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Improving physical stability of microalgae protein-based emulsions under acidic and neutral conditions via carboxymethyl chitosan complexation

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ABSTRACT

The emulsification stability of microalgae protein (MP) is limited to strongly alkaline conditions, restricting its applications in food processing. This study aims to investigate the capability of carboxymethyl chitosan (CMCS) to improve MP's emulsification stability over a wider pH range. Results indicated soluble MP-CMCS complexes formed at pH 2, 4, and 7, while aggregation of the complexes occurred at pH 8. The complexes stabilized emulsions exhibited smaller droplet sizes and higher absolute zeta potential at pH 2, 4, and 7 compared to pH 8. After 2 weeks of storage, emulsions remained stable at pH 2, 4, and 7, with significant delamination at pH 8. Laser confocal microscopy confirmed uniform droplet distribution at pH 2 and 7, with slight fusion at pH 4. The complexes stabilized emulsions exhibited higher viscosity and shear stress than MP stabilized emulsions at pH 2, 4, and 7. The stronger viscoelastic properties and higher storage moduli (G') values of MP-CMCS complexes under acidic and neutral conditions indicated stronger intermolecular interactions compared to alkaline conditions. The increase in G' and loss moduli (G") values for emulsions at pH 8 under stress highlighted the significant impact on network structure strength and viscosity in these emulsions. This study elucidated the binding interactions between MP and CMCS under various pH conditions, and demonstrated a feasible approach to improving MP's emulsification stability over a wider pH range.

1. Introduction

Microalgae have emerged as a promising and sustainable protein source due to their low freshwater consumption, minimal land requirements, and capacity to grow on non-arable water and seawater (Smetana, Sandmann, Rohn, Pleissner, & Heinz, 2017). They present a viable alternative to the non-sustainable soy resource. Proteins derived from microalgae contains comprehensive amino acid profiles essential for daily nutrition (Prandi et al., 2023), improves food quality, and impart bioactive functions to food products (Fields et al., 2020). Consequently, the application of microalgae protein in food products will remains an important research direction in the future. In 2022, Nestlé, the world's largest food manufacturer, announced its intention to develop microalgae as an ingredient in its food products. This announcement signals microalgae and their components are on the cusp of entering the mainstream food industry. Many other companies are also exploring the feasibility, scope, and potential benefits of using microalgae in their food products to promote sustainability and meet protein demands.

The role of plant proteins in food processing extends beyond their nutritional value, as they often serve as effective emulsifiers and foaming agents that enhance food's sensory qualities (Shrestha, Hag, Haritos, & Dhital, 2023). Microalgae protein (MP) has been demonstrated superior foaming properties. For example, proteins extracted from *Arthrospira platensis* exhibited higher foam overrun and stability compared to whey protein isolates, with faster adsorption kinetics at air-water interfaces and improved foam stability (Buchmann, Bertsch, Bcker, Krhenmann, & Mathys, 2019). MP also shows promising

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emulsification properties. Proteins from *Chlorella protothecoides* have demonstrated high interfacial activities in oil-in-water emulsions, maintained emulsion stability against coalescence at concentrations of 3% or higher (Dai, Hinrichs, & Jochen., 2020). Additionally, *Arthrospira platensis* proteins exhibited superior reduction in interfacial tension compared to animal-based proteins like β-lactoglobulin, lysozyme, albumin, or casein (Bertsch et al., 2023). In order to further understand the emulsification properties of MP, we have previously investigated the emulsification performance of MP in a wide pH range, and found that the emulsification stability of MP was limited to strongly alkaline pH conditions, specifically at pH 10, this undoubtedly limits its broad application in food processing (Guo et al., 2024). Therefore, there is an urgent need to explore solutions to improve the emulsification stability of MP across a wider pH range.

Carboxymethyl chitosan (CMCS) is a linear, water-solute polysaccharide and a derivative of chitosan extracted from crab shell waste. Due to its excellent biocompatibility and chemical versatility, including antibacterial properties and antioxidant activity, it is widely utilized in biological applications (Shariatinia, 2018). Additionally, CMCS, as an amphiphilic polymer, has been employed to enhance emulsifying properties, increasing the viscosity and flow resistance of emulsions, thereby improving their stability (Zhao et al., 2021). CMCS can crosslinked with gliadins (Zheng et al., 2021) and soybean lipophilic protein (Li et al., 2024) to form dense reticular structure, and then elevate the stability of emulsions to surroundings. Therefore, CMCS is speculated to potentially enhance the emulsification performance of MP. However, whether CMCS can improve MP's emulsification performance across all pH values or only at specific pH levels remains unknown.

This study aims to investigate the effect of CMCS on the emulsification properties of MP across a range of pH values. Considering the microalgae biomass producing market for food purposes is basically dominated by *Spirulina* with annual production estimated at over 12, 000 tons (Levasseur, Perré, & Pozzobon, 2020; Mu, Mehar, Mudliar, & Shekh, 2019), so the soluble protein isolated from a common *Spirulina* species (*Arthospira platensis*) were selected for study. Firstly, MP-CMCS complexes solutions with varying pH values were prepared, and structures and properties of complexes solutions were characterized and analyzed. Subsequently, the emulsions stabilized by MP-CMCS complexes was prepared, and structures, properties and stability of emulsions were investigated. This study will elucidate the binding interactions between MP and CMCS in aqueous solutions under different pH conditions, and clarify the effects of CMCS on MP's emulsification properties, as well as the underlying mechanisms driving these effects.

2. Materials and methods

2.1. Materials

Microalgae protein (MP) was extracted from the photoautotrophically cultivated spray dried *Arthospira platensis* biomass according to our previous study (Guo et al., 2024). Carboxymethyl chitosan (CMCS, substitution degree >90%, deacetylation degree >90%, molecular weight: 240 kDa) was obtained from Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Soybean oil was purchased from Zhongbai supermarket (Wuhan, China). Rhodamine B was obtained from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). All the chemicals used were of analytical grade.

2.2. Preparation of MP-CMCS complexes

The powders of MP and deionized water were mixed at a ratio of 1:100 (*w/v*) to obtained the MP solution (1%, w/v). Similarly, a CMCS solution (1%, w/v) was prepared by dispersing the CMCS powders in deionized water. Placing the MP solution and CMCS solution in a refrigerator at 4 °C overnight until the specimens were fully hydrated. The MP-CMCS complexes were prepared by mixing MP solution (1%, w/v) with CMCS solution (1%, w/v) at a ratio of 1:1 (*v*/v), the total concentration of colloidal particles in the system was 1% (*w/v*). The pH of the mixed solution was then adjusted to values of 2, 4, 7, and 8, respectively. Additionally, sodium azide (0.02%, w/v) was added to the MP-CMCS complexes to inhibit bacterial growth. Control groups consisted of 1% (w/v) MP solutions with pH values of 2, 4, 7, and 8.

2.3. Characterizations of the MP-CMCS complexes

2.3.1. UV spectrum measurement

The UV spectrum of MP-CMCS complexes were measured using a UV–vis spectrophotometer (Enspire, Perkin Elmer Company, USA) at 5 nm intervals in the range of 230–800 nm.

2.3.2. Intrinsic fluorescence measurement

The intrinsic fluorescence of MP-CMCS complexes were measured using a fluorescence spectrophotometer (F-4600, Hitachi Corporation, Japan). The excitation wavelength was set to 280 nm, and the emission spectra were recorded from 295 to 500 nm with a 2.5 nm slit width.

2.3.3. Turbidity measurement

The turbidities of MP-CMCS complexes were determined by recording the absorbances at 600 nm with a UV–vis spectrophotometer (Enspire, Perkin Elmer Company, USA).

2.3.4. Zeta-potential and particle distribution measurement

The Zeta-potential and particle distribution of MP-CMCS complexes were measured with a Nanoparticle Size and Zeta Potential Analyzer (Zetasizer Nano ZS, Malvern Instruments Ltd., UK).

2.3.5. Scanning electron microscopy (SEM)

MP-CMCS complexes solutions were dropped on the silicon wafer and dried at 40 °C. After that, the microstructure of MP-CMCS complexes was observed using a scanning electron microscope (SU 8600, Hitachi, Japan) at an acceleration voltage of 2.0 kV.

2.4. Preparation of emulsions

The MP-CMCS complexes with different pH values were mixed with soybean oil at a 1:3 ratio (*v*/v), and then homogenized at 16,000 rpm for 3 min using a high-speed dispersion homogenizer (FJ200-SH, Shanghai Specimen Model Factory, China). Emulsions prepared from the MP solution (1%, *w/v*) with different pH values according to the same procedure served as controls.

2.5. Characterization of emulsions

2.5.1. Zeta-potential and droplet distribution measurement

The Zeta-potential of emulsions was measured using a Zeta Potential Analyzer (Zetasizer Nano ZS, Malvern Instruments Ltd., UK). Droplet sizes of the emulsions were measured with a laser particle size analyzer (Master sizer3000, Malvern Panalytical Ltd., USA). Emulsions were dispersed in distilled water, and the refractive indices of the oil droplets and water were 1.45 and 1.33, respectively.

2.5.2. Microstructure of emulsions

Both optical microscopy and Confocal laser scanning microscopy (CLSM) employed to visualize the microstructure of the emulsions. The droplet morphologies were observed using an optical microscope (Nikom, Japan) at a 10× objective magnification. Samples (10 μL) were placed on slides without cover glasses to avoid droplet deformation. For CLSM, an Olympus FV1200-OSR laser scanning confocal microscope (Tokyo, Japan) with a 10× objective was used. Emulsion samples (1 mL) were stained with Rhodamine B (40 μL, 0.2%, *w/v*) and vortexed for 30 s, followed by a 30-min incubation in the dark. The stained sample (10 μL) was then placed on a concave glass slide and covered with a coverslip. The excitation wavelength used was 543 nm.

2.5.3. Rheological measurements

Rheological properties of the emulsions were measured using a shear rheometer (DHR-2, American Tower, USA) with 40 mm parallel plate geometry. The operating gap was set at 1 mm, and measurements were conducted at 25 °C. Steady shear scans were performed over a range of 0.1–100 s^{-1} to assess viscosity versus the shear frequency. Strain sweeps were conducted from 0.1% to 100% strain at a constant frequency of 1 Hz. Frequency sweeps were performed at a strain of 0.5% within the linear range, with angular frequency range from 0.1 to 10 Hz, to determine the storage modulus (G′) and loss modulus (G″).

2.5.4. Stability of emulsions

For stability evaluation, emulsions were transferred to 25 mL glass vials and stored at 4 °C for 2 weeks. The appearance of the emulsions was recorded every week using a mobile phone camera (Mate 20, Huawei Technologies Co., Ltd., China) to monitor stratification.

2.6. Statistical analysis

Statistical analysis was performed using Origin 2019 (OriginLab, Northampton, USA) and Graphpad Prism 8.0 (GraphPad Software, San Diego, USA). Results expressed as the mean ± standard deviation. One-way analysis of variance (ANOVA) was performed using SPSS 22.0 statistical software (SPSS Inc., Chicago, USA), with statistical significance set at $p < 0.05$, determined using Duncan's test.

3. Results and discussions

3.1. Complexation behavior of MP and CMCS interaction

UV–vis spectroscopy can be used to analyze the impact of the interactions between MP and CMCS on the conformation of MP under different pH conditions. MP displayed a maximum absorption peak at 620 nm when pH value was 4 and 7 (Fig. 1A). Study reported that microalgae protein exhibited a maximum UV absorption wavelength at 620 nm can be due to its C-phycocyanin composition (Yin et al., 2024), therefore, the higher absorption peak at 620 nm could be attributed to a higher C-phycocyanin content in the samples. At pH 2, the maximum absorption peak (λ_{max}) of MP shifted to 625 nm, suggesting alterations in the microenvironment of amino acid residues. The UV–vis absorption intensity of MP diminished with decreasing pH, likely due to weak electrostatic repulsion under acidic conditions leading to aggregation, thereby concealing some amino acid residues and reducing UV–vis absorption intensity (Yang et al., 2020). Notably, the maximum adsorption peak of MP demonstrated a significant red-shift at pH 8, indicating the original structure and conformation of MP were changed. The introduction of CMCS mitigated this change, potentially due to CMCS enhancing the hydrophilicity of MP's amino acid residues. At pH 2 and 7, the addition of CMCS notably decreased the absorption peak intensity of MP, suggesting that the interaction between CMCS and MP altered the structure and hydrophobic microenvironment of amino acid residues (Chen et al., 2019). A similar phenomenon has been reported, where the absorption peak intensity of soybean isolated protein decreased upon the introduction of soybean polysaccharide (Wang et al., 2020; Wang, Tao, Yin, Sun, & Li, 2020).

Tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe) residues are primary contributors to the fluorescence emission spectrum of proteins when excited at 350–360 nm and are highly sensitive to changes in the polarity of their local environment (Guo et al., 2023). The fluorescence spectra of different samples are shown in Fig. 1B. The fluorescence intensity of MP decreased as the pH shifted from alkaline/neutral to acidic, likely due to acid-induced conformational changes resulting in hydrophobic aggregation and masking of Trp and Tyr residues (Yin et al., 2021). For MP-CMCS complexes, a reduction in fluorescence intensity was observed post complexation, indicating that CMCS incorporation may have led to the burial of additional amino acid residues, causing the protein structure to become more frizzled and enclosed.

Particle size is an important index for evaluating the stability of protein solutions, as it directly reflects the dispersion of the insoluble phase in water. The droplet distribution and average particle size of MP and MP-CMCS complexes solutions were shown in Fig. 1C and D. MP solutions displayed a single-peak distribution, with uniform particle distribution at pH values of 2, 4, and 7. The addition of CMCS significantly increased the particle size of the solutions, suggesting that MP-CMCS interactions influence the size distribution. At pH values of 7 and 8, the average particle size of MP solutions was larger. Similar with previous studies, proteins exhibit smaller particle sizes at acidic pH compared to pH 7, possibly attributed to the dissociation of protein subunits in acidic conditions (Ma, Habibi, & Sagis, 2024).

Turbidity measurements offer an intuitive method to study the interactions and changes during the formation of complex systems (Li et al., 2017). As shown in Fig. 1E, the turbidity of MP solutions varied significantly over the pH range of 2–7, likely due to the isoelectric point of MP being approximately 4–5 (Chen, Chen, et al., 2019; Chen, Wang, et al., 2019). Near the isoelectric point, MP molecules exhibit minimal surface charge, resulting in weak repulsive forces and increased susceptibility to collision, coalescence, and precipitation, thereby elevating turbidity values. At pH 4 (close to the isoelectric point of MP), the negative charge on the CMCS surface induced electrostatic repulsion, and thus, the turbidity of the solution remained largely unchanged upon CMCS addition. Conversely, at pH values of 2 and 7, the introduction of CMCS led to a marked increase in the turbidity of the composite solution. Combined with the results of particle size analysis, this phenomenon can be attributed to the formation of soluble MP-CMCS complexes. The highest turbidity value was observed at pH 8, suggesting potential aggregation of the MP-CMCS complexes.

To investigate the interactions between MP and CMCS and elucidate the formation processes of MP-CMCS complexes at varying pH levels, the zeta potentials of MP and the MP-CMCS complexes were measured. As shown in Fig. 1F, the zeta potential of MP solution decreased to −2.98 mV as the pH increased to 8. MP-CMCS exhibited a similar downward trend in zeta potential with increasing pH values. Integrating the turbidity and zeta potential results, at pH 2, both MP and MP-CMCS complexes had numerous positive surface charges, leading to strong electrostatic repulsions. Consequently, the complexes co-dissolved in the aqueous solution, forming co-soluble complexes, evidenced by low turbidity ($OD600 = 1.1$) and a high zeta potential (+14.13 mV). At pH 4, the surface charges on MP particles were relatively low and near zero. CMCS chains stretched due to intramolecular repulsions, which provide strong electrostatic and steric repulsions when present on the surface of MP-CMCS complexes, thus preventing aggregation of the MP-CMCS complexes, as indicated by a zeta potential greater than zero (+7.78 mV).

Considering the turbidity and zeta potential outcomes at pH 7, MP and CMCS formed partially soluble complexes. The solubility at this pH is not attributed to electrostatic attraction, given that both MP and CMCS have the same charges. It has been reported that protein and polysaccharide molecules can also interact through covalent bonding and hydrogen bonding (Shang, Zhu, & Fan, 2013). At pH 8, MP-CMCS complexes aggregated, suggesting the hydrogen or covalent bond might be formed among the complexes. Previous studies on whey protein–pectin and oat protein–pectin complexes proposed that protein–polysaccharide complexes formation often results from multiple interacting forces (Albano & Nicoletti, 2018; Wang, Liu, Zheng, Yuan, & Yang, 2024). In summary, the interactions between MP and CMCS appear not to be electrostatic but may involve hydrogen or covalent bonds. At pH 8, further interactions within the MP-CMCS complexes led to the formation of insoluble matter, resulting in solution instability.

The microstructures of MP and MP-CMCS complexes were shown in Fig. 2A. At varying pH levels, MP exhibited porous flakes, suggesting

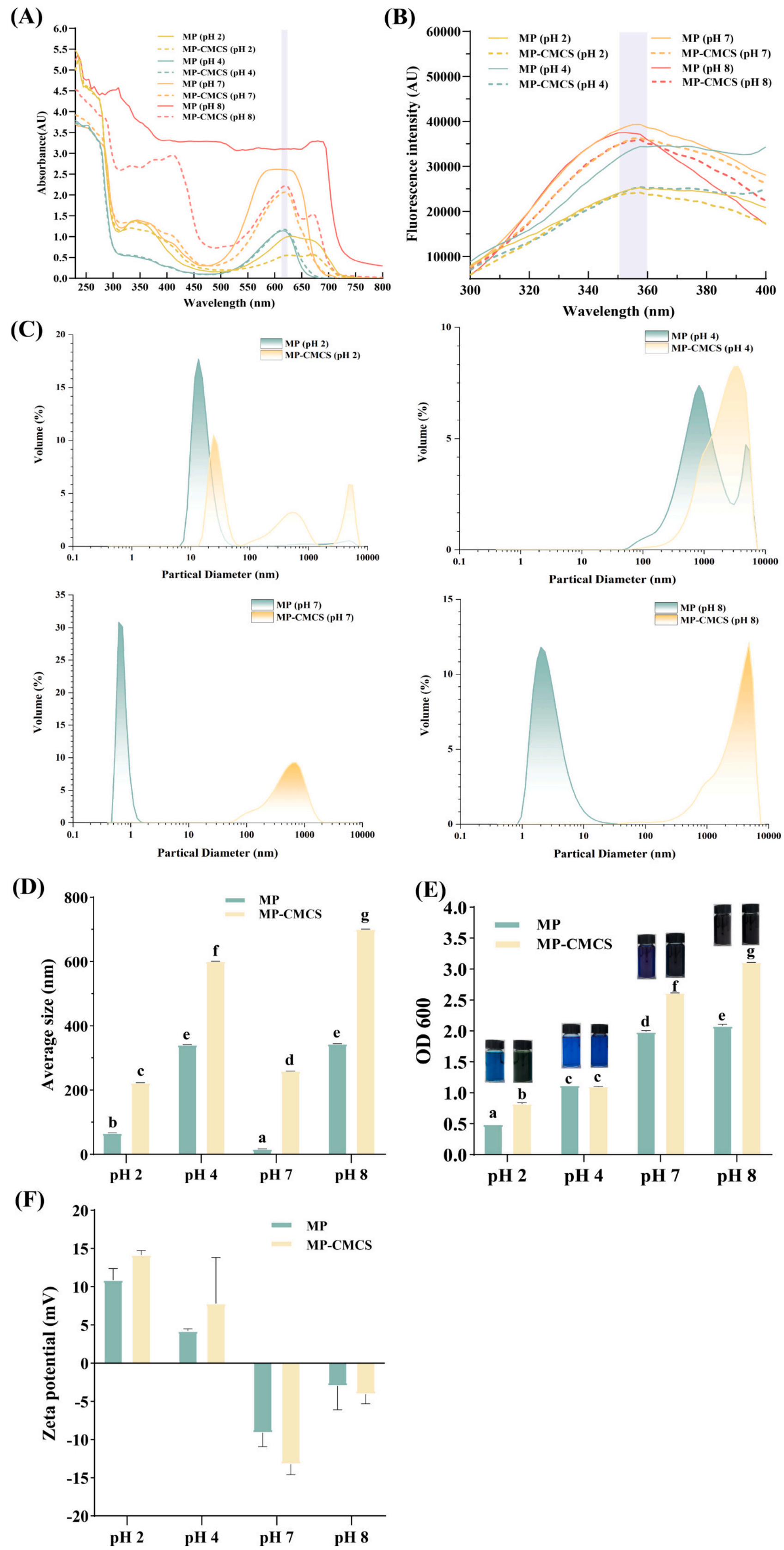

Fig. 1. Characterizations of the MP-CMCS complexes at different pH values. (A) UV spectrum. (B) Fluorescence emission spectra. (C) Particle size distribution and (D) mean particle size of MP or MP-CMCS complexes solutions. (E) Turbidity of MP or MP-CMCS complexes solutions. (F) Zeta potential of MP or MP-CMCS complexes solutions. Different lowercase letters represented significant differences between groups.

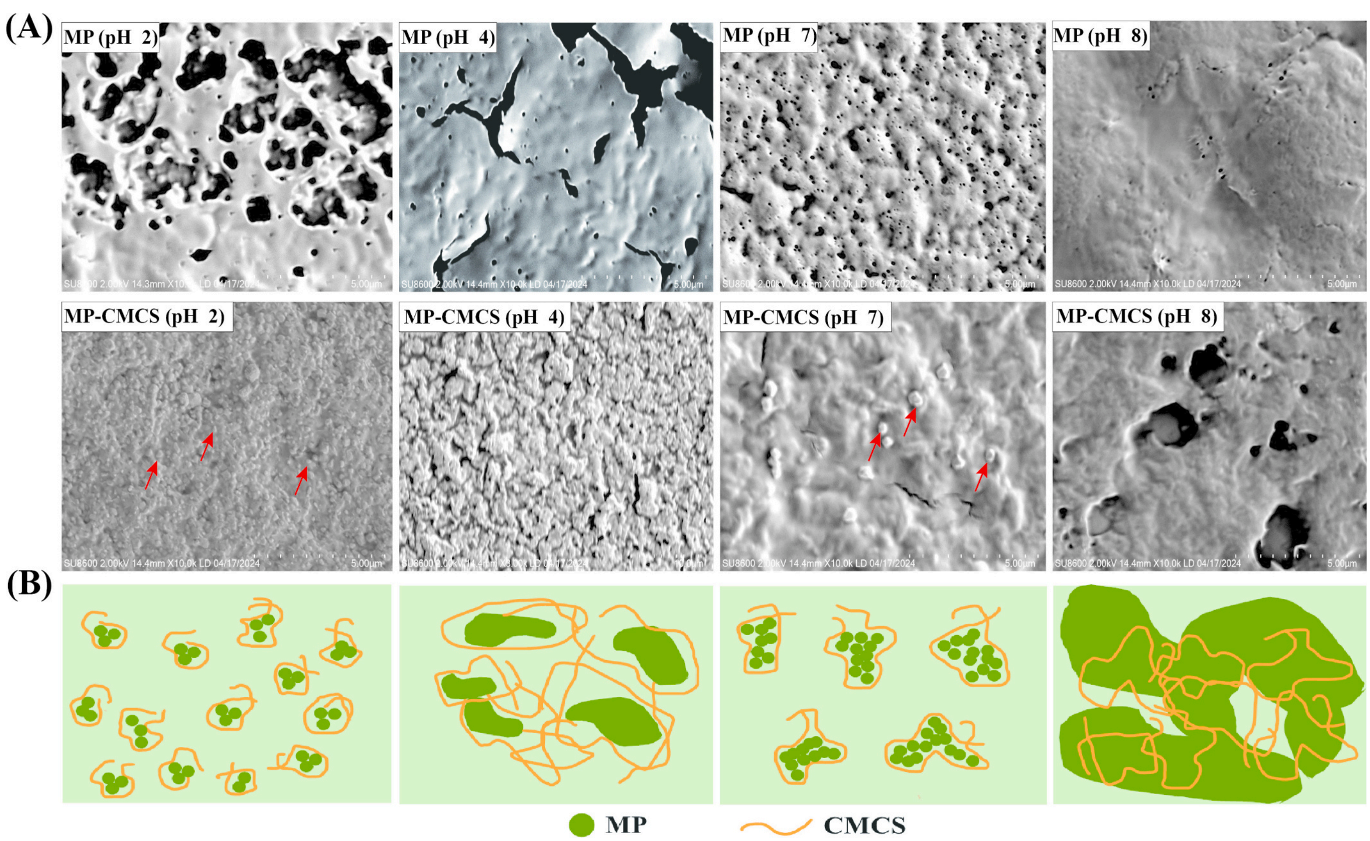

Fig. 2. SEM images (A) and schematic representation (B) of MP-CMCS complex at different pH values. Red arrows indicated MP particles. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

molecular adhesion or significant aggregation in the solution. MP-CMCS complexes displayed different morphologies. At pH 2 and 7, the complexes had many dense, smaller particles, indicating minimal adhesion and aggregation. At pH 4, MP-CMCS complexes formed relatively crumbly chunks, consistent with previous findings that MP aggregated at this pH, but CMCS can encapsulate MP and inhibited further aggregation via electrostatic repulsion. At pH 8, no particles were observed, corroborating the particle size, turbidity, and potential analyses, which suggested MP-CMCS complexes aggregation. A similar phenomenon was observed in milk protein-corn fiber gum complexes (Yadav, Strahan, Mukhopadhyay, Hotchkiss, & Hicks, 2012). Fig. 2B presented a schematic representation of MP-CMCS complexes at different pH levels.

3.2. Emulsification properties of MP-CMCS complexes

3.2.1. Droplet size and zeta potential of MP-CMCS complexes stabilized emulsions

The droplet size of an emulsion is an important indicator for estimating its stability, with smaller droplet sizes correlating with increased stability (Xie et al., 2023). As shown in Fig. 3A and B, all emulsions exhibited a single peak in droplet size distribution, indicating homogeneity of the oil droplets. The droplet size of MP-CMCS complexes stabilized emulsions was significantly smaller than MP stabilized emulsions at pH 2, 4, and 7. These results suggested that MP-CMCS complexes stabilized emulsions were more stable than MP stabilized emulsions, in other words, the presence of CMCS markedly improved the emulsification performance of MP. According to the complexation behavior analysis of MP and CMCS, at pH 2 and 7, MP and CMCS formed soluble complexes that were not prone to adhesion and aggregation. At pH 4, CMCS inhibited further aggregation of MP, allowing MP to be stably distributed on the oil droplet surface, so that the oil droplets were not easy to fuse. These findings were consistent with previous reports indicating that emulsions stabilized by protein-polysaccharide complexes exhibited smaller (Huang, Lin, Zhang, & Tang, 2022; Ling, Huang, He, & Zhou, 2024). Additionally, polysaccharide molecules are known to enhance interfacial coverage and increase the thickness of oil-water interfacial layers, leading to smaller oil droplet sizes (Zhao, Fan, Liu, & Li, 2023).

Conversely, at pH 8, the droplet size of MP-CMCS complexes stabilized emulsions was significantly larger than MP stabilized emulsions, suggesting that the addition of CMCS did not improve the emulsification properties of MP. According to the results of complexation behavior analysis of MP and CMCS, at this pH, MP and CMCS formed insoluble complexes that tended to adhere and cluster in solution, inhibiting their stable distribution on the oil droplet surface, which in turn allowed oil droplets to fuse, resulting in larger particle sizes. Previous studies had reported that emulsions stabilized by protein-polysaccharide complexes show larger droplet sizes than those stabilized by protein alone at pH 8, due to excessive aggregation of the complexes (Wang et al., 2024).

The zeta potential measurements for MP-CMCS complexes stabilized emulsions were displayed in Fig. 3C. The charge characteristics of these emulsions aligned with those of the MP or MP-CMCS complexes solutions. At pH 2, 4, and 7, the absolute zeta potential of MP-CMCS complexes stabilized emulsions was significantly higher than that of MP stabilized emulsions. This indicated that the net charges on the surface of MP-CMCS complexes stabilized emulsion droplets were higher, enhancing the repulsive forces among the droplets and thereby preventing fusion (Guo et al., 2022). Mirroring the particle size results, at pH 8, the zeta potential of MP-CMCS complexes stabilized emulsions was lower than that of MP stabilized emulsions, indicating that MP-CMCS complexes do not enhance electrostatic repulsion between oil droplets at this pH. It was reported that the surface charge of protein-polysaccharide complexes stabilized emulsion droplets was influenced by the –NH2 and –COOH groups of polysaccharide molecules, which are

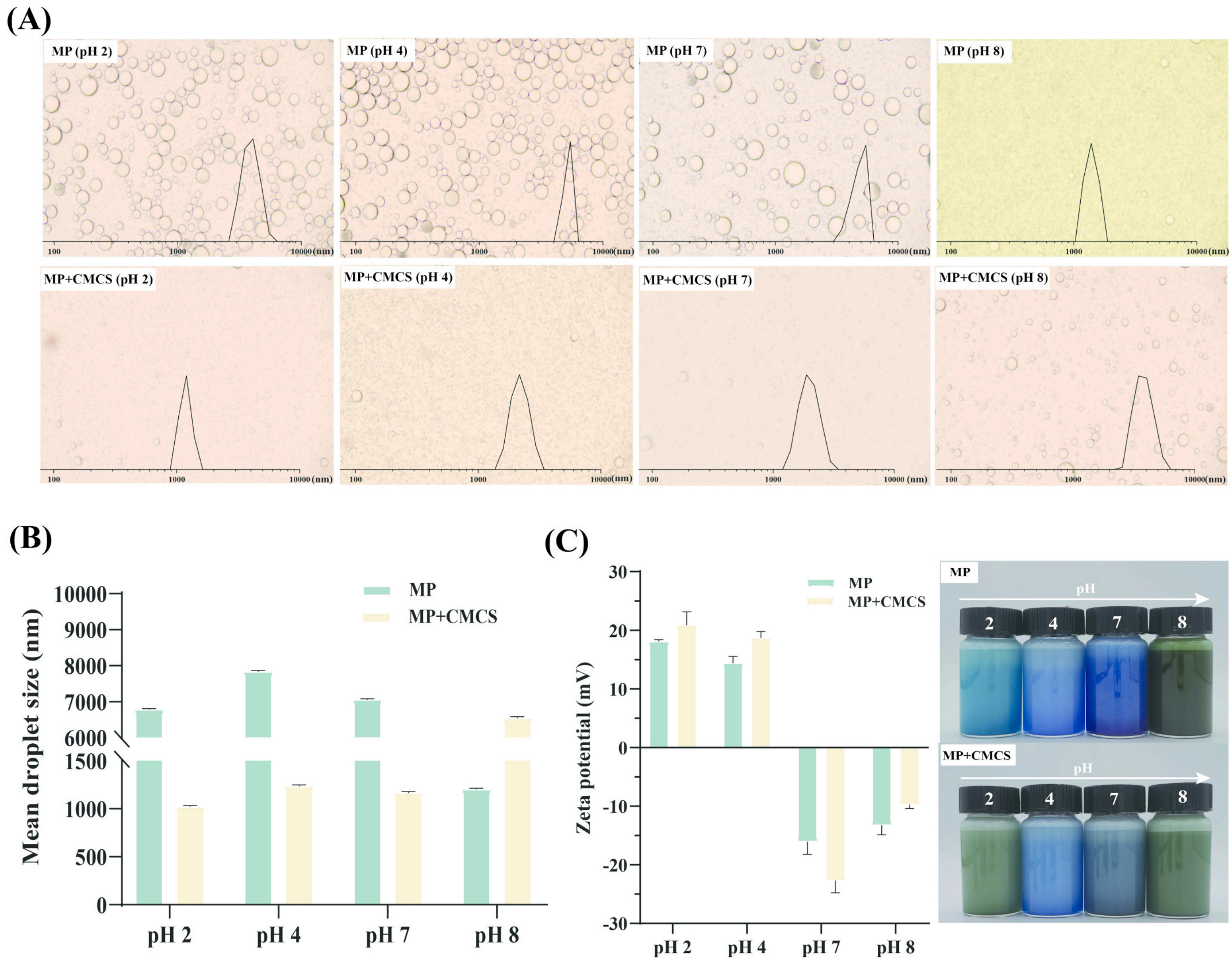

Fig. 3. Optical micrographs (A), mean droplet size (B), and zeta potential (C) of MP-CMCS complexes stabilized emulsions.

highly affected by the pH of the aqueous phase (Sriprablom, Luangpituksa, Wongkongkatep, Pongtharangkul, & Suphantharika, 2019). Therefore, in addition to the interaction between MP and CMCS, the net charge of emulsion oil droplets and their stability can also be analyzed from the perspective of pH effects on the functional groups of CMCS.

3.2.2. Visual observation of emulsion storage stability

As shown in Fig. 4A, after 2 weeks of storage, MP stabilized emulsions exhibited varying degrees of delamination at pH levels of 2, 4, 7, and 8. In contrast, the appearances of MP-CMCS complexes stabilized emulsions at pH 2, 4, and 7 were more stable than those of MP stabilized emulsions. For pure protein stabilized emulsions, the adsorption of pure protein onto the oil-water (O/W) interface, driven by hydrophobic forces, tends to fail due to protein folding. This process leads to the aggregation of hydrophobic residues within the protein molecule, contributing to emulsion instability (Xie et al., 2023). The presence of polysaccharide could modify the protein-protein interaction networks, thus leading to the differences in adsorption behavior of polysaccharide-protein complexes at the O/W interface. With the presence of polysaccharides, interactions between polysaccharides and proteins prompt protein unfolding, exposing hydrophobic residues, which enhances adsorption onto the O/W interface and improves emulsion stability (Zhao, Fan, Liu, & Li, 2022). Furthermore, the polysaccharide-protein complexes can help to form a thicker and stronger interfacial layer on the droplets (Tian et al., 2022). The coverage of polysaccharides over the protein-stabilized O/W interface can alter the net charge of the interfacial layer, thereby enhancing emulsion stability (Chang et al., 2021). At pH 8, the MP-CMCS complexes stabilized emulsions exhibited the lowest stability (distinct delamination). According to analysis results of the complexation behavior of MP and CMCS interaction, this might be caused by excessive aggregation of the complexes due to hydrogen bonding interactions.

Further, the distribution of emulsion droplets after 2 weeks of storage was observed by laser confocal microscopy (Fig. 4B). Results showed that, at pH 2 and 7, MP-CMCS complexes stabilized emulsions had uniform droplet distribution and clear boundaries, indicating no droplet fusion. However, at pH 4, the oil droplet boundaries of MP-CMCS complexes stabilized emulsions were slight ambiguous, indicating the droplet may begin to fuse. Combining the emulsion appearance and laser confocal microscopy results, CMCS can effectively improve the emulsifying properties of MP under acidic and neutral conditions.

Previous studies have demonstrated that particles composed of both hydrophilic (e.g., gelatin and cellulose) and hydrophobic compounds (e.g., zein) typically achieve a hydrophobic-hydrophilic balance (de Folter, van Ruijven, & Velikov, 2012; Li, Erni, Van Der Gucht, & De Vries, 2020). Hydrophilic compounds display a high affinity for the aqueous phase, while hydrophobic compounds exhibit a tendency to stay at the oil phase. The hydrophobicity of proteins is primarily attributed to their

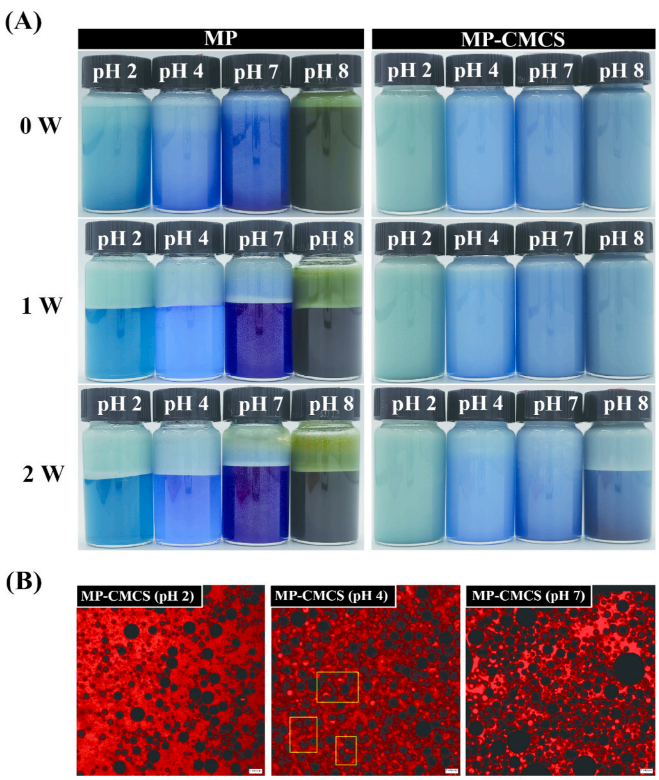

Fig. 4. Stability of MP-CMCS complexes stabilized emulsions. (A)Visual appearance after 2 weeks storage. (B) CLSM images of MP-CMCS complexes stabilized emulsions (pH 2, 4, and 7). Black color was the droplet and red color was the aqueous phase (containing soluble MP-CMCS complexes). The scale in images was 1 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

substantial non-polar amino acids (e.g., proline and alanine) (Shukla & Cheryan, 2001). Conversely, polysaccharides contain hydrophilic groups (e.g., hydroxyl, carboxyl) that form hydrogen bonds with water, imparting them with hydrophilic properties. Amphiphilic particles, which exhibit interfacial affinity, are commonly produced by the physical cross-linking of polysaccharides and proteins, forming complexes (Wang et al., 2023). Adjusting the pH can modulate protein surface hydrophobicity, thus influencing the hydrophobic-hydrophilic balance essential for stabilizing emulsions (Wang, Tao, et al., 2020; Wang, Yang, et al., 2020). In this study, MP-CMCS complexes stabilized emulsions exhibited high stability at pH values of 2, 4, and 7. This stability may be attributed not only to the formation of a network structure or thick interfacial layer by the complexes, but also to achieving a hydrophobic-hydrophilic balance at these pH levels. This balance prevented phase separation, even after two weeks of storage. Further confirmation of this hypothesis can be conducted by assessing the amphiphilicity of the complexes.

3.2.3. Rheological property

The rheological properties were assessed to understand the interactions and stability of the MP-CMCS complexes stabilized emulsions at different pHs. As shown in Fig. 5A, all emulsions exhibited shear-thinning behavior, likely due to internal structure rupture under high shear rates (Wang et al., 2021). At pH 2, 4, and 7, MP-CMCS complexes stabilized emulsions displayed higher viscosity than MP stabilized emulsions across the entire shear rate range.

The shear stress of emulsions is another flow property that provide important information about how much the force or torque is required to permanently deform droplets. As shown in Fig. 5B, when shear rate increased to 10–100 s^{-1}, MP-CMCS (pH 2), MP-CMCS (pH 4), and MP-CMCS (pH 7) showed the highest shear stress among all samples, which implies that these emulsions were probably more stable as a much stronger force was needed to initiate the flow of these emulsions (Huang et al., 2023).

The storage moduli (G′) and loss moduli (G″) as functions of shear frequency were summarized in Fig. 5C and D. For all samples, G′ exceeded G″ across the tested frequency range, indicating solid-like behavior (Zamani, Malchione, Selig, & Abbaspourrad, 2018). This can be attributed to the formation of a three-dimensional network through droplet interactions within emulsions (Dong et al., 2021). Moreover, rheological properties of the coacervates formed by protein and polysaccharide depend on the strength of their intermolecular interactions, with stronger interactions leading to stronger viscoelastic properties (Zhang, Xiao, & Huang, 2020). The G′ of MP-CMCS complexes stabilized emulsions at pH 2, 4, and 7 were higher than those at pH 8 (Fig. 5C), indicating that the interactions between MP and CMCS were stronger under acidic and neutral conditions compared to alkaline conditions. The G′ and G″ values for MP (pH 8) and MP-CMCS (pH 8) increased significantly with amplitude (Fig. 5C and D), indicating a notable impact on the network structure strength and viscosity of these emulsions under stress (Yang, Huang, et al., 2020).

Recent studies have explored the application of MP as an emulsifier in food production. For example, Rodrigues et al. (2020) utilized aqueous extracts of *Spirulina* containing phycocyanin as a substitute of additives in the ice cream production, finding phycocyanin presented emulsifying and stabilizing activity without influencing overall consumer acceptability. Almeida et al. (2021) developed an instant sauce enriched with 4% *Spirulina* sp., maintaining storage stability (appearance and taste) up to 45 days. In this study, complexation with CMCS was demonstrated a viable method for enhancing the emulsification stability of MP across a broader pH range. Future applications of MP-CMCS complex-stabilized emulsions may include fat substitutes and nutraceutical delivery (Xia, Xue, & Wei, 2021), although substantial foundational research is required to realize these potential uses. For example, it is necessary to investigate how the degree of substitution and molecular weight of CMCS influence complexes formation and emulsion stability, including the effects of these parameters on the interactions between CMCS and MP, as well as their impact on the structure and distribution of the MP-CMCS complexes at the oil-water interface.

4. Conclusion

The emulsification stability of microalgae protein (MP) is restricted under strongly alkaline conditions, particularly at pH 10. This study utilized carboxymethyl chitosan (CMCS) to form MP-CMCS complexes, significantly enhancing MP's emulsification stability under acidic and neutral conditions. At pH 2 and 7, MP and CMCS formed soluble complexes, possibly due to covalent and hydrogen bond formation. At pH 4, CMCS prevented MP aggregation and precipitation by unfolding its molecular chains, providing electrostatic and steric repulsion. At pH 8, the MP-CMCS complexes aggregated, possibly due to hydrogen or covalent bond formation. Emulsifying properties of MP were improved with the addition of CMCS at pH 2, 4, and 7, leading to enhanced emulsion stability, but were not improved at pH 8. These findings can be explained by interactions between MP and CMCS and the rheological behavior of emulsions, exhibiting higher viscosity and shear stress at pH 2, 4, and 7, thus achieving greater stability. MP-CMCS stabilized emulsions showed stronger viscoelastic properties and higher storage modulus (G') at these pH levels, indicating stronger intermolecular interactions. This study elucidated the binding interactions between MP and CMCS in aqueous solutions across different pH levels and clarified the effect of CMCS on the emulsification properties of MP, proposing a viable approach to enhance the emulsification stability of MP over a wider pH range, thus overcoming limitations in its broader application in food processing.

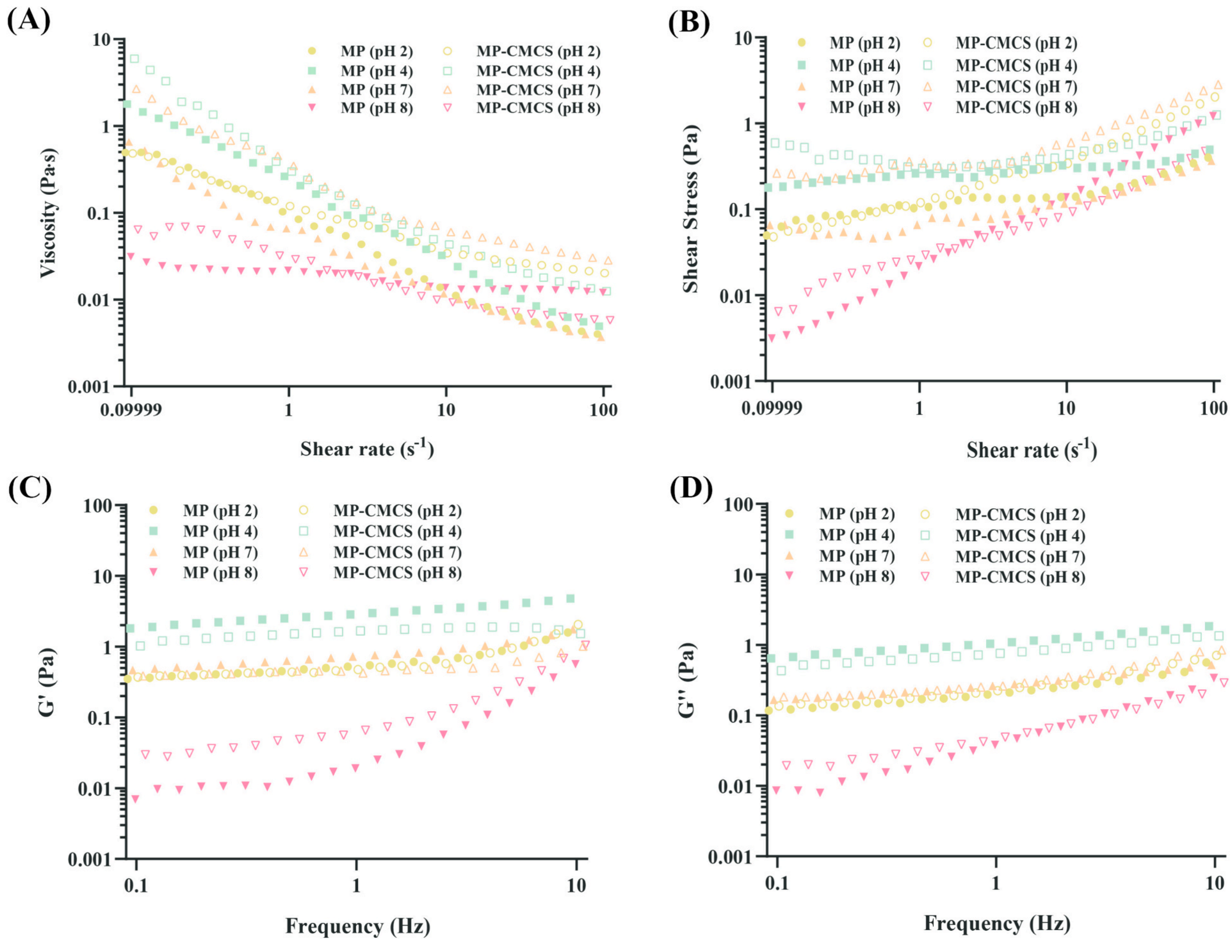

Fig. 5. Apparent viscosity (A), shear stress (B), and viscosity properties (storage modulus (G′) and loss modulus (G″)) (C and D) of MP-CMCS complexes stabilized emulsions.

CRediT authorship contribution statement

Qian Wang: Writing – original draft, Data curation. **Chunxia Li:** Writing – original draft, Data curation. **Yuqian Qiao:** Software, Methodology. **Yacheng Hao:** Methodology, Investigation. **Zhiyong Gong:** Validation, Resources. **Yongning Wu:** Formal analysis, Conceptualization. **Xiao Guo:** Writing – review & editing, Supervision. **Xin Liu:** Writing – review & editing, Funding acquisition.

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

No data was used for the research described in the article.

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Further-reading

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