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1 **Influence of casein on the formation of whey protein microparticles obtained**
2 **by dry heating at an alkaline pH**

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7 **ABSTRACT**

8 Dry heating (DH) at 100 °C for 36 h of a whey protein isolate powder conditioned at pH 9.5
9 leads to the formation of stable, large and porous whey protein microparticles (PMs),
10 resulting from the crosslinking of proteins inside the powder. These PMs could be used as
11 high-viscosity food ingredients. Casein, present as a contaminant in whey protein powders,
12 has been shown to become incorporated into the PMs. In this study, we investigated the
13 effect of adding increasing amounts of sodium caseinate to whey protein powders on the
14 formation of PMs during DH at 100 °C for 36 h. In addition, we studied PM formation during
15 DH of a micellar casein-enriched milk protein powder (Casmic). The browning index of the
16 dry-heated powders, and the size and water content of the microparticles were also
17 characterized. We confirmed that sodium caseinate was incorporated into the PMs. The
18 highest PM D[4,3] values (270 µm) were observed for powders with around 40% caseinate.
19 Powders without added caseinate displayed D[4,3] values of 150 µm. The yield of conversion
20 of proteins into PMs increased from 0.6 to 0.8 g/g with caseinate addition, whereas the
21 amount of water entrapped in the PMs decreased from around 30 to 20 g/g. PMs were also
22 formed by DH of the Casmic powder, but these particles were smaller, with sizes of around
23 80 µm. In conclusion, our study shows that the process of DH at pH 9.5 could be applied to
24 all milk proteins to obtain PMs with functional properties that could be used in the food
25 industry.

26 **KEYWORDS:** whey protein; microparticle; dry heating; aggregation; casein

27 1. Introduction

28 Whey proteins (WPs) display a range of useful functional characteristics, such as
29 rheological, foaming, emulsifying and gelation properties (Dickinson, 2015; Kharlamova,
30 Chassenieux, & Nicolai, 2018; T. Nicolai & Durand, 2013). A lot of work has been dedicated
31 to the study of WP aggregation, with the aim of increasing the functional properties of these
32 proteins. Most of these studies involved heating a solution of WPs under a range of
33 physicochemical conditions and studying the size, structure and properties of the resulting
34 aggregates (Taco Nicolai, Britten, & Schmitt, 2011). During the last two decades, an
35 increasing number of studies have investigated the effect of directly heating protein powders
36 in the presence of sugar to induce the Maillard reaction (O'Mahony, Drapala, Mulcahy, &
37 Mulvihill, 2017; Schong & Famelart, 2017). Whereas very few changes in protein structure
38 are observed during the glycation of proteins under gentle dry heating (DH) conditions
39 (Fenaille, Morgan, Parisod, Tabet, & Guy, 2003), DH treatments using high temperatures
40 and/or over long time periods can induce significant WP aggregation (Gulzar, Bouhallab,
41 Jeantet, Schuck, & Croguennec, 2011; Hiller & Lorenzen, 2010; Schmitt, Bovay, & Frossard,
42 2005).

43 Our previous studies have shown that DH for over 10 h at 100 °C of a freeze-dried or a
44 spray-dried WP powder conditioned at pH 9.5 with a water activity (a_w) of around 0.2 leads to
45 the formation of WP microparticles (Famelart, Schong, & Croguennec, 2018; Schong &
46 Famelart, 2018). By dissolving the dry-heated powder in water and observing the protein
47 microparticles (PMs), we have shown that these PMs are very large and that their size and
48 shape are influenced by the size and shape of the powder particles. The DH treatment led to
49 crosslinking of proteins inside the powder particles so that the powder was no longer soluble
50 when dispersed in water. Due to the porous structure of the powder, the PMs formed after 36
51 h of DH were able to entrap a huge amount of water (20-40 g water/g of dry PM), with a yield
52 of formation of over 0.5 g of PM/g of powder. The PMs produced by DH of the spray-dried

53 powder were spherical with a hollow structure, and were smaller and more homogeneous in
54 size than the those produced from DH of the freeze-dried powder (Famelart et al., 2018;
55 Schong & Famelart, 2018). The very high viscosity values obtained in our previous study
56 indicates that these PMs could be used as high-viscosity or food thickening agents (Schong
57 & Famelart, 2018). The suspension of particles formed by DH of the spray-dried WP powder
58 at 32 g/kg gave a viscosity of around 17 Pa.s at a shear rate of 1 s^{-1} and a temperature of 20
59 °C. This viscosity value is much higher than that obtained for other WP particles in previous
60 studies (Dissanayake et al., 2012; Inthavong, Kharlamova, Chassenieux, & Nicolai, 2016;
61 Loveday, Su, Rao, Anema, & Singh, 2012; Zhang, Arrighi, Campbell, Lonchamp, & Euston,
62 2016).

63 More recently, we studied the effect of lactose content on PM formation from freeze-dried
64 powders (Schong & Famelart, 2019). Although freeze-dried flakes are much larger than
65 spray-dried powder grains, freeze-dried powders were used as a model for spray-dried
66 powders in this study because it was more convenient to produce powders with different
67 lactose compositions by freeze-drying than by spray drying. Using this model, we showed
68 that increasing the lactose content led to increases in the rate of protein crosslinking and PM
69 formation, and to higher yields of conversion of WP into PMs to more than 0.9 g/g of powder.
70 In contrast, increasing the lactose content led to a decrease in the amount of water
71 entrapped in these PMs. It has been shown that dry-heated powder particles are able to
72 swell when placed in water, leading to particle sizes in suspension being slightly larger than
73 those in the powder. However, powder particles exposed to longer DH times and those
74 heated in the presence of higher lactose contents swelled less when placed in water, due to
75 the higher degree of crosslinking induced by DH. DH at 100 °C for 36 h without added
76 lactose, but in the presence of the trace amounts of lactose present in the WP powder
77 (around 6 g per kg powder), gave the same yield of conversion of WP into PM and led to the
78 same amount of water entrapped in the PMs as DH for only 3 h with addition of lactose at a
79 concentration of 0.42 g/10 g protein. Nevertheless, evaluation of the browning index of the

80 dry-heated powder showed that the formation of the PMs did not correlate with the progress
81 of the Maillard reaction. Indeed, the mechanism of formation and the nature of the protein
82 crosslinks are still speculative. We have found that the PMs are resistant to reduction
83 (unpublished results). Similar findings were reported by Gulzar et al. (2011), who found that
84 WP aggregates produced by DH at pH 4.5 or 6.5 were resistant to reduction, whereas
85 aggregates produced at pH 2.5 were not. Isopeptide and lysinoalanine bonds have been
86 implicated in the formation of the crosslinks as their formation is favored by high pH values
87 and severe heating (Gerrard, 2002). Furthermore, large protein aggregates have been
88 reported in the presence of lactose as a result of the Maillard reaction: these aggregates are
89 made of covalent bonds resistant to reduction and have been reported to involve the
90 degradation products of Maillard intermediates, such as dicarbonyl compounds (Friedman,
91 1996; Hiller & Lorenzen, 2010; Lan et al., 2010; Miller, Meade, & Gerrard, 2003; Zamora &
92 Hidalgo, 2005). These dicarbonyl compounds are highly reactive crosslinkers of lysine and
93 arginine residues and give rise to advanced glycation end products (Gerrard et al., 2005;
94 Miller et al., 2003). Their formation is enhanced at an alkaline pH (Martins & Van Boekel,
95 2005; Wei, Liu, & Sun, 2018). However, the large number of reactants and the complexity of
96 the pathways involved in the Maillard reaction, together with an inability to dissociate the
97 PMs into smaller protein chains for analysis, mean that it appears impossible to determine
98 the exact nature of the PM bonds.

99 The WP powder used in these previous studies was 90% protein. About 18% of these
100 proteins were caseins, present as contaminants of the WP powder. It has been shown that
101 these caseins were transferred into the PMs together with the WPs (Schong & Famelart,
102 2019). The aim of the current study was to further our understanding of the mechanism of
103 PM formation by investigating the effect of replacing WPs with casein on the formation of
104 PMs. As in our previous study investigating the effect of lactose content on PM formation,
105 freeze-dried powders were used in the current study as a convenient model for predicting the
106 behavior of caseins on spray-dried powders. Herein, we report on the changes in the

107 properties of the powder particles and PMs induced by adding sodium caseinate to WP
108 before freeze-drying and DH at 100 °C for 36 h. To gain an insight into the effect of casein
109 structure on its transfer into the PMs, a sample of micellar casein-enriched milk protein was
110 also studied after undergoing the same heating process.

111 **2. Materials and Methods**

112 Most of the materials and methods used in this study have been described in more detail
113 previously (see Schong & Famelart (2019)).

114 *2.1. Materials*

115 The commercial WP powder was obtained from a confidential source and was the same
116 powder as that used in our previous studies (Famelart et al., 2018; Schong & Famelart,
117 2018, 2019). The solid matter and protein contents of the WP powder were 965.6 ± 0.3 and
118 911 ± 7 g per kg powder, as determined respectively by the desiccation method (International
119 Dairy Federation, 2010), and by the Kjeldahl method (International Dairy Federation, 2004,
120 2014, 2016) using 6.38 as the conversion factor. Approximately 82% of this protein was WP
121 and around 18% was casein, present as a mix of α s1, α s2, β and κ caseins, as determined
122 by polyacrylamide gel electrophoresis (Schong & Famelart, 2019). The free-lactose content
123 of the powder was 5.92 ± 0.16 g per kg powder as determined by ion chromatography
124 (Gaucheron, 2003). Thus, the lactose to protein ratio was around $6.5 \cdot 10^{-3}$ g/g.

125 The commercial sodium caseinate (NaCas) powder was obtained from Lactalis
126 (Bourgbarré, France) and contained 936.9 ± 3.4 g of solid matter, $878.06 \text{ g} \pm 2.1$ of protein
127 and $2.99 \text{ g} \pm 0.03$ g of free-lactose per kg powder. Thus, the lactose to protein ratio for the
128 NaCas powder was around $3.4 \cdot 10^{-3}$ g/g.

129 The commercial micellar casein-enriched milk (Casmic) protein powder (Promilk 852B)
130 was obtained from International dairy ingredients (Arras, France) and contained 936.7 ± 0.5
131 g of solid matter, 779.8 ± 0.2 g of protein (among which 93% was micellar casein) and 29.42
132 ± 1.16 g of lactose per kg powder. The proportion of casein and WP in this powder was
133 determined by Kjeldahl analysis of non-protein and non-casein nitrogen. The lactose to
134 protein ratio was around 38.10^{-3} g/g, and was thus 6–11 fold higher than that for the two
135 other powders.

136 *2.2. Preparation of the dry-heated powders*

137 The NaCas powder was mixed with the WP powder at levels of 0, 20, 25, 33, 50 and
138 100% by weight. The casein content as a percentage of the weight of total protein was
139 calculated for each mixture based on the composition of the powders as 18, 34, 38, 44, 58
140 and 99%, respectively. These samples were then referred to as “Cas18” to “Cas99”. The
141 lactose to protein ratio decreased progressively by a factor of two from the Cas18 powder to
142 the Cas99 powder. Similarly, the dry matter content in the solution showed a 3% decrease
143 from 965.6 to 936.9 g/kg. A sample of 100% of Casmic, containing 93% casein, was also
144 prepared.

145 Solutions containing 150 g powder/kg were prepared by stirring overnight at 4 °C. The pH
146 of these solutions was then adjusted to pH 9.5 by addition of 5 mol.L^{-1} and then 1 mol.L^{-1}
147 NaOH. The solutions were then rapidly frozen and freeze-dried. The powders were finely
148 crushed, adjusted to an a_w of 0.25 ± 0.01 for two weeks and dry-heated at 100 °C for 36 h.
149 Only one solution and powder were prepared for each mixture of casein and WP, but two
150 samples of each powder solution were dry-heated and further analyzed.

151 2.3. Analysis

152 2.3.1. Powder

153 The granular size distribution of the unheated freeze-dried powder after crushing was
154 analyzed as described previously in Schong & Famelart (2019) using the QICPIC analyzer
155 and the dry disperser RODOS/L module (Sympatec GmbH, Clausthal-Zellerfeld, Germany).
156 Two measurements of the distribution of the “diameter of a circle of equal projection area” of
157 the particles were taken for each sample and the mean volume diameter (D[4,3]) was
158 deduced. Two measurements were performed for each unheated sample.

159 Samples of powder particles were transferred to a glass lamella and observed on a light
160 microscope (Olympus BX51, Rungis, France) with a x10 objective. Photographs (10 per
161 sample) were taken using a Qiclick CCD camera (Q Imaging, Microvision Instruments, Evry,
162 France) and the Archimed software program (Microvision Instruments).

163 The intensity of the Maillard reaction was estimated by monitoring changes in the
164 browning index (BI) of the powders. Briefly, the BI was deduced from L* (whiteness), a*
165 (redness/greenness) and b* (yellowness/blueness) measurements made using a CR-300
166 Chromameter (Minolta, Roissy, France). The BI was obtained according to Maskan (2001) as
167 follows:

168
$$BI = \frac{[100(x - 0.31)]}{0.17}$$
 equation 1

169
$$x = \frac{(a^* + 1.75L^*)}{(5.645L^* + a^* - 3.012b^*)}$$
 equation 2

170 Three measurements were performed for each dry-heated powder.

171 2.3.2. Proteins and protein microparticles

172 Powders were reconstituted to a final concentration of 10 g of powder.kg⁻¹ in 0.2 g.kg⁻¹
173 sodium azide in deionized water and adjusted to pH 6.5 (suspension S0). One S0
174 suspension was prepared for each dry-heated powder.

175 Samples (1 mL) of the S0 suspension were acidified by addition of 300 µL of 0.5 M
176 acetate-acetic acid buffer (pH 4.6), and then centrifuged for 15 min at 10000 g. The resulting
177 supernatants, containing residual native whey proteins (NWP), were then frozen until
178 analysis. Casein is not soluble at pH 4.6 and was partitioned into the pellet during this
179 fractionation process. The levels of native β-Lactoglobulin (β-Lg) and α-Lactalbumin (α-lac),
180 present in the supernatants were quantified by reversed phase high performance liquid
181 chromatography (RP-HPLC) as described in Schong & Famelart (2019). NWP were
182 quantified as the sum: β-Lg + α-lac. One sample was prepared for each S0 suspension and
183 three RP-HPLC injections were done for each preparation.

184 The size distribution of PMs with sizes of 0.02–2000 µm was measured in S0 suspensions
185 at 20 °C using a Mastersizer 2000 equipped with the HydroSM module (Malvern Instruments,
186 Worcestershire, UK), as described by Famelart et al. (2018). The D[4,3] was then deduced.
187 Two samples were taken from each S0 suspension and measurements on each sample
188 were carried out in triplicate. Changes in the D[4,3] of the powder particles in suspension
189 were evaluated by determining the size ratio (SR) from the D[4,3] of the PMs/D[4,3] of the
190 powder particles.

191 PMs were isolated by centrifuging around 2 g of each S0 suspension at 10 000 g for 15
192 min at 20 °C. Pellets containing sedimented PMs were then weighed in the wet state to
193 obtain the wet weight (WW) and then after drying using a Savant Speed Vac Concentrator
194 SVC-100H (Hicksville, USA) to obtain the dry weight (DW). The dry yield of conversion of the
195 powder dry matter into PMs (DY) was then determined as the dry weight of the pellet
196 obtained from 1 g of dry-heated powder. The wet yield of conversion (WY) was determined

197 as the wet weight of the pellet obtained from 1 g of dry-heated powder. As the dry matters of
198 the WP and NaCas mixtures and of the Casmic powder were made up of about 94% and
199 83% protein, respectively, the DY and WY provided good estimation of the yield of
200 conversion of proteins into particles. The water content (WC) of the PMs, determined as the
201 weight of water per 1 g of dry pellet, was calculated as follows:

$$202 \quad WC = \frac{(WW-DW)}{DW} \quad \text{equation 3}$$

203 Measurements were performed four times for each suspension.

204 S0 suspensions were diluted 10-fold in distilled water and observed by confocal laser
205 scanning microscopy (CLSM) using an inverted TE2000-E microscope (Nikon, Champigny-
206 sur-Marne, France) with a x20 oil-immersion objective. Proteins were probed with Nile Blue.
207 Ten images per sample (512 x 512 pixels², representing 636 x 636 μm²) were acquired at a
208 distance of 15 μm away from the bottom lamella.

209 *2.4. Statistical analysis*

210 The effects of powder composition on the D[4,3] and on the residual NWP content were
211 analyzed by analysis of variance (ANOVA) and, in case of significance, by the Tukey's
212 multiple comparison test for paired comparisons of means. A P value of less than 0.05 was
213 used as the level of significance. All statistical analyses were performed using the R software
214 package.

215 **3. Results and discussion**

216 *3.1. Powder characterization*

217 The BI of the powders before (0 h) and after DH for 36 h is shown in Fig. 1. For the WP
218 powder without added casein (Cas18 sample), the BI was 12 before DH and 45 after 36 h of

219 DH. These values are in accordance with those described previously: BI scores of 7-16
220 before DH and 27-54 after 36 h of DH were obtained for the same powder after same
221 treatments by Famelart et al. (2018) and Schong & Famelart (2018, 2019). The BI of all
222 powders containing a mixture of WP and NaCas was around 10 before DH and increased by
223 a factor of four after DH. The highest BI after DH was obtained for the powders with around
224 40% casein; the BI then decreased as the NaCas content of the powders increased. This
225 decrease in BI may be explained by a reduction in the lactose to protein ratio when WPs
226 were replaced by NaCas; however, the reason for the increase in BI between powders with
227 18% casein and those with 44% casein is unknown. One hypothesis is that the alkaline pH
228 applied before DH induced WP denaturation and unfolding, leading to an increased exposure
229 of the lysine residues and their glycation via the Maillard reaction. Glycation levels in caseins
230 would be expected to be higher than those in WPs as caseins are unstructured proteins and
231 do not need to be unfolded to be able to react with lactose. This could explain the initial
232 increase in BI with increased casein content. The BI of the Casmic powder increased by a
233 factor of 12 after DH, probably because this powder had a higher lactose to protein ratio
234 promoting a more intensive Maillard reaction.

235 The granular size distribution of the unheated powder is shown in Fig. 2A. The size
236 distribution of the powder particles made of WP and NaCas showed a slight shift towards
237 larger sizes with the addition of NaCas. This shift was confirmed by the significantly higher
238 $D[4,3]$ values observed for the powder particles with increasing casein content (the grey
239 points in Fig. 2C). As with the BI, a maximum for the $D[4,3]$ value was observed in powders
240 containing around 40% casein. This increase in $D[4,3]$ was probably due to higher resistance
241 of powders with higher NaCas contents (or lower lactose contents) to crushing of the freeze-
242 dried powder during preparation for DH. However, it is unclear why this increase in $D[4,3]$
243 was not linear with increasing casein content. This change in size of the powder flakes may
244 be influenced by the freeze-drying process. Firstly, freeze-drying begins with freezing of the
245 solution leading to separation of the protein solution into, first, liquid water in the form of ice

246 crystals and, second, a concentrated solution of proteins and solutes. Ice is subsequently
247 removed during the sublimation step, together with the water adsorbed onto the solutes
248 (Franks, 1998). Concentrated proteins and solutes may then interact, be denatured, and
249 either precipitate in the crystalline state or stay as an amorphous solid that can also proceed
250 to glass transition at low temperatures. We assumed that the structure of the freeze-dried
251 cake before the crushing step was made up of pores the size of ice crystals embedded in the
252 protein amorphous concentrate, and that the crushing would lead to breakage preferentially
253 through these pores. Thus, the structure of the cake would be expected to have
254 consequences on the size of the powder flakes: a larger crystal size distribution would lead
255 to a cake with larger pores, a denser amorphous protein phase, and to smaller flakes than
256 would be observed with smaller crystals. However, this hypothesis cannot be confirmed as
257 we do not know the sizes of the crystals in the different frozen mixtures. Secondly, although
258 the increase in size of the powder flakes appeared to correlate with the small decrease in
259 lactose content resulting from substitution of WP by NaCas, reduced amounts of lactose are
260 very unlikely to lead to an increase in powder flake size as decreasing the lactose content
261 has been shown previously to decrease flake sizes (Schong & Famelart, 2019). Thirdly,
262 protein powders are more rigid and brittle at temperatures below the glass transition
263 temperature (T_g) and softer and more viscous-like at temperatures above the T_g . The T_g
264 values of NaCas, Casmic and WP at an $a_w \sim 0.3$ are 61, 72 and 84 °C, respectively (Schuck,
265 Jeantet, & Dolivet, 2012). The lower the T_g of the powder, the closer the powder would be to
266 the transition towards a softer material, and the softer the material would be. Thus, we would
267 expect Cas18 to be harder than Cas99, but the observed flake sizes contradict this
268 explanation. Furthermore, differences in behavior between WP and micellar casein, with
269 relevant consequences on powder morphology, have been reported previously during spray-
270 drying (Sadek et al., 2014). Powder particles of micellar casein are wrung, and invaginated,
271 whereas those of WP are totally spherical, and present a large vacuole of occluded air and a
272 smooth surface. These differences could be ascribed to the fact that when proteins are
273 concentrated to above a critical concentration during droplet drying, casein or caseinate

274 undergo a sol-gel transition (Bouchoux et al., 2009) and globular proteins undergo a glass
275 transition (Brownsey, Noel, Parker, & Ring, 2003; Parker, Noel, Brownsey, Laos, & Ring,
276 2005), leading to development of solid-like properties. Storage moduli of 200-300 Pa for
277 casein gels and > 10000 Pa for β -Lg and bovine serum albumin gels have been reported at
278 concentrations of 100-200 g/kg for casein and > 400 g/kg for globular proteins (Bouchoux et
279 al., 2009; Brownsey et al., 2003; Parker et al., 2005). Thus, the differences in powder
280 morphology and size may have been caused by differences in the physicochemical
281 properties of the proteins, such as their transition concentration or gel firmness. However,
282 casein gels formed by concentration are softer than WP gels, a finding which again is
283 contradictory to our observation that flake size increases with increasing casein content. In
284 conclusion, the mechanism underlying the changes in flake size with altered composition of
285 the mixtures remains unclear. The observed changes in flake size were small, with the size
286 of powder flakes ranging between 5 and 500 μm and the $D[4,3]$ values ranging between 60
287 and 100 μm for all powders; however, any changes in flake size or structure could have
288 major consequences on the formation of PMs.

289 Light micrographs of powder particles from samples of Cas18, Cas58 and Cas99 after
290 DH, and from Casmic before and after DH, are shown in Fig. 3. The powder particles appear
291 as flakes, as expected for freeze-dried powders. Although microscopy could not be used to
292 provide accurate evaluations of the size distribution of the flakes, because of the very small
293 number of particles observed in each sample and the risk that these small samples were
294 unrepresentative of the bulk, we did not notice any significant size differences between the
295 flakes of powders with different NaCas contents. The Cas99 powder flakes appeared slightly
296 denser and more shrunken than flakes from powders with lower NaCas contents. In addition,
297 the Casmic powder flakes appeared much more shrunken, less translucent, and much
298 smaller than flakes from the other powders. It was of interest to note that the aspect of the
299 Casmic flakes, and indeed that of all the other powders studied (data not shown), was not
300 modified by the DH treatment. The size of the flakes from powders with WP and NaCas

301 appeared to be around 5–20 μm , whereas those from the Casmic powder were $< 10 \mu\text{m}$.
302 These estimates of flake size made from microscopic evaluations were smaller than the
303 values obtained using the Qicpic method. We consider that the Qicpic method gave the most
304 reliable estimation of flake size because using this method more than 10^6 particles were
305 characterized during one measurement.

306 *3.2. Protein microparticle characterization*

307 Photographs of S0 suspensions of dry-heated powders were taken immediately (d0, not
308 shown) and 5 d after their rehydration and quiescent storage (see the supplementary
309 information and Fig. S1). The d0 suspensions of dry-heated powders appeared cloudy, due
310 to the presence of PMs that sedimented after storage. These suspensions were yellow for
311 the Cas18-Cas99 samples, and yellow-red for the Casmic sample. In contrast, the
312 suspensions of powders before DH were translucent (not shown). Similar volumes of
313 sediment appeared to be present in the Cas18 to Cas38 samples, but the volume of
314 sediment seemed to decrease slightly in suspensions from powders with above 38% casein.
315 A far smaller volume and browner sediment was observed in the Casmic sample. These
316 findings show that DH of the powders for 36 h led to the formation of PMs that sedimented
317 with time. Evaluation of the height of the sediment on the photograph revealed that these
318 sedimented particles occupied a rather large volume: although the suspensions contained
319 only 1% dry-heated powder, the sedimented particles occupied between 10% (Casmic) and
320 24-17% (WP and NaCas) of the total volume.

321 The size distribution of the PMs varied between 4 and 1000 μm for the Cas18 suspension
322 and seemed to shift towards larger values with the addition of increasing amounts of NaCas
323 (as shown in Fig. 2B). D[4,3] values increased as the NaCas content increased from 18% to
324 around 40% and then decreased for samples with over 40% NaCas (Fig.2C). The size of
325 these PMs was 2-4 fold larger than that of the powder flakes, with the Cas34-Cas44 samples
326 having the largest PM sizes. The SR was around four for the Cas34 sample and the lowest

327 SR value, of around two, was obtained for Cas99. Swelling of the dry-heated particles when
328 placed in the aqueous phase, leading to larger sizes in suspension than those observed in
329 the powder, has already been reported in previous studies (Schong & Famelart, 2018): the
330 diameter of particles obtained by DH of spray-dried powders has been shown to increase by
331 a factor of four when placed in water. This swelling was probably due to repulsion between
332 negative charges on proteins inside the PMs. An example of this swelling is shown for a flake
333 from the Cas99 sample and occurred within around 20 s under the conditions used in the
334 current study (see supplementary Fig. S2): the dimensions of the flake increased by a factor
335 of around two, in accordance with the calculated SR of powder flakes from this sample (SR \approx
336 2). Our previous study indicated that the degree of swelling was related to the number of
337 crosslinks between proteins inside the PM: swelling was reduced after prolonged DH times
338 and in powders with higher lactose contents, i.e. under conditions where the number of
339 crosslinks increased (Schong & Famelart, 2019). However, the degree of swelling may also
340 be related to the size of the powder flakes: in the current study swelling was maximal in
341 samples with 34-44% casein. It should be noted that the maximum BI was also observed in
342 samples with around 40% casein.

343 The size of PMs from the Casmic suspension varied between around 2 and 1000 μm and
344 their $D[4,3]$ was close to the $D[4,3]$ of the powder flakes. In contrast to the WP-NaCas flakes,
345 the Casmic PMs underwent only minimal swelling when placed in water, and some of the
346 PMs appeared to be smaller than the powder particles (1-10 μm ; Fig. 2B). As these small
347 PMs swelled when placed in suspension, they may have been present in the powder, but
348 were smaller than the minimum size detected by the QICPIC method ($\sim 1 \mu\text{m}$). Although PMs
349 were formed by DH of the Casmic powder, the suspended particles appeared to be smaller
350 than the PMs observed in the other powder samples analyzed in this study. The small size of
351 the Casmic PMs probably resulted from a high degree of crosslinking associated with very
352 intense Maillard reactions promoted by the higher lactose content of this sample.

353 In conclusion, dry-heated powders with WP and NaCas swelled by a factor of 2-4,
354 whereas no swelling was observed for the Casmic powder. CLSM micrographs showing the
355 sizes of the PMs in the samples are shown in Fig. 4. No particles were visible in the powder
356 suspensions before DH (data not shown). Angular PMs, with shapes similar to those
357 observed for the powder flakes, were formed by DH of all the powders. The largest PMs
358 were observed for powders with between 34 and 44% casein. PMs from the Casmic powder
359 appeared to be much smaller than the those formed from powders containing WP and
360 NaCas. These findings are therefore in accordance with the size measurements obtained
361 using the Mastersizer method (Fig. 2).

362 The yields of conversion of the powder into PMs (DY and WY) and the WC of the PMs are
363 shown in Fig. 5. The DY value of 0.6 g/g for the WP sample without added casein was the
364 same as that reported in our previous study (0.6-0.7 g/g; Famelart et al., 2018; Schong &
365 Famelart, 2019). The DY of PMs produced from WP and NaCas powders increased slightly
366 with increasing NaCas content (+31% between Cas18 and Cas99). The fact that the DY
367 increased with the caseinate content confirmed that caseinate formed part of the PMs, with
368 the caseinate molecules being crosslinked by DH at pH 9.5. If the caseinate had not formed
369 part of the PMs, we would have expected to see a decrease in DY with increasing NaCas
370 content. It seems that the increase in DY with casein content (i.e. the slope of the DY-casein
371 content curve) was most rapid between 18% and 40% casein, and slower at higher casein
372 contents from 40 to 99%. The amount of water entrapped in the PMs (WC) from the powder
373 without added casein was 28 g/g. This value is in accordance with the WC values of 25-30
374 g/g found for the same freeze-dried powder in our previous studies (Famelart et al., 2018;
375 Schong & Famelart, 2019), but is lower than the value of 49 g/g found for the spray-dried
376 powder after the same DH treatment (Schong & Famelart, 2018). WC decreased with the
377 addition of NaCas, but this decrease was irregular: the decrease was minimal from 18 to
378 34% and from 50 to 99% casein, whereas it was maximal between 34 and 58% casein. The
379 WY from the powder without added casein was 18 g/g, compared to 18-20 g/g in previous

380 studies (Famelart et al., 2018; Schong & Famelart, 2018, 2019). The WY increased between
381 18 and 34-38% casein and then declined for powders with above 38% casein before
382 becoming almost constant between 58 and 99% casein. In conclusion, the yields and WC
383 values were slightly modified by the substitution of WP for Nacas. Replacing WP by NaCas
384 promoted the formation of more PMs, but with less water entrapped in their structures. These
385 small differences could be due to: i) changes in the size or structure of the crushed powders,
386 with flakes produced from the Cas18 powder being smaller (Fig.2C) and those produced
387 from Cas99 being denser and more shrunken (Fig.3); ii) differences in the amount of
388 unfolded protein, such as casein; iii) differences in the extent of crosslinking during DH,
389 associated with differences in lactose content. Indeed, lactose has been shown to promote
390 the rapid formation of PMs (Schong & Famelart, 2019). However, lactose content alone
391 cannot explain the changes observed with increasing casein content, as maximal values for
392 the BI, particle swelling and WY were obtained for powders with around 40% casein. As
393 discussed above, the structure of the cake would be expected to change with the size and
394 number of ice crystals formed during freezing of the protein solution, the amount of lactose or
395 any other solute, and with the physicochemical properties of the proteins, such as their Tg
396 and transition concentration and the gel firmness of their concentrated solutions. As the
397 density of the protein cake, together with the extent of the crosslinking and the protein-
398 protein and protein-water interactions responsible for swelling, define the amount of water in
399 the particles in suspension (i.e. the WC), it would be necessary to characterise all of these
400 parameters to be able to fully explain the variation in properties of the PMs produced by DH.

401 The fact that the maximum BI, powder flake and PM D[4,3] and WY values were all
402 observed at around 40% casein could also be explained by a specific interaction occurring at
403 pH 9.5 between casein and WP at this ratio, with consequences on protein behaviour. This
404 hypothesis needs to be confirmed by analysis of the proteins in solution at pH 9.5 before
405 drying and after drying and DH.

406 The Casmic powder also formed large PMs during DH, with a higher DY than the WP and
407 NaCas powders (Fig. 5). This higher DY indicates that more extensive crosslinking leads to
408 retention of the micellar casein inside the powder particles during the DH and that fewer
409 proteins were present as small aggregates or monomers in the soluble phase. These PMs
410 entrapped much less water and displayed lower WC and WY values than those observed for
411 the WP and NaCas powders (Fig. 5). The limited ability of the Casmic PMs to retain water is
412 related to the fact that the D[4,3] values of the powder and PMs were very similar, thus these
413 PMs did not swell when dissolved in water. Moreover, we have already shown that an
414 increase in the DY of WP PMs correlates with a lower WC (Schong & Famelart, 2019).

415 3.3. Denaturation of whey proteins and composition of protein microparticles

416 For powders made of WP and NaCas, the level of WP denaturation before DH and after
417 36 h of DH was almost constant, regardless of the NaCas content (Table 1). Before DH, 70%
418 of the WPs were still native, meaning that 30% were already denatured before DH. We have
419 already shown that adjusting the pH of the WP solution to 9.5, followed by drying and a_w
420 adjustment of the powder at 20 °C for 2 weeks, leads to WP denaturation: similar values of
421 50-60% (Famelart et al., 2018) and 70-80% (Schong & Famelart, 2018, 2019) residual NWP
422 were obtained before DH in previous studies. β -Lg was shown previously to be the main
423 protein impacted by the pH adjustment: the native α -lac and bovine serum albumin (BSA)
424 contents before DH were 90%, whereas that of β -Lg was only 50% (Famelart et al., 2018).
425 Indeed, alkaline-induced changes in WPs have been reported at pHs above 9, including
426 irreversible unfolding and polymerization of proteins (Monahan, German, & Kinsella, 1995;
427 Partanen et al., 2011; Taulier & Chalikian, 2001).

428 The NWP content decreased to 10-20% after 36 h of DH, and both β -Lg and α -lac were
429 denatured. These values are similar to those obtained in previous studies (Famelart et al.,
430 2018; Schong & Famelart, 2018, 2019). BSA has also been shown to be denatured by DH
431 (Famelart et al., 2018), thus we can state that most WPs are denatured by the DH treatment

432 at alkaline pH values. Finally, increasing the NaCas content did not have any effect on WP
433 denaturation during the DH process. It was not possible to analyze the levels of NWP in
434 samples from the Cas99 powder containing only 1% WP.

435 For the Casmic powder, the residual NWP content before DH was 100% (Table 1). One
436 hypothesis for the lack of denaturation of the WPs in the Casmic powder is that these WP
437 were less sensitive to the alkaline pH. Although the Casmic sample contained only 7% WP,
438 the degree of denaturation after 36 h of DH was similar to that detected in the WP and
439 NaCas powders, at around 90%. These findings show that WPs from the Casmic powder
440 were also sensitive to DH at an alkaline pH.

441 **4. Conclusions**

442 Our study shows that DH of a powder made of WP and NaCas conditioned at pH 9.5 led
443 to the production of large and highly hydrated PMs. Substitution of WP by NaCas led to small
444 changes in the formation of PMs and in their properties. The yield of conversion of protein
445 into PMs tended to increase, whereas the amount of water entrapped in these PMs tended to
446 decrease with the substitution of WP by casein. However, these changes were limited and
447 not linearly dependent on the casein content. The size of the PMs was maximal in powders
448 with around 40% casein. We showed PM formation also occurs in micellar casein-enriched
449 milk protein powders. That fact that casein has no native structure but was still able to form
450 PMs, throws doubt on the hypothesis that the role of the alkaline pH conditions used in the
451 process was to unfold proteins, as suggested in Schong & Famelart (2019). Furthermore,
452 these data suggest that it is possible to produce PMs by this process for a range of powders,
453 including whey isolate or concentrate or a milk concentrate, as casein has only a limited
454 effect on the yield and properties of the particles. Further studies are required to characterize
455 the effects of this process on spray-dried powders.

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461 size analyses.

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574

575 **Table 1**

576 Native residual whey protein (NWP) levels in S0 suspensions of powders before and after 36
 577 h of dry heating (DH) at 100 °C. Data are expressed as the % of total WP in the S0
 578 suspension.

Casein content (% w/w)	NWP	
	Before DH	After DH
18	75.4±5.1 ^a	14.6±2.1 ^a
34	77.7±7.0 ^a	14.4±2.7 ^a
38	77.2±4.3 ^a	13.9±1.2 ^a
44	71.9±0.5 ^a	14.3±2.4 ^a
58	78.6±3.7 ^a	10.9±1.6 ^a
93*	102.7±2.9 _b	8.3±0.6 ^b

579 * Micellar casein-enriched milk protein powder sample, all other samples are mixtures of
 580 sodium caseinate (NaCas) and whey protein (WP) powder.

581 ^{a,b} For each column, values marked with different letters showed a statistically significant
 582 difference in NWP content (P < 0.05).

583 **Figure captions**

584 **Fig. 1.** The browning index (BI) of powders before (grey) and after 36 h of DH at 100 °C
585 (black) as a function of the percentage casein content (w/w). The powders analyzed
586 contained either whey proteins with increasing amounts of sodium caseinate (circles) or
587 micellar casein-enriched milk proteins (crosses). Plotted lines illustrate the trend in BI with
588 increasing casein content.

589 **Fig. 2.** The granular size distribution of A) freeze-dried powder particles before dry heating
590 (DH) and B) protein microparticles in S0 suspensions after DH of the powders. Colors from
591 black to green represent powders with increasing amounts (w/w) of sodium caseinate
592 (NaCas), ranging from Cas18 with a mixture of whey proteins and 18% NaCas to Cas99 with
593 a mixture of whey protein and 99% NaCas. The red curve represents the particle size
594 distribution for the micellar casein-enriched powder (Casmic). The distributions shown are
595 representative of data obtained from two samples of each S0 suspension measured in
596 triplicate; C) The mean volume diameter (D[4,3]) of powder particles (grey) and of protein
597 microparticles (black) as a function of casein content for powders containing either whey
598 proteins with increasing amounts of sodium caseinate (circles) or micellar casein-enriched
599 milk proteins (crosses). For each curve, no statistical differences were observed between
600 samples for data points marked with the same letter ($P < 0.05$).

601 **Fig. 3.** Light micrographs of particles from powders containing whey proteins with increasing
602 amounts (w/w) of sodium caseinate (NaCas) ranging from 18% (Cas18) to 99% (Cas99) after
603 36 h of dry heating (36h DH), and of the particles from the powder containing micellar casein-
604 enriched proteins (Casmic) before DH (0h DH) and after 36 h of DH (36h DH). The
605 micrographs shown are representative of the 10 images recorded for each sample.

606 **Fig. 4** Confocal laser scanning micrographs of S0 suspensions of powders dry-heated for 36
607 h. Suspensions were prepared from: A) powders of whey proteins with increasing amounts

608 (w/w) of sodium caseinate (NaCas), ranging from 18% (Cas18) to 99% (Cas99); and B)
609 Casmic powder of micellar casein-enriched proteins. Bars = 200 μ m. The two micrographs
610 shown for each powder are representative of the 10 images recorded for each sample.

611 **Fig. 5.** Dry yield (DY) of conversion of powder dry matter into microparticles, water content
612 (WC) of the microparticles and wet yield (WY) measured as the weight of pellets from S0
613 suspensions as a function of the percentage casein content in % (w/w). The dry-heated
614 powders in the suspensions contained either whey proteins with increasing amounts of
615 sodium caseinate (circles) or micellar casein-enriched milk proteins (crosses).

Fig 1

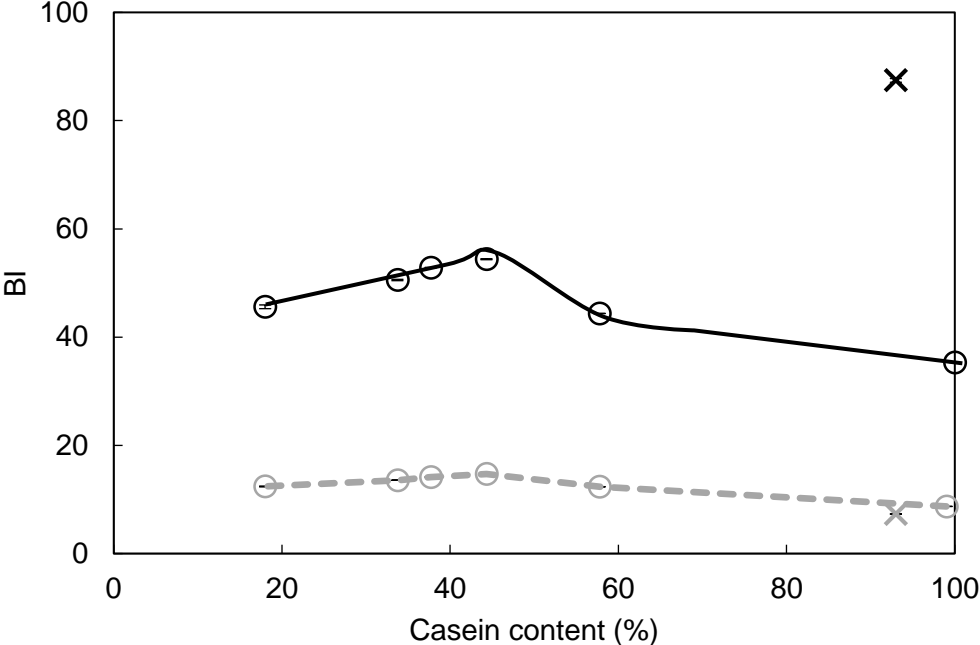


Fig. 2.

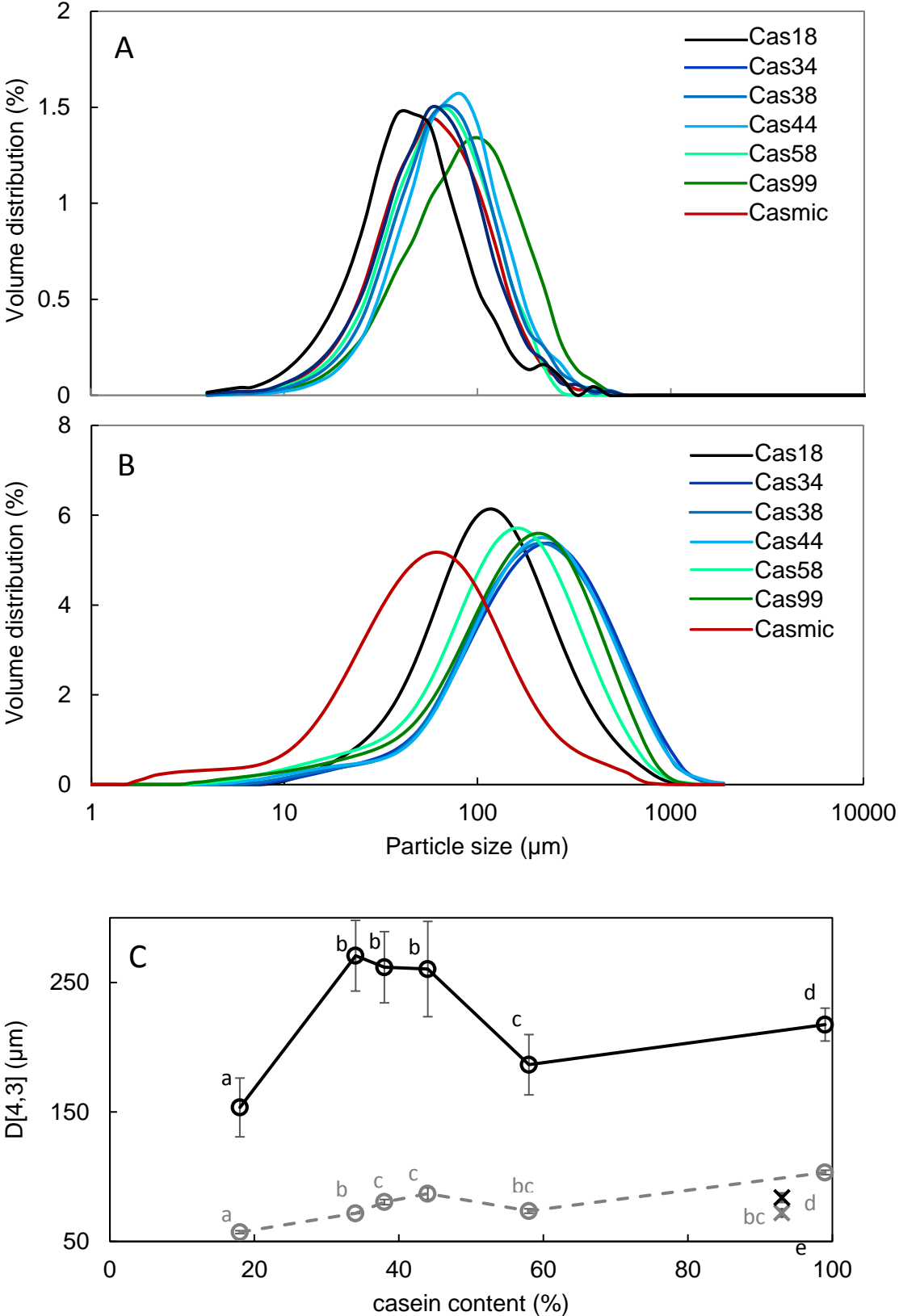
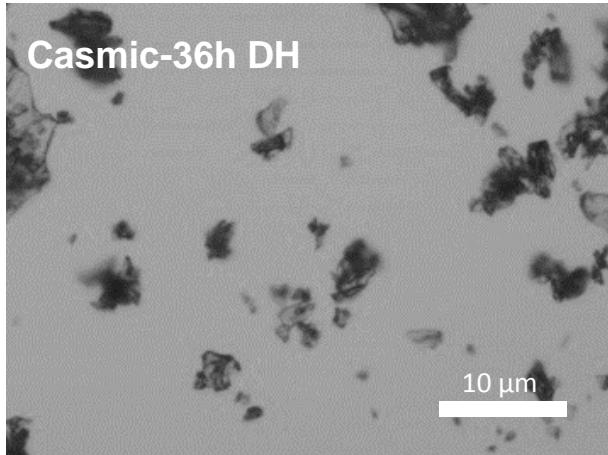
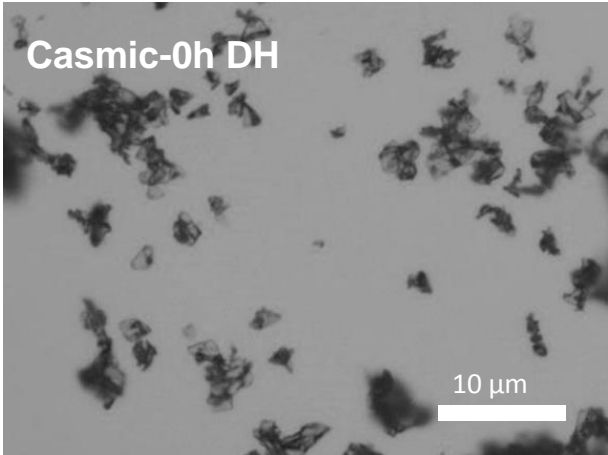
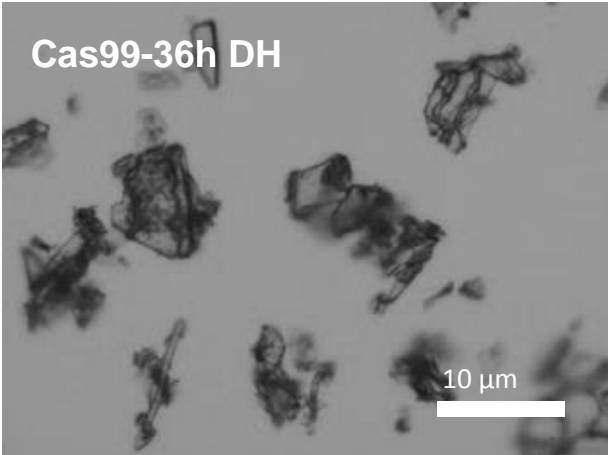
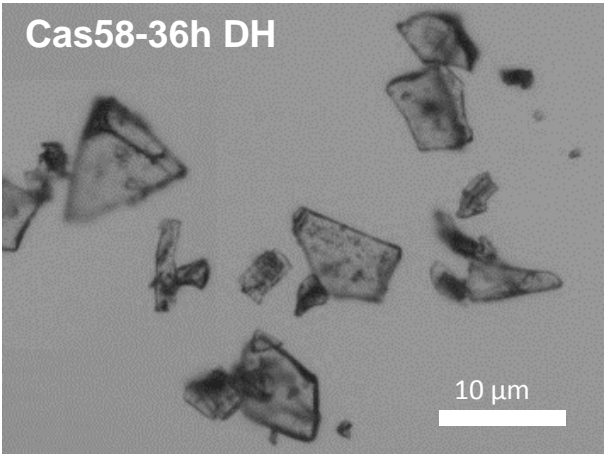
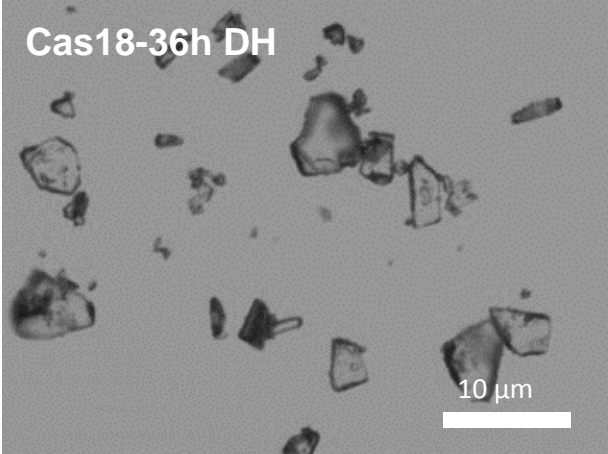


Fig. 3



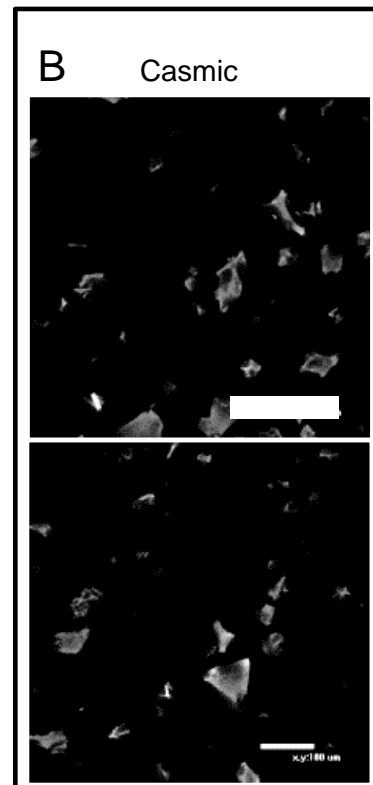
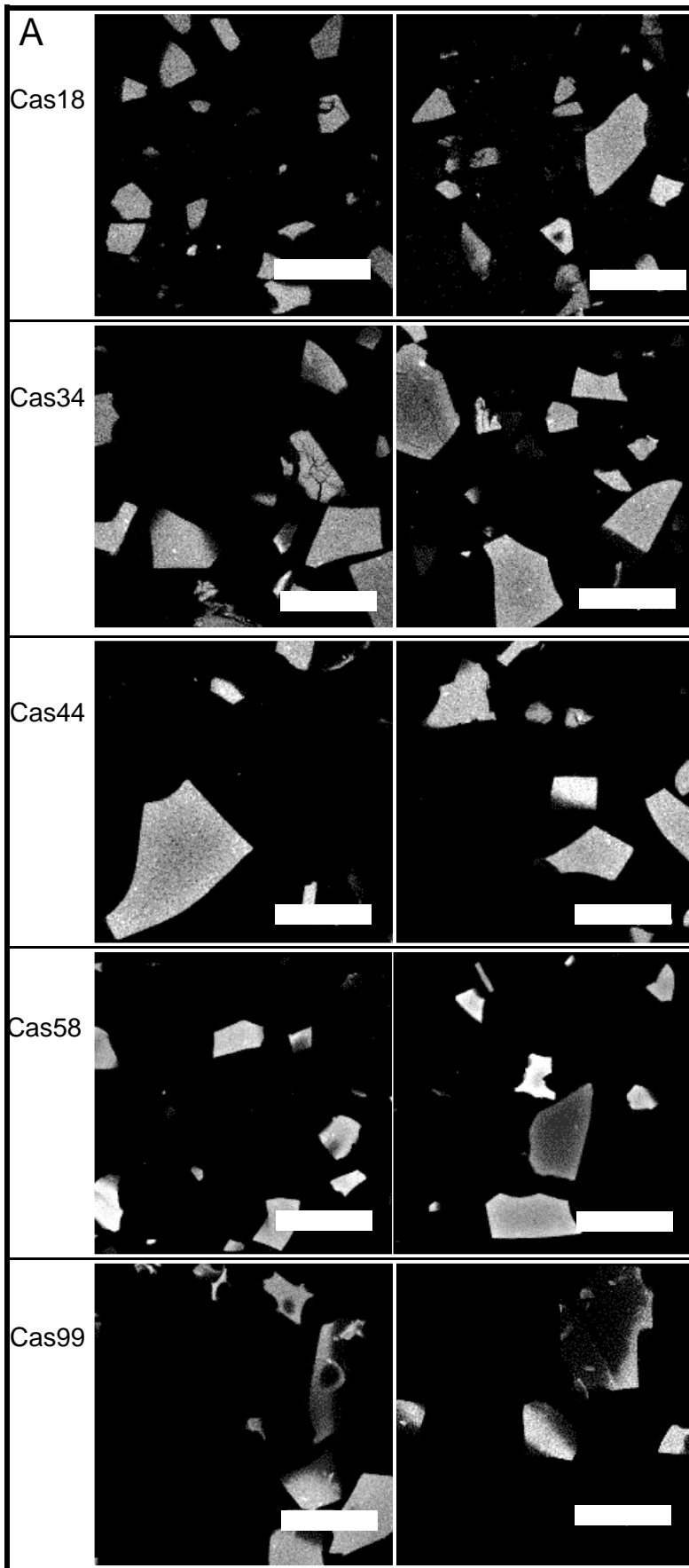


Fig. 5

