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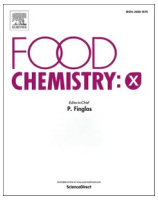

Effects of polyphenol and gelatin types on the physicochemical properties and emulsion stabilization of polyphenol-crosslinked gelatin conjugates

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ABSTRACT

Herein, six types of polyphenol-crosslinked gelatin conjugates (PGCs) with ≥ two gelatin molecules were prepared using a covalent crosslinking method with two types of polyphenols (tannic acid and caffeic acid) and three types of gelatins (bovine bone gelatin, cold water fish skin gelatin, and porcine skin gelatin) for the emulsion stabilization. The structural and functional properties of the PGCs were dependent on both polyphenol and gelatin types. The storage stability of the conjugate-stabilized emulsions was dependent on the polyphenol crosslinking, NaCl addition, and heating pretreatment. In particular, NaCl addition promoted the liquid-gel transition of the emulsions: 0.2 mol/L > 0.1 mol/L > 0.0 mol/L. Moreover, NaCl addition also increased the creaming stability of the emulsions stabilized by PGCs except tannic acid-crosslinked bovine bone gelatin conjugate. All the results provided useful knowledge on the effects of molecular modification and physical processing on the properties of gelatins.

1. Introduction

Polyphenols are natural plant antioxidants because they have at least one aromatic ring with one or more hydroxyl groups in the chemical structures (Shao, Zhang, Fang & Sun, 2014; Cao, et al., 2022). They have many health effects on human beings such as protection of the organism from external stimuli, the elimination of the formation of reactive oxygen species, the lipid profile improvement, and the systemic inflammation improvement (Shen, et al., 2020; Zhang, et al., 2022). Moreover, due to the significant antioxidant activity, polyphenol-rich diets could induce a low risk of many chronic diseases such as heart disease, cancers, diabetes, and obesity (Rasouli, Farzaei & Khodarahmi, 2017). Therefore, polyphenols are promising as nutraceuticals for the development of functional foods.

The development of protein–polyphenol products is a promising method to improve the quality of some foods. Polyphenol crosslinking could give better antioxidant activities, emulsifying properties, gelling properties, and mechanical properties to a protein due to the antioxidant polyphenol introduction and more compact molecular surface (Quan, Benjakul, Sae-leaw, Balange & Maqsood, 2019). The obtained protein–polyphenol substances can be classified into non-covalent (e.g., hydrophobic and hydrogen bonding) complexes and covalent conjugates. Covalent bond-based protein–polyphenol conjugates are generally preferable for food applications due to the more stable interactions (Poojary, Hellwig, Henle & Lund, 2023). Protein-polyphenol conjugates could be prepared via enzymatic (e.g., polyphenol oxidase, laccase, and tyrosinase) or non-enzymatic (e.g., free radical grafting, and alkaline reaction) methods (Quan, et al., 2019).

Polyphenol crosslinking has been explored to improve the emulsifying properties of gelatins (Zhang, Xu, et al., 2020). Gelatins are extracted from the tissues (e.g., bones and skins) of many animals (e.g., bovine, porcine, and fishes) (Shi, et al., 2022). Self-assembled gelatin/

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polyphenol non-covalent complexes via hydrogen bonding induced less droplet sizes and higher lipid oxidation stability of emulsions than pure gelatin (Huang, Li, Qiu, Teng & Wang, 2017). Gelatin/epigallocatechin gallate/pectin ternary non-covalent ternary complexes also induced better emulsion interfacial stability than gelatin/pectin non-covalent binary complexes because the epigallocatechin gallate promoted the dispersion of ternary complex more uniformly to reduce the agglomeration of the emulsion droplets (Huang, et al., 2023). The gelatin-oxidized polyphenol covalent conjugates with different polyphenols induced higher antioxidant activity, lower surface hydrophobicity, higher emulsion stability, and lower lipid oxidation of emulsions (Aewsiri, et al., 2009). However, the physicochemical and emulsifying properties of gelatin-polyphenol covalent conjugates remains unclear. Moreover, the effects of gelatin sources and polyphenol sources on the physicochemical and emulsifying properties of gelatins remains also unclear.

The purpose of this work was to study the protein–polyphenol crosslinking effects on the physicochemical and emulsifying properties of three types of gelatins. First, six types of polyphenol-crosslinked gelatin conjugates (PGCs) with $\geq$ two gelatin molecules were prepared and confirmed using two types of coffee-related polyphenols with different structures (tannic acid, TA; caffeic acid, CA) (Shirasago, et al., 2019) and three types of gelatins (bovine bone gelatin, BBG; cold water fish skin gelatin, CFG; porcine skin gelatin, PSG). Second, the structural properties of the obtained PGCs were determined. Third, the functional properties of the obtained PGCs were studied. Fourth, the storage stability of fish oil-loaded PGC-stabilized emulsions at different NaCl concentrations were analyzed. Finally, the storage stability of preheated fish oil-loaded PGC-stabilized emulsions was observed.

2. Materials and methods

2.1. Reagents

Fish oil (DHA + EPA $\geq$ 70 %) was purchased from Xi'an Qianyecao Co. Ltd. (Shaanxi Province, China). BBG (~240 g Bloom) was purchased from Aladdin Industrial Corp. (Shanghai, China). PSG, CFG, and TA were purchased from Sigma–Aldrich Trading Co., Ltd. (Shanghai, China). CA was purchased from Macklin Biochemical Co., Ltd. (Shanghai, China). Other chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Preparation of PGCs

PGCs were prepared by a covalent crosslinking method according to a previous work (Wei & Huang, 2019). Briefly, 4.0 g of gelatin granules were dissolved in 200 mL of ultrapure water at a temperature of 45 ℃ and a vibrating speed of 180 rpm for 60 min. After cooling down to room temperature, the pH was adjusted to 9.00 ± 0.05 using 1 mol/L NaOH and 1 mol/L HCl. Then, 0.8 g of polyphenol powder (TA or CA) were added to 200 mL of ultrapure water and the pH was adjusted to 9.00 ± 0.05. The gelatin solution and polyphenol solution were mixed at a 1:1 vol ratio and magnetically stirred (300 rpm) for 24 h. The samples were dialyzed (molecular weight cutoff: 8000–14000 kDa) in ultrapure water for 48 h and water was replaced every 6 h to remove free polyphenol molecules. The samples were freeze-dried to obtain PGC solids.

2.3. Total phenolic content

The total phenolic contents of PGCs were determined according to the Folin-Ciocalteu method using TA and CA as standards (Singleton, Orthofer & Lamuela-Raventós, 1999). Briefly, 5 mg of TA or CA standards were dissolved in ultrapure water and the total volume was set to 50 mL. The solution was diluted to a series of concentrations with water. Then, 0.5 mL of diluted polyphenol standard solution was mixed with 2.5 mL of Folin-Ciocalteu reagent (2 N, Sigma-Aldrich, Shanghai, China) and incubated in the dark for 2 h. The absorbance was measured at 760 nm against ultrapure water using a T6 UV–Vis spectrophotometer (Beijing Persee Analytics, Beijing, China) and a standard curve of absorbance vs. polyphenol concentration was obtained ($R^2 > 0.99$). The freeze-dried PGCs were dissolved in ultrapure water (1 mg/mL) and treated with Folin-Ciocalteu reagent. The total phenolic contents were expressed as millimoles of polyphenol equivalents per gram of dried conjugates.

2.4. UV spectroscopy

UV spectra of PGC solutions were examined according to a previous work (Guo, Bao, Sun, Chang & Liu, 2021). Briefly, 10 mg/mL of PGC solutions were prepared by stirring (300 rpm) the conjugates in ultrapure water for 1 h. Then, the UV spectra of the samples were measured by a T6 UV–visible spectrophotometer within the wavelength range of 200–400 nm.

2.5. SDS-PAGE

The molecular weight distribution of PGCs was examined using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) according to a previous work (Zhang, Ding, Wang & Zhong, 2020). Briefly, 2 mg/mL of PGC solutions were prepared by stirring (300 rpm) the conjugates in ultrapure water for 1 h and the pH was adjusted to 7.0 ± 0.05. Then, a mixture of 10 μL PGC solution with 10 μL SDS-PAGE buffer (2X, Sangon Biotech., Shanghai, China) was boiled for 5 min. The SDS-PAGE process was carried out using a Bis-Tris gel (8 % Sure-PAGE, GenScript, Nanjing City, Jiangsu Province, China) with a loading sample volume of 10 μL and an electrophoresis cell (DYCZ-24KS, Beijing Liuyi, China) with a voltage of 120 V for 80 min. Then, the gel was stained with Coomassie Brilliant Blue $-250 solution for 3 h and destained with a mixture of methanol/acetic acid aqueous solution (5 % methanol and 10 % acetic acid) until clear bands were shown. Finally, the gels were photographed using a digital camera.

2.6. Secondary structure analyses

The attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectra of freeze-dried PGCs were measured using an ATR-FTIR spectrometer (Spotlight 400, PerkinElmer Company, USA) with an accumulative scan time of 32, a scan resolution of 1 cm^{-1}, and a scan range of 4000–700 cm^{-1} (Zhang, Ding, et al., 2020). The spectra of 1700–1600 cm^{-1} were fitted using the PeakFit software (V4.12, SeaSolve, USA) to obtain the secondary structure percentages of the PGCs.

2.7. SEM

The freeze-dried PGCs were attached to conductive adhesives and observed using an S-3400 N Hitachi scanning electron microscope (SEM, Hitachi, Tokyo, Japan) with an accelerated voltage of 8.00 kV (Xu, et al., 2021).

2.8. Gel strength (g Bloom)

The freeze-dried PGCs were dissolved in ultrapure water (66.7 mg/mL) at a temperature of 45 ℃ and a vibrating speed of 180 rpm for 60 min and then stored at 10 °C for 16 h to form gel. The obtained samples were measured using a texture analyzer (TA-XT Plus, Stable Micro Systems Ltd., Surrey, UK) (Zhang, Ding, et al., 2020). The load cell specification was 5 kN. The flat-faced cylindrical Teflon plunger specification was 12.7 mm in diameter.

2.9. Transparency

The freeze-dried PGCs were dissolved in ultrapure water (10 mg/mL)

and the pH was adjusted to different values (3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0) (Wu, et al., 2022). Then, the samples were photographed and measured by a T6 UV–visible spectrophotometer at 600 nm.

2.10. DPPH scavenging capacity

DPPH scavenging capacities of PGCs were measured according to a previous work (Zheng, et al., 2020). Briefly, 2 mL of the PGC solution (0.5 mg/mL) was added to 2 mL of DPPH (1.2×10^{-4} mol/L) ethanol solution. The mixture was incubated in the dark for 1 h. The absorbance of the mixture was measured using a T6 UV–visible spectrophotometer at 517 nm. The DPPH scavenging capacity was calculated:

$$DPPH scavenging capacity(\%) = \left(\frac{A_0 - (A_2 - A_1)}{A_0}\right) \times 100 \quad (1)$$

where A_0 is the absorbance value of the DPPH without samples, A_1 is the absorbance value of the samples without DPPH, A_2 is the absorbance value of the samples with DPPH.

2.11. Reducing powder

Reducing powder of PGCs was measured according to a previous work (Yıldırım, Mavi & Kara, 2001). Briefly, the conjugates were dissolved in 2 % (v/v) acetic acid to form 0.25 mg/mL of conjugate solutions. Then, 1 mL of potassium ferricyanide solution (10 mg/mL) was added to 2 mL of the conjugate solution. The mixture was vortexed and

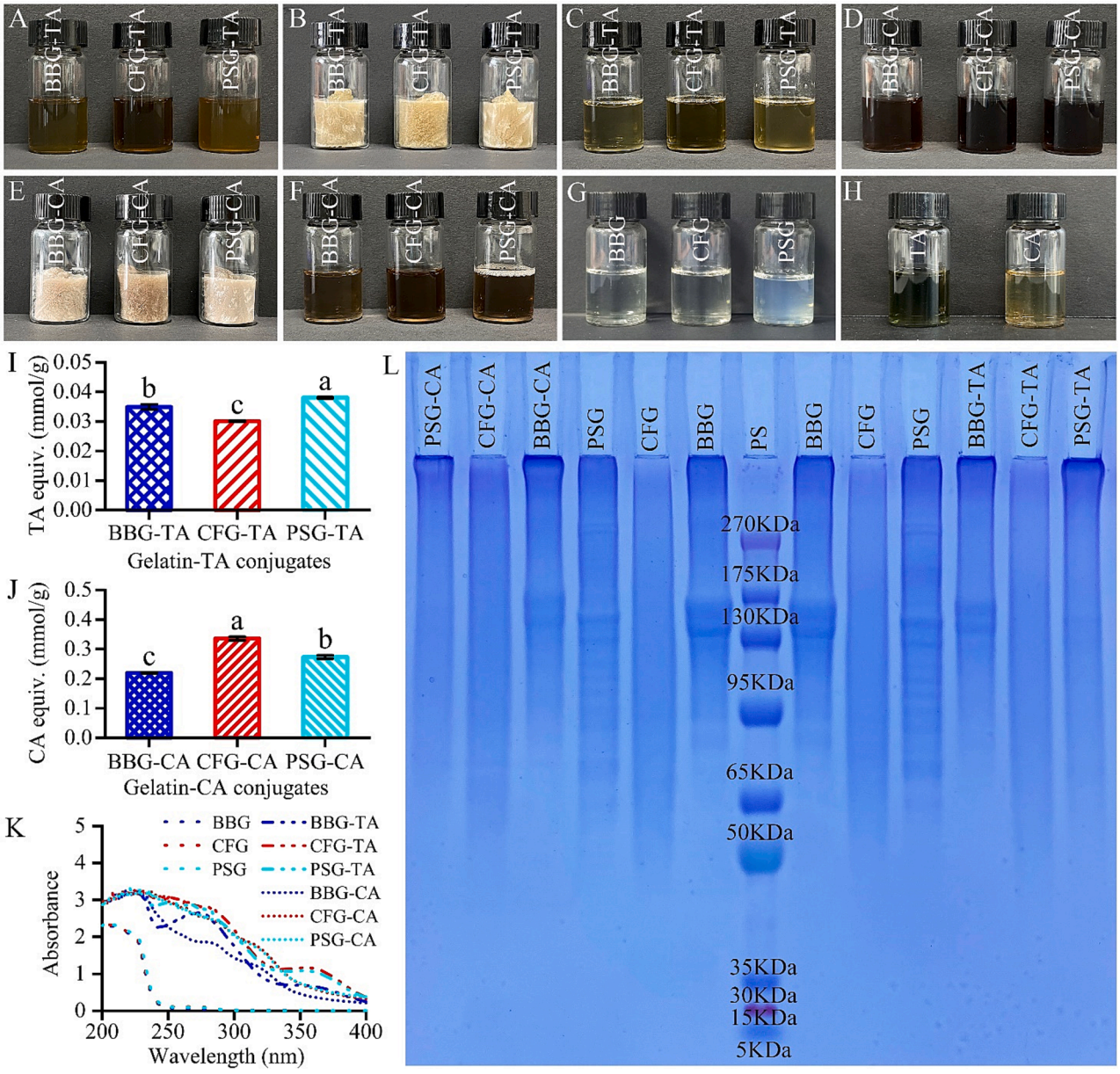


Fig. 1. Preparation and confirmation of polyphenol-crosslinked gelatin conjugates (PGCs). (A): Freshly prepared tannic acid-modified gelatin solutions. BBG-TA: Tannic acid-modified bovine bone gelatin. CFG-TA: Tannic acid-modified cold water fish skin gelatin. PSG-TA: Tannic acid-modified porcine skin gelatin. (B): Freeze-dried tannic acid-modified gelatins. (C): Redissolved tannic acid-modified gelatin solutions (2 mg/mL). (D): Freshly prepared caffeic acid-modified gelatin solutions. BBG-CA: Caffeic acid-modified bovine bone gelatin. CFG-CA: Caffeic acid-modified cold water fish skin gelatin. PSG-CA: Caffeic acid-modified porcine skin gelatin. (E): Freeze-dried caffeic acid-modified gelatins. (F): Redissolved caffeic acid-modified gelatin solutions (2 mg/mL). (G): 20 mg/mL of gelatin aqueous solutions with pH 9.00. (H): 4 mg/mL of polyphenol aqueous solutions with pH 9.00. (I–J): Total phenolic contents. (K): UV spectra. (L): SDS-PAGE results.

incubated at 50 °C for 20 min. Subsequently, 1 mL of trichloroacetic acid solution (100 mg/mL) was added to the mixture. Then, 1 mL of the resultant solution, 3 mL of ultrapure water, and 0.4 mL of ferric chloride solution (1 mg/mL) were mixed and incubated for 5 min at room temperature. Finally, the absorbance (reducing power) of the sample against ultrapure water was measured using a T6 UV–visible spectrophotometer at 700 nm.

2.12. Emulsion preparation and storage stability

PGC solutions (10 mg/mL, pH 9.0) with different NaCl concentrations (0, 0.1, or 0.2 mol/L) were mixed with fish oil at a volume ratio of 1:1. The mixtures were homogenized (11500 rpm) for 60 s using a homogenizer (ULTRA-TURRAX T10, IKA, Guangzhou, Guangdong, China) (Wu, et al., 2022). The obtained emulsions were treated without or with heating (55 or 85 °C) for 30 min. The emulsions were stored at room temperature for 28 days and photographed using a digital camera. The emulsion droplets were observed using an upright optical microscope (Shanghai Minz ML8000). Emulsion droplet sizes (400–600 droplets) were summarized from three independent experiments and the size distribution was fitted by multimodal Gaussian models. The creaming index (CI) values were obtained:

$$\mathrm{CI}\,(\%) = \frac{Serumlayerheight}{Emulsionheight} \times 100 \quad (2)$$

2.13. Statistical analysis

Three parallel samples were performed for each experiment to obtain the mean value and standard deviation. Statistical comparison of these data was analyzed using one-way ANOVA followed by Duncan's test ($p < 0.05$).

3. Results

3.1. Preparation and confirmation of PGCs

The freshly prepared (Fig. 1A and D) and redissolved (Fig. 1C and F) PGCs solutions were in green-yellow or dark brown, which was different from the colorless transparency of unmodified and transglutaminase-modified gelatins (BBG, CFG, and PSG) solutions in a previous work (Xu, et al., 2022). The freeze-dried PGCs (Fig. 1B and E) were in light yellow or light brown, which were different from the snowy white colors of the freeze-dried unmodified gelatins and slightly yellowish white colors of the freeze-dried transglutaminase-modified gelatins in a previous work (Xu, et al., 2022). According to the colors of pure gelatins (Fig. 1G) and pure polyphenols (Fig. 1H), the colors of the obtained PGCs resulted from the successful conjugation between gelatins and polyphenols.

The amounts of the bound polyphenols in PGCs were quantified by the Folin-Ciocalteu method (Singleton, et al., 1999). The total phenolic contents of TA-crosslinked gelatin conjugates followed the order (Fig. 1I): PSG-TA (0.0380 ± 0.0002 mmol/g) > BBG-TA (0.0349 ± 0.0009) > CFG-TA (0.0300 ± 0.0002). The total phenolic contents of CA-crosslinked gelatin conjugates followed the order (Fig. 1J): CFG-CA (0.336 ± 0.006 mmol/g) > PSG-CA (0.273 ± 0.006) > BBG-CA (0.219 ± 0.002). TA-crosslinked gelatin conjugates had lower total phenolic contents in mmol/g than CA-crosslinked gelatin conjugates. Therefore, the amounts of the bound polyphenols were dependent on both gelatin types and polyphenol types.

UV spectra of PGCs (Fig. 1K) were examined according to a previous work (Guo, et al., 2021). Three types of pure gelatins (CFG, PSG, and BBG) showed similar UV absorption spectra, which was similar to that of Yak skin gelatin (Xu, et al., 2017). Therefore, gelatins showed similar UV absorption intensity peaks at 200–230 nm. Compared with the pure gelatins, PGCs showed significantly higher intensities and wider peak widths. It might result from a strong interaction between gelatins and polyphenols, which buried the hydrophobic groups (Guo, et al., 2021).

SDS-PAGE results (Fig. 1L) suggested that the molecular weights of these pure gelatins were: BBG > PSG > CFG. They were consistent with previous works (Zhang, Sun, et al., 2020; Xu, et al., 2022; Yang, et al., 2023). Compared with the pure gelatins, the PGCs showed stronger bands near the bottom of the wells. Therefore, the obtained PGCs might consist of ≥ two gelatin molecules via the polyphenol crosslinking.

3.2. Structural characterization of freeze-dried PGCs

As shown in Figs. S1 and S2, PGCs showed similar spectra shapes to gelatins. Moreover, the characterized peaks of polyphenols were not shown in the spectra of the PGCs, which suggested that the intensity of polyphenol spectra was significantly lower than that of gelatins. The free polyphenol molecules were removed by the dialysis process and only the crosslinked polyphenol groups in the conjugates remained in the obtained products. Considering the molecular weights of polyphenols (CA: 180 Da; TA: 1702 Da) were orders of magnitude less than those of gelatins (Fig. 1L), it was reasonable that no obvious characterized peaks of polyphenols were shown in the spectra of PGCs.

The secondary structure percentages of the PGCs (Fig. 2A) were analyzed by fitting the ATR-FTIR spectra at 1700–1600 cm^{-1} (Fig. S3). According to the statistical comparison, all three types of gelatins and six types of PGCs showed similar random coil and α-helix percentages. Polyphenol crosslinking had obvious effects on the β-sheet, β-turn, and β-antiparallel percentages. These secondary structure percentages were dependent on both gelatin types and polyphenol types.

The microscale morphologies of freeze-dried PGCs were characterized using SEM (Zhang, Ding, et al., 2020). The PGCs showed denser microscale morphologies than untreated gelatins (Fig. 2B–J). It might result from the covalent crosslinking between polyphenols and gelatins. It was similar to morphological changes of casein film after the covalent crosslinking with TA (Picchio, et al., 2018). It could be attributed to the formation of PGCs with ≥ two gelatin molecules via the polyphenol crosslinking, which was supported by the molecular weight pattern results (Fig. 1L).

3.3. Functional properties of PGCs

As shown in Fig. 3A–C, untreated and polyphenol-crosslinked mammalian gelatins (BBG and PSG) formed gels, whereas untreated and polyphenol-crosslinked CFG did not. The behaviors of the untreated gelatins were consistent with our previous works (Zhang, Sun, et al., 2020; Xu, et al., 2022). Therefore, the gel strength values of the untreated and polyphenol-crosslinked mammalian gelatins gels were measured using a texture analyzer (Fig. 3D). The polyphenol-crosslinked mammalian gelatins gels showed lower values than the untreated mammalian gelatin gels, Therefore, the polyphenol crosslinking decreased the gel strength values of gelatins. It resulted from that the polyphenol crosslinking might negatively affect the gelatin gelation process both thermodynamically and kinetically (Wu, Chiu, Pearce & Kwei, 2001).

The transparency of polyphenol-crosslinked gelatin solutions were characterized using a digital camera (Fig. S4–S6) and a UV spectrometer (Fig. 3E–G). The results showed BBG had an isoelectric point (pI) of pH 5.0, CFG had no obvious pI, and PSG had a pI of pH 9.0. They were in accorded with our previous work (Zhang, Sun, et al., 2020; Xu, et al., 2022). It confirmed that PSG and BBG were type A and B, respectively, gelatins. After polyphenol crosslinking, the pI values of all the polyphenol-crosslinked gelatins decreased to 4–5. Previous work suggested that the phenolic compounds could react with nucleophilic groups (e.g., amino and sulfhydryl groups) in proteins under oxidizing conditions to form covalent bonds (Liu, et al., 2021). In this work, the pI value changes to 4–5 for all the polyphenol-crosslinked gelatins confirmed the polyphenolic interaction with the amino groups in

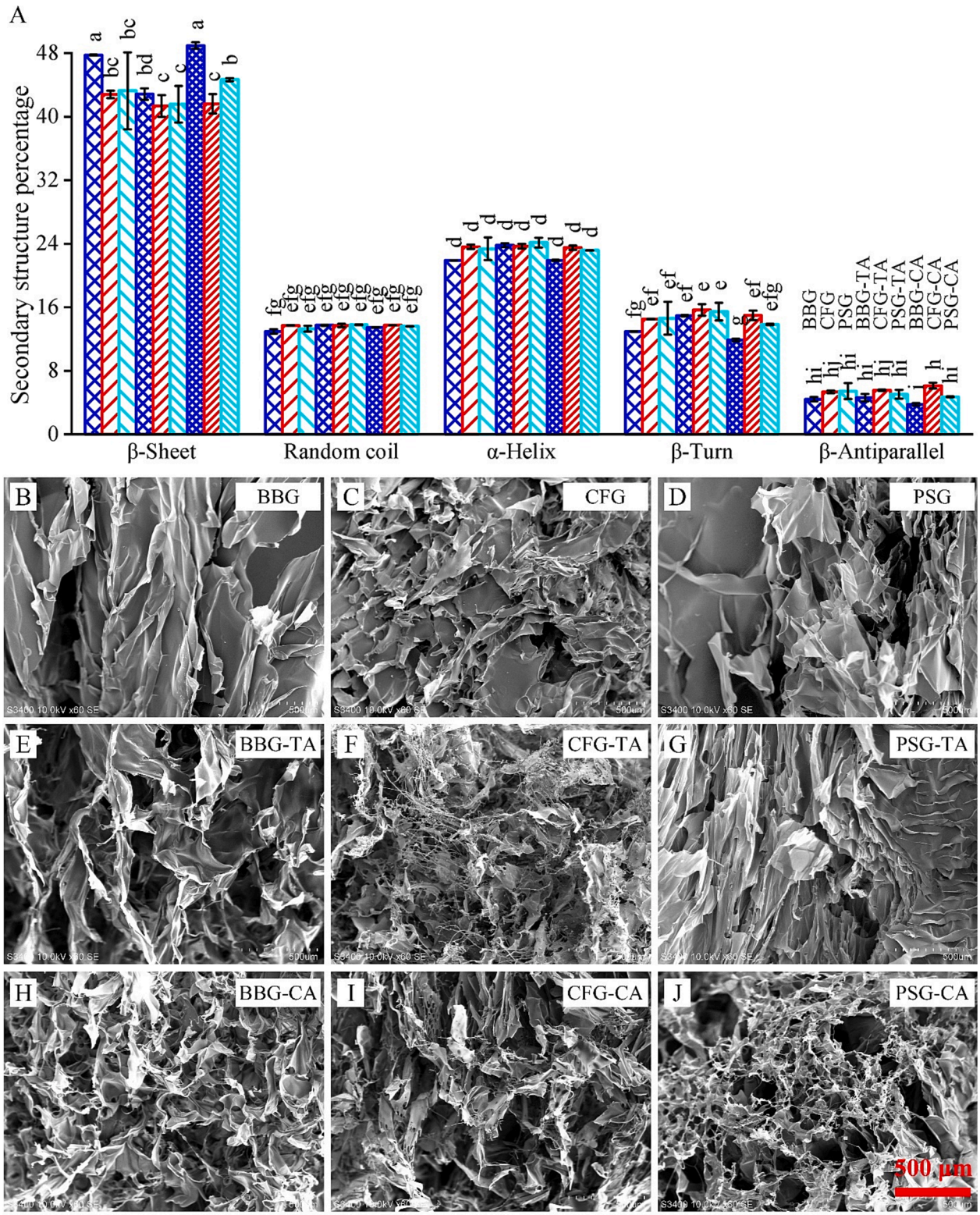


Fig. 2. Structural characterization of freeze-dried PGCs. (A) Secondary structure percentages. Different letters indicate significant differences ($p < 0.05$). (B): Scanning electron microscopy images. The red scale bar indicates 500 μm for all the images. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

gelatins.

The polyphenol crosslinking could increase the antioxidant properties of gelatins (Fig. 3H and I), which was consistent with the effect of apple polyphenol crosslinking on the antioxidant properties of gelatins in a previous work (Lin, et al., 2021). Considering the free polyphenol molecules were removed by the dialysis process, the antioxidant data (Fig. 3H and I) confirmed the successful covalent crosslinking between gelatins and polyphenols. Moreover, the antioxidant properties were dependent on both gelatin types and polyphenol types. TA induced the higher antioxidant ability than CA.

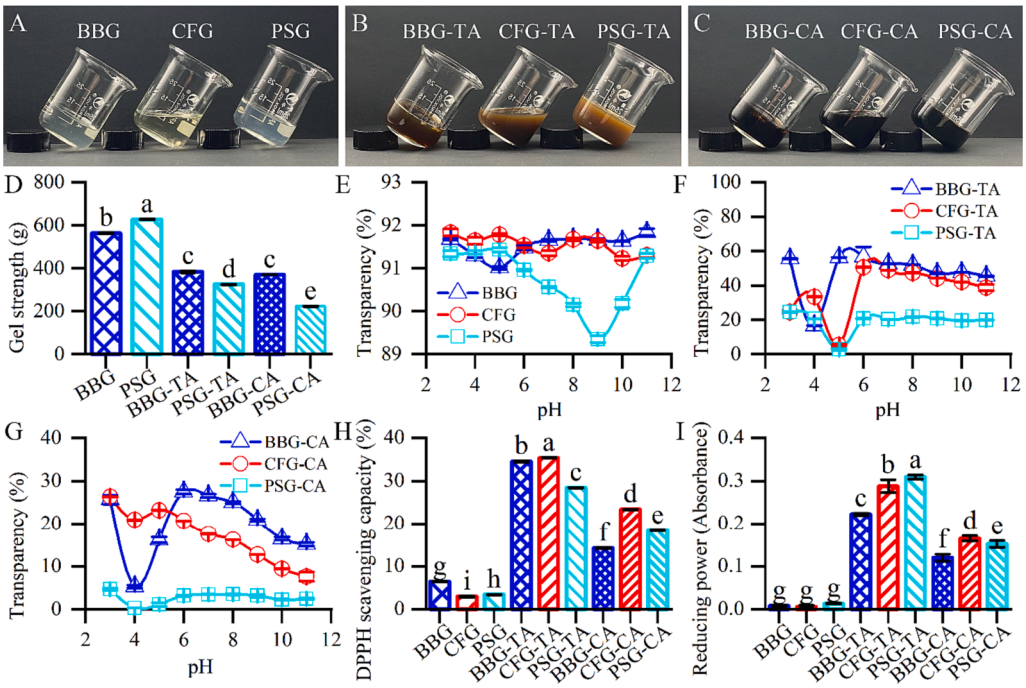


Fig. 3. Functional properties of PGCs. (A–C): PGC gels for gel strength measurements. Gelatins were used as controls. CFG and polyphenol-modified CFGs could not form gels for gel strength measurements. (D): Gel strength results. (E–G): Transparency of redissolved PGCs (10 mg/mL). (H): DPPH scavenging capacity. (I): Reducing powder. Different letters indicate significant differences ($p < 0.05$).

3.4. Storage observation of fish oil-loaded PGC-stabilized emulsions at different NaCl concentrations

Fish oil-loaded PGC-stabilized emulsions at different NaCl concentrations (0, 0.1, or 0.2 mol/L) were prepared and stored at room temperature (Fig. 4). All the freshly prepared emulsions were in milk-white liquid state and were composed of micrometer-sized droplets (Images not shown). With time, the CFG-stabilized emulsions at 0.1 and 0.2 mol/L of NaCl showed obvious droplet deformation (Fig. 4A) due to the occurrence of droplet coalescence (McClements & Jafari, 2018). Other emulsions did not show obvious droplet deformation even after 28 days of incubation (Images not shown). Therefore, polyphenol crosslinking did not change the droplet sizes and deformation behaviors of the fish oil-loaded emulsions. Polyphenol crosslinking delayed the liquid-gel transition of the mammalian gelatin-stabilized emulsions, whereas it promoted the liquid-gel transition of the CFG-stabilized emulsions. The polyphenol type had no obvious differences in the liquid-gel transition of the emulsions. The addition of NaCl promoted the liquid-gel transition of the emulsions: 0.2 mol/L > 0.1 mol/L > 0.0 mol/L.

The emulsion CI values of the pure gelatin-stabilized emulsions were dependent on gelatin types and NaCl concentrations (Fig. 4B–D). In the absence of NaCl, the mammalian gelatin-stabilized emulsions showed no obvious creaming and CFG-stabilized emulsions showed significant creaming (CI: 36.6 ± 1.3 % at day 28). It was consistent with our previous work on the BBG- and CFG-stabilized emulsions (Zhang, Sun, et al., 2020). In addition, NaCl concentrations (0–0.2 mol/L) had no obvious effects on the CI values of the pure gelatin-stabilized emulsions.

The emulsion CI values of the PGC-stabilized emulsions were dependent on gelatin types, polyphenol types, and NaCl concentrations (Fig. 4B–D). For the pure mammalian gelatin-stabilized emulsions, the polyphenol crosslinking increased CI values in the absence of NaCl: G-CA > G-TA > G. For the CFG-stabilized emulsions, the polyphenol crosslinking decreased CI values in the absence of NaCl: G > G-TA≈ G-CA. Moreover, the presence of NaCl decreased the CI values of the emulsions stabilized by PGCs except BBG-TA. The concentration of the adsorbed proteins in the oil–water interfacial layer in the emulsions decreased with the increasing ionic strength (Chen, et al., 2022). Therefore, the CI values of the emulsions decreased with the increasing NaCl concentrations (Zhang, Ding, et al., 2020). It was consistent with the effect of NaCl concentrations on the BBG-TA related emulsion in this work. However, the other PGCs might form a relatively larger gelatin conjugate network (Fig. 6A and B). Therefore, the absorbed gelatins did not decrease with the increasing NaCl concentrations. However, the interfacial layer density increased due to the presence of NaCl in the layer. So, the CI values decreased with the increasing NaCl concentrations. Further work will be necessary to analyze the underlying mechanism for this phenomenon.

3.5. Storage observation of fish oil-loaded PGC-stabilized emulsions after heating

Fish oil-loaded PGC-stabilized emulsions were heated (55 or 85 °C) for 30 min and then studied for 28 days at room temperature (Fig. 5). During the whole storage process, all the emulsions were composed of micrometer-sized droplets (Typical images at 3 h and day 7 were shown in Fig. 5A). The emulsion states at 3 h and day 3 suggested that the liquid-gel transition of the preheated emulsions was mainly dependent on gelatin types and heating temperatures. At 3 h, the BBG- and CFG-stabilized emulsions after 85 °C treatment showed quicker liquid-gel transition than those after 55 °C treatment. Therefore, heating treatment could promote the liquid-gel transition in the storage process. It was consistent with high-temperature storage could promote the liquid-gel transition of fish oil-loaded gelatin nanoparticle-stabilized Pickering emulsions (Ding, et al., 2019). Moreover, polyphenol crosslinking could delay the liquid-gel transition of the preheated emulsions (Indicated by black asterisks), which was similar to the effect of polyphenol crosslinking on the unheated mammalian gelatins-stabilized emulsions and contrary to the effect of polyphenol crosslinking on the unheated CFG-stabilized emulsion (Fig. 4).

The effect of heating pretreatments on the emulsion CI values could be analyzed by comparing the pretreated (Fig. 5B–D) with untreated emulsions (Fig. 4B–D). Heating pretreatment increased the CI values of

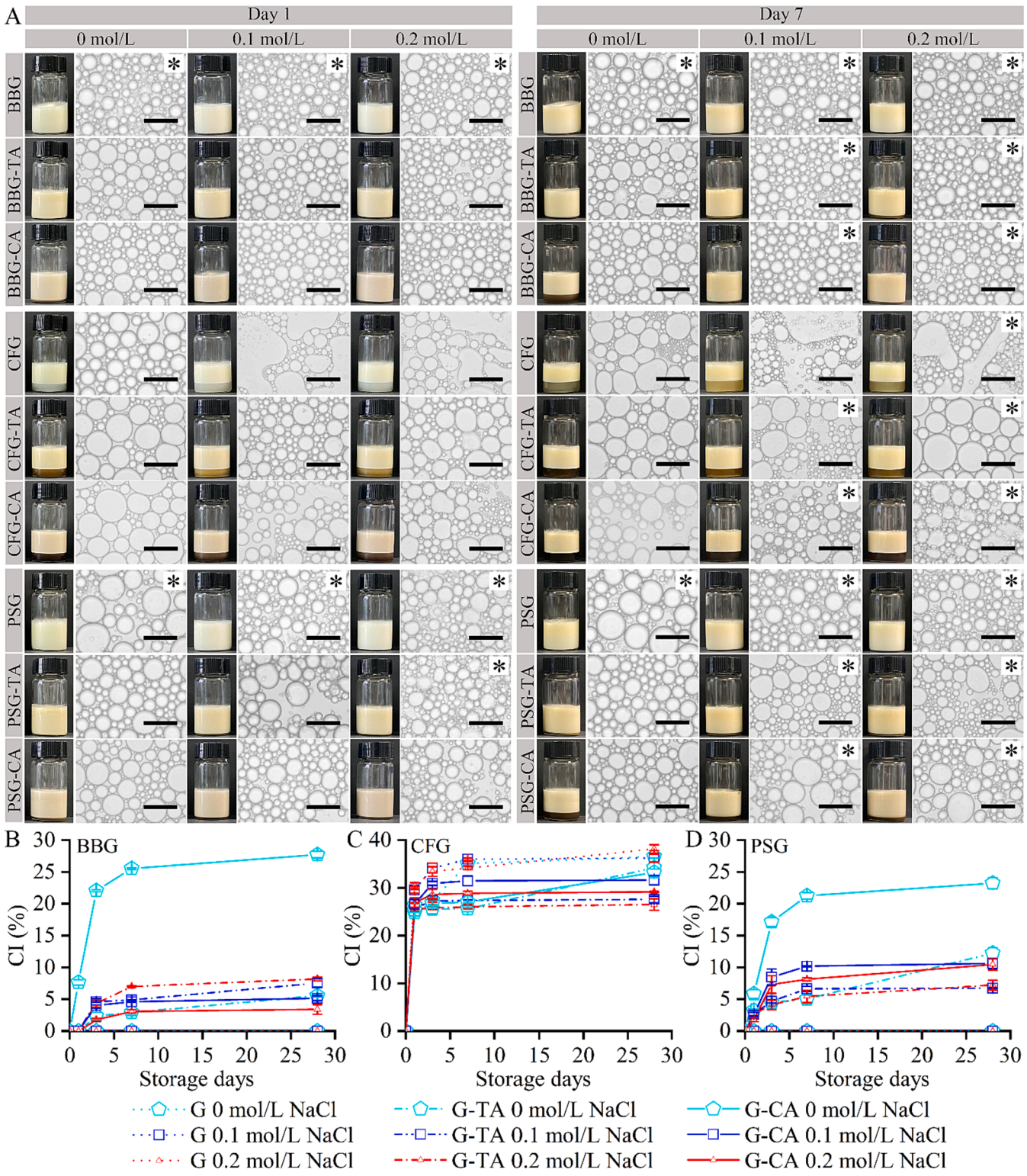


Fig. 4. Room temperature storage observation of fish oil-loaded PGC-stabilized emulsions at different NaCl concentrations. (A): Digital camera and optical microscopy images of the emulsions on days 1 and 7. Scale bars indicate 50 μm for optical microscopy images. Black asterisks on the optical microscopy images indicate emulsion gels. (B–D): Creaming index (CI) values vs. storage days.

the pure mammalian gelatin-stabilized and TA-crosslinked mammalian gelatin conjugate-stabilized emulsions (Fig. 4B, D, Fig. 5B, and D). Heating pretreatment decreased the CI values of CA-crosslinked BBG-stabilized emulsions (Fig. 4B and Fig. 5B), whereas had no obvious effects on the CI values of CA-crosslinked PSG-stabilized emulsions (Fig. 4D and Fig. 5D). Heating pretreatment decreased the CI values of the CFG-stabilized and polyphenol-crosslinked CFG-stabilized emulsions (Fig. 4C and Fig. 5C). Moreover, preheating temperatures had no obvious effects on the CI values of these emulsions (Fig. 5B–D). Therefore, the emulsion CI values of the preheated PGCs-stabilized emulsions were dependent on gelatin types, heating pretreatments, and polyphenol types.

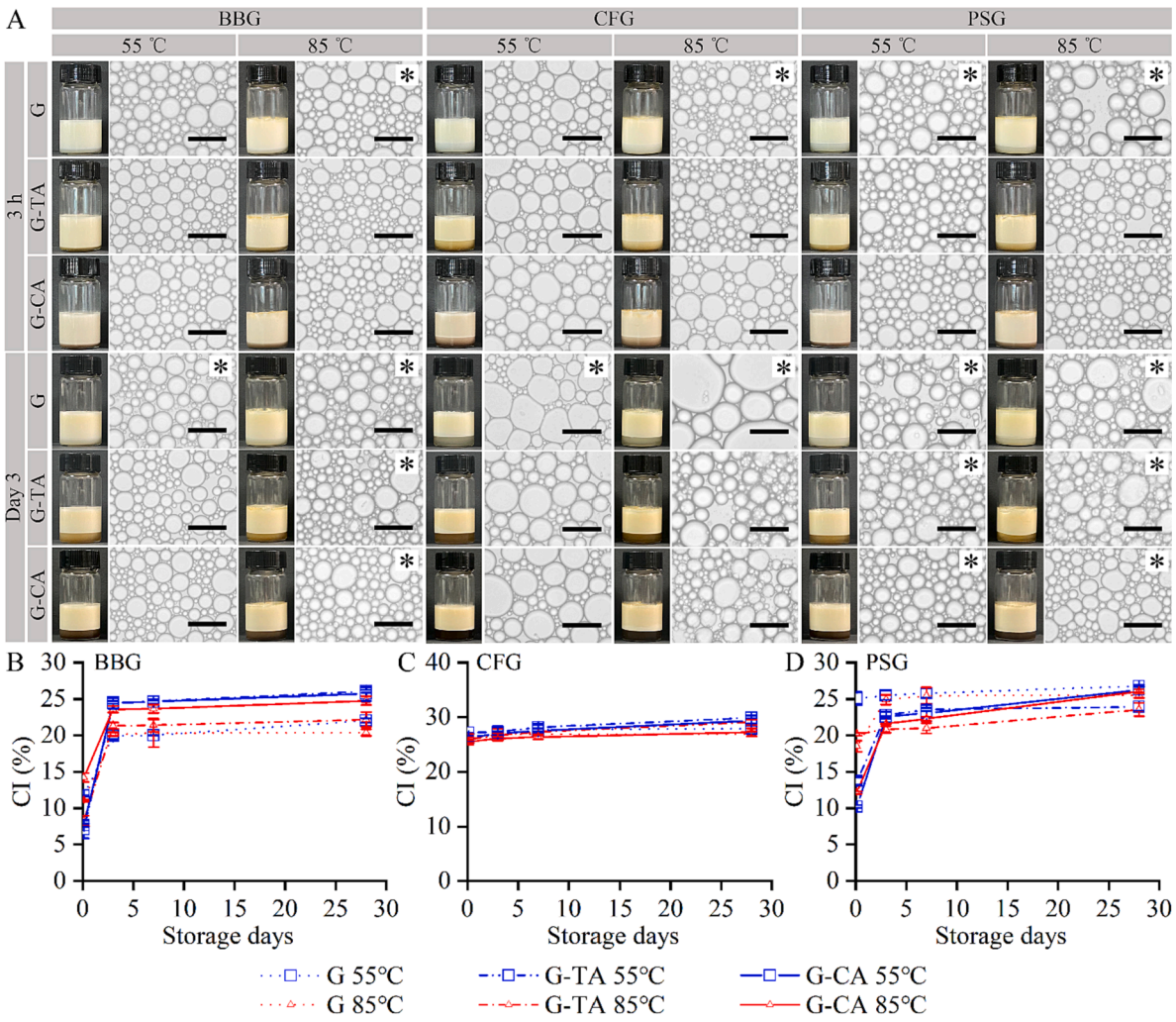


Fig. 5. Room temperature storage observation of fish oil-loaded PGC-stabilized emulsions after heating (55 or 85 °C) for 30 min. (A): Digital camera and optical microscopy images of the emulsions on days 1 and 7. Scale bars indicate 50 μm for optical microscopy images. Black asterisks on the optical microscopy images indicate emulsion gels. (B–D): Creaming index (CI) values vs. storage days.

4. Discussion

4.1. Formation mechanisms of PGCs

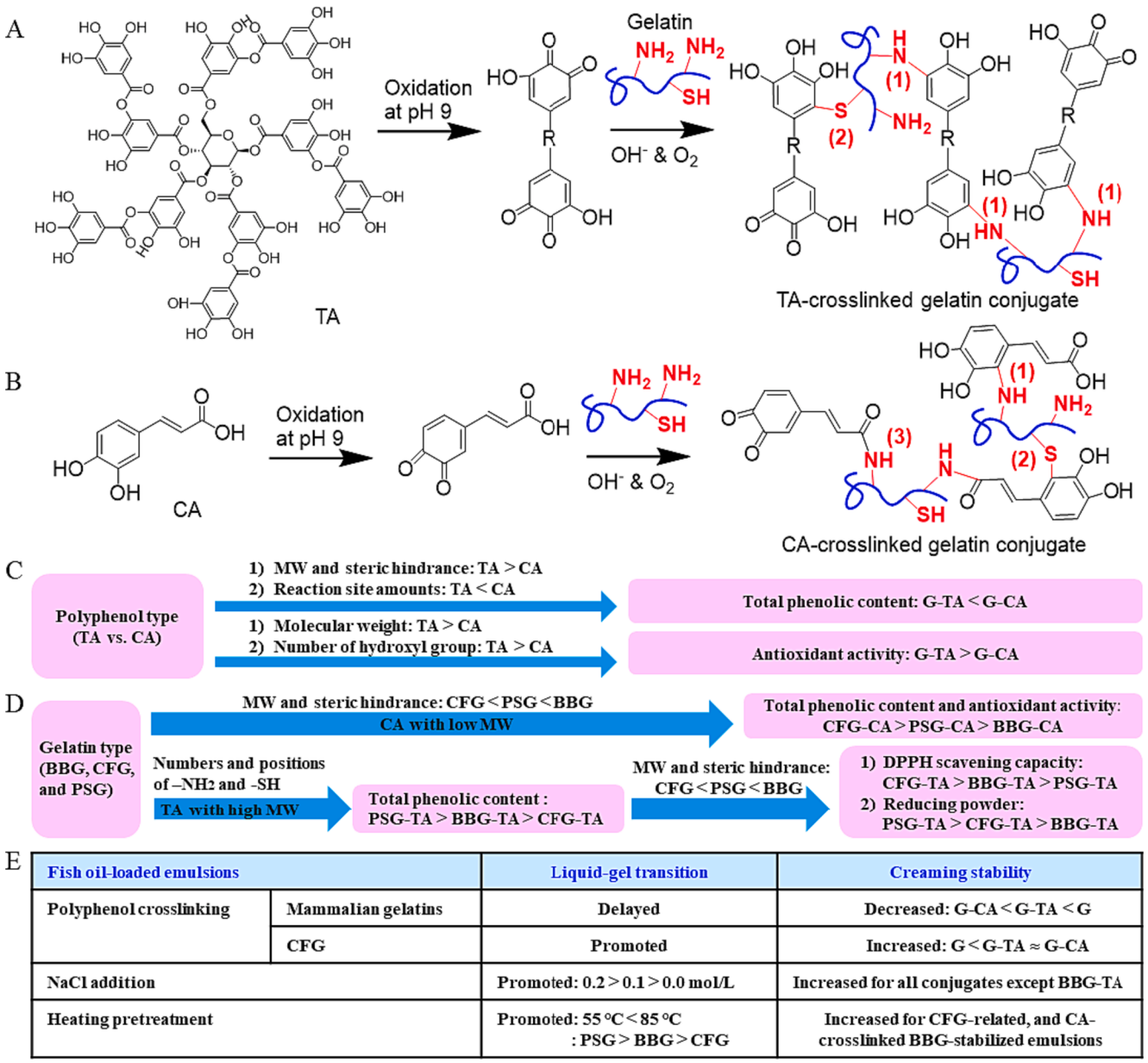
Many studies have reported the covalent interaction mechanisms between polyphenols and proteins. The nucleophilic groups (e.g., amino and sulfhydryl groups) in proteins could react with the aromatic rings of polyphenols under some special conditions (e.g., alkaline, ultrasound, and enzyme) to form protein–polyphenol conjugates (Liu, et al., 2021; Sun, Sarteshnizi & Udenigwe, 2022). In addition, it was reported that the amino group in collagen could react with the carboxyl group in caffeic acid under oxidizing conditions to fix acellular extracellular matrices (Ji, et al., 2023). The results confirmed the successful preparation of six PGCs for all the two polyphenols (TA and CA) and three gelatins (BBG, CFG, and PSG).

The formation mechanisms of the TA-crosslinked gelatin conjugates and CA-crosslinked gelatin conjugates were proposed, as shown in Fig. 6A and B, respectively. TA is a high molecular weight (MW: 1702) polyphenol with good physiological functions (e.g., antioxidant and antibacterial properties) (Xiong, Li & Yang, 2023). The amino and sulfhydryl groups of gelatin molecules could react with the aromatic rings of oxidized TA to form TA-crosslinked gelatin conjugates with ≥ two gelatin molecules (Fig. 6A). CA is low high molecular weight (MW: 180 Da) polyphenol with good physiological functions (e.g., antioxidant and antibacterial properties) (Mude, Maroju, Balapure, Ganesan & Ray Dutta, 2022). The amino and sulfhydryl groups of gelatin molecules could react with the aromatic rings and carboxyl group of oxidized CA to form CA-crosslinked gelatin conjugates with ≥ two gelatin molecules (Fig. 6B).

4.2. Effects of polyphenol and gelatin types on the properties of PGCs

Typical effective factors for protein–polyphenol interactions include polyphenol structure (steric hindrance, methylation, hydroxylation, aromatic rings), protein structure, pH, temperature, and polyphenol/protein ratio (Sun, et al., 2022). TA had higher molecular weight (MW: 1702) and more aromatic rings than CA (Fig. 6A and B). Moreover, CA provided a carboxyl group to form covalent protein–polyphenol bonds, whereas TA did not. The molecular weights of gelatins were: CFG < PSG < BBG (Fig. 1L). Therefore, both two types of polyphenols and three types of gelatins might have different reaction site amounts (polyphenols: aromatic rings and carboxyl groups; gelatins: amino and sulfhydryl groups) and different steric hindrances to affect protein–polyphenol interactions.

Polyphenol type had obvious effects on the key properties of gelatin conjugates such as total phenolic content and antioxidant activity (Fig. 6C). All three TA-crosslinked gelatin conjugates had lower total phenolic contents (mmol/g) than the corresponding three TA-



E

Fish oil-loaded emulsions		Liquid-gel transition	Creaming stability
Polyphenol crosslinking	Mammalian gelatins	Delayed	Decreased: G-CA < G-TA < G
	CFG	Promoted	Increased: G < G-TA ≈ G-CA
NaCl addition		Promoted: 0.2 > 0.1 > 0.0 mol/L	Increased for all conjugates except BBG-TA
Heating pretreatment		Promoted: 55 °C < 85 °C : PSG > BBG > CFG	Increased for CFG-related, and CA-crosslinked BBG-stabilized emulsions

Fig. 6. Schematics of the preparation and application of PGCs. (A): Preparation schematic of TA-crosslinked gelatin conjugate. (B): Preparation schematic of CA-crosslinked gelatin conjugate. (C): Effect of polyphenol type on the key properties of PGCs. (D): Effect of gelatin type on the key properties of PGCs. (E): Effect of polyphenol crosslinking, NaCl addition, and heating pretreatment on the storage of fish oil-loaded PGC-stabilized emulsions.

crosslinked gelatin conjugates (Fig. 1I and J). It might result from TA the higher steric hindrance due to higher molecular weights and less reaction site amounts (TA:aromatic rings; CA: aromatic rings and carboxyl groups) than CA. The antioxidant activity of polyphenols mainly depended on the number and positions of the hydroxyl groups (Balasundram, Sundram & Samman, 2006). TA had significantly higher molecular weights than CA. Moreover, TA had more hydroxyl groups (three) for each aromatic ring than CA (two). Therefore, though TA-crosslinked gelatin conjugates had lower total phenolic contents (mmol/g) than the corresponding CA-crosslinked gelatin conjugates (Fig. 1I and J), TA-crosslinked gelatin conjugates had higher antioxidant activities (DPPH scavenging capacity and reducing powder) than CA-crosslinked gelatin conjugates (Fig. 3H and I).

Gelatin type had obvious effects on the properties of gelatin conjugates (Fig. 6D). According to the SDS-PAGE results (Fig. 1L), gelatins had different molecular weights: CFG < PSG < BBG. It suggested the steric hindrance for the gelatin-polyphenol interaction had the same order as the molecular weights of gelatins. For the interactions between gelatins and CA with low molecular weight, the molecular weight of the gelatin was the main affecting factor. Therefore, contrary to the molecular weight order of gelatins, both the total phenolic content and antioxidant activities of the gelatin conjugates showed the same order: CFG-CA > PSG-CA > BBG-CA. For the interactions between gelatins and TA with high molecular weight, the structures of the gelatins (molecular weights, steric hindrances, numbers, and positions of amino and sulfhydryl groups) could affect the binding between gelatins and polyphenols because TA had significant steric hindrance to react with gelatins. Due to the differences in the numbers and positions of amino and sulfhydryl groups in gelatins, the total phenolic contents of the gelatin conjugates followed the order: PSG-TA > BBG-TA > CFG-TA. The antioxidant activity of polyphenols could be decreased due to steric hindrance (Platzer, et al., 2021). Due to the differences in molecular weights and steric hindrances of gelatins, the DPPH scavenging capacity order (CFG-TA > BBG-TA > PSG-TA) and reducing powder order (PSG-

TA > CFG-TA > BBG-TA) were different.

4.3. Effects of polyphenol crosslinking and NaCl addition on the stabilization of PGC-stabilized emulsions

The application of PGCs as emulsifiers is a promising research topic in the field of food science. Salt is an important food additive to improve the processing characteristics, structure, sensory quality, and shelf life of foods (Sun, et al., 2021).

The storage of fish oil-loaded emulsions was dependent on polyphenol types, gelatin types, and NaCl concentrations (Fig. 6C). The polyphenol crosslinking and NaCl addition mainly affected the liquid-gel transition (Indicated by black asterisks in Fig. 4A) and creaming stability (Fig. 4B–D). The polyphenol crosslinking delayed the liquid-gel transition and decreased the creaming stability (CI: G-CA > G-TA > G) of the mammalian gelatin-stabilized emulsions. The dominant polyphenol crosslinking might block the thiol groups to form disulfide bonds among the gelatins, and therefore, it weakened the gel strength (Tang, et al., 2017). For the mammalian gelatins, the thiol groups might be blocked, the formation of disulfide bonds were lowered, and therefore the emulsion liquid-gel transition was delayed and the emulsion creaming stability was decreased. However, the polyphenol crosslinking promoted the liquid-gel transition and increased the creaming stability (CI: G > G-TA ≈ G-CA) of the CFG-stabilized emulsions. The dominant polyphenol crosslinking might not affect the formation of disulfide bonds. The formation of polyphenol-gelatin conjugates might increase the interfacial layer thickness and density, and therefore, promoted the liquid-gel transition and increased the creaming stability. NaCl addition promoted the liquid-gel transition of the emulsions: 0.2 mol/L > 0.1 mol/L > 0.0 mol/L. In addition, NaCl addition also increased the creaming stability of the emulsions stabilized by PGCs except BBG-TA (Fig. 6D). Therefore, both polyphenol crosslinking of an emulsifier and NaCl addition were promising methods to adjust the stability of emulsion-based foods.

4.4. Effect of heating pretreatment on the stabilization of PGC-stabilized emulsions

Heating is a common step during food processing and consumption. In this work, the effect of heat pretreatment on the stabilization of PGC-stabilized emulsions was studied (Fig. 5). The results suggested that the heating pretreatment mainly affected the liquid-gel transition and creaming stability (Fig. 6E). The liquid-gel transition time decreased with the increase of heating temperature, which suggested the liquid-gel transition was promoted by heating pretreatment (55 °C < 85 °C). Moreover, the liquid-gel transition was also dependent on the gelatin types: PSG > BBG > CFG. The effect of heating pretreatment on the stability of the PGCs-stabilized emulsions was dependent on the gelatin and polyphenol types. It increased the creaming stability of the CFG-related (CFG-stabilized and polyphenol-crosslinked CFG-stabilized) and CA-crosslinked BBG-stabilized emulsions. Therefore, heating pretreatment could change the liquid-gel transition and creaming stability of the PGCs-stabilized emulsions.

5. Conclusions

In this study, six types of PGCs were prepared using two types of polyphenols (TA and CA) and three types of gelatins (BBG, CFG, and PSG). The obtained conjugates consisted of ≥ two gelatin molecules. The crosslinking behaviors were dependent on both polyphenol and gelatin types. The polyphenol crosslinking could be applied to adjust the liquid-gel transition and creaming stability of fish oil-loaded emulsions. In addition, NaCl addition and heating pretreatment could change the liquid-gel transition and creaming stability of fish oil-loaded PGC-stabilized emulsions. This work provided the basic knowledge to understand the effects of molecular modification and physical processing on the research and development of emulsion-based foods. However, the underlying mechanisms of the effects of polyphenol and gelatin types on the physicochemical properties and emulsion stabilization of polyphenol-crosslinked gelatin conjugates remains unclear. Therefore, further work is necessary to analyze the molecular structures of the PGCs in details and their effects on their properties. In addition, further work is necessary to analyze the effect of gelatin number on the properties and emulsion application of PGCs. Finally, it is also interesting to study the effect of NaCl addition on the gelatin-stabilized emulsion-based food development.

CRediT authorship contribution statement

Wenjuan Wu: Data curation, Formal analysis, Investigation, Writing – original draft. **Cuiping Shi:** Investigation. **Ye Zi:** Investigation. **Huan Gong:** Investigation. **Lijia Chen:** Investigation. **Guangyi Kan:** Investigation. **Xichang Wang:** Resources. **Jian Zhong:** Conceptualization, Data curation, Formal analysis, Supervision, Writing – review & editing.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101250.

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