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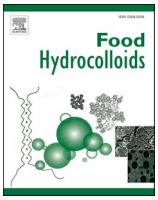

Synergistic effect of whey proteins and their derived microgels in the stabilization of O/W emulsions

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ABSTRACT

Tannic acid-crosslinked whey protein isolate (TA-WPI) microgels can physically stabilize food emulsions by adsorption to the oil-water interface. Production of these particles is often accompanied by residual un-reacted WPI, which may play a role in the stabilization of emulsions. Here, TA-WPI microgels were produced and the presence and composition of surface-active molecules was characterized using ultrafiltration. Full purification was not feasible; the final dispersion used for emulsification had microgels and free protein in a ~20:1 mass ratio, both of them enriched in β-lactoglobulin compared to the starting material. The physical characteristics of emulsions stabilized by blends of microgels and native WPI depended on the homogenization method used. When using low-shear methods (rotor-stator), microgels suppressed coalescence by bridging flocculation, which was disrupted by WPI over 14 days of storage. On the other hand, emulsions produced under high shear (microfluidizer) were very viscous, and highly flocculated, and they remained in the flocculated form after 14 days of incubation, which may be due to strong anchoring of adsorbed microgels caused by the high energy provided to the system during the homogenization procedure.

1. Introduction

Particle-stabilized emulsions, also known as Pickering emulsions (Pickering, 1907; Ramsden, 1904), show extraordinary stability compared to emulsions stabilized by conventional emulsifiers, primarily as a result of the high desorption energy required to remove particles from the interface and the thicker interfacial layers they create, which form a physical barrier against coalescence (Berton-Carabin & Schroën, 2015). Pure Pickering emulsions are surfactant-free (Leal-Calderon & Schmitt, 2008) and therefore may comply better with consumer demands for clean labels (Green et al., 2013; McClements & Gumus, 2016), which has led to efforts in designing Pickering emulsions suitable for food applications (Du et al., 2019; Ribeiro et al., 2020; Schröder et al., 2019; Wang et al., 2020).

As stabilizing particles, protein gels stand out among the available food-grade starting materials. Usually protein microgels are made by heating, leading to soft, deformable particles consisting of a water-swollen network that is internally crosslinked through a combination of hydrophobic bonds, hydrogen bonding and electrostatic interactions (Yan et al., 2020). Protein particles have a high desorption energy, and these microgels additionally have a characteristic core-shell morphology. The shell is less crosslinked than the core, and therefore can deform, giving the microgels a "fried-egg" appearance when adsorbed at interfaces (Destribats et al., 2011; Dickinson, 2017; Nicolai, 2016). This flattening effect increases the contact area in such a way that the desorption energy of microgels is typically higher than that of hard Pickering particles. The expectation is, therefore, that microgel particles are better stabilizers for emulsions than hard spheres (Murray, 2019). It is good to point out that heat treatment alters the secondary structure of proteins by molecular unfolding, which may result in hydrophobic groups initially present in the core of globular proteins to be exposed, thus improving wettability by the oil phase when present in the shell (Dickinson, 2011; Nicolai et al., 2011; Wu et al., 2015).

Whey protein is a by-product of cheese production, that is a widely

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available protein source (Li et al., 2018) containing a mixture of globular proteins such as β-lactoglobulin (β-LG), α-lactalbumin (α-LA), bovine serum albumin (BSA) and immunoglobulins (Haug et al., 2007). Microgels produced from whey protein have been reported to function as effective Pickering-like stabilisers (Destribats et al., 2014; Murray & Phisarnchananan, 2016; Wu et al., 2015; Zamani et al., 2018). In addition, we demonstrated that physical properties of microgels made of whey protein isolate (WPI) can be tuned by adding a crosslinking step using tannic acid. This enables the production of smaller particles compared to conventional WPI microgels, and that create a good physical barrier and enhance emulsion stability (Silva et al., 2021).

Due to incompleteness of the denaturation and crosslinking reactions, the production of food-grade microgels often results in mixtures of these particles with unreacted surface-active constituents. The latter cannot be easily removed and consequently may play a role in emulsion formation and interfacial stabilization (Destribats et al., 2014), which is hardly ever reported. How these interactions establish themselves typically depends on the homogenization protocol and the nature of the surface-active molecules involved.

Synergistic interactions have been observed when surface-active molecules are present at low concentrations, thus facilitating droplet break-up during homogenization and allowing time for particle adsorption to take place (Lan et al., 2007; Pichot et al., 2010), as well as possibly strengthening the interfacial layers (Gülseren & Corredig, 2013; Murray et al., 2011), and adjusting particle wettability (Binks et al., 2007; Hu et al., 2015). When concentrations of surface-active molecules are higher, antagonistic effects may become prominent due to increased competition for the interface (Gülseren & Corredig, 2013), reduction of particle desorption energy as a result of interfacial tension decrease (Murphy et al., 2018), and changes in particle wettability that may cause them to become ineffective as Pickering stabilizers (Binks et al., 2007, 2013).

In a practical food emulsion preparation setting, microgels and free proteins will be present together, which highlights the importance of understanding the interaction between the two. The present work investigates the protein composition of microgels and residual free protein after crosslinking, and studies the effects of free native whey proteins on the physical stability and interfacial properties of emulsions stabilized by tannic acid-crosslinked WPI microgels.

2. Materials and methods

2.1. Materials

Whey Protein Isolate (WPI), purity 97.0%–98.4% (BiPro, Davisco, Switzerland) and tannic acid, purity >99% (Sigma Aldrich, United States) were used as received. n-hexadecane (99%) (Alfa Aesar, Thermo Scientific, Germany) was used as model oil phase. Sodium dodecyl sulfate (SDS, >99%), dimethyl sulfoxide (DMSO, >99.7%), Nile blue A, Nile red, potassium sorbate (>99%), and aspartic acid (>98%) were purchased from Sigma Aldrich (United States). 2-Propanol (HPLC grade), n-hexane (PEC grade), and hydrochloric acid (HCL, 37–38%) were purchased from Actu-All Chemicals (Oss, Netherlands). Polyethersulfone (PES) membrane filters with a pore size of 0.03 μm were purchased from Sterlitech Corporation (United States). Acetonitrile ULC-MS (30%, Actu-All Chemicals, Netherlands) and trifluoroacetic acid (TFA) (Sigma Aldrich, United States) was used for HPSEC analysis. All solutions were made in ultrapure water (Millipore Corporation, Billerica, Massachusetts, United States).

2.2. Production and characterization of tannic acid-crosslinked WPI microgels

The production of WPI microgels followed the procedure described elsewhere (Silva et al., 2021). Briefly, a WPI solution (40 g/L) was prepared by mixing deionized water and WPI through magnetic stirring during 2 h at room temperature. The solution was incubated overnight at 4 °C to ensure complete protein hydration. Afterwards, tannic acid was slowly added to the WPI solution at room temperature in a molar ratio of 0.5:1 (tannic acid:WPI) under magnetic stirring. The solution had its pH adjusted to 5.8 by drop-wise addition of 1 M HCl and it was heated for 15 min in a water bath operating at 80 °C. The resulting microgel dispersion was then rapidly cooled to room temperature using running tap water.

The microgels were characterized for their particle size distribution and zeta potential (Zetasizer Nano ZS, Malvern Instruments, United Kingdom) using a disposable folded capillary cell (DS1080). Microgel dispersions were diluted 100-fold in ultrapure water prior to analysis to avoid multiple scattering effects. Samples were evaluated in triplicate, using a refractive index of 1.45 for the dispersed phase and 1.33 for the dispersant.

2.3. Purification and characterization of the microgel dispersion

2.3.1. Purification

To remove possible non-reacted WPI molecules from the microgel dispersion, a purification procedure based on ultrafiltration was implemented. Right after preparation, the microgel dispersion was stepwise washed 10 times in an Amicon Stirred Cell (Merck Millipore, United States), under stirring at 380 rpm and an operating pressure of 3 bar, using a PES membrane filter (0.03 μm). In every step, 50 g of filtrate were removed, after which 50 g of ultrapure water were added to keep a constant total mass of approximately 100 g inside the filtration cell. After 5 filtrations, the membrane filter was replaced by a new one, in an attempt to improve the separation performance.

2.3.2. Characterization of protein concentration (HPSEC)

The native WPI concentrations in the microgel dispersion before and after purification were determined by high-pressure size exclusion chromatography (HPSEC) (Thermo UltiMate 3000, Thermo Scientific, Germany), using a standard quaternary pump (Pump LPG-3400SD), auto sampler (WPS-3000), column-oven (TCC-3000), and photodiode array detector (PDA-3000). Samples (180 μL) were collected in glass vials and measured in duplicate. Two columns were used to perform the separation (TSKGel G3000SWXL and TSKGel G2000SWXL, Sigma Aldrich, United States) at 30 °C. Acetonitrile (30%) with 0.1% TFA was used as eluent at 1.5 mL/min flowrate, over a runtime of 20 min. Samples were detected at 214 nm wavelength and compared against an elution standard. The total native WPI concentration was estimated from the sum of the peaks corresponding to β-lactoglobulin (β-LG) dimers and the α-lactalbumin (α-LA) monomers in the chromatogram (see Fig. 2 and Fig. S1 in the Supplementary Information).

2.4. Emulsions stabilized by blends of WPI microgels and native WPI

2.4.1. Continuous phase

The continuous phases were designed to contain a fixed amount of WPI microgels, to which specific amounts of native WPI were added (or not). For emulsions prepared under low-shear homogenization, the washed microgel dispersion was diluted 10-fold using ultrapure water. Next, either 0 or 125 mg native WPI was added to 144 g of this diluted microgel dispersion (resulting in samples 0L and 125L, respectively). For emulsions prepared under high-shear homogenization, the droplets were expected to be smaller, and thus, the stabilizer content in the continuous phase was adjusted accordingly to the larger interfacial area expected to be formed during homogenization. In this case, the washed microgel dispersion was diluted 3-fold using ultrapure water. Next, either 0 or 270 mg native WPI were added to 90 g of this diluted microgel dispersion (resulting in samples 0H and 270H, respectively). Note that the amounts of WPI microgels and native WPI were rationalized based on their expected surface coverage for stabilization (mg/m^2) and the total emulsion interfacial area, which will be discussed in

section 3.3. Next to this, we prepared conventional emulsions stabilized by the same amounts of native WPI yet no TA-WPI microgels ("blank emulsions") as reference. Thus, either 0.1 or 125 mg native WPI were dissolved into 144 g of ultrapure water as continuous phase for producing conventional emulsions (samples 0B and 125B, respectively). In this case, the addition of 0.1 mg WPI was done in order to account for the remaining native WPI in the microgel dispersion (as calculated based on HPSEC determinations, see Section 2.3.2).

2.4.2. Production of emulsions

The continuous phases described in Section 2.4.1 were used to produce 10 wt% oil-water emulsions with hexadecane as the oil phase, using potassium sorbate (1 wt%) as antimicrobial agent. Initially, a coarse emulsion was made using a high-speed blender (Ultra-Turrax IKA T18 Basic, Germany) operating at 11.000 rpm for 1 min. This coarse emulsion was passed through two different devices: either a rotor-stator (low-shear) or a high-pressure homogenizer (high-shear). We used a colloid mill (IKA MagicLab, Germany) connected to a water bath at 20 °C, operating at 15.000 rpm for 2 min, and a microfluidizer (M-110Y, Microfluidics, United States) operating at 400 bar for 5 passes, respectively.

An additional set of emulsions was prepared with the continuous phase consisting only of the diluted microgel dispersion; and here the native WPI was added post-homogenization - 125 or 270 mg WPI for emulsions produced under low or high shear, respectively (samples 0/125L and 0/270H, respectively).

2.5. Droplet size distribution

The emulsion droplet size was measured by static light scattering (Mastersizer 3000, Malvern Instruments, United Kingdom). A refractive index of 1.43 was used for the dispersed phase (hexadecane) and 1.33 for the dispersant (water). An absorption index of 0.01 was applied. Emulsions that presented bimodal distribution were diluted in 1 w/w% SDS (1:1, v/v) prior to analysis to assess if flocculation was present. In that case, confocal laser scanning microscopy (CLSM), as detailed in section 2.6, was applied to make sure that SDS was not negatively impacting the emulsion's characteristics.

2.6. Microscopic evaluation

Emulsion droplets were visualized using a light microscope (Axioscope, Zeiss, Germany) at 40x magnification. For this purpose, 10 μL of an emulsion diluted in ultrapure water (1:1, v/v) were placed onto a microscopy slide and covered with a cover slide.

CLSM was used to gain more qualitative insights about the interfacial organization. Nile red (1 wt% in DMSO) and Nile blue A (1 wt% in ultrapure water) solutions were used to stain hexadecane and WPI (either microgels or native molecules), respectively. Immediately after preparation, 1 mL emulsion was added to 10 μl Nile red solution and 5 μl Nile blue A solution in test tubes protected from light. The tubes were rotated for 2 h prior to analysis to incubate the stains. Aliquots of 50 μL of stained emulsion were pipetted onto glass slides that were covered carefully by another slide and visualized in a confocal laser scanning microscope (Zeiss LSM 5 Exciter, Breda, NL). Nile red and Nile blue A were excited by a 488 nm Argon and a 633 nm Helium–Neon laser, respectively.

Finally, transmission electron microscopy (TEM) was used to study the microgel dispersions and the emulsions prepared with the microfluidizer (droplets of emulsions prepared with a colloid mill are too large to be studied with this technique as TEM has a limited, ~1 μm, penetration depth). Samples were deposited onto a freshly glow-discharged carbon coated copper grid (200 mesh). Excess solvent was removed with standard filter paper, followed by staining with phosphotungstic acid solution (2 wt%). Images were taken with a JEM1011 transmission electron microscope (JEOL, United States) operating at 80 kV in combination with a 2K × 2K SIS Veleta camera.

2.7. Statistical information

The results shown in the next section are illustrative of the general behaviour (all droplet size distributions, HPSEC signal, microscopic and CLSM images) or averages of three measurements taken on individually prepared samples (WPI concentration, ζ-potential).

3. Results and discussion

3.1. Characterization of tannic acid-crosslinked WPI microgels

The tannic acid-crosslinked WPI (TA-WPI) microgels showed unimodal size distribution ranging between 30 and 200 nm, with an intensity-weighted mean hydrodynamic diameter (z-average) of 85.4 ± 0.6 nm and a polydispersity index (PDI) of 0.11. After the ultrafiltration treatment, the microgels had a similar size distribution (Fig. 1A), confirming that this procedure did not substantially affect the microgel integrity. This is in line with exceptional stability reported in earlier studies in which WPI microgels were subjected to ultrafiltration or even harsher treatments (e.g. spray drying or sonication) and remained unharmed (Destribats et al., 2014; Schmitt et al., 2010; Silva et al., 2022), which is attributed to the internal covalent crosslinks of microgels. TEM micrographs of the filtered microgels (Fig. 1B and C) show that the particles had a spherical morphology with some surface roughness.

The zeta potential of the washed microgels was −47.8 ± 0.7 mV, which is in accordance with others for pH-values above the isoelectric point (≈4.7), which leads to dispersion stability due to electrostatic repulsion (Araiza-Calahorra & Sarkar, 2019; Destribats et al., 2014; Murray & Phisarnchananan, 2016; Wu et al., 2015; Zamani et al., 2018).

The TA-WPI microgels incorporated 64 ± 3.5% of the native WPI available in the start solution. This was estimated based on a mass balance of α-LA and β-LG as measured by HPSEC for the start solution and for the microgel dispersion before the washing step. The mass ratio between the unbound native β-LG and α-LA was reduced from 3.0 in native WPI (Fig. S1 in the Supplementary Information) to 0.9 in the unwashed microgel dispersion (Fig. 2, red chromatogram, ratio between the areas of peak 3 and 4). The ratio between β-LG and α-LA in the TA-WPI microgels is 9.5 ± 0.3. Thus, we can estimate that the microgels are composed of 90.5% β-LG and 9.5% α-LA, approximately.

Microgels cannot be produced from pure α-LA, which mainly results in the formation of precipitates and soluble aggregates (Schmitt et al., 2011). Microgel formation requires substantial amounts of β-LG, either as such or coexisting with small amounts of α-LA. The relatively higher proportion of proline embedded within the structure of β-LG (8 of the 162 amino acids) compared to α-LA (2 of the 123 amino acids) (Kilara & Vaghela, 2018) could facilitate the formation of protein-tannin complexes (Asquith & Butler, 1986; Frazier et al., 2003; Hagerman & Butler, 1981). Moreover, tannins are more likely to bind to higher molecular weight proteins (Hagerman & Butler, 1981), which would also slightly favour the complexation with β-LG dimers over α-LA monomers. The presence of a buried free thiol group, which gets exposed during protein unfolding upon heating, makes β-LG especially more vulnerable to intermolecular disulphide aggregation reactions than α-LA, which has no free thiol groups (Broersen, 2020; Schmitt et al., 2011). Additionally, the deep hydrophobic core of β-LG that becomes exposed upon heat treatment is very effective at ligand binding and could be responsible for the described hydrophobic interactions of protein-tannin complexation (Broersen, 2020; Frazier et al., 2003). Large amounts of α-LA, on the other hand, may suppress the occurrence of these hydrophobic interactions, impairing the formation of microgels (Schmitt et al., 2011).

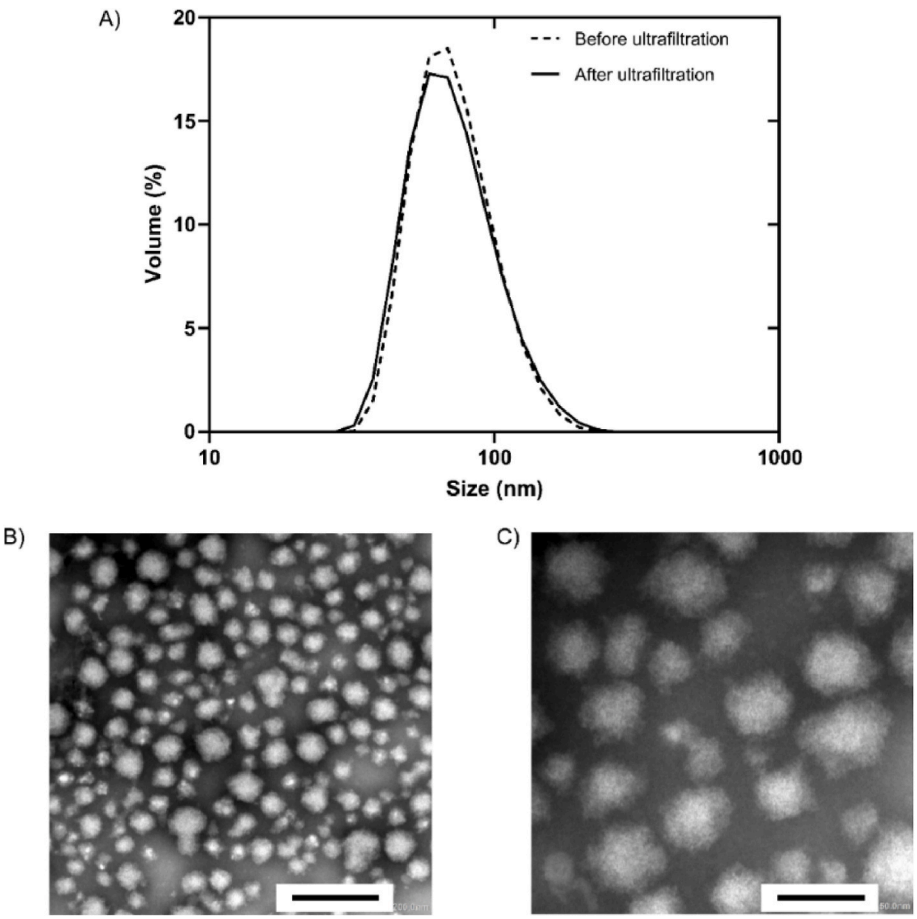

Fig. 1. Particle size distribution measured by DLS (A), and TEM micrographs of tannic acid-crosslinked WPI microgels; scale bars represent 200 nm (B) and 50 nm (C), respectively.

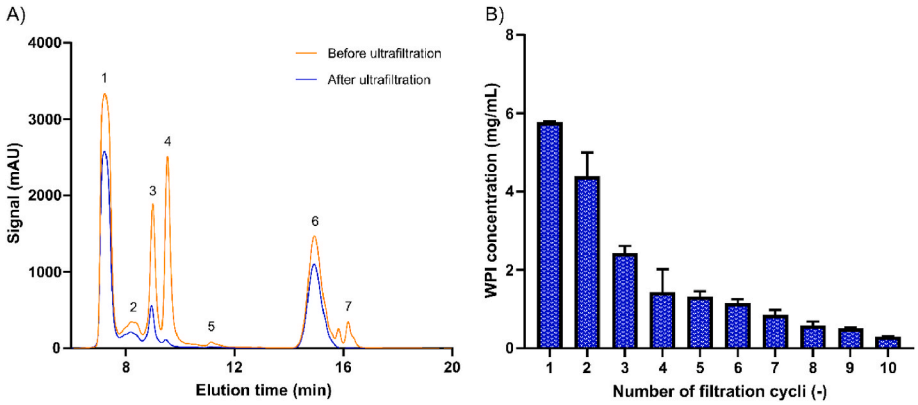

Fig. 2. Protein composition of TA-WPI microgel dispersion (40 g/L) measured by HPSEC before and after ultrafiltration: (A) Peaks correspond to (1) large protein aggregates and small microgels; (2) BSA and smaller aggregates; (3) β-LG dimers; (4) α-LA monomers; (5) peptides; (6) tannic acid; (7) minerals and salts. Note: peak 1 is not representative for all microgel particles in the sample, since larger microgels are not able to enter in the column. (B) Protein contents of filtrates obtained after each of the 10 ultrafiltration steps of the TA-WPI microgel dispersion.

3.2. Evaluation of the ultrafiltration procedure as purifying method for microgel dispersion

The effectiveness of the ultrafiltration in removing unreacted WPI molecules was evaluated by means of HPSEC analysis of the TA-WPI microgel dispersion before and after the 10-step purification. The results showed that the adopted procedure was able to remove considerable amounts of native WPI from the mixture (Fig. 2, peaks 2–5).

Interestingly, it can be observed that the ratio β-LG:α-LA, which was previously reduced from 3.0 in native WPI (Fig. S1 in the Supplementary Information) to 0.9 in the unwashed microgel dispersion, increased again during each step of ultrafiltration (Fig. S2 in the Supplementary Information) reaching a final value of 10.2 after the final (10th) step of ultrafiltration (Fig. 2, blue chromatogram), suggesting that the filter was more efficient in removing α-LA than β-LG. The higher selectivity of polyethersulfone (PES) membranes for α-LA compared to β-LG is a result of the larger size of β-LG dimers (Cowan & Ritchie, 2007), or other small aggregates that β-LG has higher tendency to form due to irreversible protein unfolding. α-LA is less likely to be involved in these reactions which allows it to renature upon cooling, returning to its initially more hydrophilic conformation (Wit & Klarenbeek, 1984), thus increasing the propensity of permeation.

The decrease in the sum of the areas of peaks 3 and 4 (Fig. 2) is equivalent to a reduction from 11.3 to 1.3 mg/mL of native WPI in the

dispersion upon filtration, which means that 90% of the unreacted WPI was effectively removed. This result indicates that even after 10 washing steps, TA-WPI microgels still coexist with some native WPI; complete purification is not possible through this procedure. In brief, the microgel dispersion after the 10th ultrafiltration step consisted of ~25.6 mg/mL TA-WPI microgels and 1.3 mg/mL free WPI; the mass ratio between microgels and residual WPI is 19.7.

3.3. Characterization of the emulsions

In order to evaluate the effect of native WPI on the characteristics of microgel-stabilized emulsions, we designed the continuous phases containing a fixed amount of TA-WPI microgels and we varied the amount of native WPI (mg/g emulsion). This was motivated because, even though only 5% of the protein material in the microgel dispersion corresponded to free WPI, this small amount can significantly influence the interfacial stabilization as small proteins molecules require a much lower surface coverage concentration (Γ) than microgels to stabilize the same area of oil-water interface, since each microgel particle consists of a large number of aggregated protein molecules. In this way, the ratio between native WPI and TA-WPI microgels is of utmost importance in the design of emulsions. Thus, we selected the amount of each stabilizer (TA-WPI microgels and native WPI) based on calculations of their individual contribution to the interfacial coverage. These calculations were performed using the compression experiments in a Langmuir trough, published in our previous paper (Silva et al., 2022). Our results showed that both native WPI and TA-WPI microgels adsorbed to an air-water interface demonstrated a maximum in dilatational modulus (ε) at surface coverages Γ^* of 1.6 and 11.6 mg/m^2, respectively. In addition, for $\Gamma < \Gamma^*$ the dilatational modulus of microgels and proteins collapse as function of the rescaled surface coverage Γ/Γ^*. Thus, we assume that microgels and proteins exhibit very similar interfacial interactions as function of Γ/Γ^*, at least up to the point at which conformational changes in the monolayer are introduced, that is at Γ^* (Silva et al., 2022). Therefore, for the present experiment we could estimate that the surface coverage concentration of microgels needs to be 11.6/1.6 = 7.3 times that of native WPI to obtain similar surface layer characteristics.

At first, our goal was to reach 67% maximum attainable (monolayer) interfacial area coverage by microgels and either 0% or 200% by native WPI (the latter for both the 125L and 270H treatments). Yet, these estimates required an *a priori* estimate of the D[3,2]. For these estimations, the D[3,2] of low-shear emulsions (~3.5 μm) was based on trial emulsions with a surplus of native protein. For the high-shear emulsions, an estimate (of ~1 μm) was made based on previous research on microfluidizer emulsification (Hinderink et al., 2019; Schröder et al., 2018).

If we ignore the dynamics of interfacial adsorption and assume that all protein material is adsorbed to the emulsion interface, we can calculate the real percentage of interfacial area that could be stabilized (at most) by either a monolayer of microgels or native protein in each prepared emulsion – after calculating the total interfacial area from the measured D[3,2] (m^2) and taking into account the estimated surface coverage concentration of each stabilizer (mg/m^2). For that evaluation, we also included the remaining amount of native WPI present in the washed microgel dispersion. The results are shown in Table 1 and indicated that the maximum attainable interfacial area coverage was different from the one initially predicted (67% microgels and 0 or 200% WPI).

Indeed, Table 1 shows that the maximum attainable interfacial area coverage of microgels decreases in presence of a high WPI content during emulsification, in particular for the high-shear emulsions (40% versus 137%). This is because native WPI reduces the interfacial tension, and, moreover, strongly contributes to interfacial stabilization during homogenization, both of which aids the formation of smaller droplets and thus, a larger amount of interfacial area to be covered by the stabilizers during homogenization. This reduces the interfacial area covered by microgels, even though the available mass of microgels is the same in each set of experiments. Adding native WPI after high-shear homogenization (sample 0/270H) also decreases the interfacial area coverage of microgels (to 93%), albeit to a much smaller extent, and is expected to be the result of preventing coalescence or promoting interfacial reorganization, which will be discussed next.

Table 1
Composition of continuous phases of emulsions stabilized by TA-WPI microgels and its blends.

Treatment	Stabilizer content (mg/g emulsion)		Maximum attainable interfacial area coverage (%)	
	Microgel	Native WPI	Microgel	Native WPI
0B	0	0.1	0	49
125B		0.9	0	233
0/125B		0.9	0	356
0L	2.2	0.1	91	33
125L		0.9	85	248
0/125L		0.9	94	275
0H	8.4	0.4	137	50
270H		3.1	40	109
0/270H		3.1	93	253

Samples were named according to shear emulsification, in which L stands for "low-shear" and H stands for "high-shear".
Samples 0L and 0H: no native WPI was added to the continuous phase.
Samples 125L and 270H: 125 and 270 mg of native WPI, respectively, was added to the continuous phase.
Samples 0/125L and 0/270H: emulsions were homogenized only in presence of the washed dispersion of TA-WPI microgels and the native WPI was added after the homogenization procedure.

3.3.1. Free protein content and physical properties of microgel-stabilized emulsions

First, the stability against flocculation and coalescence was investigated for low-shear emulsions by means of their droplet size distributions, which were determined in freshly prepared emulsions and after 14 days of storage at 20 °C (Fig. 3). The results are compared to blank emulsions, in which the same amount of native WPI was present in the continuous phase, albeit no microgels were present.

Emulsions with microgels and only the small amount of residual WPI (thus, no added WPI during homogenization: 0L and 0/125L) showed relatively large structures at day 0 compared to emulsions prepared with added WPI (125L), which turned out to be transient flocs that could be resolved by adding SDS solution, showing that the droplet size distributions in these emulsions were the same (Fig. 3A). The microgels and a very small amount of residual WPI (0L) are effective stabilisers under these homogenization conditions, and a surplus of native WPI (125L) leads to break-up of these flocs in time. Light microscopy images (Fig. 4) confirm that the individual size of droplets in all emulsions containing microgel particles as stabilizer are similar (Fig. 4A, B, and 4C); and the images also show the presence of flocs in the treatments 0L and 0/125L, in agreement with the DLS size distribution.

For the blank emulsions (0B and 0/125B), the size distribution did not change after SDS addition (Fig. 3C), indicating coalescence rather than transient flocculation. Light microscopy showed no flocs (Fig. 4D, E, and 4F), and remarkable differences in individual droplet sizes due to coalescence at low protein concentrations.

It is important to point out that, while being designed to contain the same amount of free WPI, the emulsions containing microgels summed up a higher potential for surface coverage in comparison to their respective blank emulsions. At low overall stabilizer concentration, the interfacial area formed during homogenization is not completely covered and neighbouring droplets can merge until they achieve a fully covered interface, leading to a larger droplet size for 0B and 0/125B (mode of distribution: 8.7 and 9.9 μm, respectively) compared to 125B (5.9 μm). During homogenization, the formation of droplets, adsorption of stabilizer, and re-coalescence phenomena happen simultaneously at

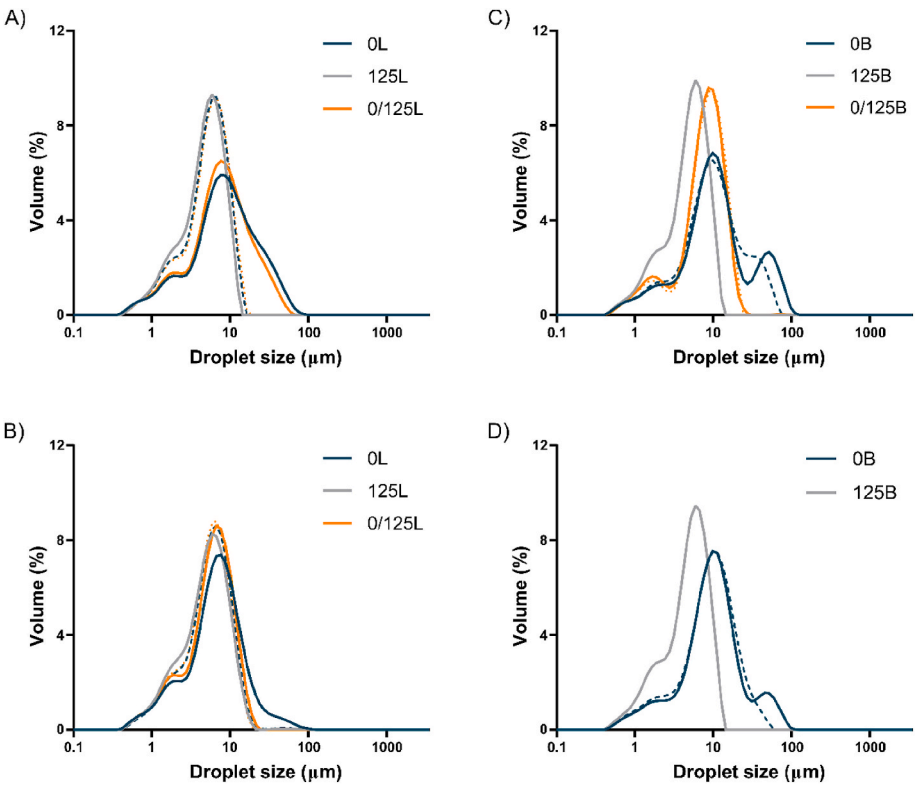

Fig. 3. Droplet size distributions of emulsions produced by low-shear homogenization (rotor-stator) on days 0 (A and C) and 14 (B and D), for emulsions with microgels (A and B) and without microgels (C and D). Samples that showed broader or bimodal distributions were diluted in 1 wt% SDS (1:1, v/v) and measured again, as represented by the dashed lines.

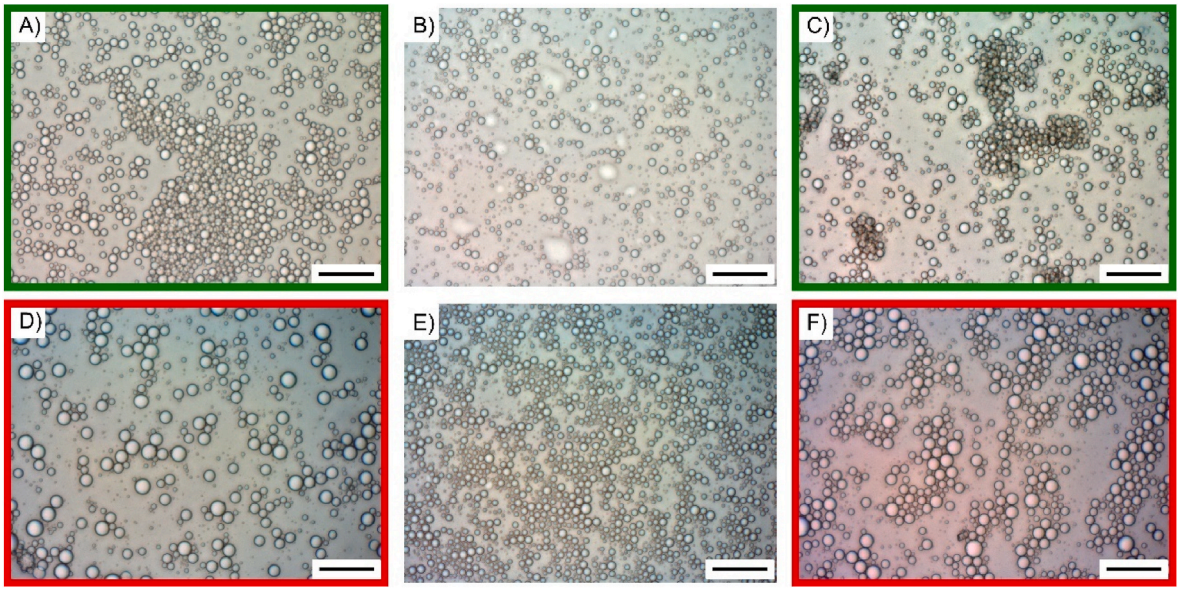

Fig. 4. Light microscope images of freshly prepared emulsions (day 0): (A) 0L; (B) 125L; (C) 0/125L; (D) 0B; (E) 125B; (F) 0/125B. Green box indicates flocculation and red box indicates coalescence. Scale bars represent 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

milliseconds time scale (Muijlwijk et al., 2017). Thus, the WPI added after homogenization is not able to prevent the early-time re-coalescence of droplets, which explains the similar droplet sizes of samples 0B and 0/125B. However, supplying additional WPI after homogenization can aid in preventing further flocculation and coalescence at slightly longer time scales.

After 14 days of storage, the flocs that were initially present in the emulsions homogenized in the presence of microgels and only low amounts of residual WPI (0L and 0/125L) were resolved. The droplet size distributions lost their shoulder between 10 and 100 μm (compare Fig. 3A and B). Moreover, the size distribution is very similar to that obtained at day 0 (after addition of SDS), which demonstrates that no

additional coalescence has taken place. For the blank emulsions, however, only sample 125B was stable against coalescence over time. Emulsion 0/125B showed macroscopic phase separation after 14 days of storage, most probably as a result of depletion interactions, and is not shown in Fig. 3D. This is supported by the fact that despite its low emulsifier content emulsion 0B did not show macroscopic phase separation.

Aiming to further investigate the flocculation behaviour of droplets over time, emulsions were characterized on days 0 and 14 by means of CLSM (Fig. 5). The CLSM images agree with the droplet size distributions of the emulsions, with flocculation being apparent in the emulsion stabilized by TA-WPI microgels at low native WPI content.

Flocculation occurred in emulsions with WPI microgels, which is a common phenomenon in Pickering emulsions (Dickinson, 2010). Particle bridging occurs in the particle-poor regime (Silva et al., 2022), when two partially covered droplets collide, which is expected to be the case for emulsions 0L and 0/125L. The strong capillary forces created by the formed menisci around the particles guarantee their positioning at the interfaces, forming a dense bridged monolayer between droplets, which prevents coalescence (French et al., 2015; Horozov & Binks, 2006). In the case of 125L the surplus of WPI during homogenization causes a full coverage preventing bridging by microgels.

The contact areas between the flocculated droplets show increased fluorescence (Fig. 5A and C at day 0), which is in accordance with microscopic observations of poorly-covered droplets reported by Destribats and Leal-Calderon (2007). These researchers suggested that increased fluorescence is indicative of particle reallocation at droplet contact zones (thus predominant in flocculated emulsions) because of dipole-dipole interactions that are favoured by the high electrostatic charge of the TA-WPI microgels.

The disruption of flocks is evidenced in CLSM images of treatments 0L and 0/125L on day 14 (Fig. 5A and C), thus confirming the size distribution results previously discussed. This could tentatively be interpreted as decreased bridging flocculation over time, which may be a result of post-emulsification interfacial reorganization. Small amounts of residual non-adsorbed WPI (0L) or native WPI added after homogenization (0/125L) may be capable of replacing part of the bridged TA-WPI microgels by reducing their desorption energy (Murphy et al., 2018; Vashisth et al., 2010). Additionally, proteins may undergo conformational changes during interfacial aging, which characteristically occurs over a time span of days to weeks for globular proteins present in WPI (McClements, 2004), and may have affected the bridged microgels, while ensuring the individual droplet integrity. It should be noted that rupture of flocks could also be augmented by flow, for example during microchannel emulsification (Silva et al., 2022) or stirring of bulk emulsions.

3.3.2. *Effect of the homogenization technique on the emulsion physical properties*

As the interfacial organization of microgel particles has been reported to be a function of the preparation pathway (Destribats et al., 2013), we produced an additional set of emulsions stabilized by blends of WPI microgels and native WPI using a high shear process. For that, we certified that the applied procedure was not promoting breakage of the microgels (data not shown), and that can be easily checked later on Fig. 7, at which microgels' size is in line with Fig. 1.

Interestingly, the emulsions produced by high-pressure homogenization appeared to have a higher viscosity than those produced by rotor-stator treatment. This was a first indication of increased flocculation of the samples. The droplet size distributions (Fig. 6) show large differences before and after the addition of SDS, confirming the strong flocculation. Post-emulsification addition of WPI did not lead to deflocculation: the size distributions of treatments 0H and 0/270H were very similar (Fig. 6A) at day 0, whereas immediate presence of WPI resulted in emulsions with smaller flocks. It is clear that the presence of native WPI during emulsification has an impact on droplet formation and reduces the extent of bridging, as was the case in their rotor-stator prepared counterpart (125L).

In addition, sample 270H had a smaller size distribution than the other two emulsions after the addition of SDS. This contrasts with the low-shear emulsion in which the blend of microgels and residual native WPI (blue dashed line on Fig. 3A) was sufficient to stabilize the smallest attainable size distribution and higher concentration of WPI did not decrease the droplet size (Fig. 3A). Through high-shear emulsification we create substantially more interfacial area as demonstrated by the average droplet size, which is 0.46 μm instead of 5.9 μm previously. In Sample 270H there is a higher potential for reducing the interfacial tension to facilitate droplet breakage (Berton-Carabin & Schroën, 2015; Binks et al., 2007; Pichot et al., 2009, 2010) and stabilizing the created interface.

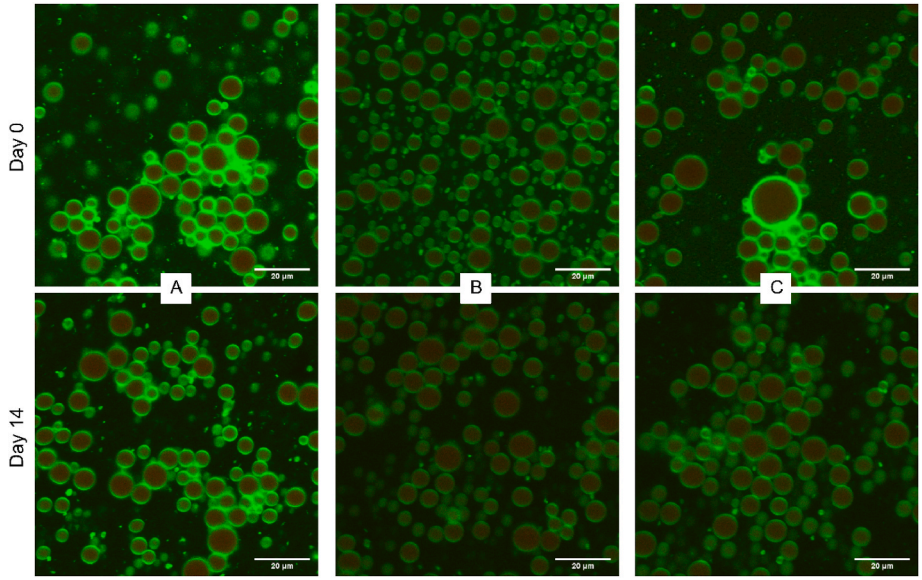

Fig. 5. CLSM images of emulsions stabilized by blends of TA-WPI microgels and native WPI on day 0 and 14. Native WPI and microgels appear in green and hexadecane appears in red. (A) 0L, (B) 125L, (C) 0/125L. Scale bars represent 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

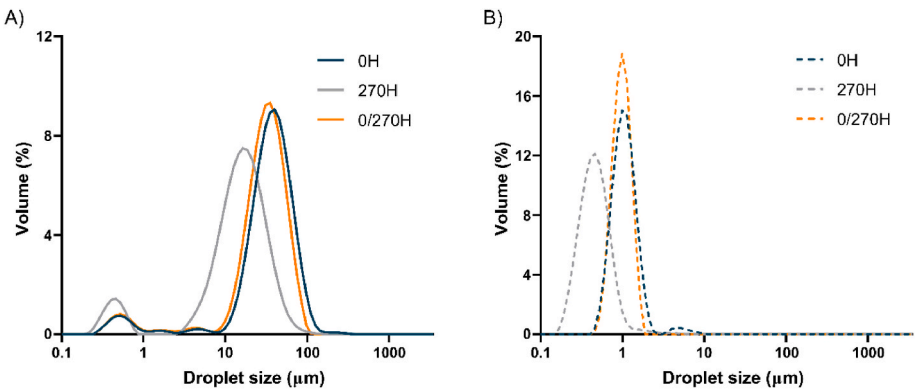

Fig. 6. Droplet size distributions of emulsions produced by high pressure homogenization on day 0: (A) freshly prepared emulsions and (B) emulsions diluted in 1 w/w% SDS (1:1, v/v).

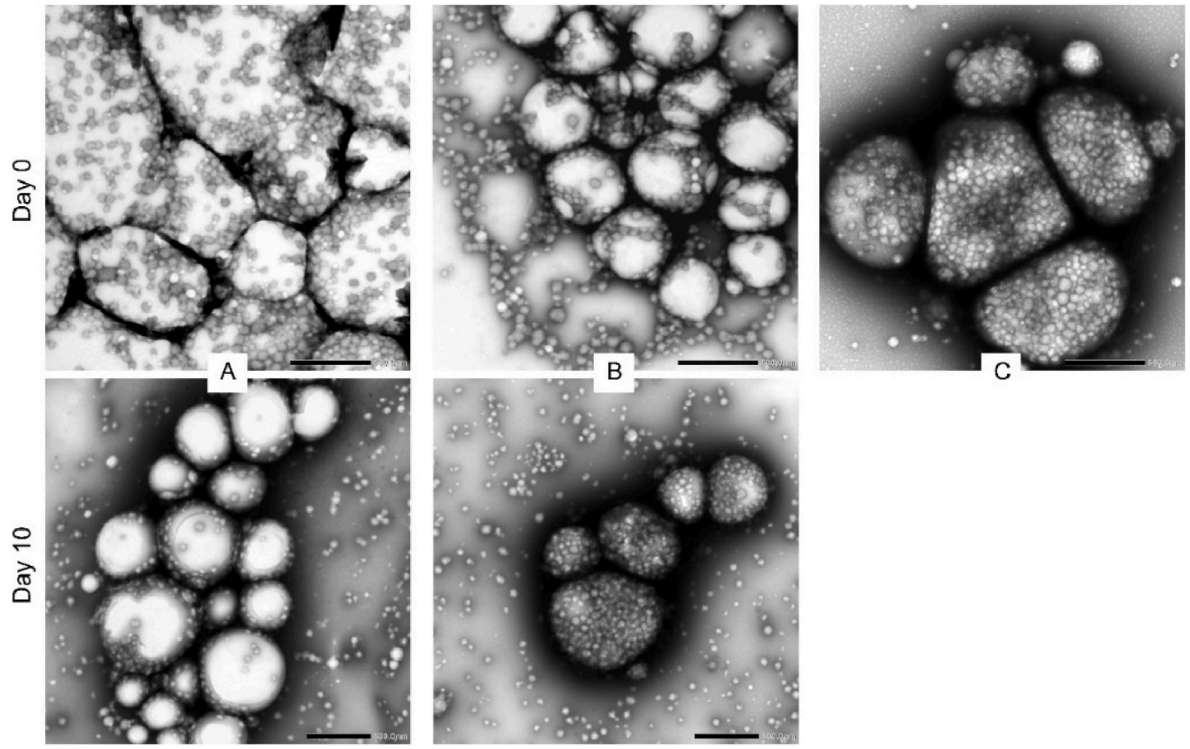

Fig. 7. TEM micrographs of emulsions produced by high pressure homogenization on days 0 and 10: (A) 0H, (B) 270H and (C) 0/270H (here, on day 10 viscosity was too high to prepare a representative sample on the TEM grid). Scale bars represent 500 nm.

After the disruption of flocks (Fig. 6B), samples 0H and 0/270H had similar droplet size distribution, and it may be expected that the interfacial composition of both samples is similar at day 0. In contrast to low-shear homogenization, the disruption of flocs was not clearly observed after extended storage time (here 10 days, see Fig. S3 in the Supplementary Information), indicating no interfacial replacement of microgels by WPI molecules over time. Indeed, TEM images of the high-shear emulsions on days 0 and 10 confirm that WPI microgels were not removed from the interface (Fig. 7) after 10 days of storage, meaning that native WPI molecules were not able to promote significant microgel desorption over time. We expect that the entrapment of droplets into bridged networks may have hindered diffusion of native molecules to the interface, and anchoring of microgels particles to the interface may be stronger in case of high-shear homogenization.

The way that microgels adsorb and arrange at the interface is directly related to the mechanical strength of the interfacial layer and consequently influence the stability of emulsions (Deshmukh et al., 2015). Flexible microgels subjected to high shear emulsification adopt deformed configurations, resulting in the presence of strongly deformed microgels in the interfacial plane. Moreover, more interfacial area is created using high emulsification energy and, in such conditions, microgels have more space to deform laterally, which leads to coverage of larger interfacial area at lower density of microgels at the interface. However, after a relaxation time, microgels may adjust their configuration in order to balance their elastic energy and interfacial tension. In the case of such adjustment leading to microgel's shrinkage, part of the interface will become uncovered and prone to bridging events (Fig. 8, right scheme). On the other hand, when using low shear emulsification, deformation of microgel particles is expected to be less, and the interfacial area created during homogenization is smaller. As a result, microgels will have much less time or space to extend. Thus, a highly dense interfacial layer is created, which accommodates fewer bridging events between droplets (Destribats et al., 2013). This allows for de-flocculation at longer time scales, as schematized in Fig. 8, left

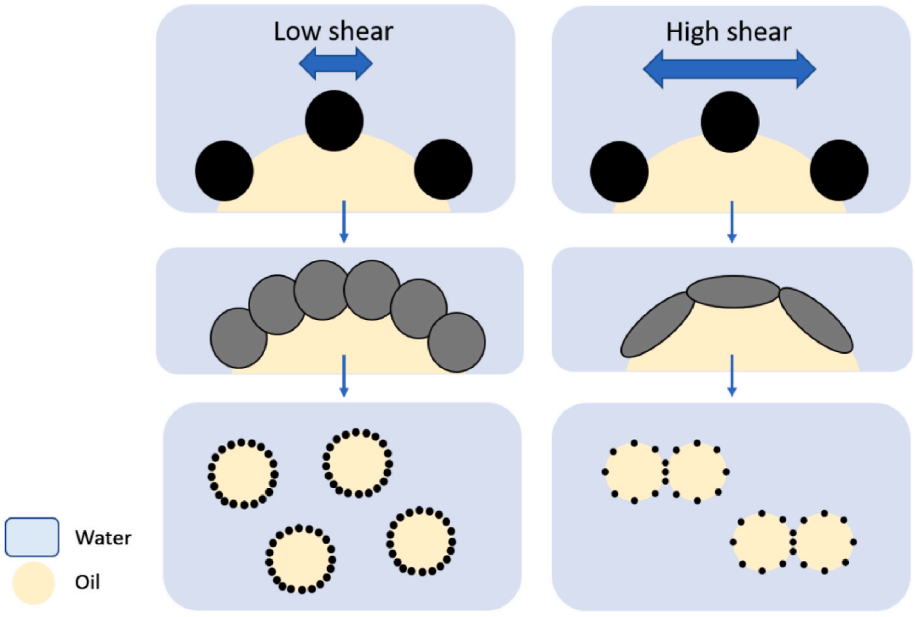

Fig. 8. Different microgel conformations under low or high shear homogenization. Black and grey structures at the droplets interface represent adsorbed microgels. Adapted from Destribats et al. (2013).

scheme. Based on our results, a synergistic effect of WPI microgels and native WPI molecules is suggested: native WPI assisted droplet formation, without impairing the adsorption of microgels nor promoting their complete desorption over time.

4. Conclusion

WPI microgels produced by heat-induced crosslinking with tannic acid have a high ratio of β-lactoglobulin to α-lactalbumin (≈10) due to preferential incorporation of β-LG. Multi-step filtration was able to remove 90% of non-reacted WPI from the microgel dispersion. The presence of residual whey proteins highlights the need to consider them in designing emulsions – especially as even low concentrations of WPI present a large potential for surface area coverage. In itself, a pure WPI microgel-stabilized system would be an asset to study physical emulsion stability from a fundamental point of view, but in any food surface-active molecules (besides the particles) will be present, and that is why we investigated the effect of free protein in these emulsions.

In hexadecane-in-water emulsions, the presence of TA-WPI microgels supressed droplet coalescence by bridging flocculation, a phenomenon well-known for particle-stabilized emulsions. For low-shear emulsions (rotor-stator) the droplet size distributions were very similar irrespective of the amount of WPI – indicating either that the small amount of residual WPI was sufficient to fully stabilize the droplets or that droplets would require more than the applied shear to decrease their size further. At low added WPI concentration, flocs initially formed were disrupted over time by the replacement of bridged particles by native WPI, while at high concentration no flocs were formed. High-shear emulsions (microfluidizer), have smaller flocculated droplets, that are stabilized by both microgels and proteins. The presence of native WPI did not impair microgel adsorption. Strong anchoring of microgels under high-shear, as wells as limited mobility of free WPI molecules in the high-viscous emulsion, may have prevented competitive adsorption of WPI and thus de-flocculation. Quantification results would be helpful to get a deeper understanding about the interfacial composition of such emulsions over time. In the present research, this was not done due to analytical limitations (quantification limit of the DUMAS equipment and interference of tannic acid on spectrophotometric analysis). However, the results presented here generates fundamental insights about the effect of native protein molecules on microgel-stabilized emulsion.

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Author statement

Jéssica Thaís do Prado Silva: Conceptualization, Investigation, Formal analysis and Writing – Original Draft; **Annabel Janssen:** Investigation and Formal analysis; **Vânia Regina Nicoletti:** Funding acquisition, Writing – Review & Editing; **Karin Schroën:** Conceptualization, Writing – Review & Editing; **Jolet de Ruiter:** Conceptualization, Formal analysis, Supervision and Writing – Review & Editing.

Declaration of competing interest

None.

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.foodhyd.2022.108229.

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