# Rennet coagulation properties of milk in the presence of oil droplets stabilised by a combination of sodium caseinate and whey protein isolate

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# Rennet coagulation properties of milk in the presence of oil droplets stabilised by a combination of sodium caseinate and whey protein isolate

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**Abstract** This manuscript describes the in situ investigation of the rennet-induced aggregation of skim milk recombined with emulsion droplets stabilised with whey protein isolate (WPI) and sodium caseinate (NaCas) in isolation or in combination. The experiments were conducted with two levels of rennet as well as with and without added $CaCl_2$. Diffusing wave spectroscopy and small deformation rheology were used to follow the gelation behaviour. In addition, scanning electron microscopy was employed to observe changes in the aggregation state of the oil droplets. We report that the presence of WPI-stabilised droplets did not alter the gelation behaviour of the casein micelles in recombined milks, even in the presence of added $CaCl_2$. In contrast, NaCas-stabilised droplets in solution impaired the rennetability of the recombined milk, even after the addition of $CaCl_2$. The mixture of both proteins at the interface was also investigated. When the WPI covering the fat droplets was gradually and systematically substituted by NaCas, any amount over 30% also impaired the rennetability of the milks. It was speculated that the increased steric repulsion of the oil droplets in conjunction with an increase in NaCas present in the serum phase in the recombined milks were the most likely contributors to the impaired rennetability of the casein micelles.

**酪蛋白酸钠和分离乳清蛋白粉结合的油滴稳定剂对乳的凝乳特性的影响**

**摘要** 本文描述了通过现场考察用分离乳清蛋白粉(WPI)和酪蛋白酸钠(NaCas)在独立和结合条件下的乳滴稳定剂调制的脱脂乳的诱导凝乳聚合特性。实验分成两种情况,一种是添加 $CaCl_2$,一种是不添加 $CaCl_2$。该实验用扩散光谱仪(DWS)和小变形流变仪追踪凝胶变化特性。另外,扫描电镜被用来观察油滴聚合状态的变化。在 WPI-稳定液滴单独存在的条件下,不会改变调制乳酪蛋白胶束胶凝特性,即使添加了 $CaCl_2$ 也不影响。相反,以酪蛋白酸钠-稳定液滴溶液会破坏调制乳的凝乳能力,即使后来添加 $CaCl_2$,效果也一样。如果将分离乳清蛋白粉和酪蛋白酸钠两种蛋白在分界面混合进行研究,当分离乳清蛋白粉(WPI)覆盖了脂肪液滴后,会逐渐的、有规律地被酪蛋白酸钠所取代,当酪蛋白酸钠量超过 30%以后也会破坏凝乳能力。据此推测增加油滴的空间排斥,同时增加调制乳乳清相中酪蛋白酸钠的量,将两者协同起来可以最大程度的破坏酪蛋白胶束的凝乳能力。

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**Keywords** Rennet coagulation · Sodium caseinate · Whey protein isolate · Fat globule · Rheology · Microstructure · Diffusing wave spectroscopy · Gelation · $CaCl_2$

**关键词** 凝乳酶凝乳 · 酪蛋白酸钠 · 分离乳清蛋白粉 · 脂肪球 · 流变学 · 微观结构 · 扩散光谱仪 · 胶凝 · 氯化钙

## 1 Introduction

The majority of cheese varieties are produced by coagulating milk by means of specific proteolytic enzymes (Horne and Banks 2003). In brief, the specific hydrolysis of κ-casein by chymosin (rennet) leads to a progressive decrease in the magnitude of the repulsive forces between the casein micelles (Tuinier and de Kruif 2002). The rennet coagulation results in the formation of strands composed of aggregated casein micelles that further associate to develop a three-dimensional continuous network (Lucey et al. 2003).

Rennet-induced milk gels can be described as a space-filling network of aggregated particles (casein micelles) entrapping fat globules in the aqueous phase and containing other non-gelling components (Dickinson and Hong 1995; Michalski et al. 2002). The rheological behaviour of filled gels is dictated not only by the deformational properties of the gel matrix and filler and the volume fraction of the filler particles but also by the size, shape, colloidal state, and interactions between the filler particles and the gel matrix (Chow 1980; van Vliet 1988; Sala et al. 2007).

It is generally agreed that native milk fat globules do not participate in the formation of the network in both acid- and rennet-induced gels, but the globules fill the space within the casein micelles gel strands (Michalski et al. 2002; van Vliet and Dentener-Kikkert 1982). Homogenisation of milk, however, decreases the average particle size of the fat globules (Olson et al. 2004) and more importantly modifies their interfacial composition (i.e. the interface is now covered with casein micelles, individual caseins and whey proteins; Sharma et al. 1996). In homogenised milk, the modified milk fat globules are actively participating in the formation of a rennet-induced casein network (Michalski et al. 2002). The fat globules–casein interactions lead to a shorter gelation time and an increase in the gel stiffness (Jana and Upadhyay 1993; Tosh and Dalgleish 1998).

Most of the research conducted so far in this area has focused on estimating the contribution of the modified milk fat globule membrane to the rheological properties of acid milk gels (van Vliet 1988; van Vliet and Dentener-Kikkert 1982; Cho et al. 1999; Xiong et al. 1991; Xiong and Kinsella 1991). During acidification, sodium caseinate (NaCas)-stabilised fat globules increase the stiffness of the gels by interacting with the casein strands. Conversely, fat globules stabilised with unheated whey proteins fill the space within the gel strands, thus not enhancing the stiffness of the gel network. Although the behaviour of whey protein isolate (WPI)- and NaCas-stabilised emulsions is understood in recombined milk during acidification, these data are not easily transferable to rennet-induced gelation. In fact, with regards to rennet-initiated milk gelation, most studies have addressed the effect of homogenised fat globules on the rheological, microstructural, and cheesemaking properties (Michalski et al. 2002; Tosh and Dalgleish 1998; Oommen et al. 2000; Metzger

and Mistry 1994, 1995; Nair et al. 2000), and much less is known about milk recombined with NaCas- and WPI-covered emulsion droplets. .

This work is aimed at comparing the effect of NaCas- and WPI-stabilised oil droplets on rennet-induced aggregation of recombined milk. The influence of the interfacial composition, when various combinations of WPI and NaCas are present, was also investigated. As differences in the size of the filler particles and their flocculation state have a dramatic effect on the development of the gel structure and on the mechanical properties of the composite gels (Michalski et al. 2002; van Vliet 1988; Dickinson and Chen 1999; McClements et al. 1993), the colloidal state of the fat globules used in this study was carefully controlled in order to be able to allow the assessment of the effect of the surface composition only on the rennet coagulation process.

In addition to the effect of the interfacial layer on the fat globules, the impact of addition of $CaCl_2$ to milk was also investigated. $CaCl_2$ affects the dynamic equilibrium existing between the colloidal and soluble calcium in milk and could affect the colloidal stability of the oil droplets, ultimately changing the rheological profiles of rennet gels.

Transmission diffusing wave spectroscopy (DWS) has been employed to observe the structural correlation between colloidal particles (utilising the turbidity parameter of the system, $1/l^*$, which is dependent on particle size, shape and charge, refractive index contrast and concentration), and their dynamics (observed by following the changes in the average diffusion coefficient and mean square displacements (MSD)] (Alexander and Dalgleish 2004; Alexander et al. 2006; Sandra et al. 2007). In addition to DWS, small deformation rheology and scanning electron microscopy (SEM) were employed to observe the effects of the interface of fat globules and the ionic conditions of the system on the rennet coagulation process.

## 2 Materials and methods

### 2.1 Preparation of recombined milks

Fresh milk was obtained from Elora Dairy Research Station (Elora, ON, Canada), and sodium azide (0.02% *w*/*v*) was added to suppress microbial growth. Milk fat was separated by centrifugation at 4,000×*g* for 20 min, at 4 °C (Beckman J2-21 centrifuge, Beckman Coulter, Mississauga, Canada). Skimmed milk was then filtered four times through Whatman fiberglass filter (Fisher Scientific, Whitby, Canada) and concentrated two times by ultrafiltration using PLGC 10k regenerated cellulose cartridge (Millipore Corp., Bedford, MA, USA).

Two times concentrated reconstituted skim milk was also prepared by dissolving low-heat skim milk powder, containing 0.6% fat, 4.8% moisture (*w*/*w*) (Parmalat Food Inc., London, Canada) to 20% (*w*/*v*) solids and Milli-Q water, stirred for 2 h at room temperature and then stored in a refrigerator. This concentrated reconstituted skim milk was used to obtain milk permeate to be used in the recombined milk experiments.

Appropriate amounts of sodium caseinate (NaCas) containing 93 g protein, 4 g moisture and 0.7 g fat (per 100 g powder) (Alanate 180, New Zealand Milk Products,

Lemoyne, PA, USA) or whey protein isolate (WPI) containing 98% protein, 4.5% moisture and 0.2% fat (dry basis) (Land O'Lakes, St. Paul, MN, USA) were dissolved in 5 mmol.$L^{-1}$ imidazole buffer at pH 6.7. Protein solutions were stirred for 2 hours at room temperature and then stored overnight at 4 °C. To prepare fat globules with various surface compositions (NaCas, WPI, and mixtures of NaCas and WPI) anhydrous milk fat (Parmalat Food Inc., London, Canada), the protein solution and imidazole buffer were mixed with a high-speed blender (PowerGen 125, Fisher Scientific, Mississauga, ON, Canada). Emulsions [1% (*w*/*w*) protein and 20% (*w*/*w*) milk fat] were passed through a homogeniser (Emulsiflex C5, Avestin, Ottawa, Canada) immersed in a temperature-controlled water bath (Versa-Bath, Fisher Scientific, USA) at 45 °C. The emulsions were homogenised with three passes at 350 bar. Emulsions were stored at refrigeration temperature until further use.

Recombined milk (total volume fraction, $\varphi=0.15$) containing casein micelles ($\varphi=0.1$) and fat globules differing in their surface compositions ($\varphi=0.05$) was prepared by blending appropriate volume of the corresponding emulsion, two times concentrated fresh skim milk and two times milk permeate. By dispersing the fat and concentrated milk in two times permeate, it was possible to ensure comparable ionic conditions between recombined milk samples and the original milk serum.

As it may be hypothesised that NaCas, either adsorbed at the oil/water interface or free in milk serum, could associate with available calcium ions and potentially affect the renneting process (Gaygadzhiev et al. 2009a, 2011), recombined milks were transferred to dialysis tubes in some experiments (MWCO, 6,000–8,000; Fisher Scientific, Whitby, ON, Canada) and dialysed against fresh whole milk with or without addition of 1 mmol.$L^{-1}$ $CaCl_2$ (final concentration in milk). The dialysis was performed with constant stirring at refrigeration temperature for 12 h. This ensured identical ionic conditions in all the recombined samples.

Enzymatic gelation was induced by Chymostar Single Strength rennet (Rhodia, Cranbury, USA) at a concentration of 0.007% (low rennet) and 0.035% (high rennet) for some samples, and at a temperature of 30 °C.

All experiments were conducted in triplicate (three separate batches of milk and emulsion).

## 2.2 Diffusing wave spectroscopy

The size of the fat globules and their stability over time when dispersed in milk serum was tested using DWS by diluting the emulsions prepared in 5 mmol.$L^{-1}$ imidazole buffer in two times milk permeate to give $\varphi=0.05$ of fat globules and ionic strength conditions typical for natural milk serum. Emulsion samples were placed in an optical glass cuvette (Hellma Canada Limited, Concord, Canada) with a 5-mm specified path length immersed in a thermostatted water bath at a temperature of 30 °C. A solid-state laser light with a wavelength of 532 nm and a power of 100 mW (Coherent, Santa Clara, CA, USA) was used in the light-scattering experiment. The averaged transmitted light intensity, acquired for 5-min time interval, was used in the calculation of the turbidity parameter, $1/l^*$. This parameter has been previously correlated to the temporal interparticle spatial correlation (Weitz et al. 1993). The apparent radius was calculated (using Stokes–Einstein equation) from the apparent diffusion coefficient, which in turn is related to the characteristic decay time, $\tau$, of the generated correlation

function. From the correlation function, the MSD, the average of the square of the distance travelled by the particles at a given time, can also be calculated.

The rennet-induced coagulation of recombined milk samples was also observed using DWS as described above. Measurements of scattered light intensity and the corresponding autocorrelation functions were collected every 4 min for 180 min for samples with low rennet (0.007%), or every 1 min for 30 min for samples containing higher concentration of rennet (0.035%). Experiments were analysed using software developed by Mediavention Inc. (Guelph, ON, Canada).

## 2.3 Rheology

The rheological behaviour of recombined milk during rennet coagulation (see above for conditions) was followed using a stress-controlled rheometer (AR 1000, TA Instruments Ltd., New Castle, USA) at 30 °C. Rheological measurements were conducted using a conical concentric cylinder geometry with the following specifications: 5,920 μm fixed gap, 15 mm radius, 14 mm rotor outer radius, and 42 mm cylinder immersed height. The samples were subjected to a time sweep test with 0.01 strain, 0.1 Hz, and an initial oscillation stress of 0.0018 Pa for 180 min. The gelation time was determined as the time needed to reach tan $\delta=1$, where $\delta=G''/G'$ ($G''$=storage modulus and $G'$=elastic modulus). After the time sweep, a frequency sweep test was conducted in the range from 0.01 to 10 Hz at an oscillation stress of 0.5 Pa. Strain sweep tests were also conducted to determine the viscoelastic region of the sample as well as the critical strain at fracture (determined as the strain after which a dramatic decrease in the value of $G'$ is observed).

## 2.4 Scanning electron microscopy

SEM micrographs we taken of the skim milk and recombined milk containing NaCas- or WPI-stabilised fat globules prepared as described above, dialysed against fresh raw milk (with no $CaCl_2$ added) and renneted at 30 °C using the 0.007% enzyme concentration. Specimens were taken at the gelation time of each sample (determined from the rheological experiments) as well as 1 h after the gel point. Sample preparation for SEM was performed as previously reported (Martin et al. 2006), with some modifications. Carbon planchets activated with a self-assembled monolayer (Martin et al. 2006) were immersed in milk samples immediately after the addition of rennet. At the appropriate times, the planchets were carefully rinsed with 10 mmol.$L^{-1}$ imidazole buffer (pH 6.7) and then transferred to 0.5% $OsO_4$ solution (0.25 g crystalline $OsO_4$ was dissolved in 25 mL of 10 mmol.$L^{-1}$ imidazole buffer at pH 6.7) and incubated for at least 12 h at room temperature. After this fixation step, carbon planchets were transferred to another fixative solution containing 1.5% glutaraldehyde in 10 mmol.$L^{-1}$ imidazole buffer for 30 min. Then, carbon planchets were progressively dehydrated in ethanol solutions (70%, 90%, and 100%) and subjected to critical point drying. The planchets were then sputter coated with gold (~30 nm) (Emitech K550X, Ashford, Kent, UK). Images were taken using a Hitachi s-4500 instrument (Hitachi, Tokyo, Japan) equipped with Quartz PCI v 5.1 imaging software (Quartz Imaging Corporation, Vancouver BC, Canada).

## 3 Results and discussion

### 3.1 NaCas- and WPI-stabilised fat globules in recombined milk

To assess the effect of surface composition of the fat globules on the rennet coagulation process, other variables that may alter the renneting behaviour of samples (such as the colloidal state and particle size of fat globules) should be taken into account. It is known that the WPI emulsification of fat globules under high ionic strength conditions, like the environmental characteristic of milk permeate, leads to the formation of flocculated fat globules (Gaygadzhiev et al. 2008). The colloidal state of the droplets will in turn affect the rheological properties of the rennet gel. Indeed, it has been demonstrated that although the WPI-stabilised fat globules do not interact directly with the casein gel matrix, the flocculated state of these fat globules causes the casein micelles to rearrange differently from when non-flocculated droplets are present, overpowering some effects that could raise from differences in the fat globules' interfacial reactivity (Gaygadzhiev et al. 2009b). For these reasons, in the present work, NaCas- and WPI-stabilised emulsions were prepared by homogenising the milk fat in protein solutions at low ionic strength buffer (5 mmol.$L^{-1}$ imidazole buffer, pH 6.7), and only after emulsification, the droplets were diluted with two times milk permeate to reach the environmental conditions typical of milk serum.

In order to investigate the renneting behaviour of emulsions containing mixed NaCas and WPI interfaces, the emulsions containing the proteins in isolation needed to be characterised and compared to skim milk first. Both NaCas- and WPI-stabilised fat globules showed no changes in turbidity and size for at least 3 h once diluted in a higher ionic strength (i.e. in permeate) (results not shown). They both yielded similar particle radii (as measured by DWS) of 156±1.8 and 173±3.1 nm, respectively. Furthermore, the turbidity parameter values (assessed at a volume fraction of fat globules φ=0.05), $1/l^*$, were also similar: 3.72±0.07 $mm^{-1}$ for NaCas-stabilised emulsion and 3.50±0.12 $mm^{-1}$ for WPI-stabilised emulsions. These results clearly demonstrate that, in addition to comparable dynamics, the spatial correlation and interparticle forces (represented by $1/l^*$ parameter) were identical for both systems. These results confirmed that, with this methodology, it was possible to prepare small oil droplets, which would not flocculate when recombined with milk serum. This allowed for a clear distinction of the behaviour of the two types of emulsion droplets during renneting, avoiding differences due to flocculation or size.

Figure 1 shows the development of the elastic moduli, $G'$, during renneting for representative runs of recombined milk containing NaCas- and WPI-stabilised fat globules ($\varphi$=0.05). The rheological behaviour of skim milk control is also shown. The average gelation time (determined at tan $\delta$=1) for recombined milk containing WPI-stabilised fat globules occurred at 80.4±4.2 min, as compared to 95.2±0.2 min for skim milk. Although this gelation time was earlier than the control, the development of $G'$ over time was comparable between these two samples as shown by the $G'$ values at 180 min (3.5±0.4 Pa for milk with WPI emulsion droplets and 4.6±0.6 Pa for control skim milk) (Table 1). The rheological profile of the sample containing WPI-stabilised fat globules differs from previously reported results (Gaygadzhiev et al. 2009b) for a similar system, where the $G'$ values were extended

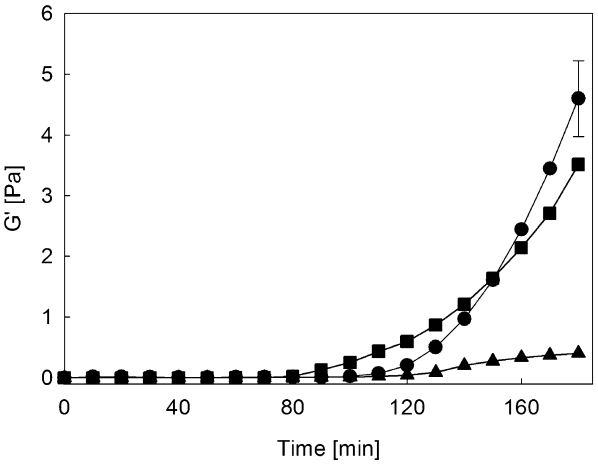


**Fig. 1** Development of storage moduli, $G'$, during the rennet coagulation process of skim milk (*circle*) and recombined milk containing whey protein isolate- (*square*) or sodium caseinate (*triangle*)-stabilised fat globules. Recombined milk samples were dialysed against skim milk

to significantly higher levels than those of the control skim milk. The discrepancy arises from the different colloidal state of the WPI-stabilised fat globules in the two systems. In the previous study, fat globules were prepared in milk serum and prone to flocculation, thereby affecting the viscoelastic properties of the gelling samples. Conversely, in the present study, the emulsions were prepared in buffer, thereby ensuring stability in the milk environment; in this case, there was minimal influence to the rheological properties of the recombined samples during renneting.

Figure 1 also illustrates the development of the elastic modulus in recombined milk containing NaCas-stabilised fat globules during renneting. This system showed rather different gelation behaviour (triangles). Contrary to the WPI-stabilised emulsion, this sample had a delayed gelation time when compared to the control skim milk. The final storage modulus 3 h after rennet addition was also statistically different and lower (Table 1) than those measured for the other two

**Table 1** Values measured during renneting of skim milk and recombined milk samples containing WPI- and NaCas-stabilised oil droplets

| | Initial $1/l^*$ ($mm^{-1}$) | Change in $1/l^*$ (min)[a] | Initial $R$ (nm) | Gelation by DWS (min)[b] | Gel point (min)[c] | $G'$ at 180 min (Pa) |
|---|---|---|---|---|---|---|
| SM | 1.53±0.02 | 34.6±0.2 | 131±2 | 87.8±2.0 | 95.2±0.2 | 4.6±0.6 |
| WPI | 6.58±0.04 | 32.1±5.7 | 270±2 | 55.1±3.2 | 80.4±4.2 | 3.5±0.4 |
| NaCas | 6.69±0.17 | 33.9±2.8 | 279±5 | 62.1±2.8 | 108±18.8 | 0.4±0.2 |
| SM+$CaCl_2$ | 1.60±0.07 | 20.8±2.3 | 115±12 | 63.7±2.3 | 82.5±3.6 | 16.5±0.5 |
| WPI+$CaCl_2$ | 6.51±0.14 | 24.0±5.7 | 269±12 | 42.3±2.8 | 65.3±1.3 | 13.9±3.3 |
| NaCas+$CaCl_2$ | 6.50±0.28 | 26.2±2.8 | 255±44 | 57.2±3.4 | 76.9±15.9 | 1.4±0.2 |

All samples were either dialysed against milk or against milk with added $CaCl_2$. Values are average of three independent experiments±standard deviation

[a] Change in $1/l^*$ indicates the time when an initial increment of $1/l^*$ is noted

[b] Gelation by DWS indicates the time corresponding to an increase in radius

[c] Gel point measured as $G''=G'$

samples. These results suggest that the rennet coagulation process was inhibited in the presence of NaCas-covered oil droplets. A similar behaviour has been previously reported, and the disruption of the calcium phosphate equilibrium of milk (caused by binding of $Ca^{2+}$ to NaCas) was proposed as a possible cause for the inhibition of structure development (Gaygadzhiev et al. 2009a). In this study, however, the ionic equilibrium of all samples was purposely kept identical by performing the dialysis against fresh milk; therefore, any effect due to differences in ionic environment can be ruled out. On the other hand, it has been recently shown that free caseins in milk affect the rennet-induced aggregation of the casein micelles by adsorbing onto the rennet-altered casein micelles and inhibiting their self-association, suggesting that adsorption of casein molecules onto the rennet-altered casein micelles, causing long-range steric repulsion forces, might be the cause of the inhibition (Gaygadzhiev et al. 2011).

To further investigate the interactions occurring during the pre-gelation stages of rennet coagulation, DWS was employed to observe the aggregation of the recombined milk samples. Figure 2 illustrates the development of the light-scattering parameters $1/l^*$ and apparent radius for (a) control skim milk and (b) recombined milk containing WPI-(squares) or NaCas-(triangles) stabilised fat globules. In the case of skim milk (Fig. 2a), the results are in full agreement with previous literature (Alexander and Dalgleish 2004; Sandra et al. 2007). The initial period after rennet addition showed no change in the values of $1/l^*$ since there was yet not enough hydrolysis of κ-casein to alter the existing interparticle forces and organisation. After this initial period, a continuous growth of the $1/l^*$ parameter could be seen, which has been attributed to a reorganisation of the casein micelles [i.e. changes in the interparticle structure factor due to the incremental removal of the stabilising 'hairy' layer (Sandra et al. 2007)]. During this time, the size of casein micelles remained about constant. After about 80 min, the $1/l^*$ values began to level off as the size of the casein micelles increased significantly. This point was considered the coagulation point of the system as determined by DWS (see Table 1).

The behaviour of the MSD for the skim milk can be seen in Fig. 2c (circles). At the initial time ($t$=0 h, filled circles), the MSD showed complete linearity, indicative of a free diffusing system, as the micelles were able to probe all accessible space in the scattering volume. At $t$=180 min (open circles), the MSD behaviour was quite different. The increase in inter-micelle interactions brought about by the cleaving of the caseino-macropeptide resulted in the formation of a gel, which in turn led to a drastic reduction in the mobility of the micelles. While at short correlation times (short length-scales), the micelles were still able to move quite unhindered; at longer length-scales, their excursions through space became limited, and the average distance accessible to the micelles was reduced. This can be seen by the asymptotic, long-time behaviour of the MSD.

Figure 2b depicts the light-scattering size and $1/l^*$ profiles of the recombined milk containing NaCas- and WPI-stabilised fat globules (triangles and squares, respectively). The scattered light intensity in these recombined systems was determined by the collective contribution of casein micelles and fat globules. However, it should be pointed out that the scattering intensity arising from the fat globules overpowers that from the casein micelles (Martin et al. 2006). Therefore, the light-scattering parameters in these binary systems represent mostly the behaviour of fat globules

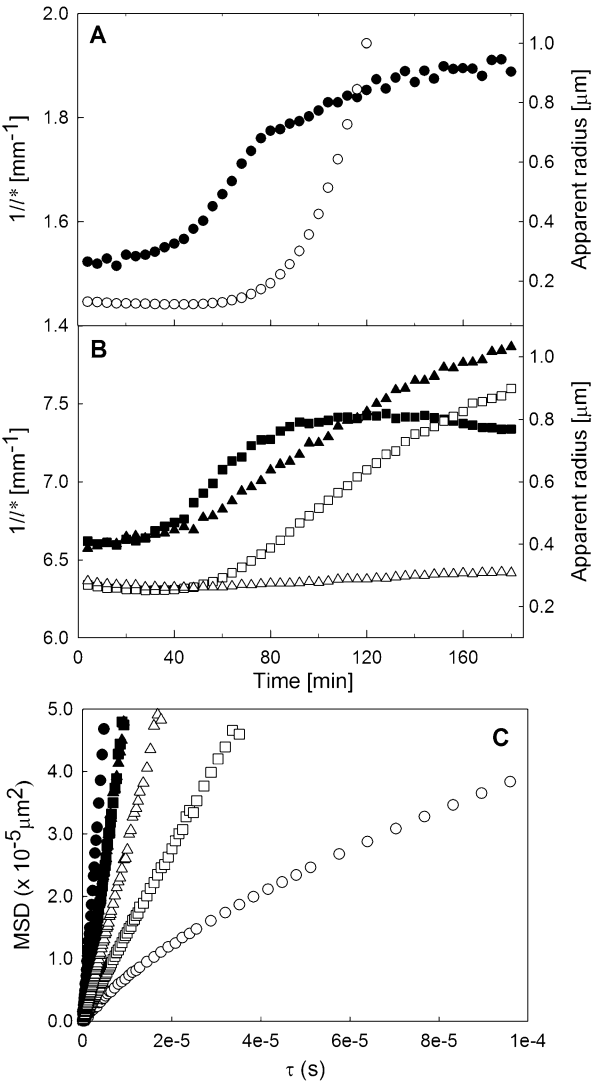


**Fig. 2** Development of $1/l^*$ (*closed symbols*) and apparent radii (*open symbols*) during the rennet coagulation process of **a** skim milk and **b** recombined milk containing whey protein isolate- (*squares*) or sodium caseinate (*triangles*)-stabilised fat globules. **c** The mean square displacement immediately after rennet addition (*closed symbols*) and after 180 min (*open symbols*). Recombined milk samples were dialysed against skim milk

during the coagulation process, as affected by the presence of casein micelles. This allows to tease out the behaviour of the oil droplets and to use them as a probe for the changes occurring to the gel network during rennet-induced aggregation, differing from rheology where the increase in the stiffness of the bulk gel is followed over time, but no information on the behaviour of the single components can be derived.

Immediately after the addition of rennet (at time $t=0$), the initial $1/l^*$ values for recombined milk containing NaCas- and WPI-stabilised fat globules were similar, but significantly higher than the turbidity parameter for skim milk. Furthermore, the initial radii of the recombined samples were noticeably higher than for the control milk (Table 1). The differences in $1/l^*$ can be readily explained by the presence of a

higher volume fraction of colloidal particles (i.e. casein micelles plus fat globules) in recombined milk samples, as well as the larger size and higher refractive index of fat globules compared to those of casein micelles. The difference in sizes is given by the fact that one system contained only casein micelles, but the other two also contained fat globules, larger than the protein particles.

The times of the initial change in the $1/l^*$ parameter after addition of rennet were not significantly different from those measured in control skim milk (Table 1). This was expected as the initial increase in interactions was driven by the action of chymosin on the surface of the micelles, and this was not affected by the presence of fat droplets (Gaygadzhiev et al. 2008). Overall, the behaviour of $1/l^*$ was similar, though in the case of NaCas-stabilised fat globules, $1/l^*$ did not show a clear levelling off within the experimental time. This might be an indication that rearrangements in the distribution of the fat globules continued to occur inside the matrix, but further investigations are needed to confirm these differences from the WPI-stabilised oil droplets system.

The onset of aggregation in the milk containing WPI-stabilised fat droplets was 55.1±3.2 (Table 1) and, in agreement with the rheological measurements shown above, significantly earlier than for the control skim milk. On the other hand, the mobility of the scattering particles (i.e. the WPI-stabilised oil droplets) behaved very differently from the control. Figure 2c shows the MSD for the WPI-stabilised emulsions (squares) at initial time (filled symbols) and 180 min after rennet addition (open symbols). Immediately after the addition of rennet, the droplets showed a linear relation between the MSD and the correlation time, very similar to that of the skim milk. The slope was slightly smaller, as the sizes are slightly larger, thereby having slower mobilities. At 180 min, the shape of the MSD showed no significant deviation from linearity, indicating that the WPI-covered fat globules were still quite mobile though the system had macroscopically gelled (Fig. 1). The noticeable decrease in slope can be attributed to a slowing down of the fat globules due to crowding, brought about by the existence of a gel matrix forming around them. However, the fact that the MSD never deviated from linearity (or the radius, Fig. 2b, shows an upper limit) clearly indicated that the fat globules were not restricted in their mobility and were still able to probe the entirety of the scattering volume. It was then concluded that the high mobility of the fat globules when compared to that of the casein micelles was due to a lack of direct interaction between the WPI-stabilised droplets and the casein network. Similar results have been observed before (Gaygadzhiev et al. 2009b). Interestingly, the value of $G'$ at 180 min after rennet addition (Table 1) was only slightly different from that of skim milk gels indicating that the casein micelles drove and carried the formation of the network and that the fat globules were mostly unaffected by its existence and did not contribute to the rheological characteristics of the gel. It is accepted in literature that the presence of other colloidal particles (fillers) modulates the mechanical properties of the gel network (van Vliet 1988; Blijdenstein et al. 2004; Ring and Stainsby 1982). Therefore, the rheological behaviour of the filled gels will depend on the structural properties of the gel matrix and of the filler particles (Tolstoguzov and Braudo 1983). Filler particles (e.g. fat globules) can be classified as ‘active’ and ‘inactive’ depending on their contribution to the gel formation (van Vliet and Dentener-Kikkert 1982). ‘Active’ fillers favour the strong interactions with the gel matrix. Conversely,

'inactive' fillers have little affinity to interact with the gel-forming species resulting in little or no contribution to the gel strength. In other words, when the emulsion droplets were fully covered by WPI, they behaved as inactive fillers (from a colloidal point of view) inside the gel.

The gelation behaviour of recombined milk containing NaCas-covered oil droplets (triangles), on the other hand, was quite different. As shown in Fig. 2b, the gelation point was drastically delayed showing little or no change in size (open triangles) over the experimental time. In accordance, the MSD (Fig. 2c) at 180 min (empty triangles) showed no deviation from linearity, and no substantial decrease in slope from the initial MSD (filled triangles), indicating that the casein-stabilised fat globules were not hindered in movement, due to the lack of formation of a gel matrix. This was also in agreement with the rheological results shown in Fig. 1, where no substantial increase in $G'$ was shown. This result is an evidence of the advantages of a multi-technique approach when trying to fully characterise the behaviour of complex systems.

To better understand the extent of the aggregation of the casein micelles during renneting, the structural state of the skim milk and recombined milk with WPI and NaCas-stabilised droplets was investigated using SEM (Fig. 3).

The structural organisation of the skim milk and recombined milk with WPI and NaCas-stabilised droplets was sampled (fixed) at their respective gelation points (as

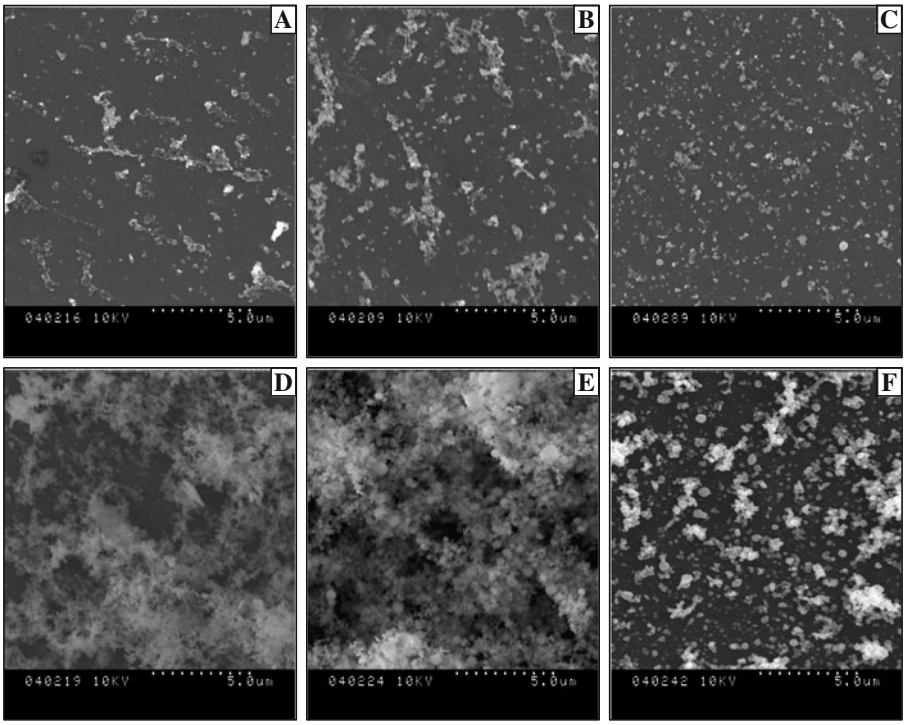


**Fig. 3** Scanning electron micrographs of skim milk (**a**, **d**) and recombined milk containing whey protein isolate- (**b**, **e**) or sodium caseinate (**c**, **f**)-stabilised fat globules. Samples were dialysed against skim milk. **a**–**c** Microstructure at the gelation point; **d**–**f** development of the microstructure 1 h after the gelation point (see Table 1)

determined by rheological experiments, see Table 1; Fig. 3a–c) and 1 h after the gelation point (Fig. 3d–f). The electron micrographs clearly demonstrated the existence of casein strands and clusters in control skim milk as well as in the two recombined milk samples right at the gelation point. The aggregation of rennet-altered casein micelles was less pronounced for recombined milk with NaCas-stabilised fat globules (Fig. 3c) compared to the other two samples, in which larger and thicker gel strands were observed. It should also be noted that the fat globules in the NaCas-containing recombined milk did not show as extensive flocculation as in the case of WPI-covered oil droplets. After the initial period of strand and cluster formation, the continuous aggregation of rennet-altered casein micelles led to the development of a space-filling gel network, which was observed for the control milk and the milk containing WPI-stabilised fat globules (Fig. 3d and e). In contrast, the recombined milk containing NaCas-stabilised fat globules showed very limited aggregation and no space-spanning gel even after 1 h from the gelation point as measured by rheology (compare Fig. 3d, f). This result is in accordance with the rheological and DWS data, showing no mobility restriction (Fig. 2c) and $G'$ development (Fig. 1) in milk with NaCas oil droplets. It should be reiterated again that, previous to rennet addition, all samples were dialysed against milk, thereby ensuring that the calcium equilibrium of the three systems was identical.

### 3.2 Effect of $CaCl_2$ addition

It has been previously reported that the addition of limited amounts of $CaCl_2$ to milk can substantially promote rennet coagulation (Lucey and Fox 1993; Bringe and Kinsella 1986; Zoon et al. 1988). Taking into account these results, the renneting behaviour of recombined milk samples was tested at a higher concentration of calcium by dialysing against fresh milk containing 1 mmol $L^{-1}$ $CaCl_2$. This dialysis step would ensure the same increase in Ca availability and environmental conditions for all samples.

Figure 4 shows the development of $G'$ during the gelation process of skim milk and the recombined milk samples discussed above, but dialysed against skim milk

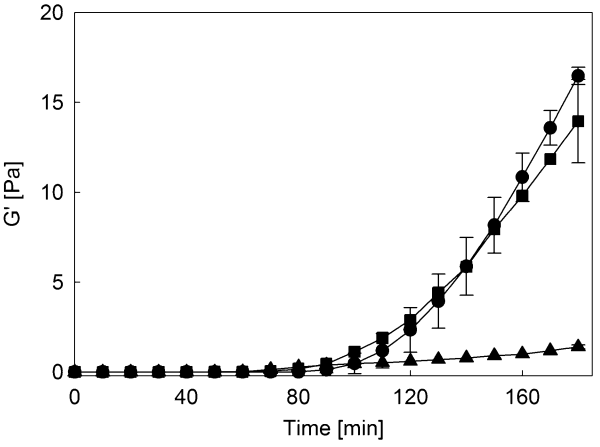


**Fig. 4** Development of storage moduli, $G'$, during the rennet coagulation process of skim milk (*circle*) and recombined milk containing whey protein isolate- (*square*) or sodium caseinate (*triangle*)-stabilised fat globules. Recombined milk samples were dialysed against skim milk containing 0.01% $CaCl_2$

with additional ionic calcium. In all cases, there was a decrease in the gelation times and a higher magnitude of the elastic moduli (see Table 1) compared to the gelation times of the same samples without added $CaCl_2$. These results were in agreement with the findings from previously published work investigating the effect of $CaCl_2$ on the rennet-induced gelation of milk (Bringe and Kinsella 1986; Zoon et al. 1988). It is known that the enhanced gelation behaviour of casein micelles in the presence of additional $CaCl_2$ results from an increase of the soluble and the colloidal calcium concentration, a decrease of the pH and charge neutralisation of the casein micelles. These effects cause the casein micelles to aggregate at a lower degree of κ-casein hydrolysis (Horne and Banks 2003). Interestingly, however, the presence of a higher amount of $CaCl_2$ did not overly improve the rennetability of recombined milk containing NaCas-stabilised fat globules (Fig. 4) since the final recorded value of $G'$ was still very low.

The results from the rheological experiment were in accordance with the light-scattering measurements (Fig. 5). The increase of particle size started at earlier times for all samples when compared to the same samples without $CaCl_2$ (compare Figs. 2 and 5). The onset of the increase in $1/l^*$ was also at earlier times (see Table 1). The general trends in turbidity and radius development of skim milk with added $CaCl_2$ were similar to those shown in Fig. 2a. This also holds true for the recombined samples containing WPI-stabilised droplets containing additional $CaCl_2$ compared to the original samples shown in Fig. 2b. After an initial period of constant $1/l^*$ and radius, the turbidity parameter started to increase earlier in time before any appreciable size increase. This is indicative of inter-micelle interactions beginning to occur due to a sufficient action of the rennet on the surface of the micelles. About 20 min after this increase in $1/l^*$, changes in the radii were observed. At this point, enough caseino-macropeptide had been removed, and aggregation of the casein micelles could take place. It can be noted that there was no difference in the development of the apparent radii in the recombined milk containing WPI-stabilised fat globules, with or without the addition of $CaCl_2$. This observation seemed to suggest that the behaviour of fat globules in both samples was not affected by the amount of soluble calcium present, as the detected light signal in recombined system is fully determined by the scattering profile of the fat globules. As mentioned above, this coagulation point was shifted forward by the presence of $CaCl_2$.

The mean square displacement for both control skim milk and WPI-stabilised recombined milk with added $CaCl_2$ at 180 min after addition of rennet (Fig. 5c, open circles and squares, respectively) also behaved similarly to those of the original systems without additional calcium ions. The mean square displacement of the skim milk sample with $CaCl_2$ showed an asymptotic behaviour, indicating arrested motion of the micelles due to their integration into a gel network. On the other hand, the WPI-stabilised recombined milk+$CaCl_2$ showed linearity at all times indicating decrease in mobility (lower slope) but otherwise still free diffusive.

The case of recombined milk containing NaCas-stabilised fat globules (open triangles) was again quite different from the other two systems, but quite similar to the original NaCas-containing milk without the addition of calcium. The overall shape of the development of the renneting system did not change dramatically, although at the later stages of the reaction (~120 min), the $1/l^*$ parameter showed a plateau. The apparent particle radius (open triangles) reached a larger apparent size

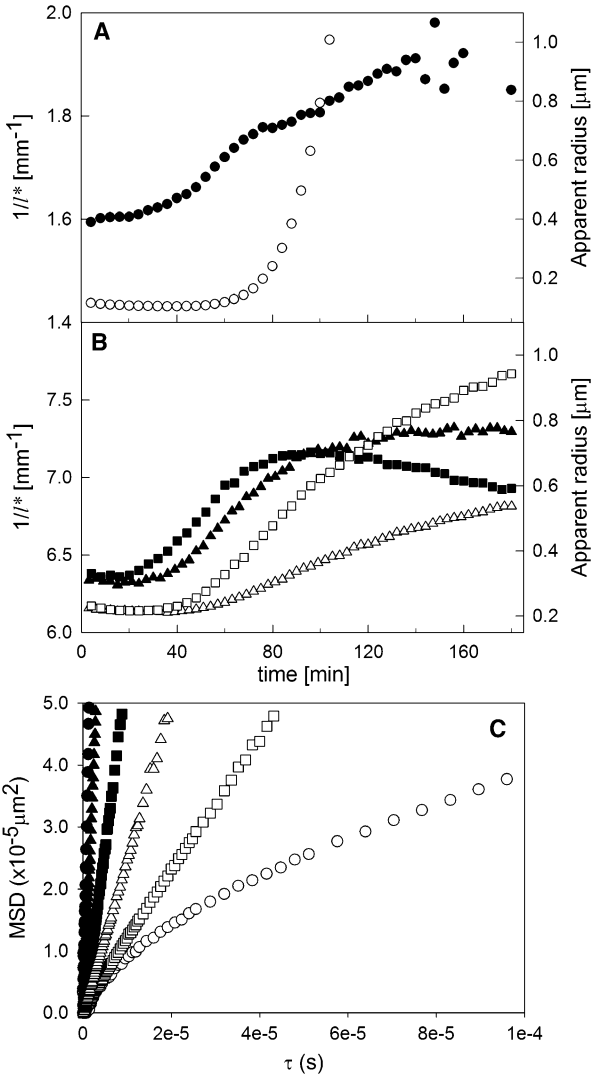


**Fig. 5** Development of $1/l^*$ (*closed symbols*) and apparent radii (*open symbols*) during the rennet coagulation process of **a** skim milk and **b** recombined milk containing whey protein isolate- (*squares*) or sodium caseinate (*triangles*)-stabilised fat globules. **c** Mean square displacements immediately after rennet addition (*closed symbols*) and after 180 min (*open symbols*). Recombined milk samples were dialysed against skim milk containing 0.01% $CaCl_2$

(~500 nm instead of ~300 nm with no added calcium) but still failed to produce substantial coagulation. The results obtained by light scattering were in full agreement with those obtained by rheology (Fig. 4). The lack of gel formation in these recombined milk samples with added calcium was also noticeable in the MSD results (Fig. 5c), which showed full linearity at 180 min and a very small decrease in slope (empty triangles).

The results obtained for the three different systems with identical ionic composition and, in the absence and presence of $CaCl_2$, are a strong indication that the inability of renneted casein micelles to coagulate in the presence of NaCas-stabilised fat globules was not caused by the lack of available soluble $Ca^{2+}$ in the milk serum.

### 3.3 Changes in the interfacial composition of the stabilised fat globules

The major differences between the recombined milk systems presented above resided in the properties of the interfacial layers of the fat globules. While the WPI-stabilised interface is characterised by a compact layer of globular proteins giving mainly electrostatic stabilisation, the NaCas-stabilised oil/water interface is covered by the flexible caseins chains yielding electrostatic and steric repulsion (Dalgleish 2006). In order to assess the relative influence of the steric stabilisation on the aggregation ability of renneted casein micelles, experiments were performed with mixed interfaces, by preparing emulsions with different ratios of WPI and NaCas. Figure 6 shows the rheological and light scattering parameters during renneting of recombined milk samples containing fat globules prepared with 1% (*w*/*v*) protein, but with different WPI and NaCas proportions. All the recombined samples were prepared as described above and dialysed against fresh milk containing 1 mmol.$L^{-1}$ $CaCl_2$, to ensure comparable serum composition.

Figure 6a shows the rheological behaviour of different recombined milk samples. The development of $G'$ was not significantly different between the recombined systems containing 100% WPI and 90% WPI+10% NaCas; however, it decreased when the oil droplets contained 70% WPI+30% NaCas. The recombined milk containing NaCas-only covered oil droplets is also shown in Fig. 6a for reference. Table 2 summarises the average gelation parameters measured by rheology and DWS. Both gelation times and $G'$ values measured at 180 min were increasingly affected by augmenting the proportion of NaCas at the interface (see Table 2). Furthermore, the $G'$ values at 180 min showed an inverse relationship with the increasing amount of NaCas, with the systems stabilised by 100% and 90% WPI+10% NaCas WPI having a $G'$ larger than an order of magnitude from that of the system with 100% NaCas (Table 2).

The development of $1/l^*$ for the milk containing oil droplets of different interfacial composition also followed the same trend as rheology. There were no significant differences for milk containing 100% WPI and 90% WPI+10% NaCas, while the sample with 70% WPI+30% NaCas had a later increase of $1/l^*$, and the one containing 100% NaCas showed the latest increase amongst them all (Table 2). The aggregation point, shown by the rapid increase in the particle size (Fig. 6c), was not statistically significant in the case of emulsions containing high amounts of WPI (100% and 90%), but showed a slower aggregation with 30% NaCas. As discussed above, milk containing 100% NaCas had a noticeably inhibited increase in radius and, in accordance with rheological measurements (Fig. 6a), showed no substantial gel development.

These results suggest that the surface composition of the fat globules affect the bulk viscoelastic properties of the recombined milk while not affecting their motion or spatial distribution at short scales. Previously reported data (Parkinson and Dickinson 2007) suggested the importance of a small proportion of NaCas in protecting WPI-stabilised emulsions during heating. This work indicated that the substitution of WPI with up to 30% of NaCas, though causing an increase in the steric repulsion of the oil droplets, did not overly affect the renneting behaviour of recombined milks. However, at higher levels of NaCas, the renneting ability of the micelles was impaired, suggesting, again, that high availability of NaCas in the system played an important role in the renneting ability of the recombined milks.

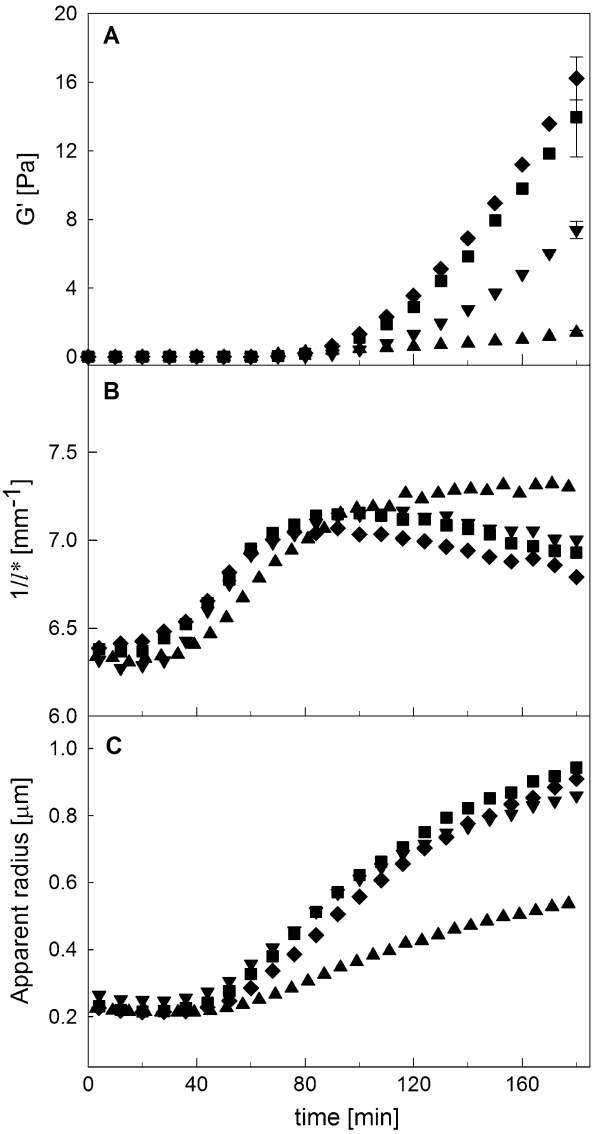


**Fig. 6** Development of **a** $G'$, **b** $1/l^*$ and **c** apparent radii during the rennet coagulation process of recombined milks containing fat globules stabilised with various proportions between whey protein isolate (WPI) and sodium caseinate (NaCas) [*square* (100% WPI+0% NaCas); *diamond* (90% WPI+ 10% NaCas); *inverted triangle* (70% WPI+30% NaCas); *triangle* (0% WPI+100% NaCas)]. All recombined milk samples were dialysed against skim milk containing 0.01% $CaCl_2$

All the experiments described above (aggregation behaviour without and with added $CaCl_2$, and substitution of the protein at the interface) were repeated with double the amount of rennet addition (results not shown). Previous experiments performed on the renneting of unheated skim milk plus excess NaCas in solution (but no oil droplets) have shown that the presence of NaCas did not affect the kinetics of the primary stage of renneting, meaning that the CMP was released with rates not significantly different from those of the skim milk control (Gaygadzhiev et al. 2011). Even under these conditions, recombined milk containing NaCas-stabilised fat globules did not show gel formation, and substantial rheological and

**Table 2** Values measured during rennetting of reconstituted milk containing oil droplets stabilised with different ratios of WPI and NaCas

| Samples dialyzed in milk+ $CaCl_2$ | Change in 1/*l** (min) | Gelation by DWS (min) | Gel point (min) | *G′* at 180 min (Pa) |
|---|---|---|---|---|
| 100% WPI+0% NaCas | 22.0±2.3 | 44.5±2.1 | 65.2±0.9 | 13.9±2.3 |
| 90% WPI+10% NaCas | 24.0±2.8 | 45.0±2.3 | 69.7±3.5 | 16.2±1.2 |
| 70% WPI+30% NaCas | 27.1±2.8 | 50.5±2.6 | 77.8±1.3 | 7.4±0.5 |
| 0% WPI+100% NaCas | 37.1±3.2 | 56.0±3.0 | 76.9±15.9 | 1.4±0.1 |

See Table 1 for details. All systems were dialysed against milk containing 1 mM $CaCl_2$. Values are average of three independent experiments±standard deviation

light scattering differences were only found when the substitution was at least 30% NaCas. Once again, these findings were in agreement with those reported above and seemed to suggest that the impaired rennetability of casein micelles by NaCas-stabilised fat globules is not caused by the lack of sufficient soluble $Ca^{2+}$ or by the kinetics of rennet coagulation process.

## 4 Conclusion

The kinetics of rennet coagulation of a system of reconstituted skim milk with WPI and NaCas-stabilised oil droplets was investigated using rheology and diffusing wave spectroscopy. All systems were also investigated after dialysis of the samples against milk+1 mmol.$L^{-1}$ $CaCl_2$, to ensure that the ionic conditions and calcium availability of the casein micelles was kept intact and comparable between recombined milk samples. The results clearly indicated that the aggregation behaviour of recombined milk containing WPI-stabilised fat globules was not significantly affected compared to the coagulation profile of control skim milk. The WPI-stabilised fat globules did not interfere with the formation of the casein network. The increased $Ca^{2+}$ and enzyme concentration significantly promoted the coagulation process of the control sample and of the recombined samples containing WPI-stabilised fat globules. These findings suggest that the development of the gel network was completely dominated by the aggregation of the casein micelles and the WPI-stabilised fat globules filled the spaces within the gel strands without being actively involved in their formation.

On the other hand, the aggregation ability of rennet-altered casein micelles was largely hindered in the presence of NaCas-stabilised fat globules. Increasing the concentration of soluble $Ca^{2+}$ and enzyme did not recover the impaired rennetability of the recombined milk containing NaCas-stabilised fat globules. The inhibition of the coagulation process was not due to a reduced availability of $Ca^{2+}$.

When emulsions were prepared with different ratios of WPI and NaCas, the rennetability of the recombined milk was not at all or slightly affected with NaCas substitution of up to 30%. However, higher ratios of NaCas/WPI showed inhibition of the casein micelles ability to form a network. The increased steric repulsion of the oil droplets and the increase of NaCas present in the serum phase in recombined

milk are most likely the contributors of the impaired rennetability of the casein micelles. Indeed, as previously reported, small concentrations of soluble caseins affect the coagulation of rennet-altered casein micelles, and it could be stipulated that at high concentration of NaCas (1% *w*/*v*) even after dilution of the emulsion droplets in recombined milk, there may be enough soluble casein to hinder gelation.

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