

Heat-induced phosphorylated ovalbumin spherical aggregates as the O/W Pickering emulsion stabilizer: self-assembly mechanisms and emulsion properties

Zhouyi Xiong^{1,2,§} , Lina Zhang^{3,§}, Jihui Wang^{1,2}, Yanxue Cai^{1,2}, Shan Xiao^{1,2} 

¹ College of Life and Health, Dongguan University of Technology, Dongguan 523808, China

² Dongguan Food Industry Science and Technology Innovation Center, Dongguan 523808, China

³ College of Food Science and Technology, Huazhong Agricultural University, Wuhan 430070, China

[§] These authors contributed equally to this work.

 Address correspondence to Zhouyi Xiong, xzy646590@163.com; Shan Xiao, xiaoshan@dgut.edu.cn

Received: March 19, 2026; Revised: April 7, 2026; Accepted: April 10, 2026

Abstract: In this study, spherical aggregates were prepared by ovalbumin (OVA) and sodium tripolyphosphate at pH 7 and 65 °C for 0, 6, 12, 18, 24 h, and the properties of phosphorylated OVA (P-OVA) spherical aggregates forming and stabilizing Pickering O/W emulsions were studied. Results showed that the P-OVA heated for 12 h showed the highest surface hydrophobicity, which was demonstrated by static contact angles. The fluorescence intensity results showed that the more chromophore were exposed to the surrounding microenvironment because of protein conformational transitions. The dynamic tracking of P-OVA spherical aggregates was performed by transmission electron microscope, and the observation demonstrated that spherical aggregate formed after 12 h of heating were closely distributed and well dispersed, showing a very dense network shape. Besides, the emulsifying ability of P-OVA spherical aggregates was demonstrated by dynamic interfacial tension, which was due to the hydrophobic interaction in protein aggregates enabling the formation of stable Pickering nano-droplets. The Pickering emulsion stabilized by P-OVA spherical aggregates showed better salt ion stability (0–100 mmol/L NaCl) and thermal stability (0–95 °C) as compared to emulsions prepared by the unheated sample. These findings demonstrate the unique properties of spherical aggregates based on P-OVA for preparing Pickering emulsions, which can provide theoretical guidance in the food industry system.

Keywords: ovalbumin; phosphorylation; spherical aggregates; conformation; Pickering emulsion

Citation: Z. Y. Xiong, L. N. Zhang, J. H. Wang, et al., Heat-induced phosphorylated ovalbumin spherical aggregates as the O/W Pickering emulsion stabilizer: self-assembly mechanisms and emulsion properties, Food Sci. Anim. Prod. 4 (2026) 9240175. <https://doi.org/10.26599/FSAP.2026.9240175>.

1 Introduction

Heat-induced aggregation of globular proteins is a promising method for preparing anisotropic macrostructures in the food industry. At pH far from the isoelectric point, the protein structure unfolds, exposing more hydrophobic amino acids, and the protein macromolecules will be aggregated to form fibrous aggregates due to various factors such as hydrophobic interactions, van der Waals forces, and intermolecular forces; at or near the isoelectric point pH, however, globular aggregates formed by dominant hydrophobic interactions and accompanying hydrogen and disulfide bonds link protein molecules to each other^[1]. Ovalbumin (OVA, 45 kDa) was the most abundant protein of the total protein content of egg white (55%). More importantly, OVA is a major globular protein in egg white, and contains free sulfhydryl groups buried inside the hydrophobic core^[2]. As a natural amphiphilic biomolecule, OVA can adsorb on the interface to undergo rapid spatial conformational rearrangement, thereby stabilizing complex multiphase food systems^[3]. A previous study has reported that OVA has successfully been prepared Pickering emulsion, which displayed excellent stability against flocculation and coalescence^[4]. However, the stability of emulsions is highly affected by environmental factors

(e.g., temperature and ionic strength), resulting in delamination during storage, which is dependent on emulsion stabilizers.

Protein composition and amino acid side chain structure determine the proteins functional and physical properties. The chemical phosphorylation modification of proteins has been shown to be an essential modification method to effectively improve the functional properties of proteins^[5]. Protein phosphorylation occurs between the phosphate group and amino acid residues via covalently bound reactions by a protein kinase. Protein phosphorylation can increase the negatively charged groups and decrease the pH of protein, which notably changes in the functional properties. Previous studies have shown that phosphorylation modification can effectively improve foaming properties, emulsifying properties, and solubility. Xiong et al.^[6] reported that the phosphorus content of egg white protein (EWP) increased ~1.05% by dry-heating at pH 4.0 and 85 °C for 5 days in the presence of pyrophosphate, the heat stability, emulsifying properties, and digestibility of EWP were improved by phosphorylation. Nayak et al.^[7] reported that there was an increase in the solubilities of water and ionic when buffalo milk proteins were phosphorylated with phosphorus oxychloride over the pH range 3.0–9.0.

Inspired by these studies, we can hypothesize that phosphorylation modification would be a viable approach to improve the functional properties of OVA as a novel Pickering emulsion in the modern food industry. In naturally phosphorylated proteins, phosphate groups have shown essential effects on protein fibrillation aggregation behaviour. Gonzalez-Jordan et al.^[6] using magic-angle spinning ³¹P nuclear magnetic resonance (MAS ³¹P NMR) to demonstrate that the migration of calcium phosphate in casein micelles varies with environmental pH and is accompanied by changes in the morphology and density of casein aggregates. Therefore, phosphorylated OVA (P-OVA) spherical aggregates prepared through controlled heat treatment may provide an effective particulate stabilizer for gel-like Pickering emulsions after microfluidization. In the present study, OVA was phosphorylated in a liquid system in the presence of sodium tripolyphosphate (STPP) which is a safe and non-toxic food additive. The effects of the reaction time on the structural and physical properties of P-OVA were evaluated. Furthermore, P-OVA spherical aggregates were analyzed to stabilize Pickering emulsions in order to explore the influence of external environmental factors on the stability of emulsions. The P-OVA spherical aggregates produced with lasting heat treatment in this study would help to explore the key phenomena, which helped to prove the significant potential of protein aggregates applied in the food, cosmetics, and even petroleum fields.

2 Materials and methods

2.1 Materials and chemicals

Eggs used in this investigation were bought in the local market of Huazhong Agricultural University. Polyethylene glycol (PEG) 8000 was made by Merck Chemicals Co., Ltd. (Shanghai, China). STPP was purchased from Aladdin Industrial Co., Ltd. (Shanghai, China). Medium-chain triglyceride (MCT) was purchased from Yuanye Biological Technology Co., Ltd. (Shanghai, China). All other chemicals used in the experiment were produced from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), and were of analytical grade.

2.2 Extract of OVA

OVA was extracted following the method of Xiong et al.^[9] with slight modification. A 100 mL egg white separated from the yolk was mixed with twice the volume of deionized water, and adjusted to pH 7.5 with 1 mol/L HCl. PEG 8000 with a concentration of 12% (*m/m*) was added to the above mixture and stirred by a magnetic stirrer for 30 min, 25 °C, then centrifuged (9 000 × *g*, 15 min, 4 °C) (Model AG-22331, Eppendorf, Hamburg, Germany). The supernatant was stored at 4 °C for 12 h until the solution was stratified. The precipitates were discarded and the supernatant was centrifuged (9 000 × *g*, 15 min, 4 °C) again. The supernatant after centrifugation was freeze-dried, then the dry powder of OVA was stored in the refrigerator at 4 °C.

2.3 Phosphorylation of OVA

OVA was dissolved at a final concentration of 20 mg/mL in STPP solution (4 g/100 mL, pH 8.0), and the pH was adjusted with 1 mol/L HCl or NaOH. The mixture was then heated in a water bath at 50 °C for 2 h. The mixture sample was kept at 4 °C and dialyzed to remove free STPP for 2 days against deionized water. The dry P-OVA powder was stored at -80 °C after lyophilized for 48 h.

2.4 Preparation of P-OVA spherical aggregates

P-OVA powder was dissolved in deionized water at 1 g/100 mL, and the pH was adjusted to pH 7.0 by 1 mol/L HCl or NaOH. Then the solution was filtered through a protein filter to remove any traces of insoluble material. Protein solutions were subsequently heated at 65 °C in a water bath (HH-2, Changzhou Guohua Electrical Appliance Co., Changzhou, China) in sealed containers without agitation to minimize evaporation. Aliquots were taken at 0, 6, 12, 18, and 24 h (P-0, P-6, P-12, P-18, P-24), immediately quenched in ice-cold water, and equilibrated to room temperature before further measurements, then stored in a refrigerator at 4 °C.

2.5 Preparation of Pickering emulsion

Pickering emulsions were prepared by mixing P-OVA spherical aggregates samples (1 g/100 mL) and MCT at oil volume fraction of 0.1 according to Ruan et al.^[10] with some modification, then homogenized using a high-shear blender (XHF-DY, Ningbo Scientz Biotechnology Co., Ltd., Ningbo, China) at 10 000 r/min for 2 min in an ice bath. The resulting Pickering O/W emulsions were further processed by a microfluidizer (Microfluidics M-110L, Microfluidics Corp., Newton, MA, USA) three times at a pressure of 9 000 psi. The total emulsion volume was 50 mL, and the pH of each prepared sample was maintained to pH 7.0 with 0.1 or 1 mol/L HCl or NaOH, then sodium azide (0.04 g/100 mL) was added to prevent microbial growth. The emulsions were stored at room temperature (25 °C).

2.6 Intrinsic fluorescence intensity measurement of P-OVA spherical aggregates

Intrinsic fluorescence intensity was determined as reported by Lü et al.^[11] with slight modifications. The P-OVA spherical aggregates solution samples were diluted with deionized water to 0.5 mg/mL and tested with F-4600 fluorescence spectrophotometer (Hitachi High-Technologies Corp., Tokyo, Japan). The excitation wavelength was set at 280 nm and the emission spectra were recorded from 300 to 500 nm (slit width: 5 nm), in order to monitor the intrinsic fluorescence contributions from Trp, Tyr, and Phe residues. The deionized water was blanking.

2.7 Surface hydrophobicity measurement of P-OVA spherical aggregates

The surface hydrophobicity of P-OVA spherical aggregates was measured referring to the method of Kazemi-Taskooh et al.^[12] with light modification. 1-Anilinonaphthalene-8-sulfonic acid (ANS) was used as the fluorescence probe. P-OVA spherical aggregates samples were diluted to the concentration of 1, 0.8, 0.6, 0.4, and 0.2 mg/mL with PBS (10 mmol/L, pH 7.0). A 16 µL of ANS which was dissolved to 8 mmol/L with PBS (10 mmol/L, pH 7.0) was added to 6 mL of prepared P-OVA spherical aggregates samples, and the mixture was left for 15 min in the dark. The blanks without P-OVA spherical aggregates samples contained 16 µL of 8 mmol/L ANS (in 10 mmol/L, pH 7.0 PBS) and 6 mL of deionized water. Fluorescence intensity was measured with an F-4600 fluorescence spectrophotometer (Hitachi High-Technologies Corp., Tokyo, Japan) at excitation and emission wavelengths of 390 and 470 nm, respectively. The initial slope of fluorescence intensity versus protein concentration plot was used as surface hydrophobicity. All the data were subtracted by the blanks.

2.8 Circular dichroism spectrum measurement of P-OVA spherical aggregates

The secondary structures of P-OVA spherical aggregates were measured using a circular dichroism spectrometer (J-1500-150, JASCO Corp., Tokyo, Japan) according to the method of Shu et al.^[13] with slight changes. All samples were diluted to 0.5 mg/mL with deionized water and loaded into a 1 mm quartz cell. The circular dichroism spectra (200–250 nm) were obtained under the test condition of continuous nitrogen filling at 25 °C and scanned in 1 nm steps with a scanning speed of 100 nm/min. The deionized water blanks were subtracted from the sample spectra. Young's equation was used to calculate the secondary structural contents. Circular dichroism is represented by the average residue ellipse value (θ) in unit of deg·cm²/dmol.

2.9 Contact angle measurement of P-OVA spherical aggregates

The contact angle of the complexes was measured by using a contact angle analyzer (OCAH200, DataPhysics Instruments GmbH, Filderstadt, Germany). Freeze-dried sample powders were compressed into tablets with the same thickness (2 mm) and placed on slides. A drop of distilled water is deposited on the tablet surface. The image of the droplet after 10 s was recorded with a video camera to measure the contact angle, and three measurements were taken for each sample.

2.10 Transmission electron microscope (TEM) observation of P-OVA spherical aggregates

The P-OVA spherical aggregates were diluted with deionized water to 1 mg/mL and prepared by negative staining. The diluted samples were applied to a copper grid, then the excess was removed after 15 s with a piece of filter paper. A drop of staining solution (2 g/100 mL potassium phosphotungstate at pH 5.6 or 2 g/100 mL uranyl acetate at pH 3.8) was added, and the excess solution was removed again with filter paper after 15 s. Prepared grids were imaged using a TEM (JEM-2100F, JEOL Ltd., Tokyo, Japan).

2.11 Foaming capacity (FC) and foaming stability (FS) measurements of P-OVA spherical aggregates

The FC and FS of P-OVA spherical aggregates were calculated in triplicate as described by Dombrowski et al.^[14]. Take 30 mL of each sample solution (10 mg/mL) in a test tube by shaking at 10 000 r/min for 1 min with an ultra-turrax T25 homogenizer (IKAL aborteknik, Staufen, Germany). The final volume after shearing and the volume after 30 min were recorded for the calculation of the foaming properties by equations (1)–(2):

$$FC (\%) = \frac{V_1}{30} \times 100 \quad (1)$$

$$FS (\%) = \frac{V_2}{V_1} \times 100 \quad (2)$$

Where V_1 is the initial volume (mL) of the foam, and V_2 is the volume (mL) of the foam after 30 min.

2.12 Dynamic interfacial tension measurement of P-OVA spherical aggregates

The dynamic interfacial tension of P-OVA spherical aggregates at the oil-water interface was performed using a drop shape analysis

tenziometer (Tracker Teclis/IT Concept, Civrieux-d'Azergues, France) at 25 °C as reported by Chen et al.^[15] with minor modification. The bulk phase (P-OVA spherical aggregates) and oil phase (MCT) were placed in the cuvette and syringe, respectively. The shape of the oil drop formed at the tip of the syringe was recorded through the experiments using a charge-coupled device camera. The dynamic interfacial tension was calculated from the shape analysis of a pendant drop according to the Young-Laplace equation. The dynamic interfacial tension of all the solutions was monitored for 7 200 s at a constant area of 25 mm² of the sample cuvette. Deionized water was used as a control aqueous phase.

2.13 Z-average measurement of Pickering emulsions

The Z-average analysis was performed at 25 °C using a dynamic light scattering (DLS) instrument (Mastersizer Nano ZS; Malvern Ins., Malvern, UK) according to the report of Lara et al.^[16] with a scattering angle. Deionized water as the dispersion medium, and Pickering emulsions were diluted to an appropriate concentration before measurement to avoid multiple scattering. The Z-average diameter and polydispersity index (PDI) were obtained from the cumulants analysis, while the Z-average distribution was recorded separately as an intensity-based distribution.

2.14 Pickering emulsions structure observation with confocal laser scanning microscope (CLSM)

CLSM observations of Pickering emulsions were performed with a CLSM (OLYMPUS FV3000, Olympus, Tokyo, Japan) according to Liu et al.^[17] with some modification. A 1 mL of the emulsion was added into 10 µL of Nile red fluorescent dye (1 g/L) and the resultant emulsions were stored at room temperature in the dark for at least 2 h to accomplish staining. The dyed emulsions were then placed on concave microscope slides and covered with glycerol-coated coverslips. The excitation and emission wavelengths were 573 and 591 nm, respectively.

2.15 Rheological characterization of Pickering emulsions

The rheological properties of the Pickering emulsions were characterized at 25 °C with a hybrid rheometer HR-3 (TA Instruments, New Castle, DE, USA) with an aluminum parallel-plate geometry (diameter 60 mm) according to the method described by Sun et al.^[18] with minor modifications. The oscillatory stress sweep was used to measure the linear viscoelastic response of each sample in the oscillatory shear condition at a frequency of 1 Hz, then the frequency sweep measurements were conducted to evaluate the change of the storage modulus (G') and loss modulus (G'') in the linear viscoelastic range of 0.01–10 Hz with 1% strain. Steady-shear measurements were performed to obtain viscosity as a function of shear rate.

2.16 Emulsion stability as affected by pH and ionic strength

Pickering emulsions were adjusted to pH 7.0, heated at room temperature (25 °C), 50, 65, 80 and 95 °C for 20 min, then cooled to room temperature. And a 1.0 mol/L NaCl stock solution was added to emulsions to obtain a NaCl concentration range of 0–100 mmol/L. The emulsion droplet size was measured as section 2.13.

2.17 Statistical analysis

Analysis of variance was performed by SPSS (SPSS Inc. IBM SPSS Statistics for Windows, Armonk, IBM Corp., NY, USA) using

Duncan's multiples comparison test. All measurements were carried out triplicate and the values were expressed as mean $\pm$ standard deviation (SD). The significance of data was defined at $P < 0.05$. All figures were generated using Origin 8.0 software (Origin Lab Inc., USA).

3 Results and discussion

3.1 Fluorescence spectroscopy analysis of P-OVA spherical aggregates

Protein fluorescence from Trp, Tyr and Phe, whose fluorescence spectra are extremely sensitive to their microenvironment, provided a useful approach to evaluate conformational changes in proteins^[19]. As shown in Figure 1A, the fluorescence spectra of P-0 and heated samples (P-6, P-12, P-18, and P-24) showed a maximum emission peak around 337 nm under the wavelength of exciting light of 280 nm. The peak wavelength of P-0 sample was 336.7 nm, and the peak wavelength of P-6 sample was slightly red-shifted (0.5 nm) after prolonged heating, indicating that the internal hydrophobic amino acids were exposed to a polar environment and the stretching of the protein molecules increased^[30]. Moreover, the fluorescence intensity of heated samples decreased significantly with increasing heating time compared with P-0, which was consistent with the findings of other researchers, probably due to phosphorylation^[21]. The phosphate group was embedded in the Trp side chain of OVA and the molecular negativity increased, resulting in a change in the conformation of the protein.

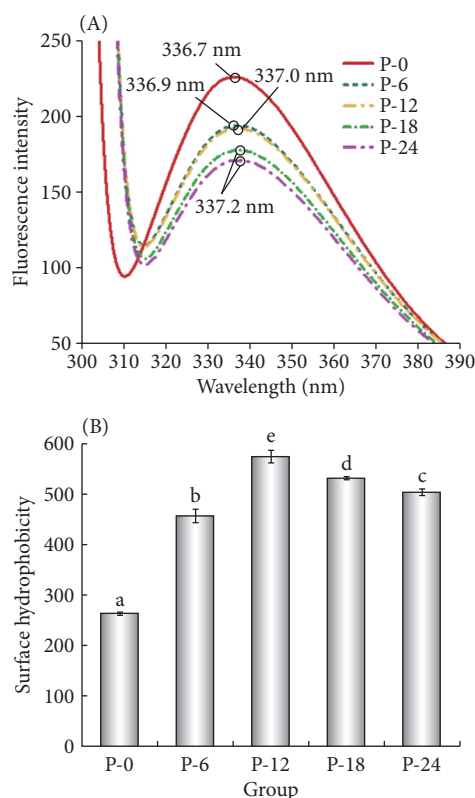


Figure 1 (A) Changes of intrinsic fluorescence intensity of P-OVA spherical aggregates. (B) Changes of surface hydrophobicity of P-OVA spherical aggregates. Values are given as the mean $\pm$ SD ($n = 3$). Different lowercase letters indicate significant differences ($P < 0.05$).

3.2 Surface hydrophobicity of P-OVA spherical aggregates

ANS is a class of fluorescent probes that are very sensitive to environmental polarity and can be used to measure fluorescence intensity to characterize the exposure of hydrophobic amino acids to protein particles^[22]. The surface hydrophobicity values of P-0 and heated samples (P-6, P-12, P-18, P-24) are shown in Figure 1B. OVA comprises 385 amino acids, more than 50% of which are hydrophobic, but most of which are hidden inside the protein. Compared to P-0 (266), the surface hydrophobicity of P-12 reached the maximum (558), 2.1-fold of P-0. Heat treatment for a specific time exposed the hydrophobic residues inside the phosphorylated oval particles and increased the hydrophobic surface value, as evidenced by circular dichroism and fluorescence spectroscopy. Except for heat treatment, the phosphorylation reaction of STPP with OVA may cause the total or partial unfolding of the protein chain, resulting in the addition of hydrophobic amino acids in the protein. However, with the heating time increased, surface hydrophobicity of P-18 and P-24 samples was lower than P-12, probably due to the more hydrophobic amino acids were exposed during the late heating period, the greater their chance of aggregation through hydrophobic interactions. Moreover, amino acid residues would covalently cross-link phosphate groups by phosphorylation, thus increasing negatively charged groups of the protein, which affected the contact of the hydrophobic groups of samples and ANS can be partially explained about the decrease of the surface hydrophobicity^[23].

3.3 Circular dichroism of P-OVA spherical aggregates

As shown in Figure 2A, there was no significant difference in the peak shape of the circular dichroism of P-0 compared with that of heated samples (P-6, P-12, P-18, P-24). An obvious positive peak band was appeared around 195 nm^[24]. And a negative band around 210–220 nm, which are characteristic of proteins containing mixed α -helix and β -sheet conformations. Compared to P-0 sample, the secondary structure changes of the protein could be divided into two phases. As shown in Figure 2B, before heating for 12 h, heated samples (P-6, P-12) had a decreased proportion of α -helix structure and an increased proportion of β -sheet structure, the α -helix relative content gradually reduced from 21.4% to 17% and the β -sheet increased from 50.2% to 52.8%. Heating of P-OVA for 6 and 12 h promoted the conformational change of the peptide chain and led to protein de-folding. Heat treatment disrupted the hydrogen bond in the α -helix structure, converted α -helix to the β -sheet and caused the protein to aggregate to form spherical aggregates^[25]. The extension of heating time to 18 and 24 h resulted in no significant changes in α -helix and β -sheet relative contents, probably due to long heating time that saturated the protein with refolding, which was not conducive to the formation of homogeneous spherical aggregates of uniform size. The β -turn relative content remained relatively low and changed only slightly, while the random coil relative content increased after prolonged heating, indicating partial unfolding and enhanced conformational disorder. The results indicated that the formation of P-OVA spherical aggregates requires conditions of high temperature and relatively long time but excessively temperature and heating time might lower the β -sheet structure relative content^[26].

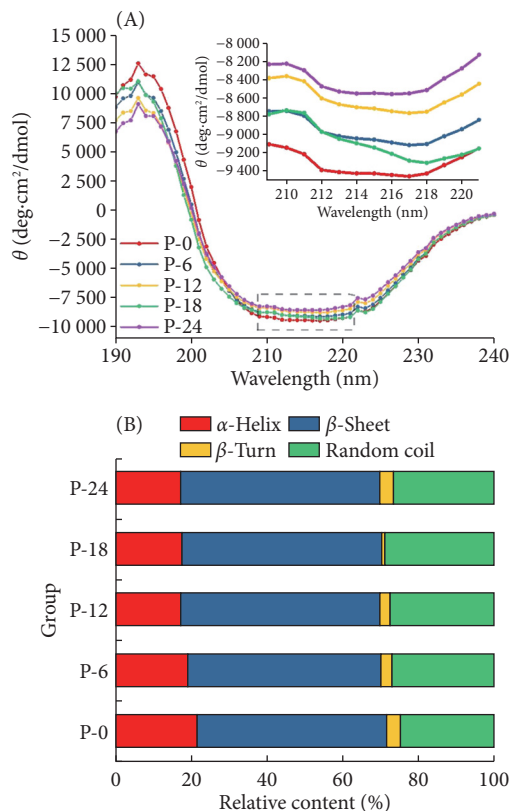


Figure 2 Changes of (A) circular dichroism spectra and (B) the relative contents of secondary structure in P-OVA spherical aggregates.

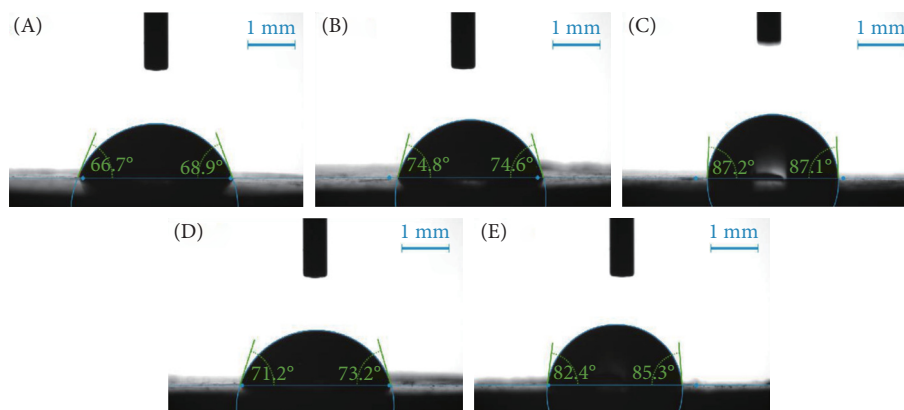


Figure 3 Changes of the static contact angle in P-OVA spherical aggregates. (A) P-0; (B) P-6; (C) P-12; (D) P-18; (E) P-24.

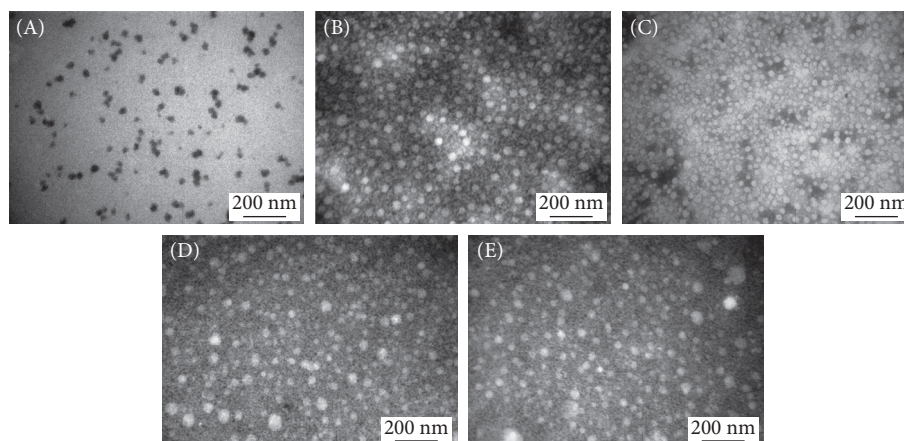


Figure 4 Changes of the microstructure in P-OVA spherical aggregates. (A) P-0; (B) P-6; (C) P-12; (D) P-18; (E) P-24.

3.4 Static contact angle of P-OVA spherical aggregates

Contact angle is an index used to characterize the surface wettability of aggregates. The wettability of spherical aggregates is a crucial property controlling the formation and stability of Pickering emulsions^[27]. As shown in Figure 3, the static contact angles of P-OVA spherical aggregates were 67.8° (P-0), 74.7° (P-6), 87.2° (P-12), 72.2° (P-18) and 83.9° (P-24), respectively, and the static contact angles of the heated P-OVA were all greater than that of the unheated P-OVA. This result indicated that heat treatment facilitated the exposure of hydrophobic groups on the protein surface, thus increasing the hydrophobicity of the protein fibre surface. The spherical particles of P-OVA stabilized Pickering emulsions by rapidly adsorbing to the oil-water interface and forming an interfacial film and the electrostatic repulsion between the particles causes the particles to orient and prevent the aggregation of oil droplets, which stabilized the entire emulsion system^[28].

3.5 Morphology observation of P-OVA spherical aggregates

As shown in Figure 4, the P-OVA was basically in the form of protein monomer particles, while P-OVA under pH 7.0 conditions showed a spherical aggregated morphology with an average diameter of 100–200 nm when heat-treated at 65 °C for different times. Similarly, it has been reported that protein aggregates form spherical aggregates when the protein is heated under neutral conditions^[29]. P-OVA by heated 6 and 12 h would undergo refolding and exposed more hydrophobic amino acids, resulting in spherical aggregates that are closely distributed and well dispersed under hydrophobic interactions, showing a very dense network

structure. As the heating time increased, a small amount of aggregation between phosphorylated protein particles was observed in Figures 4D and E. The distribution is more loosely distributed and not in tight junctions, as also evidenced by the measured Z-average diameter and the larger the average Z-average diameter formed after heating^[30].

3.6 Foaming properties of P-OVA spherical aggregates

Foam is a typical gas-water two-phase system in which proteins are adsorbed from the gas-liquid interface to the air bubble using stirring, and the molecules are joined to each other to form an interfacial film to wrap the air bubble and form a stable system of proteins. Figure 5A shows the change in foaming properties of samples under different reaction time. Heat treatment time showed a significant effect on foaming properties of P-OVA spherical aggregates. A possible explanation for this phenomenon was that heated samples (P-6, P-12, P-18, P-24) were more easily adsorbed at the gas-liquid interface, P-OVA would directly align at the interface by hydrophobic or electrostatic interaction^[31]. Sun et al.^[18] found that the FC of protein could be promoted due to the protein conformation changes with the increasing surface hydrophobicity. Surface hydrophobicity measurements showed that the heated samples protein molecules exposed more hydrophobic groups. Compared to the unheated sample ($95.49 \pm 1.53\%$), the FS of heated samples (P-6, P-12, P-18, P-24) showed a slight increase, probably because the heat treatment increased the interfacial charge at the air-water interface, thus inhibiting inter-foam aggregation by forming a structurally dense and stable interfacial film. However, with the increase of thermal time, the formed interfacial film ruptured, and the protein molecules aggregated, leading to further structural instability, which affected the FC and FS of the protein due to the limitation of the aqueous solution^[32].

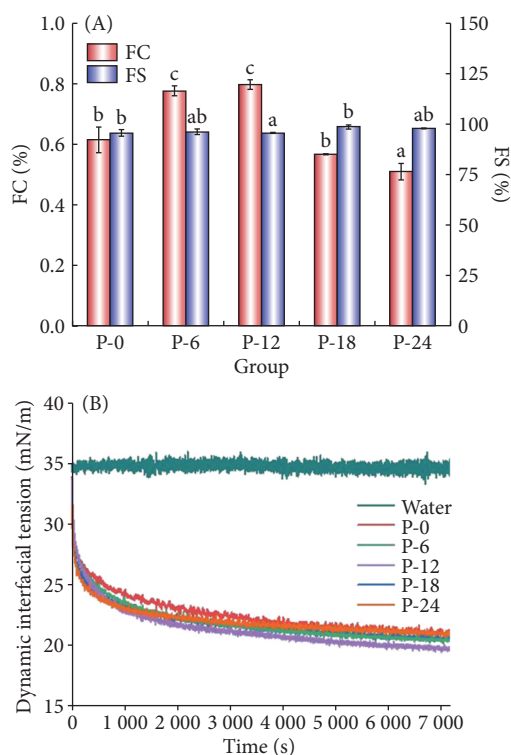


Figure 5 (A) Effect of heat treatment on the foaming properties of P-OVA spherical aggregates. (B) Time-dependence of interfacial tension of P-OVA spherical aggregates adsorbed at oil-water interface. Values are given as the mean $\pm$ SD ($n = 3$). Different lowercase letters indicate significant differences ($P < 0.05$).

3.7 Dynamic interfacial tension of P-OVA spherical aggregates

Time evolution of interfacial tension of P-OVA, P-OVA spherical aggregates, and deionized water at the oil-water interface was investigated by detecting the changes. As shown in Figure 5B, the interfacial tension of all samples decreased with the increase of adsorption time, and the rate of change of interfacial tension with time was different for different samples. The P-OVA spherical aggregates heated with different times (6, 12, 18, 24 h) remarkably lowered the interfacial tension to equilibrium values of 20.3, 19.6, 20.5, 20.9 mN/m, possible due to the reason that P-OVA is amphiphilic biomolecules with the surface activity that can reduce interfacial tension by adsorbing to the oil-water interface and forms a protective coating^[33]. It could be found that the interfacial tensions of the samples were different at different heating stages of the spherical aggregates transformation, which may be due to the more disordered structure of heated samples (P-6, P-12, P-18, P-24) compared to P-0, and the conformational changes of P-OVA spherical aggregates exposed the hydrophobic amino acids inside the molecule, allowing the P-OVA protein molecules to adsorb at the oil-water boundary through hydrophobic interactions, and the hydrophobic amino acids to rearrange more easily in MCT, resulting in a lower interfacial equilibrium. The differences in interfacial tension were likely related to the combined effects of surface hydrophobicity, particle morphology, and conformational flexibility. When the heating time reached 18 or 24 h, partial aggregation and reduced accessible hydrophobicity weakened interfacial adsorption efficiency, resulting in slightly higher equilibrium interfacial tension^[34].

3.8 Z-average and PDI of Pickering emulsions

The Z-average diameters and PDI values of Pickering emulsions stabilized by P-OVA spherical aggregates are shown in Figure 6A. Compared with the emulsion prepared from P-0, the Z-average of emulsions stabilized by heated samples increased, indicating that heat-induced spherical aggregates formed larger particulate stabilizers at the oil-water interface^[35]. The PDI decreased from 0.24 for P-0 to about 0.21 for P-6 and then increased slightly with further heating, indicating that moderate heating improved size uniformity, whereas prolonged heating caused broader Z-average distribution due to aggregation. This phenomenon was attributed to aggregation of proteins via hydrophobic interactions during heat treatment, resulting in a relatively monodisperse protein particle distribution^[36]. Figure 6B shows the Z-average distribution corresponding to different heating times of the Pickering emulsions. The heated P-OVA samples (P-6, P-12, P-18, and P-24) showed larger Z-averages and monomodal Z-average distributions compared with the unheated sample.

3.9 Morphology observation of Pickering emulsions

The CLSM images obtained for Pickering emulsions labeled using Nile red clearly confirmed the adsorption of P-OVA spherical aggregates at the oil-water interface in Figure 7. The oil droplets of P-OVA emulsions were stained red by Nile red dye. The distribution of emulsion droplets in the figure was relatively homogeneous, and the emulsions stabilized by P-OVA spherical aggregates were milky white and homogeneous under any heat treatment conditions, and there was no flocculation or agglomeration. The introduction of P-OVA caused a change in the surface charge of the protein molecules, which increased the

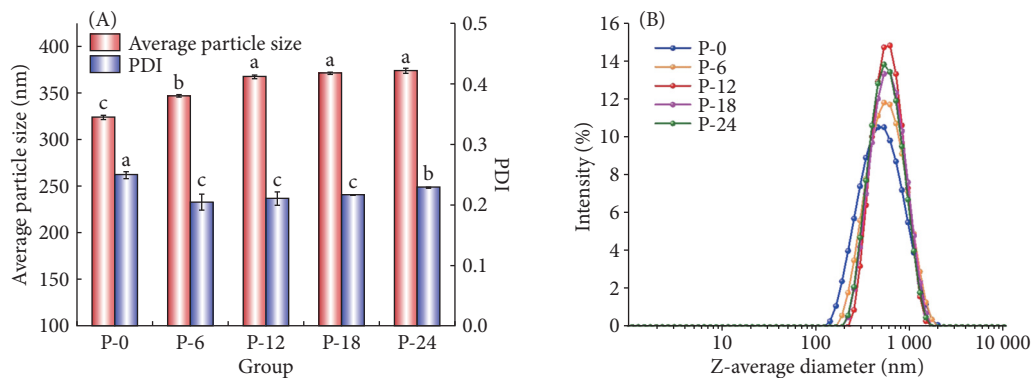


Figure 6 (A) Effect of different heating times on the average particle size and PDI of Pickering emulsions. (B) The particle size distribution of Pickering emulsion. Values are given as the mean $\pm$ SD ($n = 3$). Different lowercase letters indicate significant differences ($P < 0.05$).

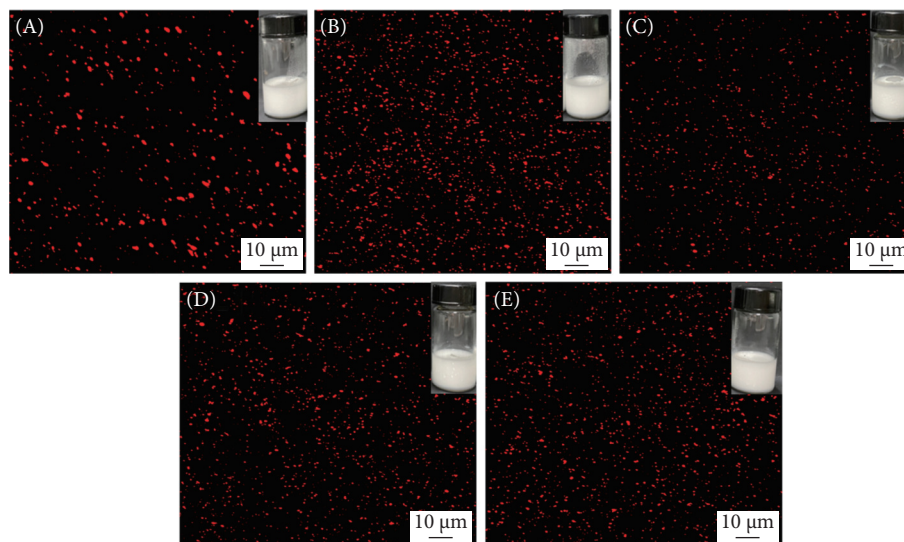


Figure 7 Micro morphology observation of the fresh Pickering emulsion stabilized by P-OVA spherical aggregates. (A) P-0; (B) P-6; (C) P-12; (D) P-18; (E) P-24.

electrostatic repulsion and reduced the surface tension, thus improving the stability of the emulsions. It is also possible that because the formation of spherical aggregates enhances the surface hydrophobicity of heated samples (P-6, P-12, P-18, P-24), such particles have stronger adsorption at the oil-water interface and form a dense interfacial film, the more homogeneous and stable Pickering emulsions are stabilized^[37]. As seen in Figures 7D–E, when the heating time was 18 h or longer, partial aggregation was observed, which may be due to the long heating time leading to overstretching of heated samples (P-6, P-12, P-18, P-24) structure and aggregation into larger particles, making the spherical aggregates stable emulsions lose their ability to resist agglomeration^[38].

3.10 Rheological characteristics of Pickering emulsions

The rheological behaviour of the emulsion was significantly influenced by the heating time of P-OVA. In order to better understand the macroscopic properties of P-OVA spherical aggregates, the effect of heating time on the rheological properties of heated samples (P-6, P-12, P-18, P-24) was investigated using a dynamic rheological approach. As shown in Figures 8A and B, G' increased with frequency for all samples, while G'' generally increased at low-to-middle frequencies but decreased at high frequency for P-6. All samples have higher values of G' than their corresponding G'' at either frequency, indicating that all samples

showed gel-like properties^[39]. No structural damage occurred under the increased frequency, suggesting that the introduction of phosphate groups may have played a role in stabilizing the protein network structure, thereby mitigating heat-induced structural disruption. Figure 8C shows the dynamic viscosity curves of P-OVA emulsions with different heating times as a function of shear rate. The viscosities all decreased with increasing shear rate, which indicates that the P-OVA spherical aggregate-stabilized emulsions all have shear thinning properties^[40]. Furthermore, the viscosity and elasticity of the emulsions were maintained, and phosphorylation did not affect the non-Newtonian fluid properties of the P-OVA spherical aggregate-stabilized Pickering emulsions.

3.11 Storage stability of emulsion droplets

In the food industry, where Pickering emulsions are applied to food systems, environmental factors are particularly important in influencing their processing. Figure 9A compares the variation of Z-average of Pickering emulsions stabilized by P-OVA spherical aggregates with different ionic strengths. The Z-average of Pickering emulsions stabilized by P-0 sample increased from 323.4 to 811.7 nm with increasing ionic strength when the NaCl concentration increased from 0 to 100 mmol/L. This may be due to the fact that the addition of salt ions creates an electrostatic shielding effect, which weakens the electrostatic repulsion between emulsions and causes aggregation between emulsions, resulting in

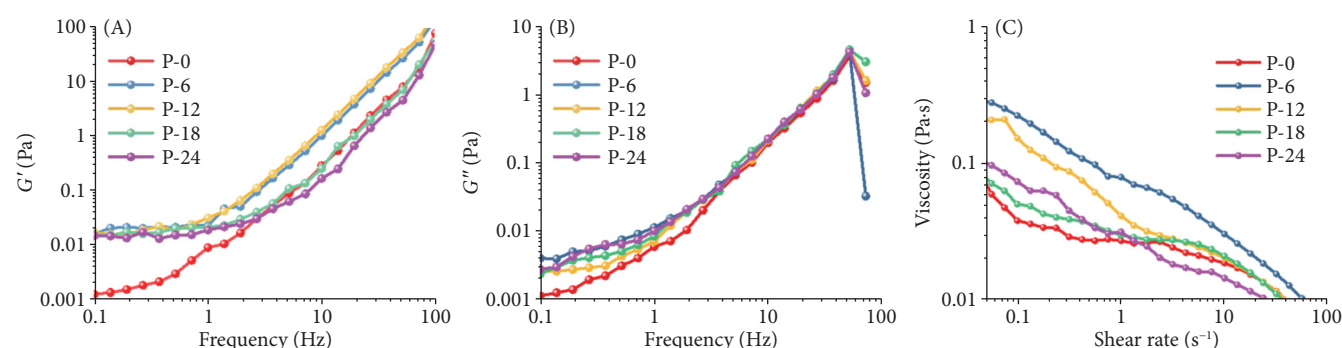


Figure 8 Effect of frequency sweep of (A) G' , (B) G'' and (C) viscosity versus shear rate of Pickering emulsion stabilized by P-OVA spherical aggregates.

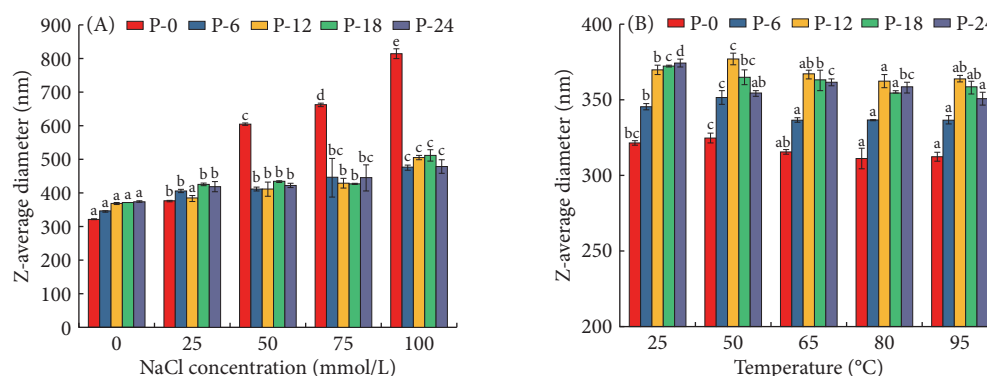


Figure 9 Particle stability at different (A) ionic strengths and (B) temperatures of Pickering emulsions stabilized by P-OVA spherical aggregates. Values are given as the mean $\pm$ SD ($n = 3$). Different lowercase letters indicate significant differences ($P < 0.05$).

an increase in the mean particle size^[41]. In contrast, Pickering emulsions stabilized by P-OVA spherical aggregates showed a non-significant effect of Z-average by salt ion concentration, which indicates that Pickering emulsions stabilized by P-OVA spherical aggregates have good salt ion tolerance and can retard the destructive effect of salt ions on emulsions, which is also related to the good emulsification properties of P-OVA spherical aggregates and the adsorption capacity of spherical aggregates at the interface of two phases to the salt ion^[42].

Figure 9B compares the changes in Z-average of Pickering emulsions stabilized by P-OVA spherical aggregates at different temperatures. The Z-average of the P-0 emulsion showed a slight decreasing or fluctuating trend with increasing temperature, whereas the emulsions stabilized by heated P-OVA spherical aggregates maintained Z-averages within a relatively narrow range. This result suggests that the heat-induced P-OVA aggregates conferred improved thermal tolerance to the Pickering emulsions. The enhanced stability may be attributed to the more flexible interfacial conformation of heat-treated protein molecules, which facilitated their adsorption and rearrangement at the oil-water interface^[43]. At the same time, the heat treatment exposed some hydrophobic groups of protein molecules on the emulsion surface, which improved the adsorption of liposomal surface proteins at the oil-water interface and increased the interfacial strength of the emulsion^[44].

4 Conclusion

P-OVA spherical aggregates were developed at pH 7.0 with an OVA concentration of 1 g/100 mL at 65 °C during a long heating time and were proved as an effective stabilizer for Pickering emulsion. A mechanism of protein aggregates formation has been

investigated and the formation of protein aggregates was contributed by the hydrophobic force. At the heat treatment time of 12 h, the P-OVA spherical aggregates exhibited the strongest surface hydrophobicity and the largest static contact angle. The interfacial tension results indicated that the P-OVA spherical aggregates had electrostatic interaction and could be well adsorbed at oil-water interface, confirming that P-OVA spherical aggregates exhibited excellent emulsification and prepared Pickering emulsion had good stability. In addition, compared to Pickering emulsions stabilized by unheated P-OVA, Pickering emulsions stabilized by P-OVA spherical aggregates showed enhanced salt ion tolerance and higher thermal stability. A better understanding of the process of protein aggregation formation using P-OVA could provide benefits for the use of P-OVA spherical aggregates as an emulsifier. The findings would be of importance for the applications of protein aggregation in functional foods and beverages in the future, making way for broader applications in the industry. It should be noted that a direct comparison with non-phosphorylated OVA under the same heating conditions was not included; therefore, the individual contributions of phosphorylation and thermal aggregation cannot be fully distinguished.

Declaration of interest statement

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.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (32572637).

Author contributions

Zhouyi Xiong: Writing—original draft, investigation, funding acquisition. Lina Zhang: Visualization, investigation, data curation. Jihui Wang: Writing—review & editing, resources, methodology. Yanxue Cai: Formal analysis, data curation. Shan Xiao: Supervision, project administration, methodology.

References

- [1] Y. Xun, Z. Y. Xiong, Y. T. Song, et al., Role of phosphate modification in enhancing ovalbumin fibril formation and functionality: insights into molecular interactions and structural dynamics, *Innov. Food Sci. Emerg. Technol.* 102 (2025) 103992. <https://doi.org/10.1016/j.ifset.2025.103992>.
- [2] M. Cheng, M. X. Pu, J. Y. Chen, et al., Multiscale non-covalent assembly of proteins and polysaccharides from mechanisms to functional enhancements and applications: a review, *Int. J. Biol. Macromol.* 327 (2025) 147381. <https://doi.org/10.1016/j.ijbiomac.2025.147381>.
- [3] Y. T. Xu, C. H. Tang, T. X. Liu, et al., Ovalbumin as an outstanding Pickering nanostabilizer for high internal phase emulsions, *J. Agric. Food Chem.* 66 (2018) 8795–8804. <https://doi.org/10.1021/acs.jafc.8b02183>.
- [4] S. S. Wu, S. B. Liu, Y. Li, et al., Recent research advances in delivery systems based on the assembly of egg white proteins: structure design and applications in the food industry, *Food Hydrocoll.* 153 (2024) 110021. <https://doi.org/10.1016/j.foodhyd.2024.110021>.
- [5] J. H. Li, M. H. Zhao, X. R. Wang, et al., Molecular structure changes induced by egg white protein storage: from interfacial properties to Pickering emulsion stability, *Food Hydrocoll.* 172 (2026) 112099. <https://doi.org/10.1016/j.foodhyd.2025.112099>.
- [6] Z. Y. Xiong, M. J. Zhang, M. H. Ma, et al., Emulsifying properties of ovalbumin: improvement and mechanism by phosphorylation in the presence of sodium tripolyphosphate, *Food Hydrocoll.* 60 (2016) 29–37. <https://doi.org/10.1016/j.foodhyd.2016.03.007>.
- [7] S. K. Nayak, S. Arora, J. S. Sindhu, et al., Effect of chemical phosphorylation on solubility of buffalo milk proteins, *Int. Dairy J.* 16 (2006) 721–727. <https://doi.org/10.1016/j.idairyj.2005.03.007>.
- [8] A. Gonzalez-Jordan, P. Thomar, T. Nicolai, et al., The effect of pH on the structure and phosphate mobility of casein micelles in aqueous solution, *Food Hydrocoll.* 51 (2015) 88–94. <https://doi.org/10.1016/j.foodhyd.2015.02.004>.
- [9] Z. Y. Xiong, M. H. Ma, G. F. Jin, et al., Effects of site-specific phosphorylation on the mechanical properties of ovalbumin-based hydrogels, *Int. J. Biol. Macromol.* 102 (2017) 1286–1296. <https://doi.org/10.1016/j.ijbiomac.2017.05.028>.
- [10] Q. J. Ruan, J. Guo, Z. L. Wan, et al., pH switchable Pickering emulsion based on soy peptides functionalized calcium phosphate particles, *Food Hydrocoll.* 70 (2017) 219–228. <https://doi.org/10.1016/j.foodhyd.2017.03.023>.
- [11] Y. Q. Lü, L. Chen, H. Z. Wu, et al., (–)-Epigallocatechin-3-gallate-mediated formation of myofibrillar protein emulsion gels under malondialdehyde-induced oxidative stress, *Food Chem.* 285 (2019) 139–146. <https://doi.org/10.1016/j.foodchem.2019.01.147>.
- [12] Z. Kazemi-Taskooh, M. Varidi, F. Shi, et al., Double-aimed superstructure: delivering nutritional divalent cations as whey protein isolate fibril mediator to stabilize tunable high internal phase Pickering emulsions (HIPPEs), *Food Chem.* 509 (2026) 148604. <https://doi.org/10.1016/j.foodchem.2026.148604>.
- [13] J. J. Shu, K. Y. Yong, F. Johler, et al., Fourier-based classification of protein secondary structures, *Biochem. Biophys. Res. Commun.* 485 (2017) 731–735. <https://doi.org/10.1016/j.bbrc.2017.02.117>.
- [14] J. Dombrowski, F. Johler, M. Warncke, et al., Correlation between bulk characteristics of aggregated β -lactoglobulin and its surface and foaming properties, *Food Hydrocoll.* 61 (2016) 318–328. <https://doi.org/10.1016/j.foodhyd.2016.05.007>.
- [15] H. L. Chen, D. J. McClements, E. M. Chen, et al., *In situ* interfacial conjugation of chitosan with cinnamaldehyde during homogenization improves the formation and stability of chitosan-stabilized emulsions, *Langmuir* 33 (2017) 14608–14617. <https://doi.org/10.1021/acs.langmuir.7b03852>.
- [16] C. Lara, S. Gourdin-Bertin, J. Adamcik, et al., Self-assembly of ovalbumin into amyloid and non-amyloid fibrils, *Biomacromolecules* 13 (2012) 4213–4221. <https://doi.org/10.1021/bm301481v>.
- [17] C. H. Liu, R. C. Wang, S. H. He, et al., The stability and gastro-intestinal digestion of curcumin emulsion stabilized with soybean oil bodies, *LWT-Food Sci. Technol.* 131 (2020) 109796. <https://doi.org/10.1016/j.lwt.2020.109796>.
- [18] J. J. Sun, C. H. Chang, Y. J. Su, et al., Impact of saccharides on the foam properties of egg white: correlation between rheological, interfacial properties and foam properties, *Food Hydrocoll.* 122 (2022) 107088. <https://doi.org/10.1016/j.foodhyd.2021.107088>.
- [19] Y. N. Ni, G. L. Liu, S. Kokot, et al., Fluorescence spectrometric study on the interactions of isoprocarb and sodium 2-isopropylphenate with bovine serum albumin, *Talanta* 76 (2008) 513–521. <https://doi.org/10.1016/j.talanta.2008.03.037>.
- [20] Y. Q. Ma, Y. Zhao, Y. Q. Jiang, et al., Effect of dry heating on the aggregation behaviour and aggregate morphologies of ovalbumin, *Food Chem.* 285 (2019) 296–304. <https://doi.org/10.1016/j.foodchem.2018.12.078>.
- [21] L. X. Ding, Q. N. Zhao, X. Zhou, et al., Changes in protein structure and physicochemical properties of egg white by super critical carbon dioxide treatment, *J. Food Eng.* 284 (2020) 110076. <https://doi.org/10.1016/j.jfoodeng.2020.110076>.
- [22] Z. Y. Xiong, T. T. Weng, Y. X. Cai, et al., Short-term phosphorylation regulates ovalbumin self-assembly into functional aggregates at the oil-water interface: structural transformation and interfacial film-forming mechanisms, *Food Hydrocoll.* 157 (2025) 111877. <https://doi.org/10.1016/j.foodhyd.2025.111877>.
- [23] G. Hu, Z. Batool, Z. X. Cai, et al., Production of self-assembling acylated ovalbumin nanogels as stable delivery vehicles for curcumin, *Food Chem.* 355 (2021) 129635. <https://doi.org/10.1016/j.foodchem.2021.129635>.
- [24] H. L. Chen, R. N. Zhao, J. J. Hu, et al., One-step dynamic imine chemistry for preparation of chitosan-stabilized emulsions using a natural aldehyde: acid trigger mechanism and regulation and gastric delivery, *J. Agric. Food Chem.* 68 (2020) 5412–5425. <https://doi.org/10.1021/acs.jafc.0c01215>.
- [25] J. L. Dong, X. Yu, L. E. Dong, et al., *In vitro* fermentation of oat β -glucan and hydrolysates by fecal microbiota and selected probiotic strains, *J. Sci. Food Agric.* 97 (2017) 5131–5138. <https://doi.org/10.1002/jsfa.8292>.
- [26] Z. Y. Xiong, T. Yu, J. R. Lü, et al., Chitin nanofiber-stabilized pickering emulsion interacting with egg white protein: structural features, interfacial properties, and stability, *Food Hydrocoll.* 149 (2024) 110866. <https://doi.org/10.1016/j.foodhyd.2024.110866>.
- [27] V. Schmitt, V. Ravaine, J. Adamcik, et al., Surface compaction versus stretching in Pickering emulsions stabilised by microgels, *Curr. Opin. Colloid Interface Sci.* 18 (2013) 532–541. <https://doi.org/10.1016/j.cocis.2013.11.004>.
- [28] E. Dickinson, F. Johler, M. Warncke, et al., Use of nanoparticles and microparticles in the formation and stabilization of food emulsions, *Trends Food Sci. Technol.* 24 (2012) 4–12. <https://doi.org/10.1016/j.tifs.2011.09.005>.
- [29] J. H. Li, H. M. Wang, Z. Wang, et al., Modification of water-soluble protein nanoparticles and their effects on the properties of Pickering emulsions: impacts of acidic and thermal

- treatments, *Int. J. Biol. Macromol.* 349 (2026) 150872. <https://doi.org/10.1016/j.ijbiomac.2026.150872>.
- [30] T. Yang, T. X. Liu, X. T. Li, et al., Novel nanoparticles from insoluble soybean polysaccharides of Okara as unique Pickering stabilizers for oil-in-water emulsions, *Food Hydrocoll.* 94 (2019) 255–267. <https://doi.org/10.1016/j.foodhyd.2019.03.035>.
- [31] L. C. Wang, H. Zhang, H. Y. Li, et al., Fabrication and digestive characteristics of high internal phase Pickering emulsions stabilized by ovalbumin-pectin complexes for improving the stability and bioaccessibility of curcumin, *Food Chem.* 392 (2022) 133055. <https://doi.org/10.1016/j.foodchem.2022.133055>.
- [32] N. Z. Wang, C. Ma, R. L. Li, et al., Synergistic modification of ovalbumin by pH-driven and metal-phenolic networks: development of dysphagia friendly high internal phase Pickering emulsions, *Int. J. Biol. Macromol.* 289 (2025) 138842. <https://doi.org/10.1016/j.ijbiomac.2024.138842>.
- [33] L. Lü, Y. J. Chi, Improvement of functional properties of ovalbumin phosphorylated by dry-heating in the presence of pyrophosphate, *Eur. Food Res. Technol.* 235 (2012) 981–987. <https://doi.org/10.1007/s00217-012-1831-7>.
- [34] S. T. Tang, J. L. Yu, L. Z. Lu, et al., Interfacial and enhanced emulsifying behavior of phosphorylated ovalbumin, *Int. J. Biol. Macromol.* 131 (2019) 293–300. <https://doi.org/10.1016/j.ijbiomac.2019.03.076>.
- [35] Q. Zeng, W. H. Zeng, Y. G. Jin, et al., Construction and evaluation of ovalbumin-pullulan nanogels as a potential delivery carrier for curcumin, *Food Chem.* 367 (2022) 130716. <https://doi.org/10.1016/j.foodchem.2021.130716>.
- [36] K. Y. Li, Y. Zhou, G. Q. Huang, et al., Preparation of powdered oil by spray drying the Pickering emulsion stabilized by ovalbumin-gum Arabic polyelectrolyte complex, *Food Chem.* 391 (2022) 133223. <https://doi.org/10.1016/j.foodchem.2022.133223>.
- [37] R. Ramzan, Z. Muhammad, F. H. Shi, et al., Stability of conventional vs. Pickering emulsions using ovalbumin-anionic starch complexes for spray-dried lipid microcapsules, *Food Chem.: X* 31 (2025) 103102. <https://doi.org/10.1016/j.fochx.2025.103102>.
- [38] L. Yuan, Y. A. Liu, J. Ge, et al., Effects of heat treatment at two temperatures on the myosin cluster of bighead carp for gel formation, *CyTA-J. Food* 15 (2017) 574–581. <https://doi.org/10.1080/19476337.2017.1321045>.
- [39] M. Quevedo, U. Jandt, U. Kulozik, et al., Investigation on the influence of high protein concentrations on the thermal reaction behaviour of β -lactoglobulin by experimental and numerical analyses, *Int. Dairy J.* 97 (2019) 99–110. <https://doi.org/10.1016/j.idairyj.2019.06.004>.
- [40] J. F. Ye, X. Hua, W. B. Zhang, et al., Emulsifying capacity of peanut polysaccharide: improving interfacial property through the co-dissolution of protein during extraction, *Carbohydr. Polym.* 273 (2021) 118614. <https://doi.org/10.1016/j.carbpol.2021.118614>.
- [41] E. Fisicaro, C. Compari, A. Braibanti, et al., Hydrophobic hydration processes thermal and chemical denaturation of proteins, *Biophys. Chem.* 156 (2011) 51–67. <https://doi.org/10.1016/j.bpc.2011.06.004>.
- [42] L. L. Liu, H. Chen, Y. G. Wang, et al., Characterization of quercetin-ovalbumin complexes stabilizing emulsions: a perspective on the complex binding mechanism, *Int. J. Biol. Macromol.* 307 (2025) 141970. <https://doi.org/10.1016/j.ijbiomac.2025.141970>.
- [43] Z. Y. Xiong, T. T. Weng, J. H. Wang, et al., Regulatory mechanisms of phosphorylation in ovalbumin self-assembly for aggregates formation: insights into size, structure, and morphology, *Food Hydrocoll.* 48 (2024) 110431. <https://doi.org/10.1016/j.foodhyd.2024.110431>.
- [44] Q. Q. Liu, Q. Zhou, Y. J. Li, et al., Spherical and fibrillar ovalbumin aggregates: tailoring oleogel-based Pickering emulsions for improved curcumin bioavailability and anti-inflammatory effects, *Int. J. Biol. Macromol.* 323 (2025) 147143. <https://doi.org/10.1016/j.ijbiomac.2025.147143>.