Acid coagulation behavior of homogenized milk: effect of interacting and non-interacting droplets observed by rheology and diffusing wave spectroscopy

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ORIGINAL PAPER

Acid coagulation behavior of homogenized milk: effect of interacting and non-interacting droplets observed by rheology and diffusing wave spectroscopy

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Abstract This study analyzes the early stages of the acid coagulation behavior of milk containing homogenized fat globules. By addition of Tween 20 to homogenized milk, it was possible to create two similar colloidal systems with completely different interfacial properties. Control experiments using skim milk demonstrated that the presence of Tween did not overly affect the acid coagulation behavior of the casein micelles. The initial stages of aggregation were also similar for the two homogenized milk systems, indicating that the casein micelles were the main driving force behind early gel formation. For the case of homogenized milk without Tween, the fat globules were fully incorporated in the network. The stiffness of the gel was higher than the control and the overall spatial distribution of the fat droplets was not largely affected by the developing gel matrix. In contrast, the homogenized milk with added surfactant contained fat globules which did not interact directly with the casein micelles but rather became trapped inside the pores of the ensuing network. This gel showed a lower elastic modulus than the homogenized milk case and free-diffusing fat globules. Although the presence of interacting and non-interacting droplets did not overly affect the coagulation kinetics of the casein micelles, the acid gels did show different final properties which fully reflected the presence of "active" or "inert" fillers. The knowledge derived from this work will be the first step towards developing novel textures for dairy gels, modulating the extent of the interactions between the fat globules and the protein network.

应用流变学和散射波光谱法观察均质乳的酸凝乳特性

摘要 本文分析了均质牛乳酸凝胶形成初期的凝聚特性。将吐温-20加到均质牛乳中，可产生具有完全不同界面性质的两种相似的胶体体系。以脱脂乳作对照组，试验结果证明了吐温-20的

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存在对酪蛋白胶束酸凝聚的影响不太大。两种均质乳体系的初期凝聚过程非常相似，表明酪蛋白胶束是凝胶形成的主要驱动力。未加吐温-20的脱脂乳，脂肪球与酪蛋白胶束的网络完全溶为一体，形成凝胶的硬度比对照组高，而且形成的凝胶网络对脂肪液滴的空间分布没有太大的影响。相反，加表面活性剂的均质乳中脂肪球并没有与酪蛋白胶束直接接触，而是被包裹在形成的凝胶网络的内部空洞中。这种凝胶比均质乳的弹性模量低。尽管，有相互作用和无相互作用脂肪球的存在并不影响酪蛋白胶束的凝聚动力学，但是，所形成的酸凝胶则具有不同的反射特性，即存在“活性”和“惰性”的物质。基于本研究的结果，可以初步探讨乳凝胶质构的形成过程，有助于调整脂肪球和蛋白网络之间相互作用的程度。

Keywords Homogenized milk · Polysorbate · Acid coagulation · DWS

关键词 均质乳 · 均质牛乳 · 聚山梨醇酯 · 酸凝聚物 · DWS

1 Introduction

Acidification is an established process commonly used in combination with heat treatment or rennet addition to prepare fermented milk products (yogurt) and acid fresh cheeses. Nowadays, there is an ample variety of acidified products with different added ingredients and manufacturing processes, thus having particular textures and sensory characteristics (Fox et al. 2004). Acidified milk products are widespread, but the molecular details of the initial changes occurring during structure formation of the acid-induced gels are still limited, especially for complex dairy products containing fat.

At the natural pH of milk (6.8), native casein micelles are supramolecular assemblies of four proteins (α_{s1}-, α_{s2}-, β-, and κ-casein), stabilized by the negative charge and steric repulsion of κ-casein (Dalgleish 1998; de Kruif 1999). The interior of the micelles is held together by hydrophobic interactions between caseins and by colloidal calcium phosphate bridges between phosphoserine residues (Horne 2009). During acidification, the pH decrease from 6.8 to 5.1 causes the release of colloidal calcium phosphate and some of the caseins are liberated into solution; the charge of individual micelles is altered and the ionic strength of the solution increases (Lucey et al. 1998a). As a result, while acidification continues, the casein micelles aggregate close to their isoelectric point, leading to the formation of chains and clusters, linked together to form a tridimensional network (Lucey et al. 1998a). Aggregation, however, occurs at an earlier pH if milk has been heat treated because of the presence of whey proteins aggregates (Lucey et al. 1998b).

The fat phase in milk contributes greatly to the microstructure of dairy products and to their flavor and texture. In whole milk, fat is emulsified in globules naturally surrounded by a milk fat globule membrane. These globules have a broad size distribution, from 0.1 to 15 μm (Mulder and Walstra 1974). The homogenization process reduces fat globules' size by the use of strong shearing forces. As a consequence, the fat surface area increases by four to ten times (Mulder and Walstra 1974), and skim milk-derived proteins adsorb to stabilize the newly formed interface against coalescence. In unheated milk, casein micelles adsorb preferentially to whey proteins due to their large size and flexible structure (Cano-Ruiz and Richter 1997; Sharma and Dalgleish 1993). In milk used for yogurt-making, homogenization is an important step of the process as it prevents fat separation during fermentation and

storage, improves consistency, increases whiteness, and reduces whey separation (Lucey et al. 1998a; Tamime and Robinson 1999).

The properties of milk gels depend on the type of interactions that occur between fat globules and the protein matrix, and these interactions are determined by the nature of the material adsorbed at the interface and the colloidal state of the droplets (Gaygadzhiev et al. 2009; Rosa et al. 2006). Extensive fundamental work has been carried out in this area, mostly employing model systems consisting of emulsion droplets of known size and composition recombined in skim milk (Cho et al. 1999; Lucey and Singh 1998). It has been previously shown that during the coagulation process, homogenized fat globules in milk interact positively with the casein micelles, as "active" (structure-forming) fillers, increasing the stiffness of the gel. On the other hand, native fat globules behave as "inert" fillers because their membrane is not able to interact directly with the destabilized casein micelles (Michalski et al. 2002; van Vliet 1988; van Vliet and Dentener-Kikkert 1982). Tween has been employed before in studies with milk, showing that Tween 20-stabilized emulsions formed droplets which did not play an active role in the structure of the casein gels (Ion Titapiccolo et al. 2010a), while Fox and Hearn (Fox and Hearn 1978) showed that Tween 80 had no effect on the heat stability–pH curve of casein micelles. In recombined milk, while casein-stabilized oil droplets behave as structure-forming, Tween 20-stabilized emulsions behave as inert fillers.

The objective of this study was to compare the behavior between active and inert fat globules in fresh homogenized milk, when it is subjected to acid coagulation. The two systems of study were homogenized milk and homogenized milk containing Tween 20, as small molecular weight surfactants are known to be able to displace proteins from the surface of oil droplets in protein emulsions (Mackie et al. 1999). This methodology will ensure two systems containing fat globules with similar size distribution but different interacting interfaces (Ion Titapiccolo et al. 2010a). When the different milks are subjected to acid coagulation, it will be possible to determine if the development of the gel-forming ability of the casein micelles is affected by the presence of the fat globules in the system and whether the different active interfaces on the droplets play a role in the development and final structure of the gels. The main techniques used will be rheology and diffusing wave spectroscopy (DWS) since the combination of these techniques will allow the observation of the early stages of aggregation in milk (Alexander and Dalgleish 2004; Gaygadzhiev et al. 2009). To avoid the effect of heat-induced whey protein aggregates, which play a major role in the formation of texture in acid milk gels, unheated milk was used for the study.

2 Materials and methods

2.1 Materials

Fresh whole milk was obtained from the Elora Dairy Research (Elora, ON, Canada), and a bacteriostatic agent (sodium azide) was immediately added at a concentration of 0.02% (*w*/*v*). The homogenization of milk was performed at room temperature in

a one-stage high pressure homogenizer (Emulsiflex C5, Avestin, Ottawa, ON, Canada) for three passes at a pressure of 34.5 MPa. After homogenization, the samples were immediately acidified except for the homogenized milk with the addition of Tween.

For experiments using skim milk, fresh whole milk was skimmed by centrifugation at 4,000×*g* for 20 min at 4 °C using a Beckman J2-21 centrifuge with JA-10 rotor (Beckman Coulter, Mississauga, ON, Canada) and filtered four times through Whatman glass fiber filters (Fisher Scientific, Whitby, ON, Canada). Skim milk samples were analyzed within a week of preparation and stored at 4 °C until analysis.

Polyoxyehylene sorbitan monolaurate (Tween 20, 1,227 $g \cdot mol^{-1}$ molecular weight, Sigma Chemical Co, St. Louis, USA) was added to skim milk and homogenized milk. Peripheral experiments were performed at two different concentrations of Tween 20 and for two different stirring times. The optimal combination was found to be at 2 g of Tween 20 solution per 100 mL of milk with a stirring time of 6 h at room temperature. This was chosen so as to avoid refrigerating the homogenized milk (and possibly modifying the interfacial properties of the fat globules) while being able to do the experiment within a day.

For the acidification experiments, D-glucono-δ-lactone (GDL; Sigma Chemical Co, St. Louis, USA) was used at a concentration of 1.5% (*w*/*v*). After GDL addition, the samples were stirred for 30 s and analyzed immediately. To measure the decrease in pH after addition of GDL of each sample, some milk was also placed in a test tube, in a water bath maintained at 30 °C. The pH of this milk was measured with pH-meter AR15 (Fisher Scientific Co, Singapore) connected to a computer software (pH-store, MFC Application, Mediavention Inc.) which recorded the pH every 10 s for the entire length of the experiment (4 h).

2.2 Gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to analyze the protein composition of the creams of the homogenized milk systems, using a Bio-Rad electrophoresis unit (Bio-Rad Laboratories Inc., Hercules, CA). To separate the cream from the serum, milk was centrifuged at 5,000×*g* for 20 min with an Eppendorf 5415D centrifuge (Brinkmann Instruments Ltd., Mississauga, ON, Canada). The cream was then dried on a filter paper for 30 min and resuspended at a final concentration of 3.5% (*w*/*w*) in milliQ water.

Each sample of cream or serum was diluted 1:2 in reducing sample buffer (containing 1 $mol \cdot L^{-1}$ Tris–HCl, pH 6.8, 10% (*w*/*v*) SDS, 75% (*v*/*v*) glycerol, β-mercaptoethanol, 1% (*v*/*v*) bromophenol blue) and heated for 5 min at 95 °C. For the electrophoresis analysis, the resolving gel had 15% acrylamide in 1.5 $mol \cdot L^{-1}$ Tris–HCl at pH 8.9, while the stacking gel contained 4% acrylamide in 0.1 $mol \cdot L^{-1}$ Tris–phosphate at pH 6.7. The electrophoresis buffer was 3% (*w*/*v*) Tris–HCl, 14.4% (*w*/*v*) glycine, 1% (*w*/*v*) SDS at pH 8.3. Aliquots of 7 μL of the samples diluted in sample buffer were loaded into the gels, and the separation was performed at 200 V for 40 min. Gels were stained with Coomassie blue in 50% (*v*/*v*) methanol and 10% (*v*/*v*) acetic acid for 30 min and de-stained in two steps: at first, using a solution of 45% (*v*/*v*) methanol and 10% (*v*/*v*) acetic acid for 1 h, then

overnight with the same solution diluted 1:1 in milliQ water. The next day, gels were scanned with a Sharp JX-330 scanner (Pharmacia Biotech).

2.3 Light scattering

Dynamic light scattering (DLS; Zetasizer Nano, Malvern Instruments, Worcestershire, UK) was used to measure the apparent average diameter of particles in milk samples. The hydrodynamic size of the particles was calculated from the average of three readings. Immediately before analysis, samples were diluted approximately 2,000 times in permeate (milk serum) and placed in the spectrometer right after dilution. Permeate was collected by ultrafiltration (PLGC 10k regenerated cellulose cartridge, Millipore Corp., Bedford, MA) of another batch of skim milk. To avoid multiple scattering while preserving the environmental conditions of the casein micelles, permeate was filtered through a 0.22-μm filter (Millipore Canada Ltd., Mississauga, ON) before use.

Integrated light scattering (Mastersizer 2000, Malvern Southborough, MA) was employed, in addition to DLS, to obtain the particle size distribution of homogenized milk samples, diluted in water. The refractive indices used were 1.46 for milk fat and 1.33 for the dispersant (water). Each measurement was obtained from the average of three readings.

DWS was used to follow the acid coagulation of all milk samples. A volume of approximately 1.5 mL of undiluted milk was placed into a flat-faced, 5 mm path length optical glass cuvette (Hellma Canada Ltd., Concord, Canada), and the temperature of the sample was maintained at 30 °C with a water bath. The light source was a solid state diode pumped Nd:YAG laser (Coherent, Santa Clara, CA) wavelength of 532 nm and power of 100 mW, and the transmitted scattered light was collected by a single fiber optic that was then bifurcated and fed to two matched photomultipliers (HC120-03, Hamamatsu, Loveland, OH) and a correlator (FLEX2K-12×2, Bridgewater, NJ). To calibrate the laser intensity, standard latex spheres of 260 nm diameter (Portland Duke Scientific, Palo Alto, CA) were used on a daily basis. During the acid coagulation experiments, correlation functions and the intensity of the transmitted scattered light were measured every 2 min for 4 h. Data were analyzed using specialized software, DWS-Fit (Mediavention Inc., Guelph, ON, Canada). In DWS, as the laser traverses the sample, the intensity of the light fluctuates as a result of the multiple scattering with the particles. The fluctuation is related to the movement of the particles and, in a free diffusing system, it is possible to calculate the radius of the particles through the Stokes–Einstein equation. If the particles in a system are not free diffusing, e.g., because they are aggregating, the decrease and final mobility of the particles can be monitored via the mean square displacement (MSD). With DWS, it is also possible to obtain information about the particles organization and interactions monitoring the turbidity parameter, l^*. This is the photon transport mean free path, which is the distance that a photon has to travel for his direction to be completely randomized, thus it is related to the positional correlation of the particles and to their light scattering properties (i.e., size, shape, and refractive index contrast). A more detailed description of the theory of DWS can be found elsewhere (Maret and Wolf 1987; Weitz et al. 1993).

2.4 Rheology

Rheological experiments were carried out with a stress-controlled rheometer AR 1000 (TA Instrument Ltd., New Castle, USA), and measurements were taken using a conical concentric cylinder geometry (5,920 μm fixed gap, 15 mm outer radius, 14 mm inner radius, and 42 mm cylinder immersed height). Acid coagulation was performed at 30 °C; the temperature was maintained connecting to the rheometer an external water bath (Isotemp 3016, Fisher Scientific, Whitby, Canada). After addition of GDL, each sample was immediately placed in the rheometer and a time sweep was run at 0.01 controlled strain, 1.0 Hz frequency, and 0.1 μN m initial torque. The gel development was then followed until each sample reached the final pH of 4.6. The gel properties are reported as a function of pH, using an acidification curve (pH versus time) tabulated for each experiment for conversion. The coagulation pH of the sample was defined in this study as the time taken for the ratio G′/G″ to be equal to 1.

2.5 Statistical analysis

All the experiments were carried out in triplicate starting from different batches of fresh milk; the values reported in this work are the means of the repetitions. All the figures presented contain graphs that are the most representative of the three replicates. Analysis of variance and least significant difference computations were carried out to determine eventual significant differences between treatments. Statistical analyses were conducted using SPlus 8.0 and differences were considered at 95% confidence level. The means of each parameter with the corresponding statistical significance are reported in Table 1. The values used for the calculations are obtained by determining the intersection point of two best-fit lines on the points just before and after a noticeable data value change in the graphs.

Table 1 Values measured during acid coagulation using rheology and diffusing wave spectroscopy, with statistical significance

	Gel point pH	Max tanδ pH	1/*l** pH	Incr radius pH	G′ at pH 4.6 (Pa)	tanδ at pH 4.6
SM	4.79^{a}	4.77^{a}	5.17^{a}	4.81^{a}	9.24^{a}	0.35^{ac}
SM-Tw	4.83^{a}	4.82^{a}	5.19^{a}	4.85^{ab}	9.37^{a}	0.36^{a}
HM	4.79^{a}	–	5.15^{a}	4.86^{bc}	13.66^{b}	0.32^{b}
HM-Tw	4.83^{a}	–	5.26^{b}	4.90^{c}	9.58^{a}	0.35^{c}

Means in the same column with no common superscript letters are different ($P<0.05$). Gel point pH measured as G″=G′; pH measured at maximum tanδ value; pH of initial increment of 1/*l**; pH value for increase in coagulation as measured by increase in radius; G′ value measured at pH 4.6 and tanδ measured also at pH 4.6

SM skim milk, *SM-Tw* skim milk+Tween, *HM* homogenized milk, *HM-Tw* homogenized milk+Tween

3 Results and discussion

3.1 Effect of Tween 20 addition to casein micelles and fat globules

The average particle size of the casein micelles in skim milk (SM) and in skim milk containing Tween 20 (SM-Tw) was measured using DLS. The addition of surfactant to milk caused a statistically significant increase of 4.5±2.6 nm in the size of the casein micelles (from 154.9±1.3 to 159.2±2.2 nm). These results agree with those found in Ion Titapiccolo et al. (Ion Titapiccolo et al. 2010b) who also performed size exclusion chromatography experiments which showed no increase of soluble caseins in serum after the addition of Tween 20. Therefore, it was considered important to study the acidification behavior of SM and SM-Tw, to determine if this small change in radius will affect the aggregation behavior of the micelles.

On the other hand, when the particle size of homogenized milk was measured using integrated light scattering, no effect of Tween 20 was noted. Figure 1 shows the size distribution of the particles present in homogenized milk (HM) and in homogenized milk containing Tween 20 (HM-Tw). Both samples show a bimodal distribution, the first peak having a maximum at about 120 nm and the second at about 1.2 μm of diameter. Homogenized milk contains two scattering particles: casein micelles, with an average diameter corresponding to the first peak of the distribution, and fat globules. Previous experiments using transmission electron microscopy have reported that homogenization creates a heterogeneous fat globule size distribution (Dalgleish et al. 1996; Ion Titapiccolo et al. 2010a) with globules either free in solution (stabilized by casein micelles at their

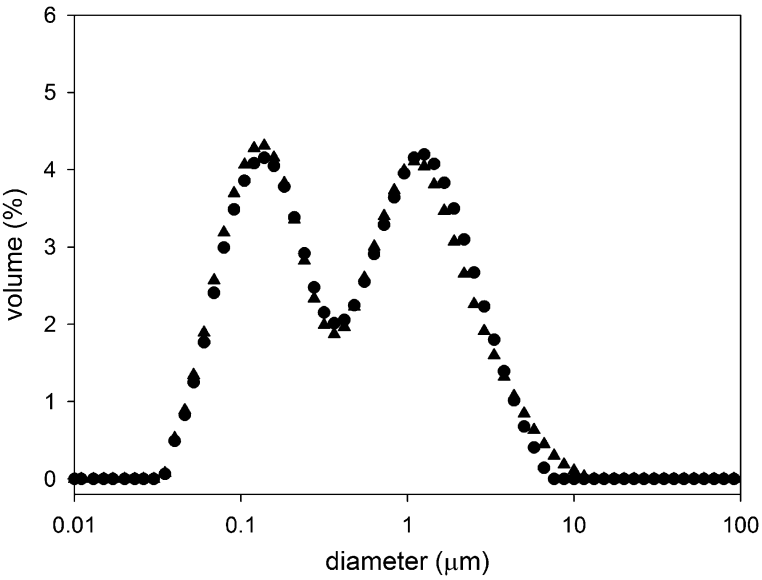

Fig. 1 Particle size distribution obtained by integrated light scattering of homogenized milk (*filled circle*) and homogenized milk with Tween (*filled triangle*)

interface) or organized in clusters, generated by bridging proteins among the smallest globules. Thus, while the first peak represents mostly the population of casein micelles, the second peak of the distribution represents the fat globules and/or clusters (Fig. 1).

While integrated light scattering did not show any significant difference in the size of the fat globules when Tween 20 was added to the homogenized milk, when the average apparent diameter was determined with DLS, it was possible to note a significant decrease in size generated by Tween 20 addition, from 272.8±10.8 to 231.4±11.4 nm. The observed decrease in the average apparent diameter might be an indication of protein displacement at the interface, but dispersion of aggregates cannot be discarded. The addition of Tween 20 to homogenized milk may also cause the disruption of fat globules' clusters, thus resulting in an increase in the number of small fat globules.

To confirm the displacement of caseins from the fat interface, the presence of residual protein on the surface of the fat globules was determined using SDS-PAGE under reducing conditions (Fig. 2). Fat globules separated from the HM (lane 2) clearly contain mostly caseins, derived from the casein micelles adsorbed at the fat interface during homogenization. In contrast, though not totally removed, very little protein is recovered in the cream phase of the HM-Tw sample (lane 3). The surfactant displaces the casein micelles from the fat/water interface; this results in the removal of most of the proteins from the surface of the globules, which become again free into solution. The competitive displacement of Tween 20 in dairy-based emulsions has been previously described (Goff and Jordan 1989; Ion Titapiccolo et al. 2010a; Mackie et al. 1999; Pugnaloni et al. 2004).

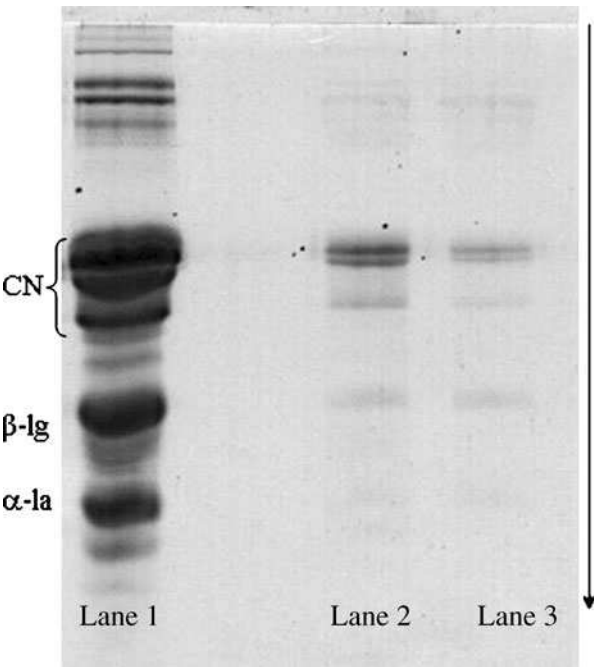

Fig. 2 SDS-PAGE carried out under reducing conditions for cream extracted from homogenized milk samples with and without Tween 20. *Lane 1* depicts the migration of the proteins present in the serum phase of homogenized milk (CN =caseins, β-lg=β-lactoglobulin, α-la=α-lactalbumin). *Lane 2* shows the separation of the cream from homogenized milk. *Lane 3* shows the separation of the cream from homogenized milk after incubation for 6 h with Tween 20. The *arrow* represents the direction of migration

3.2 Acid coagulation

The acid coagulation of homogenized milk with and without addition of 2% Tween 20 was monitored using small oscillatory rheology and DWS. The behavior of skim milk with and without surfactant was also monitored to mark any possible effects of Tween 20 on the acid coagulation properties of the casein micelles.

Changes in the storage modulus (G′) and loss tangent (tanδ, where δ is the phase angle) as a function of pH during acid coagulation are shown in Fig. 3. The elasticity of all the samples increases drastically between pH 4.85 and 4.75, indicating the coagulation point of the casein micelles (Fig. 3a).

When comparing skim milk samples with skim milk containing homogenized fat globules (HM and HM-Tw), the presence of fat globules does not markedly affect the kinetics of aggregation of the casein micelles and the formation of a protein network in unheated milk, indicating that the gelling behavior is dominated by the casein particles. Moreover, the displacement of casein micelles from the interface of the globules by addition of Tween 20 does not alter their susceptibility to acid coagulation when viewed by rheology (Fig. 3a, Table 1).

Differences in the G′ values of the milk gels measured at a final pH of 4.6 are also reported in Table 1. It has been previously shown that unheated milk forms weak gels, with low elastic modulus (Lucey et al. 1998a). The addition of Tween 20 to skim milk does not alter the final G′ of the acid gel, suggesting that this small molecular weight surfactant does not affect the aggregation and rearrangement of the casein micelles after the coagulation point. On the other hand, homogenized milk shows a significantly higher value of final G′ than skim milk. This is due to the presence of additional gel-forming particles in the form of casein-covered fat globules. Even though the fat globule is not entirely covered with caseins (there are still some non-interacting material such as fat globule membrane and whey proteins), the presence of the caseins is enough to cause these droplets to become an active interacting particle in the formation of the milk gel. These results have been observed previously by other authors (Guinee et al. 1997; Lucey et al. 1998b; van Vliet and Dentener-Kikkert 1982). It has been in fact reported that unheated skim milk containing 3.5% fat had a higher, albeit small, modulus compared to milk gels made with skim milk (Lucey and Singh 1998).

Figure 3b shows the values of loss tangent for the four samples as a function of pH during the coagulation process. Shortly after the coagulation pH, tanδ decreases to below 0.4. After the drastic decrease, tanδ decreases gradually until the final pH of 4.6. A maximum value for tanδ is partially visible only for skim milk and skim milk with Tween 20 (Fig. 3b, inset), and in both samples, the maximum is very close to the gelation pH of the samples. This maximum in tanδ has been associated to rearrangements in the gel due to the solubilization of CCP from the casein micelles, and it has been previously reported for heated milk, as the presence of protein complexes increase the coagulation pH (Lucey et al. 1998a; Lucey and Singh 1998). The HM and HM-Tw samples do not show this maximum. It is not clear at this point why this is the case. It can be speculated that there could be less rearrangements due to the presence of fat or, more likely, it is just not possible to detect the subtle effect of re-solubilization of CPP due to the presence of the fat globules.

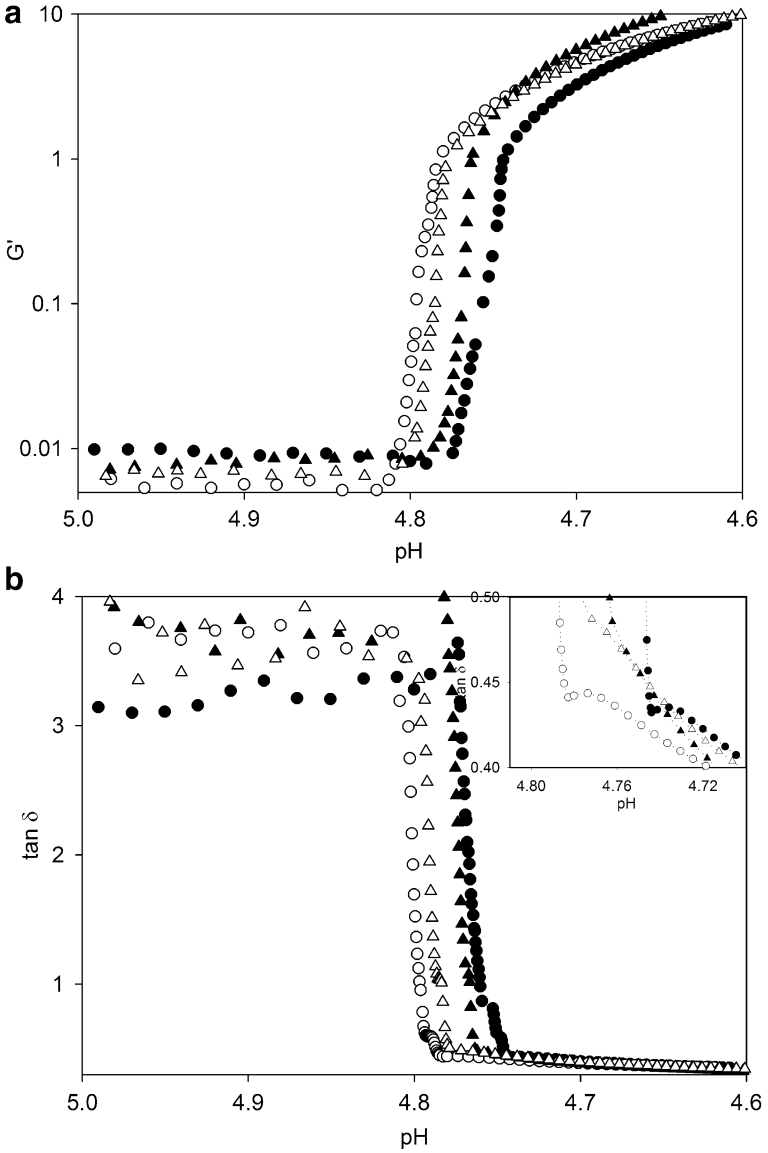

Fig. 3 **a** Development of the storage modulus, G′, and **b** development of tanδ during acidification of skim milk (*filled circle*), skim milk with Tween (*open circle*), homogenized milk (*filled triangle*), and homogenized milk with Tween (*open triangle*). *Inset* corresponds to detailed view of pH 4.8–4.72

The value of tanδ measured at pH 4.6 (see Table 1) is significantly lower in gels containing fat globules, with the lowest values shown for homogenized milk. These results confirm previously reported work using recombined milk and model emulsions, where high interacting systems showed higher stiffness of the gels and the lowest values of tanδ. On the other hand, emulsions containing Tween 60 showed a higher tanδ than gels containing interacting droplets (Cho et al. 1999). A higher value of tanδ reflects gels with a higher susceptibility to rearrangements,

and this has been related to gels with a higher tendency to syneresis (Van Vliet et al. 1991).

The differences (final value of G′ and tanδ) observed between the two homogenized milk systems (with and without Tween) arise from the presence of different components at the interface of the fat globules. In regular homogenized milk, interacting fat globules participate in the protein network, and cause the formation of a stiffer gel, with a higher water holding capacity and a lower tendency to rearrangements than skim milk gels. By the addition of Tween 20, the interface of the globules is depleted of almost all proteins. The newly formed interface, covered by small molecular weight emulsifier, is not able to interact with the casein network; consequently, the elasticity of the final gel is lower than that of the HM gel, and comparable to that of a network containing only casein micelles, while the tanδ value is intermediate between that of HM and SM.

In addition to rheological data, measurements were also performed using DWS. With this technique, the preceding stages of aggregation can be better observed by measuring the transmission parameter $1/l^*$, as well as the mobility of the scattering particles, throughout the acidification. Figure 4 shows the development of the normalized $1/l^*$ as a function of decreasing pH during coagulation of the four samples analyzed; the parameter is normalized as the value of $1/l^*$ for the systems with and without fat globules is quite different because of their different scattering properties. In the samples containing fat globules, the larger size of the globules as well as their higher refractive index contrast compared to caseins micelles dominate the scattering of light. Since we are interested in the kinetics of gel formation during acidification, and not in the absolute values of turbidity, normalization can be justified.

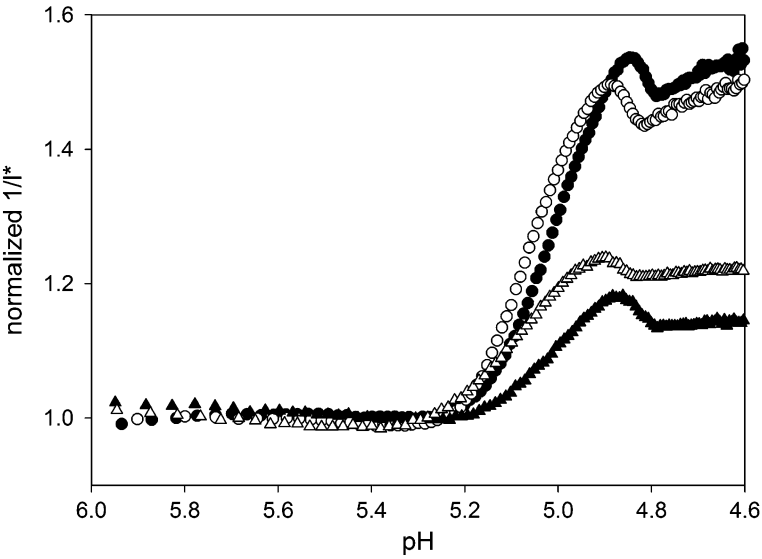

Fig. 4 Development of normalized $1/l^*$ versus decrease in pH during acidification of skim milk (*filled circle*), skim milk with Tween (*open circle*), homogenized milk (*filled triangle*), and homogenized milk with Tween (*open triangle*)

After an initial delay phase, $1/l^*$ starts to increase at about pH 5.2. This increase reflects the beginning of interparticle interactions arising from the gradual shielding of the charges around the casein micelles, hence, a decrease in steric repulsion of the particles. These interactions start at the same pH value for the skim milk samples, with or without Tween 20 as well as for the homogenized milk without Tween 20; however, HM-Tw shows interactions at a higher pH (Table 1). It can be speculated that this could be related to the fact that the presence of Tween displaces the previously fat-bound casein micelles which are now free in solution and different from the native ones. These caseins might be more reactive (some of their hydrophobic sites might now be exposed), and this leads to earlier long-range interaction potentials as well as onset of aggregation shown in the G′ results. After the initial increase, $1/l^*$ value increases rapidly and continuously up to a pH of 4.9, when the parameter decreases slightly and levels off again after a further pH drop of 0.05 units. This behavior has been reported before in acidified milk systems (Alexander and Dalgleish 2004) and is related to the development and formation of the acid gel.

All the samples show an overall similar shape in $1/l^*$, although the relative change between the beginning and the end value (at pH 4.6) for samples containing fat globules are lower than those for skim milk samples. The difference in the relative change between SM and HM samples is due to the presence of the fat globules. Since the larger contributors to the light scattering signal are the fat globules, the fact that the final and initial $1/l^*$ values are about 20% apart means that the overall spatial distribution of the fat droplets is not overly affected by the gelation of the micelles around them. In contrast, the spatial arrangement of the casein micelles in skim milk is heavily altered by their incorporation in a developing gel. Homogenized milk with Tween 20 has a behavior between the two; the overall change in $1/l^*$ is higher than HM. The protein is partly displaced from the interface (see Fig. 2) and again free in the serum, able to rearrange during coagulation. These results are in agreement with the differences in the values of tanδ. The type of bonds between adsorbed and un-adsorbed caseins (for the Tween 20 case) and between fat-bound caseins (for the homogenized case) will be different to those from the skim milk alone, primarily due to the changes undergone by the casein micelles after adsorption/desorption. The lower tanδ values of milk gels containing fat globules compared to skim milk gels highlights this difference. By Tween 20 addition to homogenized milk, the displaced caseins rearrange during gel formation, forming a gel more similar to skim milk.

Possible differences in particle aggregation and coagulation points can be measured by observing the behavior of the apparent radius measured by DWS (Fig. 5). The increase in apparent radius after gelation (measured as the intersection of two linear fits for points immediately adjacent to the aggregation point, Section 2.5) has no physical meaning, but it reflects the decrease in diffusion (or mobility) of the scattering particles. The radius at the initial pH (when the samples are still in free diffusing mode) is higher in HM than SM samples because of the presence of fat globules in addition to the smaller casein micelles. In all samples, the radius decreases slightly at early stages of acidification (inset) due to the collapse of the hairy layer of κ-casein, as previously reported (Alexander and Dalgleish 2004). Shortly after, at pH 4.85 (see Table 1), the radius starts to increase as an indication of

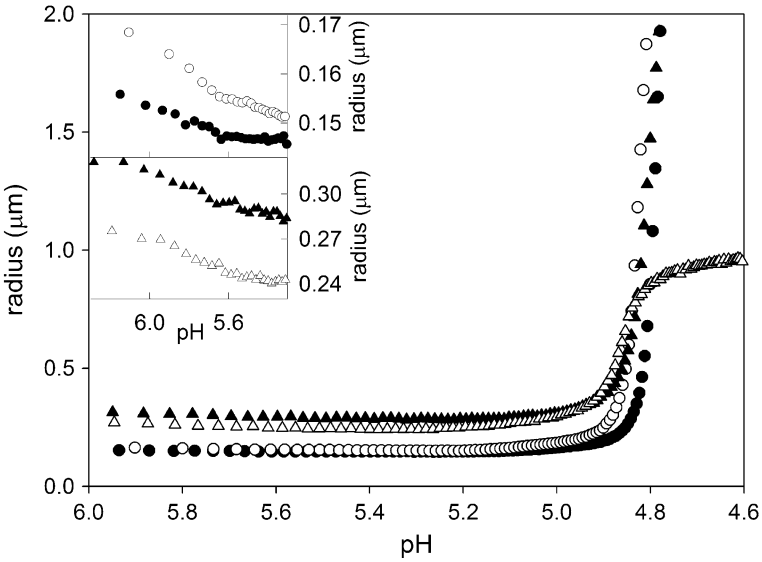

Fig. 5 Apparent radius measured with diffusing wave spectroscopy versus decrease in pH during acidification. Skim milk (*filled circle*), skim milk with Tween (*open circle*), homogenized milk (*filled triangle*), and homogenized milk with Tween (*open triangle*). The *insets* show the initial stages of the reaction in more detail

the decrease in the micelles' mobility. This can be considered as the point of aggregation. The casein particles always start to aggregate at a pH around 4.85 (see Table 1). However, while the two skim milk samples, as well as homogenized milk without Tween 20, show an exponential increase after the gelation point, the apparent size of HM-Tw levels off at about 1 μm. For the case of SM, SM-Tw, and HM, the rate of increase of size (or more correctly, the rate of decrease in diffusion) is quite similar. This is in agreement with the current understanding that HM fat globules are fully incorporated in the case in network. However, in the HM-Tw case, the particles (fat globules) seem to reach a state which remains relatively constant throughout the acidification process. A similar effect has been observed before in mixtures of skim milk and non-interacting whey protein-stabilized oil droplets (Gaygadzhiev et al. 2009). In essence, the Tween 20-stabilized fat droplets in the homogenized milk are not an integral part of the network and are hindered in their motion within the casein gel strands.

The dynamics of the scattering particles can be depicted also by observing the changes occurring to the MSD, calculated during acidification (Fig. 6). The plots are drawn for different times during the gelation process (reaction time increases from left to right). A linear dependence of the MSD with correlation time indicates free diffusive, unimpeded motion (Maret and Wolf 1987). This is clearly seen during the initial stages of acid coagulation for all the cases investigated here (symbols to the left of open triangles in Fig. 6) where both fat globules and micelles do not seem hindered in their motion, at least in the time/space scale available with this DWS setup, resulting in a linear increase of MSD with time.

As the micelles start to aggregate into small clusters, their diffusion slows down (compared to the initially free micelles and globules), and so does their mean

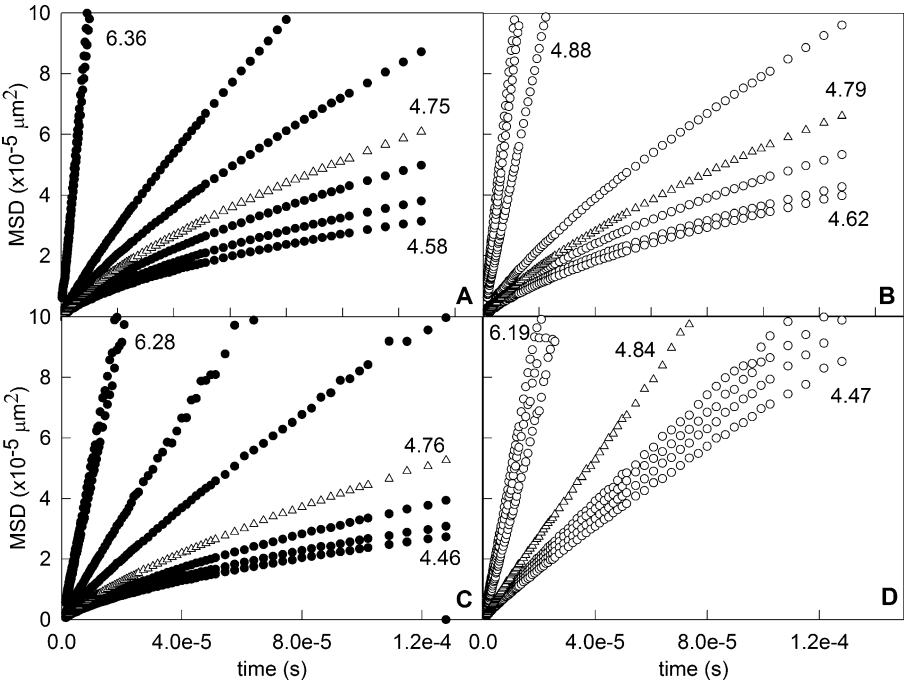

Fig. 6 Mean square displacement lines of **A** skim milk, **B** skim milk with Tween, **C** homogenized milk, and **D** homogenized milk with Tween, as the coagulation process develops (from *left* to *right*). *Numbers* correspond to selected pHs of the acidification process

excursion distances. This is also evidenced in the lines corresponding to the intermediate times during acid coagulation (but still at high pH), which still show a linear dependence between the MSD and time. In the case of SM and SM-Tw (Figs. 6a and b, respectively), the gelation point corresponds to the transition from a linear relation to the beginning of a curved one (highlighted as an empty triangle symbol). At pHs below these values and as the systems gel, the curvature of the lines is more noticeable and moving towards an asymptote at long correlation times. This reflects the arrested motion of the scattering particles as they become trapped in the network. Figure 7 shows the slope (P value, see below) of the short-time behavior of the mean square displacement for SM and SM-Tw of the systems (circles) as a function of pH. The displacement of the particles can be described by a power law relating the MSD $\propto \tau^p$ where τ is the correlation time and the p value is the exponent (Maret and Wolf 1987). These graphs show that at the gelation point (pH ~4.8) there is a quantitative change from diffusive to sub-diffusive motion, as a change in exponent from $p=1$, for free diffusive motion to p close to 0.7 is clearly seen. As already shown in Fig. 5, the overall behavior in SM-Tw is similar to that of the control SM, and surfactant molecules do not affect the mobility of the formed gel.

However, differences can be seen in the two homogenized milk systems. Homogenized milk without Tween 20 shows a somewhat similar transition from free diffusion to arrested motion when compared to SM. This is further evidence that the casein-coated fat globules are able to participate in the network formation, since, as stated before, the detected scattered light arises mostly from the fat particles. On

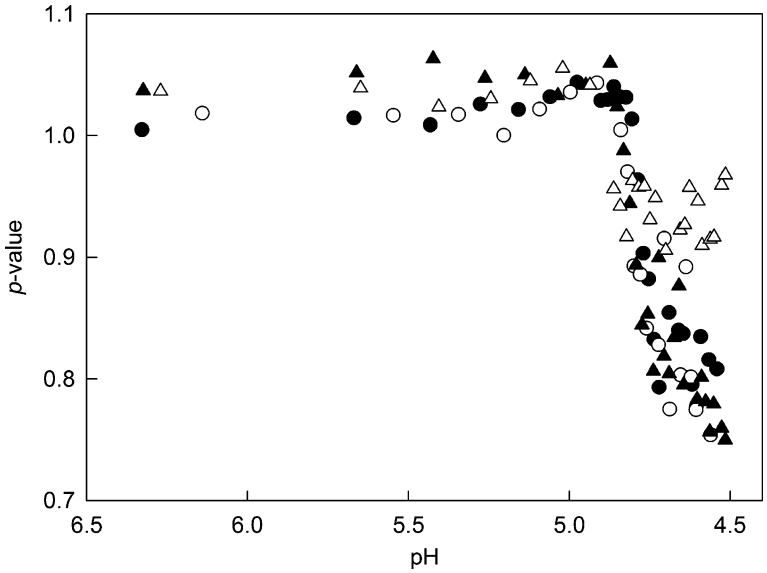

Fig. 7 Parameter *p* (see text) as a function of pH during acidification. Skim milk (*filled circle*), skim milk with Tween (*open circle*), homogenized milk (*filled triangle*), and homogenized milk with Tween (*open triangle*)

the other hand, HM-Tw shows only a very slight decrease in slope as a function of acidification at longer correlation times and minimum loss of linearity in the later stages of gelation. This behavior is consistent with the notion that Tween 20-covered fat globules do not take active part in the formation of the casein gel. The fat globules trapped by the network cages, but are relatively free to move within them. This can be further seen in Fig. 7 (triangles). While, again, HM milk shows a similar behavior to that of SM milk (p value = 1 in the pre-gelation stages and less than 1 for the system after gelation), HM-Tw is noticeably different. It is evident that the p value at low pH remains around 0.93 and much higher than for the other three systems. All throughout the development of the gel, the displacement of the fat globules change very little, consistent with the notion of inactive fillers motion hindered by the presence of gel pores. Light scattering experiments are able to detect the dynamics of these "semi-free" droplets encased in the gel pores which are unaffected by the developing and further rearrangement of the network. A similar behavior was reported during renneting of homogenized milk without and with Tween 20 (Ion Titapiccolo et al. 2010a) confirming the different role of "active" and "inert" fat globules in a case in network.

4 Conclusions

This study shows for the first time the effect of homogenized oil droplets on the beginning stages of aggregation during acidification of homogenized milk. By addition of Tween 20 to homogenized milk, it was possible to create a system with similar colloidal properties as homogenized milk but with a different fat/water interface. The initial stages of aggregation were similar in the two homogenized milk

systems, suggesting that the formation of the gel matrix in unheated samples was driven by the casein micelles either in solution or adsorbed at the interface, but that previously adsorbed micelles might react somewhat differently than native ones. However, in homogenized milk without surfactant, the fat globules participated in the formation of the network with the casein micelles, and the gel increased in stiffness when compared to a gel containing only caseins. In addition, the overall spatial distribution of the fat droplets was not largely affected by the gelation of the micelles around them, and the gel had the lowest tendency to rearrangements. In homogenized milk with Tween 20, fat globules were no longer covered with caseins and did not interact with casein micelles. The fat globules eventually became trapped inside the pores of the casein network and retained their mobility. This gel was less stiff than the gel obtained from homogenized milk, and its modulus was similar to that of a gel containing only casein micelles.

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