Structure–property relationship of pea protein fibrils in stabilization of HIPEs and the encapsulation, protection, controlled release and oral delivery of carotenoids for alleviating intestinal inflammation†

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Increasing attentions are paid to high internal phase emulsions (HIPEs) due to their unique properties. In this study, pea protein-based fibrils were used as emulsifier to stabilize HIPEs. We demonstrated that the molecular assembly pathway and interfacial behavior of pea protein-based fibrils are affected by ionic strength. And the increased abundance of highly flexible worm-like nanofibrils facilitated their adsorption and packing on oil droplets, resulting in improved emulsion properties to stabilize the HIPEs with the internal phase volume fraction as high as 90%. Based on this, high loading content of carotenoids up to 0.05 wt% in the prepared HIPEs, protection of their stability against heating, UV and iron ions, and significantly increased bio-accessibilities of the carotenoids were realized. Animal studies using a mouse model of DSS-induced colitis revealed that carotenoid loaded HIPEs can alleviate the colon injury, by downregulating the expression of inflammatory cytokines, and promoting intestinal barrier function. This work will deepen the understanding of the formation of pea protein fibrils and provide a reference for the rational use of carotenoid loaded HIPEs in IBD management.

1. Introduction

Inflammatory bowel disease (IBD) refers to chronic, recurrent inflammatory disorders of the gastrointestinal (GI) tract, with Crohn’s disease (CD) and ulcerative colitis (UC) being the most prevalent forms. Although both subtypes share similar clinical manifestations, UC is limited to the colonic mucosa, while CD can affect deeper layers of the intestinal wall throughout the GI tract. The increasing incidence of IBD worldwide over the past decade has made it a significant global public health challenge. While there are multiple effective therapeutic options available to induce and maintain remission of inflammation, there is a risk of infectious and neoplastic side effects, and some patients may not respond to therapy. Therefore, it is prudent to seek healthier approaches that can be employed to manage IBD. A strategy based on food-grade ingredients appears to be a promising candidate for IBD therapy, as it may offer a safer alternative to traditional treatments.

Recent evidence supports that carotenoids may exert positive impact on IBD. The reasons why carotenoids play a role can be summarized as follows: (a) carotenoids have been related to a number of health benefits, which have originally been ascribed to their anti-inflammatory and antioxidant properties; (b) IBD is closely linked to gut barrier functionality, and potential interactions of carotenoids with gut microbiota have been highlighted; (c) researching has shown that patients with UC have decreased levels of β-carotene in their serum; (d) carotenoids largely bypass absorption in the small intestine and are transmitted to the colon. However, these pigments have a hydrophobic nature, which results in their low solubility in water. Additionally, they are highly susceptible to degradation by factors such as light, heat, oxygen, or acids. In this sense, a gastrointestinal delivery system for carotenoids is necessary to ensure their effectiveness.

To effectively deliver carotenoids to the gastrointestinal tract, high internal phase emulsions (HIPEs) are a promising option. These emulsions are highly concentrated and have a lipid phase volume fraction that exceeds the close packing limit (typically >74%). As the volume fraction of the internal phase increases, the droplets become more tightly packed, resulting in viscoelastic semi-solid characteristics that may prolong their retention in the gastrointestinal tract.
Additionally, the high internal phase volume fraction allows for the encapsulation and protection of high levels of bioactive substances, particularly liposoluble nutraceuticals. HIPEs also exhibit better microbiological stability compared to normal emulsions by virtue of their very low water activity. As a result, HIPEs have gained significant attention and have performed well in recent years for certain food applications.

Bio-sourced HIPEs offer promising benefits for the food industry, including biocompatibility and biodegradability. Biopolymers, such as ovalbumin and globular protein, have proven to be effective emulsifiers for creating O/W HIPEs. Besides, artificial protein amyloid fibrils represent a new type of protein-based biomaterials that have garnered significant interest because of their superior emulsifying ability compared to the corresponding protein monomer. Protein amyloid fibrils are highly ordered and unbranched macromolecular structures that range in size from nanometers to micrometers. Generally, food proteins are capable of assembling into charged amyloid fibrils through hydrolysis and subsequent self-assembly at low pH and high temperature. In recent years, plant-derived proteins have become increasingly popular due to their environmental abundance, sustainability, versatility, and lower cost. However, compared to well-established amyloid fibrils from animal proteins, the use of plant-based proteins for amyloid fibrillization and subsequent emulsion stabilization remains relatively unexplored.

In the current study, we investigated the potential of using pea protein amyloid fibrils stabilized HIPEs to alleviate intestinal inflammation by encapsulating carotenoids. Initially, the effect of ionic strength on the assembly behavior of pea protein during fibrillation and the relationship between the structural changes and interfacial behavior of pea protein-based fibrils was studied. Then, based on the above results, the most stable HIPEs was prepared to encapsulate and protect three kinds carotenoids. Finally, the carotenoid loaded HIPEs were dialyzed against deionized water by using a 7000 Da dialysis bag for 3 days. CD spectra was performed on a Brighttime Chirascan J815, using a quartz cuvette of 0.1 mm optical pathlength at 25 °C. The spectra were obtained as an average of three scans in the far UV range (190–260 nm) with background subtracted.

2. Materials and methods

2.1. Materials

Pea protein was purchased from Yulong Biochemical Co., Ltd. β-Carotene, lycopene and lutein were purchased from Shanghai Yuan Ye Bio-Technology Co., Ltd. Nile Red (N815046) and Nile Blue A (N815018) were purchased from Shanghai Macklin Biochemical Co., Ltd. DSS (36–50 kDa) was purchased from MP Biomedicals (Solon, OH, USA). A mouse lipocalin-2/NGAL ELISA kit was obtained from R&D Systems (Minneapolis, MN, USA). Serum ALT and AST kits were bought from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Mouse ELISA kits of TNF-α, IL-1β, IL-6, and IFN-γ were obtained from Neobioscience Technology Co., Ltd (Shenzhen, China).

2.2. Preparation of pea proteins based amyloid fibrils

The purification and purity determination of pea protein was determined according to our reported method. To obtain pea protein amyloid fibrils, the purified pea protein powder was initially dissolved in ultrapure water containing different concentrations of NaCl (0, 25, 75, 150, 300 mM). Then the solution pH was adjusted to 2.0 with 2 M HCl. The samples were hydrated overnight at 4 °C. After that, the insoluble materials were removed by centrifugation and filtration through a 0.45 μm filter. Subsequently, the protein solutions were heated at 85 °C in an oil bath for 20 h. When the time arrived, the samples were taken out and immediately cooled in an ice bath and directly subjected to further experiments.

2.3. Atomic force microscope (AFM) experiments

AFM was performed to investigate the microstructure of pea protein based amyloid fibrils. For AFM sample preparation, diluted amyloid fibril samples (20 μL) were pipetted on freshly cleaved mica, and stand for 2 minutes, then rinsed with deionized water and dried at room temperature. The AFM images were collected using a Dimension Icon (Bruker) with tapping mode.

2.4. Circular dichroism (CD)

The prepared pea protein amyloid fibril was dialyzed against deionized water by using a 7000 Da dialysis bag for 3 days. CD spectra was performed on a Brighttime Chirascan J815, using a quartz cuvette of 0.1 mm optical pathlength at 25 °C. The spectra were obtained as an average of three scans in the far UV range (190–260 nm) with background subtracted.

2.5. SDS-PAGE electrophoresis

Gel electrophoresis of pea protein before and after fibrillation under denaturing conditions was performed, and the concentrations of the separating and stacking gels were 15% and 4%, respectively. The samples were mixed with 4× electrophoresis sample buffer at a ratio of 3 : 1 and heated for 5 min in boiling water. Then, the prepared samples (10 μL) were loaded onto the gels, and the voltage was 80 V in the concentrated gel and increased to 110 V after entering the separated gel. Gels were stained with Coomassie blue R-250, and decolorized with a solution made up of methanol/glacial acetic acid/water at a ratio of 1 : 1 : 3. Ultimately, the images of the gels were photographed by the gel imaging system (Tanon 4100, Shanghai, China).

2.6. Fabrication of pea protein amyloid fibrils-based HIPEs loading with carotenoid

The emulsion systems were prepared through mixing the protein fibrils and sunflower oil by mechanical shearing with a disperser (IKA T18 homogenizer, Germany) operating at 10 000 rpm for 2 min. The effect of different protein fibrils concentr-
tration from 0.1 to 1 wt% and internal oil volume fraction in the range of 74–96% on the fabrication of the emulsion systems were also examined.

For preparation of the carotenoid loaded emulsions, a certain amount of β-carotene, lycopene or lutein was introduced into the emulsion system through dispersing in the oil phase before mixing with the protein fibril solution. The oil phase volume fraction of the prepared HIPEs was 86% and the amyloid fibril content was 1.0 wt%. And the loading content of carotenoid in the final emulsion system ranged from 0.01 to 0.2 wt%.

2.7. Interfacial behavior

The determination of the interfacial tension and interfacial dilatational viscoelasticity was characterized with the same drop tensiometer as a function of time.29 The samples were diluted to 0.01 mg mL$^{-1}$ with Ultrapure water of pH 2 before test. With the setup, a drop is formed at the end of a capillary and the resulting drop shape is fitted by the Young–Laplace equation.30 To observe protein adsorption kinetics and interfacial layer buildup, a 3 h time sweep was performed by forming a drop with a constant area.

2.8. Characterization of the microstructure of HIPEs

Confocal laser scanning microscope (CLSM) was applied to observe the microstructure of emulsion systems by using LeicaTCS SP8. Nile Red (0.1 mg mL$^{-1}$) and Nile blue A (0.25 mg mL$^{-1}$) was added as dyes before the preparation of HIPEs. Nile red and Nile blue A were excited at 514 nm and 633 nm, respectively.

Cryo-scanning electron microscope (Cryo-SEM) was also employed to detect the microstructure of HIPEs by using SU8010 (HITACHI, Japan). HIPEs samples (~20 µL) was mounted on a copper holder and rapidly frozen by liquid nitrogen and then observed by the Cryo-SEM. The element mapping of the HIPEs was performed on a Bruker XFlash 6160 with a field emission gun operating at 20 kV.

2.9. Stability of carotenoid encapsulated in HIPEs

For these studies, the β-carotene, lycopene or lutein concentration (0.05 wt%) in these systems were fixed. β-carotene, lycopene or lutein in sunflower oil (0.05 wt%) was applied as the control.

UV light stability. To investigate the stability of carotenoid under ultraviolet irradiation, the samples of carotenoid encapsulated in the HIPEs and dispersed in bulk oil with the same concentration were exposed to ultraviolet irradiation for 6 h at room temperature. At the interval of 1.5 h, three samples were parallelly taken to determine the retention of carotenoid using the spectrophotometer method. The detection wavelength was 450 nm for β-carotene and lutein, and 472 nm for lycopene, respectively.

Thermal stability. The thermal stability of the carotenoid encapsulated in the HIPEs and dispersed in bulk oil with the same concentration was assessed by incubating samples at 90 °C for 6 h in the dark. At 1.5 h intervals, the concentration of carotenoid remaining was determined and calculated.

2.10. Bio-accessibilities of carotenoids in simulating GI digestion

The bio-accessibility of the carotenoids dissolved in oil and encapsulated in the HIPEs which was stabilized by pea protein fibrils formed at different NaCl concentrations (0, 25, 75 and 300 mM NaCl) was determined in a simulated GI digestion model. Each sample (0.5 g) was mixed with 15 mL PBS and 12 mL simulated gastric fluid (pH 1.2), which contained NaCl (2 mg mL$^{-1}$) and pepsin (3.2 mg mL$^{-1}$). Then the pH of the mixture was adjusted to pH 2.0 and continuously stirred at 120 rpm for 2 h to mimic gastric digestion. The resulting samples from the gastric digestion phase were then adjusted to pH 7.5 and mixed with simulated intestinal fluid, which contained 2.5 mL lipase solution (24 mg mL$^{-1}$) and pancreatin (2 mg mL$^{-1}$), 4.0 mL bile salt solution (93 mg mL$^{-1}$) and 7.0 mL PBS. Both the enzymes and bile salts were dissolved in PBS (pH 7.0). Finally, the pH of the system was adjusted to pH 7.5 and continuously stirred at 120 rpm for another 2 h to mimic small intestine digestion.

After simulating GI digestion model, the raw digesta was centrifuged at 10 000 rpm for 10 min to collect the middle micelle phase. To prepare the extractant, N-hexane and ethanol were mixed at a volume ratio of 3:2. The extractant was then added to the middle micelle phase in an appropriate ratio. Next, the mixture was vortexed for 30 s and centrifuged again at 10 000 rpm for 10 min. The superstratum was taken, and the carotenoid concentration was measured by an ELx800TM microplate reader. To determine the total concentration of carotenoids in the intestinal digestion, a certain amount of extractant was added to the raw digesta after the simulating GI digestion. The mixture was then extracted as described above, and the supernatant was used for measurement. The detection of lutein and β-carotene was performed at a wavelength of 450 nm, while lycopene was detected at 472 nm. The bio-accessibility of carotenoid was measured according to the equation:

$$\text{Bioaccessibility} = \frac{C_{\text{Micelle}}}{C_{\text{Digesta}}} \times 100$$

$C_{\text{Micelle}}$ is the concentration of carotenoid in the mixed micelle phase and $C_{\text{Digesta}}$ is the concentration in the total digesta collected after the small intestine phase.

2.11. Animal experimentation

C57BL/6j mice (five-week-old males) were purchased and raised in a standardization SPF animal laboratory (temperature
23 ± 2 °C, relative humidity 70–75% and 12 h light–dark cycle) with free access to food and water. Animal experiments have been approved by the Animal Experiment Center in Nanjing Agricultural University [SYXK (Jiangsu) 2011-0037], which was in line with the National Laboratory Animal Welfare Guidelines and Animal Experiment Ethics. After one week of acclimatization, mice (19–21 g) were randomly divided into six groups of 8 mice each. NC group received normal drinking water for 14 days and serve as the control group. DSS group received DSS from days 1–7 and normal drinking water from day 8 to 14. Groups D-H, D-H-Lu, D-H-Ly and D-H-βCa received DSS similarly as in DSS group; additionally, they were administered prepared HIPEs, lutein loaded HIPEs, lycopene loaded HIPEs, and β-carotene loaded HIPEs, respectively, for 7 days. For the induction of UC, mice were orally administered 2 wt% DSS dissolved in drinking water on days 1–7. The body weight, intake of food and water, stool consistency, and rectal bleeding were recorded every day as the daily clinical evaluation. At day 7 and day 14, feces collection was conducted, followed by storage at −80 °C until analysis. All the mice were sacrificed on day 14. Prior to this, blood samples were collected through cardiac puncture under anesthesia. The plasma was placed at room temperature for 4 h, and after centrifugation (3000 rpm, 20 min, 4 °C), the supernatant serum was taken and stored at −80 °C for analysis. The gut part from cecum to anus was removed to measure the length. After being washed in saline, the distal colon was cut open lengthwise into two halves: one-half was frozen and stored at −80 °C, and the other half was fixed with 4% paraformaldehyde for histological examination.

**DAI**. The disease activity index (DAI) was used for evaluation of mucosal inflammation. The hematoxylin, stool consistency and weight loss were summarized and calculated as the DAI. The scoring criteria for weight loss and stool consistency are as follows: 0, no observable blood; 1, trace blood; 2, slight blood; 3, obvious blood; 4, gross blood and 0, normal; 1, loose stool; 2, mild diarrhea; 3, diarrhea; 4, gross diarrhea. The percentage of weight change from day 0 until the end of the trial was calculated to evaluate the loss of weight (0, no weight loss; 1, 1–5%; 2, 5–10%; 3, 10–20%; 4, >20%).

**Lipocalin-2**. Quantification with ELISA. Briefly, feces collected on day 7 were reconstitute by PBS containing 0.1% Tween 20. After vortex for 30 min, the sample was centrifuged (12,000 rpm, 10 min, 4 °C) and the supernatant was collected for the quantification of lipocalin-2.

**Serum indicators for liver toxicity**. Serum ALT level was quantified using an ALT kit to preliminarily assess the hepatic functions.

**Enzyme linked immunosorbent assay**. The TNF-α, IFN-γ, IL-6, IL-1β and lipopolysaccharide (LPS) in the serum were detected using the enzyme linked immunosorbent assay (ELISA) kit according to the manufacturer’s instruction.

**Histological analysis**. Histological alteration of the gut tissues was measured by hematoxylin and eosin (HE) after fresh gut tissues were fixed with 4% paraformaldehyde for more than 24 h. The paraffin-embedded tissue was cut into 4 μm sections. The sections were viewed and pathological characteristics of the gut were photographed by a Nikon ECLIPSE TI-SR (Tokyo, Japan) fluorescence microscope.

**Colon RNA extraction and qRT-PCR analysis**. Total RNA of colonic tissue was extracted according to the operating instructions of the TaKaRa MiniBEST universal RNA extraction kit, and quantified on a NanoDrop 2000 (Thermo Fisher Scientific, USA). The ratio of the absorbance at a wavelength of 260 nm against that at 280 nm was calculated, which was between 1.8 and 2.0 for all of the samples. Then cDNA was synthesized by reverse transcription from 500 ng of RNA operated with a PrimeScript RT master mix (TaKaRa Co., Ltd). Real-time PCR was performed with SYBR Green Fast Mix (Tsingke Bio. Inc., Beijing, China) on a QuantStudio 6 Flex (Thermo Fisher Scientific Inc., USA) as described below: initially degenerated at 95 °C for 1 min, then 40 cycles, each of which at 95 °C for 10 s, 60 °C for 5 s, and 72 °C for 30 s. The relative gene expression level of target was calculated by 2−ΔΔCt, normalized with the housekeeping gene β-actin. The specific primers used in the experiment are shown in Table S1.

### 2.12. Statistical analysis

All experiments were repeated at least three times. The data were presented as the mean ± standard deviation (SD) unless stated otherwise. One-way ANOVA analysis of variance followed by Duncan’s multiple-range tests were used to calculate significant differences. Statistical significance differences were considered at p < 0.05. All analyses were performed using SPSS (IBM, New York, USA).

### 3. Results and discussion

#### 3.1. Effect of ionic strength on the assembly behaviors of pea protein-based fibrils

One of the most critical aspects of developing stable HIPEs with useful functional properties is determining the appropriate emulsifier. Recently, there has been considerable interest in developing food protein amyloid-like fibrils to form and stabilize O/W emulsions. Pea (Pisum sativum L.) protein is considered a promising protein source for constructing amyloid-like fibrils due to its high nutritional value and lack of common allergens. In our previous study, it has been confirmed that pea protein indeed went through fibrillation under appropriate conditions. In addition, researchers have shown that both the fibrillation kinetic and the final fibril morphology of artificial protein amyloid-like fibrils were largely influenced by the ionic strength, which further affected their interfacial properties. Therefore, we evaluated here the effect of ionic strength on the formation of pea protein amyloid fibrils, which is scarcely reported yet.

In the present study, according to our reported method, pea protein was purified in a facilitated and inexpensive way based on pH adjustment. After the purification of commercial pea protein, the purity of protein was elevated from 33% to 91%, and such a high purity level of protein is sufficient for the fol-
following experiment. Then, the purified pea protein was dissolved in ultra-pure water containing different concentrations of NaCl and then heated (85 °C) under acidic condition (pH 2) for 20 h, and the formed fibrils were characterized by AFM, as displayed in Fig. 1. We can see that the heating resulted in the fibril formation of pea protein at all-tested NaCl concentration (0–300 mM), but the fibril morphologies varied with different NaCl concentrations. The amyloid-like fibrils obtained in the absence of NaCl presented an aggregated state (Fig. 1A), while most of the fibrils obtained with 25 mM NaCl were short worm-like and flexible (Fig. 1B). As the ionic strength increased, some of the worm-like fibrils self-twisted to form mature linear fibrils with increasing length. When the ionic strength reached 75 mM and 150 mM, the amyloid fibrils possessed the morphology of long semiflexible and semiflexible rod-like structures, respectively (Fig. 1C and D). However, when the ionic strength is increased to 300 mM, the fibril length became shorter (Fig. 1E). These findings indicated that fibril assembly was more organized and unidirectional under lower ionic strength (25–150 mM), while higher ionic strength (300 mM) hindered the elongation of fibrils. Such similar phenomenon was also observed in the fibrillation process of rice glutelin,34 iron-bound ovotransferrin,35 hen egg white lysozyme,36 and β-lactoglobulin,37 but there were also examples to the contrary,38,39 and it was possible that the types of protein mattered. In this study, one possible explanation was that the charge shielding effect of NaCl varied with its concentration, and then influenced the molecular interaction of hydrolyzed polypeptides, thus leading to the different morphologies of pea protein fibrils. But this explanation did not fully account for the results, as the addition of only 25 mM NaCl resulted in mostly short, worm-like fibrils compared to the condition without NaCl.

To further clarify the reasons for the different performances of NaCl on the formation of pea protein amyloid-like fibrils, the changes of composition and secondary conformation of pea protein in the presence of NaCl during fibrillation were monitored. Firstly, the hydrolysis of pea protein during heating at various NaCl concentrations (0–300 mM) was analyzed by reducing SDS-PAGE (Fig. 2). There were several major subunits in pea protein with molecular weight (Mw) of ~75 kDa (convicilin), ~50 kDa (vicilin), ~38 kDa (legumin A), 31–34 kDa (vicilin) and ~15 kDa (legumin B). It can be seen that addition of NaCl has no significant effect on the composition of pea protein before the thermal treatment in acidic condition (Fig. 2A). After heating for 20 h, the subunits were degraded, and the Mw of pea protein mostly declined to lower than 15 kDa, demonstrating that polypeptides of pea protein were hydrolyzed to smaller peptides (Fig. 2B). Remarkably, the polypeptide hydrolysis patterns of pea protein by heating were slightly different at various NaCl concentrations (0–300 mM). By comparison, we can approximately see that the protein hydrolysis process was inhibited in the presence of low concentration of NaCl (25–150 mM), and the inhibitory effect was enhanced with the increase of NaCl concentration. In contrast, 300 mM NaCl promotes hydrolysis. This phenomenon suggested that the polypeptide hydrolysis of pea protein at pH 2.0 was dependent on ionic strength. Combined with the AFM results, it can be inferred that the attenuation of electrostatic repulsion and protein hydrolysis caused by the increased ionic strength (from 0 to 150 mM) promoted the correct assembly of hydrolyzed peptides to form long semi-flexible fibrils. However, the higher ionic strength (300 mM) induced faster assembly of hydrolyzed peptides was not beneficial to the elongation of fibrils, which may be due to the chaotic of building blocks and unsuitable energy balance.37,40 Furthermore, at 25 mM NaCl, the majority of fibrils observed were flexible and worm-like in shape, possibly due to weak charge shielding effect and less inhibition of hydrolysis. In spite of this, after adding 25 mM NaCl, the fluorescence intensity of ThT during the fibrillation process was consistently higher than that without NaCl (Fig. S1†), indicating that the addition of NaCl promoted the formation of pea protein fibrils.

The formation of highly-ordered β-type secondary structure (especially β-sheet) is one of the basic features for amyloid-like fibrils. Then, the secondary structures of pea protein by heating at various NaCl concentrations (0–300 mM) were characterized by using far-UV CD spectroscopy (Fig. 2C). It can be observed that with the increase of NaCl concentration, the negative bands between 210 nm and 220 nm became more obvious, implying the formation of a β-sheet structure, and the increased ionic strength facilitated the formation of ordered secondary structures. Remarkable decrease of ellipticity magnitude in the negative bands at 210 nm can be seen with NaCl concentration to be 150 mM, indicating the considerable increase in β-type secondary structure.34,41 The CD results are in consistence with the AFM results, further indicating that the presence of building peptides with a high β-type secondary structure is crucial for the assembly of fibrils.

Based on the aforementioned findings, it can be inferred that the hydrolysis of pea protein initially yields polypeptides, which subsequently undergo structural rearrangement and assemble into ordered fibrils via intermolecular interactions.
It was observed that the assembly of fibrils was significantly influenced by the ionic strength, and fibril assembly might be relevant not only to the charge screening effect of NaCl, but also to the effect of NaCl on the hydrolysis process.

### 3.2. Characterization of the interfacial properties of pea protein-based fibrils

The high aspect ratio of protein fibrils means that they can exhibit unusual emulsifying properties because of the generated capillary forces. Given the emulsifying ability of protein fibrils is dependent on interfacial properties, it is of vital importance to investigate the interfacial adsorption of pea protein fibrils. To this end, changes in interfacial tension were monitored during the adsorption process of pea protein fibrils onto the oil–water interface. In addition, recent study has shown that the size and flexibility of fibrils matter on their emulsifying properties. Thus, the fibrils formed under different concentrations of NaCl were all tested, because their size and flexibility varied as described above. It is worth mentioning that in order not to affect subsequent experiments, NaCl was removed through dialysis after the formation of fibrils. As shown in Fig. 3A, the interfacial tension progressively decreased with adsorption time in all the cases. And during the initial adsorption period, high rates of decrease in interfacial tension was observed, which should be associated with population of the bare interface by the protein fibrils. Besides, the interfacial tension was significantly lower with the fibrils formed at 25 mM NaCl than that of other samples, which means that the higher flexibility of fibrils led to a lower interfacial tension, as evidenced by the presence of more worm-like fibrils in the samples produced at 25 mM NaCl. Further comparing the situation between 25 mM and 300 mM NaCl, it is reasonable to believe that it is the worm-like fibrils rather than short fibrils that enable the pea protein fibrils more likely to pack on oil droplet surfaces. Similar results have been observed in whey protein fibrils.

Apart from interfacial adsorption, interfacial viscoelasticity is also a key factor in determining the formation and stability of emulsions. Therefore, the change of viscoelastic modulus of the interfacial membrane formed by pea protein fibrils was monitored (Fig. 3B). The results showed that, in all cases, as the time proceeded up to 10 800 s, the viscoelastic modulus of adsorbed films initially increased rapidly and then reached a plateau. This observation highlighted the role of rapid diffusion in the early stages of adsorption, which contributes to the formation of the interfacial layer. The highest interfacial modulus was associated with the pea protein fibrils formed at 25 mM NaCl, indicating that the increased surface packing of the worm-like fibrils on oil droplets led to a higher density of the interfacial films formed.

To further evaluate the emulsion properties of the pea protein-based fibrils formed at different concentrations of NaCl, the prepared fibril (1 wt%) was mixed with sunflower seed oil to obtain high internal phase emulsions (HIPEs) with
oil phase volume fraction of 82%. Then the stability of HIPEs was tested by tilting the samples (Fig. 3C). The HIPEs prepared with fibrils formed at 0, 25 and 75 mM NaCl could hold themselves as the bottles were turned upside to down, that is, these HIPEs exhibited excellent colloidal gel stability. But the HIPEs prepared with fibrils formed at higher concentration of NaCl (150 and 300 mM) flowed down as the bottle was turned upside to down, which means that the formed HIPEs is less stable. These results indicated that the emulsifying properties of pea protein fibrils formed at low concentration of NaCl was better, which was consistent with the interfacial properties mentioned above. In addition, the volume fraction of oil phase and the concentration of protein fibrils are also important parameters to determine the formation and stability of HIPEs. Based on the above results, pea protein amyloid fibrils prepared with 25 mM NaCl were selected for subsequent experiments. Here, the fibrils with protein content of 1.0 wt% showed the capability to stabilize the oil phase with fraction ratios of 82%–90% (Fig. S2A†). The sample flowed down when the volume fraction of oil phase was below or above this range. Moreover, the pea protein amyloid fibrils with protein contents ranging from 0.4 to 1.0 wt% were able to stabilize HIPEs containing the oil volume fraction ratio of 88% (Fig. S2B†). Finally, all these parameters were combined to obtain the phase diagram (Fig. S2C†). It can be found that the area of the phase diagram of the HIPEs stabilized by protein fibrils formed at 25 mM NaCl is the largest, that is, the most extensive conditions for preparation of HIPEs, suggesting that the emulsifying properties of pea protein amyloid-like fibrils formed at 25 mM NaCl was the best. Overall, these findings validate that the emulsion properties of pea protein fibrils are influenced by their size and flexibility, with the more flexible worm-like fibrils exhibiting superior emulsifying ability.

3.3. Characterization of the microstructure of the HIPEs

To confirm the above observation, the microstructure of HIPEs stabilized by pea protein fibrils formed at different concentrations of NaCl were characterized using both the CLSM and Cryo-SEM. In the CLSM experiment, a hybrid dye solution including 0.1 mg mL\(^{-1}\) Nile Red and 0.25 mg mL\(^{-1}\) Nile Blue A was integrated into the system. The fluorescent dyes in the HIPEs were excited either at 514 nm for Nile Red (for oil phase) or at 633 nm for Nile Blue A (for the protein). CLSM images are depicted in Fig. 4. Apparently, there is the presence of large oil droplets (green) in all samples. In comparison, the droplet sizes increased with the increase of NaCl concentration (Fig. 4A). And HIPEs stabilized by fibrils formed at 25 mM NaCl demonstrated smaller and more uniform diameters than other samples, thus showing higher stability as small droplet sizes could demonstrate the improved emulsion stability.45 Fig. 4B indicates that the network structure of aqueous phase (red) becomes less compact with the increase of NaCl concentration, which implied a looser connection between protein fibrils, making HIPEs less stable. It can be seen from Fig. 4C that all systems presented a typical morphology of O/W emulsions with high dispersed phase density (oil) since drops of oil (green) were formed in the continuous phase (red). As anticipated, the protein fibrils were present on the surface of the droplets, and the coating of fibrils around the oil droplet created protective films that prevented droplet coalescence by generating electrostatic and steric repulsive forces. Moreover, these results confirmed that the high stability of HIPEs prepared with fibrils formed at 25 mM NaCl came from a denser protective interfacial film formed by highly flexible worm-like fibrils.

Cryo-SEM was carried out to further observe the microstructure of samples. The Cryo-SEM images of the HIPEs prepared with pea protein amyloid-like fibrils formed at 0, 25 and 300 mM NaCl are depicted in Fig. 5. It can also be seen that the HIPEs stabilized by fibrils formed at 25 mM NaCl had small, dense and uniform oil droplets, leading to substantially less amount of ice among the oil droplets. For HIPEs stabilized by fibrils prepared with 0 and 300 mM NaCl, the oil droplet...
size was uneven, especially for the latter. And a large amount of ice among the oil droplets could be seen in the condition of 300 mM NaCl. These results were in consistence with the corresponding CLSM images.

3.4. Encapsulation and stabilization of carotenoids by HIPEs

Then we examined the potential of using the HIPEs to encapsulate and protect three widely studied carotenoids: β-carotene, lycopene and lutein. Based on the above results, the HIPEs stabilized by pea protein fibrils formed at 25 mM NaCl was selected for the subsequent experiment, and the HIPEs stabilized by fibrils formed at 0 and 300 mM NaCl were also selected as controls. In present study, the carotenoid was firstly incorporated into the oil phase (86%), which was further mixed and blended with pea protein amyloid-like fibrils (1.0 wt%) to formulate the HIPEs containing different amount of carotenoid in the range of 0.01 to 0.2 wt% (Fig. 6). After the bottles were turned upside down, if the HIPEs could stand on themselves, it indicates that they were homogeneous gel. As expected, the HIPEs stabilized by fibrils formed at 25 mM NaCl performed better, and the loading capacity of all three carotenoids was up to 0.05 wt%. While the maximum loading capacity of the HIPEs stabilized with fibrils formed at 0 and 300 mM NaCl was 0.025 wt%, 0.025 wt% for lycopene, 0.01 wt%, 0.01 wt% for β-carotene, and 0.025 wt%, 0.01 wt% for lutein, respectively. As the carotenoid concentration was increased, the color of the HIPEs changed from light yellow to orange red, which is due to the increased light absorption by the carotenoid molecules. Taking β-carotene as an example, the effect of high concentration carotenoid on the microstructure of HIPEs was observed by confocal microscopy. Here, the HIPEs was stabilized with fibrils formed at 25 mM NaCl. As shown in Fig. S3,† there were no significant changes in oil droplet size and compaction degree when the concentration of β-carotene did not exceed 0.025 wt%. However, the droplet size increased when the loading contents reached 0.05 wt%, but even then HIPEs remained stable, as shown in Fig. 6B.

Carotenoids are widely recognized to be highly vulnerable to degradation when exposed to external harsh conditions, such as light, high temperature, and iron ions, due to the abundance of conjugated double bonds. Therefore, we investigated the potential of HIPEs to enhance their stability. It can be observed that β-carotene, lycopene or lutein encapsulated in the HIPE systems stabilized with the pea protein amyloid-like fibrils can resist ultraviolet irradiation (Fig. 7A), thermal treatment (Fig. 7B), as well as iron ions (Fig. 7C) in aqueous phase. The enhanced stability of carotenoids after encapsulation with HIPEs may be related to the protective effect of the film formed by pea protein amyloid-like fibrils on the oil–water interface. Similar results were also reported in previous studies.46,47 Additionally, it is worth noting that the protective effect of HIPEs stabilized by fibrils formed at 25 mM NaCl was consistently the best in all cases. This finding is in line with the aforementioned results, which demonstrate that the highly flexible worm-like fibrils possess exceptional emulsifying properties and are capable of forming dense interfacial films. As a result, they effectively shield against external harsh conditions.

3.5. The bio-accessibilities of carotenoids in simulating GI digestion

Encapsulation with the HIPEs stabilized by the pea protein fibrils increased the bio-accessibilities of the carotenoids (0.05 wt%) dissolved in oil significantly in the simulating GI digestion (Fig. 8). In addition, the bio-accessibilities of the carotenoids, including β-carotene (Fig. 8A) and lutein (Fig. 8C) from the emulsions in simulating GI digestion showed a decreasing trend with the elevation of salt concentrations util-
lized for preparing the pea protein fibrils. However, the bio-accessibilities of lycopene encapsulated with the HIPEs stabilized by the pea protein fibrils fabricated in different concentrations of salt did not show significant difference (Fig. 8B). The enhanced bio-accessibilities of the carotenoids could be ascribed to a more homogeneous dispersion of the HIPEs stabilized by the flexible pea protein fibrils fabricated in different concentrations of salt did not show significant difference (Fig. 8B). The enhanced bio-accessibilities of the carotenoids could be ascribed to a more homogeneous dispersion of the HIPEs stabilized by the flexible pea protein fibrils induced by 25 mM salt in the simulated GI fluid, leading to higher digestion degrees of the emulsions releasing more carotenoids.

Combining the results from the visual appearance, micro-structure analysis, protective effect and enhancement on the bio-accessibilities of the encapsulated carotenoids, it could be deduced that the HIPEs formed with pea protein amyloid-like fibrils could be used as a carrier to deliver carotenoids.

### 3.6. Effect of carotenoid loaded HIPEs on intestinal inflammation

After carotenoids were successfully encapsulated and effectively protected by HIPEs, we evaluated the effect of carotenoid loaded HIPEs on intestinal inflammation at the animal level to determine whether they contributed to the alleviation of dextran sulfate sodium (DSS) induced colitis. The mice were grouped as (i) a normal control (NC group); (ii) a colitis model group treated with purified water (DSS group); (iii) a colitis model group treated with HIPEs (D-H group); (iv) a colitis model group treated with lutein loaded HIPEs (D-H-Lu group); (v) a colitis model group treated with lycopene loaded HIPEs (D-H-Ly group); (vi) a colitis model group treated with β-carotene loaded HIPEs (D-H-β-Ca group), as shown in Fig. 9A. Fecal lipocalin-2 is upregulated in IBD and can serve as a disease activity marker. Our experimental results showed that lipocalin-2 was significantly ($p < 0.05$) increased in all the DSS treatment groups, and there was no significant difference among these groups, while it could not be detected in the NC group (Fig. 9B). Serum alanine aminotransferase (ALT) activities of the D-H group, D-H-Lu group, D-H-Ly group and D-H-β-Ca group were significantly lower than those of the DSS group and almost returned to the similar activity as the NC group (Fig. 9C). As shown in Fig. 9D, the colitis disease activity index (DAI) was significantly increased after 4 days of...
DSS treatment and peaked on day 8. Upon finishing the feeding with DSS, the DAI score decreased in all tested groups, but in the DSS group, DAI score slightly increased from day 9 to day 12, and the administration of HIPEs and carotenoid-loaded HIPEs resulted in DAI score consistently lower than in the DSS group. In addition, a typical symptom of the colitis mice induced by DSS is the shortening of the colon, indicating increased inflammation. It can be observed from Fig. 9E that the length of colon in NC group, DSS group, D-H group, D-H-Lu group, D-H-Ly group and D-H-β-Ca group was 9.54 ± 0.51 cm, 7.2 ± 0.69 cm, 8.22 ± 0.86 cm, 8.37 ± 0.71 cm, 9.33 ± 0.95 cm and 8.16 ± 1.13 cm, respectively. Obviously, the length of colon was significantly shortened after DSS treatment, and administration of HIPEs and carotenoid-loaded HIPEs could alleviate DSS induced colitis. The colon of the D-H-Ly group almost recovered to a length similar to that of the NC group. Taken together, our results suggested that carotenoid-loaded HIPEs have the potential to alleviate intestinal inflammation in vivo. Meanwhile, there was no significant difference observed between HIPEs alone and carotenoid-loaded HIPEs in terms of their ability to alleviate intestinal inflammation, which could be attributed to the presence of pea protein amyloid-like fibrils or the viscoelastic semi-solid nature of HIPEs. Nevertheless, the D-H-Ly group consistently exhibited the most effective relief of colitis across all cases, suggesting that both carotenoids and HIPEs played a beneficial role in mitigating DSS-induced colitis.

Research has shown that reducing serum inflammatory cytokines is a reasonable goal for UC treatment, including TNF-α, IFN-γ, IL-6, and IL-1β, which was considered as a key factor in the pathogenesis of colitis. To verify whether carotenoid-loaded HIPEs could reverse the development related to the inflammatory response to DSS, we measured the expression levels of inflammatory factors. As shown in Fig. 10, DSS treatment caused upregulation of all these cytokines significantly, while HIPEs and carotenoid-loaded HIPEs supplementation reversed the changes. But HIPEs alone did not decrease the serum TNF-α and IL-6 levels significantly, suggesting that the positive effect of carotenoid-loaded HIPEs on DSS induced colitis mainly came from carotenoid. In addition, LPS can reflect the degree of intestinal mucosal damage and permeability changes, and the higher the LPS level, the more severe the inflammatory reactions. The level

![Fig. 10](image_url) Effects of oral treatments with carotenoids loaded HIPEs on the serum level of the cytokines for pro-inflammatory (A) TNF-α, (B) IFN-γ, (C) IL-6, (D) IL-1β, and (E) LPS. The different letters represent significant differences between different treatments ($p < 0.05$).
of LPS increased significantly after DSS treatment, but both HIPEs and carotenoid-loaded HIPEs could decrease the LPS level, again confirming their positive effect (Fig. 10E).

To evaluate the protective result of HIPEs and carotenoid-loaded HIPEs, hematoxylin and eosin staining was used to observe the changes in histopathology. As displayed in Fig. 11A, mice orally administrated DSS exhibited infiltration of inflammatory cells in the mucosa, edematous changes of epithelial cells, depletion of goblet cells and disruption of epithelial cells. In contrast to the DSS group, the HIPEs and carotenoid-loaded HIPEs intervention groups had apparent signs of recovery. These data are consistent with the above results showing that HIPEs and carotenoid-loaded HIPEs administration exerts obviously restorative effects on the status of DSS intake-induced colon damage.

Finally, we assessed the relative expression of genes associated with inflammation in the colon. The mRNA levels of lipocalin-2, IL-1β and MCP-1 were upregulated in the colon of mice from the DSS group than those from the NC group (Fig. 11B–D). However, after intervention with HIPEs and carotenoid-loaded HIPEs, the mRNA levels of lipocalin-2, IL-1β and MCP-1 were downregulated to varying degrees, suggesting that HIPEs and carotenoid-loaded HIPEs intervention has an inhibitory effect on inflammation factors, further suppressing the cascade of inflammation.

4. Conclusions

In this study, we first elucidated that the final fibril morphology was related to the ionic strength during the fibrillation process. Considering that the protein fibril would be used as emulsifier to stabilize the HIPEs, the interface behavior of the fibrils prepared under different concentration of NaCl was further explored. It is the highly flexible and worm-like fibrils prepared at 25 mM NaCl show superior emulsion properties to stabilize the HIPEs with high stability, as such fibril morphology facilitated their adsorption and packing on oil droplets. Thus, the HIPEs prepared with fibrils formed at 25 mM NaCl is selected as the focus of subsequent experiments. Three carotenoids, including β-carotene, lycopene and lutein, were encapsulated in the prepared HIPEs, respectively. Of course, the stability of carotenoids against ultraviolet irradiation, heat and iron was promoted significantly, and their bio-accessibilities increased significantly. Based on these, the relief effect of carotenoid loaded HIPEs on DSS stimulated colitis in vivo was explored. The present results indicated that carotenoid loaded HIPEs intake might alleviate DSS induced colitis by downregulating the expression of inflammatory cytokines, promoting intestinal barrier function and ultimately recovering the length of colon. And, HIPEs itself also have a positive effect on DSS induced colitis, which may be ascribed to their long time retained in the colon, serving as a barrier against bacterial invasion, but more detailed information needs to be investigated in the future.

Author contributions

Yanhua Liu: data curation; formal analysis. Ran Zhao: data curation; formal analysis. Yingqun Nian: data curation; formal analysis; financial support. Xiaorong Zhang: writing original draft; review and editing; financial support. Bing Hu: project administration, methodology; data curation; formal analysis; writing original draft; review and editing; financial support.

Conflicts of interest

There are no conflicts to declare.

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