

# Changes in the physico-chemical properties of casein micelles in the presence of sodium chloride in untreated and concentrated milk protein

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**Abstract** The addition of NaCl to milk is known to alter the mineral equilibrium and the structure of casein micelles. The objective of this study was to better understand the light scattering properties and viscosity of milk as a function of NaCl addition (0–500 mmol.L<sup>-1</sup>), for fresh skim milk and 2× concentrated milk protein. Suspensions (2×) were prepared using osmotic stressing. NaCl was added by dialyzing milk with milk serum (permeate) for 18 h. The presence of NaCl decreased the pH of milk and the zeta potential of casein micelles. When measured under diluted conditions, using dynamic light scattering, the average radius of the casein micelles showed a small increase, from 84 to 88 nm. Total calcium and phosphate in concentrated milk protein suspensions decreased with NaCl concentration, with a corresponding increase of the permeable calcium and phosphate, and of non-sedimentable caseins. In both untreated and concentrated milk protein samples, the viscosity increased with NaCl concentration. The characteristic decay time ( $\tau$ ) and turbidity measured under non-diluted conditions using diffusing wave spectroscopy showed a gradual decrease with NaCl addition. This work demonstrated that the changes to milk with addition of NaCl are mainly caused by the disruption of the internal structure of the casein micelles and changes in the composition of the serum phase, affecting the viscosity of the continuous phase as well as the refractive index contrast.

**Keywords** Osmotic stress · Casein micelle · Mineral balance · Turbidity · Diffusing wave spectroscopy

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## 1 Introduction

Caseins constitute up to 80% of total proteins in bovine milk. In native milk, they are associated into colloidal particles known as casein micelles. The composition and structure of casein micelles have been widely reported (Donato and Dalgleish 2006; Dalgleish and Corredig 2012; Holt et al. 2013). Casein micelles have a core composed primarily of  $\alpha$ <sub>S</sub>- and  $\beta$ -caseins, associated to one another by non-covalent interactions and by calcium phosphate nanoclusters of a few nanometer size, linked to the serine residues of the casein proteins (De Kruif and Zhulina 1996; Dalgleish and Corredig 2012).  $\kappa$ -casein molecules are mostly located on the surface of the casein micelle, with their hydrophilic portions partly extended into the aqueous phase, yielding a polyelectrolyte brush of great importance to the colloidal stability of the protein particles (De Kruif and Zhulina 1996).

Casein micelle stability plays a crucial role in the overall processing properties of milk, and changes in mineral balance may have important consequences to the stability of the micelles. A wide variety of studies have investigated the effect of NaCl on physicochemical characteristics of milk. The addition of NaCl to bovine milk reduces the pH of milk (Grufferty and Fox 1985; Gaucheron et al. 2000) and increases the hydration and voluminosity of casein micelles (Van Hooydonk et al. 1986; Creamer 1985). In addition, NaCl addition can increase the level of non-sedimentable calcium and magnesium (Grufferty and Fox 1985; Gaucheron et al. 2000). There are conflicting findings about the influence of NaCl on the non-sedimentable phosphate. Some researchers report that NaCl addition has little effect on the non-sedimentable phosphorus (Le Graet and Gaucheron 1999; Gaucheron et al. 2000; Huppertz and Fox 2006), while others demonstrate that NaCl significantly increased phosphorus concentration in ultrafiltrate of casein micelle dispersions (Famelart et al. 1999; Karlsson et al. 2007). Most of the studies have been carried out on reconstituted skim milk or casein concentrates (Famelart et al. 1999; Huppertz and Fox 2006), and differences may occur in the amount of soluble protein present and corresponding ions associated with the proteins.

In addition, a few studies have been conducted on the influence of salt addition on concentrated milk. Huppertz and Fox (2006) studied the effect of NaCl, directly added to milk, on various processing properties of caseins. Addition of NaCl to concentrated milk increased the pH of maximum heat stability and the maximum heat coagulation time. There were no differences in the average casein micelle size, but there was a reduced net charge on the casein micelles and a reduction of pH, also in concentrated milk. As for non-concentrated milk, soluble and ionic calcium increases by addition of NaCl, but the level of soluble inorganic phosphorus does not vary (Huppertz and Fox 2006).

In this work, the effect of NaCl on casein micelles properties was studied on fresh unheated skim milk and milk concentrated using osmotic stressing. Using this process, the concentration of casein micelles can be carried out non-invasively, unlike evaporation or ultrafiltration (Bouchoux et al. 2009). Milk proteins are selectively concentrated, while the serum phase composition remains similar to that of the original milk. The aims of this research were to further investigate the NaCl-induced changes of physicochemical properties of these milk protein concentrates, to compare them to those of fresh skim milk, and to study such properties *in situ*, without dilution, using diffusing wave spectroscopy and rheology.

## 2 Materials and methods

### 2.1 Skim milk and permeate preparation

Fresh raw milk was obtained from the Elora Dairy Research Farm of University of Guelph (Elora, ON, Canada). Sodium azide was added immediately at a concentration of 0.02% (*w/v*) (3 mmol.L<sup>-1</sup>) as a bacteriostatic agent. The milk was skimmed by centrifugation at 4,000×*g* for 25 min at 4 °C (J2-21 centrifuge, Beckman Coulter Canada Inc, Mississauga, Canada) and then filtered four times through Whatman fiberglass filter (Fisher Scientific, Mississauga, ON, Canada). The skim milk was kept refrigerated (4 °C) until use.

Skim milk permeate was prepared by ultrafiltration of skim milk (Crown Dairy, Guelph, ON, Canada) by passing it through an OPTISEP® Filter module (Smartflow Technologies, Apex, NC, USA) with 10-kDa molecular mass cutoff at ambient temperature. The acquired permeate was added with 0.02% (*w/v*) sodium immediately and kept at refrigeration temperature until it was used.

### 2.2 Concentration of milk using osmotic stressing

Polyethylene glycol (PEG) (35,000 Da, Fluka, Oakville, ON, Canada) was added to milk permeate at a final concentration of 7%. Regenerated cellulose dialysis bags (Fisher Scientific, Whitby, ON, Canada) with a molecular mass cutoff of 6–8 kDa washed with Milli-Q water and conditioned with permeate. Milk samples (40 mL) were then placed in dialysis bags and immersed in 1 L of permeates containing additional concentrations of NaCl ranging from 0 to 500 mmol.L<sup>-1</sup>. The dialysis allowed the exchange of small molecules such as water, ions, and lactose but not caseins or PEG (Bouchoux et al. 2009). Concentrated samples (2×) were obtained after dialysis for 18 h at 4 °C with stirring. When necessary, the final volumes of the 2× retentates was adjusted with ultrafiltration (UF) permeate (see above). Non-concentrated (1×) skim milk was also dialyzed in 1 L UF permeate with different NaCl concentrations as control.

### 2.3 Zeta potential and average casein micelle size

The particle size and the apparent zeta potential of the casein micelles were measured by dynamic light scattering (DLS) method (Zetasizer Nano, Malvern Instruments, Worcestershire, UK). After concentration, the milk samples were diluted 500 times with filtered (0.2-µm nylon filters, Fisher scientific) UF permeates containing the same concentration of added NaCl and analyzed. All the measurements were conducted at 25 °C.

### 2.4 Diffusing wave spectroscopy

Transmission diffusing wave spectroscopy (DWS) was used in this research which allows to investigate colloidal behavior of casein dispersion in situ without dilution. The output results from DWS for a free diffusing system is a time-dependent correlation function, with a characteristic decay time:

$$\tau = 1/k_0^2 D$$

where  $k_o$  is the wave vector ( $k_o=2\pi n/\lambda$ , where  $n$  is the refractive index of the continuous phase and  $\lambda$  is the wavelength) and  $D$  is diffusion coefficient. The photon transport mean free path  $l^*$  was also measured by DWS (Alexander and Dalgleish 2005); this parameter is related to the length scale over which the direction of the scattered light has been totally randomized. Turbidity was calculated as the inverse of the  $l^*$  parameter. The apparent radius of the casein micelles was derived from diffusion coefficient through the Stokes-Einstein relation. Standard latex spheres of diameter 260 nm (Portland Duke Scientific, Palo Alto, CA, USA) were used to calibrate the laser intensity daily.

The sample was poured into an optical glass cuvette (Hellma Canada Ltd., Concord, ON, Canada) with a 5-mm path length and then placed in a water bath maintained at 25 °C. The sample was illuminated by a solid-state laser light with a wavelength of 532 nm and a power of 350 mW (Coherent, Santa Clara, CA, USA). Scattered light intensity was collected in transmission mode as previously described (Sandra et al. 2011) and analyzed using software developed specifically for the equipment (Mediavention Inc., ON, Canada). Correlation functions and intensity of transmitted scattered light were measured at intervals of 3 min. The viscosity and refractive index of the continuous phase used to calculate the radius were  $1.021 \times 10^{-3}$  Pa.s and 1.34, respectively (Alexander et al. 2006; Alexander and Dalgleish 2005; Sandra et al. 2011).

## 2.5 Determination of viscosity

Advanced Rheometer AR 1000 (TA Instruments Ltd., New Castle, DE, USA) equipped with a peltier temperature controller was used to measure the viscosity. All samples were subjected to a steady flow test (shear rate ramp from 0 to  $100 \text{ s}^{-1}$ ), using a cone and plate geometry, with a set gap of 0.51 mm. The values are reported for  $100 \text{ s}^{-1}$ . All measurements were conducted at 25 °C.

## 2.6 Non-sedimentable protein

The soluble protein present in the casein micelle dispersion was defined as the fraction that did not sediment after ultracentrifugation at  $100,000 \times g$  for 1 h at 20 °C (Optima™ LE-80 K ultracentrifuge with rotor type 70.1Ti, Beckman Coulter Canada Inc., Mississauga, Canada). The supernatants were given two sequential filtrations using 0.45 and then 0.22  $\mu\text{m}$  (low protein binding, Fisher Scientific).

The amount of protein present in the supernatant was measured using a Dumas nitrogen analyzer (FP-528, Leco Inc. Lakeview Avenue, St. Joseph, MI) and the protein concentration was determined using 6.38 as conversion factor. The protein composition of supernatants was subsequently analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions; the resolving and stacking gel contained 15 and 4% acrylamide, respectively. Gels were run at 175 V for 1 h using Bio-Rad electrophoresis unit (Bio-Rad Power Pac HC, Hercules, CA). The gels were stained with Coomassie blue in a 5:1:4 mixture of methanol, acetic acid, and Milli-Q water for 30 min and destained in a 4.5:1:4.5 mixture of methanol, acetic acid, and Milli-Q water for 1 h for two times. Destained gels were scanned in a Bio-Rad Gel Doc EZ Imager (Bio-Rad Power Pac HC, Hercules, CA) equipped with Image Lab 3.0 (Bio-Rad Power Pac HC, Hercules, CA) software.

## 2.7 Determination of calcium and phosphate

For the determination of total calcium, aliquots (666  $\mu\text{L}$ ) of milk samples were mixed with 400  $\mu\text{L}$  of 1 M HCl and 266  $\mu\text{L}$  in an Eppendorf microcentrifuge tube using HPLC water. The mixture was then centrifuged at room temperature for 15 min at  $4,500\times g$  (Eppendorf centrifuge, 5415 D, Brinkmann Instruments Ltd., Mississauga, ON, Canada). The clear supernatants were then diluted with  $\text{HNO}_3$  acceptor solution (2 mM  $\text{HNO}_3$  solution) to 300 times. For determination of permeable calcium, the supernatants separated by centrifugation (see Sect. 2.6) were transferred to ultrafiltration tubes with molecular weight cutoff of 10 KDa; it was then centrifuged at  $4,000\times g$  for 1 h to get rid of the proteins. The obtained permeates were diluted 200 times with  $\text{HNO}_3$  acceptor solution.

The ion separation was carried out using 861 Advanced Compact IC ( $\Omega$  Metrohm ion analysis, Metrohm Ltd., Herisau, Switzerland) composed of an injection valve, a high-pressure pump, and a conductivity detector. A cation column (Metrosep C4/150, Metrohm) packed with 5- $\mu\text{m}$  silica gel with weakly acidic carboxyl acid functional groups ( $\text{RCO}_2^-$ ) was employed. Pyridine-2,6-dicarboxylic acid (99% Acros Organics, Geel, Belgium) and 70% nitric acid were used to prepare mobile phase (0.7 mM dipicolinic acid and 1.7 mM  $\text{HNO}_3$ ). The elution was monitored with a conductivity detector and the area under each peak was quantified using calibration curves prepared with calcium standards (TraceCERT, Fluka, Sigma Steinheim, Germany). Samples were eluted at a flow rate of  $0.9 \text{ mL}\cdot\text{min}^{-1}$  (Rahimi-Yazdi et al. 2010).

For the determination of total and permeable phosphate, the same sample preparation methods were adopted as calcium. However, in this case, HPLC-grade water was used as acceptor solution in the phosphate measurement, and the samples were eluted on an anion column (Metrosep A Supp5-150/4.0, Metrohm) packed with 5  $\mu\text{m}$  polyvinyl alcohol with quaternary ammonium groups. Sodium hydrogen carbonate and sodium carbonate solutions were used to prepare the mobile phase (1.0 mM sodium hydrogen carbonate and 3.2 mM sodium carbonate). Samples were eluted at a flow rate of  $0.5 \text{ mL}\cdot\text{min}^{-1}$ .

## 2.8 Statistical analysis

Three or four replicates were performed for each test. ANOVA and Turkey HSD were carried out on the experimental measurements with 95% confidence level using Minitab statistical package release 15 (Minitab Inc., State College, PA, USA).

# 3 Results

## 3.1 Influence of NaCl on pH and zeta potential of the casein micelles

The values of pH and of zeta potential of the casein micelles in milk after equilibration with NaCl are summarized in Table 1. The addition of NaCl caused a reduction in pH for both unconcentrated ( $1\times$ ) and concentrated ( $2\times$ ) milk samples, in full agreement with previous reports (Gaucheron et al. 2000; Grufferty and Fox 1985; Huppertz and Fox 2006). The pH of unconcentrated milk reduced gradually from 6.74 to 6.56 for

**Table 1** Effect of NaCl (0–500 mmol.L<sup>-1</sup>) on the pH and zeta potential of casein micelles in untreated (1×) and concentrated (2×) milk

	NaCl (mmol.L <sup>-1</sup> )	pH	Zeta potential (mV)
1×	0	6.74±0.01 <sup>a</sup>	-21.2±0.1 <sup>a</sup>
	100	6.67±0.01 <sup>b</sup>	-20.2±0.7 <sup>ab</sup>
	300	6.65±0.01 <sup>b</sup>	-19.2±0.8 <sup>b</sup>
	500	6.56±0.01 <sup>c</sup>	-16.2±0.3 <sup>c</sup>
2×	0	6.76±0.01 <sup>a</sup>	-21.9±0.2 <sup>a</sup>
	100	6.69±0.01 <sup>b</sup>	-20.3±1.0 <sup>a</sup>
	300	6.56±0.03 <sup>c</sup>	-16.1±0.2 <sup>b</sup>
	500	6.47±0.01 <sup>d</sup>	-15.4±0.9 <sup>b</sup>

Results are the average and standard deviation of three independent experiments. Within a concentration, different letters show significant differences for  $P < 0.05$

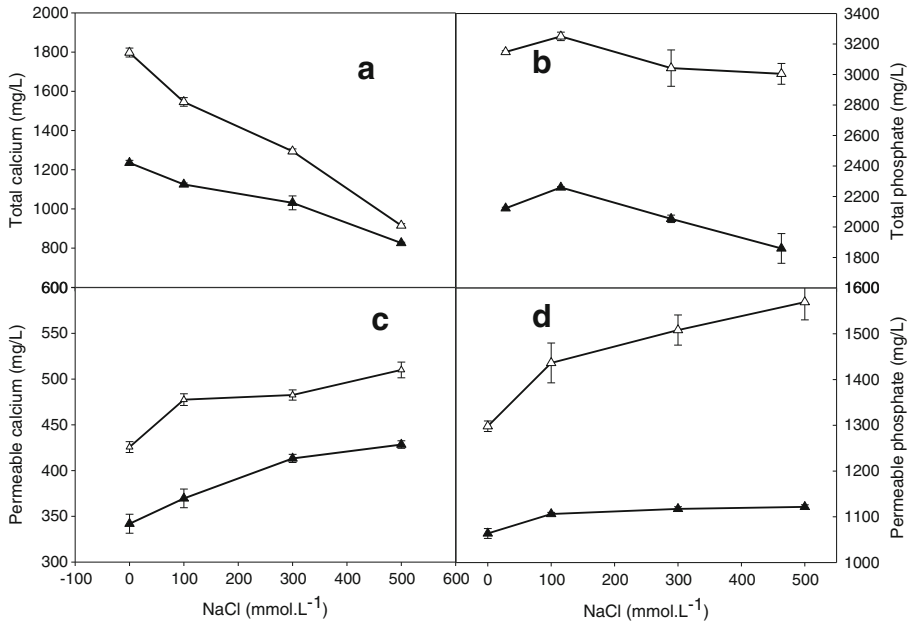
milk dialyzed against permeates containing 500 mmol.L<sup>-1</sup> NaCl; similarly, the pH of 2× concentrated milk decreased from 6.76 to 6.47.

The zeta potential of casein micelles in the absence of NaCl was about -21 mV. Addition of NaCl to unconcentrated and 2× concentrated milk protein suspensions led to a decrease of zeta potential to -16 mV (Table 1). This change is expected as the higher ionic strength of the solution will cause a decrease in the electrostatic charge and thickness of the electrical double layer (Bouchoux et al. 2009, 2010) and will reduce the electrophoretic mobility of the colloidal particles (Walstra 2003).

### 3.2 Influence of NaCl on mineral balance

Milk samples were dialyzed against milk permeate (the serum portion of milk) containing varying concentration of NaCl. By addition of PEG to the permeate, the milk was concentrated 2× when dialyzed for 18 h. Figure 1 illustrates the changes in total and soluble (defined as permeable, see Sect. 2) calcium and phosphate in the samples after dialysis against different concentrations of NaCl. In the absence of NaCl, with or without PEG in the milk permeate, the milk samples maintained the chemical potential of all ions in the native state, as previously demonstrated (Bouchoux et al. 2010). With additional NaCl, a chemical potential difference occurred between milk samples and permeates, resulting in an exchange of ions during dialysis. The total calcium in milk decreased gradually with NaCl addition (Fig. 1a). The total calcium contents were reduced from 30.9±0.3 and 44.9±0.6 mM in unconcentrated and 2× concentrated milk, respectively, to 20.6±0.1 and 23.0±0.2 mM when 500 mmol.L<sup>-1</sup> NaCl was added (Fig. 1a). Similarly, total phosphate contents, in both unconcentrated and 2× concentrated milk suspensions, showed a significant decrease after addition of 300 mmol.L<sup>-1</sup> NaCl, although to a lower extent than calcium (Fig. 1b). The total calcium decreased to 49% for 2× milk and 33% for 1× milk, while the phosphate decreased to only 4.6 and 12.4% for 2× and 1× milk, respectively.

The decrease in the total calcium concentration in milk with the addition of NaCl corresponded to an increase in permeable calcium, as clearly shown in Fig. 1c. It was concluded that with the addition of NaCl, dissociation of calcium from the casein



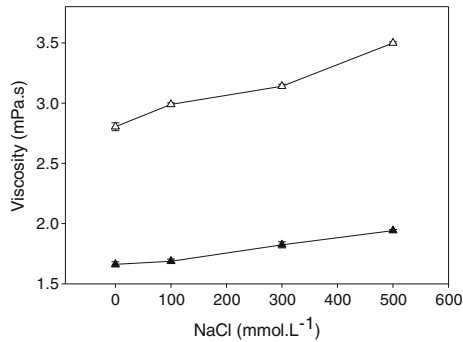
**Fig. 1** Influence of added NaCl on total calcium (a), total phosphate (b), permeable calcium (c), and permeable phosphate (d) in untreated (filled triangle) and 2× (open triangle) concentrated milk. Values are average of triplicate experiments, with the standard deviation indicated by vertical error bars

micelles occurred. Only about 32 and 22.6% of the original colloidal calcium were recovered in unconcentrated and 2× concentrated milk, respectively, in samples containing 500 mmol.L<sup>-1</sup> NaCl. On the other hand, at this NaCl concentration, there was a less than 10% increase in the amount of permeable phosphate recovered in unconcentrated milk, and about 20% (from 13.7 to 16.5 mM) for 2× concentrated milk (Fig. 1d). It was concluded that most phosphate remained associated to the proteins even after the addition of 500 mM NaCl, resulting only in a slight decrease of the total concentration of phosphate (Fig. 1b).

Previous researchers have reported that the addition of NaCl to casein micelle suspensions led to the solubilization of calcium and phosphorus from micelles and increased the casein voluminosity and hydration (Hussain et al. 2011; Karlsson et al. 2005). The increase in soluble calcium is due to the exchange between sodium and calcium during dialysis. As calcium phosphate clusters are crucial to the internal structure and contribute to rigidity of the casein micelles, the replacement of calcium by sodium within casein micelles could decrease protein-protein interactions and loosen the internal structure of the casein micelles.

### 3.3 Influence of NaCl on milk apparent viscosity

The addition of NaCl up to 500 mM progressively increased the apparent viscosity of both unconcentrated and 2× milk. Figure 2 summarizes the changes in apparent viscosity measured at 100 s<sup>-1</sup>. In untreated milk, the apparent viscosity showed a change from 1.7 to 1.9 mPa.s with 500 mM NaCl added. The apparent viscosity of 2× concentrated milk changed from 2.8 to 3.5 mPa.s. Previous work attributed the



**Fig. 2** Influence of added NaCl on the viscosity of untreated (*filled triangle*) and 2× (*open triangle*) concentrated milk. Values are average values from triplicate experiments, with the standard deviation indicated by *vertical error bars*

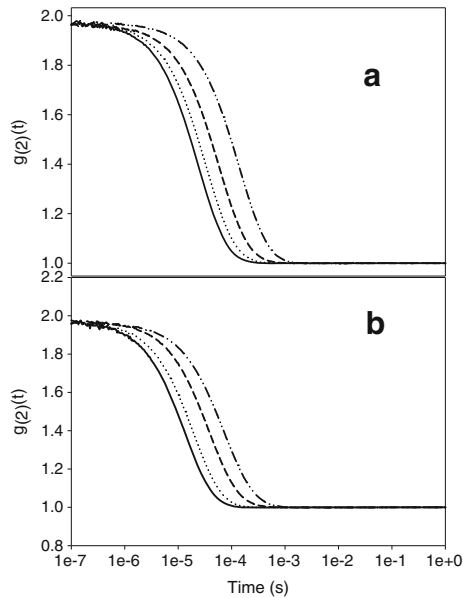
increased viscosity to an increased osmotic pressure and an effective increase in the volume fraction of the casein micelles (Carr et al. 2002). In addition, the increase of NaCl concentration will cause a decrease in the mean free distance between casein micelles because of a decrease in electrostatic repulsion, affecting the bulk milk viscosity (De Kruif 1998). These results were in full agreement with the literature and confirm that these effects also occur in milk protein concentrates. Similar results have also been reported for UF concentrates in the presence of NaCl (Karlsson et al. 2005).

### 3.4 Light scattering measurements

The light scattering properties of unconcentrated and concentrated milk were measured using dynamic light scattering, under diluted (DLS) or concentrated (DWS) conditions. Correlation functions obtained using DWS are shown in Fig. 3. In all cases, the correlation functions showed a slowing down of the characteristic decay time  $\tau$ , evidenced by a shift of curves toward longer times, with NaCl concentration. There was a difference in the  $\tau$  for unconcentrated milk compared to 2× concentrated milk proteins, and the smaller value for concentrated milk was due to the increase in the volume fraction of the casein micelles, their decreased average distance, or possible changes in the viscosity of the continuous phase. The relationship between volume fraction and diffusivity can be predicted for hard spheres using the Beenaker-Mazur formalism (Beenakker and Mazur 1983).

The average radius of the casein micelles was then derived from the diffusion coefficient (corrected for crowding) using the Stokes-Einstein equation (Beenakker and Mazur 1983). These apparent sizes were then compared to those measured by DLS, after extensive dilution of the samples in milk permeate with same amounts of NaCl (Table 2). The difference in the size values derived from DLS and DWS for control milk (with no NaCl) is due to the high dilution necessary in DLS measurement as well as the instrumental setup (Ringgenberg et al. 2012). When measured with DLS, there was an increase in size of the casein micelles from 84 to 88 nm at the high concentration of NaCl and all samples showed a monomodal distribution (not shown here). When casein micelles were measured in situ, undiluted, using DWS, the apparent radius values were much larger than those measured by DLS (Table 1). While in the absence of NaCl there was no difference in the apparent radius of the casein micelles





**Fig. 3** Typical correlation functions for untreated milk (**a**) and 2× concentrated milk (**b**) as a function of NaCl concentration: 0 (solid line), 100 (dotted line), 300 (short dash line), and 500 (dash-dot-dot line) mmol.L<sup>-1</sup>. The results shown are from individual runs and not average values

between unconcentrated and 2× concentrated milk protein, in both cases, there was an increase in the radius of the casein micelles with NaCl concentration. The change in the apparent size derived by DWS was caused by a drastic decrease in the diffusivity of the casein micelles when measured in situ, without dilution. This may have been caused by an increase in the voluminosity of the casein micelles in the presence of NaCl, or/and by an increase the viscosity of the continuous phase, due to changes in the composition of the serum phase, i.e., dissociation of protein from the casein micelle (see below).

**Table 2** Apparent radius for untreated (1×) and concentrated (2×) milk measured by DLS and DWS

	NaCl (mmol.L <sup>-1</sup> )	DLS (nm)	DWS (nm)
1×	0	84±2 <sup>a</sup>	128±1 <sup>a</sup>
	100	85±1 <sup>ab</sup>	139±2 <sup>b</sup>
	300	87±1 <sup>b</sup>	195±14 <sup>c</sup>
	500	88±1 <sup>b</sup>	571±3 <sup>d</sup>
2×	0	85±1 <sup>a</sup>	134±3 <sup>a</sup>
	100	85±1 <sup>a</sup>	143±3 <sup>b</sup>
	300	86±1 <sup>a</sup>	180±4 <sup>c</sup>
	500	90±1 <sup>b</sup>	228±3 <sup>d</sup>

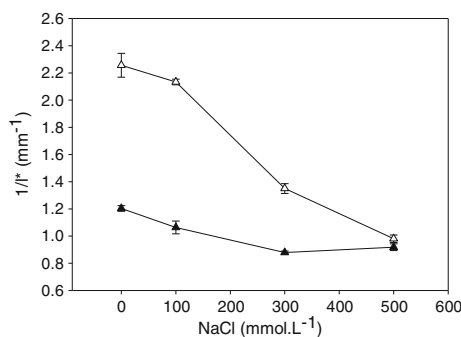
Values are means of data from triplicate experiments, ±standard deviation. Within concentration, values with a different superscript letter are significantly different ( $P < 0.05$ ). In 2× milk, diffusion coefficients were corrected according to Bennaker-Mazur formalism (Beenakker and Mazur 1983)

These results supported earlier observations by transmission electron microscope showing the presence of large rough micellar structures when NaCl is added to UF concentrate (Karlsson et al. 2005). However, it is important to note that the present work demonstrates for the first time an increase of casein micelles size (a decrease in their diffusivity) in the presence of NaCl both under diluted (DLS) and concentrated (DWS) conditions. Other researchers have reported no significant effect of NaCl on the size of casein micelles for unconcentrated milk and 2× concentrated milk (Home and Davidson 1986). However, in this case, the composition of the serum phase is similar to that of original milk, and not 2× concentrated.

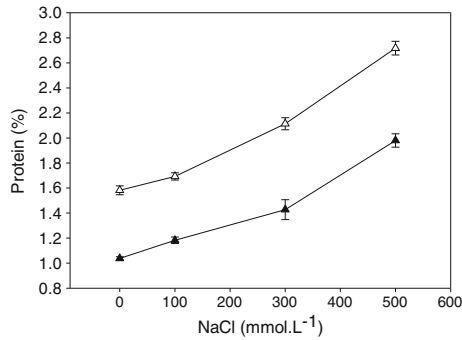
Using DWS is also possible measure the static properties (turbidity) of milk as a function of NaCl concentration. The value of  $l^*$  is a function of the refractive index contrast, the physical properties of the scattering particles, and their volume fraction, and it can also give some indication of spatial interparticle correlation. Hence, assuming no changes in the size, shape, or refractive index contrast of the scattering particles, by comparing  $l^*$  values from one sample to the next, it may be possible to identify structural differences in the dispersion (Alexander et al. 2006). The addition of NaCl clearly decreased the milk turbidity, to a much larger extent for 2× milk than for unconcentrated milk. In the case of unconcentrated milk, there were no significant differences in the  $1/l^*$  between samples with 300 and 500 mmol.L<sup>-1</sup>. The change in turbidity was larger for 2× concentrated milk; the addition of NaCl led to a progressive decrease of  $1/l^*$  value (Fig. 4), from 2.26 to 0.98 mm<sup>-1</sup>. As casein micelles are the main components that affect the turbidity of milk, all these changes also suggest a modification of the physical properties of the casein micelles, most probably caused by the increase in size and a change in of internal structure of the micelles.

### 3.5 Soluble protein content and composition

To better understand the extent of disruption of the casein micelles with addition of NaCl, the amount of non-sedimentable protein was measured in unconcentrated and 2× concentrated milk protein as a function of NaCl, as shown in Fig. 5. As expected, 2× concentrated milk had more soluble protein than untreated milk. In both cases, there was an increase in the non-sedimentable protein with NaCl, with a similar behavior. It



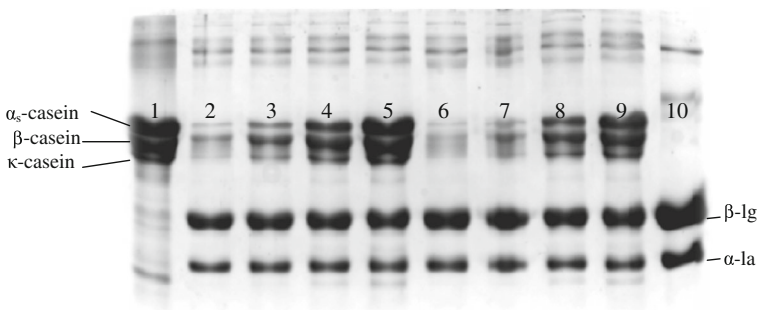
**Fig. 4** Influence of added NaCl on  $1/l^*$  of untreated (filled triangle) and 2× (open triangle) concentrated milk. Values are average values from triplicate experiments, with the standard deviation indicated by vertical error bars



**Fig. 5** Influence of added NaCl on the serum protein content of untreated (filled triangle) and 2× (open triangle) concentrated milk. Values are average values from triplicate experiments, with the standard deviation indicated by vertical error bars

was concluded that NaCl caused disruption of the casein micelles and loosening of their internal structure. The release of protein will increase the viscosity of serum phase and decrease the diffusivity of casein micelles, resulting in a larger change in the apparent radius. Nonetheless, the significant, albeit small, difference in the casein micelle radius measured by DLS under diluted conditions would suggest that both increase in medium viscosity and change in voluminosity occur with the addition of NaCl.

To better understand the composition of the soluble fraction, supernatant fractions were analyzed by SDS-PAGE electrophoresis (Fig. 6). In both 1× and 2× milk, the band intensity of  $\alpha_{S-}$ ,  $\beta$ -, and  $\kappa$ -caseins increased with NaCl addition. No significant changes were observed in the whey proteins bands. These results are in contrast with previous publications (Huppertz and Fox 2006), where the intensity of casein bands in the non-sedimentable fraction was the largest in control and decreased with NaCl addition. The discrepancy is probably caused by the use of fresh milk (present study) and reconstituted milk (earlier study).



**Fig. 6** SDS-PAGE electrophoresis of centrifugal supernatant from untreated and concentrated milk. Casein solution (lane 1) and whey protein solution (lane 10) were used as standard. Supernatants from unconcentrated milk containing 0 (lane 2), 100 (lane 3), 300 (lane 4), and 500 (lane 5) mmol.L<sup>-1</sup> NaCl and 2× concentrated milk containing 0 (lane 6), 100 (lane 7), 300 (lane 8), and 500 (lane 9) mmol.L<sup>-1</sup> NaCl. Supernatants from unconcentrated milk and 2× concentrated milk were diluted one time and two times with deionized water, respectively. Results are representative of three replicate experiments

## 4 Conclusions

It is known that  $\alpha_S$ - and  $\beta$ -caseins are highly phosphorylated and linked to calcium phosphate nanoclusters via their phosphoserine residues (Dalglish 2011).  $\kappa$ -caseins do not participate in the formation of nanoclusters as they do not possess phosphate centers. Hence, they are present on the surface of the casein micelles, providing steric and electrostatic repulsion against aggregation. After addition of NaCl, sodium can replace calcium and may alter the structure of nanoclusters. This has been previously reported to cause a change in the voluminosity of the casein micelles, calculated from the  $\eta_{\infty}$  (infinite viscosity) using the Carreau-Yasuda model (Karlsson et al. 2005). The data shown in Fig. 1 clearly demonstrated that the addition of NaCl caused partial solubilization of the colloidal calcium phosphate and weakened protein-protein interactions within the casein micelle. The size of the casein micelles increased at high concentrations of NaCl added.

With the addition of NaCl, there was an increase in the amount of non-sedimentable  $\alpha_S$ - and  $\beta$ -caseins (see Fig. 6). This partial dissociation affected the refractive index contrast of the protein particles and caused a decrease in their diffusivity, as shown by DWS measurements. Although the increased ionic strength caused partial shielding of the protein charges, with a decrease in the electrostatic repulsion between micelles, aggregation was still prevented through steric stabilization.

Casein micelle has been viewed as hard spheres from the experiment results of diffusion coefficient (Alexander et al. 2002), viscosity (Dahbi et al. 2010), and voluminosity (Liu et al. 2012). After the addition of NaCl, replacement of calcium by sodium caused a change in the internal structure and, most importantly, caused drastic changes to serum composition, as reflected by the increase in non-sedimentable protein and free calcium and phosphate. Hence, it was possible to conclude that the addition of NaCl does cause not only a change in the hydration of the casein micelles but also, and more importantly, a loosening of the internal structure, an increase in the soluble protein in the continuous phase, and a slight change in the apparent size of the casein micelles.

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