Effects of octenyl succinic anhydride chemical modification and surfactant physical modification of bovine bone gelatin on the stabilization of fish oil-loaded emulsions

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ABSTRACT

In this work, octenyl succinic anhydride (OSA) chemical modification with surfactant physical modification was combined to modify bovine bone gelatin (BBG) for emulsion stabilization at pH 6.0 (to simulate acidic food environment). OSA modification decreased the β-sheet percentage and increased β-turn percentage of BBG. Further, the combination of OSA modification with surfactant physical modification had obvious and different effects on the emulsifying properties of BBG. The creaming stability of gelatin/surfactant-stabilized emulsions was dependent on gelatin structure, surfactant type, and preparation pH. The emulsions stabilized by OBBG/Span 80 and OBBG/soybean lecithin (only blurry creaming at day 28) had significantly better creaming stability than other emulsions. These results demonstrated that the combination of OSA modification with surfactant modification could be applied to improve the emulsifying properties of BBG.

1. Introduction

Oil-in-water emulsions are excellent carriers for the encapsulation and delivery of functional active substances in the fields of food and pharmaceutics (Mwangi, Lim, Low, Tey, & Chan, 2020; Nehme, Blel, Montillet, Bellettre, & Marchal, 2021). They are important components of many foods such as milk, cream, fruit beverages, mayonnaise, and soups (Sidari & Tofalo, 2019; Yang, Li, Li, Sun, & Guo, 2020). In a typical oil-in-water emulsion, emulsifier-stabilized oil droplets at nanoscale or microscale were dispersed in a continuous aqueous phase (Liang et al., 2017; Garavand, Jalai-Jivan, Assadpour, & Jafari, 2021). All emulsions are unstable as gravitational separation (creamining and sedimentation) and particle size variation (coalescence, Ostwald ripening, and flocculation) could occur during the storage time (Novales, Papineau, Sire, & Axelos, 2003; McClements & Jafari, 2018). The development of ideal emulsifiers to increase the emulsion stability has become a hotspot in the fields of food emulsions (Ozturk & McClements, 2016; Zhang et al., 2021).

Gelatin is an excellent emulsifier model for the exploration of molecular modification methods due to its relatively weak emulsifying properties (Huang et al., 2019; Zhang, Xu, et al., 2020). Gelatin is an important natural amphiphilic polymers from many mammalian and aquatic animal by-products such as skins, hides, bones, and scales (Alipal et al., 2021). Fish oils are rich in omega-3 unsaturated fatty acids with important physiological functions. Oil-in-water emulsions are promising systems to protect against oxidation, increase water

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solubility, and mask fishy taste of fish oils. Therefore, the research and development of stable fish oil-loaded gelatin-stabilized oil-in-water emulsions have attracted many attentions in the past decades (Ding et al., 2019).

Molecular modification of emulsifiers is an efficient method to develop ideal emulsifiers for the stabilization of oil-in-water emulsions. Typical modification methods can be classified into physical, chemical, enzymatic, and combined methods (Zhang, Xu, et al., 2020). Physical and chemical modification methods are the mainstream and priority methods to improve the emulsifying properties of emulsifiers (Derkach, 2015; Ding et al., 2020b; Zhang et al., 2021). Enzymatic modification is a rising star method to improve the properties of emulsifiers (Liu et al., 2020; Glusac & Fishman, 2021). However, only few works explored the combined methods such as the combination between multilayer formation physical modification by layer-by-layer electrostatic deposition technique and enzymatic modification (Zeeb, Lopez-Pena, Weiss, & McClements, 2015). Compared with single modification methods (physical, chemical, or enzymatic modification), combined methods might ideally induce significantly higher functional improvements for an emulsifier. We expect the combined methods would attract more and more researchers to apply them for the functional improvements of emulsifiers in the future.

Some physical (Chen et al., 2020; Razavi et al., 2020), chemical (Ding et al., 2019; Huang et al., 2020), enzymatic (Luo & Zhao, 2015), and combined methods (Zeeb et al., 2015) have been explored to improve the emulsifying properties of gelatins. Our previous work found, in a typical physical modification method, gelatins and small molecular surfactants could synergistically (Span 80 and soybean lecithin (SL)) and competitively (Tween 80 and sodium dodecyl sulfate (SDS)) stabilize oil-in-water emulsions (Zhang, Ding, Zhang, et al., 2020), which were affected by the preparation pH (Zhang, Ding, Tao, Wang, & Zhong, 2020) and the adjusted pH (Zhang, Ding, Wang, & Zhong, 2020). Further, our previous work also found, in a typical chemical modification method, octenyl succinic anhydride (OSA)-modified bovine bone gelatin (OBBG) had better droplet stability and creaming stability of fish oil-loaded emulsions than unmodified bovine bone gelatin (BBG), whereas octenyl succinic anhydride-modified cold water fish skin gelatin only improved droplet stability of fish oil-loaded emulsions than unmodified cold water fish skin gelatin (Zhang, Ding, Tao, Liu, et al., 2020; Zhang et al., 2022).

The purpose of this work was to explore a combined method between octenyl succinic anhydride chemical modification and surfactant physical modification of BBG for the stabilization of fish oil-loaded emulsions at pH 6.0, which was chosen to simulate the acidic food environments. Firstly, physicochemical properties of gelatins (BBG and OBBG) with four types of small molecular surfactants (Span 80, SL, Tween 80, and SDS) were analyzed. Secondly, emulsifying parameters of fish oil-loaded emulsions stabilized by gelatin and surfactants were measured. Thirdly, macroscopic and microscopic morphologies of gelatin/surfactant-stabilized fish oil-loaded emulsions were observed. Finally, the emulsion storage stability of gelatin/surfactant-stabilized fish oil-loaded emulsions was studied.

2. Materials and methods

2.1. Synthesis of OBBG

OBBG was prepared by reacting OSA with the ε-amino group of lysine and minorly with N-terminal amino acids in gelatin polypeptide chain to convert the positively charged residues to negatively charged residues by N-acylation according to our previous work (Zhang, Ding, Tao, Liu, et al., 2020). Briefly, BBG (type B, ~240 g bloom, Aladdin Industrial Corp., Shanghai, China) was dissolved into 400 mL of ultrapure water (18 mg/mL) at 45 °C for 60 min with a stirring speed of 180 rpm. After that, the solution pH was adjusted to 8.5 with 1 M NaOH and 1 M HCl by a pH meter (Model FE28, Mettler Toledo Instruments Co., ltd., Shanghai, China). OSA (0.10 g/g of gelatin, Shanghai Yuanye, China) was slowly added into the BBG solution at 35 °C with a stirring speed of 300 rpm. The solution pH was maintained at 8.5–9.0. After 3 h, the solution pH was adjusted to 6.5 to stop the succinylation reaction. Finally, the solution was dialyzed (MW: 8000–14000 Da) for 24 h and then vacuum freeze-dried for 48 h to obtain OBBG. The degree of succinylation was measured by comparing the sample absorbance with and without succinylation after ninhydrin reaction in our previous work (Zhang, Ding, Tao, Liu, et al., 2020).

2.2. SDS-PAGE analysis

The molecular weight distribution of gelatins (BBG and OBBG) with or without small molecular surfactants (Span 80, SL, SDS, and Tween 80 from Sinopharm Chemical Reagent, Shanghai, China) were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Zhang, Sun, et al., 2020). Gelatin (2 mg/mL) with or without small molecular surfactants (4 mg/mL) at pH 7.0 were mixed with 5 × sample loading buffer (GBCBIO Technologies, Guangzhou, China) with a volume ratio of 4:1. The mixtures were boiled for 5 min and then cooled down. Subsequently, 10 μL of the mixtures were loaded into 8 % Sure-PAGE Bis-Tris gel (GenScript, Nanjing, China) and were electrophoresed for 80 min at a voltage of 120 V. Then, the gel was stained with 0.1 % (w/v) Coomassie Brilliant Blue R-250 in 25 % (v/v) isopropanol and 10 % (v/v) acetic acid for 3 h and destained with 20 % (v/v) ethanol and 10 % (v/v) acetic acid for 6–8 h. Finally, the gel was photographed by a digital camera.

2.3. ATR-FTIR analysis

The attenuated total reflectance Fourier transform infrared (ATR-FTIR) analyses of gelatins (BBG and OBBG) were performed according to our previous work (Zhang, Sun, et al., 2020). Briefly, freeze-dried gelatins were examined by an ATR-FTIR spectrometer (Spotlight 400, PerkinElmer, Waltham, Massachusetts, USA) with a wavenumber range of 500–4000 cm⁻¹. The areas of 1700–1600 cm⁻¹ were used to analyze the secondary structure percentages of gelatins by PeakFit software (V4.12, SeaSave, Framingham, MA, USA) (Xu et al., 2021).

2.4. Measurement of EAI and ESI values of gelatin/surfactant-stabilized emulsions

Gelatin (10 mg/mL) with or without small molecular surfactants (20 mg/mL) at pH 6.0 were mixed with deep sea fish oil (DHA + EPA ≥ 70 %, Xi’an Qianyecao, Shaanxi Province, China) at a volume ratio of 1:1. The mixtures were mechanically sheared at 8000 rpm and 120 s by a homogenizer (T10 with a 10 mm head, IKA, Guangzhou, Guangdong, China). Emulsion activity index (EAI) and emulsion stability index (ESI) of the emulsions (oil volumetric fraction \( \phi = 0.5 \)) were determined at 500 nm. Finally, the EAI and ESI were calculated according to the below equations:

\[
EAI (m^2/g) = \frac{2 \times (2.303 \times A_0 \times N)}{\phi \times C \times 10000}
\]

\[
ESI (\text{min}) = \frac{A_0 \times \Delta t}{A_{10}}
\]

2.5. Emulsion observation

The obtained emulsions were stored at room temperature
(20–22 °C). The emulsions (3 μL) were observed by a ML8000 upright optical microscope (Shanghai Minz Precision Instruments Co. Ltd., Shanghai, China) with a 40 × objective. The optical microscopy images from three-batch experiments were used to analyze droplet size (800–1000 droplets each sample) by Image J software (Version 2.1.0/1.53c, National Institutes of Health, USA). Multiple peak Gaussian fitting was applied to analyze the representative and statistical droplet size distributions with a bin size of 1.0 μm (Martínes et al., 2012). The creaming index (CI) values were analyzed by dividing the serum layer height by the emulsion height and multiplying 100 %.

2.6. Statistical analysis

Three parallel experiments from different batch of emulsions (For OBBG-related emulsions, OBBG were from different batch) were performed for each sample and the obtain results were shown as mean value ± standard deviation. The p-value < 0.05 was considered significant after statistical analysis by one-way ANOVA method followed by Duncan analysis in SPSS 26.0 (IBM Corp., Armonk, NY, USA).

3. Results

3.1. Physicochemical properties of gelatins with small molecular surfactants

The physicochemical properties of gelatins (BBG and OBBG) with or without small molecular surfactants were studied by SDS-PAGE and ATR-FTIR spectrometry. As shown in Fig. 1A, SDS-PAGE results showed both BBG and OBBG had two main bands (about 160 and 140 kDa, corresponding to the α1 chain and α2 chain of collagen, respectively). Therefore, both OSA chemical modification and surfactant physical mixing did not obviously change the molecular weights of gelatins, and therefore did not obviously change the molecular weight distribution of gelatins in SDS-PAGE results.

Gelatins (BBG and OBBG) showed similar ATR-FTIR spectra in 500–4000 cm⁻¹ (Fig. 1B) and 1400–1750 cm⁻¹ (Fig. 1C). By fitting the ATR-FTIR spectra in 1600–1700 cm⁻¹ (Fig. S1), the secondary structure percentages of BBG and OBBG were obtained (Fig. 1D) (Sow et al., 2017; Zhang, Sun, et al., 2020), which suggested OSA modification mainly decreased the β-sheet percentage and increased β-turn percentage of BBG. It might be an important factor for the potential functional change due to the slogan “structure determines function”.

3.2. Emulsifying parameters of fish oil-loaded emulsions stabilized by gelatins with small molecular surfactants

Fish oil-loaded emulsions stabilized by gelatin (BBG or OBBG) with or without small molecular surfactants were prepared by a simple homogenizing method at a gelatin solution pH of 6.0. EAI and ESI values of the fish oil-loaded emulsions were analyzed, as shown in Fig. 2. OSA modification had no obvious effect on the EAI value, whereas it increased the ESI value. The surfactant addition might increase (Span 80, Tween 80, and SDS) or decrease (SL) the EAI values of BBG-stabilized emulsions (Fig. 2A). The surfactant addition might increase (Tween 80), decrease (SL), or have no obvious effects (Span 80 and SDS) on the ESI values of BBG-stabilized emulsion (Fig. 2B). The surfactant addition might increase the EAI values of OBBG-stabilized emulsion (Fig. 2A). The surfactant addition might decrease (SL), increase (SDS), or have no obvious effects (Span 80 and Tween 80) on the ESI values of OBBG-stabilized emulsion (Fig. 2B). Therefore, both OSA chemical modification and surfactant physical modification had obvious and different effects on the emulsifying parameters (EAI and ESI) of gelatin/surfactant-stabilized fish oil-loaded emulsions.

Fig. 1. Physicochemical properties of gelatins (bovine bone gelatin [BBG] and octenyl succinic anhydride-modified bovine bone gelatin [OBBG]) with small molecular surfactants (Span 80, soybean lecithin [SL], Tween 80, and sodium dodecyl sulfate [SDS]). (A): SDS-PAGE pattern. PS indicate the lane of protein standard. (B–C): ATR-FTIR spectra at different wavenumber ranges of 4000–400 cm⁻¹ (B) and 1750–1400 cm⁻¹ (C). (D): Secondary structure percentages according to the peak fitting results of ATR-FTIR spectra at 1600–1700 cm⁻¹ in Fig. S1.
trimodal (Fig. 4). The emulsions stabilized by BBG, BBG/Span 80, and OBBG/SDS showed a little bit of creaming (CI = 10 %) and other emulsions were still in milk-white (Fig. 3 A and 3C) and optical microscopy (Fig. 3 B and 3D), respectively. The liquid emulsions stabilized by BBG, BBG/Span 80, and OBBG/SDS showed a little bit of creaming (CI = 10 %) and other emulsions were still in milk-white (Fig. 3 A and 3C) and optical microscopy (Fig. 3 B and 3D), respectively. The droplet stability, liquid-gel transition, and creaming stability are important characteristics for the emulsions during the transportation and preservation process. Here, the macroscopic and microscopic morphologies of the fish oil-loaded emulsions stabilized by gelatin (BBG or OBBG) with or without small molecular surfactants were analyzed by digital camera technique (Fig. 3 A and 3C) and optical microscopy (Fig. 3 B and 3D), respectively. The emulsion stabilized by BBG/SL showed obvious phase separation (from top to bottom: oil phase, emulsion phase, and water phase) at 30 min. Other emulsions were still in milk-white (Fig. 3 A: 30 min and 3C: 30 min). The sizes of the droplets were microscale (Fig. 3 B: 30 min and 3D: 30 min). The droplet sizes of the emulsions stabilized by gelatin/SDS were monomodal, whereas the droplet sizes of other emulsions were trimodal (Fig. 4). The emulsions stabilized by BBG, BBG/Span 80, and OBBG/SDS showed a little bit of creaming (CI < 10 %) and other emulsions showed no obvious creaming (Figs. 3 and 5).

### 3.3. Macroscopic and microscopic morphologies of fish oil-loaded emulsions stabilized by gelatins with small molecular surfactants

The macroscopic and microscopic morphologies of the fish oil-loaded emulsions stabilized by gelatin (BBG or OBBG) with or without small molecular surfactants were analyzed by digital camera technique (Fig. 3 A and 3C) and optical microscopy (Fig. 3 B and 3D), respectively. The emulsion stabilized by BBG/SL showed obvious phase separation (from top to bottom: oil phase, emulsion phase, and water phase) at 30 min. Other emulsions were still in milk-white (Fig. 3 A: 30 min and 3C: 30 min). The sizes of the droplets were microscale (Fig. 3 B: 30 min and 3D: 30 min). The droplet sizes of the emulsions stabilized by gelatin/SDS were monomodal, whereas the droplet sizes of other emulsions were trimodal (Fig. 4). The emulsions stabilized by BBG, BBG/Span 80, and OBBG/SDS showed a little bit of creaming (CI = 10 %) and other emulsions showed no obvious creaming (Figs. 3 and 5).

### 3.4. Emulsion storage stability

The droplet stability, liquid-gel transition, and creaming stability are important characteristics for the emulsions during the transportation and preservation process. Here, the macroscopic and microscopic morphologies of the fish oil-loaded emulsions stabilized by gelatins with or without small molecular surfactants during the storage process at room temperature for 28 days were analyzed by digital camera technique (Fig. 3 A and 3C) and optical microscopy (Fig. 3 B and 3D), respectively. The results showed the droplet sizes slightly increased with time (Fig. 3), which might be resulted from droplet coalescence behaviors (Zhang, Ding, Wang, et al., 2020).

The liquid and gel states of the fish oil-loaded emulsions during the storage process were carefully observed and marked by black asterisks in the optical microscopy images of Fig. 3. The liquid emulsions stabilized by BBG, BBG/Span 80, BBG/SDS, OBBG, OBBG/Span 80, and OBBG/SL changed into emulsion gels at day 3, 7, 14, 7, 7, and 14, respectively. The liquid emulsions stabilized by BBG/Tween 80, OBBG/Tween 80, and OBBG/SDS did not change into emulsion gels even at day 28. The results suggested the liquid-gel transition time was dependent on both gelatin structure and surfactant type at the preparation pH of 6.0: (i) OSA modification increased the liquid-gel transition times of the emulsions stabilized by gelatin and gelatin/SDS; (ii) OSA modification showed no obvious effects on the liquid-gel transition times of the emulsions stabilized by gelatin/Span 80 and BBG/Tween 80.

The creaming stability of the fish oil-loaded emulsions was observed (Fig. 3) and the CI values of the emulsions were analyzed (Fig. 5) during the storage process. The emulsions stabilized by BBG, BBG/Span 80, and OBBG/SDS showed a little bit of creaming at 30 min. The emulsions stabilized by BBG/Tween 80 and BBG/SDS showed clear creaming at 3 h. The emulsion stabilized by OBBG showed blurry creaming at day 3 and then clear creaming at day 7. The emulsions stabilized by OBBG/Span 80 and OBBG/SL showed blurry creaming at day 7 and did not change into clear creaming even at day 28. The emulsions stabilized by OBBG/Tween 80 and OBBG/SDS showed clear creaming at day 3. Therefore, the creaming stability of these emulsions was dependent on both gelatin structure and surfactant type at the preparation pH of 6.0: (i) OSA modification could delay the creaming of the emulsions stabilized by BBG and BBG/Span 80; (ii) OSA modification could inhibit the phase separation of the emulsions stabilized by BBG/SL; (iii) OSA modification could promote the creaming of the emulsions stabilized by BBG/SDS; and (iv) BBG and SDS could synergistically stabilize oil-in-water emulsions; (v) OBBG and surfactants could synergistically (Span 80 and SL) and competitively (Tween 80 and SDS) stabilize oil-in-water emulsions. Our previous works suggested BBG and surfactants could synergistically (Span 80 and soybean lecithin) and competitively (Tween 80 and SDS) stabilize oil-in-water emulsions. Compared with BBG, the obtained OBBG had no obvious effects on the initial droplet sizes and liquid-gel transition time, whereas increased the droplet stability and creaming stability of fish oil-loaded emulsions (Zhang, Ding, Tao, et al., 2020; Zhang, Ding, Zhang, et al., 2020). Therefore, the creaming stability of gelatin/surfactant-stabilized emulsions was also dependent on preparation pH.

### 4. Discussion

In a typical succinylation process, succinic anhydride molecule reacts with the ε-amino group of lysine and minorly with N-terminal amino acids in protein to form amido bond and free carboxyl group (Shilpashree, Arora, Sharma, & Singh, 2015). As shown in Fig. 6 A, our previous work suggested OSA modification could form some 12-carbon chains with free side carboxyl groups on the polypeptide chain of gelatin molecule (Zhang, Ding, Tao, Liu, et al., 2020; Zhang et al., 2022). Our previous work suggested that, at an OSA mass ratio of 0.10 g/g of gelatin, the degree of succinylation of BBG was 75.8 ± 0.7 % (Zhang, Ding, Tao, Liu, et al., 2020). Compared with BBG, the obtained OBBG had no obvious effects on the initial droplet sizes and liquid-gel transition time, whereas increased the droplet stability and creaming stability of fish oil-loaded emulsions (Zhang, Ding, Tao, Liu, et al., 2020).

Small molecular surfactants could decrease the interfacial tension of an emulsion and therefore increase the emulsion stability (Zembyla, Murray, & Sarkar, 2020). The surfactants have been applied to physically modify protein such as casein to increase emulsion stability (Jiang, et al., 2018). Our previous work suggested that BBG and four types of surfactants were synergetically (Span 80 and SL) or competitively (Tween 80 and SDS) adsorbed on the oil/water interfaces in the emulsions at pH 9.0 (Zhang, Ding, Tao, Wang, et al., 2020). Moreover, our previous work also suggested the droplet and creaming stability were also dependent on the preparation pH and adjusted pH (Zhang, Ding, Tao, Wang, et al., 2020; Zhang, Ding, Wang, et al., 2020). Preparation pH and adjusted pH could significantly decrease the droplet sizes of gelatin/Span 80-stabilized and gelatin/SL-stabilized emulsions. The effects of preparation pH and adjusted pH on the creaming stability did not...
Fig. 3. Photographs (A and C) and optical microscopy (B and D) images of fish oil-loaded emulsions stabilized by gelatins with or without small molecular surfactants during the storage at room temperature. The samples (from left to right) in the images of (A) and (C) were the emulsions stabilized by gelatin, gelatin/Span 80, gelatin/SL, gelatin/Tween 80, and gelatin/SDS. Black asterisks in the optical microscopy images indicate emulsion gels. Scale bars indicate 50 μm in the images of (B) and (D).
show obvious regular pattern.

In this work, OSA and surfactants (Span 80, SL, Tween 80, and SDS) were applied to modify BBG for the stabilization of fish oil-loaded emulsions. OBBG was obtained by modifying BBG at an OSA/gelatin mass ratio of 0.10 g/g (Fig. 6A). The isoelectric points (pI) of BBG and OBBG were about 5.0 (Zhang, Ding, Wang, et al., 2020) and < 5.0 (Zhang, Ding, Tao, Liu, et al., 2020), respectively. Considering the most food beverages are acidic and OBBG at a high degree of succinylation of 90.5 ± 0.7 % formed precipitates at pH ≤ 5.0 (Zhang, Ding, Tao, Liu, et al., 2020), we prepared the OBBG/surfactant-stabilized fish oil-loaded emulsions at pH 6.0 in this work.

The charges of both BBG and OBBG were negative at both pH 9 and 6.
and 160 k modification on the gelatin structure were studied by SDS-PAGE and gelatin structure and solution pH. As shown in Fig. 6 B, the amine group of BBG chain and the carboxyl group of OBBG chains showed different charges structure and solution pH. However, the amounts of negative charges were dependent on gelatin structure and solution pH. As shown in Fig. 6B, the amine group of BBG chain and the carboxyl group of OBBG chains showed different charges at pH 9 and 6. Therefore, the gelatins (BBG and OBBG) at pH 6 had less negative charge amounts than the corresponding gelatins at pH 6 (Fig. 6C). Moreover, BBG had fewer negative charges than OBBG at both pH 9 and 6 (Fig. 6C). The more negative charges the emulsion droplet interfaces have, the more resistance ability against aggregation the emulsion droplets have, and the more stability such as ESI values the interfaces have (Zhang, Ding, Tao, Liu, et al., 2020). Therefore, the emulsion behaviors might be dependent on gelatin structure and solution pH.

Surfactants might show different electrostatic charges at different solution pH. As shown in Fig. 6D, Span 80 and Tween 80 are non-ionic hydrophobic and hydrophilic, respectively, surfactants and are neutral at all the pH range. Amphiphilic SL is negatively charged at basic pH (e.g. pH 9) and positively charged at acidic pH (e.g. pH 6). Anionic SDS is neutral at acidic pH (e.g. pH 6) and negatively charges at other pH (e.g. pH 9). Therefore, the emulsion behaviors might be dependent on surfactant type and solution pH.

4.1. Effects of OSA modification and surfactant modification on the gelatin structure

As shown in Fig. 1, the effects of OSA modification and surfactant modification on the gelatin structure were studied by SDS-PAGE and ATR-FTIR spectrometry. The molecular weights of BBG were about 140 and 160 kDa (Fig. 1A). The molecular weight of OSA was 210.27. The

As shown in Fig. 5, the effects of OSA modification and surfactant modification on the gelatin structure were studied by SDS-PAGE and ATR-FTIR spectrometry. The molecular weights of BBG were about 140 and 160 kDa (Fig. 1A). The molecular weight of OSA was 210.27. The

Surfactant physical modification had obvious effects on the emulsifying parameters of gelatin-stabilized fish oil-loaded emulsions at pH 6.0 (Fig. 2). For the BBG-stabilized emulsions, the addition of neutral surfactants (Span 80, Tween 80, and SDS, as shown in Fig. 6D) at pH 6.0 increased the EAI values, whereas the positively charged surfactant (SL) at pH 6.0 decreased the EAI values. In addition, the addition of neutral surfactant Tween 80 increased the ESI value, neutral surfactants Span 80 and SDS had no obvious effects on the ESI values, whereas the positively charged surfactant (SL) at pH 6 decreased the ESI value. For the OBBG-stabilized emulsions, the addition of surfactants might increase the EAI values. In addition, the addition of neutral surfactant SDS increased the ESI value, neutral surfactants Span 80 and Tween 80 had no obvious effects on the ESI values, whereas the positively charged surfactant (SL) at pH 6 decreased the ESI value. Therefore, the emulsifying parameters of negatively charged BBG, whereas increased the emulsion activity (EAI value) of high negatively charged OBBG.

Fig. 5. Creaming index (CI) of fish oil-loaded emulsions stabilized by gelatins with or without small molecular surfactants during the storage at room temperature.

4.2. Effects of OSA modification and surfactant modification on the emulsifying parameters

As shown in Fig. 2, EAI and ESI values of the fish oil-loaded emulsions stabilized by gelatin with or without small molecular surfactants at pH 6.0 were analyzed. The EAI values (12.4 ± 0.5 m²/g for BBG group and 12.9 ± 0.7 m²/g for OBBG group) of the emulsions stabilized by gelatins at pH 6.0 were lower than those (22.2 ± 0.2 m²/g for BBG group and 33.5 ± 0.2 m²/g for OBBG group) of the emulsions stabilized by gelatins at pH 9.0 (Zhang et al., 2022), which might be resulted from the negative charge amount differences of gelatins at different pH (Fig. 6C) and the initial droplet sizes (Figs. 3–4). The ESI value (100.5 ± 14.9 min) of the emulsion stabilized by BBG at pH 6.0 were lower to that (188.9 ± 1.2 min) of the emulsion stabilized by BBG at pH 9.0 (Zhang et al., 2022). The ESI values (191.0 ± 33.9 min) of the emulsion stabilized by OBBG at pH 6.0 were similar to that (183.0 ± 1.2 min) of the emulsion stabilized by OBBG at pH 9.0 (Zhang et al., 2022). BBG at low pH had low emulsifying parameters than that at high pH, which was consistent with previous work on the EAI and ESI values of ovomucin (Shan et al., 2012). It was interesting that OBBG at lower pH had lower EAI value than and similar ESI value to that at high pH. Therefore, the EAI and ESI values might increase with the increase of the negative charge amounts until to a threshold and OSA modification made gelatins reach the threshold. Further, the EAI and ESI values might increase with the decrease of the initial droplet sizes of the emulsions.

4.3. Phase separation of BBG/SL-stabilized emulsion and its inhibition

The emulsion stabilized by BBG/SL showed obvious phase separation (from top to bottom: oil phase, emulsion phase, and water phase) at 30 min (Fig. 3A). They also showed the lowest EAI and ESI values among the emulsions (Fig. 2). The possible reason might be that the negatively charged BBG (10 mg/mL) and positively charged SL (20 mg/mL) could...
not form stable interfacial layer to stabilize fish oil droplets under the homogenization condition at pH 6.0 in this work (Fig. 6 E: BBG/SL). The use of negative charged gelatin and positively charged SL might form aggregates in the water phase at pH 6.0 due to the electrostatic bonding (Fig. 6 E: BBG/SL), induce the lowest EAI and ESI values (Fig. 2), and induce phase separation of the fish oil-loaded emulsions (Fig. 3 A). This phenomenon could be inhibited by adjusting the relative electric charges of the emulsifiers. Typical methods included the changes of the BBG/SL mass ratio and pH based on our previous work that fish oil-loaded emulsions stabilized by BBG (8 mg/mL) with SL (20 mg/mL) at pH 5 and 7 did not show obvious phase separation even at day 7 (Zhang, Ding, Wang, et al., 2020), and the increase of the molecular electric charges (Fig. 6 E: OBBG/SL) to form relatively stable emulsions based on the result that the emulsion stabilized by more negatively charged OBBG (10 mg/mL) with positively charged SL (20 mg/mL) did not show obvious phase separation at pH 6.0 (Fig. 3C).

4.4. Effects of OSA modification and surfactant modification on the initial droplet size and droplet coalescence

Microscopic droplet morphology is one of the important characteristics for an emulsion. The emulsion droplets at 30 min could be reasonably thought as the initial droplets in the emulsions due to the low droplet coalescence at the initial times (Fig. 3). According to Figs. 3 and 4, BBG/Span 80-stabilized droplets had higher initial droplet sizes than BBG-stabilized emulsion. Moreover, BBG/Surfactant-stabilized and BBG/Tween 80-stabilized emulsions had less initial droplet sizes than BBG-stabilized emulsion. Further, BBG/SDS-stabilized droplets showed monomodal droplet sizes, which was consistent with our previous work that BBG/SDS-stabilized emulsions at pH of 3, 5, 7 and 9 (Zhang, Ding, Tao, Wang, et al., 2020). It should be noted that the initial droplets sizes of the fish oil-loaded emulsions stabilized by BBG (10 mg/mL) with surfactants at pH 6.0 in this work were less than those of the fish oil-loaded emulsions stabilized by BBG (8 mg/mL) with surfactants (Zhang, Ding, Tao, Wang, et al., 2020). Finally, OSA modification induced less initial
droplet sizes than BBG-stabilized emulsion. Therefore, the initial droplet sizes of gelatin/surfactant-stabilized emulsions were mainly dependent on surfactant type, gelatin concentration, and OSA modification. It should be noted that the effect of gelatin concentration was analyzed by comparing this work with our previous works. Further systematic research on this point would be helpful to analyze the critical concentration of gelatin in such emulsions.

During the emulsion storage process, two or more emulsion droplets tend to fuse together to form a larger droplet when they are attractive (McClements & Jafari, 2018). This so-called droplet coalescence event could be decreased by the use of appropriate emulsifiers. In this work, all the fish oil-loaded emulsions (except the BBG/SL-stabilized emulsion) showed slow droplet coalescence (Fig. 3). Our previous work found that the fish oil-loaded emulsions stabilized by BBG (8 mg/mL) with surfactants (Span 80 or SL) at pH 3, 5, 7, and 9 showed quicker droplet coalescence than this work and the fish oil-loaded emulsions stabilized by BBG (8 mg/mL) with surfactants (Tween 80 or SDS) at pH 3, 5, 7, and 9 showed similar droplet coalescence behaviors to this work (Zhang, Ding, Wang, et al., 2020). Finally, OBBG/surfactant-stabilized emulsions showed similar droplet coalescence behaviors to BBG/surfactant-stabilized emulsions at pH 6.0. Therefore, similar to the initial droplet sizes, the droplet coalescence behaviors of gelatin/surfactant-stabilized fish oil-loaded emulsions were also mainly dependent on surfactant type and gelatin concentration.

4.5. Effects of OSA modification and surfactant modification on the emulsion liquid-gel transition

Emulsion state is one of the important characteristics for an emulsion. The emulsions after homogenization are generally liquid. Some liquid emulsions could change into emulsion gel during the storage (Ding et al., 2020a; Lin, Kelly, & Miao, 2020). Liquid and gel forms are common forms of food emulsions. As shown in Fig. 3, the gelatin/surfactant-stabilized fish oil-loaded emulsions showed different liquid-gel transition behaviors. The emulsions stabilized by BBG, BBG/Span, and BBG/SDS changed into emulsion gels, whereas the emulsion stabilized by BBG/Tween 80 did not change into emulsion gel even at day 28. They were consistent with those in our previous work at a gelatin pH of 5.0 (Zhang, Ding, Tao, et al., 2020). Further, OSA modification increased the liquid-gel transition time of the emulsions stabilized by gelatin and gelatin/SDS, whereas it had no obvious effects on the liquid-gel transition time of the emulsions stabilized by gelatin/Span 80 and BBG/Tween 80. Therefore, the liquid-gel transition times were dependent on both gelatin structure and surfactant type.

4.6. Effects of OSA modification and surfactant modification on the creaming stability

Macroscopic creaming stability is one of the important characteristics for an emulsion. The creaming stability is mainly dependent on the move speed (V_{stokes}) of the droplets in the emulsion, which could be described by Stokes’ Law (McClements, 2011; McClements & Jafari, 2018):

\[ V_{stokes} = -\frac{2g(r_p - r_i)}{9\eta_i} \]  

(3)

\[ \rho_i = \frac{\rho_o + 3\delta/p_{shell}}{r + 3\delta} \]  

(4)

where \( g \) is the gravity acceleration, \( r \) is the droplet radius, \( \rho_i \) is the density of the water phase, \( \rho_i \) is the density of the dispersed droplet phase, \( \eta_i \) is the shear viscosity of the water phase, \( \rho_{core} \) is the oil core density of the droplet, \( \rho_{shell} \) is the interfacial shell density of the droplet, \( \delta \) is the interfacial shell thickness of the droplet.

For micrometer droplets such as fish oil-loaded emulsion droplets in this work, the nanometer scale interfacial shell thickness was significantly lower than the micrometer scale initial droplet radius. Therefore, Equation (4) could be approximately described as below equation (5):

\[ \rho_i \approx \rho_{core} + \frac{3\delta(p_{shell} - \rho_{core})}{r} \]  

(5)

The creaming stability could be altered by changing the initial droplet size, density contrast, electrostatic repulsion, and steric repulsion (McClements & Jafari, 2018). Due to the introduction of 12-carbon chains with free side carboxyl groups, OSA modification might increase the interfacial shell density of the droplet (\( \rho_{shell} \)) and/or the interfacial shell thickness of the droplet (\( \delta \)). According to Eqs. (3–5), both of them could decrease the move speeds (\( V_{stokes} \)) of the droplets. Therefore, in this work and our previous works (Zhang, Ding, Zhang, et al., 2020; Zhang, Sun, et al., 2020), the emulsion stability were: OBBG-stabilized emulsions > BBG-stabilized emulsions.

Electrostatic repulsion between the emulsion droplets is mainly dependent on the molecular charges of the applied emulsifiers. The amounts of molecular charges of gelatins might be (Fig. 6C): BBG at pH 9 > OBBG at pH 6 > BBG at pH 9 > BBG at pH 6. Therefore, the interfacial layer charges and electrostatic repulsion forces of the emulsions stabilized by gelatins were: OBBG at pH 9 > OBBG at pH 6 > BBG at pH 9 > BBG at pH 6. Therefore, the emulsion stability were: OBBG-stabilized emulsion at pH 9 (Zhang, Ding, Tao, Liu, et al., 2020) > OBBG-stabilized emulsion at pH 6 (Figs. 3 and 5) > BBG-stabilized emulsion at pH 9 (Zhang, Ding, Zhang, et al., 2020; Zhang, Sun, et al., 2020) > BBG-stabilized emulsion at pH 6 (Figs. 3 and 5).

The BBG/surfactant-stabilized emulsions showed different creaming stability at pH 6.0 (Fig. 3A and 5). Compared with the corresponding emulsions at pH 9.0 (Zhang, Ding, Zhang, et al., 2020; Zhang, Sun, et al., 2020), BBG/Span 80-stabilized emulsion at pH 6.0 showed slower creaming stability, BBG/SL-stabilized emulsion at pH 6.0 even showed phase separation, and BBG/Tween 80-stabilized and BBG/SDS-stabilized emulsions showed better creaming stability. They were consistent with our previous works on the effect of pH on the BBG/surfactant-stabilized emulsions (Zhang, Ding, Tao, et al., 2020). At lower pH, BBG/Span 80-stabilized emulsion had higher initial droplet sizes and therefore had lower creaming stability according to Eqs. (3–5). At lower pH, BBG/SL-stabilized emulsion might induce quicker interfacial disorders of the emulsifiers and therefore showed faster phase separation, as discussed in the section 4.3. At lower pH, BBG/Tween 80-stabilized and BBG/SDS-stabilized emulsions might had lower molecular charges of BBG (Fig. 6C), which might promote the assembly of BBG with neutral Tween 80 or neutral SDS at the emulsion interfacial layers (Fig. 6E), and therefore, had lower interfacial shell density of the droplet and higher creaming stability according to Eqs. (3–5). For the BBG/SDS-stabilized emulsion, the smallest initial droplet sizes further increased the creaming stability (Figs. 3 and 5).

The OBBG/surfactant-stabilized emulsions showed different creaming stability at pH 6.0 (Fig. 3C and 5). BBG/Span 80-stabilized and OBBG/SL-stabilized emulsions showed slower creaming behaviors than OBBG-stabilized emulsion. OBBG/Tween-stabilized and OBBG/SDS-stabilized emulsions showed faster creaming behaviors than OBBG-stabilized emulsion. Therefore, as shown in Fig. 6E, OBBG and four types of surfactants were synergistically (Span 80 and SL) or competitively (Tween 80 and SDS) adsorbed on the oil/water interfaces in the emulsions at pH 6.0.

5. Conclusions

In this work, we explored the effects of OSA chemical modification and surfactant physical modification of BBG on the stabilization of fish oil-loaded emulsions. Both OSA modification and surfactants modification had obvious and different effects on the emulsifying properties of BBG. OBBG/Span 80 and OBBG/SL showed increased emulsifying parameter values (EAI and ESI) than corresponding BBG/Span 80 and BBG/SL, respectively. Moreover, OBBG/Span 80 and OBBG/SL showed
better emulsion stability than other gelatin-based emulsifiers at pH 6.0. This study provided important information to understand the synergistic or competitive interaction of gelatin with four types of surfactants for the stabilization of fish oil-loaded emulsions. Moreover, this study provided an efficient way to improve the stability of gelatin-stabilized fish oil-loaded emulsions at acidic pH by using the combination method of OSA chemical modification and surfactants (Span 80 and SL) physical modification of gelatin. Both gelatins and fish oils have many potential healthy functions and have widely explored in the development of functional foods and pharmaceutical preparations. Our advances would be also beneficial for the research and development of emulsifiers for stabilizing emulsions in the fields of food and pharmaceutics.

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

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