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Lactoferrin particles assembled via transglutaminase-induced crosslinking: Utilization in oleogel-based Pickering emulsions with improved curcumin bioaccessibility

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ABSTRACT

In this study, the optimal environmental condition for preparation of lactoferrin particles assembled via transglutaminase-induced crosslinking (TG-LF particles) was pH 8, 100 U/g of TG concentration, 50 °C and 2 h of crosslinking time. Contact angle of TG-LF particles was 79°. Then, corn oil-based oleogels were prepared with carnauba wax (CW), behenyl alcohol (BA) and CW-BA mixture at 1:4 ratio (MT). To investigate the effect of oleogels on oleogel-based Pickering emulsions, oleogel-based Pickering emulsions were prepared by a two-step method using different oleogels as the oil phase and the TG-LF particles as the emulsifier. In vitro digestion study revealed that CW oleogel-based Pickering emulsion had the highest lipolysis rate and curcumin bioaccessibility. This study demonstrated that TG-LF particle-stabilized oleogel-based Pickering emulsions had good performance in curcumin delivery, which provided a new idea for the preparation of Pickering emulsifier and enriched the knowledge in the field of oleogel-based Pickering emulsion.

1. Introduction

Oils and fats are important ingredients in a range of popular food products such as ice cream, sauces, margarines and shortening (Rios, Pessanha, Almeida, Viana, & Lannes, 2014). High fat intake can lead to a series of chronic diseases including cancer, obesity, cardiovascular diseases and diabetes (Ahmadi, Tabibiazar, Roufegarinejad, & Babazadeh, 2020). Oleogels, which are formed by sealing liquid oil with a three-dimensional network structure of gelators (Chaves, Barrera-Arellano, & Ribeiro, 2018), have been explored as alternatives to saturated fats and hydrogenated vegetable oils in the aforementioned foods to reduce health risks (Qi, Li, Zhang, & Wu, 2021; Zhao, Wei, & Xue, 2021). Oleogels can improve the nutritional value of food by maintaining the structure of food for a long time (Baraki et al., 2021).

In food science, emulsion is a common nutrient delivery system. Oleogel-based Pickering emulsions, which were first designed by Wei and Huang (Wei & Huang, 2019a), are a novel emulsion delivery system. Up to now, most studies on oleogel-in-water Pickering emulsions are based on comparison with oil-in-water Pickering emulsions (Qi, Zhang, & Wu, 2020; Wei & Huang, 2019a). Nevertheless, there are relatively few studies devoted to investigating the effect of different oleogels fabricated by various gelators on oleogel-based Pickering emulsions. In this study, three kinds of gelators (carnauba wax, behenyl alcohol and carnauba wax-behenyl alcohol mixture) were chosen to prepare oleogels, followed by the fabrication of oleogel-based Pickering emulsions. Carnauba wax (CW) and behenyl alcohol (BA) are common gelators. Different gelators have distinctive effects on the oleogels (Chaves, Barrera-Arellano, & Ribeiro, 2018) and further affect the oleogel-based Pickering emulsion.

Considering that proteins are excellent emulsifiers for Pickering emulsions, proteins have potential to stabilize oleogel-based Pickering emulsions (Wei, Cheng, & Huang, 2019). Lactoferrin (LF), which is widely distributed in many mammals, is a single-chain glycoprotein (Yuliarti, Lau, Wee, & Kwan, 2019). Since LF has many biological functions such as antimicrobial, scavenging harmful free radicals and regulating cell growth (Wang, Timilsena, Blanch, & Adhikari, 2019), it has been widely applied in food industry. Therefore, LF particles could be potentially selected as Pickering emulsifier. To fabricate LF particles, protein–protein crosslinking technique is adopted. Transglutaminase (TG), which is a transferase enzyme that catalyses the deamidation and

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crosslinking reactions between proteins, is used as crosslinking agent that catalyzes the formation of and ε-(γ-glutamyl) lysine isopeptide bridge between glutamine (Gln) and lysine (Lys) of LF in this study (Escamilla-García et al., 2019; Keillor, Clouthier, Apperley, Akbar, & Mulani, 2014; Ruzengwe, Amonsou, & Kudanga, 2020). Besides, TG is a safe substance that can be used in food (Romeih & Walker, 2017).

Curcumin is a low molecular weight polyphenol extracted from *Curcuma longa* L (Miskeen, An, & Kim, 2021). Curcumin is a powerful antioxidant agent that can treat many kinds of chronic diseases, which is good for human health, thereby promoting scientific interest in using it as a nutraceutical (Aliabbasi, Fathi, & Emam-Djomeh, 2021). However, the poor water solubility and lack of stability of curcumin make curcumin hard to be directly applied in food. At the same time, poor absorption and low bioaccessibility of curcumin endow curcumin a good research target for bioaccessibility enhancement (Wei, Zhu, Cheng, & Huang, 2019). Multiple tools have been developed to deliver curcumin to improve its stability and bioavailability, emulsion-based systems have been proven to be an effective tool (Wei, Zhang, & Huang, 2019). Based on these backgrounds, curcumin was selected to study nutraceutical delivery of TG-LF particle-stabilized oleogel-based Pickering emulsions.

This study started with exploration of the optimum reaction conditions of lactoferrin particles assembled via transglutaminase-induced crosslinking (TG-LF particles) through the measurement of particle size and PdI (Polymer dispersity index). The free amino group and contact angle of the optimized TG-LF particles were measured. Then, oleogels were prepared by structuring corn oil with carnauba wax and behenyl alcohols. Afterwards, three kinds of oleogel-based Pickering emulsions stabilized by TG-LF particles were prepared and characterized by droplet size and rheology. Finally, *in vitro* lipolysis and curcumin bioaccessibility in oleogel-based Pickering emulsions were examined to understand performance of these emulsions in delivering nutraceuticals. The present work may provide new ideas for nutraceutical bioaccessibility of oleogel-based Pickering emulsions.

2. Materials and methods

2.1. Materials

Carnauba wax and behenyl alcohol (1-docosanol) were purchased from Aladdin Industrial Corporation (Shanghai, China) and Shanghai Macklin Biochemical Co., Ltd (China), respectively. Curcumin was provided by Shanghai Ryon Biological Technology CO., Ltd. Corn oil was bought from a local market. Lactoferrin was purchased from Hilmar Ingredients (America). Transglutaminase (5000 U/g) was bought from Taixing Dongsheng Biotechnology Co., Ltd (China). Pepsin, pig bile salt, pancreatin and Tris were purchased from Beijing Solarbio Science & Technology Co., Ltd (China). All other chemicals were bought from Sinopharm Chemical Reagent Co., Ltd (China), unless otherwise stated.

2.2. Zeta potential of lactoferrin

Zeta potential of lactoferrin (LF) solutions (1 mg/mL) within the pH range 5–10 was measured by Nano ZS90 Zetasizer (Malvern Instruments Ltd., U.K.) with Zetasizer software v7.13 (Malvern Instruments).

2.3. Preparation of lactoferrin particles assembled via transglutaminase-induced crosslinking (TG-LF particles)

2.3.1. Effects of pHs on crosslinking process

LF (50 mg/mL) was added and dissolved in pH-adjusted Milli-Q water (pH 5, pH 6, pH 7, pH 8). Then, 4 mg of transglutaminase (TG) was added to the solutions to make the enzyme activity reach 40 U/g (''g'' refers to the content of LF in the solution, and the same mentioned later). The mixture was heated at 50 °C and stirred constantly for 2 h. Afterwards, the mixture was heated at 90 °C for 10 min to inactivate the TG. Analysis of particle size and PdI was used to judge the crosslinking condition.

2.3.2. Effects of TG concentrations on crosslinking process

Five hundred mg of LF was added and dissolved in 10 mL of Milli-Q water (pH 8). Then, different weight of TG was added to the solutions to achieve different enzyme activity (10 U/g, 20 U/g, 30 U/g, 40 U/g, 50 U/g, 60 U/g, 70 U/g, 80 U/g, 90 U/g, 100 U/g, 110 U/g). The mixture was heated at 50 °C and stirred constantly for 2 h. Afterwards, the mixture was heated at 90 °C for 10 min to inactivate the TG. Analysis of particle size and PdI was used to judge the crosslinking condition.

2.3.3. Effects of temperatures and reaction time on crosslinking process

LF (500 mg) was added and dissolved in 10 mL of pH 8 Milli-Q water. Then, 10 mg TG was added to the solutions to make the enzyme activity reach 100 U/g. The mixture was heated at the temperature of 40 °C, 45 °C, 50 °C, 55 °C, 60 °C and stirred constantly for a period of time (0.5 h, 1.0 h, 1.5 h, 2.0 h, 2.5 h). Afterwards, the mixture was heated at 90 °C for 10 min to inactivate the TG enzyme. TG-LF in the rest of this study was prepared by heating LF and TG mixture solutions (pH 8, 100 U/g, 2 h) at 50 °C with a constant stirring. Analysis of particle size and PdI was used to judge the crosslinking condition.

2.4. Characterization of LF and TG-LF particles

2.4.1. Particle size and PdI

Nano ZS90 Zetasizer (Malvern Instruments Ltd., U.K.) with Zetasizer software v7.13 (Malvern Instruments) was used to measure the particle size of TG-LF particles. First, the pH of each sample was adjusted to 3. Then, each sample was diluted 50 times to keep the concentration of TG-LF particles at about 1 mg/mL, which was the best detection concentration of the samples.

2.4.2. Free amino groups

An OPA (o-phthaldialdehyde) method was used to measure the free amino groups of LF and TG-LF particles (Wei, Cheng, Zhu, & Huang, 2019). The preparation method of OPA reagent was as follows. Firstly, OPA (40 mg) was dissolved in methanol (1 mL). Then, 100 μL of 2-mercaptoethanol, 20% (w/w) sodium dodecyl sulfate (2.5 mL), 21.5 mL of water and 25 mL of 0.1 M sodium tetraborate solution were added into the solution. Afterwards, 200 μL of 2 mg/mL LF or TG-LF was mixed with OPA reagent (4 mL). The mixture was heated at 37 °C for 2 min, followed by measurement of the absorbance at 340 nm via the UV–Vis spectrophotometer (UV2355, UNICO). The content of free amino group was calculated by L-leucine standard curve.

2.4.3. Contact angle

10 mg/mL of LF dispersion or TG-LF particle dispersion was prepared and it was dropped evenly on the glass plate. Then it was dried in an oven to form a LF or TG-LF particle film on the glass plate. With the aid of OCA (Optical contact angle) measuring system (DataPhysics Instruments GmbH, Germany), 2 μL water droplet was dropped onto the LF or TG-LF particle film to measure contact angle (Wei & Huang, 2019b).

2.4.4. Atomic force microscopy (AFM)

The morphology of TG-LF particles was investigated using the CSPM5500 AFM system (BenYuan, Guangzhou, China). TG-LF particles were first diluted to a concentration of 0.002% (w/w) with Milli-Q water, and the 10 μL sample was placed on the surface of freshly cleaved mica for 5 min, dried with nitrogen gas, and mounted on the scanner tube. Tapping mode AFM imaging was carried out with CSPM Imager software. The scanning range was 10 μm × 10 μm and the scanning frequency was 2 Hz.

2.5. Preparation of oleogels

Carnauba wax (CW), behenyl alcohol (BA) and CW-BA mixture at 1:4 ratio (MT) were selected as gelators. Afterwards, 5% (w/w) of the gelators were added into corn oil. The system was heated at 90 °C with stirring until the gelators were completely dissolved. Then, the samples were cooled at room temperature. Typical tube inversion method was used to judge whether the oleogel was formed (Chung, Simmons, Gutowska, & Jeong, 2002). After the oleogel was formed, the oleogels were stored at room temperature.

2.6. Preparation of TG-LF particle-stabilized oleogel-based Pickering emulsions

Before the preparation process, the pH of TG-LF particle dispersions was adjusted to 3. Then, TG-LF particle dispersions (50 mg/mL) were mixed with oleogels (CW oleogel, BA oleogel, MT oleogel) at the ratio of 1:1 (v/w). The mixtures were sonicated by an intelligent ultrasonic processor (SM-900A, Nanjing Shunma Instrument Equipment Co., Ltd, Nanjing, China) at a low frequency of 20 kHz for 3 min in a pulse mode (4 s on and 6 s off). Afterwards, the mixtures were homogenized at 16,050 × g for 3 min with the aid of Ultra-Turrax T25 (IKA-Werke GMBH & CO., Germany). The obtained emulsion samples were kept at room temperature.

Curcumin-loaded oleogel-based Pickering emulsions were prepared by dissolving curcumin (1 mg/g) in corn oil first. Then the curcumin-load oleogels were prepared according to the method in 2.2. Finally, curcumin-loaded oleogel-based Pickering emulsions were prepared according to the method of this section.

2.7. Characterization of TG-LF particle-stabilized oleogel-based Pickering emulsions

2.7.1. Emulsion type

The type of TG-LF particle-stabilized oleogel-based Pickering emulsion was determined by measuring its dispersibility. The emulsion was dripped into water and corn oil, respectively. Oil-in-water emulsions had good dispersibility in water but stayed immiscible in corn oil. The water-in-oil emulsion could be dispersed in oil but not dispersed in water (Wei, Cheng, & Huang, 2019).

2.7.2. Droplet size of Pickering emulsions

Droplet size of oleogel-based Pickering emulsion was measured by Intelligent laser particle sizer (Bettersize2600, Dandong Bettersize Instruments Ltd., China). The Pickering emulsion was dropped into the sample cell until the refractive reached 10%. The droplet size was measured under this condition.

2.7.3. Microstructure of Pickering emulsions

To further verify adsorption of TG-LF particles onto oil–water interfaces, fluorescence microscopy of TG-LF particle-stabilized oleogel-based Pickering emulsion was performed with a NIKON/Ni-E fluorescent microscope (Nikon, Japan). Emulsion samples were diluted 5 times with Milli-Q water at corresponding pHs and stained with rhodamine B as well as Nile red.

2.7.4. Rheological measurements of Pickering emulsions

A stress-controlled rheometer (Anton Paar Physica MCR 301, Graz, Austria), which was equipped with a Peltier plate temperature controller (Julabo, Seelbach, Germany), was used to detect the rheological properties of Pickering emulsions. All tests were carried out with a 50 mm conical plate (CP50) at a constant temperature of 25 °C, and the measuring gap was set at 0.75 mm. Stress sweep measurement was performed at 1 Hz frequency in the range of 0.01%-100% to determine the linear viscoelastic region. Frequency sweep was performed at a constant low stress level in the linear viscoelastic region (0.5%), and the frequency range was from 1 Hz to 20 Hz.

2.8. In vitro gastrointestinal digestion of TG-LF particle-stabilized oleogel-based Pickering emulsions

2.8.1. Digestion of oleogel-based Pickering emulsions stabilized by TG-LF particles

First, the pH of 2 wt‰ of NaCl solution was adjusted to 1.2 with HCl solution to obtain simulated gastric fluid (SGF). Then, 4 g of oleogel-based Pickering emulsions (containing 2 g of oleogel) was added to 20 mL SGF and incubation under magnetic stirring (210 rpm) at 37 °C. Subsequently, 32 mg of pepsin was added to the mixture to start the digestion process. After 2 h of gastric digestion, the pH of the system was adjusted to 7.5 to inactive the pepsin and cease gastric digestion.

Simulated intestinal fluid (SIF) was obtained by dissolving 1 g of bile salt, 0.111 g of $CaCl_2$ and 0.606 g of Tris into 100 mL of water, followed by adjusting the pH of the system to 7.5 with HCl (Wei, Zhang, & Huang, 2019). Then, 64 mg of pancreatin was dissolved in SIF (20 mL). Afterwards, SIF with pancreatin was added to gastric digesta to start intestinal digestion (temperature and stirring speed remained constant). During the intestinal digestion process, the pH of the system was maintained at 7.5 for 2 h by adding 0.25 M NaOH solution. The volume and time of added NaOH solution were recorded in the whole process of intestinal digestion to calculate extent and speed of lipolysis.

2.8.2. Extent of lipolysis in oleogel-based Pickering emulsion

The amount of released free fatty acids (FFA) was applied to analyze the degree of lipolysis. It was surmised that each 1 mol of triglycerides digested could release FFA and expended 2 mol of NaOH. For this reason, the fraction of released FFA could be determined as:

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

where $w_{corn\ oil}$ was the mass of corn oil (in g) in the digested emulsions, $M_{corn\ oil}$ was molecular mass of the corn oil (in g/mol), m_{NaOH} was the molarity of NaOH solution (in mol/L) and V_{NaOH} was the amount of 0.25 M NaOH added to the system (in L).

2.8.3. Curcumin bioaccessibility in oleogel-based Pickering emulsion

The intestinal digesta were centrifuged (TGL20A, Changsha Yingtai Instrument Co., Ltd, Changsha, China) at 16,050 × g at 4 °C for 30 min after *in vitro* gastrointestinal digestion. Then, the intermediate micelle phase was collected for bioaccessibility analysis of curcumin. Method for the analysis of curcumin in this study was adapted from the method described by Meng et al (Meng, Wu, Xie, Cheng, & Zhang, 2021). First, 5 mL of intermediate micelle phase was mixed with 5 mL of 95% ethyl alcohol. Afterwards, centrifuged the mixture (16,050 × g) at 4 °C for 30 min to acquire the liquid supernatant. After diluting 5 times with 95% ethyl alcohol, absorbance measurement of the solution was recorded at 428 nm by UV–Vis spectrophotometer (UV2355, UNICO).

$$\%bioaccessibility = \frac{curcumin\ content\ in\ the\ micelle\ phase}{total\ curcumin\ content\ in\ the\ formulations} \times 100\% \quad (3)$$

2.9. Statistical analysis

Each experiment was performed at least triplicate. Origin 2017 software was applied to perform all of the statistical analysis. One-way analysis of variance (ANOVA) with Fisher LSD test were applied to determine the statistical differences, and differences ($p < 0.05$) were regarded statistically significant.

3. Results and discussion

3.1. Preparation and characterization of LF and TG-LF particles

Before the preparation of lactoferrin particle assembled via transglutaminase-induced crosslinking (TG-LF particles), the properties of the LF were studied. Fig. S1 showed zeta potential of LF at different pHs. With the increase of pH, the zeta potential decreased gradually. The isoelectric point of LF was between pH 9 and pH 10. When the pH was close to the isoelectric point, the electrostatic repulsion between molecules decreased, which led to the inter-attraction and sedimentation of molecules (Destribats, Rouvet, Gehin-Delval, Schmitt, & Binks, 2014). The sedimentation of LF would hinder the cross-linking reaction with TG, so the pH below isoelectric point was selected as the reaction condition. Optimal activity of TG was at pH 5–8 (Ahammed, Liu, Wu, Khin, Yokoyama, & Zhong, 2021). Therefore, the effect of pH on crosslinking was investigated in the range of pH 5–8.

PdI reflects the dispersion index of particles. The smaller the PdI, the more uniform the particles are (Cheong, Tan, Tan, & Nyam, 2016). One prerequisite of particles as Pickering emulsifier was possessing a smaller PdI. Fig. 1a and Fig. 1b showed size distribution by intensity at different pHs. Size distribution by intensity of TG-LF particles prepared at pH 5, pH 6 and pH 7 had multimodal distribution, and the instrument showed that result quality was poor. This phenomenon might be attributed to quite small particles and the scattered distribution of particles, which led to incorrect instrument detection. The results quality of size distribution by intensity at pH 8 was good. Therefore, the reaction condition of pH 8 was better than that of pH 5, pH 6, pH 7, and pH 8 was the optimal reaction pH for the preparation of TG-LF particles. Then, the effect of TG concentration on crosslinking was studied at pH 8. Large particle size was another prerequisite for the particles as Pickering emulsifier. Larger particle size made the particles need more separation energy to separate from the oil–water interface, which endowed the Pickering emulsion stabilized by the larger particle with higher stability. It could be seen from Fig. 1c that with the increase of TG concentration, PdI of TG-LF particles had a downward trend while the particle size had an upward trend. To identify the function of TG, a control group with no TG but with the same thermal treatments was done, its particle size was only 10.533 ± 0.321 nm, which was too small to stabilize Pickering emulsions. When the concentration of TG was 110 U/g, particle aggregation and agglomeration occurred (Fig. S2). The high concentration of TG led to over-crosslinking of LF, which made the particles become larger and larger, thus producing large aggregates. Therefore, pH 8 and 100 U/g of TG concentration were considered as the optimal reaction conditions.

After the pH and TG concentration of crosslinking reaction were determined, the influence of temperature (40 °C, 45 °C, 50 °C, 55 °C, 60 °C) and time (0.5 h, 1.0 h, 1.5 h, 2.0 h, 2.5 h) on crosslinking was studied. Particle size of TG-LF particles prepared at different time and temperature was shown in Fig. 1d. All samples had narrow particle size distribution because PdI of TG-LF particle dispersions was about 0.2 at all temperatures and times (data not shown). It could be seen from Fig. 1d that there was a certain relationship between reaction temperature and particle size at the beginning of the reaction. The higher the reaction temperature, the larger the particle size. The reason was that in a certain temperature range, the increase of temperature would improve the activity of TG and Brownian motion between LF molecules, which accelerated the reaction speed (Glodowsky, Ruberto, Martorell, Mac Cormack, & Levin, 2020). In the middle and late stage of the reaction, there was a decrease in particle size. The reason for the phenomenon was that after the crosslinking was mostly completed, the larger particles would aggregate and precipitate if heating was continued, which led to the decrease of large size particles. Thus, the overall particle size became smaller. For example, it could be seen from Fig. S3 that size distribution by intensity gradually shifted to right in the first 2 h. There was a bimodal distribution at 2.5 h, which indicated that some large size particles were produced. The visual image of the TG-LF particle samples further confirmed the existence of large size particles. After the sample was tilted, there were some solid particles on the wall of the bottle. Therefore, the decrease of the particle size of the dispersion was due to the formation of large size solid particles. It was obvious that the maximum particle size appeared at 50 °C for 2 h. In summary, the best preparation conditions of TG-LF particles were pH 8, 100 U/g of TG concentration, 50 °C and 2 h of crosslinking time. Furthermore, Fig. S4 showed that TG-LF particles with sub-micrometer size were observed, supporting Nano Zetasizer measurements (160.9 nm).

Because the TG crosslinking reaction occurred on carboxamide group of glutamine and amino group of lysine (Ruzengwe et al., 2020), measurement of the free amino groups before and after the crosslinking reaction confirmed that the crosslinking reaction occurred. As shown in Fig. 2a, compared with LF, the free amino group content of TG-LF particles reduced by 65%. There were both glutamine and lysine in LF (Steijns & van Hooijdonk, 2000; Wang et al., 2019). It could be conjectured that (glutamyl) lysine isopeptide bond was formed after the crosslinking reaction, which led to the reduction of free amino groups (Fig. 2b).

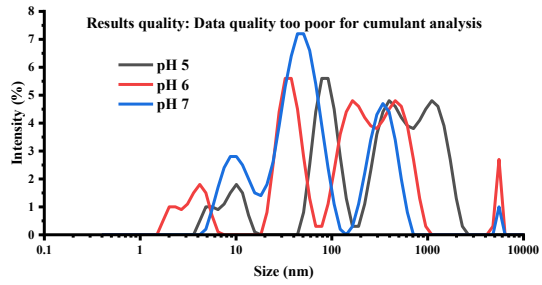
Since a prerequisite for Pickering stabilizers was the partial wettability of the particles to the oil and water phases (Xia, Xue, & Wei, 2021), the wettability of TG-LF particles was tested to evaluate whether the particles could be used as Pickering emulsifiers. The wettability of particles was judged by the analysis of contact angle, and Fig. 3 showed that contact angle of LF and TG-LF particles was 36.95 ± 0.15° and 79.30 ± 0.10°, respectively. The contact angle of TG-LF particles was greater than LF, indicating strong hydrophobicity of TG-LF particles. When the contact angle of particles was in the range of 53°–82°, the particles could be used to prepare oil-in-water Pickering emulsions (Wei, Cheng, Zhu & Huang, 2019). When the contact angle of particles was close to 90°, the formed Pickering emulsion was more stable (Xia et al., 2021). The results showed that TG-LF particles could have better performance as a Pickering stabilizer.

3.2. Preparation of oleogel

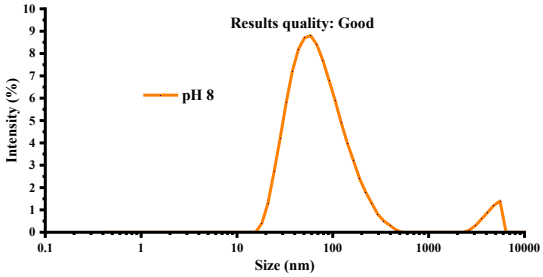
Corn oil, which is extracted from corn germ, is rich in vitamin E, phytosterols and polyunsaturated fatty acids compared with other edible oils (Wang, Sun, Yao, & Chu, 2021). Therefore, corn oil-based oleogels were developed in this study. As shown in Fig. S6, all samples did not flow after inversion, which showed that they all formed oleogels. At the gelator concentration of 5%, CW, BA and MT could be utilized to successfully prepare corn oil-based oleogels. Because the color of corn oil was lighter than gelators, the color of the oleogel was greatly affected by the gelators. It could be seen from Fig. S6 that the appearance of CW oleogel was more delicate, while BA oleogel had obvious grain structure. Both of CW and BA were low molecular weight gelators, and they formed oleogels through crystallization (Hwang, 2020; Zhao et al., 2021). Different appearance of CW oleogel and BA oleogel might be related to the different crystal structure formed by the two gelators (Callau, Sow-Kébé, Jenkins, & Fameau, 2020). After loading curcumin into corn oil, the formed oleogels could still maintain its structure.

3.3. Preparation and characterization of TG-LF particle-stabilized oleogel-based Pickering emulsions

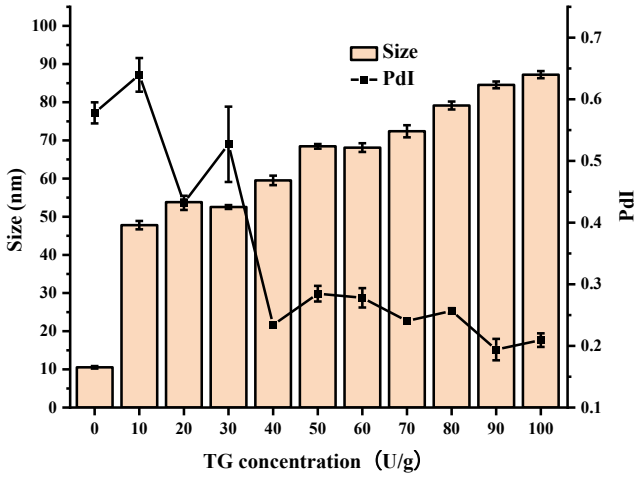
Since the particle size of TG-LF particles was not affected by pH (Fig. S5) and food was generally acidic, the pH of TG-LF particle dispersion was adjusted to 3. In this paper, two-step method of oleogel-based Pickering emulsions preparation by ultrasonic and homogenization was first developed. The former two-step method was homogenization first and then ultrasonic (Qi et al., 2021). Because of the viscosity of oleogels, a large amount of oleogels would stick to the homogenizer if homogenized directly, which made it impossible to participate in the preparation of oleogel-based Pickering emulsions. During the ultrasonic

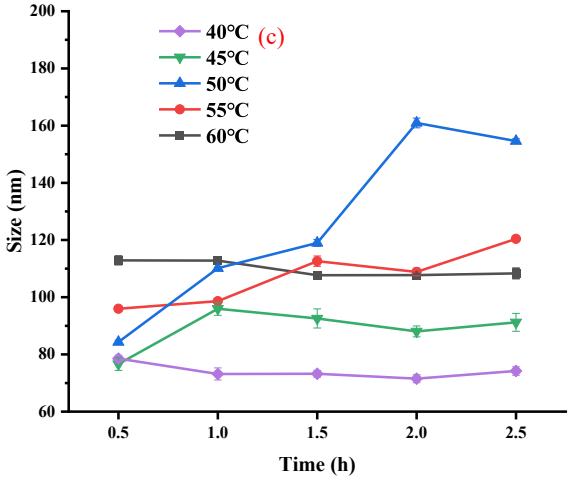


(a)



(b)





(d)

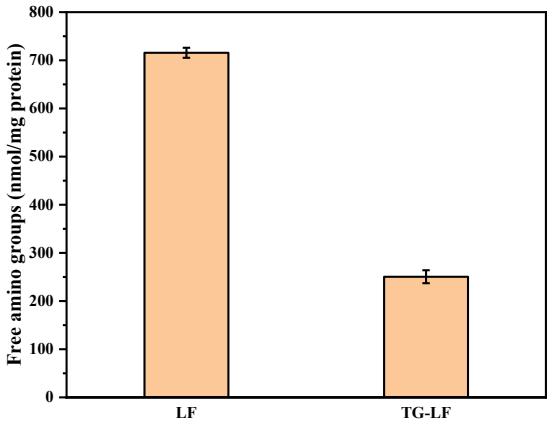
(caption on next column)

Fig. 1. (a) Size distribution by intensity of lactoferrin particles assembled via transglutaminase-induced crosslinking (TG-LF particles) prepared at different pHs (pH 5, pH 6, pH 7). (b) Size distribution by intensity of TG-LF particles prepared at pH 8. (c) Particle size and PdI (Polymer dispersity index) of TG-LF particles prepared at different TG concentrations. (d) Average particle size of TG-LF particles prepared at different time and temperatures.

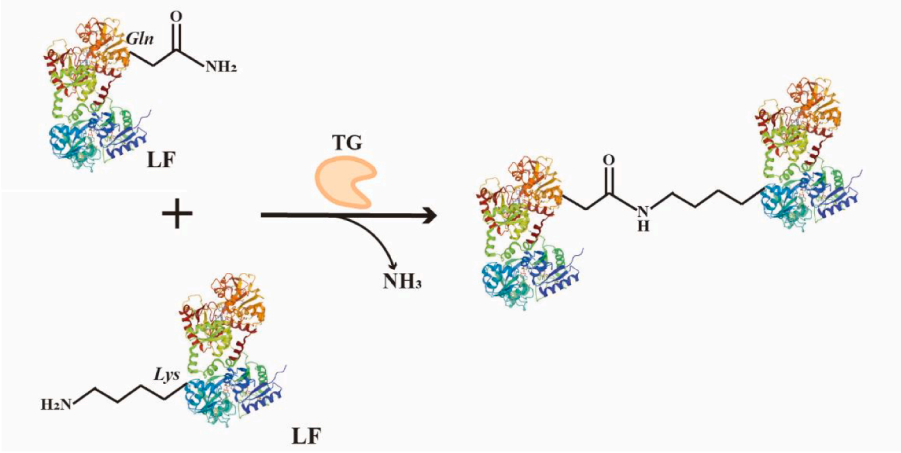
process, the thermal effect of the probe would melt the oleogels adhered to the instrument, so that all the oleogels could participate in the preparation of the oleogel-based Pickering emulsions. After ultrasonic treatment, homogenization would make the emulsion droplets more uniform. Fig. S7a showed oleogel-based Pickering emulsions prepared by different oleogels. All of the three samples could form Pickering emulsions with the emulsified phase volume fraction of 100%. Fig. S7b and Fig. S7c showed the dispersion of three oleogel-based Pickering emulsions in water and oil, respectively. Since all emulsions had good dispersibility in water and were not dispersed in oil, all of the three oleogel-based Pickering emulsions stabilized by TG-LF particles were oil-in-water Pickering emulsions, which was consistent with the measurement of contact angle. Moreover, the fluorescence images of the CW oleogel-based Pickering emulsion also demonstrated that TG-LF particles played a substantial role in stabilizing the oil-in-water Pickering emulsions (Fig. S8).

Considering that the droplet size of emulsion had great influence on the properties of emulsions, the droplet size of oleogel-based Pickering emulsions was measured. Table 1 showed the volume-weight mean diameter ($D_{4,3}$) of the Pickering emulsions prepared by different oleogels. Pickering emulsion prepared by CW oleogel had the minimum droplet size (12.07 μm). With the increase of BA content in gelators, droplet size of Pickering emulsions increased obviously. One possible reason for the phenomenon might be that wax was also an emulsifier and could stabilize oil-in-water Pickering emulsions (Li, Liu, Mei, Wang, Xu, & Sun, 2009; Xia et al., 2021). In a certain concentration range, the droplet size of emulsion tended to decrease when the concentration of emulsifiers in the system increased (Su et al., 2021). In the progress of Pickering emulsion preparation, not all of CW in the oleogels followed the oil phase into the droplet. Some of CW together with TG-LF particles could possibly be adsorbed onto the oleogel-water interface as emulsifiers, which increased the concentration of emulsifiers. Thus, the droplet size of the Pickering emulsions decreased. Another reason might be that the adsorption rate of TG-LF particles on the oleogel-water interface was different. The presence of BA might inhibit the protein adsorption at the interface. In MT oleogel-based Pickering emulsion and BA oleogel-based Pickering emulsion, some of the TG-LF particles were dispersed in the aqueous phase and not adsorbed onto the interface, resulting in a decrease of TG-LF particles absorbed on the interface. Therefore, the droplet size of MT oleogel-based Pickering emulsion and BA oleogel-based Pickering emulsion was larger than that of CW oleogel-based Pickering emulsion. Besides, the structure of oleogels might affect the droplet size of oleogel-based Pickering emulsions. As mentioned above, CW oleogel was more delicate than the other two kinds of oleogels, which made it easier to break and disperse during the process of ultrasound and homogenization. Thus, more smaller droplets were formed after the oleogel was surrounded by TG-LF particles.

Emulsion rheology was used to explore the behavior of emulsion deformation or flow (Xia et al., 2021). Investigating rheological properties of oleogel-based Pickering emulsions stabilized by TG-LF particles could help to better understand the physical and chemical properties of those Pickering emulsions. The results of strain sweep showed that storage modulus (G') and loss modulus (G'') of Pickering emulsions were stable in the 0.1%-1% strain region, which was defined as the linear viscoelastic region (LVE) for those Pickering emulsions (data not shown). Frequency sweep test was carried out in LVE (0.5% strain). Fig. 4 showed the variation of G' and G'' with respect to frequency of Pickering emulsions prepared by different oleogels. Since G' and G''



(a)



(b)

Fig. 2. (a) Free amino groups of lactoferrin (LF) and lactoferrin particle assembled via transglutaminase-induced crosslinking (TG-LF particle). (b) The cross-linking reaction catalyzed by transglutaminase between glutamine (Gln) and lysine (Lys) residues of LF (PDB ID: 1F9B).

represented the viscous and elastic parts of the Pickering emulsions, G' surpassing G'' indicated that the samples had a gel-like structure (Wei, Zhang, & Huang, 2019). It could be seen from Fig. 4 that G' all of the Pickering emulsions was larger than that of G'', which suggested that all of the three oleogel-based Pickering emulsions had gel-like structure. The reason was that both oleogel and Pickering emulsion had gel-like structure (Hwang, 2020; Wei & Huang, 2019b), so the oleogel-based Pickering emulsion retained the gel-like structure of both. G' of CW oleogel-based Pickering emulsion was the highest in the results, implying higher gel strength. This could be explained by their droplet size. As mentioned above, CW oleogel-based Pickering emulsion had the smallest droplet size, which led to more compact distribution of droplets and limited the flow of droplets. Afterwards, some of the continuous phase was trapped by the droplets. Poor liquidity and the entrapment of emulsion droplets could improve the storage modulus and viscosity of oleogel-based Pickering emulsions (Wei, Cheng, Zhu, & Huang, 2019). But G' of MT oleogel-based Pickering emulsion and BA oleogel-based Pickering emulsion was not consistent with their droplet size. This might be that the difference of their droplet size was small, so the distribution as well as flowability of droplet were similar and could not dominate its G'. The dominant factor might be the strength of the oleogel inside the droplet. As part of CW might stay at the oil–water interface, it could be speculated that the concentration of gelator inside the MT oleogel-based Pickering emulsion droplet decreased, which reduced the gel strength of oleogel inside the droplet. When the stress was applied to the MT oleogel-based Pickering emulsion, weaker gel structure made the droplet easier to flow, which led to lower storage modulus.

3.4. Lipolysis and bioaccessibility of curcumin in TG-LF particle-stabilized oleogel-based Pickering emulsions

pH-stat digestion model, an *in vitro* digestion model, was the most popular due to its easy operation (Xiao, Li, & Huang, 2015), so this model was used to evaluate the degree of lipolysis of oleogel-based Pickering emulsions and the bioaccessibility of curcumin in this study. There were two main ways in which lipase participated in the digestion of oil in the oleogel-based Pickering emulsion. One way was that void spaces might exist between the particles adsorbed on the interface of the Pickering emulsions. The lipase could pass through the void spaces and directly contacted with the internal oil phase (Tan, Sun, Lin, Mu, & Ngai, 2014). The other way was that the lipase directly digested the particles at the Pickering emulsion interface, thereby destroying the particle interface layer and allowing the lipase to enter into contact with the oil

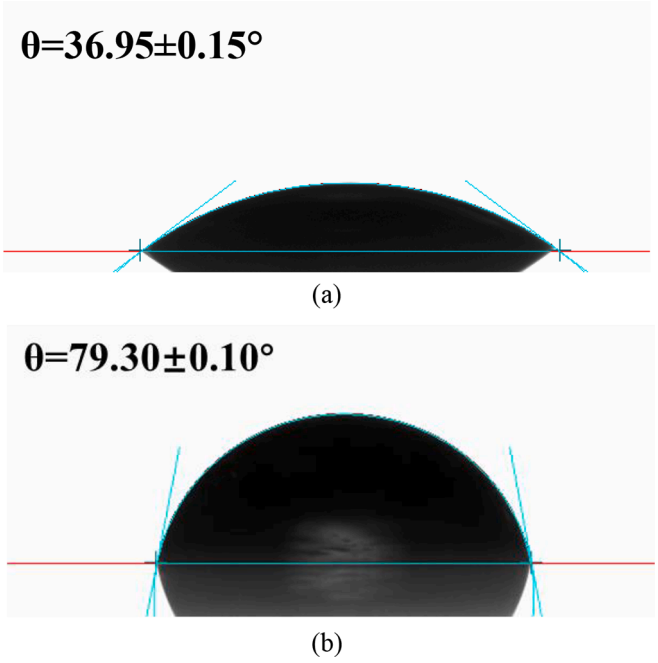


Fig. 3. Contact angle of lactoferrin (a) and lactoferrin particle assembled via transglutaminase-induced crosslinking (b).

Table 1
Volume-weighted mean diameter ($D_{4,3}$) of the Pickering emulsions prepared with different oleogels.

Emulsions prepared with	$D_{4,3}$ of the samples (μm)
CW oleogel	12.07 ± 0.25
MT oleogel	42.25 ± 1.96
BA oleogel	62.65 ± 1.34

Note: carnauba wax (CW), behenyl alcohol (BA) and CW-BA mixture at 1:4 ratio (MT).

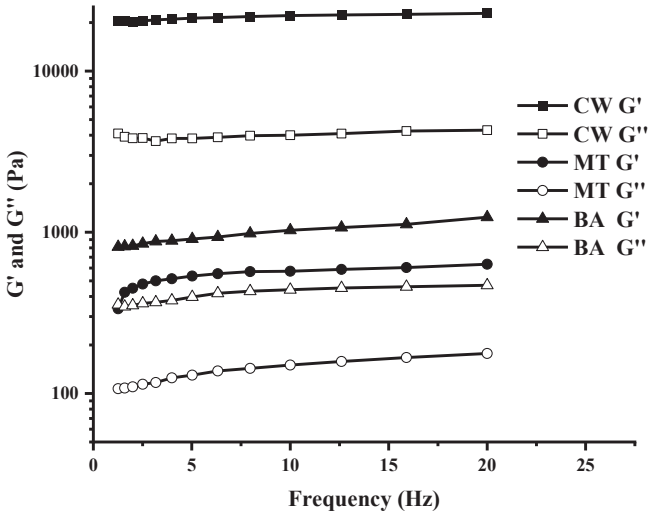
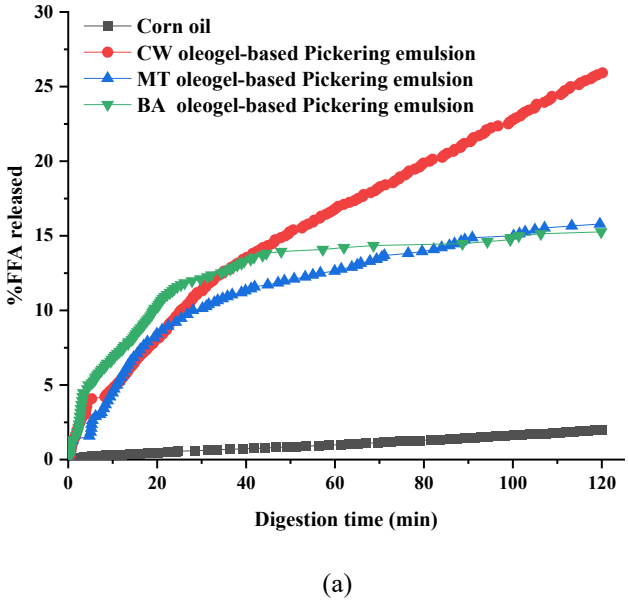


Fig. 4. Storage modulus (G′) and loss modulus (G″) of oleogel-based Pickering emulsions prepared by different oleogels (carnauba wax (CW) oleogel, behenyl alcohol (BA) oleogel, CW-BA mixture at 1:4 ratio (MT) oleogel).

(Sarkar, Murray, Holmes, Ettelaie, Abdalla, & Yang, 2016). Fig. 5a showed the release of free fatty acids (FFA) in oleogel-based Pickering emulsions prepared by CW oleogel, MT oleogel and BA oleogel,



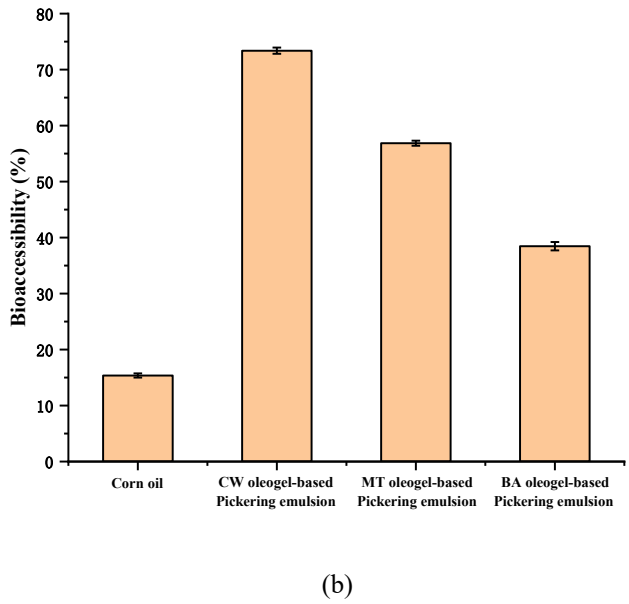


Fig. 5. (a) Release profile of free fatty acids (FFA) in corn oil and different Pickering emulsions. (b) The bioaccessibility of curcumin in corn oil and different Pickering emulsions after *in vitro* digestion.

respectively. It could be seen that all of three oleogel-based Pickering emulsions maintained higher FFA release rates than pure corn oil during initial digestion, and the final amount of FFAs released from the CW oleogel-based Pickering emulsion was higher than that from MT oleogel-based Pickering emulsion and BA oleogel-based Pickering emulsion, which indicated that the degree of lipolysis of CW oleogel-based Pickering emulsion was higher. MT oleogel-based Pickering emulsion and BA oleogel-based Pickering emulsion had similar degree of lipolysis. The difference of lipolysis could be explained by the following theories. First, in the early stage of digestion, the digestion rate was kept at a high level due to the large amount of emulsion droplets. With the progress of digestion, the number of droplets among the three oleogel-based Pickering emulsions began to differ, which might lead to the change of digestion rate. Second, the CW oleogel-based Pickering emulsion had smaller droplet size than the other two oleogel-based Pickering

emulsions, which endowed emulsions with a larger interface area. Because lipid digestion was a kind of interface phenomenon (Wei, Zhu, et al., 2019), larger interface area could promote the full contact between lipase and oil, which led to higher lipolysis rate.

In the process of curcumin-loaded oleogel-based Pickering emulsions preparation, curcumin could be completely dissolved in corn oil. In the process of Pickering emulsions digestion, the internal oil phase would be converted to free fatty acids and mono monoacylglycerols, and then they formed mixed micelles. With the progress of lipolysis, curcumin, which was originally dissolved in the oil phase, was transferred to the mixed micelles and became the core of the micelles (Yu & Huang, 2012). Curcumin in the core of micelles was considered to be bioaccessible (Wei, Cheng, Zhu, & Huang, 2019). Curcumin bioaccessibility of Pickering emulsions prepared by CW oleogel, MT oleogel and BA oleogel was shown in Fig. 5b, respectively. By comparison, it was found that the lipolysis rate and bioaccessibility of curcumin of the oleogel-based Pickering emulsions had the same trend. The curcumin bioaccessibility of oleogel-based Pickering emulsions was higher than that of pure corn oil. Curcumin bioaccessibility of CW oleogel-based Pickering emulsions was the highest, followed by MT oleogel-based Pickering emulsions and BA oleogel-based Pickering emulsions. The higher curcumin bioaccessibility was mainly related to the high rate of lipolysis. Higher lipolysis rate led to more free fatty acids, which was conducive to the formation of more micelles. Because hydrophobic curcumin was bioaccessible after dissolving into the core of micelles, the more micelles there were, the higher bioaccessibility of curcumin was. However, the difference of lipolysis rate between MT oleogel-based Pickering emulsion and BA oleogel-based Pickering emulsion was small, but the difference of curcumin bioavailability between them was large. It might be that the gelators affected the bioavailability of curcumin. After digestion, CW in MT might be attached to the mixed micelles because CW was difficult to digest (de Freitas, de Sousa, Soares, da Silva, Benjamin, & Guedes, 2019), which might attract more curcumin into the micelles core, thus improving its bioavailability.

4. Conclusion

In summary, the optimal environmental condition for preparation of TG-LF particles was pH 8, 100 U/g of TG concentration, 50 °C and 2 h of crosslinking time. The determination of free amino group showed that crosslinking reaction occurred between LF under the catalysis of TG. TG-LF particles had better wettability than LF. Carnauba wax, behenyl alcohol and carnauba wax-behenyl alcohol mixture (at 1:4 ratio) at 5% concentration could be applied to successfully prepare oleogels. Three types of oleogel-based Pickering emulsions stabilized by TG-LF particles were successfully prepared by a two-step method. Droplet size of CW oleogel-based Pickering emulsion was the smallest among the three Pickering emulsions, which led to increase in both extent of lipolysis and curcumin bioaccessibility. The obtained result may fill the research gap of the influence of oleogels on oleogel-based Pickering emulsions and provide a new approach for preparation of oleogel-based Pickering emulsions with an excellent nutraceutical delivery efficiency.

CRediT authorship contribution statement

Tianhang Xia: Investigation, Software, Data curation, Writing - original draft. **Yuxing Gao:** Investigation, Data curation, Writing - review & editing. **Yu Liu:** Investigation. **Zihao Wei:** Conceptualization, Methodology, Resources, Writing - original draft, Writing - review & editing, Project administration, Funding acquisition, Supervision. **Changhu Xue:** Resources, Funding acquisition, Supervision.

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.foodchem.2021.131779.

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