stable under neutral conditions. With the extension of digestion time, the CS will be desorbed from the second layer interface. Otherwise, the interfacial properties of the bilayer emulsion would be affected by the digestive enzymes and the bile salts, which made the RBPH-FA interface layer expose to the digestive environment, then the nutrient was released gradually [11]. The *in vitro*

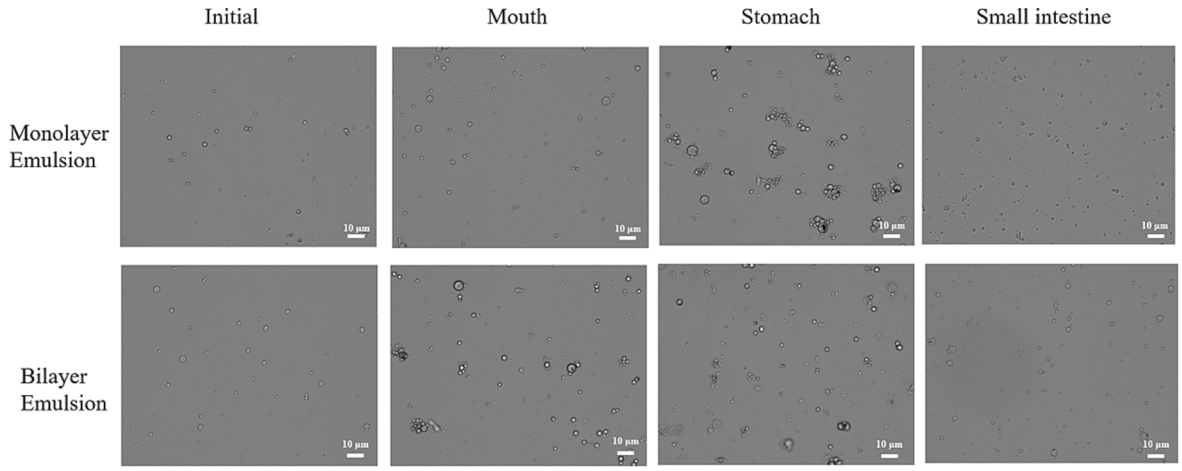

Fig. 11. Microstructure of β-carotene emulsion *in vitro* simulated digestion.

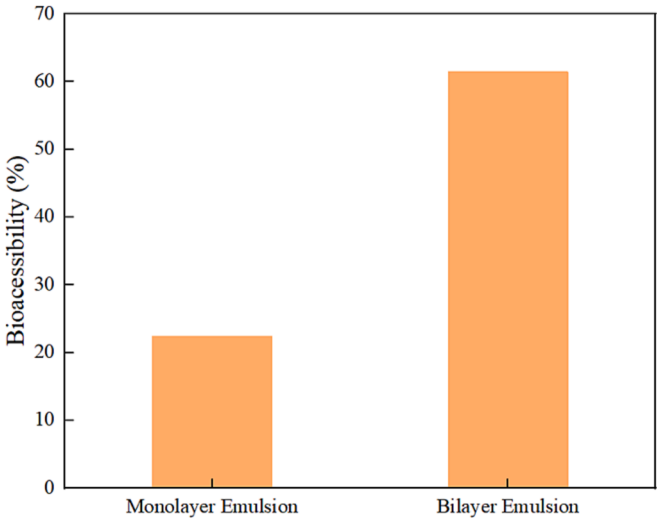

Fig. 12. Bioaccessibility of β-carotene emulsion.

digestion process of emulsions was showed by the optical microscope observation in Fig. 11. The results confirmed the droplets changes of monolayer emulsion and bilayer emulsion during *in vitro* simulated digestion.

3.8.2. Bioaccessibility

Bioaccessibility is the degree that the nutrients are absorbed and utilized by intestinal epithelial cells in the form of mixed micelles in GIT. It is necessary to deliver the β-carotene by the stable emulsion system, which could resist the external adverse environment and improve the bioaccessibility of it.

From the results (Fig. 12), the bioaccessibility of β-carotene in bilayer emulsion was significantly higher than that in monolayer emulsion. This was because the CS interface layer on the surface of the bilayer emulsion can resist digestive enzymes in the stomach and the extreme environment, it could deliver the β-carotene to the small intestine steadily. In the gastric stage, the interface proteins of monolayer emulsion were hydrolyzed by pepsin and the interface layer was destroyed, resulting in lipid droplets exposed to the digestive environment and the β-carotene was destroyed prematurely. The bilayer emulsion formed by CS prolonged the retention time of the emulsion in the digestive tract [45]. Some studies reported that the bilayer emulsion fabricated by electrostatic layer-by-layer deposition of CS could improve the bioaccessibility of lipid-soluble nutrients [46].

4. Conclusions

In this study, the bilayer emulsion was prepared by high intensity ultrasonic emulsification under acidic condition (pH 5.0), with RBPH-FA as the first layer and chitosan as the second layer. The results showed that the chitosan could stabilize the conjugate emulsion and form "armor" on the interface to prevent the emulsion aggregation. The chitosan could provide electrostatic repulsion to maintain a "safe distance" between droplets. Chitosan could improve the interfacial stability of conjugate emulsions under acidic conditions by forming a network structure in the continuous phase and generating steric hindrance. The chitosan-stabilized conjugate emulsion had good storage stability, oil oxidation resistance and the ability to resist the changes of external complex environmental factors. The bilayer emulsions provided effective protections to the β-carotene during delivery and significantly improved the bioaccessibility of β-carotene compared to monolayer emulsions.

CRediT authorship contribution statement

Tengyu Wang: Conceptualization, Investigation, Methodology, Formal analysis, Writing – original draft, Visualization. **Shirang Wang:** Investigation, Validation, Formal analysis, Writing – review & editing. **Lijuan Zhang:** Investigation. **Jiapeng Sun:** Software. **Tianhao Guo:** Data curation. **Guoping Yu:** Conceptualization, Supervision, Project administration, Writing – review & editing, Funding acquisition. **Xiufang Xia:** Resources, Methodology, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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