



HAL
open science

Stabilization of amylopectin-pullulan water in water emulsions by Interacting protein particles

João P.E. Machado, Isabelle Capron, Rilton de Freitas, Lazhar Benyahia,
Taco Nicolai

► To cite this version:

João P.E. Machado, Isabelle Capron, Rilton de Freitas, Lazhar Benyahia, Taco Nicolai. Stabilization of amylopectin-pullulan water in water emulsions by Interacting protein particles. Food Hydrocolloids, 2022, 124, part B, pp.107320. 10.1016/j.foodhyd.2021.107320 . hal-03533846

HAL Id: hal-03533846

<https://hal.inrae.fr/hal-03533846>

Submitted on 5 Jan 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

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

3

4

5 João P. E. Machado^{a,b}, Isabelle Capron^c, Rilton A. de Freitas^b, Lazhar Benyahia^a, and
6 Taco Nicolai^a

7

8 ^a Le Mans Université, IMMM UMR-CNRS 6283, 72085 Le Mans Cedex 9, France

9 ^b BioPol, Chemistry Department, Federal University of Paraná, 81.531-980 Curitiba,
10 Paraná, Brazil.

11 ^cUR1268 Biopolymères, Interactions et Assemblages, INRA, F-44316 Nantes, France

12

13

14

15 Corresponding author:

16 E-mail: Taco.Nicolai@univ-lemans.fr

17 ORCID Taco Nicolai: 0000-0002-7206-1975

18

19

20 **Abstract**

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

35

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

37

38

40 1. Introduction

41 Thermodynamically incompatible mixtures of aqueous soluble polymers give rise to
 42 aqueous two-phase systems (Frith, 2010; Gonzalez Ortiz et al., 2020), which are common in food
 43 products that contain different biopolymers that form distinct phases on mesoscopic scales. When
 44 solutions of two incompatible polymers are mechanically mixed above a certain concentration,
 45 they form water in water (W/W) emulsions with one polymer phase dispersed as droplets in a
 46 continuous phase rich in the other polymer. W/W emulsions have properties that differentiate
 47 them from conventional oil/water (O/W) emulsions. One of the most important differences is that
 48 since both phases are aqueous solutions, the interfacial tension in W/W emulsions is orders of
 49 magnitude lower and becomes zero at the critical point (Firoozmand et al., 2009; Scholten et al.,
 50 2002). Another feature of W/W interfaces is that their width is on the same length scale as the
 51 correlation length of the polymers in the phases (Nicolai & Murray, 2017). These characteristics
 52 inhibit the use of molecular surfactants as stabilizers for W/W emulsions. However, the addition
 53 of solid particles has been shown to stabilize W/W emulsions in some cases (Dickinson, 2019;
 54 Esquena, 2016; Nicolai & Murray, 2017; Sarkar & Dickinson, 2020).

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

$$61 \quad \Delta G = -\pi R^2 \gamma_{AB} (1 - |\cos \theta|)^2 \quad (1)$$

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

$$64 \quad \cos(\theta) = \frac{(\gamma_{PA} - \gamma_{PB})}{\gamma_{AB}} \quad (2)$$

65 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
 66 particles do not adsorb at the interface. The most favorable condition for adsorption is when γ_{PA}
 67 $= \gamma_{PB}$, i.e. when the particles partition equally between the phases.

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

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

80 One type of particle that has been used in the past to stabilize W/W emulsions is whey
81 protein microgels (MG) (De Freitas et al., 2016; Gonzalez-Jordan et al., 2017, 2018; Hazt et al.,
82 2020; Nguyen et al., 2013, 2015). At neutral pH these protein particles are negatively charged and
83 repel each other. However, when the net charge density of the proteins is reduced by reducing the
84 pH towards their isoionic point (IP=5.0 (Kharlamova et al., 2016)) hydrogen bonding and
85 hydrophobic interactions drive aggregation of the particles (Schmitt et al., 2010). Gonzalez-
86 Jordan et al., (2017) investigated the effect of adding MG on the stability of W/W emulsions
87 formed by mixing dextran (DEX) and poly(ethylene) oxide (PEO) at different pH. Between pH
88 6.0 and pH 3.5, the microgels aggregated at a rate that depended on the pH. Closer to the IP, large
89 clusters of MG were formed rapidly during mixing that migrated to the interface and extended
90 into the dextran phase. Further from the IP, aggregation was slower, allowing the formation of a
91 continuous protein layer at the droplet interface that subsequently gelled. However, MG at the
92 interface of different droplets could also bind to each other, leading to clustering of droplets.

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

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

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

125

126 **2. Materials and methods**

127

128 **2.1 Materials**

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

141 weight-average molar masses, M_w , determined were the following: ALG = 7.9×10^4 , KC = $8.8 \times$
142 10^4 , PEC = 3.7×10^6 , PUL = 3.0×10^5 , and AMP = 1.6×10^8 g mol⁻¹.

143 Whey protein isolate (BiPRO®) was procured from Davisco Foods International, Inc (Le
144 Sueur, MN, USA). Size exclusion chromatography showed that the sample contained 65% β -Ig
145 and 20% α -lac. The remaining 15% consisted of other whey proteins and a small amount of
146 caseins. Protein microgels (MG) were obtained and characterized as described in detail by Phan-
147 Xuan et al. (2011). Briefly, 4 wt% WPI in aqueous solution at pH = 5.90 was heated for 15 h at
148 80 °C. After this treatment, approximately 65 % of the WPI had formed microgels. The MG were
149 analyzed by static and dynamic light scattering from which it was found that the average
150 hydrodynamic radius was 120 nm, the radius of gyration was 125 nm, and the molar mass was
151 3.3×10^8 g/mol. The remaining 35 % was composed of small strand-like aggregates formed by
152 heating that do not have W/W interfacial properties (Nguyen et al., 2013).

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

172 2.2 Methods

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

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

180

181 **3. Results**

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

188

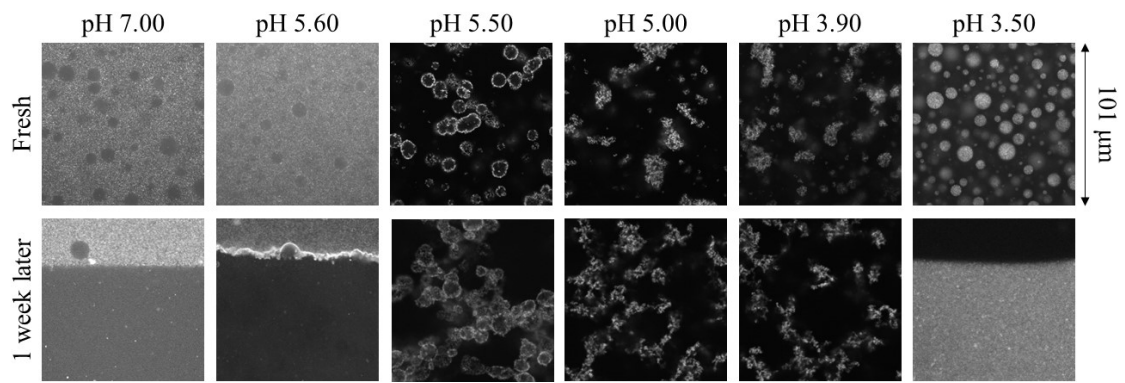
189 **3.1 Effect of pH on the microstructure of AMP/PUL emulsions in the presence of MG**

190 *3.1.1 pH reduction with HCL while stirring*

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

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

221



222

223

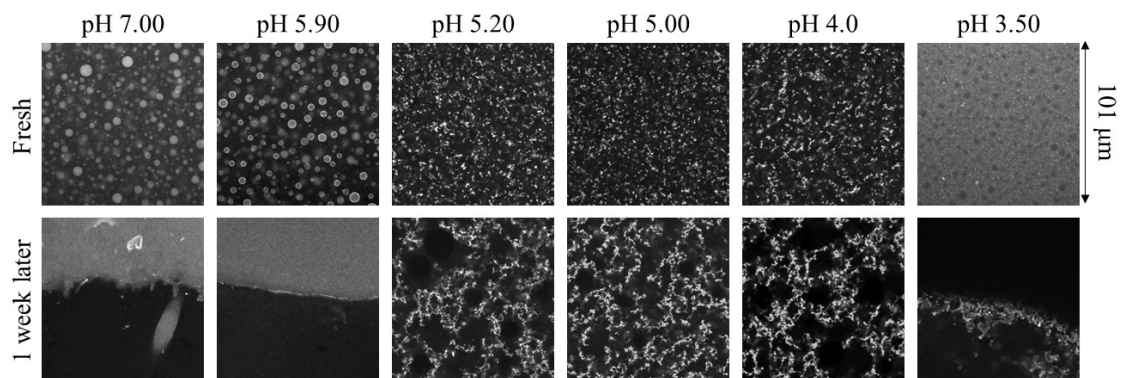
224 *Figure 1. CLSM images of A/P emulsions in the presence of 0.4 wt% MG at different pH set by*
 225 *adding HCL as shown in the figure. The fluorescence from the labelled MG is shown in white.*
 226 *The top and the bottom rows correspond, respectively, to images taken just after preparation and*
 227 *1 week later. All images are on the same scale (101 μm x 101 μm). Note that the images at pH*
 228 *7.00, 5.60, and 3.50 were taken at the interface between the macroscopic PUL (top) and AMP*
 229 *(bottom) phases .*

230

231 In P/A emulsions, the droplets are filled with MG at pH 7.0, and no MG were observed
 232 at the interface. Probably due to the higher local concentration of MG inside the droplets, the
 233 aggregation started at pH 5.9 instead of pH 5.5 for A/P emulsions, see Fig. 2. At $\text{pH} \geq 5.8$ and pH
 234 ≤ 3.5 macroscopic phase separation occurred within one day, see Fig. S2 of the supplementary
 235 information.. At pH 5.6 the droplets were non-spherical as for A/P emulsions at pH 5.5 showing
 236 that MG formed a gelled layer at the droplet interface, results not shown. However, the gelled

237 interface was not enough to prevent coalescence. Careful observation showed that excess MG
 238 (mostly in the form of aggregates) partitioned to the continuous AMP phase at $\text{pH} < 5.6$. Between
 239 $\text{pH} 5.6$ and 4.0 , no clear layer of MG at the droplet interface was observed, but the MG formed a
 240 weak network in the AMP phase that trapped the PUL droplets for at least 1 week so that the
 241 emulsion remained macroscopically homogeneous, see Fig. S2 of the supplementary
 242 information,. Although the network was strong enough to inhibit creaming of the PUL droplets,
 243 the emulsion still freely flowed when tilted.

244



245

246 *Figure 2. CLSM images of P/A emulsions in the presence of 0.4 wt% MG at different pH set by*
 247 *adding HCl as indicated in the figure. The top and the bottom rows correspond, respectively, to*
 248 *images taken just after preparation and 1 week later. Note that the images at pH 7.00, 5.90 and*
 249 *3.50 after one week were taken at the interface between the macroscopic PUL (top) and AMP*
 250 *(bottom) phases. All images are on the same scale (101 $\mu\text{m} \times 101 \mu\text{m}$).*

251

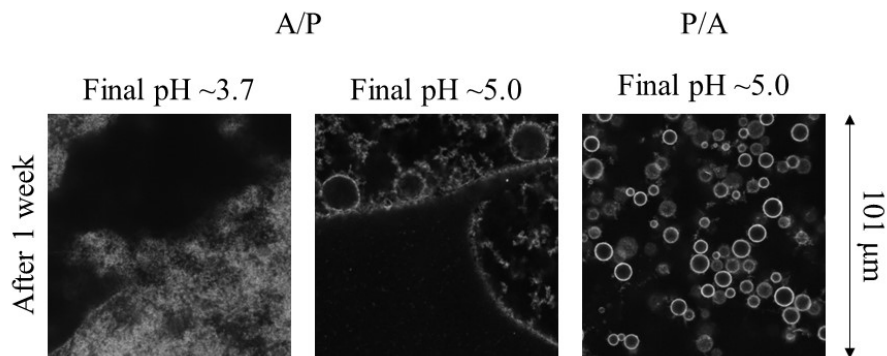
252 3.1.2 *in-situ* pH reduction with GDL under quiescent conditions

253 By adding GDL to the emulsions, it was possible to observe how the microstructure
 254 changes while the pH decreases *in-situ*. Video 1 of the supplementary information shows the
 255 evolution of an A/P emulsion. Images taken from the video at different pH are shown in Fig. S3
 256 of the supplementary information. As the pH decreased, the MG, which were situated in the
 257 dispersed phase, first started to aggregate at pH close to 5.5 and formed small clusters. With
 258 decreasing pH, the clusters grew and migrated towards the interface, where they remained
 259 irreversibly adsorbed. No transfer of MG to the AMP phase was observed at lower pH, which
 260 apparently requires mechanical mixing. As the pH decreased further towards the final value of
 261 $\text{pH} 3.7$, the AMP droplets covered with MG clusters flocculated, see fig. 3. If less GDL was added
 262 so that the final pH was 5.0, macroscopic phase separation into continuous AMP and PUL phases
 263 occurred before a stable MG layer was formed around the AMP droplets, with MG clusters

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

267 The evolution of a P/A emulsion after the addition of GDL was very different, see video
268 2 of the supplementary information. In this case the MG situated in the dispersed phase
269 accumulated at the interface below pH 5.9 without forming clusters. Again no transfer of MG
270 from the PUL to the AMP phase was observed. At the final pH 5.0 the layer of MG stabilized
271 PUL droplets for at least one week, see fig. 3. The droplets were relatively small with diameters
272 less than 10 μm , and no sedimentation was observed after a week. The different behavior of P/A
273 emulsions compared to A/P emulsions can be explained by the fact that in P/A emulsions, the MG
274 are concentrated within the droplets and can therefore adsorb more easily at the interface that is
275 nearby. In addition, the viscosity of the AMP phase was found to be ten times higher than that of
276 the PUL phase, which slows down coalescence when AMP is the continuous phase.

277



278

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

280

281 **3.2 Effect of adding anionic polysaccharides**

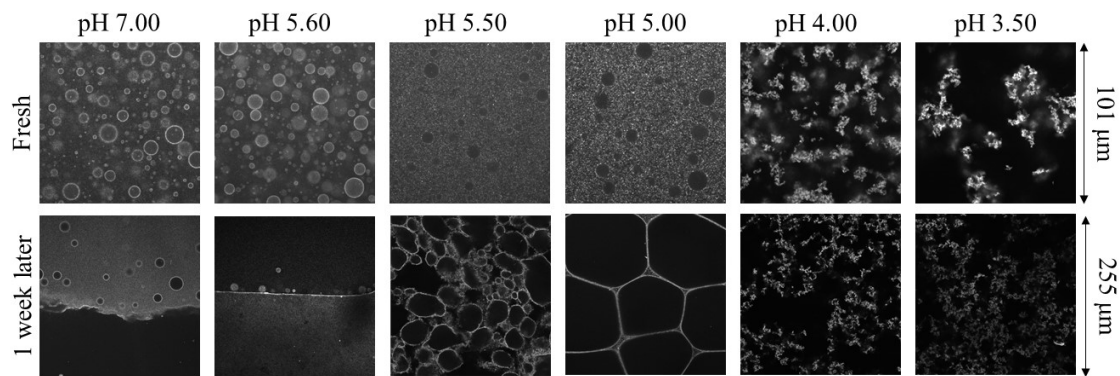
282

283 *3.2.1 pH reduction with HCL while stirring*

284 Fig. 4 shows the morphology of A/P emulsions at different pH in the presence of 0.05
285 wt% alginate (ALG). In this case, the MG adsorbed at the interface already at pH 7.0, as was
286 reported elsewhere (Machado et al., 2021). The microstructure remained the same down to pH
287 5.6, but the MG partitioned more strongly to the AMP phase with decreasing pH. However,
288 between pH 5.5 and pH 5.0 the fraction of MG that adsorbed rapidly at the interface decreased,
289 and the excess MG partitioned more strongly to the PUL phase, i.e. the opposite of what happened
290 without adding ALG. In addition, no aggregation of MG was observed. This behavior can be

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

298



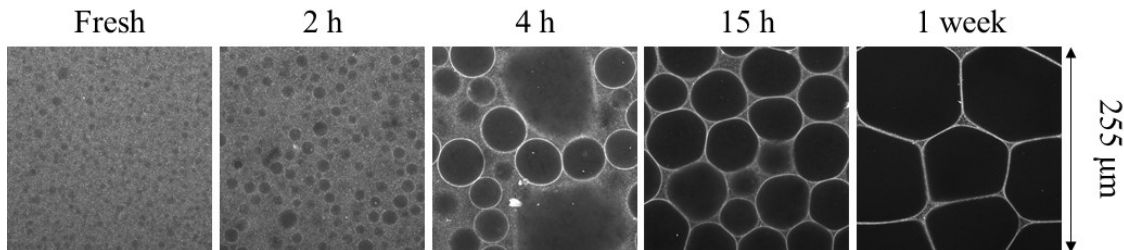
299

300 *Figure 4. CLSM images of A/P emulsions with 0.05 wt% ALG and 0.4 wt% MG at different pH*
 301 *set by adding HCl as indicated in the figure. The top and the bottom rows correspond,*
 302 *respectively, to images taken just after preparation and 1 week later. Note that the images at pH*
 303 *7.0 and 5.6 after one week were taken at the interface between the macroscopic PUL (top) and*
 304 *AMP (bottom) phases and that the scale is not the same for the images in the top and bottom rows.*

305

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

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



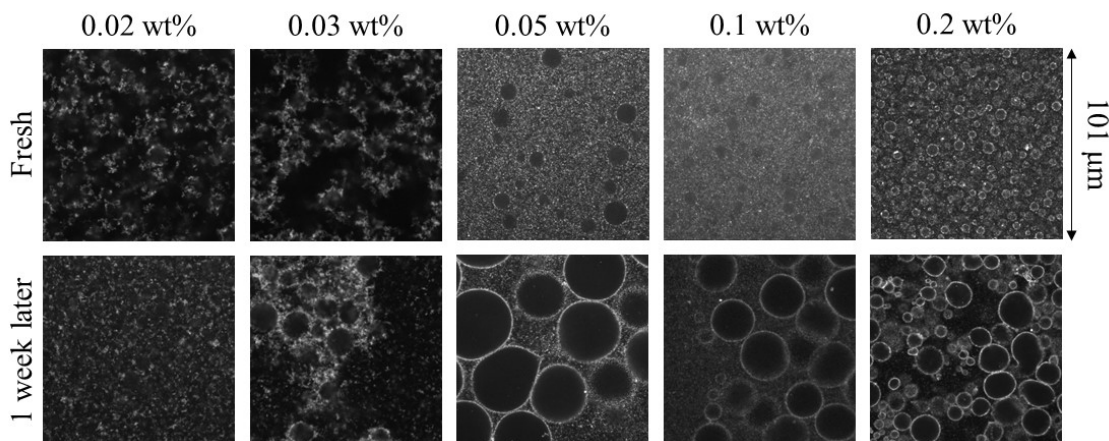
322

323 *Figure 5. CLSM images showing the evolution with time of A/P emulsions with 0.4 wt% MG and*
 324 *0.05 wt% ALG at pH 5.0. All images are on the same scale (255 μm x 255 μm).*

325

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

337



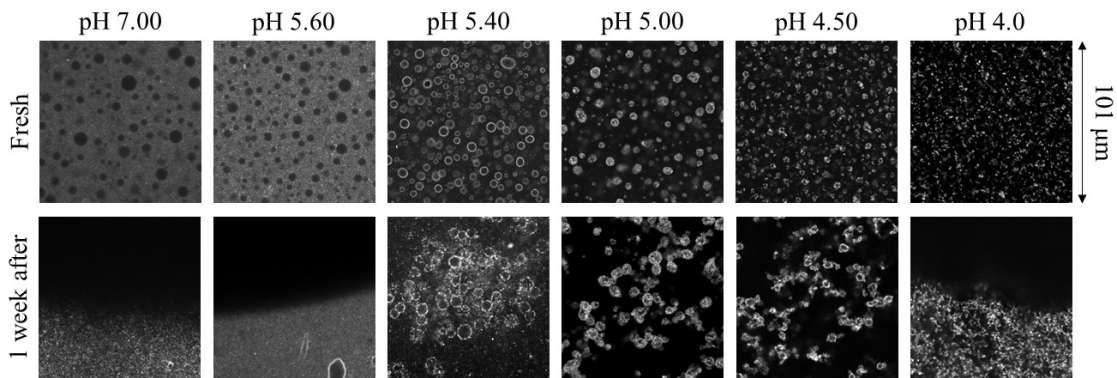
338

339 *Figure 6. CLSM images of A/P emulsions at pH 5.0 set by adding HCl with 0.4 wt% MG and*
 340 *different concentrations of ALG as indicated in the figure. The top and the bottom rows*
 341 *correspond, respectively, to images taken just after preparation and 1 week later. All images are*
 342 *at the same scale (101 $\mu\text{m} \times 101 \mu\text{m}$).*

343

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

352

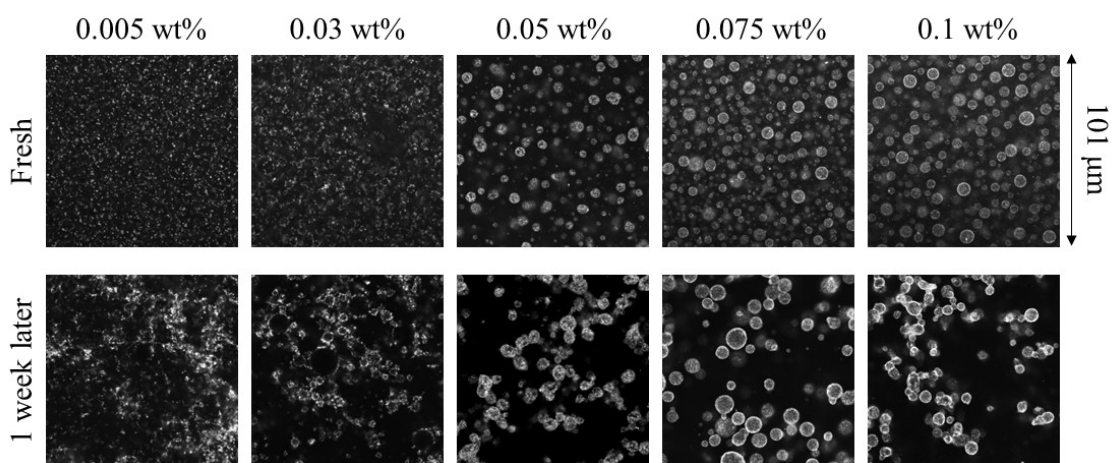


353

354 *Figure 7. CLSM images of P/A emulsions with 0.05 wt% ALG and 0.4 wt% MG at different pH*
 355 *set by adding HCL as indicated in the image. The top and the bottom rows correspond,*
 356 *respectively, to images taken just after preparation and 1 week later. Note that the images at pH*
 357 *7.0, 5.6 and 4.0 after 1 week were taken at the interface between the macroscopic PUL (top) and*
 358 *AMP (bottom) phases. All images are on the same scale (101 $\mu\text{m} \times 101 \mu\text{m}$).*

359

360 The effect of the ALG concentration on P/A emulsions at pH 5.0 is shown in fig. 8. Below
 361 0.05 wt% the behavior was similar to that without ALG suggesting that complexation with ALG
 362 was insufficient to inhibit aggregation of the MG. At higher ALG concentrations the behavior
 363 was similar to that at 0.05 wt% with the formation of stable PUL droplets that associated into
 364 larger clusters or a weak gel.



365

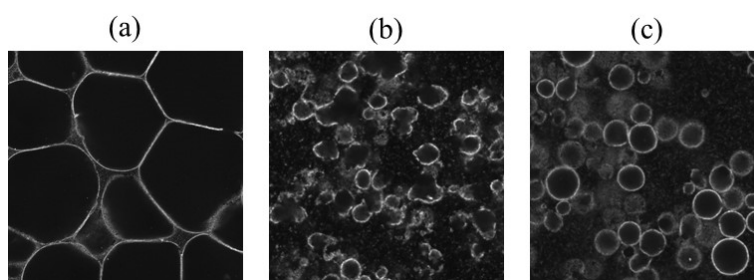
366 *Figure 8. CLSM images of P/A emulsions at pH 5.00 with 0.4 wt% MG and different*
 367 *concentrations of ALG as indicated in the figure The top and the bottom rows correspond,*
 368 *respectively, to images taken just after preparation and 1 week later. All images are at the same*
 369 *scale (101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

370

371 3.2.2 pH reduction under quiescent conditions

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

383

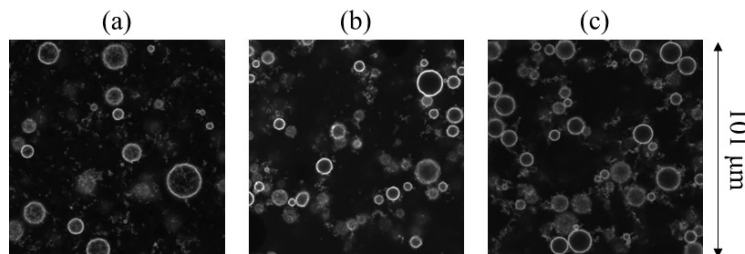


384 Figure 9. CLSM images of A/P emulsions at pH 5.0 set by adding GDL with 0.4 wt% MG and
385 0.05 wt% ALG (a), KC (b) or PEC (c). The images were taken one week after preparation. The
386 scale of image (a) is $255\ \mu\text{m} \times 255\ \mu\text{m}$ whereas that of (b) and (c) is $101\ \mu\text{m} \times 101\ \mu\text{m}$.

387

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

394



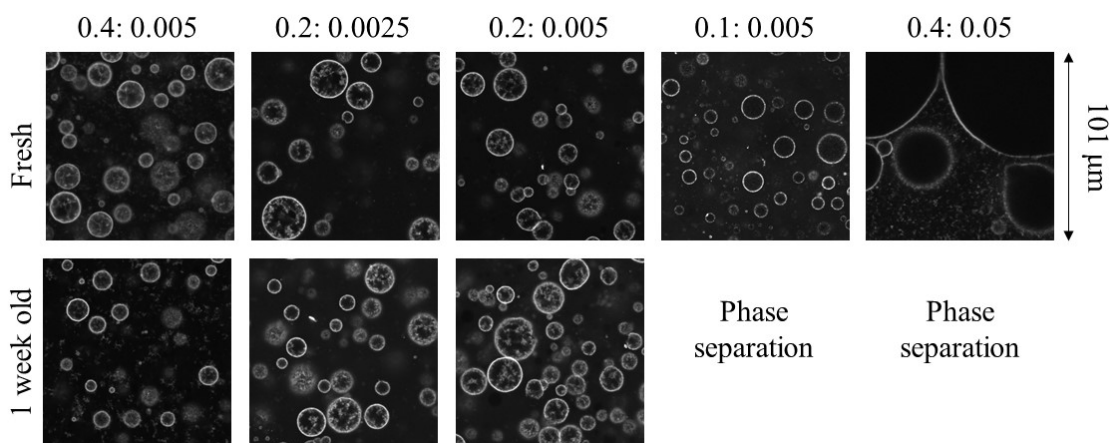
395

396 Figure 10. CLSM images of A/P emulsions at pH 5.0 set by adding GDL with 0.4 wt% MG and
397 0.05 wt% ALG (a), KC (b), or PEC (c). The images were taken one week after preparation. All
398 images are at the same scale ($101\ \mu\text{m} \times 101\ \mu\text{m}$).

399

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

408



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

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

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

451

452 **5. Conclusion**

453

454 The stability and morphology of W/W emulsions can be controlled by adding MG
455 particles and tuning the interaction between the particles. The partition of protein microgels in
456 AMP-PUL emulsions depends on the pH and changes from preferentially to the PUL phase at pH
457 > 5.0 to preferentially to the AMP phase at pH < 5.0. The change in the interfacial tension between
458 the MG and the phases causes the MG to adsorb at the W/W interface below pH 5.6. Decreasing
459 the pH towards the pI reduces electrostatic repulsion between the MG and leads to aggregation of
460 the MG at the interface and in the AMP phase causing the formation of stable weak emulsion gels
461 that flow when tilted. If the pH is decreased *in-situ* without shaking, the MG cannot migrate to
462 the AMP phase but remain trapped at the W/W interface. In this case, weak emulsion gels are
463 formed in A/P emulsions, whereas in P/A emulsions a strong gelled MG layer is formed around
464 the AMP droplets that remain freely dispersed in the PUL phase for at least a week. Addition of
465 small amounts of anionic polysaccharides can be used to modulate the interfacial tension of the
466 particles with the phases as well as the interaction between the particles.

467

468

469 **Acknowledgements**

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

474

475

476 **Authors contributions**

477 João Pedro Elias Machado: Experiments, writing original draft;

478 Taco Nicolai: Conceptualization, data curation, writing, reviewing, and editing.

479 Lazhar Benyahia: Reviewing and editing

480 Rilton Alves de Freitas: Reviewing and editing

481 Isabelle Capron: Reviewing and editing

482

483 **References**

484 Aveyard, R., Clint, J. H., & Horozov, T. S. (2003). Aspects of the stabilisation of emulsions by
485 solid particles: Effects of line tension and monolayer curvature energy. *Physical Chemistry*
486 *Chemical Physics*, 5(11), 2398–2409. <https://doi.org/10.1039/b210687f>

487 Balakrishnan, G., Nicolai, T., Benyahia, L., & Durand, D. (2012). Particles trapped at the droplet
488 interface in water-in-water emulsions. *Langmuir*, 28(14), 5921–5926.
489 <https://doi.org/10.1021/la204825f>

490 Copeland, L., Blazek, J., Salman, H., & Tang, M. C. (2009). Form and functionality of starch.
491 *Food Hydrocolloids*, 23(6), 1527–1534. <https://doi.org/10.1016/j.foodhyd.2008.09.016>

492 De Freitas, R. A., Nicolai, T., Chassenieux, C., & Benyahia, L. (2016). Stabilization of Water-in-
493 Water Emulsions by Polysaccharide-Coated Protein Particles. *Langmuir*, 32(5), 1227–1232.
494 <https://doi.org/10.1021/acs.langmuir.5b03761>

495 Dickinson, E. (2019). Particle-based stabilization of water-in-water emulsions containing mixed
496 biopolymers. *Trends in Food Science and Technology*, 83(October 2018), 31–40.
497 <https://doi.org/10.1016/j.tifs.2018.11.004>

498 Esquena, J. (2016). Water-in-water (W/W) emulsions. *Current Opinion in Colloid and Interface*

499 *Science*, 25, 109–119. <https://doi.org/10.1016/j.cocis.2016.09.010>

500 Firoozmand, H., Murray, B. S., & Dickinson, E. (2009). Interfacial structuring in a phase-
501 separating mixed biopolymer solution containing colloidal particles. *Langmuir*, 25(3), 1300–
502 1305. <https://doi.org/10.1021/la8037389>

503 Frith, W. J. (2010). Mixed biopolymer aqueous solutions - Phase behaviour and rheology.
504 *Advances in Colloid and Interface Science*, 161(1–2), 48–60.
505 <https://doi.org/10.1016/j.cis.2009.08.001>

506 Gonzalez-Jordan, A., Benyahia, L., & Nicolai, T. (2017). Cold gelation of water in water
507 emulsions stabilized by protein particles. *Colloids and Surfaces A: Physicochemical and*
508 *Engineering Aspects*, 532(January), 332–341. <https://doi.org/10.1016/j.colsurfa.2017.04.073>

509 Gonzalez-Jordan, A., Nicolai, T., & Benyahia, L. (2018). Enhancement of the particle
510 stabilization of water-in-water emulsions by modulating the phase preference of the particles.
511 *Journal of Colloid and Interface Science*, 530, 505–510.
512 <https://doi.org/10.1016/j.jcis.2018.04.088>

513 Gonzalez Ortiz, D., Pochat-Bohatier, C., Cambedouzou, J., Bechelany, M., & Miele, P. (2020).
514 Current Trends in Pickering Emulsions: Particle Morphology and Applications. *Engineering*,
515 6(4), 468–482. <https://doi.org/10.1016/j.eng.2019.08.017>

516 Hazt, B., Bassani, H. P., Elias-Machado, J. P., Aldinucci Buzzo, J. L., Silveira, J. L. M., & de
517 Freitas, R. A. (2020). Effect of pH and protein particle shape on the stability of amylopectin–
518 xyloglucan water-in-water emulsions. *Food Hydrocolloids*, 105769.
519 <https://doi.org/10.1016/j.foodhyd.2020.105769>

520 Kharlamova, A., Inthavong, W., Nicolai, T., & Chassenieux, C. (2016). The effect of aggregation
521 into fractals or microgels on the charge density and the isoionic point of globular proteins. *Food*
522 *Hydrocolloids*, 60, 470–475. <https://doi.org/https://doi.org/10.1016/j.foodhyd.2016.04.013>

523 Khemissi, H., Bassani, H., Aschi, A., Capron, I., Benyahia, L., & Nicolai, T. (2018). Exploiting
524 Complex Formation between Polysaccharides and Protein Microgels To Influence Particle
525 Stabilization of W/W Emulsions. *Langmuir*, 34(39), 11806–11813.
526 <https://doi.org/10.1021/acs.langmuir.8b02383>

527 Levine, S., & Sanford, E. (1985). Stabilisation of emulsion droplets by fine powders. *The*
528 *Canadian Journal of Chemical Engineering*, 63(2), 258–268.
529 <https://doi.org/10.1002/cjce.5450630211>

530 Liu, D., Zhou, P., & Nicolai, T. (2020). Effect of Kappa carrageenan on acid-induced gelation of
531 whey protein aggregates. Part I: Potentiometric titration, rheology and turbidity. *Food*

532 *Hydrocolloids*, 102(November 2019), 105589. <https://doi.org/10.1016/j.foodhyd.2019.105589>

533 Machado, J. P. E., Benyahia, L., & Nicolai, T. (2021). Effect of adding a third polysaccharide on
534 the adsorption of protein microgels at the interface of polysaccharide-based water in water
535 emulsions. *Journal of Colloid and Interface Science*.
536 <https://doi.org/10.1016/j.jcis.2021.06.053>

537 Nguyen, B. T., Nicolai, T., & Benyahia, L. (2013). Stabilization of Water-in-Water Emulsions by
538 Addition of Protein Particles. *Langmuir*, 29(34), 10658–10664.
539 <https://doi.org/10.1021/la402131e>

540 Nguyen, B. T., Wang, W., Saunders, B. R., Benyahia, L., & Nicolai, T. (2015). pH-responsive
541 water-in-water pickering emulsions. *Langmuir*, 31(12), 3605–3611.
542 <https://doi.org/10.1021/la5049024>

543 Nicolai, T., & Murray, B. (2017). Particle stabilized water in water emulsions. *Food*
544 *Hydrocolloids*, 68, 157–163. <https://doi.org/10.1016/j.foodhyd.2016.08.036>

545 Nishinari, K., Kohyama, K., Williams, P. A., Phillips, G. O., Burchard, W., & Ogino, K. (1991).
546 Solution Properties of Pullulan. *Macromolecules*, 24(20), 5590–5593.
547 <https://doi.org/10.1021/ma00020a017>

548 Peinado, I., Lesmes, U., Andrés, A., & McClements, J. D. (2010). Fabrication and morphological
549 characterization of biopolymer particles formed by electrostatic complexation of heat treated
550 lactoferrin and anionic polysaccharides. *Langmuir*, 26(12), 9827–9834.
551 <https://doi.org/10.1021/la1001013>

552 Phan-Xuan, T., Durand, D., Nicolai, T., Donato, L., Schmitt, C., & Bovetto, L. (2011). On the
553 crucial importance of the pH for the formation and self-stabilization of protein microgels and
554 strands. *Langmuir*, 27(24), 15092–15101. <https://doi.org/10.1021/la203357p>

555 Santipanichwong, R., Suphantharika, M., Weiss, J., & McClements, D. J. (2008). Core-shell
556 biopolymer nanoparticles produced by electrostatic deposition of beet pectin onto heat-denatured
557 β -lactoglobulin aggregates. In *Journal of Food Science* (Vol. 73, Issue 6).
558 <https://doi.org/10.1111/j.1750-3841.2008.00804.x>

559 Sarkar, A., & Dickinson, E. (2020). Sustainable food-grade Pickering emulsions stabilized by
560 plant-based particles. *Current Opinion in Colloid and Interface Science*, 49, 69–81.
561 <https://doi.org/10.1016/j.cocis.2020.04.004>

562 Schmitt, C., Moitzi, C., Bovay, C., Rouvet, M., Bovetto, L., Donato, L., Leser, M. E.,
563 Schurtenberger, P., & Stradner, A. (2010). Internal structure and colloidal behaviour of covalent
564 whey protein microgels obtained by heat treatment. *Soft Matter*, 6(19), 4876–4884.

565 <https://doi.org/10.1039/c0sm00220h>

566 Scholten, E., Tuinier, R., Tromp, R. H., & Lekkerkerker, H. N. W. (2002). Interfacial tension of
567 a decomposed biopolymer mixture. *Langmuir*, 18(6), 2234–2238.
568 <https://doi.org/10.1021/la0114373>

569 Singh, R. S., Saini, G. K., & Kennedy, J. F. (2008). Pullulan: Microbial sources, production and
570 applications. *Carbohydrate Polymers*, 73(4), 515–531.
571 <https://doi.org/10.1016/j.carbpol.2008.01.003>

572 Tester, R. F., Karkalas, J., & Qi, X. (2004). Starch - Composition, fine structure and architecture.
573 *Journal of Cereal Science*, 39(2), 151–165. <https://doi.org/10.1016/j.jcs.2003.12.001>

574

Graphical abstract

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

João P. E. Machado, Isabelle Capron, Rilton A. de Freitas, Lazhar Benyahia, and Taco Nicolai

