**Pickering emulsions immobilized within hydrogel matrix with enhanced resistance against
harsh processing conditions and sequential digestion**

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ABSTRACT. Protein particles stabilized Pickering emulsions are unstable under harsh processing conditions and vulnerable to premature lipid phase release during gastric digestion. The objective of this study is to encapsulate kafirin nanoparticles-stabilized Pickering emulsions (KPE) within hydrogel matrix to develop orally administrated Pickering emulsions with enhanced storage and sequential release properties. By premixing KPE and sodium alginate with different volume ratios, emulsion hydrogels (EGs) with various emulsion fractions were immobilized within the calcium ions crosslinked sodium alginate hydrogel matrix thereafter. Ultra small-angle x-ray scattering (USAXS)/ small-angle x-ray scattering (SAXS) analyses suggested that encapsulation of KPE resulted in shrinkage in hydrogel network mesh size, and the emulsion interface evolved from smooth to course one as volume ratio of sodium alginate to KPE increased. When incubated under alkaline (i.e., pH=8.5) or high-temperature (i.e., 60 °C) conditions which were previously reported to cause severe structural collapse for KPE, the coalescence and lipid phase release in EGs were largely inhibited. EGs were also found to be less subjective to stimulated gastric digestion, while the collapse of EGs and thus the release of lipid phase took place in simulated intestinal fluid. The volume fraction of alginate in EGs did not affect the ultimate free fatty acid (FFA) release extent, but it had a negative correlation with the bioaccessibility of lipophilic nutraceutical. This study highlights the potential of designing hydrogel carrier for the oral administration of Pickering emulsion with ease of preparation, improved processing stability and controlled digestion profile.

KEYWORDS: Pickering emulsion; emulsion hydrogel; microstructure; stability; digestion profile

1. Introduction

Pickering emulsions, emulsion droplets stabilized by colloidal particles (Pickering, 1907), exhibited peculiar advantages over conventional emulsions in terms of outstanding stability against coalescence and Oswald ripening (Dickinson, 2010), the capacity of stabilizing emulsions with high internal phase (Ikem, Menner, & Bismarck, 2008) as well as introducing novel rheological (Xiao, Wang, Gonzalez, & Huang, 2016) and thus sensory properties. For these reasons, Pickering emulsion based formulations have gained renewed research interest in fields of food, material, cosmetics and pharmaceuticals (Xiao, Li, & Huang, 2016; Tang, Quinlan, & Tam, 2015; Yi, Yang, Jiang, Liu, & Jiang, 2011). Among which, edible particles stabilized Pickering emulsions were expected to function as promising vehicles for encapsulation, protection and delivery of bioactive nutraceuticals through oral administration route (Jia, et al., 2015; Wang, et al., 2015; Wu, et al., 2015). However, formulations that can meet challenges during the processing procedures of food/pharmaceutical products and serve for functional delivery purposes are far from readily available. In our previous research, we established a kafirin protein nanoparticles stabilized Pickering emulsion (Xiao, et al., 2016). Although it exhibited long-term stability towards coalescence and possessed protection effects towards lipid phase during storage, they could not withstand high temperature and alkaline treatments. And the emulsion droplets lost their structure integrity due to proteolysis in gastric digestion fluid (Xiao, Li, & Huang, 2015). Design Pickering emulsion based delivery systems with enhanced resistance against the harsh processing and gastric conditions, so as to deliver active agents to specific locations within gastrointestinal tract, is one of the urgent challenges for edible particles stabilized Pickering emulsions.

Previous research efforts in enhancing stability and controlled release of lipids in

conventional emulsion systems involved multilayer emulsion systems via coating lipid droplets with biopolymer coatings (Bortnowska, 2015), emulsion - filled hydrogel beads through physical or chemical crosslinking processes (Chen, et al., 2007; Gulsen & Chauhan, 2005; Guo, Ye, Lad, Dagleish, & Singh, 2014; Li & McClements, 2011; Shingel, Roberge, Zabeida, Robert, & Klemberg-Sapieha, 2009) etc. In these cases, small molecular weight surfactant or surface-active biopolymers in primary emulsions stabilize the non-polar phase and/or function as network forming component.

In this study, we intend to immobilize Pickering emulsion droplets within hydrogel strips using a physical crosslinking method, which may fulfill the application potential of protein particles stabilized Pickering emulsion as an oral drug delivery vehicle with programmed release profile. To this end, alginate, an un-branched binary copolymer of β (1/4) linked β -D mannuronic acid (M) and α -L-guluronic acid (G) (Draget, 2009), was selected as the coating layer and hydrogel formation matrix due to its ability to form mechanically consistent hydrogel in the presence of divalent cations as well as evidenced resistance towards gastric digestion (Fajardo, et al., 2012; Li, Hu, Du, Xiao, & McClements, 2011; Liu, Liu, Liu, Li, & Liu, 2013). To elucidate the microstructure and digestion profile of as-prepared emulsion hydrogel systems (EGs), curcumin, a lipid-soluble nutraceutical with intrinsic fluorescence (Xiao, Nian, & Huang, 2015), was chosen as a lipophilic encapsulate marker.

The primary Pickering emulsion stabilized by kafirin protein nanoparticles (KPE) was mixed with different volume ratios of sodium alginate solution. The as-prepared mixture was then dripped into 1% CaCl_2 solution to induce network formation among Ca^{2+} and anionic G-G rich sequences in sodium alginate. Instead of forming spherical hydrogel beads, which is the most commonly adopted morphology for calcium-alginate hydrogel, we injected the mixture into bulk

CaCl₂ solution by a pipette in a continuous and fast manner, the resultant emulsion embedded hydrogels (EGs) were thus in a strip form. Fluorescence microscopy as well as the combined ultra-small-angle X-ray scattering and small-angle X-ray scattering (USAXS/SAXS) were carried out to reveal the multilevel microstructure of EGs. The stability of EGs under harsh processing conditions, which were reported to cause structural damage to KPE (Xiao et al., 2015a), was then evaluated. Release of lipid was evaluated using *in vitro* simulated gastric fluid and simulated small intestine fluid with the presence of pepsin and pancreatin, respectively. The impacts of emulsion hydrogel composition on the extent of lipid digestion and bioaccessibility of curcumin were also examined. Results gained in this work will fill the gap between knowledge in edible particles stabilized Pickering emulsion and their practical applications in oral delivery systems.

2. Materials and methods

2.1. Materials

Kafirin protein with a purity of 90% was extracted from whole sorghum grain and characterized in our lab (Xiao et al., 2015b). Sodium alginate (Fine food & pharmaceutical grade, lot number: IL-1-150, viscosity 20-50 mPas for a 1% solution at 20 °C) was purchased from Kimica Corporation (Chuo-Ku, Tokyo, Japan). Curcumin (82% curcumin, 15% demethoxycurcumin (D-Cur) and 3% bisdemethoxy-curcumin (BD-Cur), Mw 361.05) was a gift from Sabinsa Corporation (Piscataway, NJ, USA) and used without further purification. Pure Wesson vegetable oil (ConAgra Foods. Inc., USA) was purchased from a local market and used without further purification. Glacial acetic acid, analytical grade HCl and NaOH were purchased from Alfa Aesar (Ward Hill, MA). Calcium chloride dehydrate (CaCl₂·2H₂O), sodium chloride, bile salts, Pepsin from porcine gastric mucosa (P7125, ≥400 units/mg protein), pancreatin with 8

× USP specification, Tris maleate iron (II) chloride, ammonium thiocyanate, cumene hydroperoxide, 2-thiobarbituric acid, 1,1,3,3-tetraethoxypropane were purchased from Sigma-Aldorich (St. Louis, MO, USA). Sodium taurodeoxycholate (Na TDC) was purchased from CalBiochem (La Jolla, CA). Water purified by Milli-Q system was used for sample preparation.

2.2. Pickering emulsion preparation

Curcumin encapsulated KPE were formulated as described in our previous paper (Xiao, et al., 2015a). Briefly, curcumin was dissolved in vegetable oil to the final concentration of 3 mg/mL. Kafirin protein was first dissolved in acetic acid and then added dropwise into bulk water to form nanoparticles suspension, dialysis was then conducted in Spectra/Por® 7 RC dialysis tubes (MWCO: 10 kDa) to remove excess acetic acid. Pickering emulsions (total volume 6 mL) were prepared by homogenizing 3 mL vegetable oil (containing 3 mg/mL curcumin) with 3 mL kafirin particle suspension (1% w/v) in a glass vial (1.3 cm in internal diameter and 6 cm in length) under high-speed homogenizer unit (model IKA-ULTRATURRAX T25 digital, IKA 190 Works, Inc., Wilmington, NC, USA) with an 10 mm dispersion probe at 13, 000 rpm for 3 min. The as-prepared Pickering emulsions showed a wide size distribution range (10 – 120 µm), and the volume averaged size (D_{43}) was around 45 µm.

2.3. Preparation of Pickering emulsion encapsulated alginate hydrogel

Sodium alginate (1.5 wt%) was dissolved with DI water at 40 °C and then mixed for 4 h under stirring. Subsequently KPE and the sodium alginate solution were mixed at volume ratio of (1:1, 1:2, 1:4, 1:8) under stirring for 5 min. Ultrasonication (FS- 28 solid-state ultrasonicator with sonic power, 225 W; sweep frequency, 40 kHz, Fisher Scientific, Pittsburgh, PA, USA) was applied for 1 min to disrupt any flocculated droplets formed during the mixing process. The mixture was injected manually by a 1 mL pipette tip into bulk CaCl_2 solution (1 wt%) in a fast

and continuous manner, the tip was immersed in bulk CaCl_2 solution when injection took place. The hydrogels were allowed for hardening in CaCl_2 solution for another 30 min before further experiment. The obtained hydrogels were named as EG11, EG12, EG14, EG18, respectively. Sodium alginate based hydrogel without KPE encapsulation was prepared parallel as a control and abbreviated as AG. Experiments were performed in triplicate for each formulation.

2.4. Optical microscopy observation

Optical microscopy observation of emulsions was visualized using a Nikon Eclipse TE 2000-U with a Q-Imaging camera. Emulsions or emulsion hydrogels were deposited onto a concave glass slide and covered by a coverslip before imaging at 100 magnification under bright and fluorescence fields. The droplet size of emulsion was analyzed via Image J2x 2.1.4.7 by measuring a minimum of 50 droplets for each sample. The volume averaged emulsion droplet size (D_{43}) was calculated by equation: $D_{43} = \frac{\sum_1^n D_i^4}{\sum_1^n D_i^3}$.

2.5. USAXS/SAXS measurement

USAXS measurements were carried out at sector 9 ID-C at the Advanced Photon Source (APS), Argonne National Laboratory. Beam energy was 18 keV and the USAXS instrument can be used to collect scattering data over a q range as low as $0.0001 \text{ \AA}^{-1}$ with q resolution of $0.00008 \text{ \AA}^{-1}$. To accommodate the need for high- q scattering measurements of samples that scatter X-rays weakly, a pinhole camera (PILATUS 100K) covering the q range from $0.05 \text{ \AA}^{-1}$ to $1.2 \text{ \AA}^{-1}$ was in conjunction with the USAXS range. The intensity curves obtained in both ranges were combined by merging their overlapping regions. The combination allowed characterization of samples with hierarchical structure and microstructures at multiple length scales (Ilavsky, et al., 2013). Data reduction, de-smearing and modeling were performed within the Nika 2D and Irena 2 packages (Ilavsky, 2012; Ilavsky & Jemian, 2009) for data evaluation and to get modeling macros for Igor

Pro (Wavemetrics, Lake Oswego, OR). In this way intensity data $I(q)$, where $q = 4\pi \sin(\theta/2)/\lambda$, with θ the scattering angle, and λ the wavelength of the radiation, were obtained. During experiment, samples were transferred to silicone insulators (JTR8R-A2-1.0, lot #666105, purchased from Grace Bio Labs) combined with two microscope cover slides to create single use container with a 1 mm X-ray beam path length.

2.6. Structural stability of EGs under harsh processing conditions

After the hardening process, one set of EGs was subjected to storage at 60°C for 4h. Another set of EGs was incubated in pH 8.5 solution and stored at room temperature for 7 days. At the end of each treatment, samples were centrifuged at 5000 rpm for 10 min and filtered through a Whatman® Grade-2 filter paper. The released oil phase was rinsed with 100% ethanol, and absorbance of ethanolic extract after proper dilution was measured at 425 nm using a microplate reader (BioTek Instruments, Winooski, VT) in conjunction with KC4 data reduction software. The measured absorbance was converted to curcumin concentration using a standard curve prepared by measuring the absorbance of a series of known concentrations of curcumin in ethanol. The release percentage of curcumin was calculated by dividing the curcumin content in the ethanolic extract over curcumin encapsulated within the primary emulsion. Changes in structure of EGs at the end of treatment were also recorded by microscopy observation.

2.7. Simulated gastric digestion

One liter of simulated gastric fluid (SGF) was prepared by dissolving 5 g sodium chloride and adjusting pH to 1.5 via 5 M HCl. One gram of emulsion gel sample or KPE sample was mixed with 9 mL SGF and incubated at 37 °C under magnetic stirring (230 rpm) for 10 min. 32 mg pepsin was dissolved in 1 mL SGF and added to the mixture to start the digestion process. Mechanical stirring was applied throughout the digestion process. Digestions were stopped after

2 hours' incubation by raising the pH to 7.5 to inactive the pepsin. At the end of gastric digestion, the percentage of released curcumin and changes in emulsion structure were recorded as described in preceding section.

2.8. Release of free fatty acid and bioaccessibility of lipid encapsulate

The digestion profile in stimulated small intestine fluid (SIF) was measured by determining the amount of free fatty acids (FFA) released from emulsion gel samples using a pH titration method (Xiao et al., 2015a). Briefly, 1 g pancreatin was mixed with 5 mL fed-state buffer (50 mM Tris maleate, 150 mM NaCl, 5mM CaCl₂, 20 mM NaTDC, 5 mM Phosphatidylcholine), centrifuged and kept on ice. Emulsion gel samples (containing 0.5 mL KPE) or KPE (0.5 mL) was mixed with 10 mL fed state buffer under a water bath at 37.0 °C for 10min and then adjusted to pH = 7.5 using 1M NaOH. One mL pancreatin preparation was added to start the lipolysis. During lipolysis, the pH was maintained at 7.50 ± 0.02 by direct titration with 0.25 N NaOH manually. The volume of consumed NaOH over time was recorded throughout the lipolysis experiments, and was used to calculate the concentration of free fatty acids generated by lipolysis. A blank assay was carried out with AG in the absence of lipid phase, the titration value was subtracted from the reported values. The extent of lipolysis was defined as the percentage of triglycerides digested in the *in vitro* lipolysis experiments. It was assumed that digestion of one mole of triglycerides released exactly two moles of free fatty acids (FFA) and consumed two moles of NaOH. Consequently, FFA can be calculated as:

$$\%FFA = 100 \times \frac{V_{NaOH} \times m_{NaOH} \times M_{lipid}}{w_{lipid} \times 2} \quad (1)$$

Here V_{NaOH} is the volume of sodium hydroxide required to neutralize the FFA produced (L), m_{NaOH} is the molarity of the sodium hydroxide solution used (in M), w_{lipid} is the total mass of

triacylglycerol oil initially present in the digestion cell (in g), and M_{lipid} is the molecular mass of the triacylglycerol oil (in g/mol).

Curcumin bioaccessibility was determined based on our previously reported methodology (Xiao, et al., 2015a). Aqueous mixture after SIF digestion was collected at centrifuged (Type 60 Ti rotor, 180,000 g, Beckman Coulter) at 40,000 rpm for 40 min, the middle micelle layer was collected and analyzed for curcumin content. The bioaccessibility of curcumin was calculated as:

$$\%Bioaccessibility = (C_{aqueous} \times V_{aqueous})/w_{lipid} (2)$$

Where the numerator and denominator were mass of solubilized curcumin and mass of curcumin in lipid phase. Microstructure changes at the end of digestion process were observed under optical microscopy observation.

2.9. Statistical analysis

All the data obtained were averaged values of triplicate determinations and subjected to statistical analysis of variance (ANOVA) using Origin 9.0. Least significant differences ($p < 0.05$ or $p < 0.001$) were accepted among the treatments.

3. Results and discussion

3.1. Formation and characterization of EGs

Our previous work suggested that kafirin nanoparticles spread at emulsion droplet interface in a loose manner with a low surface coverage rate (Xiao et al., 2016), which was responsible for its instability under harsh processing conditions. In this study, we intended to improve its stability by introducing surface deposition of polymer and followed by immobilization within a hydrogel network. Right after mixing KPE with different volume ratios of sodium alginate solution, the liquid mixtures were observed under optical microstructure (Fig.1a). No significant change in volume averaged emulsion droplet size (D_{43}) occurred after mixing, and there was no

significant difference in D_{43} among different mixtures, according to statistic analysis via Image J (Data not show). This confirmed in the first place that the initial solvent mixing process did not jeopardize the stabilization of KPE.

Alginate based hydrogels were commonly formed into spherical beads in previous literatures, and they were usually fabricated through a syringe needle with a slow dripping rate. Herein, we employed a much time efficient way of operation by continuously dripping the liquid mixture via a 1 mL pipette tip into the bulk liquid phase containing Ca^{2+} ion. The injected liquid mixture solidified through immediate contact with surrounding Ca^{2+} ions. Due to the continuous dripping manner, instead of forming spherical beads, the resultant emulsion gels (EGs) held a cylinder shape (Fig.1b). Their diameters fell within a narrow range of 0.8 to 1.2 mm under a wide selection of injection speeds and compositions of primary mixtures. With regard to microstructures of the as-prepared EGs, emulsion droplets, indicating by the green fluorescent spheres containing curcumin, were immobilized within the alginate hydrogel matrix in a highly concentrated manner (Fig. 1c). The droplet size of embedded emulsion droplets remained almost the same after room temperature storage for over 14 days, demonstrating good stability against coalescence. Compared to the hydrogel beads formation process, this protocol was more time and cost efficient, and it is good enough to achieve our ultimate goal of improving the storage and digestion performance of Pickering emulsion, which we will discuss in later sections.

3.2. Structure and interactions within EGs revealed by USAXS/SAXS

To unveil the structural response of alginate hydrogel to the encapsulation of KPE and changes in emulsion interface after immobilization, we utilized combined USAXS and SAXS techniques. In Fig. 2, USAXS/SAXS spectra for EGs are plotted in a double logarithmic scale. The scattering by fractal-like structure is described in USAXS/SAXS curves by $\log I = c + b$

log Q , the slopes (b) of straight-line segments in different q ranges determine the: mass fractal dimension (d_m) (when $|b| < 3$, $d_m = |b|$) or surface fractal dimension (d_s) (when $3 < |b| < 4$, $d_s = 6 - |b|$) (Grigoriev, Luboradzki, & Gronkowski, 2006; Sorensen, Oh, Schmidt, & Rieker, 1998).

Scattering intensity in high Q range ($0.02 \text{ \AA}^{-1}$ to $1 \text{ \AA}^{-1}$) for all the hydrogel samples was originated from primary “egg-box” junctions and their lateral condensations along the alginate chains (Donati & Paoletti, 2009; George & Abraham, 2006; Stokke, et al., 2000). In the central Q domain ($0.001 \text{ \AA}^{-1}$ - $0.02 \text{ \AA}^{-1}$), d_m of AG shifted from 1.80 to 1.42 as Q decreased, reflecting the alginate chain conformation transformed from rod-like to plate-like conformation as the hydrogel network extended. The critical shoulder peak position in this range (q_m') corresponded to a correlation length of 21.6 nm in size, calculated by Bragg’s law through $2\pi/q_m = d_m$ (q_m is the critical position of the shoulder peak) (Sorensen, et al., 1998), probably the length of crosslinking inhomogeneity in swollen hydrogels (Tirumala, Ilavsky, & Ilavsky, 2006) or average network mesh size ξ (Grassi, et al., 2009). When incorporating KPE into the alginate hydrogel matrix, EGs exhibited intermediate d_m values ranging from 1.68 to 1.46. Meanwhile, the correlation length at q_m' decreased from 21.6 nm to 19.0 nm when the volume fraction of KPE increased up to 1:2 or 1:1. A reasonable explanation for this phenomenon is: the crowding effect as well as the decrease in electrostatic repulsion among alginate chains in the presence of KPE induced an increase in local concentration of Ca^{2+} and local aggregation among alginate polymer chains, thus leading to shrinkage in alginate hydrogel network.

At ultra small Q region ($10^{-4} \text{ \AA}^{-1}$ - $10^{-3} \text{ \AA}^{-1}$), AG presented a slope of -1.41, which corresponding to a d_m of 1.41, indicating an oblate like or rod like morphology. The curve reached a plateau at ultra small Q region ($0.0001 \text{ \AA}^{-1}$ - $0.0003 \text{ \AA}^{-1}$), due to the finite size ξ of the

mass-fractal domain (1142 nm) (Perissinotto, Awano, Donatti, de Vicente, & Vollet, 2015). This value corresponded well with the dimension of cage-like interconnected alginate hydrogel pores (around 1 μm in size). Contrary to AG, EGs exhibited excess power law scattering at low Q region in USAXS, the upturn of intensity profile at $Q < 0.0005 \text{ \AA}^{-1}$ confirmed the presence of large emulsion droplets (larger than 6.28 μm) in hydrogel matrix. The slopes of EGs fell into the range of 3-4, indicating scattering in this range corresponding to the surface structure at the emulsion droplet interface. The d_s value of EG11 equaled 2.44, indicating a relatively smooth surface (Grigoriev, Temeriusz, Chmielewska, Gronkowski, & Mirkowska, 2007). As the volume ratio of sodium alginate to KPE increased, d_s progressively increased (with EG12: $d_s = 2.53$; EG14: $d_s = 2.77$; EG18: $d_s = 2.84$), indicating a trend of increase in interface roughness. This phenomenon further confirmed the existence of sodium alginate deposition at emulsion droplet interface driven by short-range electrostatic attraction. Higher volume ratio of sodium alginate during gel formation process lead to higher interface aggregation density, thus a rougher interface.

3.3. Structural stability against processing conditions

In our preceding researches, the physicochemical stability of KPE during storage conditions was investigated (Xiao et al., 2015a). Our results suggested that KPE was less stable in alkaline conditions than in acid or neutral conditions, one weeks' storage in pH 8.5 buffer induced 44.1% oil release, and 4 hours' storage in 60 $^{\circ}\text{C}$ environment induced over 50% oil release. Herein, we evaluated the resistance of EGs towards these harsh processing conditions by monitoring the microstructure of KPE within hydrogel matrix and the release percentage of encapsulated curcumin.

As shown in Fig.3a, regardless of the volume fraction of KPE, alkaline incubation induced

increase in emulsion droplet size. And this trend was also observed after 4 hours' high temperature storage (Fig. 3b). To quantitatively analyze their influence on the emulsion microstructure, emulsion droplet sizes right after preparation were compared with those after each treatment. Image J particle analysis function was applied to analyze the droplet size in each digital image (at least three locations were recorded for each formulation), to minimize the inaccuracy caused by out of focus effect, only the particles in focus was calculated in each image slice. The mean volume diameter (D_{43}), which is sensitive to the existence of large droplet size, was then calculated based on at least 30 droplets for each formulation. As presented in Fig.3c, D_{43} increased in all formulations after both alkaline and heating incubation. Among which the most remarkable change occurred in the EG11 formulation, the emulsion droplet size almost doubled after heating incubation.

As discussed in our previous paper (Xiao, et al., 2015a), alkaline solution detrimentally changed the contact angle of kafirin nanoparticle layer and high temperature overweighed the resistance of Pickering emulsion towards coalescence. The alginate based hydrogel matrix are generally considered as highly permeable: its pore size would allow the free diffusion of water soluble components with molecular weight as high as 300 kDa (Pothakamury & BarbosaCanovas, 1995; Tanaka, Matsumura, & Veliky, 1984). Thus, kafirin particle layer was still easily subjected to detrimental alkaline solution and emulsion droplets suffered from heating solution. Although, coalescence was observed in as-prepared EGs, the coalescence rate was largely inhibited as compared to free KPE, no phase separation was observed. At the end of treatments, the content of released curcumin was negligible in all formulations. Apparently, increase in bulk viscosity, the confinement of emulsion droplets and structure integrity of alginate hydrogel network under harsh processing conditions contributed to this significant improvement. In addition, we suspect

that the deposition of alginate upon the kafirin nanoparticle stabilized interface may also play a role in enhancing the emulsion stability. If this was the case, alginate played a dual role of network forming component and stabilizer for the nonpolar phase.

3.4. Digestion in SGF

The digestion profile of emulsion hydrogels and adsorption fate of encapsulated nutraceutical in gastrointestinal (GI) tract are critical criteria in assessing formulation's potential as oral delivery system. In this study, we simulated the digestion behavior of curcumin encapsulated EGs after oral intake via *in vitro* simulated gastric and intestinal digestion. As a comparison, free KPE was subjected to the same digestion process.

As shown in Fig.4a, free KPE underwent complete phase separation at the end of gastric digestion, and 89.0% of curcumin previously encapsulated within KPE were recovered in the released oil phase. Obviously, bare kafirin nanoparticle layer could not survive through proteolysis by pepsin. As for KPE immobilized within hydrogels, the penetration of pepsin into hydrogel matrix was inevitable, but sodium alginate was reported to inhibit pepsin activity *in vitro* by up to 53.9% (Chater, Wilcox, Brownlee, & Pearson, 2015; Sunderland, Dettmar, & Pearson, 2000; El Aouad, et al., 2011). Therefore, limited degree of pepsin digestion and thus less severe emulsion coalescence as compared to free KPE were expected in EGs. As seen in Fig.4a, instead of undergoing severe phase separation, EGs were sheared into smaller fragments induced by mechanical stirring during gastric digestion and less than 10% curcumin were released from EGs. Microstructures of the corresponding hydrogel fragments were presented in Fig. 4b. All EGs exhibited more severe coalescence than those subjected to either 7 days' alkaline incubation or 4 hours' heating treatment as suggested by the enlargement in emulsion droplets. The bridging effect among adjacent emulsion droplets in EG18 (highlighted in red

circle in Fig. 4b) confirmed the ongoing process of pepsin digestion induced coalescence. In formulation with the least volume ratio of sodium alginate, EG11, loss in rigid hydrogel boundary was also observed, which explained its highest curcumin release percentages among all EGs. This further confirmed the critical role of alginate polymer in improving the resistance of EGs towards pepsin digestion.

3.5. Digestion in SIF

Alginate was reported to have no significant inhibitory effect on pancreatin (Chater, et al., 2015), and the majority of alginate gel network would disintegrate during intestinal digestion (Rayment, et al., 2009) via replacing the calcium ions by chelating agents or sodium ions (Bhagat, Mendes, Mathiowitz, & Bhargava, 1994). Hence the main effect of alginate hydrogel during simulated intestinal digestion would be the retard in penetration of enzyme or bile salts onto the emulsion interface and the diffusion of free fatty acid out of the matrix. As suggested by the FFA release profile of both KPE and EGs in Fig. 5a, the ultimate FFA release rate has no significant differences among all formulations, expect that the release profiles of EGs were in a sustained release manner as compare to a burst release manner of KPE. When fitting the release profile to a Korsmeyer-Peppas model, the exponent index of KPE equaled 0.15, indicating a Fickian diffusion dominant release behavior, while the exponent indexes of EGs fell into the range of 0.39-0.47, suggesting a combined effects of diffusion and erosion.

Along with the free fatty acid release process, lipid phase were able to generate mixed micelles, through which the originally dissolved curcumin will be solubilized in the micelle core and thus become bioaccessible. The ultimate FFA release rates as well as the corresponding bioaccessibility of curcumin in the lipid phase were then presented in Fig.5b and liner correlations between these two sets of data and composition of EGs were performed in Fig.5c.

Compared with KPE, EGs, except for EG18, showed enhanced bioaccessibility. No strong correlation existed between the composition of EGs and the ultimate FFA values, while a negative correlation was found between the volume fraction of sodium alginate in EGs and the bioaccessibility of curcumin.

This phenomenon raised the possibility that although the majority of alginate hydrogel lost structural integrity in simulated intestinal fluid, there were some insoluble alginate hydrogel pellets left after digestion, which adsorbed and prevented free curcumin from solubilizing by micelles. Representative microstructures of EGs at the end of SIF digestion proved the existence of curcumin entrapped alginate pellets, which were irregular pellets showing green fluorescence in Fig.5d. This phenomena was more significant in case of EG18, instead of entering into the soluble micelles, large quantity of curcumin was resided in the gel residues, which explained the declined bioaccessibility of curcumin. This finding reminded us that a balance point between enhanced processing stability and better nutraceutical delivery efficiency should be identified during the formulation process of the Pickering emulsion hydrogel systems.

4. Conclusions

In this study, Pickering emulsion hydrogel were fabricated through immobilizing kafirin nanoparticle stabilized Pickering emulsion within calcium cross-linked sodium alginate hydrogel matrix. The encapsulation of emulsion droplets within hydrogel matrix lead to the shrinkage in hydrogel network mesh size and deposition of sodium alginate at emulsion droplet interface. Compared to KPE, coalescence among emulsion droplets under alkaline or heating incubation was largely inhibited. Although coalescence due to the digestion of pepsin to kafirin protein was still detected in EGs, the release in lipid phase was largely diminished as compared to that of KPE. The majority of EGs were kept intact until the complete digestion in simulated intestinal

fluid. The volume fraction of hydrogel matrix did not affect the ultimate FFA release rate. A negative correlation between the volume fraction of sodium alginate and the bioaccessibility of curcumin was detected due to the retention of free curcumin onto alginate hydrogel residues. Overall, the formulated EGs offered an effective solution for utilizing edible particles based Pickering emulsions as potential oral delivery vehicles with ease of preparation, improved processing stability and controlled digestion profile.

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Figure captions

Fig. 1. (a) Optical microscopy images of mixture of KPE and sodium alginate solution with different volume ratios (1:1, 1:2, 1:4, 1:8); (b) Digital pictures of EGs and AG after dripped into CaCl_2 solution; (c) Microstructure of EGs under bright and fluorescence fields of optical microscopy, AG without encapsulation of emulsion droplets were included as a control. Scale bar (red rectangular) = 100 μm

Fig.2. (a) Intensity profile of USAXS/SAXS spectra for EGs and AG. The curves are separated by a shift of $\log(I)=1$ for clarity.

Fig.3. Representative optical microscopy images of EGs after (a) alkaline and (b) heating treatment under bright and fluorescence fields; (c) Quantitative analysis of D_{43} before and after each treatment. Scale bar (red rectangular) = 100 μm

Fig.4. (a) Release percentage of curcumin after SGF incubation, inserted are digital photos of KPE and EGs before and after SGF digestion; (b) Optical microscopy of EGs after digestion under bright and fluorescence fields, red circle highlighted the ongoing bridging effect of emulsion droplet. * to *** represent significant difference between samples at level of $P < 0.05$ and $P < 0.001$. Scale bar (red rectangular) = 100 μm

Fig.5. (a) Free fatty acid release profiles of KPE and EGs; (b) Ultimate FFA values and curcumin bioaccessibility of KPE and EGs; (c) Correlations between EG composition and FFA or Bioaccessibility, lines of best fit were also presented; (d) Digital images of EGs after SIF digestion, enlarged are representative images of digestion end products. Scale bar (red rectangular) = 100 μm

Figure.1

Figure.2

Figure.3

Figure.4

Figure.5