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PFG-NMR self-diffusion in casein dispersions: effects of probe size and protein aggregate size

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Abstract

The self-diffusion coefficients of different molecular weight PEGs (Polyethylene glycol) and casein particles were measured, using a pulsed-gradient nuclear magnetic resonance technique (PFG-NMR), in native phosphocaseinate (NPC) and sodium caseinate (SC) dispersions where caseins are not structured into micelles. The dependence of the PEG self-diffusion coefficient on the PEG size, casein concentration, the size and the mobility of casein obstacle particles are reported. Wide differences in the PEG diffusion coefficients were found according to the casein particle structure. The greatest reduction in diffusion coefficients was found in sodium caseinate suspensions. Moreover, sodium caseinate aggregates were found to diffuse more slowly than casein micelles for casein concentrations > 9 g/100 g H_2O. Experimental PEG and casein diffusion findings were analyzed using two appropriate diffusion models: the Rouse model and the Speedy model, respectively. According to the Speedy model, caseins behave as hard spheres below the close packing limit (10 g/100 g H_2O for SC (Farrer & Lips, 1999) and 15 g/100 g H_2O for NPC (Bouchoux et al., 2009)) and as soft particles above this limit. Our results provided a consistent picture of the effects of diffusant mass, the dynamics of the host material and of the importance of the casein structure in determining the diffusion behavior of probes in these systems.

Keywords: Diffusion, casein micelle, sodium caseinate aggregates, PEG, Rouse model, Speedy model.

I. Introduction

Molecular transport, as characterized by diffusion coefficients, is a key feature of food processes and particularly of dairy processes. For example, the transformation of milk into cheese involves many operations such as coagulation, draining, salting and ripening, in which water and solute diffusion are important parameters that affect the microbiological and sensorial stability of cheese. Molecular transport behavior will obviously be different depending on the composition and microstructure of the dairy matrix. Caseins make up to 80% of the protein content of milk (Holt, 1992). Native phosphocaseinate (NPC) and sodium caseinate (SC) are two casein systems that exhibit differences in structure. In a native phosphocaseinate solution, caseins exist as large colloidal particles called “casein micelles”, which contain the four caseins, α_{s1}, α_{s2}, β and κ, in the proportions of 3:1:3:1, and ~8% in mass of phosphate and calcium ions (Holt, 1992). The structure of the casein micelle has been studied for over 40 years and quite precise descriptions are available, although they are still controversial (Horne, 2006). It is commonly accepted that micelles are roughly spherical core-shell particles with outer diameters ranging from 50 to 500 nm (Dalgleish, Spagnuolo, & Douglass Goff, 2004; de Kruif, 1998; McMahon & McManus, 1998). The core is now generally described as a homogeneous network of caseins in which calcium phosphate nanoclusters are uniformly distributed (Horne, 2002; Marchin, Putaux, Pignon, & Leonil, 2007; McMahon & Oommen, 2008). The shell is essentially made of κ-casein parts with C-terminal sides, which protrude into the aqueous phase of the milk and provide steric and electrostatic stabilization of the particles (Horne, 1996; Sandra, Alexander, & Dalgleish, 2007). Casein micelles are very porous, highly hydrated and sponge-like colloidal particles containing approximately 3.4 g H_2O/g protein (Morris, Foster, & Harding, 2000). Even if these two systems contain the same relative amount of caseins, SC systems are quite different from casein micelles with respect to structure (HadjSadok, Pitkowski, Nicolai, Benyahia, & Moulai-Mostefa, 2008; Lucey, Srinivasan, Singh, & Munro, 2000; Radford & Dickinson, 2004) and interactions because they do not contain calcium phosphate nanoclusters (Farrer et al., 1999). Caseins are present as individual molecules

50 in these dispersions, or as reversible self-assembled aggregates with a radius of ~ 11 nm (HadjSadok et
51 al., 2008; Lucey et al., 2000; Radford et al., 2004).

52 Among the different methods of approach, pulsed field gradient nuclear magnetic resonance (PFG-
53 NMR) provides a convenient means for measuring translational diffusion (Price, 1997). This technique
54 was used because it permits nondestructive, fast and precise polymer self-diffusion coefficient
55 measurements. Numerous PFG-NMR studies have been performed on dairy matrices such as NPC and
56 SC systems. Some of these studies have focused on investigating the dynamic properties of casein
57 proteins in relation to the protein concentration by observing changes in their self-diffusion coefficients.
58 For example, (Tan & McGrath, 2010) measured casein self-diffusion coefficients in SC suspensions
59 with casein concentrations ranging from 2 to 20% w/w. (Mariette, Topgaard, Jonsson, & Soderman,
60 2002) measured casein diffusion in NPC suspensions for different casein concentrations ranging from
61 0.03 to 0.19 g/g. (Le Feunteun, Ouethrani, & Mariette, 2012) monitored the diffusion of casein particles
62 during the renneting of a concentrated casein micelle suspension (14% w/w). Other studies, however,
63 have concentrated on measuring self-diffusion coefficients of PEG polymer probes of different
64 molecular weights in NPC suspensions and gels. A first study was accomplished in NPC suspensions
65 and rennet gels by (Colsenet, Soderman, & Mariette, 2005). This study was expanded by (Le Feunteun
66 & Mariette, 2007) who investigated the translational dynamics of PEG polymers with molecular weights
67 varying from 6.10^2 to 5.10^5 in casein suspensions and in gels induced by acidification, enzyme action
68 and a combination of both. The main findings of these studies were that probe diffusion was affected
69 by: (i) the casein concentration, i.e., PEG diffusion decreases with increasing casein concentration; (ii)
70 the size of the PEG, i.e., for a given casein concentration, PEG diffusion decreases as polymer size
71 increases; and (iii) the state of the matrix, solution or gel, i.e., PEG diffusion increases after coagulation.
72 This effect was greater when the size of the probe was larger.

73 The diffusion coefficients of PEG observed in NPC suspensions and gels were explained by assuming a
74 model with two diffusion pathways, one around and one through the casein particles. According to the

75 “two pathways” diffusion model, variations in the diffusion rate of a molecule depend only on its ability
76 to diffuse through the casein particles and the volume fraction occupied by them. This model implies
77 that the diffusion of larger molecules is more affected by the presence of casein particles because they
78 can less easily diffuse through them. These large probes could diffuse only around casein micelles.

79 The aim of the study presented here was to improve the understanding of PEG diffusion in casein
80 systems (NPC and SC) both from the experimental and theoretical points of view. In this paper, PEGs
81 with different molecular weights and casein protein self-diffusion coefficients were measured in both
82 casein systems in order to provide some answers to the following questions:

83 (i) Does the intra-micellar diffusion mechanism adopted in NPC suspensions exist? This question
84 is tackled by comparing the diffusion behavior of PEGs in NPC and SC dispersions. The
85 particularity of the SC system resides in the fact that the extra-micellar diffusion mechanism is
86 the only one to be considered in these suspensions. PEGs cannot diffuse inside SC aggregates
87 because of their small size. Thus, according to the interpretation cited above, small PEG
88 diffusion should be more rapid in the SC system since they encounter fewer obstruction effects
89 when diffusing around casein aggregates in SC suspensions.

90 (ii) What is the effect of casein protein size and mobility in determining the diffusion behavior of
91 PEG polymers?

92

93

II. Experimental

Materials. Native phosphocaseinate powder was prepared at the INRA laboratory (Rennes) and the sodium caseinate powder was provided by Armor Protéines (Saint-Brice en Coglès, France). The detailed composition of the powder is given in Table 1. PEGs with different molecular weights (M_w) and low polydispersity indices (M_w/M_n) were obtained from Varian Laboratories (Massy, France). Sodium azide (Merck, Darmstadt, Germany) and sodium chloride (Sigma-Aldrich, Steinheim, Germany) with purities above 99% were used without purification.

	Total solids (g/Kg)	Total nitrogen matter (g/Kg)	Non-casein nitrogen (g/Kg)	Non-protein nitrogen (g/Kg)	Pure caseins (g/Kg)
NPC (%)	100	88.20	6.90	0.20	81.30
SC (%)	100	95.20	0.84	0.17	94.36

Table 1. Composition of NPC and SC powders.

Dispersion preparation. Rehydration of the powders with NaCl/water solution (0.1 M) was performed at room temperature over 36 h under constant stirring for micellar casein, and at 40°C over 24 h for sodium caseinate. Sodium azide was added (0.02% w/w) to each solution to prevent bacterial development. The solutions were studied without pH adjustment. The pH of SC solutions was 6.6±0.04 for casein concentrations ranging from 1 to 24 g/100 g H_2O, while the pH of the micellar casein dispersion was higher, ranging from 7.1 at 3 g/100 g H_2O to 6.9 at 22 g/100 g H_2O. Once the powder was totally rehydrated, 0.1% w/w of PEG was added to casein suspensions, regardless of the molecular weight. The dry matter of all casein suspensions was controlled by measuring variations in weight after

drying in an oven for 24 h at 103°C. Casein concentrations were calculated from values of dry matter content in each casein suspension and the pure casein percentage of the dry matter (Table 1).

Dynamic light scattering. Dynamic light scattering measurements of casein particles in NPC and SC solutions were performed on a Malvern Zetasizer Nano ZS instrument (Malvern Instruments, Worcestershire, UK). Size distribution of casein particles in solutions was obtained by measuring the light scattered by casein particles illuminated with a laser beam (scattering angle = 173°, T° = 20°C) using the NNLS analysis method (nonnegative least squares). The measured data were reported in a log-normal intensity distribution.

NMR measurements. A PFG-NMR experiment yields a series of decaying 1D-spectra. The chemical shift resolution of NMR, which enables identifying the different chemical environments that exist within a molecule and/or studying the different constituents of a mixture is thus preserved. As an example, Figure 1 presents a ^{1}H-PFG-NMR spectrum of the casein suspension we studied. It shows that the three main constituents of our sample were well-separated by ^{1}H-NMR. The signal coming from water molecules, which was suppressed with the PFG-NMR sequence we used, resonate at 4.7 ppm. The sharp peak at 3.6 ppm is the signal coming from the 0.1 % (w/w) of a 32530 g/mol polyethylene glycol (PEG). All the other signals come from the protons of the casein molecules.

PEG and casein self-diffusion measurements were performed on a 500 MHz spectrometer equipped with a dedicated field gradient probe (DIFF30 from Bruker, Wissembourg, France) with a static gradient strength of 1200 (±0.2) G/cm for an amplifier output of 40 A. Diffusion spectra were acquired with a stimulated echo sequence using bipolar gradients (STE-BPP) and a 3-9-19 WATERGATE pulse scheme to suppress the water signal. Experiments were carried out with 16 different values of g, ranging from 20 to 900 G/cm, with δ = 1 ms (for PEG measurements) and δ values ranging from 1 to 2.2 ms (for casein measurements). Sixteen scans were carried out and the recycle delay was set at 5 T_1. Depending on the molecular weight of the PEG studied, Δ was adjusted to obtain a diffusion distance z of ~1.5 µm

in the casein suspension, in accordance with the Einstein equation, $z = (2\ D_{PEG}\ )^{1/2}$. This procedure enabled the molecular probes to cover much greater distances than the casein micelle diameter.

NMR processing methods. All the data processing was performed with Matlab and Table Curve software. Monte-Carlo simulations were used for error calculations with 200 iterations. In a PFG-NMR experiment using the BPP sequence, the echo intensity, I, is described by:

$$I / I_0 = \sum_i p_i \exp(-k\ D_i) \quad \textbf{(1)}$$

with

$$k = \gamma^2 g^2 \delta^2 (\Delta - \delta/3 - \tau/4) \quad \textbf{(2)}$$

where I_0 is the signal intensity in the absence of gradients, γ the gyromagnetic ratio (for protons, $\gamma = 26.7520 \times 10^7$ rad.T^{-1} .s^{-1}), g the amplitude of the gradient pulse, δ the gradient pulse duration, Δ the time between the leading edges of gradient pulses, τ the time between the end of each gradient and the next radiofrequency pulse, D_i the self-diffusion coefficient of the ith component, and p_i the fractional proton number of the ith component. The magnetization decay was analyzed using a monoexponential fitting (i=1) for PEGs and a biexponential fitting (i=2) for casein self-diffusion. The standard error in PEG and casein diffusion coefficients estimated by the fitting procedure was less than 10%.

Normalization of diffusion coefficients. The self-diffusion coefficients presented in Table 2 were used to normalize the diffusion coefficients measured in SC suspensions. Since the amount of soluble compounds is greater in NPC powder than in SC powder, their contribution to PEG diffusion hindrance in the aqueous phase cannot be neglected in NPC suspensions. To consider the effects of casein alone, we normalized the probe self-diffusion coefficients measured in NPC samples by the probe self-diffusion coefficient measured in the aqueous phase that includes these soluble compounds.

III. Results and Discussion

1. Dynamic light scattering

DLS was used to determine the size distribution of particles in suspensions of native phosphocaseinate (3 g/100 g water) as well as sodium caseinate (1 g/100 g water) containing 100 mM NaCl. Figure 2 indicated that particles of native phosphocaseinates presented a single broad population distribution from ~ 68 to ~ 459 nm in diameter. The mean casein micelle diameter was 187 nm. These results are in very good agreement with values already reported by several authors (Dalgleish et al., 2004; de Kruif, 1998; McMahon et al., 1998). In contrast, sodium caseinate solution was found to contain two distinct populations, a major one (98% of the total volume) with an average hydrodynamic diameter of approximately 22 nm, and a small weight fraction (2%) of particles with an average diameter of approximately 200 nm. Since large particles scatter more light than small particles, the relative scattering intensity of these particles can be strong, even if their weight fraction is small (Chu, Zhou, Wu, & Farrell, 1995). In the absence of NaCl salt, caseins are known to be mostly present in the form of individual molecules (HadjSadok et al., 2008). When electrostatic repulsion is screened by the addition of 100 mM NaCl, the hydrophobic parts of the casein molecules associate, leading to the formation of small micellar aggregates that are probably star-like particles (HadjSadok et al., 2008). Our results are in very good agreement with those of (HadjSadok et al., 2008; Panouille, Benyahia, Durand, & Nicolai, 2005). In the presence of 100 mM of NaCl and under the same conditions of temperature and pH, caseins were found to have a hydrodynamic diameter of approximately 22 nm. A second population of larger particles with R_h ~100 nm was also observed. However, the nature of these large particles is as yet unknown, but it is clear that they are not residual native casein as supposed by Nash et al. (Nash, Pinder, Hemar, & Singh, 2002) since they do not precipitate during ultracentrifugation.

2. Diffusion by NMR

2.1. Self-diffusion of PEGs in water

The molecular weight, polydispersity index and self-diffusion coefficients measured for all PEGs studied in water/0.1M NaCl are presented in Table 2. Hence, the hydrodynamic diameter radius of the PEGs can be calculated with the help of the Stokes-Einstein equation:

$$R_h = \frac{k_B T}{6\pi\eta D} \quad \textbf{(3)}$$

where T is the temperature in Kelvin, K the Boltzman constant (1.38 x 10^{-23} JK^{-1}), and η the viscosity of the aqueous phase (1x10^{-3} Pa at 20°C).

M_w	M_w/M_n	D ($m^2.s^{-1}$)	R_h (nm)
615	1.07	2.70E-10	0.79
7920	1.04	6.77E-11	3.16
21300	1.06	3.99E-11	5.37
32530	1.06	3.06E-11	7
93000	1.06	1.62E-11	13.22

Table 2. Self-diffusion coefficients and the corresponding hydrodynamic radii of various PEGs. Data obtained from NMR diffusion measurements in a H_2O/NaCl solution (0.1 M) at 20°C.

2.2. Self-diffusion of PEGs in SC suspensions

The PEG self-diffusion coefficients (615, 7920, 21300, 32530 and 93000 g/mol) were measured at 20°C in SC suspensions with casein concentrations ranging from 1.26 to 24.04 g/100 g water (Figure 3). At high protein concentrations, the solutions became highly viscous. The absence of restricted diffusion at the length-scale studied (~1.5 µm) was verified by measuring the self-diffusion coefficients of the 93000 g/mol PEG in a concentrated casein suspension at different diffusion times, thus probing a range

214 of distances. The results obtained (data not shown) demonstrated that the PEG self-diffusion coefficient
215 was the same, regardless of the distance traveled between 0.65 and 1.97 µm.

216 PEG diffusion coefficients were found to be dependent on both casein concentration and PEG size. PEG
217 diffusion decreased with increasing casein concentration, and for a given concentration, PEG diffusion
218 decreased as their size was important. The change in diffusion coefficients was more significant for the
219 probes of higher molecular weight and larger size, giving D/D_0 = 0.39, 0.13 and 0.016 for 615, 7920 and
220 93000 g/mol PEG, respectively, at a casein concentration of 24 g/100 g H_2O.

221 The effect of varying the NaCl concentration between 0 and 100 mM on the 7920 g/mol PEG self-
222 diffusion coefficients for a fixed concentration of 5 g/100 g of H_2O was also studied. Reducing the ionic
223 strength from 100 mM to 0.05 and 0 mM led to a decrease in the PEG-reduced diffusion from
224 0.692±0.01 to 0.679±0.01 and 0.620±0.01, respectively (data not shown). As stated above, if
225 monovalent salt is added so that electrostatic interaction is screened, the hydrophobic parts of the
226 individual casein molecules of R_h~3 nm associate, leading to the formation of small casein aggregates of
227 R_h~11 nm. The association number is determined by the balance of hydrophobic and electrostatic
228 interactions, which explains the observed diffusion increase with increasing ionic strength. Thus, these
229 results clearly showed that probe self-diffusion is dependent on the protein size: the smaller the particle
230 size, the slower the probe diffusion rate will be. This finding is in very good agreement with numerous
231 numerical simulations and studies in polymer systems (Colsenet et al., 2005; Gong, Hirota, Kakugo,
232 Naria, & Osada, 2000; Saxton, 1987; Tremmel, Kirchhoff, Weis, & Farquhar, 2003), which have
233 highlighted the effect of obstacle size or density on probe diffusion. It was found that the probe
234 diffusion coefficient strongly decreases with decreasing protein size or increasing protein density, as the
235 density is higher for a given composition with smaller particle sizes.

236 **2.3. Comparison of PEG diffusion in NPC and SC suspensions**

237

Figure 3 compares the PEG-reduced self-diffusion coefficient in NPC and SC suspensions as a function of casein concentration for different PEG molecular masses. D_{PEG} decreased with increasing casein concentrations for both NPC and SC. These results obtained in NPC suspensions are consistent with values already reported by Le Feunteun et al. (Le Feunteun & Mariette, 2008) for the 620 and 96750 g/mol PEGs in a concentrated NPC suspension (empty triangle and diamond shapes). However, the casein structure affected the intensity of the decrease. PEG diffusion was much more attenuated in the SC system. For example, at a casein concentration of 12 g/100 g H_2O, the decrease in D_r for the 615 g/mol, 7920 and 93000 g/mol PEGs was 0.76, 0.7, 0.51 in NPC suspensions and 0.61, 0.35, 0.12 in SC suspensions, respectively.

All these differences in diffusion behavior may be explained by the loss of the peculiar structure of the casein micelle in SC dispersions. These differences should thus, first of all, be attributed to the difference in the casein protein size. In polymer theory, the diffusion of polymer chains can be divided into two regions based on the chain length relative to pore size R (de Gennes, 1979b; Doi & Edwards, 1986). The first region for a Gaussian chain is observed when the gyration radius of the chain $R_g < R/2$, where the diffusion is describe by the Rouse model. The second behavioral region is observed when $R_g > R/2$, where the reptation theory, proposed by de Gennes, describes the movement of an unattached chain by Brownian motion in a many-chain or gel system. A simple power law provides a description of the solute diffusion coefficients versus molecular weight:

$$D = A.M^{-\alpha} \quad \textbf{(4)}$$

where A is a pre-exponential factor and α a characteristic exponent.

This equation is often used to describe the self-diffusion of polymer chains with α varying from 0.55 for dilute systems to 2 in concentrated systems. The Rouse model is a well-established model for non-entangled polymer chains. The diffusion of a high molecular weight polymer in an unentangled system, or a diluted solution, is described as follows by the Rouse model: $D \sim M_w^{-1}$. The reptation theory was

complementary to the works of Rouse and is expressed as $D \sim M_w^{-2}$. An intermediate mechanism was proposed by Favre et al. (Favre, Leonard, Laurent, & Dellacherie, 2001) to possibly explain the somewhat intermediate situation between negligible partial drainage of the solvent in an ideal statistical sphere (-0.5 or 0.6 exponent value) and the wormlike displacement of a linear molecule in a network of fixed obstacles (-2 exponent value). According to these authors, it can be assumed that an intermediate stage corresponding to an ellipsoidal solute shape may occur when system mesh size approaches the solute radius.

Equation 4 was applied to our data. The plots of D_{PEG} versus PEG molecular weight (M_w) in casein suspensions for different casein concentrations are given in Figure 4. All data were fitted with Equation 4, and values of α were extracted for each concentration. Figure 5 shows the different α values obtained in NPC and SC suspensions with casein concentrations varying between 0 (water + NaCl) and 24 g/100 g H_2O.

It can be seen that the exponent, which is close to the theoretical value of 0.6 for PEG diffusion in water (de Gennes, 1979a), gradually shifted towards higher values when the casein concentration (i.e., network density) increased. However, in the case of SC systems, the exponent shifted more rapidly towards higher values. For the NPC system, the α value varied between 0.58 and 0.84, with casein concentrations varying from 2.88 to 22.4 g/100 g water. The values of α, around 0.7, obtained for casein concentrations between 3 and 15 g/100 g H_2O, show that although the diffusion was reduced by casein micelles for these PEGs, this obstruction mechanism had no constraints on their spatial organization and they thus retained a spherical random coil form. If PEGs had diffused inside the micelle, the α values would have been higher since a PEG would be forced to change its conformation in order to diffuse inside the micelle. However, for casein concentrations > 15 g/100 g H_2O, the α values showed that PEGs adopted an ellipsoidal random coil form at this concentration and that the casein system mesh size approaches the solute radius. For SC systems, α values varied between 0.58 and 1.42, with casein concentrations varying from 1.26 to 24.04 g/100 g water. In addition, for a casein concentration > 6

287 g/100 g H_2O, PEGs assumed the aspect of statistically ellipsoidal random coils and there radius
288 approached the system mesh size.

289 The analysis above suggests that SC systems have higher densities than NPC systems for the same
290 casein concentration, which is partially responsible for the strong reduction in the diffusion in SC
291 suspensions with an increase in casein concentration. These results also suggest that the casein networks
292 (NPC and SC systems) are not dense enough to induce reptation, but are dense enough so that the solute
293 conformations corresponding to elongated shapes are favored for the diffusion step to be effective.

294

295 **2.4. Protein diffusion in NPC and SC suspensions**

296

297 The diffusion coefficient of casein micelles (NPC) and sodium caseinate aggregates (SC) were also
298 measured as a function of casein concentration. Due to the presence of a small amount of soluble
299 compounds (amino acids, peptides) in the NPC and SC powders, their contribution to the proton NMR
300 signal of casein can therefore not be neglected. Consequently, all attenuation curves of the NMR echo
301 signal for casein particles showed a non-linear decay and were fitted with a bi-exponential model
302 providing two diffusion coefficients. A first diffusion coefficient with a value of approximately 10^{-11}
303 m^2s^{-1} was calculated in both systems. According to the Stokes Einstein (SE) relation, this diffusion
304 coefficient corresponds to particles with a hydrodynamic radius equal to approximately 3 nm. Diffusion
305 coefficients of the same order have also been measured in NPC suspensions by other authors using the
306 PFG-NMR technique (Le Feunteun et al., 2012; Mariette et al., 2002). This diffusion coefficient was
307 attributed to the soluble compounds, a conclusion supported by diffusion measurements of these
308 compounds in the serum phase extracted by ultracentrifugation. The diffusion coefficient obtained
309 ($6.44e^{-11}$ m^2s^{-1}) was equal to the value estimated at zero casein concentration. The second diffusion
310 coefficient, which represented the main fraction of the echo attenuation, can therefore be attributed to

311 the casein particles. Figure 6 shows the variation of casein particle diffusion coefficients as a function of
312 casein concentrations in NPC and SC systems. Diffusion data were extrapolated to infinite dilution in
313 order to estimate the casein particle size from the SE relation. The radii obtained using this equation
314 were consistent with the casein size distribution determined by DLS and were equal to 12 nm for
315 sodium caseinate aggregates and 96 nm for casein micelles. These findings are in very good agreement
316 with several PFG-NMR (Le Feunteun et al., 2012; Mariette et al., 2002), inelastic light scattering and
317 dynamic wave spectroscopy studies (Alexander, Rojas-Ochoa, Leser, & Schurtenberger, 2002;
318 Gaygadzhiev, Corredig, & Alexander, 2008) where the values of casein micelle diffusion reported
319 ranged from $2x10^{-12}$ down to $2x10^{-13}$ m^2s^{-1}, depending on the composition of the dairy suspension and
320 on its casein concentration. Sodium caseinate aggregate diffusion measurements were also consistent
321 with values already measured by (Tan et al., 2010) in SC dispersions using the PFG-NMR technique.

322 The experimental values of casein diffusion were fitted to the following empirical equation proposed by
323 Speedy (Speedy, 1987) to describe the self-diffusion of non-interacting hard spheres in a hard sphere
324 fluid:

325 $$D = D_0\left(1-\frac{\phi}{0.571}\right)\left(1+\phi^2(1.459-11.04\phi^2)\right) \quad \textbf{(5)}$$

326

327 The volume fraction occupied by the hard spheres is $\phi = CxV$, where C is the casein concentration
328 (g/ml) and V is the voluminosity occupied by the casein particles (ml/g). As seen in Figure 7, the
329 experimental values of casein-reduced self-diffusion coefficients as a function of casein concentration
330 can be fitted with the Speedy model up to a casein concentration of approximately 9 g/100 g H_2O for the
331 SC system and 15 g/100 g H_2O for the NPC system. Above these concentrations, the model failed to
332 describe the casein diffusion data. These results suggest that SC and NPC dispersions still behave as
333 hard-sphere fluids up to a casein concentration that matches the random-close packing of these two

334 systems. This is in accordance with findings from previous studies that showed that the hard sphere
335 model could be a valuable model for casein micelles (Bouchoux et al., 2009; Gaygadzhiev et al., 2008;
336 Anne Pitkowski, 2007) and aggregating sub-micelles (Farrer et al., 1999; Panouille et al., 2005;
337 Pitkowski, Durand, & Nicolai, 2008). At higher concentrations, there is another regime (soft spheres) in
338 which casein micelles and casein submicelles deform, deswell and interpenetrate as the casein
339 concentration increases. The values of voluminosity calculated from the fitting for casein micelles and
340 sodium caseinate aggregates were 3.4±0.05 and 7±0.4 ml/g, respectively, in close agreement with
341 previously estimated values. Reported voluminosity values for casein micelles at the pH of milk vary
342 widely, i.e., V= 1.5 to 7.1 ml/g (Walstra, 1979), depending on the method employed. Recently, (De
343 Kruif & Huppertz, (2012); Jeurnink & De Kruif, (1993); Morris, Foster, & Harding, 2000) reported a
344 voluminosity of 4.2-4.4 ml/g on the basis of viscosity measurements. Moreover, voluminosity values
345 varying between 4 and 6 ml/g for casein submicelles estimated by neutron scattering and measurement
346 of the intrinsic viscosity have been reported (Panouille et al., 2005; Stothart & Cebula, 1982).

347 Analysis of the casein self-diffusion coefficients measured (Figure 6) revealed that for casein
348 concentrations < 9 g/100g H_2O, caseins diffused faster in SC suspensions than in NPC dispersions due
349 to their differences in size. Increasing the concentration resulted in a rapid decrease in the self-diffusion
350 coefficient of sodium caseinate aggregates (SC). The rate of change slowed down considerably once the
351 level of approximately 10 g/100 g H_2O sodium caseinate was reached. However, above this
352 concentration, sodium caseinate aggregates are known to close pack, leading to a strong increase in the
353 viscosity (Farrer et al., 1999; Pitkowski et al., 2008). Using previously measured NPC and SC solution
354 viscosities (Pitkowski et al., 2008; Anne Pitkowski, 2007), the inverse normalized viscosities were
355 compared with our normalized diffusion coefficient. As shown in Figure 8, casein micelles and sodium
356 caseinate aggregates diffused in both systems according to the SE relation. A positive deviation from
357 this relation was observed for sodium caseinate aggregates with casein concentrations > 12 g/100 g

358 H_2O. The differences in diffusion behavior between casein micelles and sodium caseinate aggregates
359 can thus be attributed to differences in protein size and dispersion viscosity.

360 PEG-reduced diffusion data were also compared with the SE relation (data not shown). In both casein
361 systems, PEGs were found to diffuse faster than expected from the solution viscosity. This indicates that
362 the reduction in the PEG diffusion coefficient was largely a function of the space occupied by the matrix
363 and minorly affected by the macroviscosity of the dispersions. Moreover, obstacle mobility should be
364 taken into account for a complete description of probe diffusion. This issue has been investigated, via
365 simulations methods, by many authors (Saxton, 1987; Tremmel et al., 2003). When the obstacles are
366 immobile, their effect may be described by percolation theory, which states that the long-range diffusion
367 constant of the tracers goes to zero when the area fraction of the obstacles is greater than the percolation
368 threshold (critical concentration). In contrast, if the obstacles are themselves mobile as for caseins, the
369 diffusion constant of the tracers depends on the area fraction of obstacles and the relative jump rate of
370 tracers and obstacles. In this case, long-range diffusion of a tracer is not prevented, but only retarded.
371 The extent of the diffusion retardation certainly depends on the diffusion coefficient of the obstacles and
372 the size of the tracer. This may explain the fact that long-range diffusion occurred at all of the casein
373 concentrations studied, even though the onset of close-packing of the casein sub-micelles (SC) and
374 casein micelles (NPC) occurs at casein concentrations of approximately ~10 g/100 g H_2O and ~ 15
375 g/100 g H_2O, respectively.

376 In light of these results, the question arises as to whether the intra-micellar diffusion mechanism already
377 adopted in NPC suspensions exists or not. Previous studies have shown that the PEG diffusion in NPC
378 suspensions could be explained by considering two characteristic length scales of structure (Colsenet et
379 al., 2005; Le Feunteun et al., 2007). For large PEGs, the free volume fraction unoccupied by casein
380 micelles is the prevailing element, whereas for small PEGs, the micelle internal porosity is the
381 preponderant factor. This model has made it possible to explain the effect of probe size in particular.
382 Such an explanation was proposed because casein particles cannot be considered as impenetrable

383 particles since they are known to be porous and highly hydrated (Morris et al., 2000). On the other hand,
384 PEGs are flexible, easily deformable and can change their shape according to their environment, as
385 shown in this study and proven by (Griffiths, Stilbs, Yu, & Booth, 1995). They can therefore diffuse
386 through small spaces compared to their hydrodynamic diameter by adopting a more elongated
387 conformation. However, our experimental results show that an extra-micellar mechanism, which is the
388 only one to be considered in the case of SC suspensions, is sufficient to explain the difference in the
389 values of the observed diffusion coefficient according to the probe size. These results therefore let us
390 assume that intra-micellar diffusion would be negligible and that the data can be simply explained by
391 taking probe size, obstacle size and mobility into account.

392

393 **IV. Conclusion**

394 The aim of this work was to critically examine the diffusion of PEGs and caseins in NPC and SC
395 dispersions by combining both experimental and theoretical approaches. It has been shown that in NPC
396 and SC systems:

397 • Caseins behave as hard spheres in a fluid and their self-diffusion is inversely proportional to the
398 solution viscosity measured macroscopically up to a casein concentration that matches the
399 random-close packing of these two systems. Consequently, sodium caseinate aggregates diffuse
400 more slowly than casein micelles when the casein concentration exceeds 9 g/100 g H_2O. Casein
401 voluminosity values obtained by fitting the casein experimental diffusion data to the Speedy
402 model were found to be in close agreement with previous values found in the literature.

403 • Two drastically different diffusion behaviors of PEGs were obtained in relation to differences in
404 casein obstacle size (inter-particle distance) and mobility between the two casein systems. A SC
405 suspension with a casein particle size equal to 12 nm and slow mobility has a considerably

406 stronger hindering effect than a NPC suspension with a casein particle size equal to
407 approximately 100 nm. Diffusion data were explained using the classical power law used in the
408 Rouse model.

409 The results obtained challenge the “two pathways” diffusion model already proposed to explain the
410 diffusion of a probe in NPC suspensions (Colsenet et al., 2005; Le Feunteun et al., 2007) and indicate
411 that the extra-micellar diffusion mechanism is the only mechanism to be considered, regardless of the
412 size of the probe.

413

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References

Alexander, M., Rojas-Ochoa, L. F., Leser, M., & Schurtenberger, P. (2002). Structure, dynamics, and optical properties of concentrated milk suspensions: an analogy to hard-sphere liquids. *Journal of Colloid and Interface Science, 253*(1), 35-46.

Bouchoux, A., Debbou, B., Gesan-Guiziou, G., Famelart, M. H., Doublier, J. L., & Cabane, B. (2009). Rheology and phase behavior of dense casein micelle dispersions. *Journal of Chemical Physics, 131*(16).

Chu, B., Zhou, Z., Wu, G., & Farrell, H. M., Jr. (1995). Laser light scattering of model casein solutions: effects of high temperature. *Journal of Colloid and Interface Science, 170*(1), 102-112.

Colsenet, R., Soderman, O., & Mariette, F. (2005). Effect of casein concentration in suspensions and gels on poly(ethylene glycol)s NMR self-diffusion measurements. *Macromolecules, 38*(22), 9171-9179.

Dalgleish, D. G., Spagnuolo, P., & Douglass Goff, H. (2004). A possible structure of the casein micelle based on high-resolution field-emission scanning electron microscopy. *International Dairy Journal, 14*, 1025-1031.

de Gennes, P. G. (1979a). Brownian motions of flexible polymer chains. *Nature, 282*, 367-370.

de Gennes, P. G. (1979b). Scaling concepts in polymer physics. In. Ithaca, New York: Cornell University Press.

de Kruif, C. G. (1998). Supra-aggregates of casein micelles as a prelude to coagulation. *Journal of Dairy Science, 81*(11), 3019-3028.

de Kruif, C. G., & Huppertz, T. (2012). Casein Micelles: Size Distribution in Milks from Individual Cows. *Journal of Agricultural and Food Chemistry, 60*(18), 4649-4655.

Doi, M., & Edwards, S. F. (1986). The theory of polymer dynamics. In. Oxford: Oxford University Press.

Farrer, D., & Lips, A. (1999). On the self-assembly of sodium caseinate. *International Dairy Journal, 9*(3-6), 281-286.

Favre, E., Leonard, M., Laurent, A., & Dellacherie, E. (2001). Diffusion of polyethyleneglycols in calcium alginate hydrogels. *Colloids and Surfaces a-Physicochemical and Engineering Aspects, 194*(1-3), 197-206.

Gaygadzhiev, Z., Corredig, M., & Alexander, M. (2008). Diffusing wave spectroscopy study of the colloidal interactions occurring between casein micelles and emulsion droplets:comparison to hard-sphere behavior. *Langmuir, 24*(8), 3794-3800.

Gong, J. P., Hirota, N., Kakugo, A., Narita, T., & Osada, Y. (2000). Effect of aspect ratio on protein diffusion in hydrogels. *The Journal of Physical Chemistry B, 104*(42), 9904-9908.

Griffiths, P. C., Stilbs, P., Yu, G. E., & Booth, C. (1995). Role of molecular architecture in polymer diffusion: a PGSE-NMR study of linear and cyclic poly(ethylene oxide). *The Journal of Physical Chemistry, 99*(45), 16752-16756.

HadjSadok, A., Pitkowski, A., Nicolai, T., Benyahia, L., & Moulai-Mostefa, N. (2008). Characterisation of sodium caseinate as a function of ionic strength, pH and temperature using static and dynamic light scattering. *Food Hydrocolloids, 22*(8), 1460-1466.

Holt, C. (1992). Structure and stability of bovine casein micelles. *Advances in Protein Chemistry, 43*, 63-151.

Horne, D. S. (1996). The hairy casein micelle: Evolution of the concept and its implications for dairy technology. *Netherlands Milk and Dairy Journal, 50*(2), 85-111.

Horne, D. S. (2002). Casein structure, self-assembly and gelation. *Current Opinion in Colloid & Interface Science, 7*(5), 456-461.

Horne, D. S. (2006). Casein micelle structure: Models and muddles. *Current Opinion in Colloid & Interface Science, 11*(2-3), 148-153.

Jeurnink, T. J. M., & De Kruif, K. G. (1993). Changes in milk on heating: viscosity measurements. *Journal of Dairy Research, 60*(2), 139-150.

Le Feunteun, S., & Mariette, F. (2007). Impact of casein gel microstructure on self-diffusion coefficient of molecular probes measured by H-1 PFG-NMR. *Journal of Agricultural and Food Chemistry, 55*(26), 10764-10772.

Le Feunteun, S., & Mariette, F. (2008). PFG-NMR techniques provide a new tool for continuous investigation of the evolution of the casein gel microstructure after renneting. *Macromolecules, 41*(6), 2071-2078.

Le Feunteun, S., Ouethrani, M., & Mariette, F. (2012). The rennet coagulation mechanisms of a concentrated casein suspension as observed by PFG-NMR diffusion measurements. *Food Hydrocolloids, 27*(2), 456-463.

Lucey, J. A., Srinivasan, M., Singh, H., & Munro, P. A. (2000). Characterization of commercial and experimental sodium caseinates by multiangle laser light scattering and size-exclusion chromatography. *Journal of Agricultural and Food Chemistry, 48*(5), 1610-1616.

Marchin, S., Putaux, J.-L., Pignon, F., & Leonil, J. (2007). Effects of the environmental factors on the casein micelle structure studied by cryo transmission electron microscopy and small-angle x-ray scattering/ultrasmall-angle x-ray scattering. *Journal of Chemical Physics, 126*(4), 45101.

Mariette, F., Topgaard, D., Jonsson, B., & Soderman, O. (2002). ^{1}H NMR diffusometry study of water in casein dispersions and gels. *Journal of Agricultural and Food Chemistry, 50*(15), 4295-4302.

McMahon, D. J., & McManus, W. R. (1998). Rethinking casein micelle structure using electron microscopy. *Journal of Dairy Science, 81*(11), 2985-2993.

McMahon, D. J., & Oommen, B. S. (2008). Supramolecular structure of the casein micelle. *Journal of Dairy Science, 91*(5), 1709-1721.

Morris, G. A., Foster, T. J., & Harding, S. E. (2000). Further observations on the size, shape, and hydration of casein micelles from novel analytical ultracentrifuge and capillary viscometry approaches. *Biomacromolecules, 1*(4), 764-767.

Nash, W., Pinder, D. N., Hemar, Y., & Singh, H. (2002). Dynamic light scattering investigation of sodium caseinate and xanthan mixtures. *International Journal of Biological Macromolecules, 30*(5), 269-271.

Panouille, M., Benyahia, L., Durand, D., & Nicolai, T. (2005). Dynamic mechanical properties of suspensions of micellar casein particles. *Journal of Colloid and Interface Science, 287*(2), 468-475.

Pitkowski, A. (2007). *Processus de gelification des caseines en presence de polyphosphates.* PHD thesis, University of Le Mans

Pitkowski, A., Durand, D., & Nicolai, T. (2008). Structure and dynamical mechanical properties of suspensions of sodium caseinate. *Journal of Colloid and Interface Science, 326*(1), 96-102.

Price, W. S. (1997). Pulsed-field gradient nuclear magnetic resonance as a tool for studying translational diffusion: Part 1. Basic theory. *Concepts in Magnetic Resonance, 9*(5), 299-336.

Radford, S. J., & Dickinson, E. (2004). Depletion flocculation of caseinate-stabilised emulsions: what is the optimum size of the non-adsorbed protein nano-particles? *Colloids and Surfaces A Physicochemical and Engineering Aspects, 238*(1), 71-81.

Sandra, S., Alexander, M., & Dalgleish, D. G. (2007). The rennet coagulation mechanism of skim milk as observed by transmission diffusing wave spectroscopy. *Journal of Colloid and Interface Science, 308*(2), 364-373.

Saxton, M. J. (1987). Lateral diffusion in an archipelago. The effect of mobile obstacles. *Biophysical Journal, 52*(6), 989-997.

Speedy, R. J. (1987). Diffusion in the hard-sphere fluid. *Molecular Physics, 62*(2), 509-515.

Stothart, P. H., & Cebula, D. J. (1982). Small-angle neutron scattering study of bovine casein micelles and sub-micelles. *Journal of Molecular Biology, 160*(2), 391-395.

Tan, H. L., & McGrath, K. M. (2010). The microstructural and rheological properties of Na-caseinate dispersions. *Journal of Colloid and Interface Science, 342*(2), 399-406.

Tremmel, I. G., Kirchhoff, H., Weis, E., & Farquhar, G. D. (2003). Dependence of plastoquinol diffusion on the shape, size, and density of integral thylakoid proteins. *Biochimica et Biophysica Acta-Bioenergetics*, *1607*(2-3), 97-109.

Walstra, P. (1979). Voluminosity of bovine casein micelles and some of its implications. *Journal of Dairy Research*, *46*(2), 317-323.

Figure legends

Figure 1. Example of an NMR spectrum obtained during a self-diffusion measurement. It stems from a concentrated casein suspension that contains 0.1 % (w/w) of a PEG polymer and 12 g/100g H_2O of caseins.

Figure 2. Lognormal particle size distribution for a NPC (solid line) and a SC (dotted line for intensity distribution/dashed-dotted line for volume distribution) suspension in the presence of 100 mM NaCl.

Figure 3. Reduced self-diffusion coefficients of different PEGs (615 ♦, 7920 ■, 21300 -, 32530 + and 93000▲) as a function of casein concentrations in NPC (solid lines) and SC (dashed lines) suspensions. Empty diamond and triangle correspond to the 620 and 93000 g/mol PEG diffusion already measured by Le Feunteun et al. in a concentrated NPC suspension (16.8 g/100 g H_2O).

Figure 4. Power law representation of the diffusion coefficient of PEG (7920, 21300, 32530 and 93000 g/mol) showing different molecular weights in water and (A) NPC suspensions for various casein concentrations (from top to bottom): 2.88, 6.43, 9.22, 11.86, 15.35 and 22.4 g/100 g H_2O (B) in SC suspensions for various casein concentrations (from top to bottom): 1.26, 2.25, 4.19, 5.20, 6.21, 9.11, 11.87, 17.45 and 24.04 g/100 g H_2O.

Figure 5. Exponent (α) of a power law curve fit obtained after regression of PEG diffusion coefficients (D) in NPC (■) and SC (♦) suspensions vs. molecular weight (M_w). Error bars represent the uncertainties estimated by the fitting procedure.

Figure 6. Comparison of casein effective diffusion in NPC (♦) and SC suspensions (•).

Figure 7. Casein-reduced diffusivity D/D_0 vs. casein concentration in NPC (■) and SC (♦) systems has been fitted to the Speedy model (solid line). In the insert panel we show the same behavior but in log-linear scale.

Figure 8. Casein concentration dependencies of the casein-normalized diffusivities D_r (filled shapes) and the inverse suspension viscosities (η_s) normalized by the solvent viscosity (η_0) (empty shapes) for SC (square) and NPC suspensions (diamonds).