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Resveratrol-loaded α-lactalbumin-chitosan nanoparticle-encapsulated high internal phase Pickering emulsion for curcumin protection and its *in vitro* digestion profile

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ABSTRACT

The use of antioxidant-loaded protein-polysaccharide nanoparticle in stabilizing and delivering curcumin with high internal phase Pickering emulsions is comparatively scarce. Resveratrol (RES)-loaded α-lactalbumin (ALA)-chitosan (CHI) particles were fabricated and used for curcumin-loaded high internal phase Pickering emulsions (HIPPEs) stabilization and delivery. CLSM illustrated that RES-ALA-CHI nanoparticles were effectively adsorbed on oil/water (O/W) interface and a gel-like structure was formed surrounding oil droplets. All HIPPEs exhibited excellent physical stability. CUR retention was 75.4 % for HIPPEs with RES-ALA-CHI colloidal particles, which was appreciably higher than that with ALA-CHI colloidal particles (63.9 %) after 30 days storage. Compared to bulk medium-chain triglyceride (MCT), both lipolysis extent and curcumin (CUR) bioaccessibility were pronouncedly enhanced with HIPPEs-based delivery systems. But both HIPPEs (51.4 % and 43.7 %) exhibited lower extent of lipolysis than conventional emulsions (90.4 %). The occurrence of RES significantly restrained the lipolysis. These results demonstrated that HIPPEs could be excellent delivery systems for delivering lipophilic curcumin.

Introduction

Curcumin (CUR), a substance mainly found in turmeric (*Curcuma longa*), has been extensively used as a nutrition supplement and food coloring in food systems (Tsuda, 2018). Recent studies have shown that CUR has promising health-promoting effects such as anti-oxidation, anti-inflammation, anti-obesity, cancer prevention, and neuro-protection. Unfortunately, the chemical and structural nature endows CUR with low water solubility, poor stability especially in neutral and alkaline solutions or exposed to UV light, and low oral bioaccessibility, which seriously limits the applications as dietary supplements or food enrichment.

More recently, there is increasing interest in fabricating particles to stabilize emulsions (Pickering emulsions) since they are far more stable than conventional emulsions. Generally, particles are considered to adsorb onto oil/water (O/W) interface irreversibly to provide a steric barrier to prevent oil droplets from destabilization (Tavernier, Wijaya, Van der Meeren, Dewettinck, & Patel, 2016). Compared with solid particles, colloidal particles fabricated with naturally occurring biopolymers including polysaccharides and proteins are desirable in food systems mainly attributed to their clean-label, cost-effective, biocompatible, and biodegradable properties (Araiza-Calahorra & Sarkar, 2019; de Folter, van Ruijven, & Velikov, 2012; Mwangi, Lim, Low, Tey, & Chan, 2020). Chitin, cellulose, and native starch are mostly used polysaccharides for Pickering emulsions stabilization (Ribeiro, Morell, Nicoletti, Quiles, & Hernando, 2021). Nevertheless, the effectivity of adsorption onto O/W interface as superior stabilizers may not be high for hydrophilic and large polysaccharides. Chemical or physical modifications are necessary to expand their utilization.

Recently, Ca^{2+}-cross-linked whey protein nanoparticles are fabricated for effectively stabilizing and delivering β-carotene (BC) with high internal phase Pickering emulsions (HIPPEs)-based delivery systems (Yi, Gao, Zhong, & Fan, 2020). *In vitro* Caco-2 cells studies confirm whey protein nanoparticles have no cytotoxicity even at 20 mg/mL. Generally,

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HIPPEs are considered as an emulsion-based delivery system having an internal phase volume no<0.74 (v/v). In O/W Pickering emulsions, internal phase refers to oil phase. Nevertheless, obvious coalescence, or oiling-off occurred for HIPPEs stabilized with whey protein nanoparticles when the pH was close to the isoelectric point (IEP, approximately pH 4.8–4.9). HIPPEs stabilized with prolamine colloidal particles including zein, and gliadin are prone to coalescence due to the water insolubility (Li, Zhu, Pan, Meng, Zhang, & Chen, 2019; Zhou et al., 2018). One of the effective approaches to control the interfacial properties of proteins molecules is to interact with polysaccharide to develop protein-polysaccharide colloidal particles. As an effective Pickering stabilizer, it could be moderately wetted by both the water and oil phase, facilitating the accumulation of the colloidal particles at the O/W interface and exhibiting the maximal desorption energy (Araiza-Calahorra and Sarkar, 2019). HIPPEs with whey protein-high methoxyl pectin complex revealed pronouncedly better stabilization against coalescence than that with whey protein at the pH close its IEP (Wijaya, Van der Meeren, Wijaya, & Patel, 2017). In fact, Protein-polysaccharide complex-based colloidal particles (microgels) including whey protein isolate-lactose conjugate, β-lactoglobulin fibril-gum Arabic complex nanoparticle (Gao et al., 2019), and gliadin-chitosan colloid particles (Zhou, Zeng, Yin, Tang, Yuan, & Yang, 2018) have been extensively synthesized and utilized to fabricate stable Pickering emulsions or HIPPEs.

The incorporation of antioxidant in protein-polysaccharide nanoparticles is believed to enlarge their applications as high-efficient stabilizers in food systems due to the pronounced increase of antioxidative activity. However, knowledge about the use of antioxidant-loaded protein-polysaccharide nanoparticles in stabilizing and delivering curcumin with HIPPEs is comparatively scarce. Recently, resveratrol (RES)-loaded α-lactalbumin (ALA)-chitosan (CHI) nanoparticle with high stability, ideal wettability, and strong antioxidant activity was fabricated in our lab (Liu, Gao, Yi, Fan, Wu, & Zhang, 2020). RES, a natural lipophilic polyphenol, exhibited plentiful biological, and functional activities (including anti-oxidation, anti-cancer, anti-inflammation, and anti-ageing), which is beneficial to human health (Delmas, Jannin, & Latruffe, 2005; Gülçin, 2010; Hsieh & Wu, 2010). Among all significant biological activities of RES, antioxidant activity is one of the most significant. Linoleic acid peroxidation in emulsion can be pronouncedly inhibited up to 89.1 % in the presence of only 30 μg/mL RES (Gülçin, 2010). No reports on RES-ALA-CHI colloidal particles in stabilizing and delivering CUR-loaded HIPPEs have been found.

The goal of this study is to investigate the influences of RES-loaded ALA-CHI nanoparticles on the formation, storage stability, simulated digestion fate of CUR-loaded HIPPEs. Our previous study demonstrates that the water solubility and *in vitro* antioxidative property of RES have been pronouncedly improved with ALA-CHI colloidal particles (Liu, Gao, et al., 2020), suggesting RES-ALA-CHI colloidal particles may be outstanding stabilizers for CUR protection and controlled release since RES on the O/W interface exhibits great capacity in efficiently scavenging free radicals, chelating pro-oxidants, and interacting with digestive enzymes in gastrointestinal digestion tract.

Materials and methods

Materials

Pure ALA (>95 % protein based on a dry basis, ALA > 90 %, ash < 3.5 %, fat < 0.5 %, and lactose < 0.2 %) was purchased from Agropur Ingredients Inc. (Appleton, WI, U.S.). Corn oil and medium chain triglycerides (MCT) and were bought from Yihai Kerry (Shanghai, China). CUR (>94 % purity), and commonly used inorganic salts like $CaCl_2$ and NaCl were provided by Macklin (Shanghai, China). CHI (Mw approximately 100 kDa, about 75–85 % degree of deacetylation (DD), RES (≥98 % purity), Nile red, pepsin, bile salts, pancreatin and Nile blue A were bought from Sigma-Aldrich (Shanghai, China).

Fabrication of ALA-CHI and RES-ALA-CHI nanoparticles

ALA-CHI nanoparticles were fabricated based on the approach published previously.(Liu, Gao, et al., 2020) Firstly, ALA and CHI (1.0 %, w/v) were solubilized in ultrapure water and ethanoic acid solution (1.0 %, w/v). The stock solutions were kept in a refrigerator overnight to make sure the complete hydration of ALA and CHI. For ALA-CHI colloidal particles fabrication, various amounts of CHI solutions diluted to certain concentrations were added dropwise to ALA solution to obtain various ALA:CHI mass ratio (1:1, 2:1, 5:1, and 10:1, w/w). After that, the pH was adjusted to 5.0, and 6.5 and mixed for about 2 h to lead to the formation of ALA-CHI nanoparticles.

For RES-ALA-CHI colloidal particles fabrication, a certain quantity of RES solution (dispersed in ethanol) and ethanol were added dropwise to ALA-CHI colloidal particles solutions. The final RES content was set at 400 μg/mL with a final ethanol content of 2 % (w/v). Subsequently, the pH was adjusted to 5.0, and 6.5. The solutions were further magnetically stirred for about 6 h in the dark environment. Then, free RES was eliminated through centrifugation (10000g, 20 min) with a benchtop centrifuge. Free RES was precipitated at the bottom of the centrifuge tube.

Encapsulation efficiency (EE), and loading amount (LA) of RES in nanoparticles were assessed according to the following formulas:

$$\mathrm{EE}\ (\%) = \left[\frac{(\text{total quantity of RES} - \text{free RES})\mu g}{(\text{total quantity of RES})\mu g} \times 100\right] \quad (1)$$

$$\mathrm{LA}\ (\mu g/mg) = [\frac{(\text{encapsulated quantity of RES})\ \mu g}{(\text{total quantity of nanoparticle})\ mg} \quad (2)$$

RES concentration was investigated by a UV2600 spectrophotometer (Shimadzu, Japan) at 305 nm (Fan, Liu, Gao, Zhang, & Yi, 2018). After EE and LA measurement, both ALA-CHI and RES-ALA-CHI nanoparticles were lyophilized (Scientz, Ningbo, China), and kept in fridge for further use.

HIPPEs fabrication

HIPPEs were fabricated through mixing oil phases (edible MCT) with aqueous phases (ALA-CHI or RES-ALA-CHI colloidal particles solutions) at the mass ratio of 80:20 (w/w) using a T-25 digital homogenizer (12000 rpm, IKA-Werke, Germany) (Yi, Gan, Wen, Fan, & Wu, 2021). The concentrations of ALA-CHI or RES-ALA-CHI colloidal particles in aqueous phase were set at 0.24 %, 0.60 %, 1.2 %, and 2.4 % (w/w). For oil type optimization, corn oil and MCT were used as oil types for HIPPEs development at the mass ratio of 80:20 (oil phase: aqueous phase, w/w). For CUR-loaded HIPPEs preparation, CUR was dispersed in MCT under magnetically stirring for approximately 2–3 h to make sure the full dispersion at 25 °C. The newly fabricated HIPPEs were kept in a fridge and subjected to characteristics determination soon.

HIPPEs characterization

Microscopy

The morphologies and sizes of the HIPPEs encapsulated with ALA-CHI and RES-ALA-CHI complex particles were analyzed using a Zeiss light microscopy (Axio Scope A1 pol, Germany) (Yi et al., 2020). A portion of (approximately 2 μL) HIPPEs were added onto a glass slide, covered with a cover slip. After that, the glass slides were used for observation. All the images of HIPPEs were photographed after one day storage.

Particle size and size distribution measurement

The particle size and size distribution of HIPPEs were determined by a Malvern Mastersizer2000 analyzer (Malvern Instrument ltd., England). All HIPPEs were diluted with ultrapure water before measurement to avoid multiple scattering effects. The refractive indexes of MCT

and ultrapure water were set at 1.45 and 1.33, respectively.

Zeta-potential measurement

Zeta-potential values of HIPPEs were obtained according to the electrophoretic mobility using a Nano ZSE Zetasizer (Malvern Instrument ltd., England). All HIPPEs were diluted 100-fold before analysis. The detections were performed at ambient temperature with 1 min equilibration.

Confocal laser scanning microscopy (CLSM)

The characteristics and morphologies of HIPPEs were detected using a LSM880 confocal microscope (Carl Zeiss, Germany). Each HIPPEs (5 mL) was simply and gently mixed with approximately 20 μL Nile Red (0.1 %, w/v) and Nile Blue A (0.1 %, w/v) isopropyl alcohol solution. The photos were obtained at the excitation wavelengths of 488 nm and 633 nm.

Rheology

The viscoelastic properties (apparent viscosity, elastic modulus (G') and viscous modulus (G'')) of the HIPPEs stabilized with ALA-CHI, and RES-ALA-CHI colloidal particles were investigated with an AR-1000 rheometer (TA Instruments ltd., U.S.) according to a recently published protocol (Yi et al., 2020). The apparent viscosities of HIPPEs versus shear rates (0.1 to 100 s^{-1}) were recorded with the TA software at 25 °C. A plate with 25 mm diameter was applied and the gap between plates was set at 0.800 mm. The frequency sweep (1 Pa stress, 0.1 to 100 rad/s) was carried out in the linear viscoelastic region of HIPPEs.

Physical stability of CUR-loaded HIPPEs

The physical stability of CUR-loaded HIPPEs during 75 days storage at ambient temperature (25 °C) was determined by microstructure appearance and particle size change determination. Sodium azide (0.02 %, w/v) was used as an antibacterial agent during storage.

CUR retention rates in HIPPEs

For CUR-loaded HIPPEs heat stability at 50 °C, aliquots (0.1 g) of HIPPEs at certain intervals during heat treatment were collected, and vortexed with 5 mL of ethanol. Then, the sample was centrifuged (2000 rpm, 10 min) with a bench top centrifuge (Thermo-Scientific, USA) to remove any possible sediments at 10 °C. Lastly, the supernatant was collected and used for CUR quantification. CUR content was determined by a UV-2600 spectrophotometer (Shimadzu, Japan) at 428 nm (Yi, Fan, Zhang, Wen, Zhao, & Lu, 2016). A calibration curve, obtained through detecting the absorbance as a function of CUR contents, was used for CUR quantification. MCT fully dissolved with the same content of CUR in HIPPEs has been used as control.

In vitro lipid digestion profile

In vitro lipid digestion profiles of CUR-loaded HIPPEs were investigated with a previously reported *in vitro* gastrointestinal digestion model (Yi, Gan, et al., 2021). Firstly, 0.5 g of CUR-loaded HIPPEs (0.4 g oil in total), ultrapure water (7.5 mL) and simulated gastric fluid (10 mL containing 3.2 mg/mL pepsin (with an enzymatic activity of ≥ 400 units/mg of protein) and 0.15 M NaCl) were mixed, and the pH was adjusted to 2.0 immediately. The mixture was incubated for 1 h under moderate magnetic stirring at 37 °C with a circulating water bath. pH of the digesta was immediately adjusted to 7.0 after gastric digestion. After that, lipolysis was initiated by adding simulated intestinal fluid (15 mL containing 1.0 mg/mL pancreatin (8 × US Pharmacopeia (USP) specifications), 20.0 mg/mL bile extract and 5 mM $CaCl_2$, pH 7). The pH of the digesta was maintained at 7.0 with the addition of NaOH (0.25 M) dropwise during 2 h incubation. Lipid digestion profile was evaluated through analyzing the amount of added NaOH. Lipolysis extent (%) was quantified using the following formula:

$$Lipolysis(\%) = \frac{V_{NaOH} \times C_{NaOH}}{2M_{lipid}} \times 100$$

where V_{NaOH} is NaOH volume added dropwise. C_{NaOH} is 0.25 M. M_{lipid} is the average molecular weight (Mw) of MCT.

CUR bioaccessibility

The aqueous phase rich in CUR micelles was obtained by centrifugation (10000 rpm, 20 min, 10 °C) of the digesta after whole digestion based on previous studies (Chen, McClements, Wang, et al., 2018; Chen, McClements, Zhu, et al., 2018). Aliquots (0.2 mL) of the micelles solution were collected with a syringe, vortexed with ethanol (about 5 mL), and centrifuged at 2000 rpm for 20 min using a bench top centrifuge (Thermo-Scientific, USA) to remove any possible sediments. The supernatant was collected and detected using a UV-2600 spectrophotometer (Shimadzu, Japan) at 428 nm. A previously described calibration curve of absorbance versus CUR concentration in ethanol was used for CUR contents determination. CUR bioaccessibility was quantified through dividing the quantity of CUR transferred into micelles during digestion by the amount of CUR in initial HIPPEs with the following formula:

$$CUR\ Bioaccessibility\ (\%) = \frac{\text{amount of CUR in micelles}}{\text{the initial amount of CUR added into the digestion system}} \times 100$$

Statistics

Statistical analyses were assessed using an ANOVA procedure with a SPSS software 22.0 (SPSS Inc., U.S.) using a level of significance of 0.05 ($P < 0.05$).

Results and discussion

Characteristics of RES-ALA-CHI colloidal particles

Mean particle size, zeta-potential, polydispersity index (PDI), EE, and LA of RES-ALA-CHI colloidal particles at two different pHs (5.0 and 6.5) were investigated, as illustrated in Table 1S. A same trend of particle size changes was observed with the increase of ALA:CHI mass ratios at both pHs (5.0, and 6.5), consistent with our previous reports (Liu, Gao, et al., 2020). At pH 5.0, the mean particle size of RES-ALA-CHI nanoparticle reduced from 365.2 nm to 286.7 nm increasing ALA:CHI mass ratios from 1:1 to 5:1, but boosted from 286.7 nm to 396.3 nm increasing the ALA:CHI mass ratios from 5:1 to 10:1 ($p < 0.05$). RES-ALA-CHI colloidal particles (5:1, ALA:CHI) exhibited the lowest Z-average diameters (286.7 nm and 211.2 nm) at both pHs (5.0 and 6.5) ($p < 0.05$). The addition of excessive CHI molecules would result in the excessive adsorption of CHI molecules on ALA molecules, which would

contribute to the pronounced enhancement of mean particle size and PDI value (Zhang, Sun, Fan, Li, & He, 2018). Moreover, bridging effects of CHI with ALA would result in the occurrence of complex coacervation through electrostatic interaction in the absence of sufficient CHI (10:1) (Zhang et al., 2018). PDIs showed similar trends with mean particle sizes with the variations of the mass ratio.

At pH 5.0, zeta-potentials of ALA-CHI colloidal particles progressively reduced from 40.1 mV to 35.1 mV increasing ALA:CHI mass ratios from 1:1 to 10:1. Smaller quantity of CHI could result in less available CHI biopolymers bound onto ALA side chains, which contributes to the decrease of positive zeta-potential. EE exhibited a remarkable decrease increasing ALA: CHI mass ratio, whereas LA displayed a pronounced enhancement. The decrease of CHI concentration would lead to the occurrence of ALA aggregation and complex coacervation, which would be detrimental to RES encapsulation, resulting in the decrease of EE. No pronounced differences of LA were found between two ALA:CHI mass ratios (5:1 and 10:1) at both pH values.

In addition, according to the wettability and contact angle results of ALA and ALA-CHI colloidal particles (Yi, He, & Fan, 2021), The contact angle of ALA was found to be 72.0° (pH 5.0), while the value of ALA-CHI complex particles was augmented to 89.6°($p < 0.05$). The contact angle of ALA was declined from 61.7° to 50.2° when increasing pH from 5.0 to 6.5, demonstrating the reduction of hydrophobicity of ALA molecules at pHs away from the isoelectric point (IEP) ($p < 0.05$). At pH 5.0, the contact angle of ALA-CHI nanoparticles was markedly higher than that at pH 6.5. It is reported that particles (microgels) possessing a 90° contact angle on O/W interface exhibit the maximum desorption energy (Binks & Lumsdon, 2000). Nevertheless, the lower contact angle (indicating higher hydrophilicity) would facilitate the particles to disperse in water phase, contributing to the formation of unstable HIPPEs. This implied that ALA-CHI nanoparticle (5:1, pH 5.0) exhibited the highest hydrophobicity, indicating it possessed the great potential as an excellent Pickering stabilizer for HIPPEs stabilization. What's more, it has been demonstrated that ALA-CHI nanoparticle (5:1, w/w) at pH 5.0 has remarkably greater emulsifying activity and stability than that at pH 6.5 (Liu, Fan, Wu, Lu, & Yi, 2020). ALA-CHI nanoparticle (5:1, pH 5.0) was optimized and selected as a superior Pickering stabilizer for HIPPEs fabrication since their contact angle was 89.6°, remarkably higher than that at pH 6.5, suggesting it could be wetted by both the oil and water phase.

Impact of ALA-CHI concentration and oil types on the formation of HIPPEs

A stable Pickering emulsion encapsulated with just 0.12 % (w/w) ALA-CHI colloidal particles at an oil fraction of 60 % (w/w) can be successfully fabricated (data not shown). Higher oil fraction would increase the loading amounts of encapsulated nutraceuticals in Pickering emulsion-based delivery systems. However, stable HIPPEs cannot be synthesized with low concentrations of ALA-CHI or RES-ALA-CHI nanoparticles (0.12–1.2 %, w/w) when increasing the oil fraction from 60 % to 80 % (w/w), as illustrated in Fig. 1. This result demonstrated that the amounts of ALA-CHI colloidal particles were not sufficient to completely cover all lipid droplets formed during high-speed mixing, resulting in the occurrence of oiling-off, Ostwald ripening and coalescence. Gel-like stable HIPPEs could be formed with 2.4 % (w/w) ALA-CHI nanoparticles, suggesting stabilizer molecules were sufficient to completely cover all lipid droplets (Fig. 1). This information demonstrated that ALA-CHI nanoparticles were suitable stabilizers for HIPPEs stabilization. ALA-CHI and RES-ALA-CHI colloidal particles at the concentration of 2.4 % (w/w) were used for CUR-loaded HIPPEs fabrication.

Generally, oil type is also considered to dominate the formation of stabilization of HIPPEs with biopolymers-based Pickering stabilizers. Stable HIPPEs with ALA-CHI and RES-ALA-CHI colloidal particles can be fabricated with MCT oil as dispersing phase at a mass fraction of 80 %, but not corn oil. This might be primarily due to the relatively low density of corn oil since corn oil exhibited higher volume than MCT oil at the same weight. Higher concentrations of colloidal particles were expected to fully cover all corn oil droplets. Oil droplet size of a high-speed homogenized emulsion was generally determined by the balance between droplet break-up and recoalescence. Colloidal particles dominated the process which was to lower the resistance to droplet break-up by reducing the interfacial tension. At the same colloidal particle concentration, HIPPEs with corn oil was expected to have higher particle size due to droplet recoalescence. Higher oil droplets size would contribute to faster and higher extent of instability (Ostwald ripening and coalescence) during emulsion preparation. Furthermore, viscosity differences between corn oil and MCT oil exhibited some impacts on the ability of forming stable emulsions even though they are not high. It has been reported that the viscosity of corn oil is slight greater than that of coconut oil (Noureddini, Teoh, & Davis Clements, 1992). Since viscosity would dissipate energy during high-speed mixing, limiting oil droplet formation to bigger particle sizes (Gupta, Narsimhan, Hatton, & Doyle, 2016). Therefore, MCT oil was used as dispersing phase for CUR loading and HIPPEs fabrication.

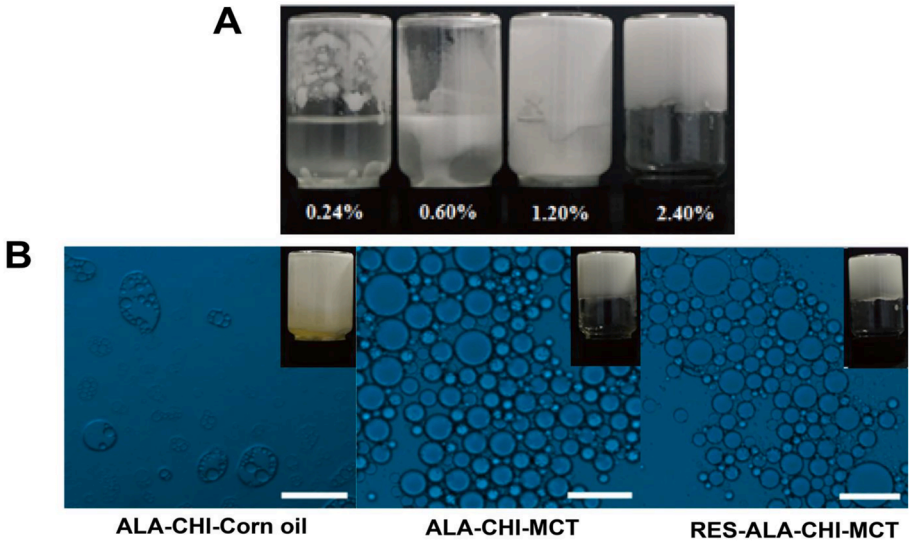

Fig. 1. Effects of Pickering stabilizer concentrations (0.24, 0.60, 1.2, and 2.4 %) on the visual appearance of HIPPEs encapsulated with ALA-CHI nanoparticles (A); and effects of oil types (corn oil, and MCT) on the visual appearance of HIPPEs encapsulated with ALA-CHI, or RES-ALA-CHI complex particles (B). Oil phase mass ratio (φ, w/w) is 0.8. Optical microscopy photograph of HIPPEs (A: ALA-CHI, corn oil; B: ALA-CHI, MCT; C: RES-ALA-CHI, MCT) under 400 × magnification. Scale bar is 20 μm.

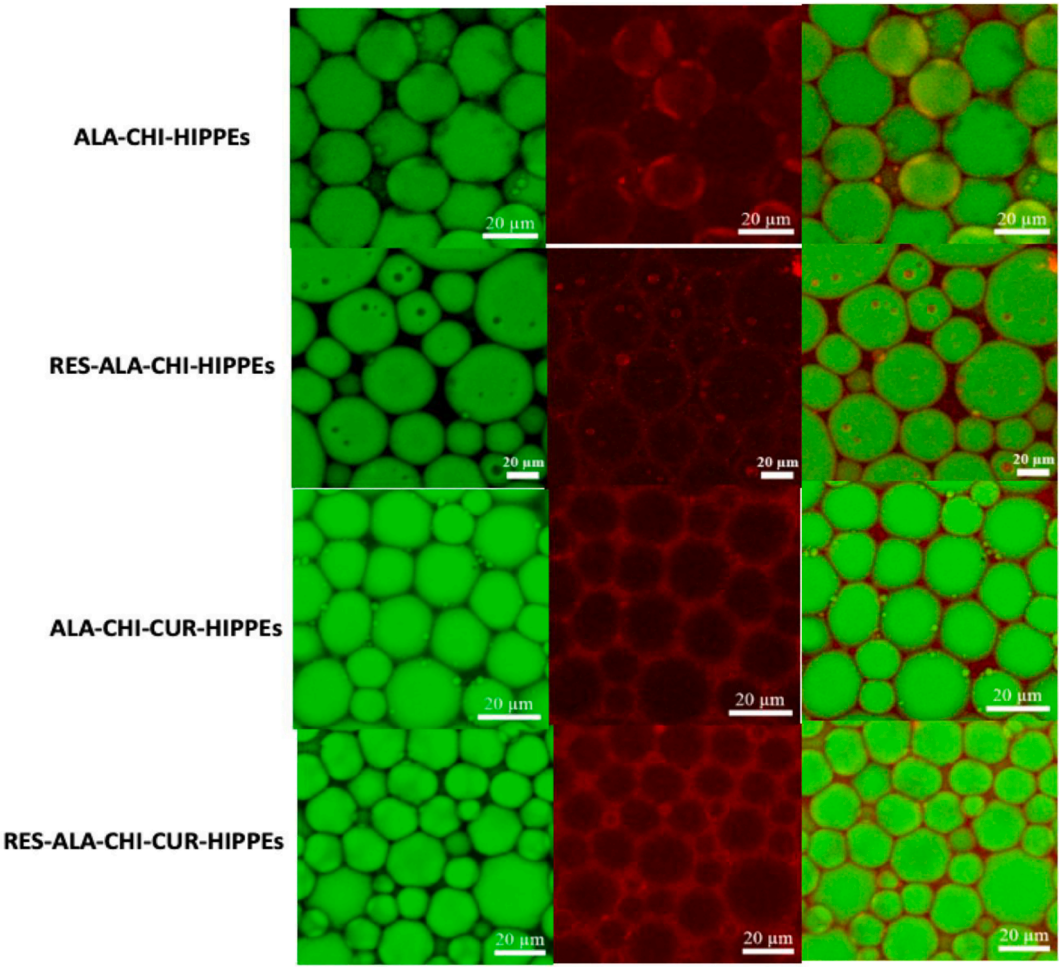

Fig. 2. CLSM photos of HIPPEs with 80 % MCT or CUR dissolved MCT as the internal phase stabilized by ALA-CHI colloidal nanoparticles or RES-ALA-CHI nanoparticles (2.4 wt%). MCT was dyed with Nile Red (green), and ALA was dyed with Nile Blue A (red). The excitation wavelengths for Nile Red and Nile Blue A were set at 488 nm and 633 nm, respectively. Images on right column were combined images of those on the left and middle in the same line. Scale bar is 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

CLSm

The microstructure of HIPPEs and CUR-loaded HIPPEs at 80 % (w/w) oil fraction stabilized with ALA-CHI and RES-ALA-CHI-based colloidal particle was studied by CLSM, in which oil droplets were dyed with Nile red and colloidal particles were dyed with Nile blue A (Fig. 2). The images demonstrated that oil droplets exhibited green fluorescence and colloidal particles layer outside exhibited red fluorescence, and oil droplets were dispersed homogenously and fully covered by red layers. These results indicated both ALA-CHI and RES-ALA-CHI colloidal particles can irreversibly adsorb onto the O/W interface and provide physical barriers to oil droplets against coalescence and Ostwald ripening. ALA-CHI and RES-ALA-CHI colloidal particles may be merged into a viscoelastic film on O/W interface, leading to the full covering surrounding oil droplets, evidencing the Pickering stabilization mechanisms of ALA-CHI and RES-ALA-CHI-based colloidal particles. Similar results were also observed for HIPPEs stabilized with starch/chitosan complex particle that a gel-like, and thick network could be formed by interacting colloidal particles, trapping oil droplets (Yan, McClements, Zhu, Zou, Zhou, & Liu, 2019).

Rheology

The rheological behavior of CUR-loaded HIPPEs (80 %, oil fraction (w/w)) encapsulated by ALA-CHI and RES-ALA-CHI-based nanoparticles was evaluated. A shear-thinning behavior was found for HIPPEs increasing shear rate independently of Pickering stabilizer types (Fig. 3A). This was probably due to the disturbance of the gel-like network structure and distortion of lipid droplets. Interestingly, the incorporation of CUR appreciably increased the apparent viscosity of oil droplets at the same condition. The lipophilic backbone of CUR is mainly composed of an aliphatic chain conjugated with two aromatic rings. However, the attachments of polar groups like carobonyl on the aliphatic chain and hydroxyl on aromatic rings endowed CUR molecule some amphiphilic characteristics (Sharma, Gescher, & Steward, 2005). It is reasonable to expect CUR molecules to adsorb onto O/W interface of oil droplets in HIPPEs. Polyphenol could act as a crosslinking agent due to its polyhydroxyl structure, facilitating the cross-linking between ALA and CHI, or between ALA and ALA on O/W interface. Polyphenol, especially in oxidized state, could react with amino acid residues of proteins including amino and sulfhydryl groups, resulting in the formation of covalent C—N or C—S bonds and protein cross-links (Quan, Benjakul, Sae-leaw, Balange, & Maqsood, 2019). This results could contribute to the enhancement of viscosity of emulsions and O/W interfacial viscoelasticity (Shavandi, Bekhit, Saeedi, Izadifar, Bekhit, & Khademhosseini, 2018).

As displayed in Fig. 3B, all HIPPEs possessed remarkably higher storage modulus (G') than loss modulus (G'') regardless of frequency range, demonstrating the formation of a gel-like network structure of HIPPEs. Both G' and G'' exhibited small increases increasing frequency.

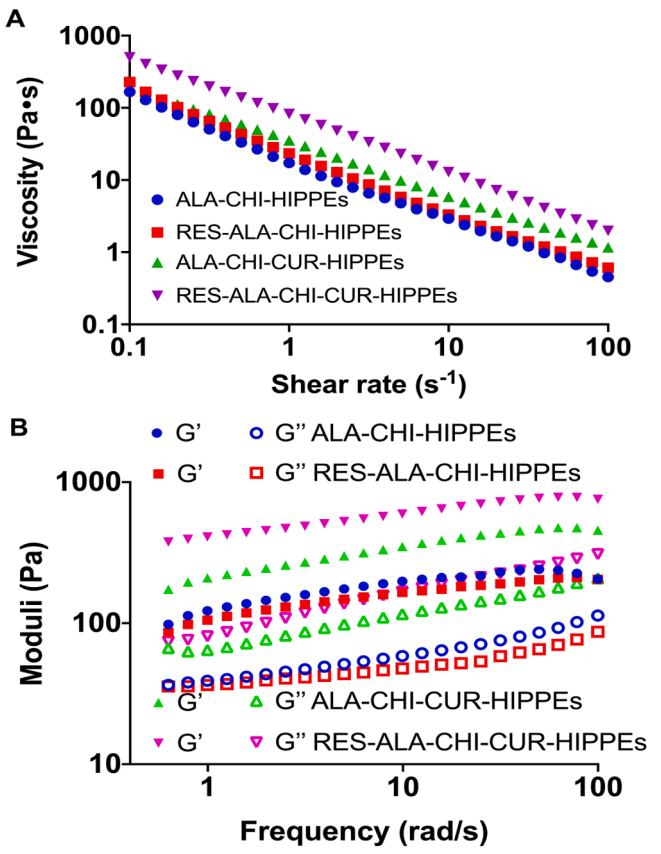

Fig. 3. Rheological results of HIPPEs encapsulated with ALA-CHI and RES-ALA-CHI complex particles. Apparent viscosity of HIPPEs (A). G′: storage modulus, G′′: loss modulus (B).

In addition, G' values were in the following order: RES-ALA-CHI-CUR-HIPPEs > ALA-CHI-CUR-HIPPEs > ALA-CHI-HIPPEs > RES-ALA-CHI-HIPPEs at the same frequency. HIPPEs demonstrated a weak solid-like behavior, where elastic modulus (G') presented higher values than viscous modulus (G") during the whole frequency range analyzed. The incorporation of CUR probably contributes to the cross-linking of biopolymers on O/W interface, which leads to the increase of the thickness, stiffness and viscoelasticity of biopolymers layers. Because polyphenol could act as a crosslinking agent due to its polyhydroxyl structure, which facilitates the cross-linking between ALA and CHI, or between ALA and ALA on O/W interface. Previous studies demonstrated that polyphenol, especially in oxidized state, could react with amino acid residues of proteins including amino and sulfhydryl groups, resulting in the formation of covalent C—N or C—S bonds and protein cross-links (Quan et al., 2019).

Physical stability of CUR-loaded HIPPEs

The visual appearance, microstructure and oil droplets diameter variation were detected to estimate the physical stability of CUR-loaded HIPPEs with ALA-CHI and RES-ALA-CHI colloidal particles during storage (75 days). As displayed in Fig. 4A, almost all initial lipid droplets were evenly dispersed and spherically shaped regardless of Pickering stabilizer types (upper row). After 75 days storage, no significant variations were found for HIPPEs, demonstrating HIPPEs could maintain its physical stability against coalescence, Ostwald ripening, or oiling-off, although slight coarsening of the oil droplets was observed. The formation of a solid-like structure was probably the primary reason. Particle size results (Fig. 4B) showed that oil droplets in HIPPEs were bimodally distributed with a major peak at 20 μm and a small peak at 1.5 μm. After 75 days storage, the particle size distributions exhibited slight shifts and moved to higher particle size ranges, indicating the increase of particle diameter of oil droplets. Intensity of the peak at 1.5 μm exhibited a slight increase and moved to approximately 4.0 μm. Furthermore, the particle size distribution became bigger. Even so, no oiling-off phenomena were found, suggesting the great physical stability of HIPPEs with ALA-CHI and RES-ALA-CHI colloidal particles. Due to the high oil fraction, a continuous, and gel-like network wrapping around the closely packed oil droplets was formed, leading to the pronounced enhancement of network strength and physical stability of HIPPEs (Fig. 4), consistent with the rheological property results (Fig. 3).

Chemical stability of CUR-loaded HIPPEs

CUR, sensitive to environmental stress, is liable to be hydrolyzed, oxidized and degraded at neutral or mildly alkaline conditions. Our previous study demonstrated that over 40 % of CUR can be oxidized and degraded after only half hour storage at neutral pH value, and only 23 % was remaining after 4 h storage at 50 °C (Fan, Yi, Zhang, & Yokoyama, 2018). In this study, the retention rates of CUR in HIPPEs encapsulated by ALA-CHI and RES-ALA-CHI colloidal particles during one month storage at 50 °C were evaluated, as illustrated in Fig. 5. CUR-loaded Bulk MCT was used as control. With the increase of storage time, CUR retention in all samples gradually decreased. Approximately 10.4 % of CUR lost after only 2 days of storage and over 86 % of the CUR in bulk MCT degraded after 30 days, implying that CUR experienced rapid degradation. The hydrophobic backbone of CUR is mainly composed of an aliphatic chain with two attached aromatic rings. The occurrence of oxidative and hydrolytic reactions could result in the degradation of CUR and the formation of degradation products including bicyclopentadione, ferulic acid, and feruloylmethane. After encapsulation, about 98.2 % CUR was remained after 2 days storage for ALA-CHI colloidal particles-stabilized HIPPEs and the retention was 78.5 % after 16 days storage, suggesting CUR's chemical stability was pronouncedly enhanced using HIPPEs-based delivery systems. After 20 h storage, CUR retention was about 60 % when encapsulated in sodium dodecyl sulfate (Leung, Colangelo, & Kee, 2008). CUR in Pickering emulsion stabilized with chitosan-sodium tripolyphosphate nanoparticles was reported to exhibit pronounced chemical stability than those nanoparticle-based delivery systems and only 14 % of the CUR was degraded after 24 h of storage (Shah et al., 2016). In another study, the time required for 50 % CUR degradation in Pickering emulsion stabilized with silica nanoparticles was about 87 h (Tikekar, Pan, & Nitin, 2013). Compared to these studies, our results exhibited pronounced enhancement in the chemical stability of CUR in HIPPEs where approximately 98.2 % of CUR was remaining after 2 days at 50 °C. Firstly, amino acid residues of ALA including tyrosine, phenylalanine, and tryptophan residues could scavenge peroxyl radicals and oxygen, hence restraining CUR from oxidation (Bayram, Pekmez, Arda, & Yalçın, 2008). Furthermore, CHI used in our study has been widely regarded as a biopolymer possessing effective antioxidative activity (Anraku et al., 2018). Lastly, compared to Pickering emulsion (0.44 μm) by Tikekar et al., the larger oil droplet diameter (20.0 μm) and thus bigger interfacial area in our study may result in better protection effects (Tikekar et al., 2013).

At the same condition, CUR retention was only 45.3 % in ALA-stabilized conventional emulsions after 30 days storage, pronouncedly lower than those in HIPPEs, suggesting HIPPEs were better delivery system than conventional emulsion in protecting nutraceuticals from oxidation and degradation ($p < 0.05$). Our recent study also demonstrated a similar result in beta-carotene-loaded HIPPEs with Ca^{2+}-cross-linked whey protein nanoparticle (Yi et al., 2020). The reason of CUR in HIPPEs with highest retention is that the interfacial layer formed with colloidal particles plays a vital role in retarding the interaction between CUR and pro-oxidants as a feasible physical barrier. In addition, the increase of oil fraction would contribute to the remarkable improvement of Pickering emulsions' viscosity, which would restrain the irregular

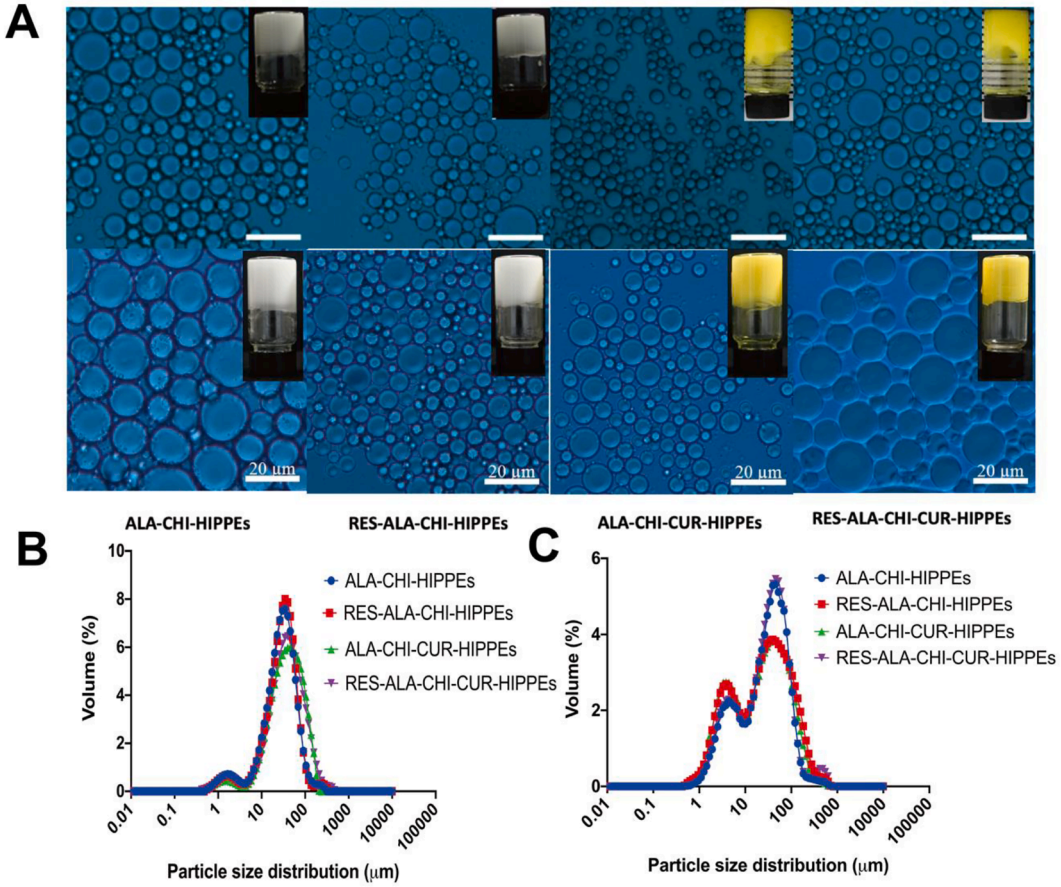

Fig. 4. Appearance and microstructure images of HIPPEs with ALA-CHI and RES-ALA-CHI complex particles, and CUR-loaded HIPPEs with ALA-CHI and RES-ALA-CHI complex particles before and after 75 days storage (A). Droplets size distribution of HIPPEs with ALA-CHI and RES-ALA-CHI complex particles, and CUR-loaded HIPPEs with ALA-CHI and RES-ALA-CHI complex particles before (B) and after 75 days storage (C) at room temperature.

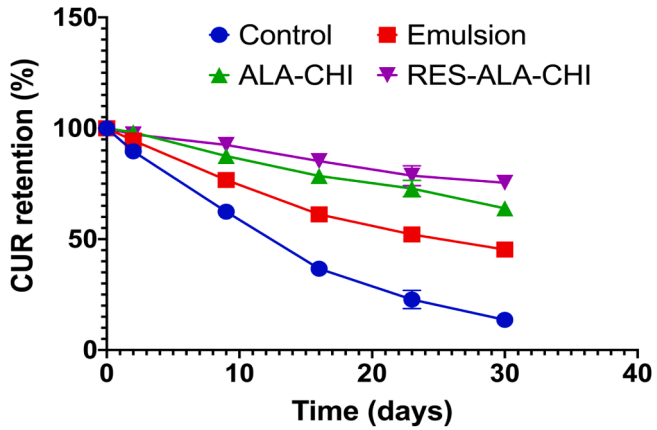

Fig. 5. CUR retention rates in HIPPEs stabilized with ALA-CHI and RES-ALA-CHI complex particles after one month storage at 50 °C. CUR-loaded Bulk MCT (with the same concentration in HIPPEs) was used as control, and CUR-loaded conventional emulsion was used for comparison.

Brownian movement of lipid droplets. Moreover, the formation of the gel-like network could effectively trap the oil droplets and inhibit the degradation of CUR initiated by free radicals (peroxyl radicals), oxygen, or metal ions (Fe^{3+}) on O/W interface.

Interestingly, CUR in HIPPEs with RES-ALA-CHI colloidal particles exhibited a markedly higher retention than that with ALA-CHI colloidal particles, suggesting RES-ALA-CHI colloidal particles were better stabilizers than ALA-CHI colloidal particles. For example, CUR retention was 63.9 % for HIPPEs with ALA-CHI colloidal particles after 30 days storage, whereas the value rapidly increased to 75.4 % with RES-ALA-CHI colloidal particles ($p < 0.05$). RES, located on the O/W interface because of its amphiphilicity, could act as an effective antioxidant due to the presence of phenolic structure in scavenging free radicals (peroxyls) and chelating pro-oxidants, and protect CUR from oxidation.

Lipolysis

The rate and extent of lipolysis of HIPPEs encapsulated with ALA-CHI and RES-ALA-CHI colloidal particles were evaluated as a function of digestion time (120 min), as illustrated in Fig. 6A. CUR in MCT was used as control. Both the rate and extent of lipolysis in HIPPEs were pronouncedly higher than those of control, demonstrating that HIPPEs-based delivery system could promote the lipid digestion independent of Pickering stabilizer types. Generally, the lipolysis rate was considered to be highly associated with the surface area of oil droplets. Generally, the increase of surface area facilitated the lipid digestion. Compared to bulk MCT, the surface area of oil droplets in HIPPEs was pronouncedly increased after encapsulation. After 120 min digestion, the lipolysis extent of bulk MCT was only 32.3 %, whereas the values for HIPPEs were increased to over 40 % regardless of Pickering stabilizer types ($p < 0.05$). Interestingly, pronounced differences in lipolysis rate and extent of HIPPEs stabilized with ALA-CHI colloidal particles and RES-ALA CHI colloidal particles were observed. Both the rate and extent of lipolysis in HIPPEs with RES-ALA-CHI colloidal particle were remarkably lower than those with ALA-CHI colloidal particles. Lipid digestion extents were 51.4 % and 43.7 % for HIPPEs stabilized with ALA-CHI and RES-ALA-

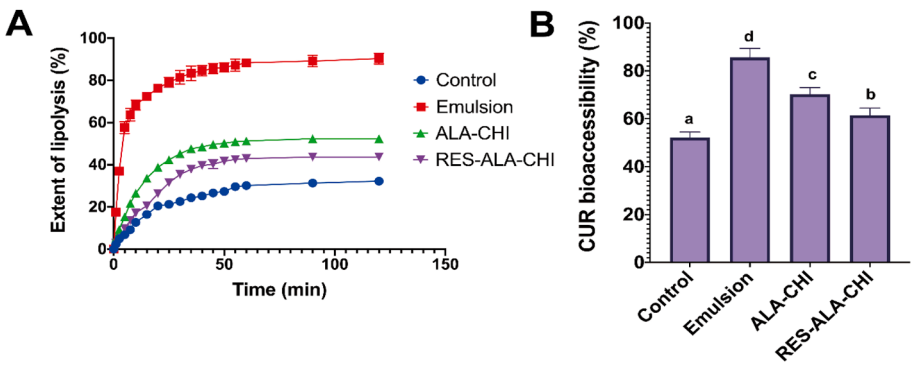

Fig. 6. Extent of lipid digestion of HIPPEs (A) and bioaccessibility of CUR in HIPPEs (B) stabilized with ALA-CHI and RES-ALA-CHI colloidal particles during *in vitro* simulated gastrointestinal digestion. CUR-loaded Bulk MCT (with the same concentration in HIPPEs) was used as control, and CUR-loaded conventional emulsion was used for comparison.

CHI colloidal particles after digestion, respectively ($p < 0.05$). This result demonstrated that the occurrence of RES could remarkably inhibit the lipolysis of oil droplets. The adsorption and anchoring of RES on O/W interface may interact with digestive enzymes including lipase, which decrease its enzyme activity and retard the lipolysis (Lasa et al., 2012). Lipolysis extents of both HIPPEs were pronouncedly lower than that of conventional emulsions. The extent of lipolysis of CUR-loaded conventional emulsions with ALA was found to be 90.4 %. The reason is that the physical barriers restricted the permeability of lipase and the interaction between lipase and oil droplets. The interfacial layer (colloidal particles) could create a pore-like nanostructure among oil droplets, which could limit the permeability of lipase. Furthermore, a gel-like structure of HIPPEs with high viscoelasticity should be another reason. Firstly, the increase of oil fraction would lead to the pronounced improvement of HIPPEs' viscosity, which would effectively decrease the approachability of lipase to oil droplet and restrain oil droplets digestion. Furthermore, the formation of the gel-like network could effectively restrain the Brownian movement of MCT droplets and the anchoring of lipase on O/W interface. Briefly, the combination of the Pickering mechanism and encapsulated RES on oil/water interface were the primary approaches to control lipolysis and release of CUR under simulated *in vitro* digestion.

In vitro release of CUR

CUR bioaccessibility of CUR-loaded HIPPEs stabilized with RES-ALA-CHI and ALA-CHI colloidal particles was illustrated in Fig. 6B. CUR dissolved MCT oil was used as control. CUR bioaccessibility was only 52.2 % for bulk MCT oil, whereas the values were increased to 70.3 % and 61.4 % for HIPPEs with RES-ALA-CHI and ALA-CHI colloidal particles, respectively ($p < 0.05$). The relatively higher bioaccessibility of CUR in bulk MCT oil in this research than other studies is primarily attributed to the different oil types and delivery systems. Compared to the non-emulsified bulk MCT oil, CUR bioaccessibility exhibited pronounced increase after encapsulation in HIPPEs regardless of Pickering stabilizer types, suggesting HIPPEs may be suitable delivery systems for hydrophobic nutraceuticals delivering since a more effective transfer of CUR from oil droplets to mixed micelles was observed. The accessibility of lipase to triglyceride has been retarded by the less interfacial area of bulk MCT, which would restrain the transfer of CUR into micellar phase and reduce its bioaccessibility. Pickering emulsions including HIPPEs have been previously fabricated to enhance the bioaccessibility of encapsulated CUR. CUR bioaccessibility was reported to be approximately 28.8 % in HIPPEs with ovotransferrin-gum Arabic particles (Wei & Huang, 2020). About 60 % of CUR in Pickering emulsion stabilized with silica nanoparticles were released within 2 h of *in vitro* simulated digestion (Tikekar et al., 2013). The final release rates of the encapsulated CUR in Pickering emulsion with chitosan-tripolyphosphate nanoparticles were in the range of 69 and 74 % depending on the oil fractions MCT (Shah et al., 2016). These previous reports also evidenced the enhancement effects of Pickering emulsions on CUR's bioaccessibility.

Interestingly, the bioaccessibility of CUR in HIPPEs with RES-ALA-CHI colloidal particles (61.4 %) was pronouncedly lower than that with ALA-CHI colloidal particles (70.3 %), suggesting the incorporation of RES possessed remarkable impacts on the lipid digestion and CUR micellization from oil droplets to mixed micelles ($p < 0.05$). This finding demonstrated that the release of CUR during *in vitro* digestion can be controlled through the incorporation of functional polyphenols. Moreover, CUR's bioaccessibility was found to be positively related to the final extent of lipolysis of HIPPEs. In comparison to HIPPEs, conventional emulsions with ALA had the highest CUR bioaccessibility (85.6 %). The differences in CUR bioaccessibility among different delivery systems may be primarily due to different oil fractions and microstructure characteristics.

Conclusion

In this study, RES-ALA-CHI colloidal particles (mass ratio 5:1) with 89.6° wettability, 286.7 nm Z-average diameter, 38.2 mV zeta-potential value and 208.5 μg /mg RES LA were fabricated at pH 5.0 and used for CUR-loaded HIPPEs stabilization and delivery. Colloidal particles fabricated with RES, ALA, and CHI could effectively stabilize CUR-loaded HIPPEs with a high oil fraction of 0.80. A gel-like 3D network trapping oil droplets was formed, evidenced by the combination of optical microscopy and CLSM, endowing HIPPEs with high viscoelasticity and physical stability. Chemical stability of CUR was pronouncedly enhanced with HIPPEs-based delivery systems, and HIPPEs with RESALA-CHI colloidal particles exhibited the highest oxidant stability. Compared to bulk MCT oil, ALA-CHI colloidal particle-stabilized HIPPEs pronouncedly enhanced both lipolysis extent and CUR bioaccessibility. The occurrence of RES significantly restrained the lipolysis. When compared with conventional emulsions, both HIPPEs exhibited remarkable decreases of both lipolysis rate and extent. The occurrence of RES on O/W interface may interact with digestive enzymes including lipase, which decrease its enzyme activity and retard the lipolysis. These results may provide great insights into potential utilization of HIPPEs constructed with biopolymers and antioxidants in hydrophobic nutraceutical encapsulation, protection, and delivering.

CRediT authorship contribution statement

Yuting Fan: Investigation, Funding acquisition, Methodology, Writing – original draft. **Dixue Luo:** Software, Methodology. **Jiang Yi:** Supervision, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2022.100433.

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