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The heterogeneous substructure of casein micelles evidenced by SAXS and NMR in demineralized samples

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41 1 INTRODUCTION

42

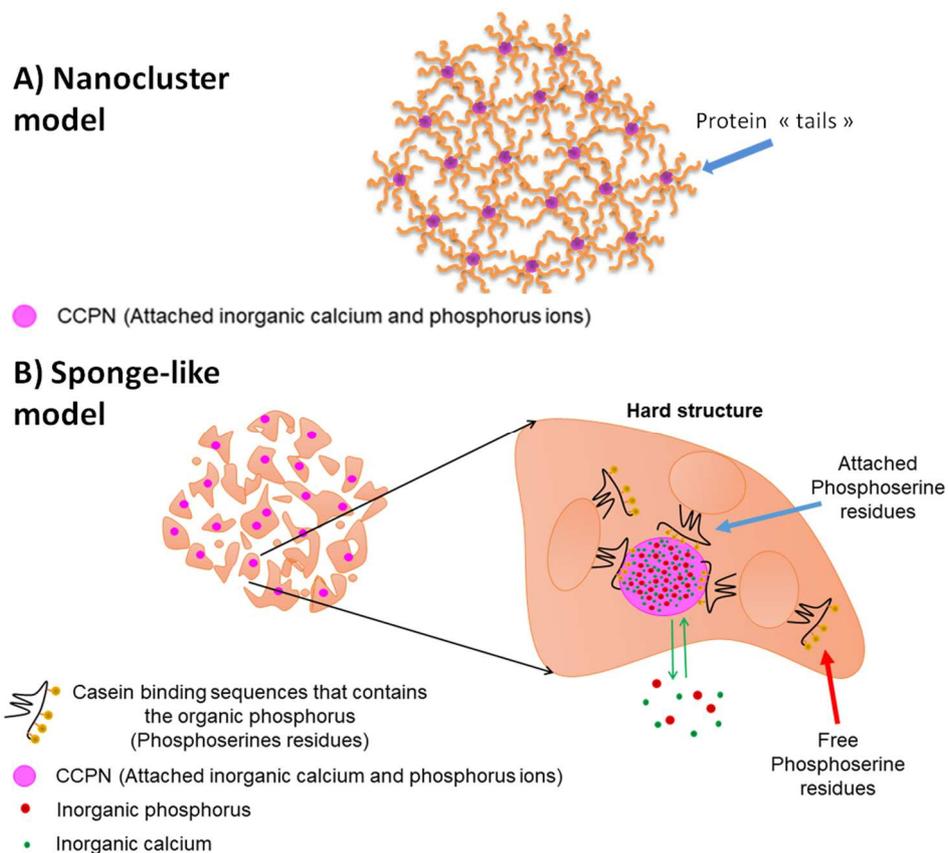
43 The casein proteins are primary constituent in cow's milk and represent ~80%
44 of total protein content. These proteins are associated with colloidal calcium
45 phosphate (CCP) into major molecular assembly present in the cow's milk and most
46 of the dairy products called casein micelles (Walstra et al., 2006). The micellar casein
47 presents excellent digestibility, higher amino acids supplement and especially leucine
48 (Vickery & White, 1933). Because of its industrial and dietary importance, the
49 characteristics and relation between its structure and the physico-chemical
50 environment have been widely studied (Belicium et al., 2012; Crowley et al., 2014;
51 Dahbi et al. 2010; Silva et al. 2013).

52 The calcium phosphate nanoclusters play a pivotal role as a molecular glue in
53 the casein's assembly into casein micelle (De Kruif & Holt, 2003). The casein
54 micelles show high degree of dissociation upon loss of calcium phosphate and if the
55 loss of calcium phosphate is >45%, it induces the formation of caseinate (Bouchoux
56 et al., 2009; Ingham et al., 2016). While the partial loss (between 5%-45%) of CCP
57 induces internal restructuring of CMs (Boiani et al., 2018; Broyard & Gaucheron,
58 2015; Kort et al., 2011; McCarthy et al., 2017; Ramchandran, Luo, & Vasiljevic, 2017)
59 but no major changes have been observed in the micellar size (~5%) (Ingham et al.,
60 2016).

61 Despite the studies done towards understanding the role of CCPs in details,
62 the influence of CCPs in micellar caseins is still a matter of debate among scientific
63 community. The recent studies by De Kruif et al. (2014) supports the so called
64 "Nanocluster Model" of casein micelles (figure 1). In this model, as proposed by De
65 Kruif et al. (2014), the CCPs are presented as central body covered by their protein
66 counterparts (caseins) and many of such small assemblies are clustered together via
67 the interaction of the free dangling tail part of the caseins to the neighboring
68 assemblies (figure 1). The small-angle x-ray scattering (SAXS) and Neutron
69 scattering (SANS) data support this "Nanocluster Model" of CMs assembly. Among
70 other features, SANS data show that the composition of micelles is quite
71 homogenous at the medium size scale (>10nm) but inhomogeneities are observed
72 near the small size scale of ~4nm.

73 The work of Bouchoux et al. (2010) proposed a complexification of the model
74 (we will call here the sponge-model) at the medium scale range. This work shows

75 that the CMs are composed of several domains of ~20nm in size (called “Hard”
 76 regions) formed by protein assemblies to CCPs or free protein assemblies without
 77 CCPs and water cavities of similar sizes. Their data show that, under osmotic stress,
 78 the casein micelles are compressed as a sponge, while some regions (attributed to
 79 the presence of large voids) collapse first leaving unchanged the so called “Hard”
 80 regions in the first degree of compression. The presence of these inhomogeneities
 81 contrasts to what have been stated by De Kruif, C. G. (2014); that the micelle is
 82 homogenous gel at the medium size scale. Such homogenous gels in the size scale
 83 of tens of nanometers, as presented by the “Nanocluster Model”, should display a
 84 continuous variation of the internal structure of the micelle as a function of the
 85 reduction of the micellar volume.
 86



87

Figure 1 – (A) Schematic representation of the so-called nanocluster model as proposed by De Kruif, C. G. (2014): a casein micelle formed by small nanocluster units composed of a CCP and shell of proteins where the tails of these proteins interact with the neighbor nanoclusters. (B) Schematic representation of a CM as proposed by the “sponge-like model”. (Right) Zoom into a so-called “Hard” region representing casein micelles binding sequences (phosphoserine residues) interaction with calcium phosphate nanoclusters (CCP) through organic phosphorous from phosphoserine residues. Green arrows represent the equilibrium between calcium and phosphorous ions between the CCP (Colloidal phase) and the soluble phase. One can notice that all nanoclusters (CCP) are “enveloped” by proteins represented in brown (which constitutes the “hard” structures).

88

89 In the present work, the primary objective is to fill in the gaps between
90 aforementioned models and data, also to better understand and provide new insights
91 towards the role of CCPs in the casein micelle's structure and dynamics. To achieve
92 the set goals, this study has associated SAXS data with ³¹P solid state nuclear
93 magnetic resonance (NMR) to understand and improve the knowledge regarding the
94 specific impact of different degrees of demineralization on the local structure of
95 clusters. Moreover, fluorescence spectroscopy has been used to better understand
96 how hydrophobic interactions between proteins evolve with the different levels of
97 demineralization.

98

99 **2 MATERIALS AND METHODS**

100

101 **2.1.1 Samples identification**

102

103 The casein-based powders studied in this work were manufactured and
104 supplied by Ingredia S.A (Arras, France). The powders were produced at industrial
105 pilot plant of Ingredia using traditional drying procedures (Pierre et al., 1992; Schuck
106 et al., 1994).

107 The powders were mostly composed of casein proteins and over 82% (w/w) of
108 protein content proof was reported by Ingredia, representing over 90% of the total
109 nitrogen content from powders (see Table 1, SI). Four different compositions of
110 powders at different degree of demineralization were used in the present study,

- 111 • 0% calcium-demineralized powder (from here on “«native»”) was
112 used as control,
- 113 • 4.47% calcium-demineralized powder (DM-05),
- 114 • 9.16% calcium-demineralized powder (DM-10),
- 115 • 25.73% calcium-demineralized powder (DM-25),

116

117 The four powder formulations were identified as described previously as the
118 closest to the real demineralization values (rounded off to the real demineralization
119 value). It is also important to mention that the process used to generate and
120 rehydrate all the powders, might have some impact on the structure of casein

121 micelles (Nogueira et al., 2020). For this reason, the «native» sample will always be
122 displayed within quotation marks.

123

124 **2.1.2 Demineralization and powder production**

125

126 To produce such calcium-demineralized CM-rich powders, one starts from
127 acidified skimmed milk at three different pHs (6.4, 6.2 and 5.9) using lactic acid. This
128 process allows the excision of certain percentage of calcium from CMs, as described
129 in literature (Broyard & Gaucheron, 2015).

130 The calcium-demineralized CMs-rich powders start from skimmed milk, which
131 was acidified to three different pHs (6.4; 6.2; and, 5.9) by the addition of lactic acid.
132 This process permits the excision of part of the calcium from the CMs, as described
133 by the literature (Broyard & Gaucheron, 2015).

134 The present study follows the protocol applied by Silva et al., (2013), which
135 reports a decrease of about 6%, 14% and 23% of the total calcium from CMs
136 concentrates, which was acidified with hydrochloric acid and to three pH values of
137 6.4, 6.1 and 5.8. To ensure that the complete equilibrium is achieved, the acidified
138 skimmed milk was left at 10°C for 10hours (maturation time).

139 The maturation time is followed by a protein concentration step, which consists
140 of ultrafiltration process with a membrane cut-off of 10 kDa. The ultrafiltration process
141 concentrates the milk proteins via the selective removal of water and soluble salts
142 (Carvalho & Maubois, 2010). The protein concentrates are then submitted to
143 membrane separation techniques, which follows two subsequent microfiltration. The
144 first microfiltration was aimed at removing the bacterium and the membrane cut-off of
145 1.4µm was used, while the second microfiltration was performed with the 0.1µm pore
146 size membrane to remove soluble constituents of the concentrates such as whey
147 proteins, lactose and soluble minerals, which simultaneously concentrates the CMs
148 at higher degree (Pierre et al., 1992).

149

150 **2.1.3 Powder rehydration**

151

152 The casein-rich powders were rehydrated with deionized water to 14.0 g
153 (protein) x 100 g⁻¹ (water) submitted to stirring at 500 r.p.m at 50 °C/1 hour. Three
154 drops of antifoam silicon solution were added to each powder dispersion at the

155 beginning of the rehydration to prevent the foam formation. The pH was adjusted to
156 7.0 with NaOH 1M. After pH adjustment, the samples were homogenized at 10000
157 r.p.m for 5 minutes using a rotor-stator homogenizer, Polytron PT 10-35a
158 (Kinematica, France). Antimicrobial agent sodium azide was added to 0.3 g L⁻¹
159 (Sigma Aldrich, France) to prevent microbiological growth.

160

161 **2.1.4 Fluorescence measurements**

162

163 To measure changes in the number of hydrophobic interaction in the CMs, the
164 Fluorescence spectroscopy (using tryptophan as a probe) has been used. The
165 Fluorescence analysis method used is the one used in our previous publication
166 (Nogueira et al., 2020), using a Fluoromax-4 spectrofluorometer (Jobin Yvon, Horiba,
167 NJ, USA). The angle of the excitation radiation set at 60° and temperature controlled
168 by Haake A25 AC200 temperature controller set to 20° C (Thermo-Scientific,
169 Courtaboeuf, France). The samples were poured into a 3 mL quartz cuvette and the
170 emission spectra of tryptophan residues in a wavelength from 305 to 450 nm after
171 excitation set at 290 nm were analyzed.

172

173 **2.1.5 CMs structure organization observed by Small-angle X-ray scattering** 174 **(SAXS)**

175

176 In the present study, the SAXS method was applied to investigate the
177 structure of the CMs at different levels. As described in the literature (Bouchoux et al.
178 2010) the SAXS spectra of the CMs is representative of three levels, which
179 correspond to different structures from the micelle: i) the level 0, which is the micellar
180 envelope (~100 nm of diameter) corresponding to a Q range 0.0065 to 0.0010 nm⁻¹
181 and, in the present concentration, to the inter-micellar distance; ii) the level 1, which
182 corresponds to smaller structures (~20 nm of diameter) described as “Hard-regions”,
183 which are related to incompressible structures (Ingham et al., 2016), once submitted
184 to osmotic pressure, within the micelle corresponding to a Q range of 0.042 to
185 0.006 nm⁻¹; and iii) the level 2, which arises mainly from the structure of proteins
186 associated to CCP (Ingham et al., 2016), assigned to an apparent “shoulder-region”
187 at a Q range between 0.042 to 0.27 nm⁻¹. The quantification of the intensity of this
188 last “shoulder” have been measured by averaging the SAXS intensity between 0.07

189 and 0.15 nm⁻¹ (the center of the “shoulder”) for each sample. The values in relative
190 percentage are given in table 1.

191 The SAXS measurements were conducted as described in (Nogueira et al.,
192 2020). All SAXS acquisitions were performed at room temperature (~25 °C) at the
193 French national synchrotron facility SOLEIL in Gif-sur-Yvette, France; on the SWING
194 beamline operating at ~12 keV of photon energy. The SAXS intensities were
195 recorded on a detector placed at ~0.5 m and 6.5 m from the sample. For each
196 sample, data was recorded at short exposure time (typically ~0.2 s) to prevent any
197 radiation damage.

198

199 **2.1.6 Nuclear Magnetic Resonance (NMR) Spectroscopy quantification of** 200 **attached phosphoserines and nanoclusters phosphorous**

201

202 The four different phosphorus species present (Boiani et al., 2016), (Peixoto et
203 al., 2017), (Boiani et al., 2018) (Gonzalez-Jordan et al, 2015) in the CMs rich powder
204 formulations based on their localization and interactions with the micellar components
205 were analyzed and characterized by NMR method. The phosphorus species are
206 identified into four classes based on which CMs part they belong to. The first species
207 is the “Organic” phosphorus of phosphoserine amino acids attached to CCP cluster;
208 the second species is also an “Organic” phosphorus of identical origin but not
209 interacting with CCP clusters (more dynamic because of free phosphoserine
210 residues); “inorganic” soluble phase phosphorus is classified as third species and
211 “inorganic” phosphorus forming or bound to the CCP cluster is the fourth distinct
212 species present in the CMs. Noteworthy, here the appellation of “free” (organic and
213 inorganic) phosphorous refers the phosphorous that are linked to only one or multiple
214 calcium ions (but not to a cluster) as well for the phosphorous in its anionic form
215 since both of these forms (linked to calcium ions or not) are indistinguishable in the
216 NMR spectra, in the present conditions. Indeed, the binding of calcium ions to a
217 single phosphate is always much faster than the NMR measurement resulting in a
218 peak that represents the average signal between these two species (linked to
219 calcium ions or not).

220 The NMR analysis was conducted as described by Nogueira et al., (2020)
221 using a Bruker AVANCE I; 9.4T (1H: 400 MHz; ³¹P: 161.9 MHz) spectrometer, which

222 was used to measure the ^{31}P spectra, proton (^1H), cross-polarization (CP) and the t1
223 (direct correlation between ^{31}P and ^1H).

224 More specifically, Bruker AVANCE I; 9.4T (^1H : 400 MHz; ^{31}P : 161.9 MHz)
225 spectrometer was used to measure the ^{31}P spectra, proton (^1H), cross-polarization
226 (CP) and the t1 (direct correlation between ^{31}P and ^1H). The 4 mm probe heads were
227 set with samples and submitted to 700 Hz of Magic Angle Spinning (MAS) speed.
228 Quantitative experiments at 25°C of ^{31}P were done with high power decoupling-
229 recycle delay: 30s; 90° pulse; RF field (^{31}P): 65 kHz; ^1H decoupling (RF field: 60 kHz;
230 SPINAL64); 1024 accumulations. The chemical shifts were given in parts per million
231 (ppm) concerning the analysis of H_3PO_4 (85%) for ^{31}P NMR spectra at 0ppm.

232 In the quantitative spectra, the area of the different peaks in the spectrum
233 corresponds to the relative abundance of each phosphorus species obtained from
234 the ^{31}P -NMR analysis. The NMR spectra was simulated using the DMFIT software
235 (Peixoto et al., 2017; Massiot et al., 2002) to access the area of each peak that
236 corresponds to the different species of phosphorus present, that forms the ^{31}P -NMR
237 spectra, in the CMs dispersions. The peak's decomposition is presented in the
238 (Figure1, SI). As a control for the demineralized samples, the signal from «native»
239 sample was used, where 100% of the signal was considered as representative signal
240 for the attached phosphoserine and CCP.

241 The peaks decomposition is presented in figure 1 in supporting information.
242 The signal from the «native» sample used as representative of the 100% of the
243 signal for the attached phosphoserine and CCP and was used as a control for the
244 demineralized samples. The fit is made following a standardized protocol already
245 described (Peixoto et al., 2017) based in a first step of manual fit followed by an
246 automatic adjustment made by DMFIT software.

247 Concomitantly, a NMR ^1H - ^{31}P cross-polarization experiment have been made
248 to study the calcium/phosphorous concentration in the CCP by studying the NMR
249 signal of the organic phosphorous from the phosphoserines residues, attached to the
250 CCP. The principle is that, in our conditions organic phosphorous from the
251 phosphoserines residues display a chemical shift anisotropy (CSA) shape is sensitive
252 to strength of the hydrogen bond network as well as the ionic environment of the
253 phosphorous (Gardiennet-Doucet, Assfeld, Henry, & Tekely, 2006). The ^1H - ^{31}P
254 cross-polarization experiments strengthen specifically the signal of the chemical shift
255 anisotropy (CSA) signal of the attached phosphoserines phosphorous (in contrast to

256 the signal of inorganic phosphorous that does not display a methyl proton at its close
257 environment) (Peixoto et al., 2017) and, by doing so, allows a better access the CSA
258 parameter of the organic phosphorous.

259

260 **2.1.7 Statistical analysis**

261

262 The data presented in this study were analyzed using an analysis of variance
263 (ANOVA) followed by a Tukey test at $p < 0.05$; and a principal component analysis
264 was performed for the data from the fluorescence spectroscopy.

265 **3 RESULTS and DISCUSSION**

266

267 **3.1 Demineralization impacts on the internal organization of the CMs structure**

268

269 **3.1.1 The stability of hydrophobic interactions evaluated through fluorescence** 270 **spectroscopy**

271

272 In the present study, no significant difference of fluorescence intensity, or λ -
273 max (the wavelength at which the tryptophan of the samples exhibit the maximum of
274 its absorbance), has been observed between the «native» CMs and the three
275 calcium-demineralized CMs samples (Figure 3, SI).

276 These results demonstrate that in comparison to the «native» samples, even
277 the most demineralized samples (DM-25) do not display a significant change in the
278 molecular environment of the tryptophan. This indicates that demineralization does
279 not have any significant effect on the hydrophobic interactions between proteins, at
280 least in the present level of demineralization. The CCPs are attached to the
281 hydrophilic or charged structures of the CMs, which are primarily composed of
282 phosphoserine residues including some glutamate residues (Walstra et al., 2006). In
283 our understanding, the demineralization that induces depletion of CCPs from the
284 CMs is responsible for the changes in the charge and hydrophilic interactions
285 between the internal proteins that form the CMs. In Literature (Horne, 2017).

286

287 **3.1.2 Organic and inorganic ^{31}P distribution and the environment by NMR**

288

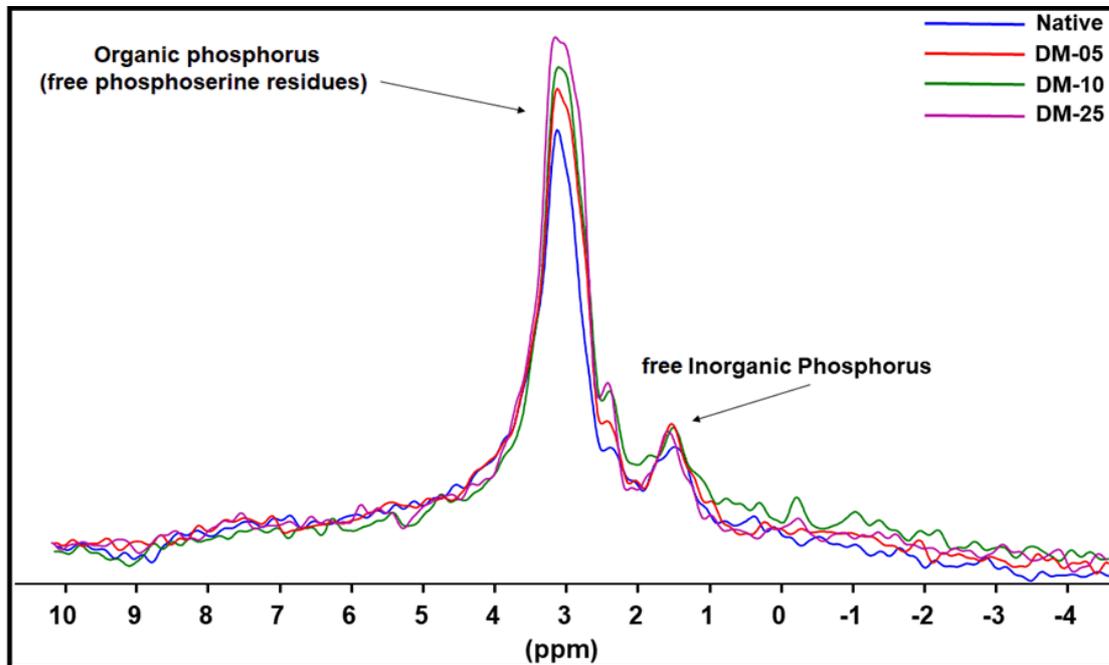
289 Since CCPs act as a cross-linking centers in casein micelles, the decrease in
290 the amount of CCP is an essential factor governing the CMs structure (De Kruif &
291 Holt, 2003). The Calcium and phosphorous ions within the CCPs are in equilibrium
292 with the ions in the soluble phase, and this equilibrium depends on the chelating
293 properties of the local structure of the micelle (Bijl et al., 2019).

294

295 The ^{31}P -NMR spectra corresponding to the organic phosphorus from the free
296 phosphoserine residues and inorganic phosphorus present in the soluble phase (free
297 inorganic phosphorous) can be seen in figure 2. As it can be clearly observed, that

298 more demineralized is the sample (DM-25), the greater is the amount of free
299 phosphoserine residues.

300



301

302 **Figure 2** – Zoom in the region displaying the sharper peaks in the ^{31}P nuclear magnetic resonance
303 (NMR) spectra from casein samples with different demineralization levels, being: («native») = »native»
304 casein; (DM-05) = 4.47% demineralized casein; (DM-10) = 9.16% demineralized casein; (DM-25-
305 25) = 25.73% demineralized casein.

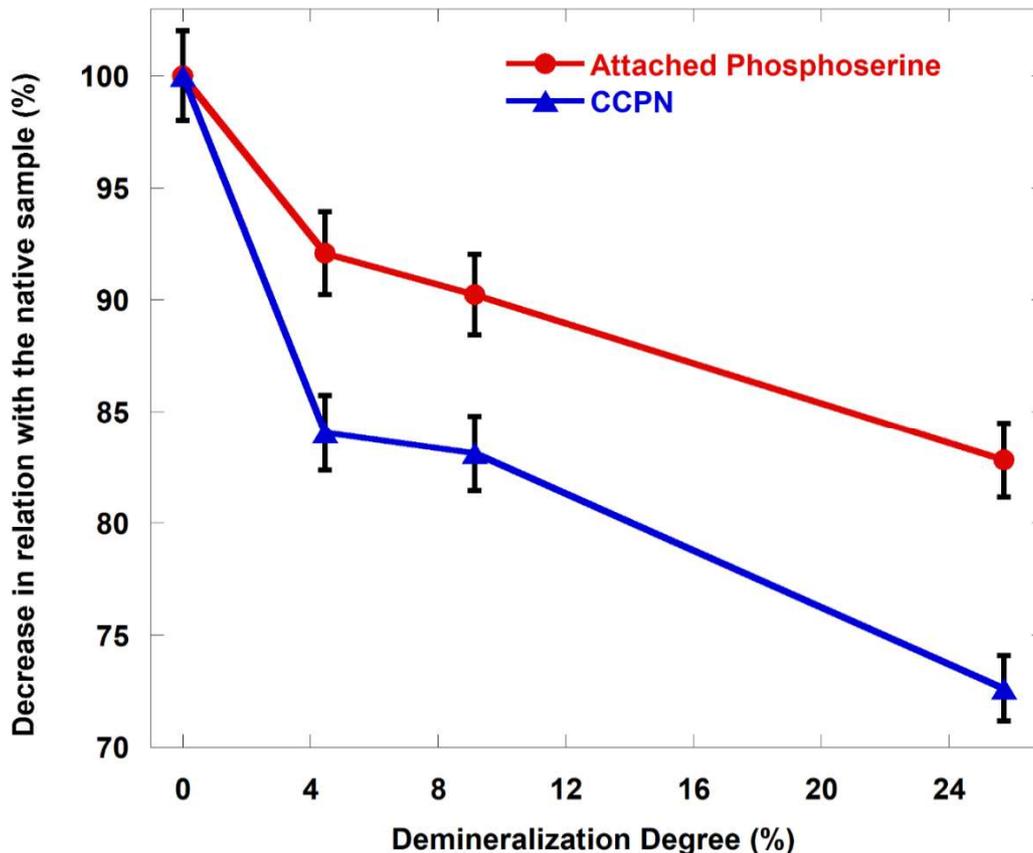
306

307 Concomitantly to the increase in the number of free phosphoserine residues
308 as observed represented in Figure 2, for the demineralized samples, a decrease in
309 the number of attached phosphoserine residues (figure 3) and attached inorganic
310 phosphorous is observed.

311 The decrease in the number of attached phosphoserines residues has been
312 reported to be related to the loss of phosphoserine residues attached to the CCPs as
313 a result of CM demineralization (Famelart et al., 2009). It is important to take into
314 account that some amount of the measured free ^{31}P in all samples is likely to come
315 from the κ -caseins' phosphorous which are not bound to CCP. In figure 3, it can be
316 observed that the amount of inorganic attached phosphorus displays the same trends
317 as the CCP.

318 Quantitatively, the results from ^{31}P -NMR (figure 3) reveals, that the decrease
319 of attached phosphoserines and attached CCPs, as a function of demineralization
320 display a nonlinear relation. As a function of the demineralization level, There is a
321 more substantial loss of attached species (phosphoserine residues and CCP) for the

322 less demineralized samples than for the most demineralized sample. Indeed, the
323 difference in the total calcium content between the «native» and the two less
324 demineralized samples (DM-05 and DM-10) is only 5% and 10%, but this represents
325 more than 15% and 17% of the loss in the attached inorganic phosphorus. In
326 contrast, for the most demineralized sample (DM-25) the total loss in calcium content
327 (-25%) is quite equivalent to the loss of attached inorganic phosphorus (CCP).
328



329

Figure 3 - Decrease in the number of attached phosphoserine (P-Ser) residues (●) and CCP (▲) as a function of the different demineralization degrees of casein dispersions. These results were obtained from the differences obtained from the fitting of the ³¹P-NMR of a «native» casein micelle dispersion and three different degrees of demineralization (DM-05) 04.47% less calcium; (DM-10) 9.16% less calcium and (DM-25) 25.73% less calcium.

330 It is important to notice that in the first step (from DM-05 to DM-10) the loss of
331 attached phosphoserines residues is less accentuated (about -7%) than the loss of
332 CCP phosphorous (between -15-17%). In contrast, for the more demineralized
333 samples the loss seems rather similar for both, phosphoserines and CCP
334 phosphorous.

335 The present study is in accord with our previous work (Peixoto et al., 2017)
336 and the signal of the attached phosphorus from the phosphoserine residues, displays
337 a clear CSA (chemical shift anisotropy) shape (Figure 1, SI). As explained in the
338 experimental section, the shape of the CSA is sensitive to strength of the hydrogen
339 bond network around the phosphoryl oxygens as well as the close ionic environment
340 (Gardiennet-Doucet et al., 2006). This makes the CSA signal highly informative about
341 the proximity of cations as calcium around the phosphorous from the
342 phosphoserines. The data presented in supporting information shows that
343 demineralization induces only small changes in the CSA signal shape of attached
344 phosphoserines suggesting that, even in strongly demineralized samples, most of the
345 remaining CCP cluster keeps a near-»native« composition in terms of Ca/P ratio.

346 Since this last result indicates that, even in the most calcium-demineralized
347 sample (DM-25), the Ca/P ratio in the CCP remains close to the «native» sample, the
348 observed loss of inorganic phosphorus is likely to correspond to equal loss of calcium
349 from the clusters. Thus, such result indicates that demineralization does not strongly
350 affect the properties of the remaining clusters in the demineralized samples in terms
351 of size and composition. Indeed, smaller clusters after the loss of about 25% of
352 inorganic and organic phosphorous as displayed by our data should display a higher
353 surface tension, which might impact the hydrogen bond network around the CCP.
354 Instead, the data indicate that the remaining clusters in demineralized samples
355 display a quite «native»-like order and composition. It is possible that a loss of only
356 15% of calcium phosphate ions in the clusters would not induce a detectable change
357 in the H-hydrogen network around the surface of the cluster. However, it is unlikely
358 that a loss superior to 25% of the ions would induce any detectable change in CSA,
359 considering if this loss is homogenous for all clusters. Thus, the scenario more
360 compatible with our data is that demineralization has induced a complete depletion of
361 some clusters at least in the case of the most demineralized sample.

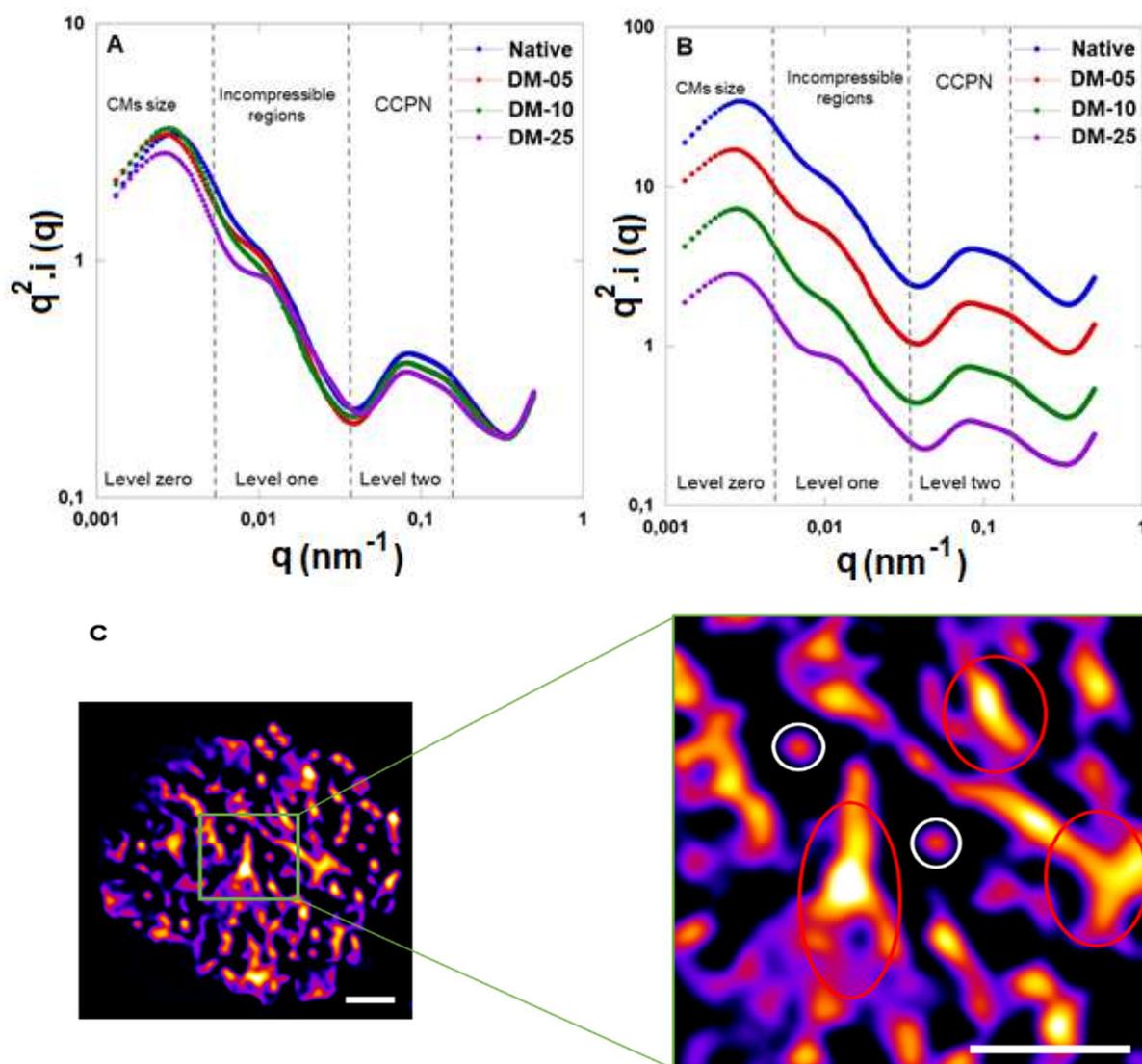
362

363 **3.1.3 Internal structures reorganization evaluated through SAXS:**

364

365 In this section, SAXS data analysis has been used to study the impact of
366 demineralization over the cluster size, as well as over CM structures (~5 to 50 nm).
367 Each “shoulder” of the SAXS profile (figure 4) can be assigned to a characteristic
368 structure of the micelle (see details in the experimental section).

369 As it concerns level 2 proteins associated with the “sequestered” CCP (P-
 370 CCP), one can notice that demineralization induces some decrease in intensity of the
 371 corresponding “shoulder” (figure 4a corresponding to level two), but there is no
 372 detectable shift. This same feature have been observed in previous works in
 373 presence of calcium chelator agents or in lowering of pH (Ingham et al., 2016). Such
 374 feature is interpreted as a loss of local inhomogeneous protein structure around the
 375 cluster.
 376



377
 378 **Figure 4** - Internal characterization obtained through Small Angle X-Ray Scattering (SAXS) of casein
 379 samples with different demineralization levels being: («native») = »native« casein; (DM-05) = 4.47%
 380 demineralized casein; (DM-10) = 9.16% demineralized casein; (DM-25) = 25.73% demineralized
 381 casein. (A) = SAXS spectra from all the samples, Results from an average of three repetitions and its
 382 duplicates. (B) = SAXS spectra from the separated samples*;
 383 * To obtain this graphic representation, the intensities were multiplied by a factor (~x3) only to present
 384 samples separately.

385 (C) Schematic representation of an internal structure of a CMs, being the red circles representing the
386 «Hard» structures that contain the CCP and the white circles the CCP present in the “void” regions,
387 scale bar correspond to 50 nm. The three levels are represented in figure 4A, being: Level zero, which
388 corresponds to the CMs size; Level one, which represents the “hard” incompressible structures, and
389 level two that are characteristic of the CCP region.

390 One can also notice, that such a relative decrease in the “shoulder” intensity
391 correlates quite well with the relative decrease in the amount of phosphorous in the
392 CCP clusters, which is detected by NMR as a function of the demineralization level.
393 Indeed, as it is the case for the amount of organic and inorganic phosphorus
394 measured by NMR (figure 3), from the «native» sample to DM-05. There is a
395 significant loss of SAXS intensity for this “shoulder”, but not a loss as significant as
396 for the other demineralized samples (DM-10 or DM-25).

397 Looking now at the second “shoulder,” the one associated with the so-called
398 “Hard” structures (~10 to 40 nm) in the CM (Bouchoux et al., (2010), (Ingham et al.,
399 2016). The detected decrease in intensity of this “shoulder” seems to be uncorrelated
400 to the decrease in the intensity of the CCP “shoulder.” Indeed, upon comparing the
401 «native» sample with DM-05, no remarkable changes in the intensity or the shape of
402 the profile of the “Hard” structures has been observed. It seems that the substantial
403 loss of CCPs detected by SAXS and NMR did not affect much of the micellar
404 structure at this scale range. In contrast, passing from DM-05 to DM-10 and,
405 subsequently, to DM-25, there is a small and progressive shift of the “shoulder” of
406 these “Hard” structures to larger Q. These shifts indicate that in the samples with
407 highest degree of demineralization, an internal reorganization of these “Hard”
408 structures has been induced.

409 The interpretation concerning the last “shoulder,” the one at the largest scale
410 and the lowest Q, is not possible from our data alone. Since this signal represents
411 mixture of signals coming from the CM’s radius of gyration (the form factor of the CM)
412 as well a change in the average inter-micellar particle distances in solution (the
413 structure factor). This last factor is susceptible to change of micelle size, which will
414 decrease the average inter-micellar distance in the solution and also with a change in
415 stickiness and repulsiveness between the CMs, which will affect the distribution of
416 inter-micellar distances in the solution.

417

418

419 **4. Discussion**

420

421 According to presented data in current study there is no significant
422 modification of the hydrophobic interactions between samples. It has been suggested
423 that hydrophobic interaction play a role in inter-protein interaction in casein (De Kruif,
424 C. G., 2014). Thus, the presented data suggest that demineralization did not change
425 much the protein-protein interactions in the samples.

426 Our data from current study also show that the measured loss of ions from
427 CCP (as detected by NMR) as well the decrease in attached phosphorylated
428 residues correlate quite well with the decrease of the P-CCP “shoulder” in SAXS
429 data. This observation agrees with the nanocluster model since, according to this
430 model CCP acts as a coordination center for the local protein network attached to it.
431 Thus, in this model the loss of the interaction between the phosphorylated center of
432 casein and the ions from the CPPs should always induce an observable loss of local
433 protein network around the CCPs.

434 In our study, NMR data indicate that the loss of ions from the CCPs does not
435 decrease the overall size of the CCPs but rather completely removes some CCPs
436 from the micelles leaving the remaining ones quite intact. This scenario is also
437 supported by the physical-chemistry of such objects (Bijl et al., 2019; Cross et al.,
438 2005; Holt, C., 2004). Indeed, the loss of the local protein network around CCPs
439 means that some protein “capping” the CCP have been detached. It has been clear
440 from published work, that a reduction in the amount of capping proteins around a
441 nanocluster is rather a factor that favors the instability of the CCP clusters (Bijl et al.,
442 2019; Cross et al., 2005; Holt, C., 2004). The fewer phosphorylated residues (from a
443 protein or a peptide) “capping” a CCP, the larger and unstable the CCP should be
444 (Holt et al., 2004). Thus, it is unlikely that the decrease in the amount of ions and
445 sequestering proteins forming the P-CCPs would induce a global decrease of the
446 CCPs size. Rather than that, it is more likely that the first clusters to lose ions (and
447 the capping proteins) will become quickly unstable leading to their complete excision
448 from the micelles (agreement with NMR data).

449 Indeed, this scenario is coherent with the fact that the presented NMR data do
450 not show a simple correlation between the total loss of calcium and phosphorus in
451 the samples and the specific loss of calcium and phosphate from the CCPs. The less
452 demineralized samples (DM-05 and DM-10) display a greater loss of these ions from

453 the CCPs (about 15% and 17% respectively) than the measured loss of calcium
454 phosphate in the samples (which is -5% and -10% respectively).

455 This means that there is a difference in equilibrium in terms of bound/free ions
456 for the CCPs between the less demineralized samples and the most demineralized
457 ones. Such change in equilibrium indicates a structural difference in the sequestered
458 P-CCPs. In the «native» sample, CCPs are likely to be composed of some fraction
459 (around 15-17%) of CCPs displaying fewer “capping” phosphorylated residues. Such
460 CCPs are the first ones to be removed from the micelle by demineralization, since
461 they are the less stable ones. This scenario explain quite well why NMR detected a
462 less important loss of attached phosphorylated proteins in the less demineralized
463 (comparing the «native» to the DM-05) samples than for the most demineralized
464 ones (comparing the “DM-10” to the DM-25). Since the first clusters to be removed
465 are likely to be the ones with less “capping” proteins, which explains their greater
466 instability. Their depletion will generate a weaker loss of attached phosphorylated
467 residues than the depletion of the more stable ones.

468 The data show, that the measured loss of ions from CCP (as detected by
469 NMR) as well the decrease in attached phosphorylated residues correlate quite well
470 with the decrease in intensity of the CCP “shoulder” in SAXS data (table 1).
471 Noteworthy, a quasi-linear relation between the amount of proteins capping the
472 phosphate centers and the intensity of the CCP “shoulder” observed in SAXS, in this
473 demineralization range, is expected by the model used to interpret SAXS data
474 (Ingham et al., 2016). This observation supports the nanocluster model view that all
475 the CCP in the micelles acts as a coordination centers for the local protein network
476 attached to it. Thus, in this model the loss of the interaction between the
477 phosphorylated center of caseins and the ions from the CPPs should always induce a
478 rather important loss of protein inhomogeneities around the CCPs.

479

	SAXS loss in intensity of the CCP « shoulder » (%)	NMR Amount of P Ser (%)
« Native »	100	100
DM-05	92	93
DM-10	92	92

480 **Table 1.** Comparison between the amount of attached phosphorous from phosphoserines
481 (PSer) as quantified by NMR and the intensity of the CCP “shoulder”. The intensity of the “native”
482 samples is considered as 100%.

483 In the literature, much of discussion about the composition, the structure and
484 or the spatial distribution of the nanoclusters in the micelle have not considered the
485 possibility of the presence of CCPs with different stabilities (Dalgleish, 2011, Bhat,
486 Dar, & Singh, 2016; Broyard & Gaucheron, 2015). Probably, the main reason for this
487 is that most articles studying the evolution of the structure of CCP upon
488 demineralization have been relying on the quantification of the evolution of the
489 number of colloidal/free ions or/and structural data from SAXS or TEM (Bhat, Dar, &
490 Singh, 2016; Broyard & Gaucheron, 2015; Dalgleish, 2011; Dalgleish & Law, 1989;
491 Griffin, Silva, et al., 2013; Lyster, & Price, 1988; Udabage, McKinnon, & Augustin,
492 2000). From SAXS and TEM data alone is very hard to correlate the decrease in the
493 micellar CCP content and the structural modification of the micelle and, so far at our
494 knowledge, ³¹P NMR have not been used to studied samples with displaying a range
495 of demineralized levels as it have been done in this work.

496 The question now is the relation between the depletion of those small-scale
497 structures with the observed structural changes in a large scale “Hard” structures.
498 Bouchoux et al.’s (2010) data indicate that the signal of those structures corresponds
499 to large inhomogeneities in the CM. De Kruif, (2014)’s SAXS model did not take into
500 account the “Hard Structure” “shoulder” observed in SAXS data, probably because
501 SANS data in the same work (De Kruif, (2014) have showed that the micelles display
502 a rather homogenous structures at medium to large scales (>8 nm). Nevertheless,
503 more recent work (Ingham et al. 2016) have taken into account this shoulder in their
504 model and concluded on the note that their data is consistent with the interpretation
505 of Bouchoux et al. (2010): the intermediate-q feature is due to some medium scale
506 inhomogeneities within the micelle (Ingham et al. 2016) which also is supported by
507 cryo-TEM data (Trejo et al. 2011).

508 Ingham et al. (2016) have showed that the “Hard” region shoulder in SAXS
509 data increases in intensity after some hours at 25°C, without any change in intensity
510 elsewhere in the spectrum. Moreover, it has also been observed that micelles from
511 different sources can display different intensities in this region (Day, L. et al., 2017).
512 These data suggest a scenario where regions of the micelle could be re-structured to

513 form these “Hard regions” due to the destabilising factors such as calcium
514 concentration, pH, temperature and time.

515 In highly demineralized samples (Ingham et al., 2016), the removal of 22% to
516 45% of minerals (addition of 5 mM and 10 mM of EDTA respectively) induces only
517 very small changes in the SAXS signal of the “Hard” structure. One must conclude
518 that there is a reasonable amount of proteins located in “Hard structures” and those
519 proteins are not directly interacting with the CCP clusters, as the nanoclusters model
520 would suggest, but rather they are stabilized by a larger protein-protein network. So
521 any modification in the small scale structures (the P-CCPs) would not necessarily
522 induce a change in such large scale inhomogeneity.

523 Indeed, in the present data, only the samples with higher degree of
524 demineralization display a significant decrease in the intensity of these “Hard”
525 regions. Thus, the observed local depletion of the P-CCP structures does have an
526 almost any impact in the decrease of these large scale inhomogeneities. Indeed, for
527 the less demineralized samples it is likely that the caseins previously attached to
528 CCP, somehow still part of a large scale inter-protein network. In this way the
529 excision of the CCP always strongly affect the small scale structure of the CM (the P-
530 CCP centers) but it does not affect (at least for the less demineralized samples) as
531 strongly, the large scale inhomogeneities. Thus, this specific behavior of “hard”
532 region observed in this work is strong evidence that the micelles are composed of a
533 medium scale protein-protein network generating these inhomogeneities.

534

535 **5 CONCLUSIONS**

536 This study aimed to evaluate the impact of the demineralization on the internal
537 organization of the CMs structures of a dense casein micelle dispersion. Our data
538 quite clearly indicate that demineralization does not induce a change in
539 hydrophobicity of the micelle (at least at the demineralization levels studied), which
540 suggests that no hydrophobic interactions substantial protein-protein reorganization
541 occurs. It also shows that casein micelles display some clusters with strong stability
542 than others indicating a structural heterogeneity in the complex CCPs (and proteins
543 associated) found in the micelle. The loss of the less stable CCPs induces only a
544 structural change at the smaller scale. In contrast, the loss the more stable clusters
545 induce structural changes at the both, small as well at the medium scales. The data

546 from current study is an improvement in the sponge-like model described by
547 Bouchoux et al., (2010) and at the same time adds more information about the role of
548 the nanoclusters in keeping the structure of the micelle.

549

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551

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557

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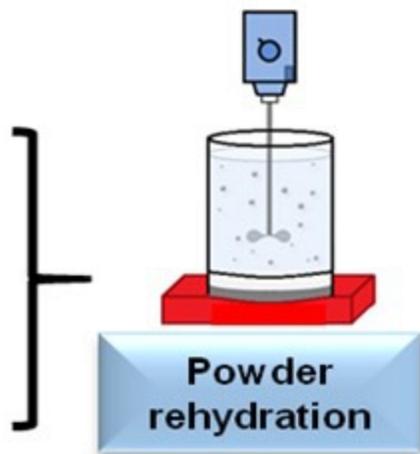
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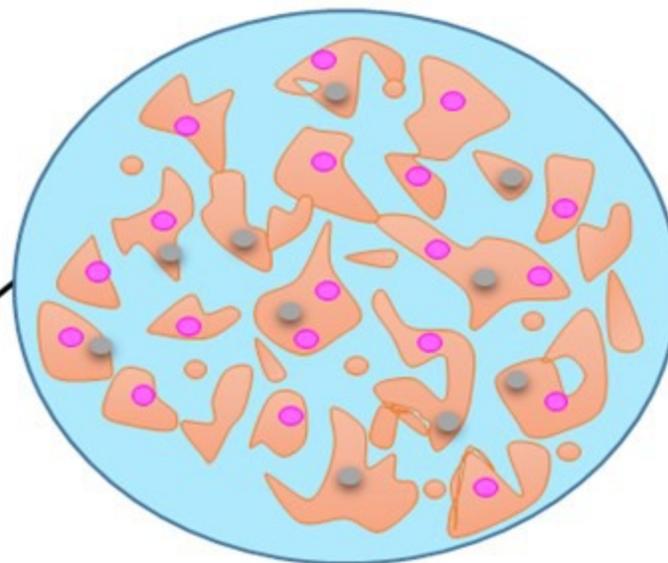
Casein-rich powders with different demineralization degree (%)

0%
5%
10%
25%

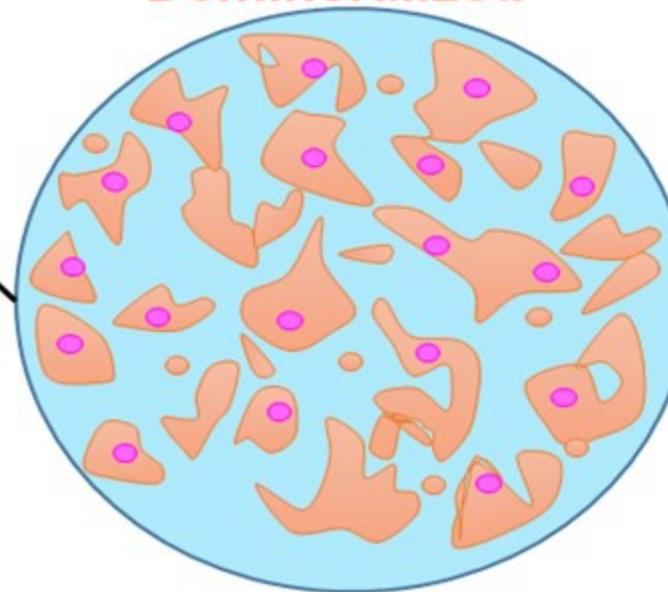


Rehydrated to 14% of protein

Native



Demineralized



 So-called "Hard structures"

 More instable colloidal calcium phosphate nanoclusters (CCPN)

 More stable colloidal calcium phosphate nanoclusters (CCPN)