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Abstract

Water in water emulsions were prepared by mixing aqueous solutions of amylopectin (AMP) and pullulan (PUL) in the presence of whey protein microgels (MG). Attractive interaction between the MG was introduced by decreasing the pH from neutral towards their isoelectric pH either by adding HCl while stirring or progressively *in-situ* by adding glucono-δ-lactone (GDL). Decreasing the pH led to a change in the preference of the MG from the PUL phase to the AMP phase and to adsorption of the MG at the interface. The morphology of the emulsions was observed using confocal laser scanning microscopy. The morphology and stability of the emulsions depended strongly on the pH and differed when AMP droplets were dispersed in the PUL phase or vice versa. In some cases, stable weak emulsion gels were formed that flowed when tilted. In others, droplets remained dispersed in a liquid phase stabilized by a gelled interface layer of MG. The interaction between the MG was further modulated by adding small amounts of anionic polysaccharides that formed complexes with the MG below pH 5.6. This was found to influence the partitioning of the MG between the phases, as well as the stability and morphology of the emulsions.

Keywords: Water-in-water emulsion; Pickering; microgel; aqueous two phase; polysaccharide

1. Introduction

Thermodynamically incompatible mixtures of aqueous soluble polymers give rise to aqueous two-phase systems (Frith, 2010; Gonzalez Ortiz et al., 2020), which are common in food products that contain different biopolymers that form distinct phases on mesoscopic scales. When solutions of two incompatible polymers are mechanically mixed above a certain concentration, they form water in water (W/W) emulsions with one polymer phase dispersed as droplets in a continuous phase rich in the other polymer. W/W emulsions have properties that differentiate them from conventional oil/water (O/W) emulsions. One of the most important differences is that since both phases are aqueous solutions, the interfacial tension in W/W emulsions is orders of magnitude lower and becomes zero at the critical point (Firoozmand et al., 2009; Scholten et al., 2002). Another feature of W/W interfaces is that their width is on the same length scale as the correlation length of the polymers in the phases (Nicolai & Murray, 2017). These characteristics inhibit the use of molecular surfactants as stabilizers for W/W emulsions. However, the addition of solid particles has been shown to stabilize W/W emulsions in some cases (Dickinson, 2019; Esquena, 2016; Nicolai & Murray, 2017; Sarkar & Dickinson, 2020).

Particles adsorb at the interface, thus procuring steric hindrance against droplet coalescence, known as the Pickering effect. The driving force of interfacial particle adsorption was first described for oil-water interfaces (Aveyard et al., 2003; Levine & Sanford, 1985) and later applied to W/W emulsions (Balakrishnan et al., 2012). The free energy gained by adsorption (ΔG) of a spherical particle depends on the particle radius (R) and the contact angle (θ) between the particle and the interface:

$$\Delta G = -\pi R^2 \gamma_{AB}(1 - |\cos\theta)|)^2 \quad (1)$$

The contact angle is determined by the difference between the interfacial tension of the particle with phase A (γ_{PA}) and B (γ_{PB}) and that between the phases (γ_{AB}):

$$\cos(\theta) = \frac{(\gamma_{PA} - \gamma_{PB})}{\gamma_{AB}} \quad (2)$$

From Eq. 2, it follows that $|\cos(\theta)| < 1$ only if $\gamma_{AB} > |\gamma_{PA}-\gamma_{PB}|$, implying that if $\gamma_{AB} < |\gamma_{PA}-\gamma_{PB}|$ the particles do not adsorb at the interface. The most favorable condition for adsorption is when $\gamma_{PA} = \gamma_{PB}$, i.e. when the particles partition equally between the phases.

Recently, we showed that the partition of particles between two polysaccharide phases can be controlled by adding small amounts of a third polysaccharide that partitions between the phases and does not have a specific interaction with the particles (Machado et al., 2021). The reason is that the presence of the third polysaccharide within a phase modifies the interfacial

tension of particles with that phase. In this manner, particles could be induced to adsorb at the interface in situations where they did not adsorb in the absence of the third polysaccharide. Equal partition could be achieved by fine-tuning the concentration of the third polysaccharide, and it was shown that in that case, the particles remained adsorbed at the interface upon dilution down to very close to the binodal. It was found, however, in that study as well as in others (Gonzalez-Jordan et al., 2018; Nguyen et al., 2013) that the adsorption of particles at the interface is not a sufficient condition to inhibit the coalescence of dispersed droplets. It was speculated that interaction between the particles at the interface is also required.

One type of particle that has been used in the past to stabilize W/W emulsions is whey protein microgels (MG) (De Freitas et al., 2016; Gonzalez-Jordan et al., 2017, 2018; Hazt et al., 2020; Nguyen et al., 2013, 2015). At neutral pH these protein particles are negatively charged and repel each other. However, when the net charge density of the proteins is reduced by reducing the pH towards their isoionic point (IP=5.0 (Kharlamova et al., 2016)) hydrogen bonding and hydrophobic interactions drive aggregation of the particles (Schmitt et al., 2010). Gonzalez-Jordan et al., (2017) investigated the effect of adding MG on the stability of W/W emulsions formed by mixing dextran (DEX) and poly(ethylene) oxide (PEO) at different pH. Between pH 6.0 and pH 3.5, the microgels aggregated at a rate that depended on the pH. Closer to the IP, large clusters of MG were formed rapidly during mixing that migrated to the interface and extended into the dextran phase. Further from the IP, aggregation was slower, allowing the formation of a continuous protein layer at the droplet interface that subsequently gelled. However, MG at the interface of different droplets could also bind to each other, leading to clustering of droplets.

Flocculation of protein microgels close to the IP can be avoided by adding polysaccharides (Peinado et al., 2010; Santipanichwong et al., 2008). It was shown that that anionic (κ-carrageenan), cationic (chitosan) and neutral (xyloglucan) polysaccharides formed complexes with MG in a pH range close to the IP, which adsorbed at the W/W interface in DEX/PEO emulsions, but did not bind to each other so that the droplets remained individually dispersed (De Freitas et al., 2016; Khemissi et al., 2018). Thus, we may conclude that the addition of polysaccharides influences the behavior of protein microgels in this W/W emulsion at all pH, but differently when electrostatic repulsion between the polysaccharides and the microgels is strong compared to when electrostatic attraction leads to complexation. In both cases, the interfacial tension between the phases and the particles is influenced by the presence of polysaccharides, but when complexes are formed the nature of the particles themselves is modified and, therefore, their interaction with each other.

Here we present an investigation of W/W emulsions formed by mixing two neutral food-grade polysaccharides: amylopectin (AMP) and pullulan (PUL), in the presence of whey protein

microgels. AMP is a highly branched polysaccharide present in starch (Copeland et al., 2009; Tester et al., 2004), whereas PUL is a linear polysaccharide obtained from a yeast-like fungus (Nishinari et al., 1991; Singh et al., 2008). We recently investigated this system at neutral pH and found that the MG was strongly partitioned to the PUL phase and only adsorbed at the interface at high PUL and AMP concentrations (Machado et al., 2021). However, as mentioned above, after adding small amounts of a third polysaccharide, the MG adsorbed at the interface even very close to the binodal. Unfortunately, the adsorption of the MG did not significantly improve the stability against coalescence, which we suggested was due to a lack of sufficiently strong attractive interaction between the MG at the interface. Therefore, the objective of the present study was to evaluate the effect of introducing attraction between whey protein microgels (MG) by decreasing the pH. The interaction was further modulated by adding anionic polysaccharides that form complexes with the MG. We will show that these emulsions can be rendered stable by introducing attractive interactions between the particles in a controlled manner. The pH was decreased either by adding the required amount of HCl under stirring or *in-situ* by adding glucono-δ-lactone (GDL) that slowly degrades, releasing H^+. The method of pH decrease and the amount of added polysaccharide on the structure and stability of the W/W were found to be very important. It will be demonstrated that very stable emulsions can be obtained by *in-situ* pH decrease and fine-tuning the amount of added polysaccharide.

2. Materials and methods

2.1 Materials

Amylopectin (from maize, batch: SLBP9703V), alginate (alginic acid sodium salt , batch: MKBZ5563V) (ALG), and κ-carrageenan (KC) (batch: BCBX5072) were purchased from Sigma-Aldrich. Pullulan and low-methylated pectin (PEC) from lemon peel were kindly provided by Hayashibara co and Cargill, respectively. KC and AMP were purified before use. An aqueous KC solution at 0.5 wt% was dialyzed using 13.5 kDa dialysis bags first against 0.1 mol L^{-1} NaCl and then against ultrapure water (Milli-Q) for 2 days, regularly refreshing the outside water to remove residual K^+. Subsequently, the solution was freeze-dried. An AMP solution at 5 wt% was prepared in a mixture of dimethyl sulphoxide (DMSO) and water 95:5 v/v. The solution was centrifuged, and AMP in the supernatant was precipitated by adding 3 volumes of ethanol. The precipitate was rinsed with acetone and diethyl ether and dried for 2 days under vacuum at 30 °C. Both PEC and ALG solutions were centrifuged to remove insoluble aggregates. The polysaccharides were characterized by size-exclusion chromatography with on-line light scattering detection, and the

weight-average molar masses, M_w, determined were the following: ALG = 7.9×10^4, KC = 8.8×10^4, PEC = 3.7×10^6, PUL = 3.0×10^5, and AMP = 1.6×10^8 g mol^{-1}.

Whey protein isolate (BiPRO ®) was procured from Davisco Foods International, Inc (Le Sueur, MN, USA). Size exclusion chromatography showed that the sample contained 65% β-lg and 20% α-lac. The remaining 15% consisted of other whey proteins and a small amount of caseins. Protein microgels (MG) were obtained and characterized as described in detail by Phan-Xuan et al. (2011). Briefly, 4 wt% WPI in aqueous solution at pH = 5.90 was heated for 15 h at 80 °C. After this treatment, approximately 65 % of the WPI had formed microgels. The MG were analyzed by static and dynamic light scattering from which it was found that the average hydrodynamic radius was 120 nm, the radius of gyration was 125 nm, and the molar mass was 3.3×10^8 g/mol. The remaining 35 % was composed of small strand-like aggregates formed by heating that do not have W/W interfacial properties (Nguyen et al., 2013).

The pH of all polymer solutions was set to 7.0 before use by dropwise addition of NaOH or HCl (0.1 and 0.01 mol L^{-1}). Emulsions were prepared by mixing stock solutions of the various ingredients in the required amounts using a vortex mixer. No effect on the order of mixing was observed. Two emulsions were investigated in detail according to the phase diagram reported elsewhere (Machado et al., 2021) and reproduced as fig. S1 in the supplementary information. One composed of a dispersed phase containing 1.4 wt% PUL in a continuous phase of 7.8 wt% AMP (P/A) and one composed of a dispersed phase containing 1.2 wt% AMP in a continuous phase of 5.1 wt% PUL (A/P). The amount of PUL in the AMP phase was negligible, but the phase diagram showed that the AMP phase contained approximately 0.8 wt% PUL. The volume fraction of the dispersed phase was in both cases 13 %. These mixtures were situated on the same tie-line, and therefore, the interfacial tension between the PUL and AMP phases was the same. Unless otherwise specified, the concentration of total added protein was 0.4 wt%. The pH was decreased either by adding dropwise an HCl solution while stirring with a vortex or by adding a fresh solution of GDL. Hydrolysis of GDL leads to the release of H^+ and to the formation of gluconate ions. The pH of the samples was measured as function of time after addition of GDL in parallel with the microscopic observations. The final pH of an aqueous solution of GDL depends only on the initial GDL concentration, as long as it is significantly above the pK_a =3.8 of GDL, and the rate of dissociation is linear until approximately half of the GDL is dissociated (Liu et al., 2020). At room temperature, it takes approximately 24h for the GDL to degrade fully.

2.2 Methods

A Zeiss LSM800 (Carl Zeiss Microscopy GmbH) adapted with a water objective was used to obtain confocal laser scanning microscopy (CLSM) images. Two water objectives (HC×PL APO 63× and HC×PL APO 25×) were used. The samples were inserted in acrylic wells

plates or in a hermetically-sealed concave slide covered with a glass slip to prevent the water from evaporating for long time observations. Rhodamine B at approximately 5 $mg.L^{-1}$ was used to label the MG physically. Excess rhodamine partitioned preferentially to the AMP phase. The rhodamine B was excited at 580 nm and detected in a range between 580 and 800 nm.

3. Results

We will first show the effect of decreasing the pH on AMP/PUL emulsions containing 0.4 wt% MG and then the influence of adding different amounts of anionic polysaccharides. In both cases, we will compare reducing the pH by adding an aliquot of a concentrated HCl solution while stirring under a vortex with reducing the pH *in-situ* under quiescent conditions by adding GDL. Fluorescent labelling of the MG allowed us to visualize the distribution of the MG between the phases and the interface using confocal laser scanning microscopy.

3.1 Effect of pH on the microstructure of AMP/PUL emulsions in the presence of MG

3.1.1 pH reduction with HCL while stirring

As was reported by Machado et al. (2021), MG did not adsorb at the interface of the emulsions at pH 7.0 at the concentrations studied here and almost fully partitioned to the PUL phase. The effect of setting the pH to lower values by adding HCl is shown in Fig. 1 for an emulsion of AMP droplets dispersed in a continuous PUL phase (A/P). Between pH 7.0 and 5.7 the MG remained dispersed in the PUL phase. At pH 5.6 a few MG adsorbed at the interface with the excess still in the PUL phase. At pH 5.5 most MG adsorbed at the interface, and the excess MG were situated in the AMP phase. The droplets were no longer spherical, indicating that the MG layer had formed a sufficiently strong gel to resist the driving force of the interfacial tension to render the droplets spherical. At lower pH, the MG rapidly formed large flocs inhibiting the formation of spherical AMP droplets. At pH 3.9, more or less deformed droplets could be distinguished, whereas at pH 3.5 all MG were situated within the AMP droplets, and no clear layer was observed at the interface. At $pH \leq 5.6$ and $pH \geq 3.5$ the emulsions quickly destabilized, forming continuous PUL and AMP phases with the MG situated in the PUL and the AMP phase, respectively. In the intermediate pH range, the AMP domains containing the MG clusters did not coalesce, but were bound to each other forming large clusters or even a weak space spanning network. In the latter case, the emulsions remained visually homogeneous during at least one week, but flowed when tilted, implying that the network was strong enough to resist the effect of buoyancy of the droplets, but not of gravity on the macroscopic sample. We note that aggregation of the MG in pure AMP and PUL phases was observed in the same pH range as in the emulsions.

It is remarkable that simply neutralizing the negative charges on the proteins causes a shift in preference of the MG from being in contact with PUL to being in contact with AMP. According to Eq. 4, it is the decrease of the interfacial tension between MG and AMP relative to that between MG and PUL which drives the MG to the interface. The difference decreases when the pH is decreased to pH 5, but further decrease of the pH increases the difference again, this time with $\gamma_{PUL\text{-}MG} > \gamma_{AMP\text{-}MG}$, until at pH ≤ 3.5 the MG no longer adsorb at the interface. These observations resemble those reported for W/W formed by mixing DEX and PEO, mentioned in the introduction (Gonzalez-Jordan et al., 2017). However, for that system the MG adsorbed at the interface already at pH 7.0 and the excess MG remained in the DEX phase down to pH 3.5 even though a shift in the partitioning towards the PEO phase with decreasing pH was found for native proteins.

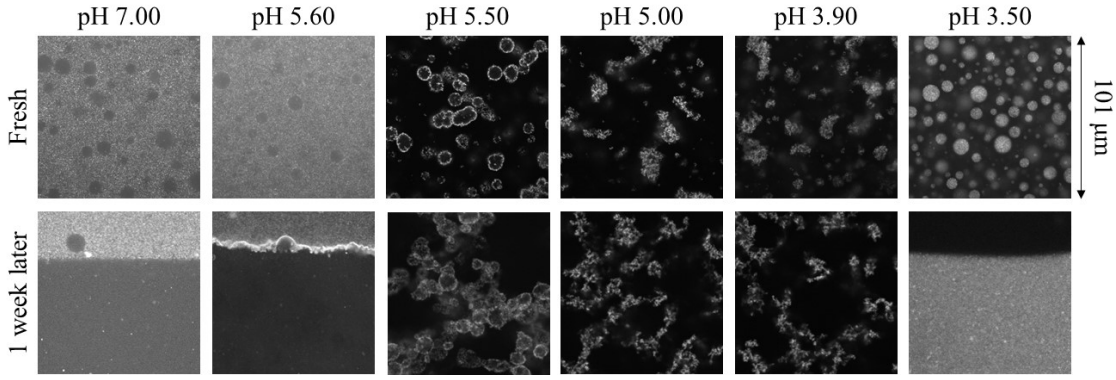


Figure 1. CLSM images of A/P emulsions in the presence of 0.4 wt% MG at different pH set by adding HCL as shown in the figure. The fluorescence from the labelled MG is shown in white. The top and the bottom rows correspond, respectively, to images taken just after preparation and 1 week later. All images are on the same scale (101 μm x 101 μm). Note that the images at pH 7.00, 5.60, and 3.50 were taken at the interface between the macroscopic PUL (top) and AMP (bottom) phases .

In P/A emulsions, the droplets are filled with MG at pH 7.0, and no MG were observed at the interface. Probably due to the higher local concentration of MG inside the droplets, the aggregation started at pH 5.9 instead of pH 5.5 for A/P emulsions, see Fig. 2. At pH ≥ 5.8 and pH ≤ 3.5 macroscopic phase separation occurred within one day, see Fig. S2 of the supplementary information.. At pH 5.6 the droplets were non-spherical as for A/P emulsions at pH 5.5 showing that MG formed a gelled layer at the droplet interface, results not shown. However, the gelled

interface was not enough to prevent coalescence. Careful observation showed that excess MG (mostly in the form of aggregates) partitioned to the continuous AMP phase at pH < 5.6. Between pH 5.6 and 4.0, no clear layer of MG at the droplet interface was observed, but the MG formed a weak network in the AMP phase that trapped the PUL droplets for at least 1 week so that the emulsion remained macroscopically homogeneous, see Fig. S2 of the supplementary information,. Although the network was strong enough to inhibit creaming of the PUL droplets, the emulsion still freely flowed when tilted.

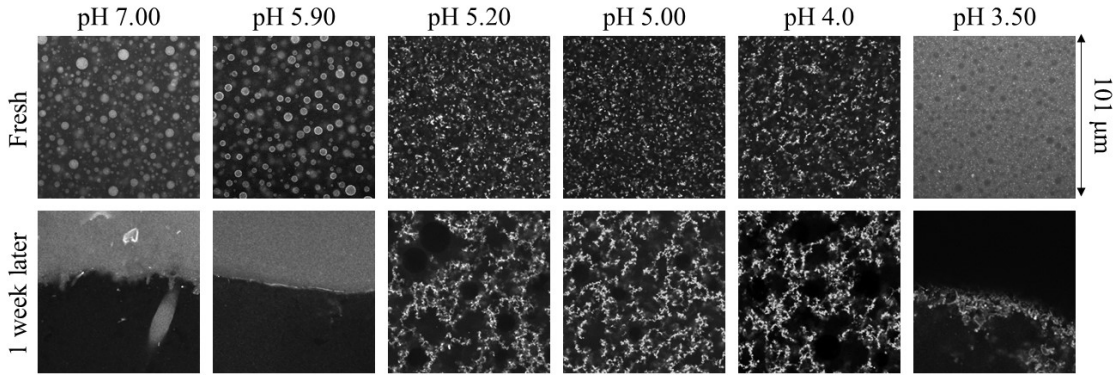


Figure 2. CLSM images of P/A emulsions in the presence of 0.4 wt% MG at different pH set by adding HCl as indicated in the figure. The top and the bottom rows correspond, respectively, to images taken just after preparation and 1 week later. Note that the images at pH 7.00, 5.90, and 3.50 after one week were taken at the interface between the macroscopic PUL (top) and AMP (bottom) phases. All images are on the same scale (101 µm x 101 µm).

3.1.2 in-situ pH reduction with GDL under quiescent conditions

By adding GDL to the emulsions, it was possible to observe how the microstructure changes while the pH decreases *in-situ*. Video 1 of the supplementary information shows the evolution of an A/P emulsion. Images taken from the video at different pH are shown in Fig. S3 of the supplementary information. As the pH decreased, the MG, which were situated in the dispersed phase, first started to aggregate at pH close to 5.5 and formed small clusters. With decreasing pH, the clusters grew and migrated towards the interface, where they remained irreversibly adsorbed. No transfer of MG to the AMP phase was observed at lower pH, which apparently requires mechanical mixing. As the pH decreased further towards the final value of pH 3.7, the AMP droplets covered with MG clusters flocculated, see fig. 3. If less GDL was added so that the final pH was 5.0, macroscopic phase separation into continuous AMP and PUL phases occurred before a stable MG layer was formed around the AMP droplets, with MG clusters

dispersed in the PUL phase, see fig. 3. This observation shows that stabilization below pH 5.6 necesitates that the pH is reduced very rapidly, e.g. by adding HCl, so that phase separation has no time to develop at higher pH during slow reduction of the pH.

The evolution of a P/A emulsion after the addition of GDL was very different, see video 2 of the supplementary information. In this case the MG situated in the dispersed phase accumulated at the interface below pH 5.9 without forming clusters. Again no transfer of MG from the PUL to the AMP phase was observed. At the final pH 5.0 the layer of MG stabilized PUL droplets for at least one week, see fig. 3. The droplets were relatively small with diameters less than 10 µm, and no sedimentation was observed after a week. The different behavior of P/A emulsions compared to A/P emulsions can be explained by the fact that in P/A emulsions, the MG are concentrated within the droplets and can therefore adsorb more easily at the interface that is nearby. In addition, the viscosity of the AMP phase was found to be ten times higher than that of the PUL phase, which slows down coalescence when AMP is the continuous phase.

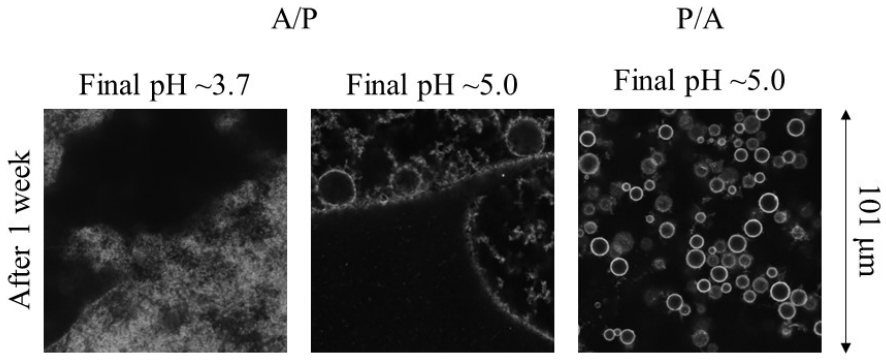


Figure 3. CLSM image of A/P and P/A emulsions with 0.4 wt% MG one week after adding GDL.

3.2 Effect of adding anionic polysaccharides

3.2.1 pH reduction with HCL while stirring

Fig. 4 shows the morphology of A/P emulsions at different pH in the presence of 0.05 wt% alginate (ALG). In this case, the MG adsorbed at the interface already at pH 7.0, as was reported elsewhere (Machado et al., 2021). The microstructure remained the same down to pH 5.6, but the MG partitioned more strongly to the AMP phase with decreasing pH. However, between pH 5.5 and pH 5.0 the fraction of MG that adsorbed rapidly at the interface decreased, and the excess MG partitioned more strongly to the PUL phase, i.e. the opposite of what happened without adding ALG. In addition, no aggregation of MG was observed. This behavior can be

explained by the fact that ALG prefers the PUL phase, considering that ALG complexes with the MG below pH 5.6. We note that formation of complexes between MG and κ-carrageenan (KC) (Khemissi et al., 2018) and PEC (Machado et al., 2021) was demonstrated to occur below pH 5.6. The effect of adding ALG on the stability of the MG as a function of the pH was the same in pure AMP and PUL phases as in the emulsions. Complexation also explains why the MG did not form large clusters in this pH range. However, at pH ≤ 4.80, aggregation of the MG was observed, which was perhaps induced by the bridging of the MG by ALG chains.

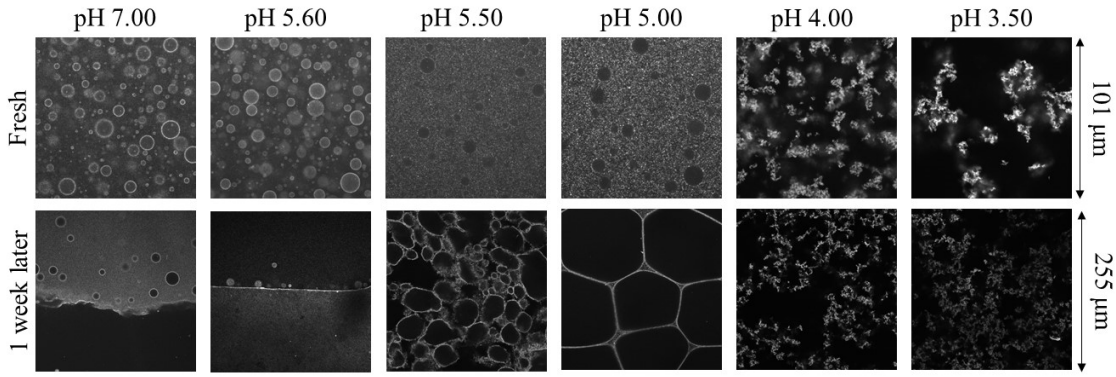


Figure 4. CLSM images of A/P emulsions with 0.05 wt% ALG and 0.4 wt% MG at different pH set by adding HCl as indicated in the figure. The top and the bottom rows correspond, respectively, to images taken just after preparation and 1 week later. Note that the images at pH 7.0 and 5.6 after one week were taken at the interface between the macroscopic PUL (top) and AMP (bottom) phases and that the scale is not the same for the images in the top and bottom rows.

At pH ≥ 5.6, the droplets coalesced, and macroscopic phase separation was observed within one day. Between pH 5.5 and 5.0, more MG migrated to the interface during ageing forming a gelled layer. Stable more or less deformed droplets were formed that sedimented into a compact bottom layer. The droplets were much larger than in fresh emulsions implying that they coalesced before the accumulation and the structuration of MG/ALG complexes at the interface was sufficient to inhibit further coalescence. We investigated this process in more detail after setting the pH to 5.0 and found that coalescence slowed down after 4h, but the dense suspension of sedimented droplets continued to coarsen very slowly, see Fig. 5. Clearly, in this pH range the complexes were formed in the bulk phases and only slowly adsorbed at the W/W interface. Most likely, the complexes at the interface adhered to each other strongly slowing down further coalescence.

One might expect that if the complexes were formed separately in the continuous phase before mixing with the dispersed phase, they could migrate more quickly towards the interface after emulsification. However, when the complexes were allowed to form in the pure PUL phase for 24 h at pH 5.0 before mixing with the AMP phase, they did not adsorb at the interface, and the emulsions destabilized within 24 h after preparation.

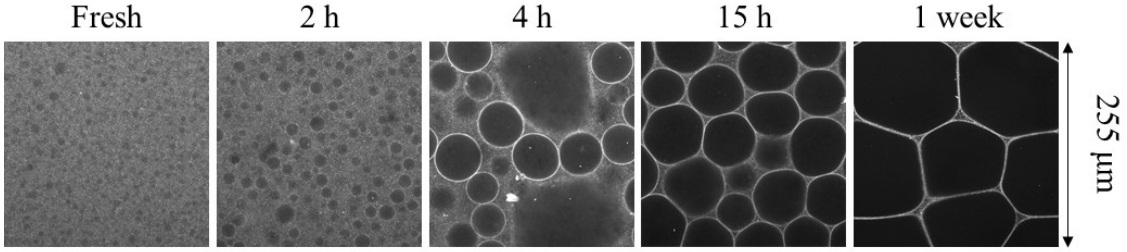


Figure 5. CLSM images showing the evolution with time of A/P emulsions with 0.4 wt% MG and 0.05 wt% ALG at pH 5.0. All images are on the same scale (255 µm x 255 µm).

Fig. 6 shows the effect of varying the ALG concentration at pH 5.0. In the presence of less ALG (0.03 wt%), the complexes aggregated rapidly and adhered to the interface in the form of clusters, as was found in the absence of ALG. However, excess clusters of complexes remained in the PUL phase even when as little as 0.05 wt% ALG was added, whereas excess pure MG clusters partitioned to the AMP phase below pH 5.6. It shows that complexation even with little ALG was sufficient to render the MG compatible with PUL. When more than 0.05 wt% ALG was added, smaller droplets were observed after one week of standing, and the MG layer appeared more strongly gelled. Apparently, the attraction between the MG increased again with high ALG concentrations, perhaps caused by bridging of ALG between the MG. Similar behaviors were observed when ALG was substituted by PEC or KC, see figures S4 and S5 of the supplementary information.

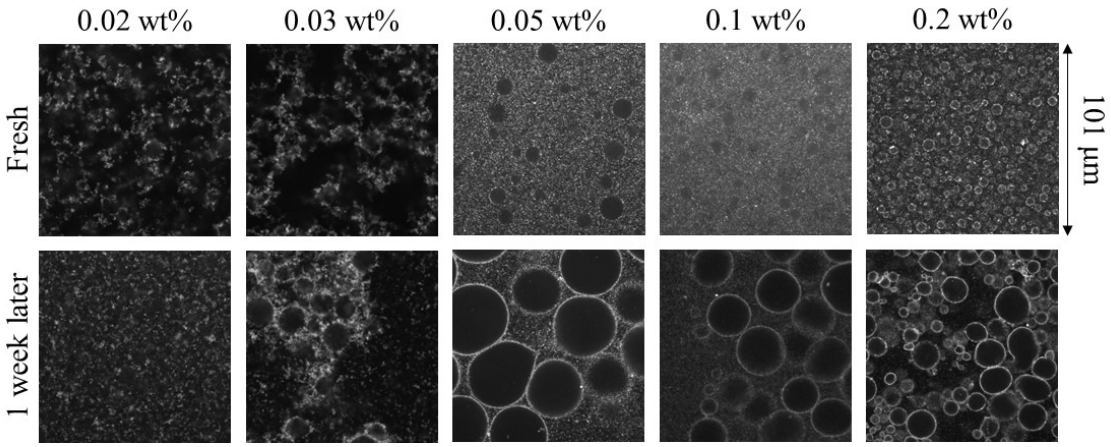


Figure 6. CLSM images of A/P emulsions at pH 5.0 set by adding HCl with 0.4 wt% MG and different concentrations of ALG as indicated in the figure. The top and the bottom rows correspond, respectively, to images taken just after preparation and 1 week later. All images are at the same scale (101 μm × 101 μm).

The microstructure of P/A emulsions with 0.05 wt% ALG at different pH is shown in Fig. 7. For this emulsion, the MG at pH 7.0 partitioned to the AMP phase, and no interfacial layer was observed. We suggested elsewhere that this was due to the higher concentration of the ALG in the PUL phase when the volume fraction of the latter is smaller (Machado et al., 2021). When complexes are formed below pH < 5.6, a layer of MG is clearly visible around the PUL droplets, which decreased in size with decreasing pH. At pH 5.0 and lower, excess MG partitioned to the PUL phase. The droplets remained stable for at least one week, but stuck to each other, forming a weak network or large flocs that sedimented.

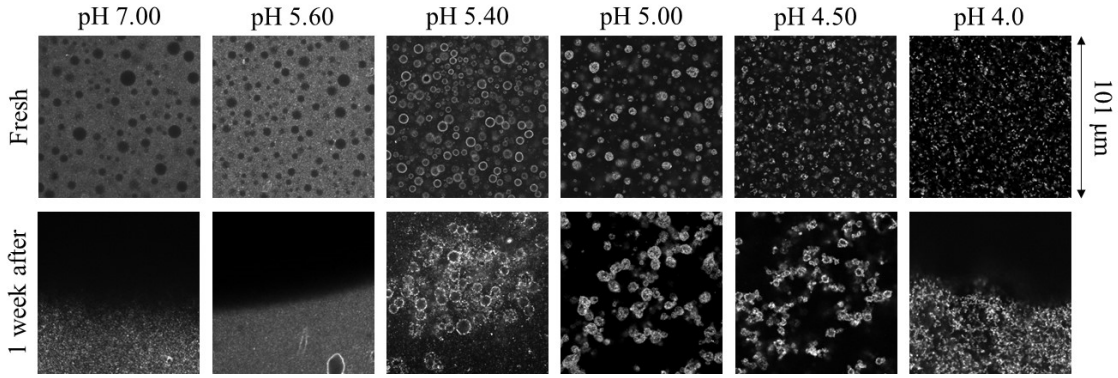


Figure 7. CLSM images of P/A emulsions with 0.05 wt% ALG and 0.4 wt% MG at different pH set by adding HCL as indicated in the image. The top and the bottom rows correspond, respectively, to images taken just after preparation and 1 week later. Note that the images at pH 7.0, 5.6 and 4.0 after 1 week were taken at the interface between the macroscopic PUL (top) and AMP (bottom) phases. All images are on the same scale (101 μm x 101 μm).

The effect of the ALG concentration on P/A emulsions at pH 5.0 is shown in fig. 8. Below 0.05 wt% the behavior was similar to that without ALG suggesting that complexation with ALG was insufficient to inhibit aggregation of the MG. At higher ALG concentrations the behavior was similar to that at 0.05 wt% with the formation of stable PUL droplets that associated into larger clusters or a weak gel.

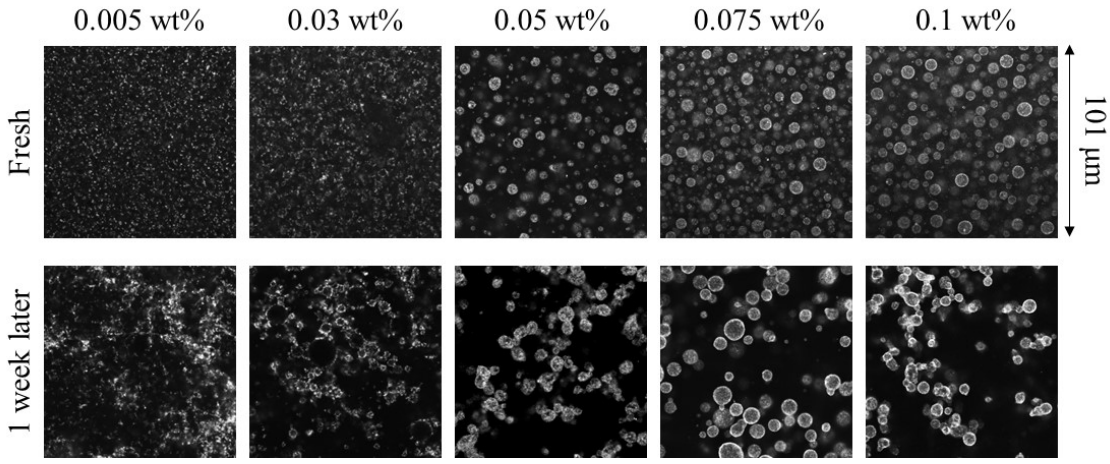


Figure 8. CLSM images of P/A emulsions at pH 5.00 with 0.4 wt% MG and different concentrations of ALG as indicated in the figure The top and the bottom rows correspond, respectively, to images taken just after preparation and 1 week later. All images are at the same scale (101 μm × 101 μm).

3.2.2 pH reduction under quiescent conditions

In the presence of 0.05 wt% ALG, KC or PEC, the MG were situated at the interface of A/P emulsions already at pH 7.0, and when the pH was reduced to pH 5.0 *in-situ* under quiescent conditions they remained there, see video 3 of the supplementary information. Images taken from the video at different pH are shown in fig. S6 in the supplementary information. This means that complexes are formed below pH 5.6 with the MG already situated at the interface. In order to reduce the time needed to reach pH values were complexation occurred, GDL was added to solution prepared at pH 5.7. However, this did not inhibit coalescence during the decrease of the pH so that large droplets were formed that sedimented into a dense layer, see Fig. 9. The size of the AMP droplets depended somewhat on the type of polysaccharide that was added. There appears to be no major difference between the structure of A/P emulsions whether it was set at pH 5.0 using HCl or using GDL.

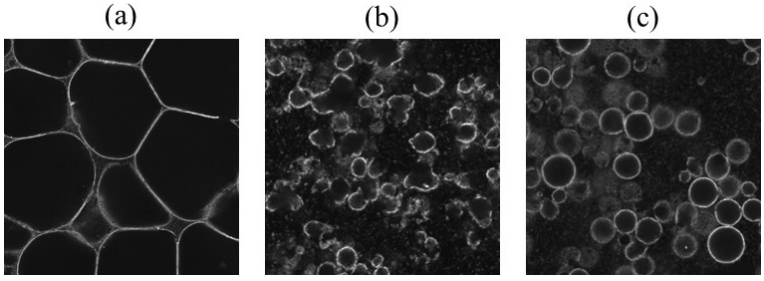


Figure 9. CLSM images of A/P emulsions at pH 5.0 set by adding GDL with 0.4 wt% MG and 0.05 wt% ALG (a), KC (b) or PEC (c). The images were taken one week after preparation. The scale of image (a) is 255 μm × 255 μm whereas that of (b) and (c) is 101 μm × 101 μm..

For P/A emulsions, a lower anionic polysaccharide concentration was chosen, because at 0.05 wt% the MG are no longer at the interface at pH 7, but partition to the AMP phase. In the presence of 0.005 wt% ALG, KC, or PEC the MG are at the interface at pH 7.0, and quiescent *in-situ* decrease of the pH led to the formation of a gelled layer of MG around spherical AMP droplets similar to what was observed in the absence of anionic polysaccharide, see Figure 10. The droplets were relatively small and remained stable in suspension for at least a week.

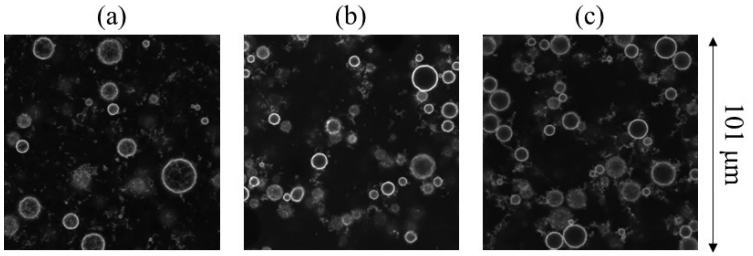
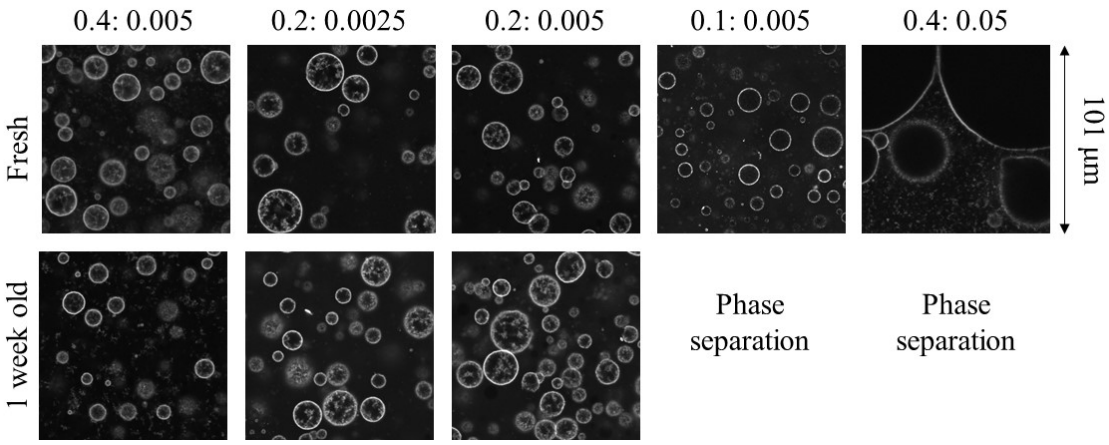


Figure 10. CLSM images of A/P emulsions at pH 5.0 set by adding GDL with 0.4 wt% MG and 0.05 wt% ALG (a), KC (b), or PEC (c). The images were taken one week after preparation. All images are at the same scale (101 μm × 101 μm).

A few measurements at different MG and ALG concentrations were done, see Fig. 11. At 0.2 wt% MG, similar stable PUL droplets were formed, but at 0.1 wt% MG, macroscopic phase separation occurred, indicating that the MG concentration in the interfacial layer was insufficient to inhibit coalescence. There was little effect of ALG on the size and stability of the PUL droplets when its concentration was low. However, at 0.05 wt% ALG the emulsion was not stable, and macroscopic phase separation was observed even though the MG did absorb at the interface when the pH was decreased *in-situ.* Apparently, the attraction between the complexes at the interface containing more ALG was not strong enough to avoid coalescence.



409

410 *Figure 11. CLSM images of P/A emulsions right after preparation (top row) and 1 week later*
411 *(bottom row) with different MG:ALG ratios indicated above each column. GDL was used to*
412 *reduce the pH from 7.0 to ~5.0. All images are at the same scale (101 µm × 101 µm).*

413

414 **4. Discussion**

415 The present investigation shows that subtle changes in the strength of the interaction
416 between the particles can have a strong effect on the morphology and stability of W/W emulsions.
417 Decreasing the pH below 5.6 caused the MG to adsorb at the W/W interface, but also caused
418 attractive interaction between the MG. If the attraction is too strong, it leads to clustering of the
419 MG before forming a smooth layer at the interface. This can lead to the formation of a weak gel
420 that traps the dispersed phase, but does not inhibit the emulsion from flowing easily when tilted.

421 Interestingly, the MG partitioned to the the AMP phase when the pH was set to 5.0 or
422 below, whereas at higher pH they partitioned to the PUL phase. The reason for this inversion is
423 not clear to us at this moment. An important observation is, however, that if the pH is decreased
424 without mechanical mixing, the MG did not migrate from the PUL phase to the AMP phase, but
425 remained trapped at the interface. For this reason, the behavior of the emulsions was very different
426 when the pH was reduced with or without shaking, in particular, that of the P/A emulsions. When
427 pH of P/A emulsions was decreased *in-situ* to pH 5.0, PUL droplets with diameters smaller than
428 10 µm were stabilized by a smooth interfacial gel layer of MG. Remarkably these droplets did
429 not bind to each other to form larger flocs, but remained well dispersed in solution for at least one
430 week. Preliminary measurements have shown that this system is stable to dilution to below the
431 binodal and even to increasing the pH to 7. More research is currently being done to establish in
432 more detail the properties of this interesting food-grade W/W emulsion.

433 The strength of the interaction between the MG at a given pH can be modified by adding
434 small amounts of anionic polysaccharides that form complexes with the MG. Complexation can

inhibit clustering of the MG and reduces the strength of the attraction between the MG at the interface. Consequently, droplets with smooth interfaces were formed at pH values where large-scale flocculation of MG occurred in the absence of polysaccharides. The effect of adding anionic polysaccharides was similar for ALG, KC, and PEC, but it depended strongly on the concentration. At high concentrations is can reduce the attraction between the MG to such an extent that the layer no longer inhibits coalescence.

Here we did not investigate the effect of adding salt, but it is evident that the strength of the interactions between MG can also be modulated by adding salt, as was shown by Gonzalez-Jordan et al. (2017). In this manner, it should be possible to introduce attractive interaction between the MG at pH > 5.6. Another possible extension to this work is to use fractal protein aggregates instead of MG. A comparison between the effect of adding MG and fractals on the stability of W/W emulsions was reported by Gonzalez-Jordan et al., 2016. Fractals have a lower density, and therefore less protein is required to cover the interface. In addition, the strength of the interaction between fractals and their interaction with anionic polysaccharides is different. Clearly, much more research is needed in order to fully understand and exploit particle stabilization of W/W emulsions.

5. Conclusion

The stability and morphology of W/W emulsions can be controlled by adding MG particles and tuning the interaction between the particles. The partition of protein microgels in AMP-PUL emulsions depends on the pH and changes from preferentially to the PUL phase at pH > 5.0 to preferentially to the AMP phase at pH < 5.0. The change in the interfacial tension between the MG and the phases causes the MG to adsorb at the W/W interface below pH 5.6. Decreasing the pH towards the pI reduces electrostatic repulsion between the MG and leads to aggregation of the MG at the interface and in the AMP phase causing the formation of stable weak emulsion gels that flow when tilted. If the pH is decreased *in-situ* without shaking, the MG cannot migrate to the AMP phase but remain trapped at the W/W interface. In this case, weak emulsion gels are formed in A/P emulsions, whereas in P/A emulsions a strong gelled MG layer is formed around the AMP droplets that remain freely dispersed in the PUL phase for at least a week. Addition of small amounts of anionic polysaccharides can be used to modulate the interfacial tension of the particles with the phases as well as the interaction between the particles.

Acknowledgements

This research was financed by the regional program Food for Tomorrow /Cap Aliment; Research, Education and Innovation in Pays de la Loire, which is supported by the French Region Pays de la Loire and the European Regional Development Fund. The CLSM analyses were conducted within platforms “Matière molle” at IMMM.

Authors contributions

João Pedro Elias Machado: Experiments, writing original draft;

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Graphical abstract

Stabilization of Amylopectin-Pullulan Water in Water Emulsions by Interacting Protein Particles

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