Pickering emulsions stabilized by soybean protein isolate/chitosan hydrochloride complex and their applications in essential oil delivery

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Running title: Stabilization and applications of Pickering emulsions

Abstract: In this work, fabrication of soybean protein isolate (SPI)/chitosan hydrochloride (CHC) composite particles stabilized O/W Pickering emulsions using soybean oil as an oil phase was optimized by examining the effects of pH, SPI/CHC mass ratio, SPI/CHC composite particle concentration and oil phase fraction on the stability of the emulsions. The results showed that under the conditions of SPI/CHC mass ratio 1:1, pH 4 and particle concentration 2%, the SPI/CHC composite particles could stabilize the emulsions with oil phase fraction up to 80%. At an oil phase fraction of 60%, the emulsions had a minimum particle size. The microstructure, storage and oxidation stabilities and rheological properties of the emulsions were determined. Using this SPI/CHC composite particle-stabilized Pickering emulsion template, citrus essential oil (CEO) Pickering emulsion (CEOP) was prepared. CEOP was found to markedly inhibit two food-related microorganisms, *Staphylococcus aureus* and *Escherichia coli*. In addition, the CEOP emulsion dilution (containing 4500 μL CEO/L) not only improved the water solubility of CEO, but also effectively retarded the browning and bacterial growth of fresh-cut apple. The SPI/CHC-stabilized Pickering emulsion template constructed in this work provides a promising alternative for the delivery of antimicrobial essential oils in the food industry.

Keywords: Soybean protein isolate-chitosan hydrochloride; Pickering emulsions; Citrus essential oil

1. Introduction

Emulsions play an important role in the food and pharmaceutical industries, such as for the encapsulation and delivery of bioactive compounds [1, 2]. Traditional emulsions are stabilized by surfactants or polymers. They are generally categorized into macroemulsions (200 nm–200 μm), nanoemulsions (20–200 nm) and microemulsions (4–200 nm) [3]. Nanoemulsions possess a higher level of kinetic stability than macroemulsions and microemulsions. However, nanoemulsions are thermodynamically unstable systems, and can be destabilized through one or several mechanisms including creaming or sedimentation, flocculation, coalescence and Ostwald ripening [3]. Although highly physically stable nanoemulsions can be obtained by the use of appropriate surfactant, lipid composition and emulsification technique, it is still in demand to develop alternative systems for the delivery of bioactive compounds as a supplement. Pickering emulsions are the emulsions stabilized by natural colloid particles that adsorb at the interface between two phases of the emulsions [4], they shield droplets at high concentrations of dispersed phase by rendering stabilization against coalescence and Ostwald ripening [5], thus possessing higher stability during anti-coagulation, fat floating and Ostwald ripening compared with the traditional emulsions [6]. The colloidal particles prepared by natural biomolecules are non-toxic, biocompatible and ecologically acceptable, thus their potential applications in the food industry are attracting more and more attention [7, 8]. However, the hard shell of Pickering emulsions is difficult to equilibrate during preparation to form the desired droplet size [9]. Their emulsification stability is closely

related to the concentration and size of solid particles [10]. In recent years, the rapid development of material technology has resulted in an increase in the number of available particles. Protein-polysaccharide composite particles stabilized emulsions have been extensively studied and provide better emulsion stability than using protein alone [9]. Pickering emulsions using protein-polysaccharide complex as stabilizers can be used to encapsulate tartaric acid and other bioactive ingredients, and showed good antibacterial and antioxidant properties in the fruit preservation [11]. For instance, Pickering emulsion loaded with cinnamon-perilla essential oil (C-PEO) stabilized by chitosan-collagen complexes showed good antimicrobial properties and sustained-release ability on chilled fish fillet preservation [12]. Therefore, it has attracted more research attention. However, selection of protein and polysaccharide is crucial to effectively improve the stability of Pickering emulsions.

Soybean protein isolate (SPI), prepared from skimmed soybean meal by alkali/acid precipitation (isoelectric point 4.5) [13], is the most important plant-derived protein in the food industry due to its abundant and inexpensive source. Combination of SPI with polysaccharide was reported to enhance stability of Pickering emulsions [13]. The SPI/chitosan (CS) complex was reported to effectively stabilize emulsions [14], but the oil fraction was relatively low, only up to 40%. Chitosan hydrochloride (CHC), a water-soluble product obtained by the protonation modification on the amino groups of chitosan (CS) with hydrochloric acid [15]. Compared with CS, CHC not only retains the properties of chitosan, but also is water soluble, thus use of CHC instead of CS for complexation with SPI is more convenient for practical manipulation [15,16].

In addition, CHC possesses strong cationic property, thus is expected to become a drug carrier under mild preparation conditions [17, 18].

Citrus essential oil (CEO) is extracted from orange peel, and its main component D-limonene has good antibacterial, antioxidant, anti-inflammatory and anti-tumor effects [19]. Thus, CEO, as a natural antimicrobial agent, has a promising application in food industry, such as the sanitization and preservation of vegetables and fruits. However, CEO is prone to evaporation or decomposition when exposed to light, oxygen and heat during food processing and storage, drug preparation and antibacterial membrane preparation, which greatly limits its application. In order to overcome the sensitivity of essential oils to the environment, various improvement methods have been developed, among which the method using emulsion to stabilize essential oils has attracted more and more attention [11,20]. Fabrication of essential oil-based Pickering emulsions and nanoemulsions using various stabilizers has been reported [3], and the potential applications of essential oil emulsions have also been widely investigated, for example, as drug carriers, larvicidal agent carriers, antioxidant carriers, antimicrobial agents and ingredient in active packaging [21,22].

In this study, fabrication of SPI/CHC composite particles stabilized Pickering emulsions using soybean oil as an oil phase was investigated by examining the effects of pH, SPI/CHC mass ratio, SPI/CHC composite particle concentration and oil phase fraction on the stability of the emulsions. Using such SPI/CHC composite particles stabilized Pickering emulsion template, CEO Pickering emulsion (CEOP) was prepared,

its antibacterial and antioxidant properties and preservative effect on fresh-cut apples were also studied.

2. Materials and methods

2.1. Materials

The soybean protein isolate (SPI) and the chitosan hydrochloride (CHC, deacetylation degree 80-90%, molecular weight 150,000-250,000 Da) were provided by Zhengzhou Qihuaton Chemical Products Co. Ltd. China and Shanghai Maclean Biochemical Technology Co. Ltd. China, respectively. Citrus essential oil (main component is D-limonene) was purchased from Borui Spice Co. Ltd., China. The bacterial strains were cultivated in our laboratory. Apples were purchased from local Yonghui supermarket, Hangzhou, China.

2.2. Complexation of SPI with CHC

The SPI stock solution (4%, w/v) was prepared by dissolving in deionized water with stirring (800 r/min) and assistance of ultrasound for 4 h and to ensure full dissolution. The CHC stock solution (1%, w/v) was prepared by dissolving in deionized water bath and stored at 4 ℃ for later use.

The SPI-CHC composite solution (total SPI/CHC concentration 0.05 % (w/v), SPI:CHC=1:1) was prepared by mixing SPI solution with CHC solution for 4 h with stirring, followed by adjusting to different pH value (2-7) with HCl or NaOH. The SPI-CHC composite solution was freeze-dried to yield off-white powder.

2.3. Characterization of SPI/CHC composite particle

2.3.1. Zeta (ζ) - potential, particle size and polydispersity index (PDI)

The ζ-potential, particle size and PDI of the above SPI-CHC composite solutions (SPI:CHC=1:1, total SPI/CHC concentration was 0.05%), SPI solution (0.05 %, w/v) and CHC solution (0.05 %, w/v) with different pH values (2-7) were determined.

2.3.2. Scanning electron microscope (SEM)

Freeze-dried samples were directly mounted on the double-sided adhesive and coated with a gold layer to prevent charging. SEM images were recorded using a scanning electron microscope (ZEISS Sigma 300, Germany) at an accelerating voltage of 3.0 kV.

2.4. Construction of SPI/CHC composite particles-stabilized Pickering emulsion template

Referring to a previous literature with slight modifications [23], Pickering emulsions were obtained by homogenizing a mixture of soybean oil and SPI/CHC composite particles using a high-speed shear (12000 rpm, 4 min). Conditions for the preparation of SPI/CHC composite particles stabilized Pickering emulsions were optimized by the following single factor tests.

Effects of SPI/CHC mass ratio (1:4, 1:2, 1:1, 2:1, and 4:1) on the stability of emulsions was investigated under the conditions of pH 4, SPI/CHC complex particle concentration 2% (w/v) and oil phase fraction (φ) 50%. The effect of pH (3-7) on the stability of emulsions was investigated under the conditions of SPI/CHC mass ratio 1:1,

SPI/CHC complex particle concentration 2% (w/v) and oil phase fraction 50%. The effect of SPI/CHC complex particle concentration (0.25%，0.5%，1%，1.5%，2% (w/v)) on the stability of emulsions was investigated under the conditions of SPI/CHC mass ratio 1:1, pH 4 and oil phase fraction 50%. The effect of oil phase fraction (10%, 20%, 30%, 40%, 50%, 60%, 70%, and 80%) on the stability of emulsions was investigated under the conditions of SPI/CHC mass ratio 1:1, pH 4 and SPI/CHC complex particle concentration 2% (w/v).

2.5. Characterization of Pickering emulsions

2.5.1. Appearance and storage stability of the emulsions

When the SPI/CHC composite particle stabilized Pickering emulsion was dropped into water, the droplets dispersed quickly, while the emulsion shape remained in the oil, which proved that the emulsion was O/W type emulsion (Fig.S1). The emulsions prepared under different conditions were stored at 4 ℃. The change in appearance of the emulsions during the storage period was recorded by photographing at the same time intervals. The creaming index (CI) was calculated using the following equation (1). Each measurement was conducted for three technical replications.

$$CI\ (\%) = (Hs/Ht) \times 100 \qquad (1)$$

where Hs was the height of the serum layer and Ht was the total height of the emulsion.

2.5.2. Particle size of Pickering emulsion

The particle size of the emulsions characterized by both area average diameter $d_{3,2}$ and volume average diameter $d_{4,3}$, was measured by a laser particle size analyzer (Leica TCS-SP8, Germany).

2.5.3. Morphology and microstructure of the Pickering emulsions

An optical microscope was used to observe the morphology of a drop of freshly prepared emulsions. Confocal Laser Scanning Microscopy (CLSM) was used to observe the microstructure of the emulsions. The sample staining method was as follows: 500 μL freshly prepared emulsions prepared under the conditions of SPI/CHC mass ratio 1:1, SPI/CHC complex particle concentration 2% (w/v), oil phase fraction φ 50%, and different pH values (3-7), was stained with 20 μL Nile red and Nile blue mixed fluorescent dye in the dark; Nile red and Nile blue were excited at 488 nm and 633 nm, respectively. The scanning frequency was 100 Hz, and the scanning density was 1024×1024.

2.5.4. Rheological properties of SPI/CHC Pickering emulsions

The apparent viscosity, elastic modulus (G') and loss modulus (G'') of the emulsions were characterized and analyzed using a rotational rheometer (Discovery HR-2, USA). Appropriate amounts of samples were placed between the horizontal plates (diameter: 20 mm), and the test temperature was set as 25 ℃, the scanning frequency was fixed at 1 Hz, and the apparent viscosity curve was recorded at shear rates ranging from 0.01 to 100 s^{-1}. Frequency sweeps were carried out at a strain of 0.5% and the change curves of the modulus were recorded at scanning frequencies ranging from 0.1 to 10 Hz to ensure that all measurements were in the linear viscoelastic region

[24].

2.5.5. Oxidation stability of SPI/CHC Pickering emulsions

A certain volume of freshly prepared emulsions was placed in a centrifuge tube and stored at 37 ℃ in the dark for 15 days to allow the oxidation of emulsions. During this period, appropriate amount of sample was taken every 3 days to determine the content of peroxide values (POV value) and thiobarbituric acid reactive substances (TBARs value) to evaluate oxidation degree of the emulsions [25]. Each sample was measured three times in parallel.

2.6. Preparation of citrus essential oil Pickering emulsions (CEOP)

After considering the storage stability and rheological properties of the emulsions, citrus essential oil Pickering emulsion (CEOP) was prepared under the optimized conditions for the preparation of SPI/CHC complex stabilized Pickering emulsion template obtained above. All the homogenization processes were performed using homogenizer (FJ200S, Hangzhou, China) at 12,000 rpm for 4 min.

2.7. Characterization of CEOP

2.7.1. Antibacterial assay

Staphylococcus aureus (*S. aureus*) and *Escherichia coli* (*E. coli*) were selected to determine the antibacterial activity of the emulsions. The minimum inhibitory concentration (MIC) of CEO and CEOP was determined by tube gradient dilution method [26]. Briefly, 50 μL CEOP (containing 30 μL CEO) was added to the test tube, and the mixture was thoroughly mixed with the liquid medium. Half of the mixture was added to the next test tube and thoroughly mixed, and the gradient dilution was carried

out similarly, so that the concentration of CEOP in the 6 test tubes decreased gradient. Finally, 40 μL of bacterial suspension of the test strain was added to each test tube. At the same time, blank control (containing only bacterial suspension and medium), the experimental group and control group were incubated at 37 ℃ for 24 h, and all groups were observed at the end of the incubation period.

2.7.2. Antioxidant assays

Oil phase was prepared by mixing CEO with soybean oil at ratios of 0:1, 1:3, 1:1, 3:1 and 1:0. The aqueous phase containing SPI/CHC particles was mixed with the oil phase at an oil phase fraction of 60% to explore the effect of essential oil content in emulsions (final CEO concentration 0, 15, 30, 45 and 60%) on its antioxidant activity.

The DPPH radical scavenging activity of CEOP was measured referring to a previous report [27]. Emulsion (0.1 mL) was thoroughly mixed with DPPH ethanol solution (4.0 mL, 0.1 mM) at 25°C for 30 min in the dark. The absorbance value of the solution was measured at 519 nm. The control group was ethanol solution without DPPH, and ultrapure water was used as blank control instead of sample. DPPH radical scavenging activity ($DPPH_{scav}$) was calculated using formula (2):

$$DPPH_{scav}\,(\%) = \left(1 - \frac{A_{sample} - A_{control}}{A_{blank}}\right) \times 100 \qquad (2)$$

The hydroxyl radical scavenging rate was determined using salicylic acid method [28]. Sample solution was mixed with same volume of $FeSO_4$ solution (6 mM), H_2O_2 solution (3%), salicylic acid-ethanol solution (9 mM) and distilled water. After incubation at 37 ℃ for 30 min, absorbance of the resulting solution was measured at 517 nm. The control group was prepared without addition of H_2O_2 solution, and the

blank group was prepared without addition of sample solution. Hydroxyl radical scavenging activity (•OH_{scav}) was calculated using formula (3):

$$\bullet OH_{scav}\ (\%) = (1 - \frac{A_{sample} - A_{control}}{A_{blank}}) \times 100 \qquad (3)$$

Same volume of ABTS solution (7 mM) and potassium persulfate solution (2.45 mM) were mixed and left in the dark for 12-16 h to generate ABTS free radical [29]. ABTS working solution was prepared by mixing the ABTS free radical solution with PBS (pH7.4) at a ratio of 1:30-50 to make the absorbance of the solution between 0.7±0.02 at 734 nm. Sample solution (0.3 mL) was mixed with 3 mL ABTS working solution, the reaction was carried out in the dark for 6 min, and absorbance of the reaction mixture was then measured at 734 nm. The sample was replaced by distilled water to make the blank control. The ABTS free radical scavenging activity ($ABTS^{\bullet+}_{scav}$) was calculated using formula (4):

$$ABTS^{\bullet+}{}_{scav}\ (\%) = (1 - \frac{A_{sample}}{A_{blank}}) \times 100 \qquad (4)$$

2.8 Fresh-keeping effect of CEOP on fresh-cut apples

2.8.1. Appearance quality changes

The apple was freshly cut into 0.5 cm thick slices and immediately soaked in CEOP dilution (CEO concentration 1500-5500 μL/L). Water group and SC group were made by soaking apple slices in distilled water and complex particle solution, respectively, for comparison. The apple slices were drained after soaking for 1 min, and then transferred to a sterile sealed bag and stored at 4°C for 9 days. The morphological changes of apple slices were recorded by photographing [30].

2.8.2. Color change determination

The color of the apple slice surface was determined by recording their L* (lightness), a* (redness and greenness) and b* (yellowness and blueness) values on a CR-400 colorimeter (Konica Minolta, Japan) [30], which was calibrated by the standard white plate provided. The color measurements were performed for each sample every 2 days during 9 days of storage. All measurements were made in triplicate. The total color difference (ΔE) was used to evaluate the anti-browning potential of different treatments. The ΔE is calculated as follows:

$$\Delta E = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}} \quad (5)$$

2.8.3. Degree of browning

Freshly cut apples (2.0g) were dipped in double-distilled water (40 mL), and then trichloroacetic acid (10 mL, 10%) was added. The apples were kept in a water bath at 35 ℃ for 2 h. After filtration, the absorbance was measured at 420 nm. Each treatment was performed in parallel three times [31].

2.8.4. Polyphenol oxidase (PPO) and peroxidase (POD) activity assays

The PPO and POD activity of CEOP was measured referring to a previous report [32]. A mixture of freshly cut apples (5.0 g), sodium phosphate buffer (45.0 mL, 0.2 M, pH7.0) and polyvinyl pyrrolidone (5.0 g) was ground into homogenates on an ice bath. After centrifugation (10000 r/min, 5 min), the supernatant was used for the PPO and POD activity assays. Briefly, the supernatant (0.8 mL) was mixed with catechol (2.4 mL, 0.02 mol/L), and the absorbance of the reaction mixture was recorded at 420 nm every 10 s in the first 1 min, and then was recorded every 30 s for 3 min. The change

of the absorption value in unit time is proportional to the unit PPO enzyme activity.

The supernatant (0.2 mL) was mixed with 2.8 mL guaiacol solution (25 mM), followed by the addition of 200 μL H_2O_2 solution (0.5 M) to initiate the reaction. The absorbance value was recorded every 10 s at the wavelength of 470 nm for continuous measurement to obtain at least 15 data points. Each treatment was repeated three times. The change of the absorption value in unit time is proportional to the unit POD enzyme activity.

2.8.5. Determination of total bacterial count (TBC)

The total bacterial count was determined by employing the coating counting method. Briefly, fresh-cut apples (10 g) were chopped, ground and homogenized with 90 mL water, yielding a 10^{-1} diluent, which was further diluted with sterile saline (0.9% NaCl, w/w) to a series of sample diluents (10^{-2}, 10^{-3}) using the tenfold-dilution method. Each sample diluent (100 μL) was plated on sterile nutrient agar medium, which was was incubated at 37 °C for 48 h. The TBC was counted on days 0, 3, 6 and 9. All assays were performed in triplicate.

2.9. Statistical analysis

All the experiments were conducted at least three parallels. The experimental results were statistically analyzed using variance test analysis (SPSS Statistics 19 software). Significant differences between the groups with different treatments were examined using t-test. The difference was considered to be significant if $P < 0.05$.

3. Results and discussion

3.1. Characterization of SPI/CHC composite particles

The complexation of SPI with CHC was investigated by the determination of zeta-potential and particle size change of SPI-CHC (mass ratio 1:1) composite solution (SC) at different pH values. As presented in Fig. S2, CHC is always positively charged at pH 2-7 and SPI is negatively charged at pH above 4.5; although SPI is also positive charge predominant at pH 2-4.5, there would be still a number of negatively charged groups (such as carboxyl groups) on the protein [33]. Thus, SPI/CHC complexes can form under weak acid condition by electrostatic attraction. At pH value lower than 6, the particle size of SPI/CHC complexes was small (291-701 nm), and decreased with the decrease of pH value (Table S1), indicating a good stability of SPI/CHC complexes, which was due to the electrostatic repulsion between the particles with positive zeta-potential (Fig. S2). At pH 7, the repulsive force between the particles decreased due to the decrease of zeta-potential, which leads to the coagulation of particles and an increase of particle size [34]. PDI is an index that characterizes the homogeneity of particle size distribution. The PDI of SPI/CHC complexes formed at pH 2-5 was low (0.297-0.454), indicating a good particle dispersion.

The surface morphology of SPI, CHC and SPI/CHC complexes prepared under the condition of pH 4 was observed by SEM (Fig. S3). SPI was found to be uneven and spherical, with an inward concave and clustered surface. CHC appeared as a flat block with a rough surface, indicating that there were high-level structural interactions inside, thus possessing a good stability. The SPI-CHC complexes had a rough surface with a

few voids.

3.2. Fabrication of Pickering emulsions

3.2.1 Effect of SPI/CHC mass ratio on the stability of Pickering emulsions

Fig.1A$_1$ shows the Pickering emulsions prepared with different SPI/CHC mass ratios after stored for 1 day and 30 days. After stored at 4ºC for 30 days, no oil phase was exuded from the emulsified layer of all the emulsions. However, in the case of SPI/CHC ratio 4:1, a small amount of precipitation appeared at the bottom, indicating the relatively lower stability compared with the cases of other SPI/CHC ratios. Creaming index (CI value) is an important index of emulsion stability. As shown in Fig.1B$_2$, the CI values of the emulsions prepared with different SPI/CHC mass ratios did not change significantly after 30 days of storage, and remained at high level of over 92%, with the exception of SPI/CHC ratio 4:1, indicating that the storage stability of emulsions prepared with SPI/CHC ratios of 1:4, 1:2, 1:1 and 2:1 was higher than that with SPI/CHC ratio 4:1. This result agrees with that of appearance observation. This is possibly because too many SPI particles could not be completely covered by CHC, resulting in Brownian motion during storage and diffusion to the aqueous phase, which was then deposited to the bottom due to the gravity. With the increase of CHC content in complex particles, the CI value of the emulsions (SPI/CHC 1:2 and 1:4) gradually increases, because CHC has a strong positive charge under acidic conditions, forming electrostatic repulsion around the emulsion droplets and avoiding droplet flocculation [35]. Fig.1C$_1$ shows an optical microscope observation of the Pickering emulsions stabilized by composite particles with different SPI/CHC mass ratios. When the

proportion of CHC was increased, the emulsion droplets were dispersed first and then condensed. It is assumed that the repulsive force caused by the strong electric charge of CHC made the droplets dispersed, while excessive CHC led to the flocculation of polysaccharide molecules and condensation of the emulsions. At the SPI/CHC mass ratio of 1:1, the droplets of emulsions were small and evenly distributed in the visual field. As shown in Fig.1D$_1$, in the case of SPI/CHC mass ratio 1:1, emulsions had the smallest droplet size, which is consistent with the morphology diagram of the emulsions under the microscope in Fig.1C$_1$. Therefore, the SPI/CHC complex particles with mass ratio of 1:1 was best choice for the stabilization of Pickering emulsions.

3.2.2. Effect of pH on the stability of Pickering emulsions

As shown in Fig.1A$_2$, the emulsions were light yellow and uniform after 1 day. After 30 days of storage, no oil phase was exuded from the emulsions with pH values of 3 to 6, and a small amount of precipitates were observed at the bottom of the emulsions with pH 5 and 6, while oil phase precipitated on the surface of the emulsion with pH value of 7 was found to be demulsified and oil-leaked, indicating the instability of the emulsions. The larger the pH value, the stronger the condensation of protein and polysaccharide, and the fewer particles effectively adsorbed on the oil-water interface, resulting in the accumulation of oil droplets. The particles not adsorbed on the interface precipitate to the bottom due to the action of gravity, and the larger particle aggregates cannot provide a huge specific surface area covering the oil-water interface, leading to demulsification and oil leakage after a long time [36]. Fig.1B$_2$ shows the changes of CI values of the emulsions prepared under different pH values during the storage. After

storage for 30 days, the CI values of the emulsions prepared at pH 3 and 4 changed little, and remained at high level, indicating the good stability of the emulsions. In contrast, in the cases of pH 5 and 6, the CI values of the emulsions were relatively lower, and changed more obviously, which could be due to the fact that the charge of particles was getting lower at these pH values, leading to coagulation of the emulsions. This is because at pH 5 and 6, the charge of CHC decreases, while the charge of SPI changes from positive to negative, thus the net charge of SPI/CHC particles decreases. This is confirmed by the ξ-potential determination (Fig. S2). Fig.1C$_2$ shows optical microscope images of particle-stabilized Pickering emulsions at different pH values. With the increase of pH value, the size of the emulsion droplets increased. At pH 4, the emulsion droplets possessed even and small size and regular shape, which could be attributed to the electrostatic repulsion and hydrogen bonds between SPI/CHC complex particles. This result is consistent with the that of Yuan et al. [35]. In addition, with the increase of pH, the interaction between the molecular chains of SPI and CHC gradually decreased and the macromolecular chains gradually expanded, which led to a decrease in the interaction force maintaining the network structure formed by the SPI/CHC particles, resulting in a decrease in the emulsion stability [37]. As presented in Fig.1D$_2$, the particle size of emulsions increased with the increase of pH value, which is consistent with the result of above optical microscope observation. Therefore, stable Pickering emulsions can be prepared by adjusting the pH value of the composite system.

3.2.3. Effects of SPI/CHC particle concentration on the stability of Pickering emulsions

Fig.1A_3 presents the Pickering emulsions stabilized by the SPI/CHC (mass ratio 1:1) particles with a concentration of 0.25~2%. After storage for 30 days, no demulsification and oil leakage were observed in all the emulsions, indicating the good emulsifying capacity of the SPI/CHC particles. With the increase of particle concentration, the CI value of the emulsions increased (Fig.1B_3), indicating the higher stability. As depicted in Fig.1C_3, with the increase of particle concentration, the size of the Pickering emulsion droplets decreased. This is because increased particle concentration on the oil−water interface reduces the free energy, making the system more stable, thus reducing the droplet size [38]. This result agrees with that of previous work [38]. Decreased droplet size of Pickering emulsions increases the total interfacial area of complex particles. This is because when the particle concentration is low, only a part of the interface is stabilized. After the shear action stops, the oil droplets that are not adsorbed by the particles condense to reduce the area of the oil-water interface. Due to the irreversible adsorption of particles, coagulation stops once the interface area is reduced to be completely covered by particles [39]. Fig.1D_3 shows the particle size of the particle-stabilized Pickering emulsions with different particle concentrations. With the increase of particle concentration, the particle size of emulsions gradually decreases. The results presented in Fig.1A_3-Fig.1D_3 all indicate that stability of the emulsions increased significantly with increasing particle concentration. This could be because the increase of particle concentration increased the absorption of particles at the oil-water interface and formed a dense interfacial film, thereby increasing the spatial steric hindrance between the droplets and preventing the coalescence of the droplets to a

certain extent. In addition, the increase in particle concentration also enhanced the three-dimensional network structure formed by the particles, leading to an increase of emulsion stability [40]. Thus, the Pickering emulsions stabilized by 2% of SPI/CHC particles possessed the best stability.

3.2.4. Effect of oil phase fraction on the stability of Pickering emulsions

As shown in Fig.1A$_4$, the emulsification layer increased with the increase of oil phase fraction. In the cases of oil phase fraction 60%-80%, the emulsions were even and ropy semi-solids, which did not flow when inverted. This could be because the increase of oil content increased the oil-water interface area, which increased the interaction between particles, leading to the formation of firm gel network structure [41]. It was also found that the CI value of the emulsions increased with increasing oil phase fraction (Fig.1B$_4$). In the cases of low oil phase fraction (10%-40%), CI value was at low level (Fig.1B$_4$), and the emulsions were unstable. This could be because the particles that were not covered with droplets existed in the emulsion gap during the emulsification, and spread to the water phase during storage, resulting in the reduction of emulsified layer. In the cases of high phase fraction (50%-80%), the emulsions had a high CI level (Fig.1B$_4$), thus possessing a good stability. Fig.1C$_4$ presents the optical microscope images of particle-stabilized Pickering emulsions at different oil phase fractions. The increase of oil phase fraction increases total area of the oil-water interface and enhances the interfacial tension, which increases the rearrangement of the composite particles adsorbed on the interface. Therefore, the more particles for stabilization of the interface, the smaller the size of oil droplets. In the cases of oil phase

fraction 10%-30%, the size of the emulsion droplets was relatively large. In the cases of oil phase fraction 40%-60%, with the increase of oil phase fraction, the interface area of emulsion droplets increased and the particle size of emulsions became smaller. Compared with the oil phase fraction of 60%, the size of the emulsion droplets with oil phase fraction of 70% and 80% increased due to the extrusion and agglomeration of the droplets. In addition, the composite particles formed more bridging flocculation on the interface, forming some droplets clusters, which were not easy to be dispersed. Thus, oil phase fraction 60% was optimal for the fabrication of the SPI/CHC particles stabilized Pickering emulsions.

3.3 Microstructure of Pickering emulsions

The pH value has a great influence on the formation and charge of SPI/CHC particles. The Confocal Laser Scanning Microscopy (CLSM) images visually illustrated the influence of particles on the stability of interface between oil phase and water phase with the change of pH value (Fig. 2). CLSM images further demonstrated that the Pickering emulsions were stabilized by the adsorption of complex particles and excess particles distributed in the continuous phase on the oil-water interface. At pH 3, there was no obvious green signal around the emulsion droplets, and the droplets were more dispersed. This is because when the highly charged SPI/CHC composite particles (zeta potential +39.55 mV at pH 3) (Fig.S2) are close to the oil-water interface, a repulsive force between them leads to difficult or weak adsorption, resulting in instability of the emulsions [42]. At pH 4-6, the green signal at the oil droplet interface is obvious, and the network structure formed by SPI/CHC complex in the continuous

phase stabilized the Pickering emulsions. The emulsions were found to aggregate at pH 7, as the SPI/CHC composite particles had a low charge and were easy to be adsorbed on the surface of the oil droplets, resulting in thickening of the interface layer. However, the composite condensation between SPI and CHC formed insoluble complexes with different sizes, which caused the instability of the emulsions. This result is consistent with the previous results on the morphology of Pickering emulsion. As shown in Fig.2D, the adsorption of SPI/CHC particles (green) on the oil phase (red) interface further proved that the Pickering emulsions stabilized by the particles were oil-in-water emulsions.

3.4 Rheological properties of Pickering emulsions

Rheological properties are one of the most important indices to characterize the stability and functionality of Pickering emulsion. As illustrated in Fig.3A, all emulsions exhibited shear thinning property, and the apparent viscosity decreased with the increase of shear rate, indicating that the adhered oil droplets dispersed under the action of shear force, which confirmed the existence of bridging flocculation structure in the emulsions [43]. When the pH value was 3, the repulsive force between particles led to the dispersion of emulsion droplets and the viscosity was low; when the pH value was 4-7, the network structure formed by electrostatic action hindered the shear to a certain extent. However, with the increase of pH value, the decrease of particle charge led to the decrease in the force maintaining the network structure, the accumulation of emulsion and the decrease of viscosity [37]. The elastic modulus (G′) and viscosity modulus (G″) of the Pickering emulsions stabilized by SPI/CHC soluble composite

particles were determined by rheometer with frequency (0.1~10 Hz) (Fig.3B). All the G′ values of the emulsions prepared under different pH values were found to be greater than those of the corresponding G″ values, indicating that the emulsions had the gel characteristics. This result agrees with that of a previous report [44]. The G′ value gradually increased with increasing pH from 3 to 7, suggesting a progressive enhancement in the gel-like structures. Chitosan has been demonstrated to possess good gelling property [45], thus the gel characteristics of the Pickering emulsions studied in this work could be attributed to the presence of chitosan hydrochloride.

3.5 Oxidation stability of Pickering emulsions

The primary oxidation rate of Pickering emulsion can be detected by measuring the concentration of POV. As presented in Fig. 4A, POV values of all samples did not change significantly after 6 days with the exception of the case at pH 3. This could be because the SPI/CHC complexes formed a solid shell around the oil droplets, which acted as a barrier to prevent the contact of oil with oxygen. The POV value of the emulsions prepared at pH 3 increased significantly after 9-15 days, increasing from 25 mg/L to 70 mg/L. This is possibly because the SPI/CHC composite particles attached to the surface of oil droplets could not form a stable membrane structure during the accelerated oxidation process of oil, due to the large repulsion between the SPI/CHC composite particles. In the other cases, POV value of emulsions was negatively correlated with pH value, which could be because the charge of particles gradually decreased with the increase of the pH value, leading to the thickening of the oil-water interface layer, thus reducing the movement of the oxidant [46].

The secondary oxidation rate of the Pickering emulsions was detected by measuring TBARs concentration. As shown in Fig. 4B, the change trend of TBARS values in the pH range of 3-7 was similar to that of primary oxidation products. At the late stage of storage, the oxidation rate of oil was accelerated, which might be caused by the instability of emulsion structure and more and more inducing factors [47].

3.6 Antibacterial activity of the citrus essential oil Pickering emulsions (CEOP)

CEOP was prepared using the above Pickering emulsion template under the conditions of SPI/CHC mass ratio 1:1, pH 4, SPI/CHC composite particle concentration 2% and oil phase fraction 60%.

3.6.1 Minimum inhibitory concentration (MIC) assays

Previous studies have reported that essential oils have strong antimicrobial activity [48]. The MIC values of both pure CEO and CEOP (containing the same amount of essential oil) against Gram-negative pathogenic bacterium *E. coli* were found to be 1.875 μL/mL. The emulsions without essential oil embedded did not show obvious antibacterial properties. For the Gram-positive pathogen *S. aureus*, the antibacterial effect of CEOP was stronger than that of pure CEO, and the MIC values were 0.938 μL/mL and 1.875 μL/mL, respectively, indicating that the SPI/CHC complex particles in CEOP played a certain role. CEOP emulsions possessed a stronger inhibitory effect on Gram-positive bacteria than Gram-negative bacteria, which might be attributed to the different cell wall composition of the two types of bacteria. This finding is consistent with that of Viuda Martos et al. [49]. The presence of lipopolysaccharide prevents hydrophobic substances from entering the interior of the bacteria [50, 51]. It

was reported that the oregano essential oil (OEO) Pickering emulsion stabilized by cellulose nanocrystals had a MIC value of 12.5 μL/mL (real concentration of OEO contained is 0.25 μL/mL) against both *E. coli* and *S. aureus*, which was higher that of pure OEO (0.125 μL/mL) [10].

3.7 Antioxidant capacity of CEOP

All the emulsions showed antioxidant capacity, as shown in Fig. 5. The scavenging rates of DPPH radical, hydroxyl radical and ABTS radical of Pickering emulsions without essential oil were 37.14±3.15%, 29.58±5.13% and 38.71±2.84%, respectively. With the increase of essential oil content, the free radical scavenging ability of the emulsions was gradually enhanced. When the CEO content in CEOP reached 60%, the scavenging rates of DPPH radical, hydroxyl radical and ABTS radical reached 94.68±2.54%, 93.64±3.45% and 92.84±2%, respectively. Poor water solubility of the antioxidant components (e.g. D-limonene, γ-Terpineol, etc.) in CEO limits its bioavailability, while Pickering emulsion delivery system increases their solubility, thus the antioxidant activity of CEOP is positively correlated with the content of essential oil. In addition, CHC molecule contains amino and hydroxyl groups, thus possessing potent free radical scavenging ability [52]. Therefore, it is likely that CHC in CEOP also contributed to its antioxidant property.

3.8 Preservative effect of CEOP on freshly cut apples

3.8.1 Morphological changes of fresh-cut apples during storage

The soaking treatment model of CEOP emulsion dilution (CEO concentration 1500-5500 μL/L) was used to evaluate its fresh-keeping effect on fresh-cut apples. As shown in Fig.6A, the apples in all groups presented normal appearance after 1h of treatment, without mildew, browning and obvious damage. The apples in CEOP groups after 9 day-storage at 4 ℃ showed no obvious mildew and were superior to the blank control. In CEOP-treated groups, the efficiency in retarding the browning of apple slices increased with the increase of CEO concentration up to 4500 μL/L, thereafter decreased. The apple slices in CEO group showed obvious browning after the 3 days, and showed dark brown after 6 days, suggesting that the poor solubility and stability of pure essential oils lead to their inability to be applied to the preservation of fresh-cut fruits by soaking [53]. Besides, the degree of color change was significantly higher in the SC group than that in the blank group. This is possibly because proteins in SPI/CHC composite particles promote the deterioration of apples in the absence of essential oils. This finding is consistent with that reported by Jiang et al. [11]. In addition, effect of the emulsions on the taste is also an important factor determining the feasibility of their application. After 9-day storage, no obvious CEO odor was detected in all the samples treated with diluted emulsions of 1500-5500 μL/L CEO, indicating that CEOP developed in this study not only possessed preservative effect, but also avoided the influence of the addition of essential oil on the flavor and texture of the fruits.

3.8.2 Effect of CEOP on color change of fresh-cut apple

The surface color change of freshly cut apple slices after 9 days of storage at 4 ℃ is shown in Fig.6. Color changes of the freshly cut apple slices based on the

determination of CIE L*a*b* values are presented in Table 1. The L^* value of apples in all groups basically decreased with the increase of storage time, indicating the browning of the sample; and the CEO group showed the most obvious decreasing trend. The decreasing rate of L^* value in CEOP treatment groups was obviously less than that of the blank group ($P < 0.05$); and among these groups, the L^* value in CEOP-4500 group (CEO concentration 4500 μL/L) decreased slowest. After the 3-day storage, the a^* value of the control group had an obvious upward trend, while the increasing trend of the treatment group remained stable until the ninth day. The increase in a^* value (red) may be due to the increased respiration rate and enzymatic browning, which resulted in the mass loss of fruits [54]. The b^* value of the treatment group did not change significantly during the storage ($P < 0.05$), while the b^* value of the blank group decreased but remained in the positive range (yellow). Total color difference (ΔE) of all groups showed an upward trend with the increase of storage time. During the storage, the ΔE values of blank control group, SC group and CEO group were all greater than 10 on the 9th day, while the ΔE values of the CEOP groups were all less than 5, indicating that CEOP could effectively maintain the color and quality of fresh-cut apples ($P < 0.05$). This result is consistent with that of a previous report [55]. Among the CEOP treatment groups, the group with CEO concentration of 4500 μL/L possessed the best preservative effect.

3.8.3 Browning degree of freshly-cut apples during storage

The degree of browning can be expressed by the absorbance of the filtrate containing the browning products of each group. As shown in Table 2, the browning

degree of the blank group increased rapidly during the storage (0-9 days), while that of the CEOP groups increased slowly in the first 3 days and slightly increased in the following 6 days. After 5th day, CEOP groups showed obvious effect in retarding the browning of apple samples compared with blank group, CEO group and SC group ($P < 0.05$). Therefore, CEOP stabilized by SPI/CHC composite particles can effectively inhibit browning reaction and extend the shelf life of freshly-cut apple slices. On the 9th day, the browning index of CEOP groups decreased with CEO concentration from 1500 to 4500 μL/L, but increased when CEO concentration increased to 5500 μL/L.

3.8.4 Effect of CEOP on polyphenol oxidase and peroxidase in freshly-cut apples

With the extension of storage time, PPO activity in the samples increased continuously (Table 2). Compared with the blank group, the activity of PPO in the CEOP groups was inhibited, which was possibly because CEOP formed an oxygen barrier on the surface of freshly cut apples. This result was consistent with the results of antioxidant assays (Fig.5). POD activity in the blank control group was higher than that the treatment group, and the changing trend was the same as that of the PPO activity (Table 2). The morphology and appearance of fresh-cut apples during storage also showed that CEOP treatment could inhibit browning more effectively. This phenomenon may be due to the inhibition of POD activity under acidic conditions (pH 4.0 for emulsion preparation) [32]. Among the CEOP groups, the treatment with CEO concentration of 4500 μL/L had the best anti-browning effect, but the treatment with CEO concentration of 5500 μL/L had poor anti-browning effect, which was consistent with the effect of some natural anti-browning agents [54]. In summary, the anti-

browning effect of CEOP on fresh-cut apples could be attributed to its inhibition on PPO and POD activities.

3.8.5 Total bacterial count in fresh-cut apples during storage

In order to evaluate the inhibitory effect of CEOP on the bacterial growth in fresh-cut apples, the total bacterial count (TBC) in fresh-cut apples with different treatment were determined during storage. As presented in Table 2, the TBC value in each group increased rapidly with the time ($p < 0.05$). The increasing rates of TBC in CEOP-treated groups were significantly lower than that of water group, SC group and pure CEO group ($p < 0.05$). This indicates that citrus essential oil plays a good role in the inhibition of bacteria. CEO contains a variety of components, among which D-limonene is the most abundant, which has bactericidal, insecticidal and anti-inflammatory activities [19]. The TBC decreased with the increase of CEO concentration in CEOP up to 4500 μL/L, thereafter TBC increased. Thus, CEOP (4500) treatment was the most effective in inhibiting the bacterial growth, TBC was found to be 3.32lg CFU/g after 9 days of treatment, which was lower than the Water group (4.33 lg CFU/g), CEO control group (4.13 lg CFU/g) and SC control group (4.11 lg CFU/g). Pure CEO did not exhibit a good antibacterial effect, which could be due to the fact that pure CEO had poor water solubility and fast evaporation, which hampered its interaction with the cell membrane [56].

4. Conclusion

In this work, a template of the O/W Pickering emulsions using soluble SPI/CHC composite particles as stabilizer was constructed. The conditions for the fabrication of

Pickering emulsions were optimized as: pH 4, SPI/CHC mass ratio 1:1, composite particle concentration 2% and oil phase fraction 60%. This emulsion template was used to load citrus essential oil to obtain a stable and biologically active Pickering emulsions. The emulsions have certain antibacterial and antioxidant properties. In fresh-cut apple preservation study, citrus essential oil Pickering emulsions had better preservation effect than pure essential oil. The Pickering emulsion template developed in this work could be used for the loading of active substances to improve their bioavailability and activity, thus having potential applications in food and pharmaceutical industries.

Conflicts of Interest

The authors declare no competing financial interest.

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Tables

Table 1. Color changes of fresh cut apples with different treatments during the storage at 4 °C based on the CIE $L^*a^*b^*$ values.

	Time	Treatments							
	(d)	Water	SC	1500	2500	3500	4500	5500	CEO
L^*	1	59.78 ± 0.21^{Ca}	61.16 ± 1.11^{BCa}	63.07 ± 1.36^{ABa}	62.71 ± 0.60^{Ba}	61.87 ± 0.52^{Ba}	64.95 ± 1.27^{Aa}	61.53 ± 1.20^{BCa}	52.17 ± 1.57^{Da}
	3	57.82 ± 0.26^{Cb}	55.16 ± 1.65^{Db}	61.66 ± 0.89^{ABa}	60.26 ± 1.24^{Bab}	60.36 ± 0.84^{Bb}	62.64 ± 0.93^{Aab}	59.22 ± 1.71^{BCb}	47.29 ± 1.32^{Eb}
	5	55.69 ± 0.12^{Cd}	51.18 ± 1.41^{Dc}	58.39 ± 1.33^{Bb}	58.73 ± 1.18^{Bb}	58.14 ± 1.95^{Bb}	61.52 ± 1.22^{Aab}	56.85 ± 1.59^{BCc}	40.16 ± 1.21^{Ec}
	7	54.25 ± 1.60^{Cd}	47.01 ± 1.24^{Dd}	61.98 ± 1.42^{Aa}	60.81 ± 1.74^{Aa}	58.40 ± 0.40^{Bb}	61.52 ± 1.52^{Aab}	57.4 ± 1.27^{Bbc}	38.68 ± 1.45^{Ecd}
	9	53.94 ± 0.20^{Cd}	45.68 ± 1.39^{Dd}	57.35 ± 1.49^{Bb}	58.87 ± 1.32^{ABb}	56.75 ± 1.31^{Bb}	59.43 ± 1.65^{Ab}	54.90 ± 0.76^{BCd}	36.93 ± 1.35^{Ed}
a^*	1	-1.59 ± 0.51^{Ba}	-2.51 ± 0.05^{Ca}	-2.33 ± 0.47^{Ca}	-3.12 ± 0.14^{Ca}	-2.78 ± 0.34^{Ca}	-4.17 ± 0.17^{Da}	-2.47 ± 0.38^{Ca}	-0.05 ± 0.47^{Aa}
	3	-0.96 ± 0.45^{Ba}	-0.66 ± 0.18^{Bb}	-2.25 ± 0.36^{Da}	-2.22 ± 0.55^{Db}	-2.48 ± 0.47^{Da}	-3.77 ± 0.33^{Ea}	-1.54 ± 0.48^{Cb}	1.07 ± 0.38^{Ab}
	5	-0.57 ± 0.08^{Ca}	0.86 ± 0.26^{Bc}	-1.87 ± 0.47^{Db}	-2.45 ± 0.21^{Dab}	-2.08 ± 0.58^{Db}	-3.59 ± 0.26^{Ea}	-1.36 ± 0.37^{Db}	3.73 ± 0.40^{Ac}
	7	-0.17 ± 0.20^{Ca}	1.92 ± 0.37^{Bd}	-2.65 ± 0.46^{Ea}	-2.92 ± 0.47^{Ea}	-2.23 ± 0.35^{Ea}	-3.51 ± 0.37^{Ea}	-1.36 ± 0.27^{Db}	4.83 ± 0.40^{Ad}
	9	1.15 ± 0.57^{Cb}	3.33 ± 0.48^{Be}	-1.93 ± 0.59^{Db}	-2.12 ± 0.12^{Db}	-2.26 ± 0.36^{Db}	-2.85 ± 0.17^{Db}	-1.47 ± 0.42^{Db}	4.80 ± 0.39^{Ad}
b^*	1	20.91 ± 0.53^{Aa}	19.64 ± 0.14^{Ba}	20.26 ± 0.12^{Ab}	19.43 ± 0.04^{Bb}	20.36 ± 0.17^{Ab}	19.44 ± 0.03^{Bb}	21.53 ± 0.43^{Aa}	21.66 ± 0.32^{Aa}
	3	21.05 ± 0.61^{ABa}	19.65 ± 0.37^{Ba}	19.30 ± 0.26^{Bc}	19.84 ± 0.52^{Bb}	20.24 ± 0.56^{Bb}	19.58 ± 0.58^{Bb}	21.58 ± 0.69^{Aa}	19.55 ± 0.41^{Bb}
	5	21.57 ± 0.38^{Aa}	16.74 ± 0.30^{Bc}	20.74 ± 0.14^{Aa}	21.28 ± 0.10^{Aa}	21.31 ± 0.26^{Aa}	20.93 ± 0.08^{Aa}	21.73 ± 0.10^{Aa}	17.20 ± 0.22^{Bd}
	7	21.58 ± 0.51^{Aa}	17.76 ± 0.24^{Db}	19.91 ± 0.32^{BCb}	19.63 ± 0.38^{Cb}	20.37 ± 0.19^{Bb}	19.53 ± 0.59^{Cb}	21.35 ± 0.39^{Aa}	18.06 ± 0.42^{Dc}
	9	20.52 ± 0.43^{Ab}	17.29 ± 0.12^{Db}	20.03 ± 0.37^{Bb}	19.95 ± 0.40^{Bb}	19.54 ± 0.21^{Cc}	19.44 ± 0.65^{BCb}	20.66 ± 0.54^{Ab}	17.43 ± 0.42^{Dcd}
ΔE^*	1	1.61 ± 1.39^{Ba}	0.75 ± 0.73^{Ba}	1.73 ± 1.05^{Ba}	1.32 ± 1.43^{Ba}	0.48 ± 0.44^{Ba}	0.84 ± 1.17^{Ba}	1.40 ± 1.36^{Ba}	4.30 ± 1.53^{Aa}
	3	3.42 ± 0.44^{Bab}	6.53 ± 1.19^{Ab}	3.18 ± 0.82^{Bab}	3.42 ± 0.53^{Bab}	1.68 ± 0.77^{Cb}	1.06 ± 0.98^{Ca}	1.90 ± 1.43^{BCa}	8.18 ± 1.32^{Ab}
	5	5.61 ± 0.10^{Cb}	11.06 ± 1.24^{Bc}	5.22 ± 1.14^{Cb}	4.57 ± 1.13^{Cb}	4.05 ± 1.28^{Cb}	1.42 ± 1.26^{Da}	4.04 ± 1.05^{Cb}	16.37 ± 1.82^{Ac}
	7	6.96 ± 1.19^{Cbc}	15.04 ± 1.05^{Bd}	3.62 ± 0.59^{Da}	3.56 ± 0.72^{Dcd}	3.67 ± 0.54^{Dab}	2.42 ± 1.36^{Cab}	3.61 ± 1.18^{Da}	17.91 ± 1.49^{Acd}
	9	9.46 ± 1.39^{Cc}	16.65 ± 1.15^{Bd}	6.20 ± 1.49^{Dab}	5.30 ± 1.34^{Db}	5.42 ± 1.20^{Dd}	5.66 ± 1.19^{Dab}	5.95 ± 0.69^{Db}	19.68 ± 1.37^{Ad}

Note: Different lowercase letters indicate significant difference ($P < 0.05$) between groups with the same treatment at different times. Different capital letters indicate significant difference ($P < 0.05$) between groups with different treatment at the same time.

Table 2. Preservative effects of different treatments on fresh-cut apples during storage.

	Time	Treatments							
	(d)	Water	SC	1500	2500	3500	4500	5500	CEO
Browning indexes	1	0.04±0.01^{Ba}	0.05±0.01^{Ba}	0.04±0.01^{Ba}	0.04±0.02^{Ba}	0.04±0.01^{Ba}	0.04±0.01^{Ba}	0.04±0.02^{Ba}	0.14±0.01^{Aa}
	3	0.08±0.01^{BCb}	0.08±0.02^{BCb}	0.05±0.01^{Ca}	0.05±0.01 ^{Ca}	0.05±0.01^{Ca}	0.04±0.01^{Ca}	0.10±0.01^{Bb}	0.37±0.05^{Ab}
	5	0.14±0.01^{Bc}	0.15±0.01^{Bc}	0.11±0.01^{Cb}	0.11±0.02^{Cb}	0.10±0.01^{Cb}	0.12±0.02^{Cb}	0.11±0.02^{Cb}	0.42±0.03^{Ac}
	7	0.20±0.01^{BCd}	0.24±0.01^{Bd}	0.12±0.01^{CDb}	0.11±0.02^{CDb}	0.12±0.01^{Db}	0.13±0.02^{Db}	0.14±0.03^{Cc}	0.42±0.06^{Acd}
	9	0.29±0.02^{BCe}	0.32±0.01^{Be}	0.20±0.03^{Dc}	0.18±0.02^{Dc}	0.17±0.03^{DEc}	0.16±0.03^{DEc}	0.24±0.05^{Cd}	0.45±0.04^{Ad}
PPO activity (unit/g/min)	1	0.22±0.02^{Ba}	0.20±0.2^{Ba}	0.21±0.04^{Ba}	0.23±0.04^{Ba}	0.21±0.06^{Ba}	0.22±0.06^{Ba}	0.21±0.01^{Ba}	0.61±0.02^{Aa}
	3	0.74±0.03^{Bb}	0.71±0.04^{Bb}	0.73±0.03^{Bb}	0.70±0.09^{Bb}	0.56±0.01^{BC}	0.51±0.03^{Cb}	0.80±0.03^{Bb}	1.58±0.01^{Ab}
	5	0.96±0.04^{BCb}	1.09±0.03^{Bc}	0.81±0.02^{Cb}	0.81±0.03^{Cbc}	0.75±0.01^{Cbc}	0.71±0.02^{Cbc}	1.07±0.04^{Bbc}	1.80±0.02^{Abc}
	7	1.51±0.03^{Bc}	1.64±0.02^{Bd}	1.16±0.06^{Cc}	0.88±0.07^{Dc}	0.81±0.03^{Dc}	0.82±0.04^{Dc}	1.15±0.04^{Ccd}	1.99±0.09^{Ac}
	9	1.63±0.03^{Bc}	1.71±0.03^{Bde}	1.21±0.03^{Ccd}	1.15±0.06^{CDd}	1.01±0.07^{Cd}	1.03±0.04^{Cd}	1.55±0.06^{BCd}	2.36±0.06^{Ad}
POD activity (unit/g/min)	1	0.16±0.02^{Aa}	0.18±0.03^{Aa}	0.15±0.04^{Aa}	0.11±0.02^{Aa}	0.11±0.02^{Aa}	0.11±0.05^{Aa}	0.15±0.04^{Aa}	0.24±0.05^{Aa}
	3	0.38±0.03^{Bb}	0.35±0.02^{Bb}	0.16±0.03^{Ca}	0.12±0.05^{Ca}	0.10±0.01^{Ca}	0.12±0.03^{Ca}	0.18±0.03^{Ca}	1.41±0.05^{Ab}
	5	0.53±0.04^{Bc}	0.57±0.05^{Bc}	0.47±0.02^{Bb}	0.45±0.04^{Bb}	0.39±0.02^{BCb}	0.36±0.05^{BCb}	0.37±0.06^{BCb}	1.51±0.07^{Abc}
	7	0.70±0.01^{Bd}	0.80±0.01^{Bd}	0.49±0.05^{BCb}	0.55±0.01^{BCbc}	0.45±0.04^{Cbc}	0.45±0.04^{Cbc}	0.64±0.01^{Bc}	1.72±0.04^{Ac}
	9	0.97±0.05^{Be}	1.1±0.06^{Be}	0.75±0.06^{Cc}	0.75±0.03^{Cc}	0.64±0.01^{CDc}	0.64±0.03^{CDc}	0.78±0.08^{Ccd}	1.8±0.05^{Acd}
TBC (lg CFU/g)	0	<1	<1	<1	<1	<1	<1	<1	<1
	3	3.09±0.03^{Ab}	2.95±0.08^{Bb}	2.48±0.04^{Eb}	2.35±0.06^{Db}	2.01±0.05^{Fb}	1.83±0.05^{Gb}	1.92±0.06^{Db}	2.73±0.07^{Cb}
	6	3.92±0.08^{Ac}	3.63±0.06^{Bc}	3.23±0.09^{Dc}	3.12±0.07^{Ec}	2.86±0.03^{Fc}	2.57±0.12^{Hc}	2.63±0.13^{Gc}	3.55±0.12^{Cc}
	9	4.83±0.05^{Ad}	4.51±0.05^{Bd}	3.87±0.12^{Cd}	3.61±0.11^{Dd}	3.42±.008^{Ed}	3.03±0.09^{Ed}	3.26±0.06^{Cd}	4.47±0.16^{Bd}

Note: Different lowercase letters indicate significant difference ($P < 0.05$) between groups with the same treatment at different times. Different capital letters indicate significant difference ($P < 0.05$) between groups with different treatment at the same time.

Figure captions

Fig. 1. Effect of different ratio of SPI/CHC mass, pH, particle concentration, and oil phase fraction on storage stability of the Pickering emulsions. (A) Appearance observation; (B) creaming index; (C) surface morphology under optical microstructure; (D) particle size.

Fig. 2. CLSM imaging of Pickering emulsions under different pH conditions (A. series for oil phase dyeing; B. series for protein staining; C. mixed staining; D. 100× image; Series 1-5 correspond to pH 3-7 emulsions, respectively).

Fig. 3. Rheological properties of Pickering emulsion. (A) Viscosity, (B) $G^{'}$ and $G^{''}$

Fig. 4. Oxidation stability of Pickering emulsions. (A) POV value; (B) TBARs value. Different lowercase letters indicate significant difference ($P < 0.05$) between groups with the same treatment at different times. Different capital letters indicate significant difference ($P < 0.05$) between groups with different treatment at the same time.

Fig.5. Antioxidant ability of emulsion with different ratio of CEO. (A) DPPH• scavenging capacity; (B) •OH scavenging capacity (C) $ABTS^{\bullet+}$ scavenging capacity. Different letters indicate significant difference ($P < 0.05$) between groups with different treatment.

Fig.6. Visual appearance of fresh cut apple slices treated with water, emulsion with different CEO concentrations during the storage at 4 °C.

Fig. 1.

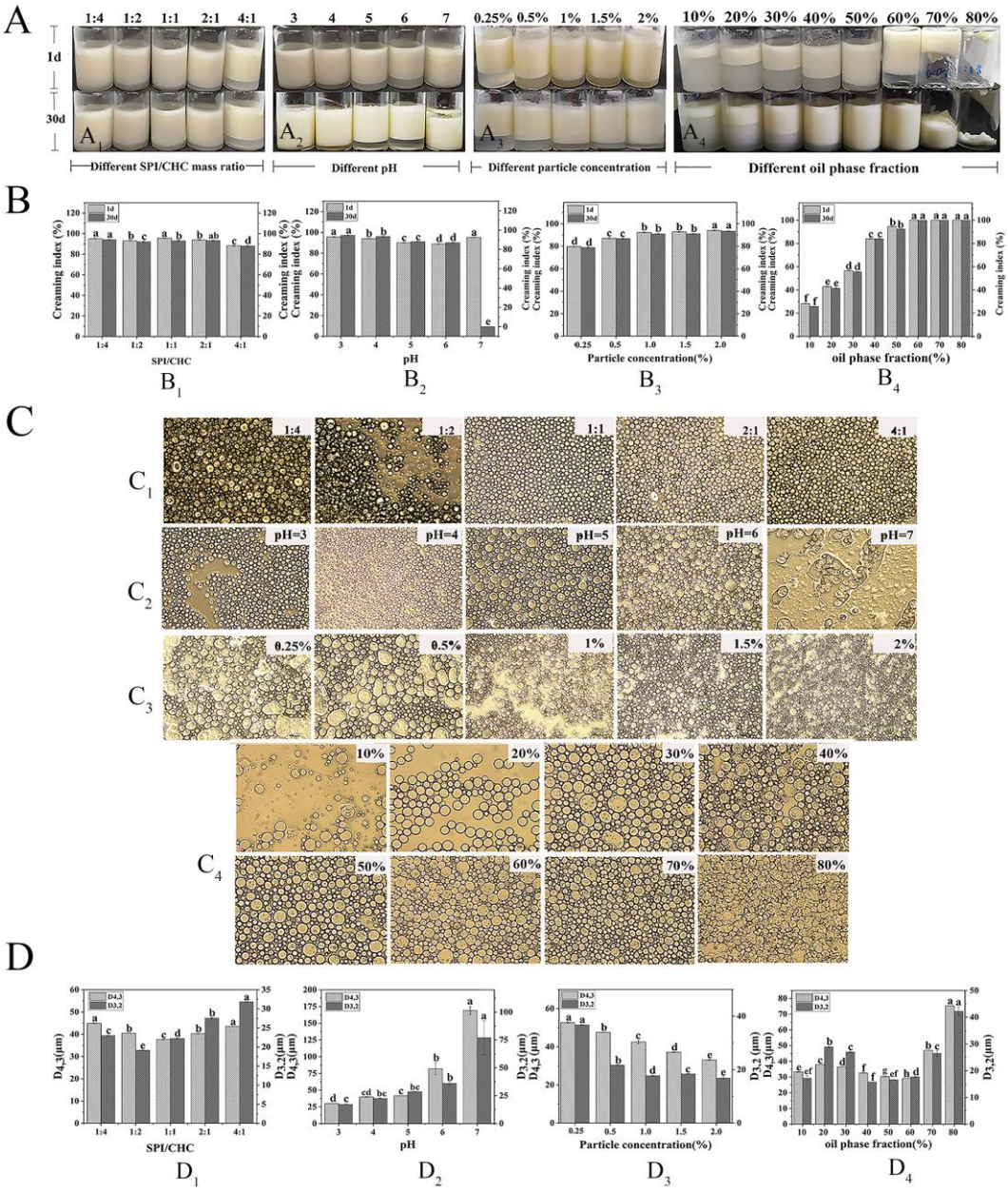

Fig. 1. Effect of different ratio of SPI/CHC mass, pH, particle concentration, and 0fraction on storage stability of the Pickering emulsions. (A) Appearance observation; (B) creaming index; (C) surface morphology under optical microscope; (D) particle size. Different letters indicate significant difference ($P < 0.05$) between groups with different treatment.

Fig.2.

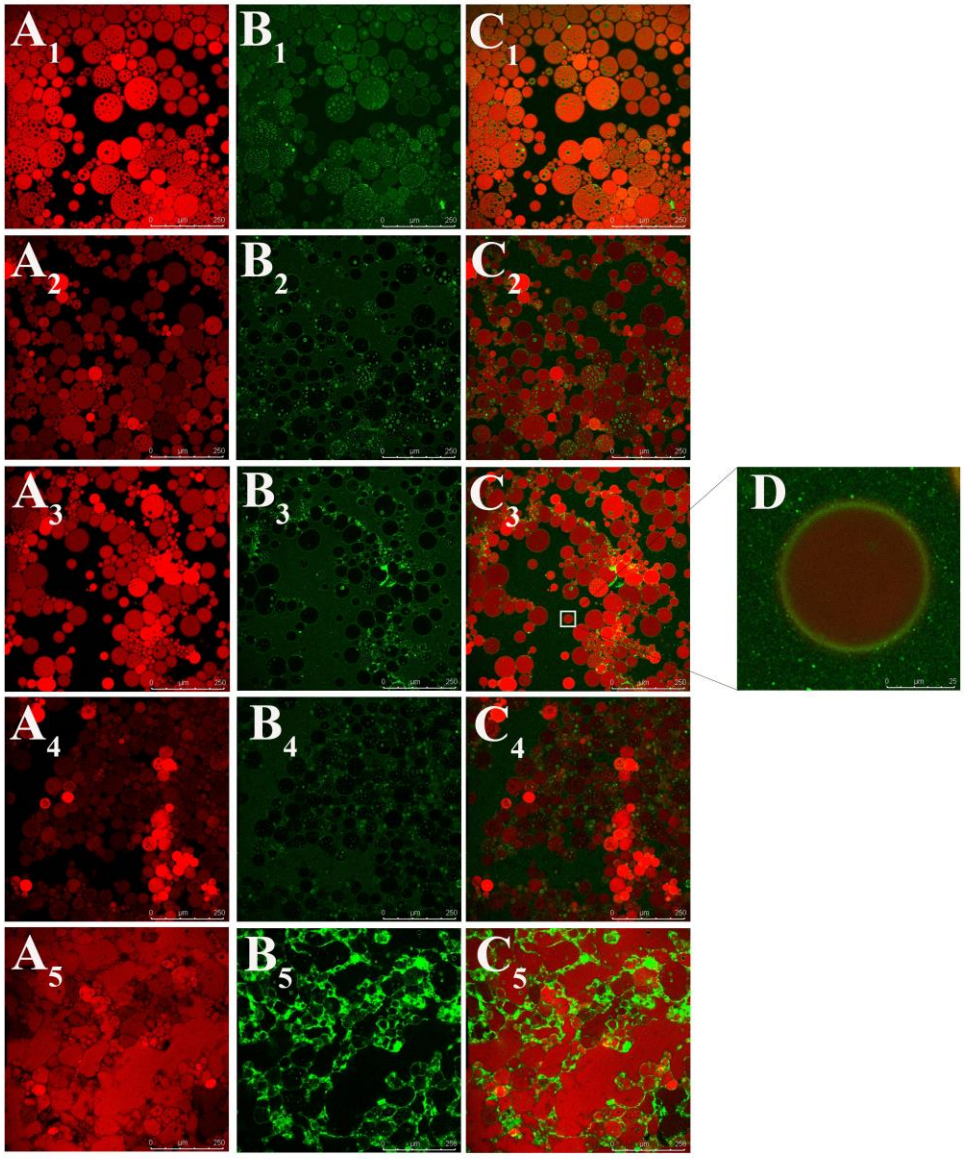

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Fig. 3.

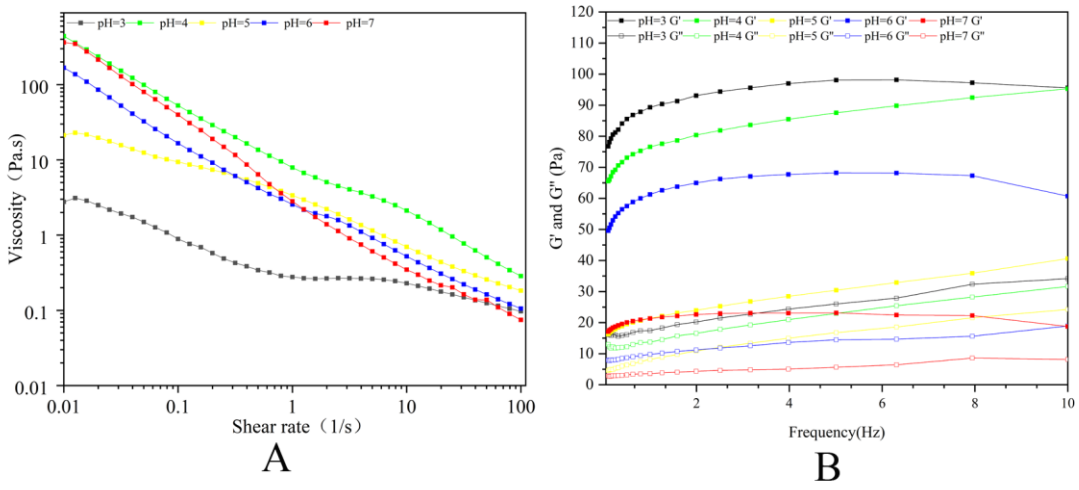

Fig. 3. Rheological properties of Pickering emulsion. (A) Viscosity, (B) G$^{'}$ and G$^{''}$

Fig. 4.

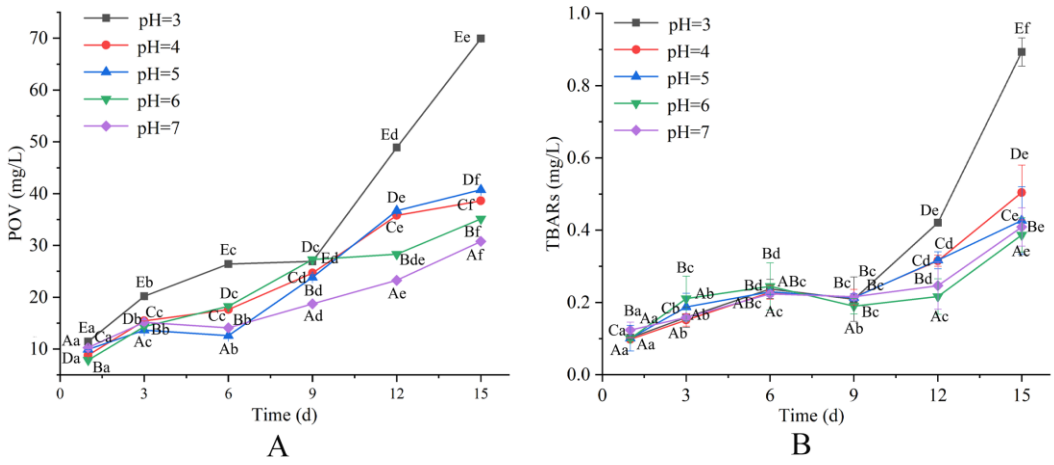

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Fig. 5

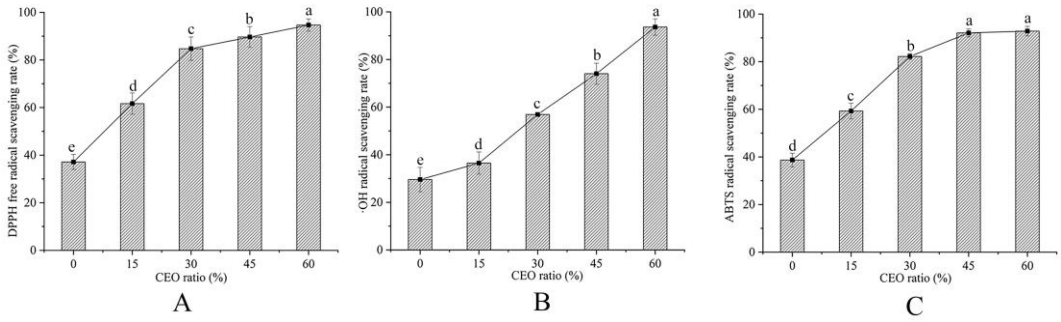

Fig.5. Antioxidant ability of emulsion with different ratio of CEO. (A) DPPH• scavenging capacity; (B) •OH scavenging capacity (C) $ABTS^{\bullet+}$ scavenging capacity. Different letters indicate significant difference ($P < 0.05$) between groups with different treatment.

Fig. 6

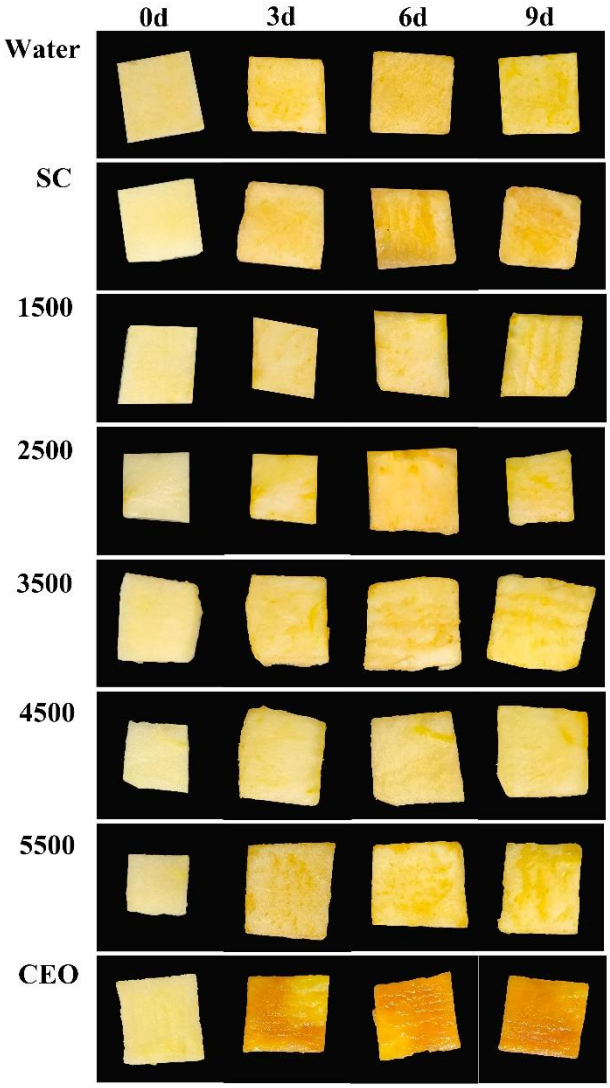

Fig.6. Visual appearance of fresh cut apple slices treated with water, emulsion with different CEO concentrations during the storage at 4 °C.

Supplementary Materials

Pickering emulsions stabilized by soybean protein chitosan/ hydrochloride isolate complex and their applications in essential oil delivery

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ORCID: 0000-0003-4510-3639 (T. Zhou)

Running title: Stabilization and applications of Pickering emulsions

Contents

Fig. S1.

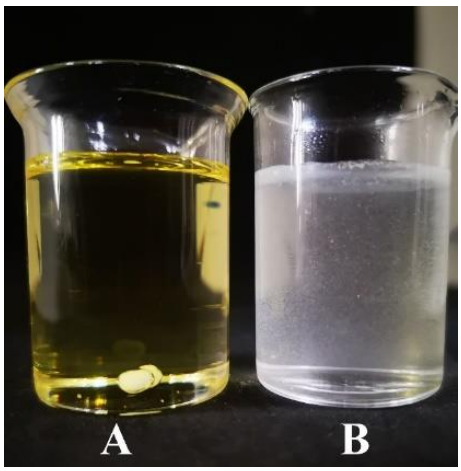


Fig. S1. Dispersion of emulsion in water/oil (A. oil; B. water).

Fig. S2.

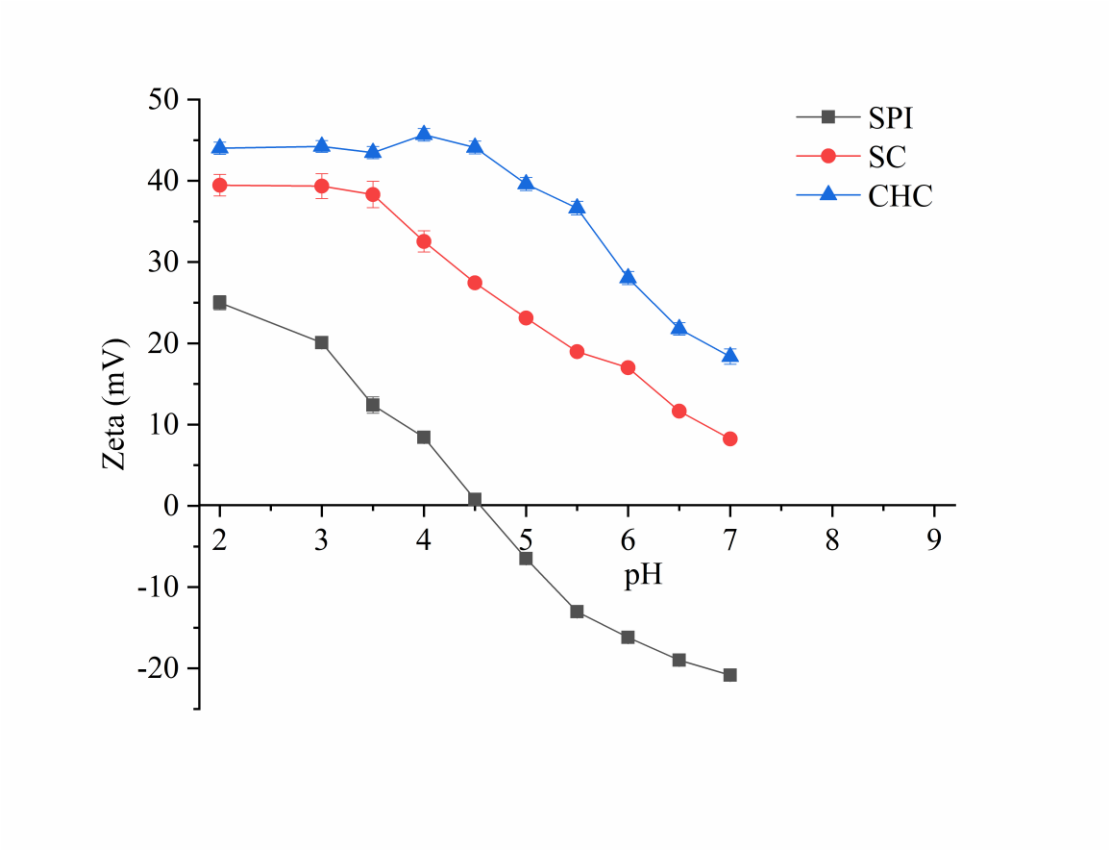
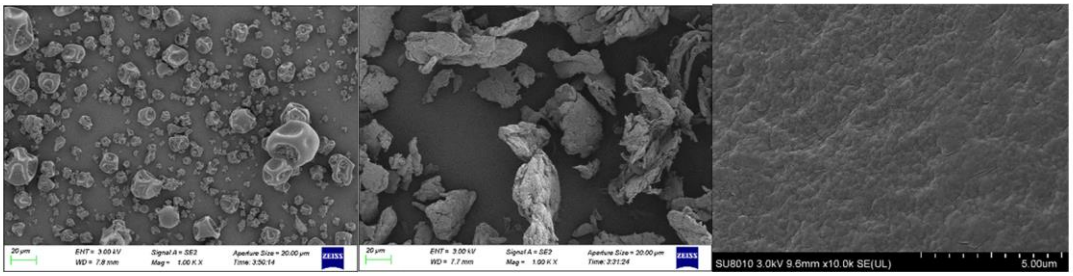


Fig. S2. Effect of pH on zeta of SPI, CHC and SPI-CHC complex solutions.

Fig. S3.



A B C

Fig. S3. SEM image of SPI (A), CHC (B), SPI/CHC complex (C).

Table S1.

pH	Size (nm)	PDI
2	291.50±38.19	0.297±0.029
3	330.70±17.95	0.283±0.092
4	399.33±23.75	0.433±0.047
5	474.39±35.08	0.454±0.037
6	701.10±41.23	0.720±0.180
7	1782.00±98.18	1

Table S1. Particle size and PDI of SPI/CHC solution at different pH.