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1 **Interfacial and (emulsion) gel rheology of hydrophobised whey proteins**

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7 Ashkan Madadlou, Marie-Helene Famelart, Stéphane Pezennec, Florence Rousseau, Juliane

8 Flourey, Didier Dupont*

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13 *STLO, UMR 1253, INRA, Agrocampus Ouest, 35000 Rennes, France*

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19 *Corresponding author. Tel.:

20 *E-mail address:* didier.dupont@inra.fr (D. Dupont)

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27 ABSTRACT

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29 Hydrophobisation of whey proteins, followed by erythritol addition into the hydrophobised
30 protein dispersion, enables formation of water-in-water (W/W) emulsions of the proteins and
31 alginate. In this study, we investigated the interfacial and gel rheology of whey proteins as
32 affected by hydrophobisation, heat denaturation and erythritol addition. Additionally, the gel
33 rheology of the resultant W/W emulsion was assessed. Hydrophobisation shortened the linear
34 viscoelastic region and decreased the surface storage modulus (G'_s), the flow stress and the
35 flow strain of protein layer at the air-liquid interface. Erythritol addition into the
36 hydrophobised protein caused a further reduction of G'_s . In accordance with interfacial
37 rheology, protein hydrophobisation and erythritol addition decreased the dynamic moduli of
38 acid-induced protein gels. Frequency sweep tests indicated that the gelled emulsion had
39 higher dynamic moduli than all WPI gels. The higher firmness of the emulsion gel was
40 ascribed, based on microstructural images, to micro-phase separation of alginate droplets.

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43 **1. Introduction**

44

45 Different types of whey proteins, such as β -lactoglobulin (β -lg) and bovine serum
46 albumin, as well as whey protein products such as whey protein isolate (WPI) and whey
47 protein concentrate (WPC), are widely used in various fields of food technology, including
48 foam and emulsion stabilisation (Jaishankar, Sharma, & McKinley, 2011), fat replacement,
49 and gel formation (Zhang, Arrighi, Campbell, Lonchamp, & Euston, 2018). Each application
50 may require modification of one or several functional properties of whey proteins. Enzymatic
51 hydrolysis, heat-induced polymerisation (Foegeding, Davis, Doucet, & McGuffey, 2002),
52 high pressure processing (Liu, Powers, Swanson, Hill, & Clark, 2005), and chemical
53 functionalisation (Zhao, Ma, Yuen, & Phillips, 2004) are examples of whey protein
54 modifications.

55 Recently, WPI was hydrophobised through a process consisting of two consecutive
56 steps including a pre-acetylation stage followed by a combined acetylation/heat treatment
57 period. The process resulted in acetylation of $\geq 90\%$ of available amino groups of whey
58 proteins. Details about the structural characterisation of the hydrophobised WPI have been
59 provided elsewhere (Madadlou, Flourey, & Dupont, 2018). The modified product was then
60 used to form a water-in-water (W/W) emulsion of alginate and WPI, which was attributed to
61 the adsorption of protein particles to the liquid-liquid interface. The interfacial tension
62 between the two incompatible biopolymer phases was further modulated by addition of
63 erythritol, a low-calorie sugar alcohol (Madadlou, Saint-Jalmes, Guyomarc'h, Flourey, &
64 Dupont, 2019).

65 Adsorption of macromolecules and particles to the liquid/liquid interface during
66 emulsification forms a viscoelastic layer, which can be assessed by shear rheology
67 components (elasticity and viscosity). Shear rheology characterises the response of the

68 (particle-laden) interface to a shape deformation caused by an external mechanical
69 perturbation, in a similar way done for bulk rheology (Mendoza et al., 2014). Inter-particle
70 attractive and repulsive forces such as electrostatic and hydrophobic interactions govern the
71 interface rheology. Indeed, interfacial rheology is very sensitive to the structure and
72 composition of a protein layer formed at the interface, as well as to the nature of interactions
73 within the layer (Dickinson & Matsumura, 1991). Therefore, it was presumed that probing
74 interfacial rheology is advantageous for assessing the emulsification behaviour of whey
75 proteins as influenced by hydrophobisation.

76 Rheological analysis is also helpful to study the gelation properties of whey proteins.
77 Cold-set gelation of whey proteins is of great value in forming particles that can modify
78 viscosity (Zhang et al., 2018), and encapsulate heat-labile cargos (Farjami, Madadlou, &
79 Labbafi, 2016; Hashemi, Madadlou, & Salami, 2017). This property can also be used to form
80 whey protein-stabilised emulsion gels for fortification and technological purposes. For
81 example, such emulsions can be incorporated into cultured milk products like yoghurt to
82 improve texture (Boutin, Giroux, Paquin, & Britten, 2007). The cold-set gelation process
83 takes place by a two-step consecutive procedure. Initially, the proteins are heat-denatured at
84 non-gelling conditions (i.e., at low ionic strength, low protein content and at pH values far
85 from the proteins' isoelectric point, pI), which causes formation of whey protein soluble
86 aggregates. Then the protein dispersion is gelled at ambient temperature by adding salts
87 and/or adjusting pH toward the pI (Ju & Kilara, 1998; Meydani, Vahedifar, Askari, &
88 Madadlou, 2019). The rheology of such gels is governed by the interaction between the
89 aggregating protein particles, which is highly dependent on the aggregate chemistry (Alting,
90 Hamer, De Kruif, & Visschers, 2003). Therefore, the modification of whey proteins via
91 hydrophobisation is expected to alter the cold-set gelation behaviour and the gel rheology of
92 WPI.

93 The first objective of the present study was to analyse the interfacial rheology of WPI
94 at the air-liquid interface as influenced by heat denaturation and hydrophobisation treatments,
95 as well as, erythritol (E) addition into the hydrophobised WPI. Subsequently, bulk rheological
96 experiments were carried out to assess the acid-induced cold-set gelation process and the gel
97 properties of the hydrophobised WPI (with and without E) and the alginate-supplemented E-
98 added hydrophobised WPI (i.e., W/W emulsion).

99

100 **2. Materials and methods**

101

102 *2.1. Materials*

103

104 WPI was received as a gift from Lactalis ingredients (Bourgarré, France). It
105 contained, according to the information provided by the manufacturer, 90% protein, 5.1%
106 water, 3.0% lactose, and 1.97% ash. Reversed-phase high-performance liquid
107 chromatography (RP-HPLC) analysis indicated that the α -lactalbumin and β -lactoglobulin
108 content of the WPI was 16% and 56% of total protein, respectively. Erythritol was purchased
109 from Vita-World GmbH (Taunusstein, Germany). Alginic acid sodium salt from brown algae
110 was procured from Sigma-Aldrich (SKU W201502, Sigma-Aldrich Chemie S.a.r.l., Saint-
111 Quentin Fallavier, France). Sodium hydroxide and hydrochloric acid were bought from
112 Sigma-Aldrich. Ultrapure, 15 m Ω -resistivity water prepared by reverse osmosis was used
113 throughout the study and all chemicals were analytical grade.

114

115 *2.2. Protein modification, emulsion preparation and gelation*

116

117 WPI was hydrophobised following the method described in our previous report
118 (Madadlou et al., 2018). Briefly, WPI powder was hydrated (65 mg mL^{-1}) for 18 h and
119 supplemented with acetic anhydride at a ratio of 0.18 g g^{-1} protein. After 30 min acetylation
120 while the pH was kept over 8.0 through successive additions of 1 M NaOH, the WPI
121 dispersion was heated at $85 \text{ }^\circ\text{C}$ for 20 min. A non-acetylated but heat-denatured WPI sample
122 was also prepared by sequentially adding 1 M HCl (200 μL), and 1 M NaOH (300 μL) to a
123 native WPI dispersion (9.5 mL, pH 6.7) to set pH to 8.25 ± 0.05 , and subsequent heat
124 treatment at $85 \text{ }^\circ\text{C}$ for 20 min. The sequential addition of the acid and alkali was carried out to
125 mimic the fall and rise of pH during the hydrophobisation process. After heat treatment, the
126 pH value of the hydrophobised and heat-denatured WPI samples was adjusted to 7.50 ± 0.05
127 using approximately 20 μL of either 1 M HCl or 1 M NaOH.

128 The alginate-in-WPI W/W emulsions were prepared according to the method
129 described elsewhere (Madadlou et al., 2019) with minor changes. At first, erythritol was
130 added (100 mg mL^{-1}) into the hydrophobised WPI dispersion and stirred, followed by addition
131 of sodium alginate solution (30 mg mL^{-1}) into WPI-erythritol mixture at a volume ratio of 1:9.
132 The mixture was vortexed at a rotating frequency of 40 Hz (Bioblock Top Mix 11118, Fisher
133 Scientific, Waltham, MA, USA) for 90 s to obtain the W/W emulsion.

134 The hydrophobised (with and without added erythritol) and heat-denatured WPI
135 samples, as well as the alginate-in-WPI mixed (i.e., W/W emulsion) system ($\text{pH } 7.50 \pm 0.1$)
136 were gelled at $37 \text{ }^\circ\text{C}$ by acidification. Glucono- δ -lactone (GDL) was added (5, 10 and 20 mg
137 mL^{-1}) into the samples to gradually hydrolyse into gluconic acid, causing acid gel formation.
138 The samples were stored at $37 \text{ }^\circ\text{C}$ for 24 h before analyses. The pH value of the samples
139 during gelation was monitored using a digital pH meter (CyberScan Series pH 110, Eutech
140 Instrument, Singapore). The accuracy of the pH measurement was ± 0.02 pH unit.

141

142 2.3. *Interfacial shear measurements*

143

144 A stress-controlled rheometer (Discovery Series Hybrid Rheometer (DHR) HR-2,
145 New Castle, DE, USA) using the double wall ring (DWR) geometry was used to perform the
146 interfacial shear analyses including strain amplitude sweep and creep tests. The WPI sample
147 was poured into a round trough and an annular ring made of a platinum–iridium alloy was
148 positioned at the liquid–air interface. The DWR geometry has been thoroughly described
149 (Vandebriel, Franck, Fuller, Moldenaers, & Vermant, 2010) and provides certainty that high
150 values of the Boussinesq number are attained. Therefore, the contribution of the induced sub-
151 phase flow to torque is negligible and the interfacial torque dominates (Jaishankar &
152 McKinley, 2013). Based on time sweep tests at a strain amplitude of 0.1% and an angular
153 frequency of 10 rad s⁻¹ and on the literature review (Jaishankar et al., 2011), all steady state
154 experiments were carried out after waiting for 3 h at 20 °C. WPI dispersions (56 mg mL⁻¹)
155 were loaded into the trough, the annular ring was positioned at the interface and after the rest
156 period, the experiments were carried out.

157 It is noteworthy that in the current study, the interfacial shear rheology of WPI
158 dispersions was analysed at the same concentration as emulsion preparation was done.
159 Commonly, the interfacial rheology tests are performed at much lower concentrations of
160 amphiphilic compounds to make sure that a monolayer of the compounds is present at the
161 interface. However, as noted earlier (Lexis & Willenbacher, 2014), the structure of the
162 interfacial layers of proteins depends largely on concentration; therefore, a correlation
163 between properties such as foaming and emulsification, and interfacial rheology is achieved
164 only when the interfacial layers at both conditions (i.e., emulsion formation and interfacial
165 rheology test) are comparable. Nonetheless, as the thickness of the interfacial layer may not
166 be negligible anymore, the measured moduli are treated as apparent values. Similar

167 concentrations have already been used for the interfacial rheological analysis of bovine serum
168 albumin (Jaishankar & McKinley, 2013; Jaishankar et al., 2011; Sharma, Jaishankar, Wang,
169 & McKinley, 2011).

170 After amplitude sweep tests, creep tests were performed on WPI interfacial layers over
171 short durations. In stress-controlled rheometry, for sufficiently viscoelastic fluids, even if they
172 are simple Newtonian fluids, a ringing event (i.e., damped oscillations) is observed at the start
173 of a creep test (Ewoldt & McKinley, 2007). The coupling of fluid elasticity and instrument
174 inertia causes periodic oscillations which decay exponentially due to viscous dissipation
175 (Jaishankar & McKinley, 2013). The creep ringing logarithmic decrement, Δ and ringing
176 frequency, ω_* (Nagarkar et al., 2009) of WPI interfacial layers were calculated (where
177 applicable) using the following equations:

$$178 \quad \Delta = 2 \ln \left[\frac{(J_1 - J_2)}{(J_3 - J_2)} \right] \quad [\text{Eq. 1}]$$

$$179 \quad \omega_* = \frac{1}{(t_3 - t_1)} \quad [\text{Eq. 2}]$$

180 where J_1 , J_2 , and J_3 are the compliance values of the 1st peak, 1st valley and 2nd peak of the
181 creep ringing data, respectively (Nagarkar et al., 2009) and t_3 and t_1 are the times of the 2nd
182 peak and 1st peak, respectively.

183

184 2.4. Particle size and ζ -potential of WPI dispersions during gelation

185

186 The hydrodynamic diameter and ζ -potential of protein particles during gelation were
187 approximated by dynamic light scattering (DLS) following the methods of Ju and Kilara
188 (1998) and de Jong, Klok, and van de Velde (2009a) for acid-induced gelation of whey
189 proteins. A Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) was used for
190 this purpose. Protein samples (at 37 °C) were supplemented with GDL (20 mg mL⁻¹ for
191 hydrophobised WPI and 5 mg mL⁻¹ for heat-treated WPI), and stored at 37 °C (de Jong, Klok,

192 & van de Velde, 2009b) for different durations, i.e., 130 min for the hydrophobised WPI
193 samples and 35 min for the heat-denatured WPI. At various time intervals, the samples were
194 diluted 100 fold with deionised water and subjected to DLS analysis. Dilution did not have a
195 remarkable influence (less than 0.2 unit) on the pH value of the samples. The refractive index
196 of dispersant (water) was 1.33, and the dispersant viscosity was set to 0.69 mPa s. A laser-
197 backscattering angle of 173° and a wavelength of 633 nm were applied in size measurements.

198

199 2.5. *Rheological analyses of WPI and alginate-WPI gel samples*

200

201 The gelation time (T_{ig}) and rheological properties of WPI and emulsion samples were
202 determined using an Anton Paar Physica MCR 301 rheometer (Anton Paar GmbH, Graz,
203 Austria) equipped with a cone-plate geometry (diameter 49.96 mm and cone angle 1.99°).
204 Samples supplemented with GDL (20 mg mL⁻¹ for the hydrophobised WPI and the emulsion
205 and 5, 10, and 20 mg mL⁻¹ for the heat-denatured WPI) were poured onto the plate, followed
206 by introducing the cone. Temperature was kept at 37 °C. Low-density paraffin was added
207 around the samples to prevent evaporation. Changes in storage modulus (G'), loss modulus
208 (G''), and damping factor ($\tan \delta$) during gelation of the samples were measured at an angular
209 frequency of 1 rad s⁻¹ and 0.1% strain. Gelation time (T_{ig}) was arbitrarily defined as $G' = 1$ Pa
210 (Andoyo, Guyomarc'h, & Famelart, 2016). Gels were oscillated in situ for 24 h to obtain a
211 fully developed network. Then, a frequency sweep test was performed to determine the effect
212 of time scale of deformation on the rheological properties when the samples were formed
213 (Lee & Lucey, 2003). Frequency was increased from 0.0628 to 628 rad s⁻¹ at 0.1% strain and
214 37 °C.

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216 2.6. *Confocal laser scanning microscopy*

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Confocal laser scanning microscopy (CLSM) observation of gel microstructure was performed using the ZEISS LSM 880 inverted confocal microscope (Carl Zeiss AG, Oberkochen, Germany) set at magnification 63× (Plan Apochromat objective, oil immersion, NA 1.4) using the Airyscan detection unit. The dispersion of WPI and emulsion containing GDL were mixed with a 1% (w/v) solution of Fast Green at a ratio of 9:1. Each sample (1 mL) was injected into the chamber of a gel cassette® system (Brocklehurst, Mackie, Steer, & Wilson, 1998) and vertically incubated at 37 °C for 24 h. After gelification, Airyscan images were acquired using a He/Ne laser with a wavelength of 633 nm, using with a main beam splitter MBS488/561/633 and no additional emission filter. The zoom was set at 4 for gel imaging and 1.8 for W/W emulsion sample. For further image analysis of W/W emulsion, ten micrographs of 1336×1336 pixels (1 μm = 18.17 pixels) were taken on different regions in a constant z-position. Zen Black 2.1 (Version 13.0.0.0) software was used to process the acquired datasets using the 2D mode at default settings of the Airyscan processing function, including a Wiener filter deconvolution.

Airyscan confocal micrographs of the W/W emulsions were converted to binary images using the “Default” thresholding algorithm of Fiji-win64 software. The segmentation procedure was validated by visual comparison of the resulted binary image with its original image. The particle areas were measured using the Analyze Particles measurements tool (in μm²). Corresponding diameters were calculated, based on the hypothesis that all particles were spherical.

2.7. *Statistical analysis*

241 Samples were prepared in triplicate and the experiments were done at least two times.
242 The results were analysed by one-way ANOVA with SPSS software version 16 (IBM
243 software, New York, NY, USA) using Duncan's test at a significance level of $P < 0.05$.

244

245 **3. Results and discussion**

246

247 *3.1. Interfacial rheology*

248

249 *3.1.1. Amplitude sweep*

250 The dynamic moduli of air-WPI interfaces were first measured over time at a fixed
251 strain amplitude and oscillation frequency. The interfacial shear storage (G'_s) and loss (G''_s)
252 moduli reached equilibrium 2–3 h after sample loading, which indicates that the structure of
253 interface reached steady state (Jaishankar & McKinley, 2013). Then, small amplitude
254 oscillatory strain sweeps were carried out to find out the onset of non-linearity of the samples
255 viscoelastic response. Typical curves of the results of strain sweep tests are illustrated in Fig.
256 1. In agreement with the literature on native β -lactoglobulin (Ridout, Mackie, & Wilde, 2004),
257 the G'_s of the air-liquid interfacial layer of native WPI had a value of about 13 mN m^{-1} (Table
258 1). Heat denaturation and hydrophobisation decreased the G'_s value of the air-liquid interfacial
259 layer of WPI. Native β -lactoglobulin, as the major whey protein, when adsorbed at interface
260 polymerises over time, concomitant with lack of any appreciable exchange between adsorbed
261 polymers and non-adsorbed monomers. The polymerisation takes place by the gradual
262 exposure of the free –SH groups embedded in the interior of the molecule, triggering
263 progressive formation of disulphide crosslinks. It develops a remarkable extent of β -
264 lactoglobulin oligomers at interface 2 h after interfacial adsorption (Dickinson & Matsumura,
265 1991).

266 In contrast to native proteins, extensive conformational changes and disulphide bond
267 formation take place before interfacial adsorption in heat-processed proteins (Meydani et al.,
268 2019), i.e., the heat-denatured and hydrophobised WPI samples. This reduces the extent of
269 permanent crosslinks formation after interfacial adsorption. Furthermore, the greater absolute
270 values of negatively signed ζ -potentials of heat-denatured and hydrophobised WPIs in
271 comparison with native WPI at comparable pH values (Madadlou et al., 2018) suggest a
272 stronger electrostatic repulsion between protein particles at the interfacial layer, reducing the
273 stiffness and hence the G'_s of the layer. Likewise, a smaller extent of crosslinking between
274 protein molecules was argued to explain the lower interfacial viscosity of β -lactoglobulin at
275 the air-water interface when compared with oil-water interface (Roth, Murray, & Dickinson,
276 2000).

277 The point at which G'_s deviated by more than 10% from a constant value was
278 considered as the onset of non-linearity (TA Instruments, New Castle, DE, USA:
279 <http://www.tainstruments.com/pdf/literature/RS23.pdf>), which took place at a strain
280 amplitude of 1.8%, 1.6%, and 1.2% for the air-liquid interfacial layers of native, heat-
281 denatured and hydrophobised WPI samples, respectively. For these three samples, a shorter
282 linear region coincided a lower G'_s value (Table 1).

283 Although erythritol addition slightly decreased the G'_s and G''_s of the hydrophobised
284 WPI interfacial layer, the reduction was not statistically significant (Fig. 1; Table 1).
285 Likewise, the phase-shift angle, δ of the layer was not influenced by erythritol addition (Table
286 1). Furthermore, the δ values of the air-liquid interfacial layers of the hydrophobised WPI
287 samples (either E-free or E-added) were not different from that of the heat-denatured sample.
288 In contrast, native WPI had a remarkably smaller δ in the linear region. Within the linear
289 region, G'_s was larger than G''_s for all samples, indicating that the interfacial layers were
290 predominately elastic (Langevin, 2000; Sharma et al., 2011).

291 All interfacial layers underwent a yielding process at high strain amplitudes. The
292 process is shown by a steep collapse in the magnitude of G'_s and the domination of G''_s beyond
293 a certain amplitude (Jaishankar et al., 2011). The values of oscillation flow stress (τ_f) and
294 flow strain (γ_f) of the WPI interfacial layers were affected by heat denaturation and
295 hydrophobisation. The crossovers of G'_s and G''_s , which indicate the flow points of the
296 interfacial layers, are specified by the circles in Fig. 1, and the corresponding τ_f and γ_f values
297 are reported in Table 1. In accordance with the influence on G'_s , heat-denaturation and
298 hydrophobisation decreased the values of τ_f and γ_f of the interfacial layer, and erythritol
299 addition into the hydrophobised WPI did not influence the flow-point criteria (i.e., τ_f and γ_f)
300 of the air-liquid interfacial layer. Again, hydrophobisation caused a greater reduction of the
301 flow-point criteria than heat denaturation.

302

303 3.1.2. Creep ringing

304 The creep compliance of the interfacial layers as a function of time at different
305 magnitudes of interfacial stress, τ_s over a few seconds is illustrated in Fig. 1. A typical creep
306 ringing behaviour, i.e., damping free oscillation, is observed for the interfacial film of native
307 WPI. In the linear regime, strain is a linear function of stress; hence, the compliance curves
308 should overlap for an ideal linear viscoelastic material. This happened at times shorter than 2
309 s for the interfacial layer of native WPI and indicates that the elastic component of the sample
310 controls the first instant response, causing comparable ringing behaviours irrespective of the
311 value of applied τ_s (Goudoulas & Germann, 2016). Likewise, the creep compliance curves of
312 polyacrylamide solutions (Goudoulas & Germann, 2016) and bovine serum albumin
313 interfacial films (Jaishankar et al., 2011) deviated at periods longer than 2 s and 5 s,
314 respectively, indicating the onset of non-linear behaviour and the domination of viscous
315 component.

316 Creep ringing was weakly observed for the air-liquid interfacial film of the heat-
317 denatured WPI. In addition, the compliance curves of this sample (heat-denatured WPI)
318 commenced to deviate only after 0.8 s. The interfacial films of the hydrophobised WPI and
319 the E-added hydrophobised WPI did not show any creep ringing property. Indeed, to observe
320 creep ringing, the sample must have sufficient elasticity, so that $G_J > G_{critical}$, where G_J is the
321 modulus of spring in the Jeffreys model (Ewoldt & McKinley, 2007). Therefore, the absence
322 of the ringing property in the air-water interfacial layers of the hydrophobised WPIs (with and
323 without added erythritol) agrees with the results of the strain amplitude oscillatory test.
324 Δ and ω_* values of the interfacial layer of native WPI-air were 0.125 ± 0.010 and 1.17 ± 0.06
325 Hz, respectively. The short time creep response is quadratic in time and according to Struick's
326 formula, the interfacial elastic modulus is proportional to the square of ω_* (Jaishankar et al.,
327 2011):

$$328 \quad G'_s \approx \alpha_s \omega_*^2 \left[1 + \left(\frac{\Delta}{2\pi} \right)^2 \right] \quad [\text{Eq. 3}]$$

329 The parameter $\alpha_s = \frac{I}{b}$ is an instrumentation parameter and remains unchanged for the
330 samples because the total moment of inertia (I) of the system and the factor b which is
331 determined by the geometry are identical. The ratio of the square values of ω_* of native WPI-
332 air layer was 2.25, which is very close to the ratio of the samples G'_s values in the linear
333 region, i.e., 2.35. This indicates that the linear elastic modulus can be directly determined
334 using the data from the creep ringing test.

335

336 3.2. Buffering capacity and particle size of WPI during acid gelation

337

338 Interfacial rheology analyses indicated that hydrophobisation decreased the dynamic
339 moduli of air-liquid interfacial layers of WPI. Next, we assessed the influence of

340 hydrophobisation on the acid-induced cold-set gelation properties of WPI using bulk
341 rheology. The WPI samples and the W/W emulsion produced were gelled with GDL. The pH
342 value of the heat-denatured WPI supplemented with GDL at a level of 20 mg mL⁻¹ decreased
343 to ≈3.8, after acidification for 130 min, whereas its value for hydrophobised WPI
344 supplemented with a comparable GDL concentration (20 mg mL⁻¹) decreased only to 5.07
345 after an identical period of time (results not shown). On the other hand, the acidification
346 profiles (pH versus time) of the heat-denatured and hydrophobised WPI samples
347 supplemented with GDL at the respective concentration of 5 mg mL⁻¹ and 20 mg mL⁻¹ were
348 very close. Therefore, the buffering capacity (i.e., resistance to pH changes) of whey proteins
349 increased due to hydrophobisation.

350 The carboxylic acid group of amino acids has a pK_a value of approximately 2.3, which
351 is significantly lower than the pK_a value (4.8) of acetic acid due to negative inductive
352 (electron withdrawing) effect on the carboxylic acid group by the –NH₃⁺ group. Accordingly,
353 the carboxyl acid group of α-amino acids is about 300 times stronger an acid than acetic acid.
354 In proteins and peptides, the pK_a of terminal α-carboxyl groups is 3.1–3.8 (intermediate
355 between the carboxylic acid groups of acetic acid and glycine) because of the lower negative
356 inductive effect of proximal peptide bond rather than the –NH₃⁺ group in an α-amino acid. It is
357 noteworthy that the β and γ carboxylic acid groups of aspartic (pK_a = 3.9) and glutamic (pK_a
358 = 4.3) acids are somewhat insulated from these electronic effects by intervening saturated
359 carbons (Barrett & Elmore, 1998). Hydrophobisation, which occurred through the acetylation
360 of a great majority (>90%) of free amino groups of whey proteins (Madadlou et al., 2018),
361 decreased the inductive effect and increased the pK_a values of terminal carboxylic acid
362 groups. A large amount of acid or base is needed to change pH near the pK_a, so that buffer
363 solutions are stronger at pH levels near their pK_a values (Shimadzu Corporation, 2015). The
364 hydrophobised WPI that had a higher pK_a value was a stronger buffer and required a 4-fold

365 higher amount of GDL to undergo an acidification profile comparable with that of the heat-
366 denatured WPI.

367 During formation of acid-induced protein gels, reduction of pH towards the pI
368 decreases the net negative charge on protein particles. Then, the particles begin to crosslink,
369 forming clusters of different but finite size. At a certain threshold of interconnection, the final
370 gel network is formed (Madadlou, Emam-Djomeh, Mousavi, Mohamadifar, & Ehsani, 2010).
371 The particle size of WPI dispersions was measured during acidification immediately after
372 GDL addition. The hydrophobised protein particles had a mean hydrodynamic diameter of
373 approximately 82 nm, 3 min after GDL addition. Fig. 2A indicates that during the
374 acidification, the particle size of hydrophobised WPI initially decreased, followed by a
375 progressive increase over the rest of acidification. Plotting the mean hydrodynamic diameter
376 of protein particles versus mean pH during acidification until gel formation time (Fig. 2B)
377 indicated that the size of hydrophobised protein particles decreased with decreasing pH to
378 approximately 5.5 (identified by an arrow in Fig. 2B). This pH value corresponded to 30 min
379 after GDL addition. Likewise, the particle diameter of heat-denatured WPI showed a small
380 reduction in the beginning of acidification, followed by a remarkable increase (Fig. 2A). As
381 soon as GDL was added, the pH value of WPI dispersions sharply decreased (results not
382 shown), and then reached a plateau after gel formation over long durations. The initial
383 reduction and the subsequent increase of protein particle size during acid gelation, which were
384 observed for both heat-denatured and hydrophobised WPI dispersions are attributed
385 respectively to protein particles shrinkage due to the decrease of intra-particle electrostatic
386 repulsion and succeeding aggregation of the particles.

387 Erythritol addition did not influence the initial particle size of hydrophobised WPI
388 dispersion, which suggests that erythritol did not interact electrostatically with WPI particles
389 (Madadlou et al., 2019). It did not also affect the aggregate size as a function of pH reduction

390 (Fig. 2B). However, erythritol addition postponed the onset of aggregation of protein particles
391 to a lower pH value (approximately 5.4) and caused formation of comparatively smaller
392 aggregates just before the gelation point (Fig. 2A).

393

394 3.4. Gelation time

395

396 Different WPI samples supplemented with GDL (20 mg mL⁻¹ for the hydrophobised
397 WPI samples, and 5, 10, and 20 mg mL⁻¹ for the heat-denatured WPI) were gelled at 37 °C on
398 a rheometer and the corresponding gelation times are illustrated in Fig. 3A. The heat-
399 denatured WPI gelled approximately 5, 13, and 42 min after adding GDL at respective
400 concentrations of 20, 10, and 5 mg mL⁻¹. The G' values of the heat-denatured WPI gels
401 prepared using GDL at 20 and 10 mg mL⁻¹ levels decreased after a peak value (Fig. 3B). The
402 reduction of G' was substantial for the heat-denatured WPI supplemented with 20 mg mL⁻¹
403 GDL, and is attributed to gel disintegration due to over-acidification, which reduced pH to
404 values well below the proteins pI.

405 Gelation time (t_g) of whey proteins was influenced by hydrophobisation, as well as by
406 erythritol and alginate addition (Fig. 3A). Hydrophobised WPI required about 125 min to gel
407 (pH 5.07 ± 0.01) after adding GDL at 20 mg mL⁻¹. Erythritol addition, which did not alter the
408 pH value of the hydrophobised WPI, postponed the t_g (Fig. 3A). The E-added hydrophobised
409 WPI gelled approximately 134 min (pH 5.06 ± 0.02) after GDL supplementation (20 mg mL⁻¹
410 ¹), which is 9 min longer compared with the counterpart without erythritol. On the contrary,
411 alginate addition into the E-added hydrophobised WPI resulted in a shorter t_g (94 ± 2 min) in
412 comparison with both E-added and E-free hydrophobised WPIs.

413

414 3.5. Dynamic rheology of the WPI gels

415

416 The gels developed in situ on the rheometer were subjected to frequency sweep test to
417 gain an insight into their time-dependent behaviour. This rheological analysis is useful to
418 gather information on the internal structure of samples (polymer solutions, gels, emulsions,
419 etc.) and the stability of dispersions during storage. The results of the frequency sweep test
420 are illustrated in Fig. 4. The values of G' and G'' of all samples increased with increasing
421 frequency and G' was greater than G'' at any frequency for all samples, which suggests
422 significant protein-protein interactions at the gels (Zhang et al., 2018) and domination of the
423 elastic component to the viscous one (Madadlou, Khosroshahi, & Mousavi, 2005). However,
424 the difference between the G' and G'' of all gels was smaller than 1 log cycle, indicating that
425 the gels were rather weak (Kamal, Foukani, & Karoui, 2017).

426 In a manner similar to that of the interfacial rheology results, the hydrophobised WPI
427 gel had lower G' values than the heat-denatured WPI gel throughout the whole frequency
428 range. Moreover, erythritol addition decreased the maximum G' value of the hydrophobised
429 WPI gel in the frequency sweep analysis from approximately 5 kPa to 4 kPa. The G'' of the
430 WPI gel decreased at a comparable extent (≈ 1.27 fold) due to erythritol addition (Fig. 4). Both
431 E-free and E-added hydrophobised gels had a final (i.e., after 24 h) pH value of 4.75 ± 0.1 .
432 Erythritol might interrupt gel network development (Cai, Feng, Regenstein, Lv, & Li, 2017;
433 Torres, Raymundo, & Sousa, 2013). We hypothesise that erythritol increased the hydration of
434 protein particles, reducing the formation of non-covalent bonds (He, Azuma, Hagiwara, &
435 Kanno, 2006) between the particles, impairing gel development. This may explain why the E-
436 added hydrophobised WPI had relatively smaller aggregates than E-free sample just before
437 the gelation point. Although erythritol did not interact with whey proteins (Madadlou et al.,
438 2019), it could increase protein hydration because of excluded volume effect, a phenomenon
439 that weakly depends on the native or denatured state of proteins (Ebel, Eisenberg, &

440 Ghirlando, 2000). Addition of carbohydrates such as sugar alcohols to native protein solutions
441 is known to delay the heat-induced aggregation and gel formation (Wijayanti, Bansal, &
442 Deeth, 2014).

443 In accordance with the influence on gelation time, alginate addition increased the G'
444 and G'' values of the E-added hydrophobised WPI gel. One may assume that alginate gelation
445 along with whey proteins accelerated the gelation and increased gel firmness. Alginate can gel
446 without the aid of calcium (and other multivalent cations) when pH is gradually lowered (for
447 example by GDL) because the fraction of unionised carboxyl groups on the uronic acid
448 residues increases and water is squeezed out (Yotsuyanagi, Yoshioka, Segi, & Ikeda, 1991).
449 Gradual pH reduction is requisite as an abrupt acidification leads to precipitation of alginic
450 acid rather than gel formation. It has been estimated that a minimum alginate concentration of
451 3.5 mg mL^{-1} is needed to obtain a GDL-induced gel (Draget, Skjåk Bræk, & Smidsrød, 1994),
452 which is remarkably lower than the alginate concentration employed in the current study (30
453 mg mL^{-1}), although alginate would partition between the protein-rich and alginate-rich phases
454 of the emulsion (Madadlou et al., 2019).

455 To assess alginate gelation, we added different concentrations of GDL to the alginate
456 solution (30 mg mL^{-1}) and found that a GDL concentration of 1.1 mg mL^{-1} decreased pH after
457 about 90 min (similar to the emulsion t_g) to 5.15 ± 0.04 , which is comparable with the
458 emulsion pH at gelation point. Nevertheless, the alginate solution did not gel. The alginate-
459 added WPI gel had a final (after 24 h) pH of approximately 4.75, similar to that of heat-
460 denatured and hydrophobised WPI gels, associated with the protein buffering capacity
461 (Léonard, Husson, Langella, Châtel, & Saurel, 2016). Also, de Jong et al. (2009a) observed a
462 constant pH, independent of polysaccharide type and amount for the acid-induced gels of
463 WPI/polysaccharide mixtures. It was presumed that the continuous and disperse phases of the
464 W/W emulsion had equal pH values (Singh, Medronho, Miguel, & Esquena, 2018). Adding

465 higher concentrations of GDL, namely 1.4 and 1.7 mg mL⁻¹, decreased pH (after 90 min) to
466 approximately 4.85 and 4.75, respectively; however, gelation did not happen. Indeed, acid
467 gelation of alginate happens at pH values below the pK_a of the uronic acid residues which is
468 approximately 3.7 (Bu, Kjøniksen, & Nyström, 2005).

469 Although the WPI powder used in the current study contained a small amount of
470 minerals, which could cause alginate hardening/gelation when pH was reduced, the shorter t_g
471 and higher dynamic moduli of alginate-added gel compared with the alginate-free WPI
472 systems is mainly attributed to the microstructural organisation of the composite gel.
473 Likewise, a higher G' of an alginate/caseinate aqueous two-phase system gel than individual
474 gels of either alginate or caseinate was ascribed to the development of a more organised and
475 interconnected structure in the mixed gel (Léonard et al., 2016). Hence, the microstructural
476 features of the samples were assessed to explain the difference between the dynamic moduli
477 of the alginate-added gel and the alginate-free WPI gels.

478

479 3.6. *Microstructure of the WPI gels*

480

481 The microstructures of well developed (i.e., stored for 24 h) gel samples were imaged
482 by CLSM and the typical images are depicted in Fig. 5. The polysaccharide-free WPI gels had
483 homogenous microstructures, composed of a protein-rich phase (green) and a serum phase.
484 The latter is observed as black micro-channels interconnected throughout the gel matrix.
485 Microscopic images (Fig. 5) showed that alginate was dispersed as droplets in a continuous
486 matrix of proteins. Micro-phase separation of protein-polysaccharide pairs is usual and known
487 to increase the firmness of the subsequently formed protein gels (Zand-Rajabi & Madadlou,
488 2016). Micro-phase separation concentrates the incompatible polymers in separated domains,
489 causing higher effective concentrations than nominal (Beaulieu, Turgeon, & Doublier, 2001).

490 The diameter of approximately 1700 droplets were measured and the result is illustrated in
491 Fig. 5. The emulsion gel droplet size was highly inhomogeneous and droplets as large as 20
492 μm were present. It is also observed in Fig. 5D that some adjacent alginate droplets were in
493 contact and partially coalesced. Modification of the gelation method might be helpful to
494 confine the droplet disproportionation kinetics.

495

496 **5. Conclusions**

497

498 Hydrophobisation of whey proteins through acetylation decreased the liquid-air
499 interfacial viscoelasticity of WPI. Air is a very hydrophobic medium. Hence, concurrent
500 reduction of the interfacial elasticity (observed in the current study) and interfacial tension
501 (observed in a previous study; Madadlou et al., 2019) of hydrophobised WPI at the liquid-air
502 interface indicates that more hydrophobic protein particles which accumulated at the interface
503 were less elastic. An opposite process took place in the alginate-protein emulsions, i.e. more
504 elastic but less hydrophobic protein particles stabilised the all-aqueous interfaces. This
505 conclusion relies on the assumption that at least two populations of particles with different
506 hydrophobicities were present at the protein phase. It is noteworthy that macroscopic phase
507 separation of non-gelled W/W emulsions is conventionally slowed down by accumulation of
508 third component particles at the water-water interface. However, as communicated in an
509 earlier report (Madadlou et al., 2019), stable W/W emulsions can be made using a pair
510 consisting of only hydrophobised protein and a co-charged polysaccharide without a third
511 component. The fabrication of the two-component stable W/W emulsions is thrilling. Should
512 the above-mentioned conclusion be correct, one may design a wide range of two-component
513 (protein and polysaccharide only) stable and fluid (not gelled) W/W emulsions by masterful
514 manipulation of the shape, size, and surface hydrophobicity of protein particles. Acidification

515 of the emulsion produced soft gels which might be used for encapsulation of drugs,
516 nutraceuticals and enzymes as two-phase oil-free systems. In addition, the emulsion could be
517 utilised to perform reactions at the interface of two discrete, but interfacially touching,
518 aqueous phases. Addition of erythritol to the hydrophobised WPI dispersion, which facilitated
519 emulsification, resulted in an inferior interfacial and gel viscoelasticity. Therefore, it is
520 possible to tune the interfacial and gel viscoelasticity of hydrophobised WPI and the W/W
521 emulsions using a non-caloric sweetener.

522

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524

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530

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Figure legends

Fig. 1. Interfacial strain sweep (A, C, E, G) and creep (B, D, F, H) tests of whey protein isolate (WPI) samples: A and B, native WPI; C and D, heat denatured WPI; E and F, hydrophobised WPI; G and H, erythritol-added hydrophobised WPI. The interfacial shear storage (G'_s) and loss (G''_s) moduli are shown by ● and ■, respectively. The dashed line (strain sweep results) indicates the onset of non-linear viscoelasticity and the dashed circle specifies the crossover of the dynamic moduli. The creep tests were performed at three different interfacial stress, τ_s values of 0.0649, 0.324, and 0.649 $\mu\text{Pa m}$.

Fig. 2. The particle diameter, as a function of (A) time, and (B) pH, and ζ -potential (C) of WPI samples (▲, heat-denatured; ●, hydrophobised; ■, erythritol-added hydrophobised) during acidification with glucono δ -lactone (GDL) at 37 °C. The heat-denatured and hydrophobised WPI samples were gelled by the addition of GDL at a level of 5 and 20 mg mL^{-1} , respectively. The arrow specifies the onset of protein particles growth in the hydrophobised WPI solution as pH decreases. Error bars represent standard deviation.

Fig. 3. Evolution of the storage modulus (G') of whey protein isolate (WPI) solutions during acidification with glucono δ -lactone (GDL) for (A) up to 150 min and (B) over 1500 min. Symbols in panel A are: ▲, ◆, and ✕, heat-denatured WPI at 5, 10 and 20 mg GDL mL^{-1} , respectively; ●, ■ and ●, hydrophobised WPI, erythritol-added hydrophobised WPI and emulsion, respectively, at 20 mg GDL mL^{-1} (error bars represent standard deviation). Symbols in panel B are: ◇ and ✕, heat-denatured WPI at 10 and 20 mg GDL mL^{-1} , respectively.

Fig. 4. Mean dynamic moduli of different whey protein isolate (WPI) samples: ▲ and △, heat-denatured WPI; ● and ○, erythritol-free hydrophobised WPI; ◆ and ◇, erythritol-added hydrophobised WPI (diamond); ■ and □, alginate-in-erythritol-added hydrophobised emulsion. Solid symbols indicate storage modulus (G') and the hollow symbols indicate loss modulus (G'').

Fig. 5. CLSM images of acid-induced WPI gels and alginate-in-WPI emulsion gel: A, heat-denatured WPI; B, hydrophobised WPI; C, erythritol-added hydrophobised WPI; D, alginate-in-erythritol-added hydrophobised WPI. Panel E presents the results of droplet size analysis of the emulsion gel.

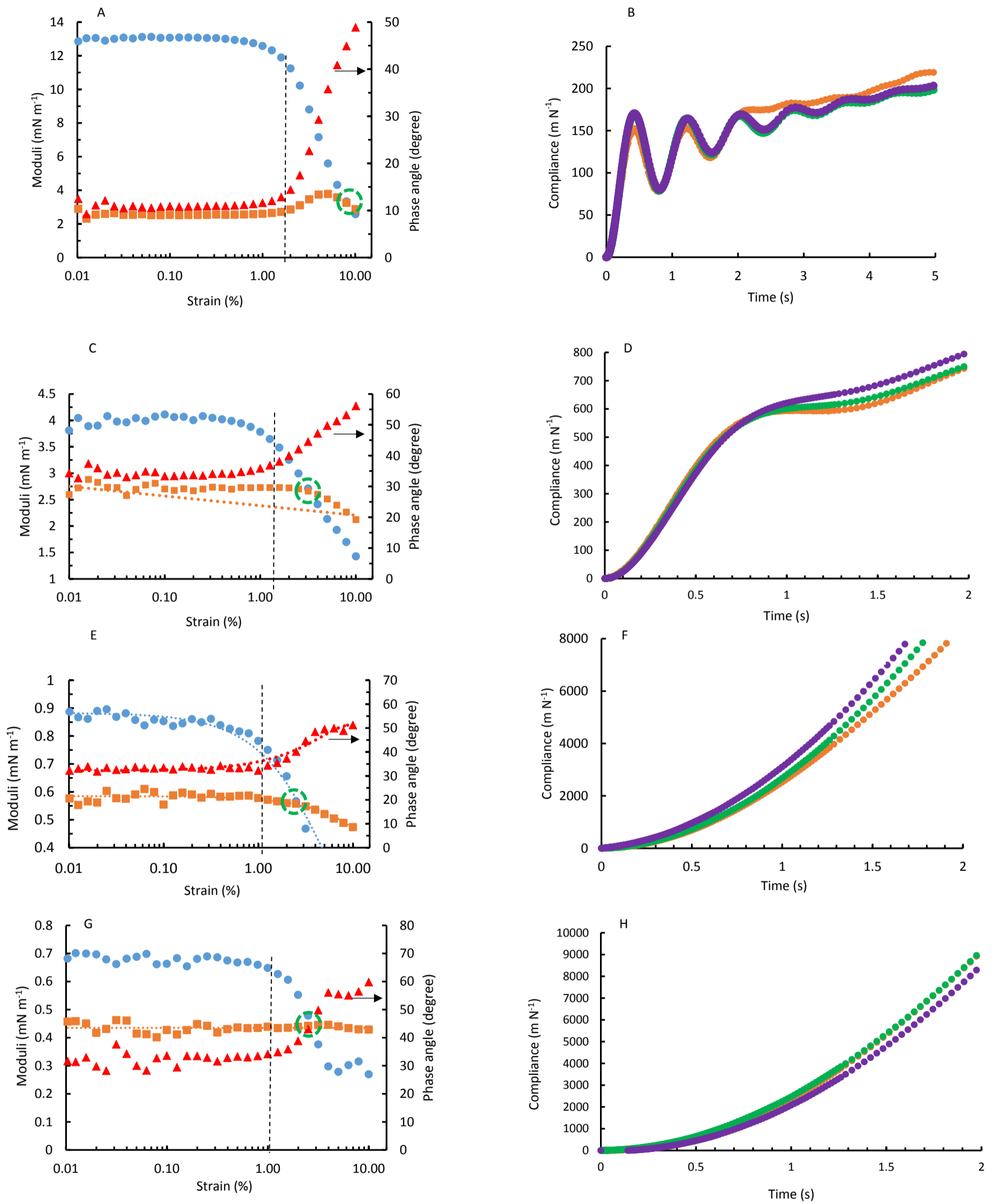


Figure 1

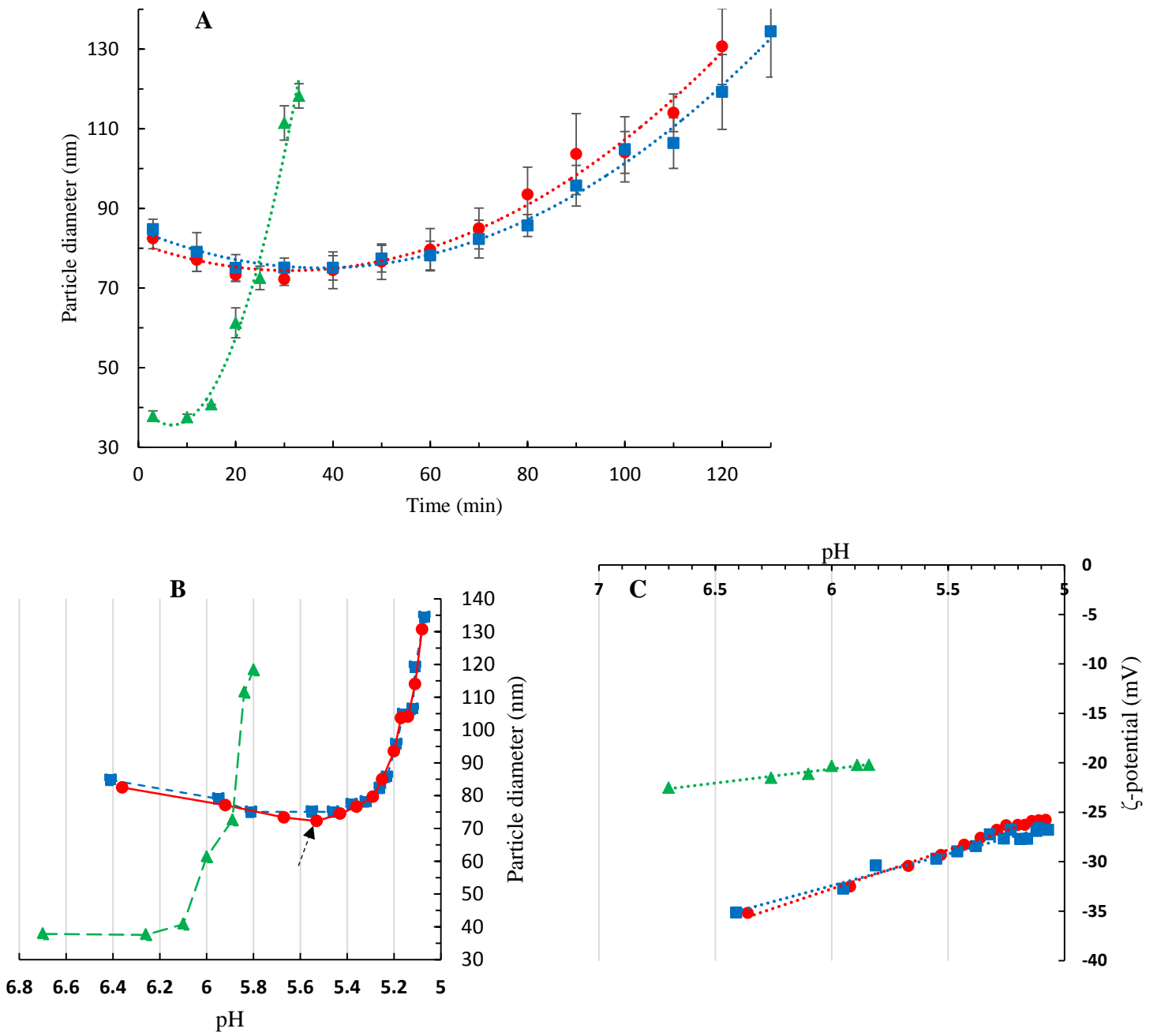


Figure 2

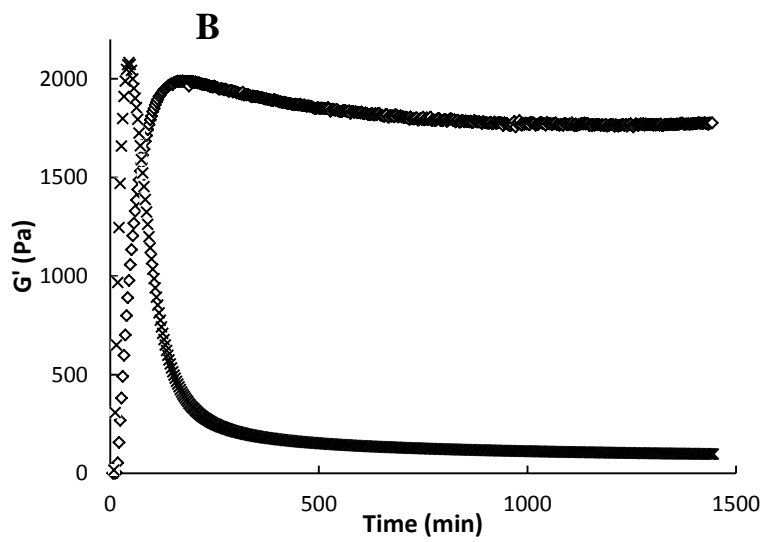
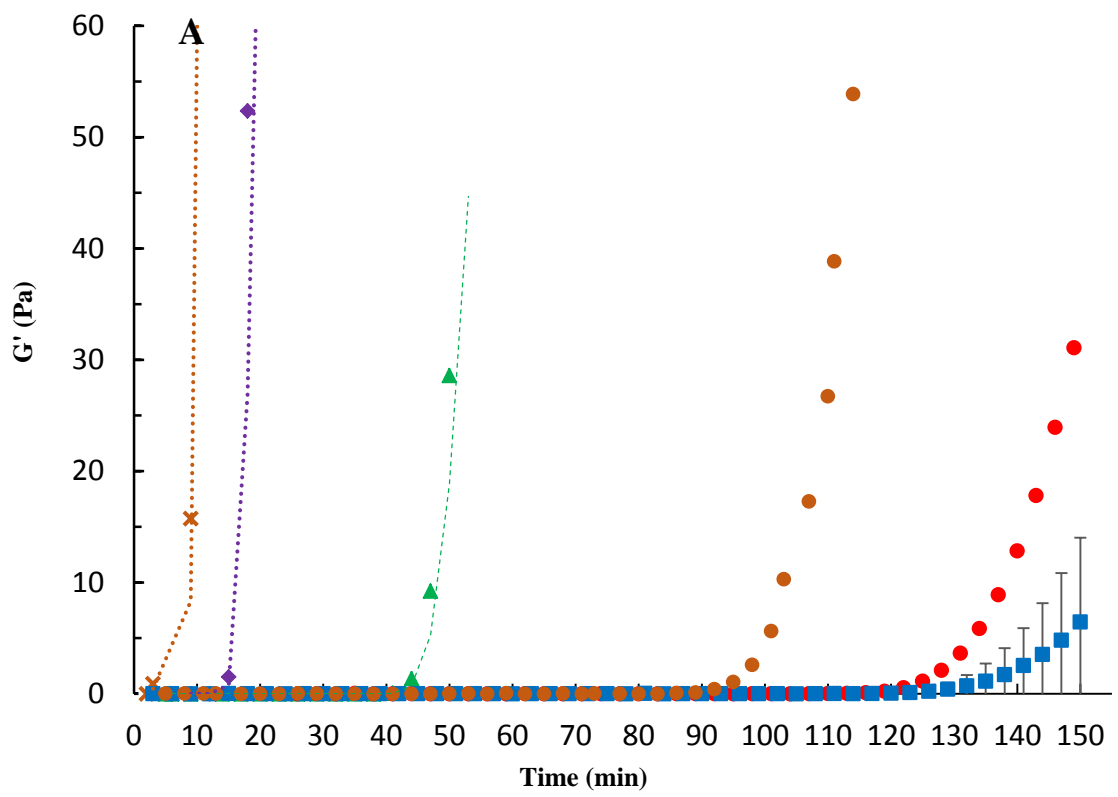


Figure 3

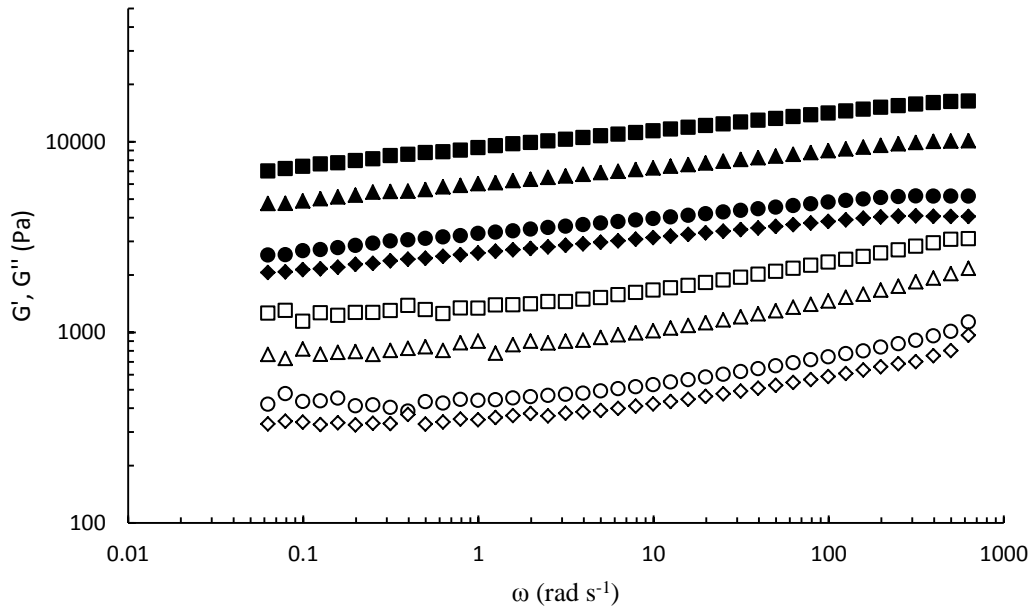


Figure 4

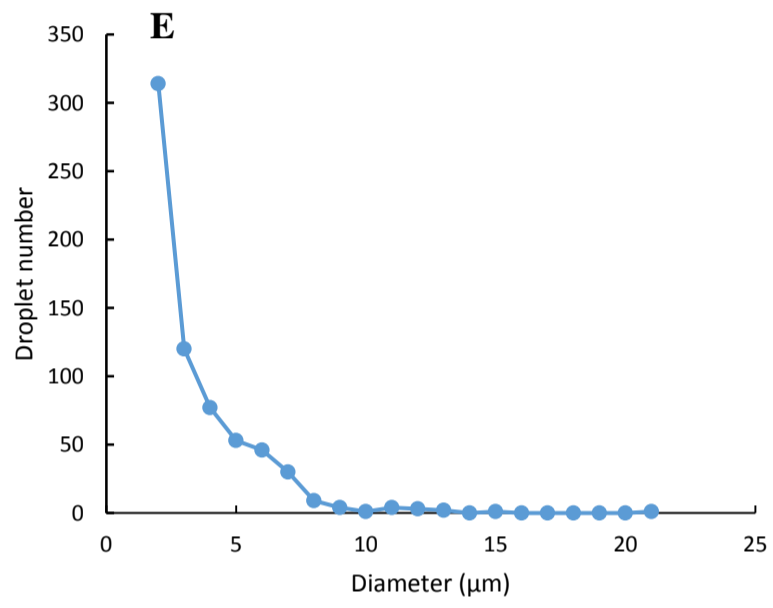
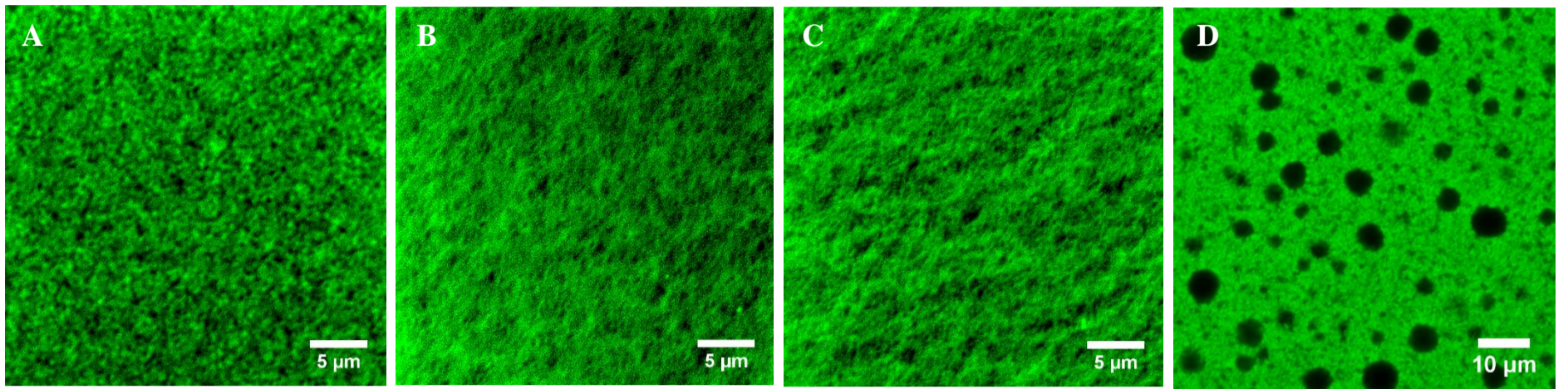


Figure 5

Table 1Interfacial properties of whey protein isolate (WPI) samples. ^a

Interface	G'_s (mN m ⁻¹)	δ_l (°)	τ_f (μ N m ⁻¹)	γ_f (%)
Native WPI-air	12.95 ^a \pm 0.36	10.9 ^b \pm 0.6	3.8	8.0
Heat-denatured WPI-air	4.40 ^b \pm 1.3	30.3 ^a \pm 2.8	1.2	3.1
Hydrophobised WPI-air	0.86 ^c \pm 0.13	30.9 ^a \pm 2.7	0.2	2.6
Erythritol-added hydrophobised WPI-air	0.63 ^c \pm 0.11	30.5 ^a \pm 2.1	0.2	2.6

^a Abbreviations are: G'_s , storage modulus in the linear viscoelastic region; δ_l , phase-shift angle in the linear viscoelastic region; τ_f , flow stress; γ_f , flow strain of the interfacial layer. The τ_f and γ_f values were acquired from Fig. 1, which shows typical curves of the strain sweep test at the liquid-air interface of different WPI samples; means with different superscript letters within a column differ significantly ($P < 0.05$).