Comparison of milk protein concentrate, micellar casein, and whey protein isolate in loading astaxanthin after the treatment of ultrasound-assisted pH shifting

X. P. Wang,1 C. F. Wang,1* X. Q. Zhao,1 M. J. Ma,1 Z. H. Li,1 H. Jiang,1 X. N. Zhang,1 and C. Z. Yuan2*
1School of Food Science and Engineering, Qilu University of Technology (Shandong Academy of Science), Jinan 250353, China
2Department of Obstetrics and Gynecology, Qilu Hospital of Shandong University, Jinan, China

ABSTRACT

Milk proteins can be used as encapsulation walls to increase the bioavailability of active compounds because they can bind hydrophobic, hydrophilic, and charged compounds. The objective of this study was to investigate the effects of astaxanthin (ASTA) encapsulation and the functional properties of milk protein and ASTA nanocomposites by an ultrasound-assisted pH-shifting treatment of different milk proteins, including milk protein concentrate (MPC), micellar casein (MCC), and whey protein isolate (WPI). The ultrasound-assisted pH-shifting treatment of milk protein helped to improve the encapsulation rate of ASTA. Therein, MCC showed great improvement of encapsulating ASTA after co-treatment with the raised encapsulated rate of 5.11%, followed by WPI and MPC. Furthermore, the nanocomposites of ASTA with milk protein exhibit improved bioavailability, antioxidant capacity, and storage stability. By comparison, MCC-encapsulated ASTA has the best storage stability, followed by MPC, and WPI-encapsulated ASTA has the least stability over a 28-d storage period. The results of intrinsic fluorescence and surface hydrophobicity showed that milk protein underwent fluorescence quenching after binding to ASTA, which was due to the hydrophobic sites of the protein being occupied by ASTA. In general, the nanocomposites of milk protein and ASTA fabricated by using an ultrasound-assisted pH-shifting treatment have the potential to be better nano-delivery systems for ASTA in food, pharmaceuticals, and dietary supplements.

Key words: milk protein, ultrasound-assisted pH shifting, astaxanthin, nanocomposites, functional properties

INTRODUCTION

In recent decades, with the increasing interest in developing healthy foods containing natural bioactive compounds (Bustamante et al., 2016), astaxanthin (ASTA) has attracted much attention as a valuable antioxidant with anti-inflammatory (Kwak et al., 2021) and anticancer (Zhou et al., 2021) properties. However, ASTA has a highly unsaturated, conjugated structure, which reduces its bioavailability (Ambati et al., 2014). In addition, the poor water solubility of ASTA limits its dispersion in aqueous food media, further contributing to its low oral bioavailability (Morales et al., 2021). These drawbacks highly limit the application of ASTA in food, pharmaceuticals, and dietary supplements. Currently, various technologies are being used to slow ASTA degradation and improve its bioavailability. These include various methods of microencapsulation using different shell substances, including pectin, chitosan, starch, alginate, maltodextrin, cellulose, and so on (Kittikaiwan et al., 2007; Bustos-Garza et al., 2013; Bustamante et al., 2016; Vakarelova et al., 2017; Feng et al., 2018). Because some microencapsulated materials, such as cellulose, do not have any specific enzymes that can break down the shell, these structures can occasionally be difficult to digest (Feng et al., 2018). Chitosan was successfully employed in another study to shield ASTA from oxidation (Higuera-Ciapara et al., 2004; Kittikaiwan et al., 2007), however this method is unsuitable for many food applications due to chitosan’s insolubility in water at neutral pH and its sparse release.

Proteins can be used to manufacture bioactive compound delivery systems because of their binding to hydrophobic, hydrophilic, and charged bioactive compounds and their ability to stabilize compounds’ biological activity (Pan et al., 2014). The mechanism of protein binding of hydrophobic bioactive compounds lies in several noncovalent interactions, including hydrogen bonding, hydrophobic interactions, electrostatic interactions, and van der Waals forces, in which hydrophobic interactions between β-LG and ASTA were...
the dominant force that maintained the binding affinity (Liu et al., 2021a). Improved stability, water solubility, increased encapsulation rate, extended shelf life, and bioavailability of lipophilic medicines during gastrointestinal digestion are benefits of protein-based food delivery vehicles (Chen et al., 2014). According to a study, when molecules of curcumin were connected to nonpolar regions (the aromatic AA residues) of whey protein isolate (WPI) by hydrophobic interactions, their solubility increased by 11,355 times and their antioxidant activity increased by 7 times when compared with raw curcumin crystals (Liu et al., 2016). Similarly, after β-carotene is embedded in WPI-based microparticles, its stability, antioxidant activity, and resistance to gastric protein degradation significantly improved (Rodrigues et al., 2020). Curcumin may be encapsulated in emulsions stabilized with whey protein emulsions, strengthening its bioaccessibility, antioxidant activity, and storage stability (Pan et al., 2020). Casein micelles have been reported to bind curcumin molecules by hydrophobic interactions with a binding constant of 1.48 × 10^4 M⁻¹; the resulting complex exhibited higher water dispersibility, bioavailability, and antitumor proliferation ability (Barick et al., 2021). Overall, milk proteins have inherent properties that make them excellent candidates for the encapsulation of bioactive compounds in various environments, and WPI are employed in most of the studies on milk protein delivery. However, the utilization of milk protein concentrate (MPC) and micellar casein (MCC) in such delivery systems is rarely reported, and no studies have been conducted to compare their capacity to deliver bioactive substances.

It is generally known that physicochemical techniques may alter the structural characteristics and functional characteristics of proteins (Uluko et al., 2016). Protein subunits may be altered by ultrasound, which can also increase protein solubility and surface hydrophobicity (Jiang et al., 2017). A pH transformation treatment may improve the nutritional proteins’ functional properties. This is comprised of first changing the pH of the initial proteins to an overly alkaline or acidic level before restoring it to a neutral level to allow the protein molecules to progressively undergo full or partial unfolding and refolding (Liang et al., 2018). Our prior research has shown that ultrasound-assisted pH shifting may successfully enhance the physicochemical characteristics and foaming capacity of milk proteins, particularly MCC, and has a wide range of potential applications in the food business (Zhao et al., 2022). The method of combining pH shifting and sonication has been applied to the protein-based delivery of bioactive substances. Xu et al. (2021) modified WPI by pH shifting combined with sonication, which greatly improved the water solubility and antioxidant capacity of resveratrol upon delivery. However, the study of modifying the structure of milk proteins to encapsulate ASTA by pH shifting combined with the ultrasonication method has not been reported.

In this work, ASTA was loaded with 3 kinds of milk proteins treated by ultrasound-assisted pH shifting to investigate the effects of ultrasound-assisted pH treatment on the role and structural-functional changes of milk protein-embedded astaxanthin and the differences in astaxanthin embedding between different milk proteins. The aim was also to further improve the ASTA encapsulation rate, bioaccessibility, and storage stability of nanocomposites. This research advances the possible use of ASTA in the creation of functional dairy products by illuminating the relationship between ASTA and milk protein.

**MATERIALS AND METHODS**

No human or animal subjects were used, so this analysis did not require approval by an Institutional Animal Care and Use Committee or Institutional Review Board.

**Materials**

The MPC (about 80% protein content) and MCC (about 90% protein content) were obtained from a commercial supplier, Leprino Foods Nutrition, and WPI (about 91% protein content) was donated by Agropur. All milk protein powder was stored at −80°C. Pepsin and trypsin were purchased, respectively, from Aladdin Reagent Co. Ltd. (Shanghai, China) and Solarbio Technology Co. Ltd. (Beijing, China). And bile salt extract, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were from Maclin Biochemical Technology Co. Ltd. (Shanghai, China). Astaxanthin (≥98% pure) was obtained from Yuanye Biotechnology Co. Ltd. (Shanghai, China), and dimethyl sulf oxide, dichloromethane, and methanol were provided by Fuyu Fine Chemical Co. Ltd. (Tianjin, China). Potassium persulfate was purchased from Damao Chemical Reagent Factory, and sodium hydroxide (NaOH), FeSO₄·7H₂O, and H₂O₂ were from Sinopharm Chemical Reagent Co. Ltd. Hydrochloric acid (HCl), Salicylic acid, and 8-anilino-1-naphthalenesulfonic acid (ANS) were supplied by Shanghai Macklin Biotechnology Co. Ltd. (Shanghai, China).

**Fabrication of the Composite Nanoparticles**

The pH of these solutions was raised to 11 using NaOH after the MPC, MCC, and WPI powders were...
dissolved in Milli-Q water (Direct-Q 5 UV, Millipore S.A.S.) to a protein concentration of 5 mg/mL. For 30 min, they were kept in an agitated state. Fifty milliliters of protein solution was mixed with 1 mL (0.6 mg/mL) of ASTA-dimethyl sulfoxide solution, and the mixture was allowed to stand for 30 min. Additionally, using ultrasound equipment (JY92-IIIN, Ningbo Scientz Biotechnology Co. Ltd.) equipped with a 12-mm horn, solutions were sonicated for 5 min at 20 kHz and 300 W. After applying HCl (1 mol/L) to bring the pH of the solutions to 7.0 (+0.02), they were then left at room temperature for half an hour. The nanocomposites of milk protein and ASTA prepared here were named A/MPCPU, A/MCCPU, and A/WPIPU. The nanocomposites without sonication and pH-shifting treatment were used as controls and named A/MPC, A/MCC, and A/WPI. All samples were stored in a refrigerator at 4°C until further analysis.

**Determination of ASTA Encapsulation Rates**

Astaxanthin encapsulation rates were estimated according to the method of Wang et al. (2022) with slight modifications. Five milliliters of nanocomposites were centrifuged at 1,000 × g for 10 min at 25°C. The supernatant was then collected and dissolved in 9 mL of organic solvent; the volume ratio of dichloromethane to methanol was 2:1. After centrifugation at 1,000 × g for 10 min at 25°C, the absorbance of the solution was measured at 476 nm using a UV spectrophotometer. A standard curve of ASTA developed under the same circumstances was used to calculate the ASTA content. The encapsulation efficiency of ASTA nanoparticles was calculated by the following equation:

\[
\text{Encapsulation rate} = \frac{C_1 - C_2}{C_1} \times 100\% ,
\]

where \(C_1\) is the total amount of ASTA in the solution, and \(C_2\) is the unencapsulated ASTA content.

**Particle Size, Zeta Potential, and Turbidity**

The Zetasizer Nano ZS90 instrument (Malvern Instruments Limited, Worcestershire, UK) was used to determine the mean particle size distribution of protein and ASTA nanocomposites. To avoid numerous scattering effects, protein sample solutions were 5-fold dilution with Milli-Q water before to analysis.

Employing a spectrophotometer (Yoke Instrument Co. Shanghai, China), the turbidity of the samples was determined. Milli-Q water served as the blank. The turbidity of each sample was indicated by its absorbance at 600 nm. At a temperature of 25°C, all measurements were made.

**Fluorescence Spectroscopic Analysis**

The intrinsic fluorescence of nanocomposites was determined using a fluorescence spectrophotometer in accordance with Liang and Subirade (2012). The prepared sample solution was diluted with phosphate buffer saline (PBS, 0.1 mol/L, pH 7.4) to a concentration of 0.1 mg/mL. The widths of the slits used for excitation and emission were 2.5 and 5 nm, respectively. The excitation wavelength of 280 nm was used to record the fluorescence emission spectra, which ranged from 290 to 450 nm.

**Surface Hydrophobicity**

The method of Han et al. (2020) was slightly adjusted to evaluate the surface hydrophobicity using the ANS as a fluorescent probe. The sample solution was diluted with PBS (0.1 mol/L, pH 7.4) to 0.5 mg/mL. For every 6 mL of the diluted sample, 30 L of an ANS solution (8 mmol/L, pH 7.4) was added. The reaction solution’s fluorescence intensity was measured after it had been allowed to react for 15 min in complete darkness using the following parameters: an excitation wavelength of 390 nm, an emission wavelength of 470 nm, and a slit width of 5 nm.

**Antioxidant Activity**

DPPH, ABTS⁺, and hydroxyl radical-scavenging ability were used to measure the antioxidant capabilities of nanocomposites. The test for DPPH radical scavenging was carried out using the method reported by Dai et al. (2017) with a few modifications. Briefly, 2 mL of the DPPH solution (0.1 mmol/L) was mixed with 6 kinds of nanocomposites (1.25 mg/mL) of equivalent aliquots. A dimethyl sulfoxide solution with the same ASTA concentration was used as a control. After being incubated at room temperature in the dark for 30 min, the reaction solution was centrifuged at 10,000 g for 10 min at 25°C. Then, using UV-visible spectroscopy, the absorbance of the reaction solution was determined at 517 nm. The fraction of the DPPH radical-scavenging percentage was calculated as follows:

\[
\text{RSA(\%)} = \frac{A_1 - A_0}{A_0} \times 100\% ,
\]

where \(A_0\) is the absorbance of the sample solvent, and \(A_1\) is the absorbance of the sample.
The ABTS radical-scavenging activity was measured using the method that Wang et al. (2012) previously reported. Milli-Q water was used to dilute the nanocomposites twice. Protein nanocomposites or ASTA-dimethyl sulfoxide solution were combined in equal parts with the ABTS solution. The absorbance value at 734 nm was ascertained right away after 10 min of incubation at room temperature. Equation 2 was used to get the radical scavenging percentage for ABTS.

The hydroxyl radical-scavenging activity was assessed using the method described by Yuan et al. (2013). After mixing 1.0 mL of FeSO₄ (9 mM), 1.0 mL of H₂O₂ (10 mM), 1.0 mL of salicylic acid-ethanol solution (9.0 mM), and protein nanocomposites, the resulting mixture was diluted 2-fold with Milli-Q water. After 30 min of incubation at 37°C, the absorbance at 517 nm was measured. Milli-Q water, instead of salicylic acid-ethanol, was used to prepare the control, and Milli-Q water, instead of the sample solution, served as the blank. The scavenging activity was obtained using Equation 2.

Bioaccessibility of ASTA in In Vitro Simulated Digestion

The method of Wang et al. (2022) was used to simulated in vitro digestion studies with a few minor adjustments. Simulated gastric juice (SGF) was prepared by mixing 2 g/L NaCl, 7 mL/L HCl and 1.6 g/L pepsin and adjusting the pH to 1.5. The SGF containing pepsin was mixed with protein solutions, and the pH was adjusted to 2. The mixture was incubated under successive stirring at 37°C at 150 rpm for 2 h. Simulated intestinal fluid (SIF) was prepared by dissolving 880 mg of NaH₂PO₄ and 876 mg NaCl in 100 mL of ultrapure water, followed by the addition of bile salt extract (4 mg/mL) and pancreatin (7.5 mg/mL). SIF (10 mL) was combined with the gastric phase’s final mixture (10 mL). The resultant mixture’s pH was raised to 7, and it was then put in a water bath at 37°C with constant shaking for 2 h. The same gastrointestinal procedures were applied to ASTA dispersed in dimethyl sulfoxide for comparison.

Following in vitro digestion, the bioaccessibility of ASTA was assessed using a modified version of the procedure outlined by Boonlao et al. (2020). The raw digest was briefly centrifuged at 1,000 × g for 15 min at 25°C. The resulting supernatant fraction was collected after centrifugation. Six milliliters of the organic solvent (dichloromethane: methanol 2:1, vol/vol) were used to extract 4 mL of ASTA. Before measuring the solution’s absorbance at 476 nm using a UV-visible spectrophotometer, the solution was centrifuged at 1,000 × g for 10 min at 25°C. Equation 3 was used to determine ASTA bioaccessibility.

\[
\text{Bioaccessibility} (%) = \frac{C_1}{C_0} \times 100\%, \quad [3]
\]

where \( C_1 \) is the concentration of ASTA in the supernatant, and \( C_0 \) is the initial concentration of ASTA in the solution.

ASTA Retention Rate During Storage

The samples were put in brown glass vials and kept in the fridge at 4°C for 28 d. The particle size and potential of the samples, as well as the retention rate of ASTA in the sample solution, were measured once every 7 d. ASTA was extracted by mixing 1 mL of the sample solution with 9 mL of the organic solvent (dichloromethane: methanol 2:1, vol/vol). The solution’s absorbance was measured at 476 nm after centrifugation at 1,000 × g for 10 min at 25°C. The ASTA retention rate was calculated using Equation 4:

\[
\text{ASTA retention rate} \% = \frac{B_0}{B_1} \times 100\%, \quad [4]
\]

where \( B_0 \) is the concentration of ASTA in the sample during storage, and \( B_1 \) is the initial concentration of ASTA in the solution.

Statistical Analysis

All experiments with milk protein-embedded ASTA were performed in 3 replicates. Standard deviation and mean are used to show the data. One-way ANOVA was used for the statistical analysis in SPSS (version 22; IBM Corp., Armonk, NY) with a significance level of \( P < 0.05 \). Graphs were created using the Origin Pro 8.5 program (OriginLab).

RESULTS AND DISCUSSION

ASTA Encapsulation Rate

As shown in Figure 1, we observed a significant increase in encapsulation efficiency of ASTA embedded in proteins after treatment with ultrasound-assisted alkaline pH shifting for the end goal of ASTA delivery. ASTA is lipid soluble and has poor solubility in water. However, when ASTA was mixed with a milk protein solution, its dispersion in aqueous media was improved due to the interaction between milk proteins and ASTA. For the 3 samples of untreated milk proteins with different compositions, WPI showed the highest encapsulation rate of ASTA, reaching 88.63%, which was likely attributable to its functional properties, such as good
solubility of WPI. This can be attributed to the functional properties of WPI, major components of WPI, β-LG and α-LA, have a large portion of hydrophilic AA residues on the surface to remain highly soluble at the corresponding pI (Damodaran, 2017). The MCC component has a high quantity of casein, which has a lower solubility in comparison to WPI, which results in an inability to evenly distribute it in water, leading to a reduced ASTA encapsulation rate (Lu et al., 2015; Panthi et al., 2021). After an ultrasound-assisted pH-shifting treatment, the 3 different compositions of milk proteins were observed the significant changes on embedding in ASTA, with the greatest increase in the embedding rate of MCC, which raised by 5.11%. Although WPI showed the highest encapsulation rate, we observed no significant difference in the encapsulation rates of the 3 proteins after an ultrasound-assisted pH-shifting treatment. The exposure of hydrophobic sites of the proteins and the more structural changes after the treatment with ultrasound-assisted alkaline pH shifting was beneficial to the binding of the proteins to hydrophobic active substances, that is, it strengthened the binding rate between the proteins and ASTA (Xu et al., 2021). At the same time, co-treatment resulted in favorable improvements in the structure of milk proteins and their functional properties, such as solubility. These improvements would also contribute to the encapsulation of ASTA by milk proteins (Du et al., 2022; Jiang et al., 2022b).

**Particle Size**

Figure 2 shows changes in the particle size of the protein and ASTA nanocomposites after an ultrasound-assisted pH-shifting treatment. The particle size of MPC (Figure 2a and 2d) and MCC (Figure 2b and 2e) underwent significant reduction by treating them with ultrasonication and pH shifting. This discovery was primarily due to the severe mechanical disrupting forces produced by ultrasound and the increase in electrostatic repulsion between protein molecules under extremely alkaline circumstances (Jiang et al., 2022a). Ultrasonication was applied after unfolding of proteins with high pH treatments, which opened up their tight structure, making them more susceptible to breakdown by external forces (Lee et al., 2016). For WPI (Figure 2c and 2f), after conducting pH shifting and ultrasound treatment, no particle fragmentation was observed. However, we observed the aggregation in extreme alkaline pH treatments, which was in line with the experimental findings of our earlier investigation (Zhao et al., 2022). The particle size of MPC and MCC did not change significantly after the addition of ASTA to the milk protein without combined treatment. In contrast, a significant increase in particle size occurred after the binding of WPI (Figure 2c and 2f) to ASTA, which indicated that whey protein had more affinity for ASTA than casein. It is speculated that WPI has a large portion of hydrophilic AA residues on the surface, and a complex of WPI and ASTA with hydrophilic head (WPI) and hydrophobic tail (ASTA) was generated first, which created a phospholipid-like surface characteristic. Subsequently, more automatic self-assembly of WPI and ASTA complexes is generated (Liu et al., 2021b). Three proteins with treatment showed a significant increase in particle size after the addition of ASTA, which was according to the increase in ASTA encapsulation rate after co-treatment. This result is consistent with the study of Xu et al. (2021), who reported increased particle size in nanocomposites manufactured from an ultrasound-assisted pH treatment of WPI and gelatin-loaded resveratrol. This finding can be attributed to alkaline pH shifting resulting in stronger intramolecular electrostatic repulsions and protein unfolding, which exposed the hydrophobic AA residues. Meanwhile, ultrasound treatment exposed proteins’ hydrophobic domains previously buried in these macromolecules. These exposed hydrophobic regions can bind more ASTA, resulting in an increase in particle size. Therefore, it is reasonable to relate this slight increase in particle size with modifications in milk proteins loaded with ASTA.
Zeta Potential and Turbidity

The absolute value of the zeta potential always corresponds to the stability degree of the system, with higher absolute values suggesting reduced collision and agglomeration tendencies (Zhang et al., 2021b). From Figure 3a, it can be seen that the absolute value of the zeta potential for A/MPCPU and A/MCCPU increased significantly, whereas it decreased for A/WPIPU ($P < 0.05$). This is due to the shear stress and cavitation effects of the ultrasound, which causes the structure of the milk protein-ASTA complexes was driven to unfold (Chen et al., 2022), and the protein was split into smaller particles, the surface area was increased, and more charged groups were exposed (Geng et al., 2022). Moreover, with the increase in absolute zeta potential, the frontal electric repulsion between particles intensified, resulting in better separation of particles, which contributed to the increased stability of the complex solution (Gao et al., 2023). These findings and theories were supported by the results derived from the particle size analysis in this study. Hence, it was obvious that the treatment of ultrasound-assisted pH shifting showed a strong influence on the structure and stability of the complex. The conformation of the protein and the exposure of the hydrophobic region on the surface after the combined treatment facilitated the interaction of the protein with ASTA to form a more stable complex. The decrease in the absolute value of the zeta potential of A/WPIPU was due to the generation of macromolecular aggregates in the whey protein after an ultrasound-assisted pH shift treatment, which reduced the stability of the solution.

The turbidity and visual appearance of all samples of protein nanocomposites are shown in Figure 3b. The turbidity of the nanocomposites is related to the ultrasound-assisted pH-shifting treatment. A/MPCPU and A/MCCPU treated by the combined ultrasound-assisted pH shifting displayed much lower turbidity as compared with untreated samples (A/MPC and A/MCC); the former was more transparent than the latter. The combined treatment induced a reduction in the particle size of milk proteins and improved their solubility, which caused a decrease in the scattering factor of proteins. Therefore, the turbidity of the nanocomposites embedded by MPC and MCC showed a significant decline after the combined treatment. The previous study also reported that poor solubility for MPC and MCC was attributed to a poor rate of water transfer into the high-protein milk powders (Mimouni et al., 2010; Sikand et al., 2011), and after ultrasonic treatment, the rate of water transfer to the protein was accelerated, increasing the solubility of MPC and MCC, thereby reducing turbidity (Han et al., 2020). In this study, WPI presents lower turbidity due to its higher solubility and smaller particle size, and the addition of ASTA had little effect on its turbidity. The visual appearance of A/WPI and A/WPIPU offered
no significant changes. This observation is consistent with the results of particle size and zeta potential. After the combined treatment, the particle size of A/MPCPU and A/MCCPU decreased significantly, and the system was more stable, whereas the particle size of A/WPIPU increased and the stability decreased slightly.

**Intrinsic Fluorescence Spectroscopy**

Protein tertiary structure alterations may be seen in an intrinsic fluorescence spectrum. When stimulated with the appropriate energy, the aromatic AA residues (Trp, Tyr, and Phe) exhibit fluorescence properties (Xu et al., 2016). The microenvironment’s polarity has a significant effect on the wavelength’s intensity (Vlasova et al., 2014). As shown in Figure 4, the combined ultrasound-assisted pH-shifting treatment increases the endogenous fluorescence intensity of MPC, MCC, and WPI, with WPI being the most significant. This phenomenon suggests that extremely alkaline environments and ultrasound treatments may lead to changes in the tertiary structure of milk proteins and that these treatments can cause protein unfolding and subsequent movement of exposed tryptophan residues to a more hydrophobic environment (Zhang et al., 2021a). The polarity of the tryptophan residue microenvironment under the alkaline shifting condition causes a minor red shift in the max of FI in the co-treated WPI (about 4 nm) as compared with natural WPI (Figure 4c). Regardless of whether an ultrasound-assisted pH-shifting treatment was performed, a significant decrease in endogenous fluorescence intensity of all 3 milk proteins and ASTA nanocomposites occurred after encapsulation of ASTA. Furthermore, the changes in fluorescence reduction of MPC and MCC after treatment with ASTA addition were found the greater than that of WPI, which could be due to the fact that the combined treatment increased the solubility of casein and enhanced molecular interactions, including hydrophobicity (Liang et al., 2021). The encapsulation of ASTA caused the quenching of endogenous fluorescence of milk proteins, further proving that we observed hydrophobic interactions between ASTA and milk proteins (Xu et al., 2021). This observation is consistent with the interaction of ASTA with β-La (Liu et al., 2021b).

**Surface Hydrophobicity**

Ultrasound-assisted pH shifting increased the surface hydrophobicity of milk proteins, especially the surface hydrophobicity of WPI presented a significant increase ($P < 0.05$) compared with untreated samples (Figure 5). The unfolding of the protein, which exposed hydrophobic AA residues, was the cause of the rise in surface hydrophobicity (Zhang et al., 2021a). Compared with the samples without ASTA, we observed a significant drop in the surface hydrophobicity of 3 milk proteins binding with ASTA, indicating a decrease in hydrophobic sites on the protein surface.

The hydrophobic molecule ASTA interacting to the hydrophobic spots on the protein surface and competing with the hydrophobic probe ANS for binding may be the cause of the reduction in surface hydrophobicity. The findings of the fluorescence spectroscopy are
consistent with the reduction in surface hydrophobicity. These results are similar to previous reports on the interaction of β-LG with apigenin (Zhu et al., 2020) and the interaction of xylitol with whey protein (Kong et al., 2020).

Antioxidant Capacity and Bioaccessibility

The antioxidant capacities of nanocomposites were evaluated using DPPH, ABTS$^+$, and ·OH measurements (Table 1). As shown in Table 1, the antioxidant activity of the milk protein was significantly increased ($P < 0.05$) after binding to ASTA as compared with free ASTA, which is related to the antioxidant properties of the protein itself. It can also be seen that the ultrasound-assisted pH-shifting treatment plays a positive role in the antioxidant properties of nanocomposites. By inference, the combined treatment induced a stronger interaction between the protein and ASTA, resulting in better binding. In this manner, the antioxidant properties of ASTA embedded in proteins were enhanced after treatment with ultrasound-assisted alkaline pH shifting. These results prove that ASTA loaded into milk proteins does not undergo an alteration of internal structure and potential biological activity. The chemical antioxidant activities of the ASTA-hydroxypropyl-β-cyclodextrin (HPCD) inclusion complex and free ASTA were investigated by Yuan et al. (2013). The ASTA-HPCD complex had a stronger DPPH radical-scavenging activity at low concentrations than ASTA or ascorbic acid, but as concentration increased, this activity reduced. After phenols from grape seed extract were embedded in whey protein concentrate (WPC) as wall material, their antioxidant capacity improved significantly by 70% (Yadav et al., 2020). Therefore, milk protein as an embedding carrier is more conducive to the improvement of antioxidant activity.

Free ASTA presented poor bioavailability due to its low water solubility, which directly influences its uptake by organisms. Therefore, it is crucial to explore the effect of the carrier on the bioavailability of ASTA. As seen in Table 1, free ASTA showed a low bioavailability of 15.66%. After encapsulating ASTA in milk proteins using ultrasound-assisted pH shifting, the bioavailability of A/MPC$_{PU}$, A/MCC$_{PU}$, and A/WPI$_{PU}$ nanocomposites reached 44.44%, 43.96%, and 55.96%, respectively, with a significant increase in the bioavailability of ASTA. The increase in the bioavailability of ASTA suggests that milk proteins may be an effective ASTA delivery vehicle. Similar results were obtained from other studies. Using WPC as a stabilizer, Zanoni

Figure 4. Changes in the intrinsic fluorescence of milk proteins and their nanocomposites. MPC = milk protein concentrate; MCC = micellar casein; WPI = whey protein isolate. The nanocomposites of milk protein and ASTA prepared were named A/MPC$_{PU}$, A/MCC$_{PU}$, and A/WPI$_{PU}$. The nanocomposites without sonication and pH-shifting treatment were used as controls and named A/MPC, A/MCC, and A/WPI. A.U. = absorbance unit.
et al. (2019) reported that nanoparticles were created by emulsification-solvent evaporation process beginning with HP oleoresin, which dramatically increased the bioavailability of ASTA.

Storage Stability

The protein and ASTA nanocomposites were stored at 4°C for 28 d, protected from light; the stability of the nanocomposites during storage was evaluated by measuring the average particle size, zeta potential, and retention of ASTA during storage (Figure 6). We observed a slight decrease in the particle size of the untreated MPC and MCC nanocomposites during storage (Figure 6a), probably because the proteins without treatment are more stable during storage when they remain in their natural state. The particle size of the co-treated samples increased slightly during storage, indicating that some aggregation occurred between the nanocomposite particles. In the treated nanocomposites, the protein formed a new “molten globular state” structure during the treatment, which may be less stable than the protein in its natural state during storage. The increase in particle size may be responsible for the decrease in the absolute value of the zeta potential (Figure 6c), which leads to a decrease in electrostatic repulsion between the nano-micelle particles (Liu et al., 2021a). The changes in zeta potential also indicate changes in the interfacial composition and structure of the nanomicelle particle surface during storage. As far as the retention rate of ASTA during storage is concerned, its retention decreased from 100% on the start day to 72.16% after 28 d of storage (Figure 6d). Compared between the 3 types of milk proteins, the retention of ASTA encapsulated by treated MCC was consistently the highest throughout the storage period, followed by untreated MCC, treated MPC, treated WPI, untreated MPC and WPI, with the stability of their encapsulated ASTA decreasing in that order. It can be deduced that treated MCC had the best encapsulation of ASTA, with the stability remaining close to 98% after 28 d of storage, whereas the treated WPI with the retention rate of 85.39%. The co-treated nanocomposites had a better retention rate and slower degradation of ASTA compared with the untreated nanocomposites. The particle size distribution after 28 d of storage showed (Figure 6b) that the particle size of the treated nanocomposites was smaller, and their stability was better. These results suggest that the treatment of milk proteins by ultrasound-assisted pH shift is profitable for the stability of hydrophobic bioactive compounds and slows their degradation during storage.

Figure 5. Changes in surface hydrophobicity of milk proteins and their nanocomposites. MPC = milk protein concentrate; MCC = micellar casein; WPI = whey protein isolate. The nanocomposites of milk protein and ASTA prepared were named A/MPCPU, A/MCCPU, and A/WPIPU. The nanocomposites without sonication and pH-shifting treatment were used as controls and named A/MPC, A/MCC, and A/WPI. A.U. = absorbance unit.
Table 1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and OH radical scavenging activity of nanocomposites and bioaccessibility of astaxanthin (ASTA)

<table>
<thead>
<tr>
<th>Sample</th>
<th>DPPH (%)</th>
<th>ABTS (%)</th>
<th>OH (%)</th>
<th>Bioaccessibility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASTA</td>
<td>4.18 ± 0.52e</td>
<td>2.57 ± 0.32d</td>
<td>0.68 ± 0.08g</td>
<td>15.66 ± 1.26e</td>
</tr>
<tr>
<td>A/MPC</td>
<td>32.73 ± 0.52d</td>
<td>78 ± 1.21g</td>
<td>56.54 ± 0.56d</td>
<td>41.13 ± 0.83d</td>
</tr>
<tr>
<td>A/MPCPU</td>
<td>40.67 ± 1.03c</td>
<td>92.29 ± 0.65f</td>
<td>59.35 ± 0.35f</td>
<td>44.44 ± 1.34e</td>
</tr>
<tr>
<td>A/MCC</td>
<td>33.5 ± 1.16e</td>
<td>73.43 ± 0.73d</td>
<td>61.4 ± 0.75g</td>
<td>40.72 ± 1.26d</td>
</tr>
<tr>
<td>A/MCCPU</td>
<td>43.34 ± 0.85a</td>
<td>95.14 ± 0.56c</td>
<td>65.18 ± 1.61a</td>
<td>43.96 ± 1.48a</td>
</tr>
<tr>
<td>A/WPI</td>
<td>31.71 ± 1.10d</td>
<td>85.14 ± 0.56d</td>
<td>34.28 ± 1.04e</td>
<td>52.11 ± 1.27b</td>
</tr>
<tr>
<td>A/WPIPU</td>
<td>34.67 ± 1.02c</td>
<td>97.14 ± 1.53d</td>
<td>37.5 ± 1.25e</td>
<td>55.96 ± 1.55a</td>
</tr>
</tbody>
</table>

a–gValues with different letters in the same column are significantly different (P < 0.05).

1MPC = milk protein concentrate; MCC = micellar casein; WPI = whey protein isolate. The nanocomposites of milk protein and ASTA prepared were named A/MPCPU, A/MCCPU, and A/WPIPU. The nanocomposites without sonication and pH-shifting treatment were used as controls and named A/MPC, A/MCC, and A/WPI.

Figure 6. Average particle size (a), particle size distribution on d 28 (b), zeta potential (c), and ASTA retention (d) of nanocomposites during storage. MPC = milk protein concentrate; MCC = micellar casein; WPI = whey protein isolate. The nanocomposites of milk protein and ASTA prepared were named A/MPCPU, A/MCCPU, and A/WPIPU. The nanocomposites without sonication and pH-shifting treatment were used as controls and named A/MPC, A/MCC, and A/WPI. Different lowercase letters (a–d) indicate significant differences (P < 0.05) between samples. Error bars represent SD. The particle size distributions of d 7, 14, and 21 are presented in Supplemental Figures S1, S2, and S3 (https://doi.org/10.6084/m9.figshare.24467518.v1).
As summarized in Figure 7, hydrophobic interactions dominate the mechanisms of all 3 milk protein carriers for encapsulation astaxanthin, and there may be other noncovalent interactions, such as electrostatic interactions. The co-treatment is to enhance the binding of ASTA and protein by increasing the hydrophobic sites of several proteins to improve the embedding rate. Despite the fact that WPI had the highest encapsulating rate, MCC showed the greatest improvement on embedding ASTA after co-treatment. Meanwhile, the co-processing also reduces the particle size of MCC and MPC and improves the bioavailability and storage stability of the 3 nanomaterials. In particular, the best storage stability of ASTA were observed in MCC after treatment of ultrasound-assisted pH shifting.

**Figure 7.** Schematic representation of the embedded mechanism of astaxanthin with 3 types of milk proteins before and after treatment. MPC = milk protein concentrate; MCC = micellar casein; WPI = whey protein isolate. The nanocomposites of milk protein and ASTA prepared were named A/MPC<sub>PU</sub>, A/MCC<sub>PU</sub>, and A/WPI<sub>PU</sub>. The nanocomposites without sonication and pH-shifting treatment were used as controls and named A/MPC, A/MCC, and A/WPI.
CONCLUSIONS

A hydrophobic substance, ASTA, was delivered by milk proteins treated with ultrasound-assisted pH shifting. The results showed that ASTA binds to the hydrophobic sites of the protein surface, which proved that the encapsulation mechanism was mainly through hydrophobic interactions. Treatment with ultrasound-assisted pH shift improved the bioaccessibility of ASTA and effectively slowed down the degradation of ASTA during storage, resulting in a higher retention of ASTA. Importantly, MCC was most influenced by ultrasound-assisted pH shifting than other 2 proteins, with strong change of particle size and zeta potential, and the treated MCC was particularly advantageous for the antioxidant capacity and storage stability of ASTA. It can be concluded that both protein type and treatment technique were shown to have significant relationships to the result, and ultrasound-assisted pH shift treatment of the fabricated milk proteins for ASTA delivery provides an effective strategy to improve its solubility, stability, and bioavailability in water, which is important to address the challenges of ASTA application in hydrophilic food systems.

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ORCIDS
X. P. Wang https://orcid.org/0000-0003-0890-5210
C. F. Wang https://orcid.org/0000-0001-7992-6524
X. Q. Zhao https://orcid.org/0000-0002-4548-6361
H. Jiang https://orcid.org/0000-0003-0013-7173