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Structure and stability of nanogel particles prepared by internal cross-linking of casein micelles

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Abstract

Cross-linking all caseins within the casein micelles with the enzyme transglutaminase creates nanogel particles consisting of a covalently linked casein network from which micellar calcium phosphate (MCP) can be removed without compromising structural integrity. These casein nanogel particles show similar light, neutron and X-ray scattering behaviour to native casein micelles, indicating similarity in size and substructure. Casein nanogel particles are more stable to heat-induced coagulation, but less stable to acid-induced coagulation than native casein micelles. Changing the MCP content of casein nanogel particles to levels between 0% and 150% of its original concentration strongly affected colloidal stability of the particles. Stability to both heat- and acid-induced coagulation increased with decreasing MCP content. Casein nanogel particles offer applications not only in traditional dairy products, but also in products and applications where the integrity and biocompatibility of the nanogel particle is important.

Keywords: Casein micelle; Nanogel; Transglutaminase; Micellar calcium phosphate

1. Introduction

In bovine milk, caseins constitute the main protein fraction and are present predominantly in the form of hydrated association colloids called casein micelles. Casein micelles contain ~3.4 g H_2O g^{-1} dry matter (Morris, Foster, & Harding, 2000), which consists of ~94% protein and ~6% inorganic materials, referred to as micellar calcium phosphate (MCP; de Kruif & Holt, 2003). Casein micelles have an average radius of ~100 nm (de Kruif & Holt, 2003) and a micelle of this size is thought to contain ~900 structural elements called nanoclusters (Holt, de Kruif, Tuinier, & Timmins, 2003), which contain a core of amorphous calcium phosphate, surrounded by a shell of α_{s1}-, α_{s2}- and β-casein caseins, whose centres of phosphorylation participate in the core via ionic interactions (de Kruif & Holt, 2003; Little & Holt, 2004). The existence of these nanoclusters is supported by data of neutron and X-ray scattering (de Kruif & Holt, 2003; de Kruif et al., in preparation; Holt et al., 2003; Marchin, Putaux, Pignon, & Leonil, 2007; Pignon et al., 2004). The question how nanoclusters associate to form casein micelles is still a matter of debate; cross-linking of nanoclusters by caseins containing multiple centres of phosphorylation and weak protein–protein interactions, including hydrophobic and electrostratic interactions and hydrogen bonding, are likely to be involved (de Kruif & Holt, 2003; Horne, 1998). The micelles are sterically stabilized by what is traditionally termed a 'hairy layer' of κ-casein, and can be considered a polyelectrolyte brush in a medium of high ionic strength (de Kruif & Zhulina, 1996). Colloidal instability of casein micelles can be induced by removal or collapse of the brush and is reviewed by Holt and Horne (1996) and de Kruif (1999).

Due to its abundance and relative ease of isolation, the industrial potential of caseins in food and non-food applications is widespread. In food products, caseins, mostly in the form of caseinates, are used to improve functional properties, such as water binding, viscosity,

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structure, texture, emulsification and foaming (Rollema, 2003). Non-food uses of caseins include casein glue, casein wool, casein bone or ivory and casein photoresist, to produce the so-called shadow masks in TV-tubes (de Kruif, 2003). In this article, we will present a more recent application of caseins, i.e., as biocompatible nanogel particles.

Nanogel (1–1000 nm) and microgel (1–1000 μm) particles are gel particles prepared from synthetic or natural polymers (Hans & Lowman, 2002; Stieger, Pedersen, Linder, & Richtering, 2004a; Stieger & Richtering, 2003; Stieger, Richtering, Pedersen, & Lindner, 2004b). These particles have become an increasingly important area in research in drug delivery, because of their ability to deliver a wide range of drugs to various parts of the body for a sustained period of time (Hans & Lowman, 2002). Other applications of nano- or microgel particles include coatings and adhesives, composite manufacture and low-shrinkage dental polymers. For many of their intended uses, the particles required extremely high stability, which is derived from intra-particle cross-linking of the polymers (Hans & Lowman, 2002).

In this article, we studied nanogel particles prepared by enzymatic cross-linking of micellar caseins. Due to their low level of secondary and tertiary structure, caseins form excellent substrates for cross-linking by transglutaminase (TGase), which can covalently link protein-bound glutamine and lysine residues. The rate of cross-linking of caseins is higher in sodium caseinate than in skim milk (Lorenzen, 2000). In sodium caseinate, where the caseins exist in small solvent-mediated aggregates, the susceptibility of caseins to cross-linking decreases in the order κ-CN > α_s-CN > β-CN (Tang, Yang, Chen, Wu, & Peng, 2005), whereas in milk, where casein micelles predominate, the rate of cross-linking decreases in the order κ-CN > β-CN > α_s-CN (Hinz, Huppertz, Kulozik, & Kelly, 2007; Sharma, Lorenzen, & Qvist, 2001; Smiddy, Martin, Kelly, de Kruif, & Huppertz, 2006). Enzymatic cross-linking of milk proteins is reviewed in detail by Jaros, Parschefeld, Henle, and Rohm (2006a). Cross-linking progressively increases the intra-micellar stability (O'Sullivan, Kelly, & Fox, 2002b; Smiddy et al., 2006) and cross-linking of all caseins converts casein micelles from association colloids to nanogel particles (Huppertz, Smiddy, & de Kruif, 2007). The studies presented in this article provide further information on the structure and colloidal stability of these casein nanogel particles.

2. Materials and methods

2.1. Sample preparation

Skim bovine milk was prepared by reconstituting Nilac low-heat skim milk powder (NIZO Food Research, Ede, The Netherlands) in demineralised water at a level of 9 g 100 g^{-1}. Serum protein-free milk was prepared by reconstituting serum-protein free skim milk powder, prepared by diafiltration (see Huppertz et al., 2007), in demineralised water at a level of 8.4 g 100 g^{-1}. Milk dialysate was prepared by exhaustively dialysing a 50 g L^{-1} lactose solution against 2 × 20 volumes of reconstituted skim milk for 48 h at 20 °C. Sodium azide (0.5 g L^{-1}) was added to all samples to prevent microbial activity.

For dynamic and static light scattering and small-angle X-ray scattering measurements, residual lipid globules were removed from the serum protein-free micelle suspensions. For this purpose, casein micelles were pelleted by ultracentrifugation, resuspended in milk dialysate, followed by adjustment of the casein content to 2.5 g 100 g^{-1}. This procedure is outlined in detail by Huppertz et al. (2007). Samples prepared as such will be referred to as casein micelle suspensions.

2.2. Enzymatic cross-linking

Samples of milk, serum protein-free milk or casein micelle suspensions were pre-warmed to 30 °C and Activa TG TGase (declared activity 1000 unit g^{-1}; Ajinomoto, Hamburg, Germany) was added at a level of 0.5 g L^{-1}. Samples were incubated at 30 °C for 24 h prior to inactivation of the TGase by heating at 70 °C for 10 min. Samples were subsequently cooled to 20 °C in ice-water. A control sample, without added TGase, was treated in the same manner in all cases. SDS–PAGE under reducing conditions was used to determine the level of residual monomeric casein in the sample.

2.3. MCP-adjustment

For adjustment of the MCP content, TGase-treated samples were cooled to 5 °C, followed by adjustment of their pH to ~4.5 or 5.5, 6.0 with 1 M HCl or to 8.0 or 9.0 with 1 M NaOH; a control sample was kept at its original pH of ~6.6. Samples were subsequently subjected to dialysis against 2 × 20 volumes of skim milk for 48 h at 5 °C. The concentration of total, micellar and non-micellar calcium was determined by atomic absorption spectroscopy, as described by Huppertz et al. (2005).

2.4. Dynamic and static light scattering

Following 100-fold dilution in milk dialysate, filtration (5 μm pore size) and centrifugation (1000 × g for 5 min), suspensions of native and cross-linked casein micelles were analysed simultaneously by dynamic light scattering (DLS) and static light scattering (SLS) using an ALV Compact Goniometer System (ALV-Laser; Vertriebsgesellschaft Gm-bH, Langen, Germany) equipped with four detector units (ALV/GCS-4) and two ALV-5000/E multiple tau digital correlators. A Coherent Verdi V2 diode-pumped laser (Coherent, Inc., Santa Clara, CA, USA) was used, operating with vertically polarised light with a wavelength of $\lambda = 532.0$ nm. Measurements were performed at 16 or 32 scattering wave vectors in the range

$4.7 \times 10^{-3} < Q < 3.0 \times 10^{-2}\,\mathrm{nm}^{-1}$ ($Q = 4\pi n_s \sin(\theta/2)/\lambda$, where n_s is the refractive index of the medium and θ is the angle of observation). The cuvette holder was controlled at $25.0 \pm 0.1\,^{\circ}\mathrm{C}$. DLS measurements were performed until at least 10^7 photons were collected at each angle. The diffusion coefficient (D; in $\mathrm{m^2\,s^{-1}}$) was calculated from each auto-correlation function using a cumulant fit and the hydrodynamic radius (R_H) was calculated using the Stokes–Einstein equation:

$$D = \frac{k_B T}{6\pi\eta R_H}, \tag{2.1}$$

where k_B is the Boltzmann constant, T is the absolute temperature of the liquid and η is the viscosity of the medium in mPa s.

2.5. Small angle neutron scattering

For small angle neutron scattering (SANS) measurements, serum protein-free milk powder was reconstituted at a level of $8.4\,\mathrm{g\,100\,g^{-1}}$ in D_2O and treated with TGase as outlined in Section 2.2. Following inactivation of the enzyme, native and cross-linked micelles were pelleted by centrifugation at $100{,}000 \times g$ for 60 min at 20 °C. The pellets were resuspended in their ultracentrifugal supernatants which had first been clarified of any residual proteins and lipid globules by filtration through a 10 kDa cut off membrane. Resuspended casein micelles were kept stirring for 24 h at 5 °C and subsequently centrifuged at $500 \times g$ for 20 min at 5 °C to remove any undissolved materials. The protein content of the centrifuged casein micelle suspension and milk serum was determined using the Kjeldahl method, and the casein content of the micelle suspension was adjusted to $20\,\mathrm{g\,L^{-1}}$ casein, by addition of clarified milk serum. Prior to SANS measurements, samples were adjusted to a casein content of $10\,\mathrm{g\,L^{-1}}$ and, in some cases, 35% (v/v) deuterated ethanol. Samples were placed in quartz cells with a path-length of 2 mm and analysed by SANS at the ISIS facility (Chilton, UK) using the LOQ, which uses neutrons at a wavelength of 0.22–1.00 nm and allows detection in the Q-range of $\sim$0.08–2.5 $\mathrm{nm^{-1}}$. SANS measurements were performed at 25, 40, 60 or 80 °C.

2.6. Small-angle X-ray scattering

Small-angle X-ray scattering (SAXS) measurements were performed at the Dutch–Belgian beam-line at the European Synchrotron Radiation Facility in Grenoble (France). The cuvettes contained 19.65 $\mathrm{mm^3}$ of sample. The wavelength of the X-rays was equal to 0.093 nm. The detector was a two-dimensional (512×512 pixels) gas-filled detector placed at 5 m distance from the sample. The scattering wave vector (Q) was between 0.1 and 1.7 $\mathrm{nm^{-1}}$ (corresponding to a range of observable length scales between 62.8 and 3.7 nm in real space). The temperature of the samples was maintained at 25 °C.

2.7. Heat-induced coagulation of milk

The heat stability of milk samples and casein micelle suspensions was measured subjectively, by determining the heat coagulation time (HCT) at 140 °C as a function of milk pH (6.3–7.3, at 0.1 pH unit increments), using the method of Davies and White (1966). All HCT-pH profiles shown are typical profiles; experiments were repeated on three individual milk samples and were reproducible to the extent that similar trends were observed in all cases. Uncertainties derive from the extremely high pH sensitivity of the HCT-pH profile.

2.8. Acid-induced coagulation of casein micelles

Samples were equilibrated at 32 °C for 30 min; subsequently, glucono-δ-lactone (GDL) was added to the casein micelle suspension at a level of $15\,\mathrm{g\,L^{-1}}$, the sample was stirred for 1 min to dissolve the GDL, and subsequently incubated at 32 °C. Acid induced coagulation of casein micelles was monitored by diffusing wave spectroscopy (DWS), as outlined by Vasbinder, van Mil, Bot, and de Kruif (2001). The time at which the auto-correlation curve had decayed to 50% of its maximum plateau level was defined as the relaxation time, $\tau_{1/2}$. In DWS, a relaxation time is, as in classical DLS, directly related to a particle diffusivity and therefore to particle size and interaction via the generalised Stokes–Einstein relation. During the initial stages of flocculation, the relaxation time is therefore a direct measure of particle growth (i.e., size). All data were normalised relative to its control, which is the $\tau_{1/2}$ value of the sample equilibrated at 32 °C, prior to the addition of GDL.

2.9. Zeta-potential

The electrophoretic mobility of MCP-adjusted TGase-treated casein micelle suspensions, diluted 1:500 in milk dialysate, was determined by Laser Doppler Electrophoresis, at an applied voltage of 120 V and a modulation frequency of 250 Hz, respectively, using a Malvern Zetamaster (Malvern Instruments Ltd., Malvern, UK). The electrophoretic mobility (U_e) was converted into zeta-potential (ζ) using Henry's equation:

$$U_e = \frac{2\varepsilon\zeta f(\kappa a)}{3\eta}, \tag{2.2}$$

where ε is the dielectric constant of the medium, η is the viscosity of the medium and $f(\kappa a)$ is Henry's function. In aqueous media and at moderate electrolyte concentration, $f(\kappa a) = 1.5$, which is referred to as Smoluchowski's approximation.

3. Results and discussion

3.1. Structure and stability of cross-linked casein micelles

SDS–PAGE analysis of the samples used in this study indicated that after treatment with TGase no monomeric

casein remained (data not shown), indicating that all casein was cross-linked, as previously reported by Huppertz et al. (2007). Casein micelle size, determined by DLS (Fig. 1A), SLS (Fig. 1B) or SAXS (Fig. 1D) was not affected by incubation with TGase, indicating that TGase-induced cross-linking of casein micelles is exclusively intra- and not inter-micellar. SANS and SAXS are extremely valuable techniques to elucidate the substructure of colloids and casein micelles (Hansen et al., 1996; Holt et al., 2003; Pedersen, 1997; Pedersen & Schurtenberger, 2004). The negligible effect of enzymatic cross-linking on the scattering of neutrons (Fig. 1C) and X-rays (Fig. 1D) suggests that the substructure of cross-linked casein micelles is similar to that of native casein micelles.

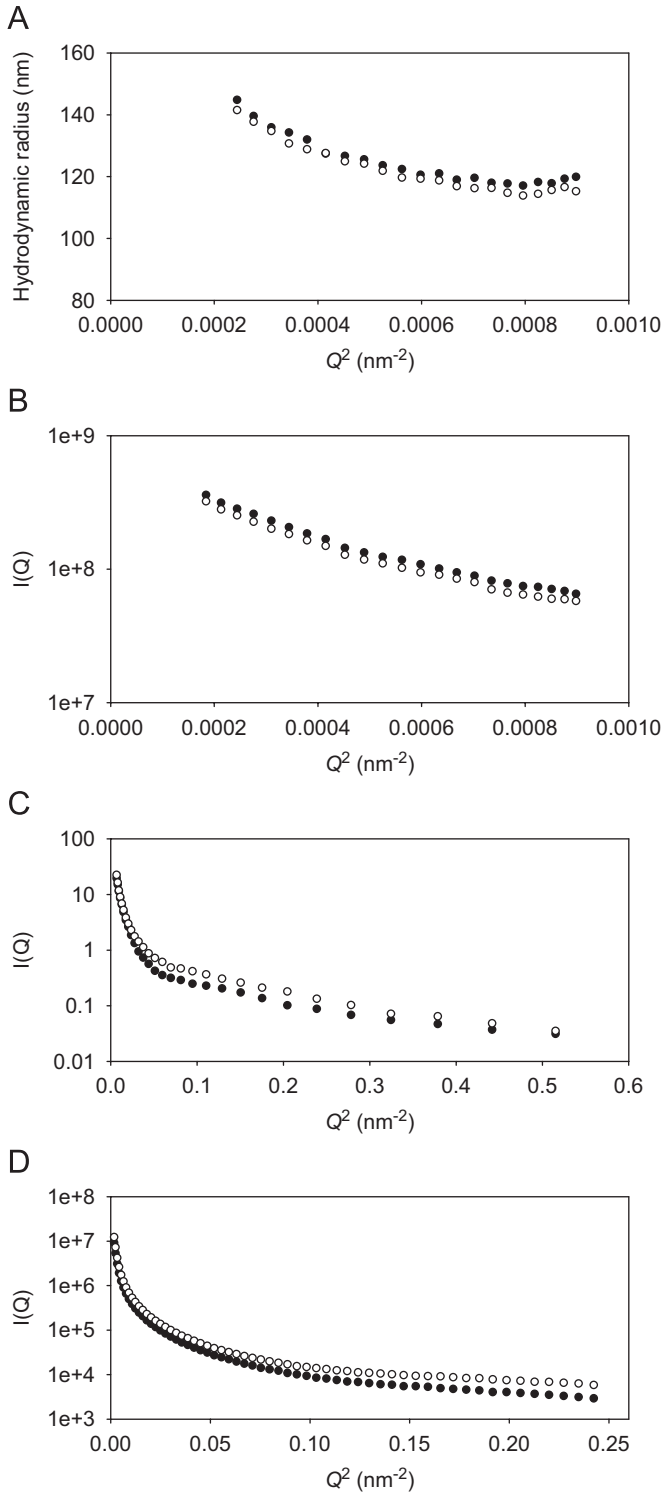

Fig. 1. Hydrodynamic radius from dynamic light scattering (A) or scattering intensity ($I(Q)$) from static light scattering (B), small-angle neutron scattering (C) or small-angle X-ray scattering (D) as a function of scattering wave vector squared (Q^2) of native casein micelles (●) or casein micelles cross-linked with transglutaminase (○).

Intra-micellar cross-linking increases the stability of caseins micelles against disruption on increasing solvent quality, disrupting protein–protein interactions or solubilisation of MCP increased (O'Sullivan et al., 2002b) and this stability increases with increasing extent of cross-linking (Smiddy et al., 2006). When all caseins are cross-linked, casein micelles are completely stable against disruption by urea and/or citrate (Huppertz et al., 2007). The changes in turbidity of a suspension of completely cross-linked casein micelles on addition of urea and citrate can be accounted for completely when accounting for the following (Huppertz et al., 2007): (1) urea and citrate increase the refractive index of milk serum and reduces the refractive index increment (dn/dc) of casein micelles in milk serum; (2) citrate solubilises MCP, as a result of which the dry mass of the micelle decreases; and (3) solubilisation of MCP decreases dn/dc of the micelles, because MCP has a higher dn/dc than micellar casein, as well as because of the increased concentration of calcium and phosphate in the serum phase increases its refractive index. This suggests that no urea- or citrate-induced disruption occurs for completely cross-linked casein micelles (Huppertz et al., 2007).

SLS, DLS and SANS measurements further show that cross-linked micelles are not disrupted in the presence of urea and/or citrate, but may show swelling as a result of changes in solvent quality (Huppertz et al., 2007). Swelling behaviour is also illustrated in Fig. 2 in which SANS spectra are presented of cross-linked casein micelles in the presence of 35% (v/v) deuterated ethanol at 25–80 °C. Although the spectra are somewhat noisy, it is evident that the ordinate value is not affected by temperature, whereas scattering intensity decays faster as a function of Q at higher temperature. These observations are consistent with a swelling nanogel particle for which the scattering intensity is proportional to $(dn/dc)^2$, mass concentration and molar mass. All these three parameters do not change, so the Q-dependence is represented by the scattering form factor $P(Q)$, which decays faster for a larger particle. Heating a native casein micelle dispersion in the presence of ethanol, leads to a disruption of the casein micelles as a result of increased solvent quality (O'Connell, Kelly, Auty, Fox, & de Kruif, 2001a; O'Connell, Kelly, Fox, & de Kruif, 2001b), which is now prevented by the enzymatic cross-linking. Based on the findings described above, micelles in which all caseins are cross-linked can be described as a covalently linked protein network from which MCP can be

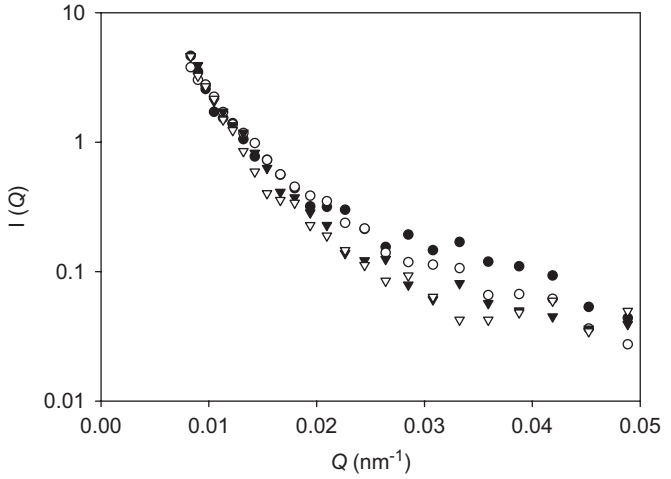

Fig. 2. Scattering intensity ($I(Q)$) as a function of the scattering wave vector (Q) for small-angle neutron scattering measurements of a suspension of casein microgel particles in deuterium oxide containing 35% (v/v) deuterated ethanol at 25 °C (●), 40 °C (○), 60 °C (▼) or 80 °C (▽).

solubilised and which displays swelling behaviour on changes in solvent quality, leading to classification of them as casein nanogel particles (Huppertz et al., 2007).

3.2. Colloidal stability of casein nanogel particles

3.2.1. Acid-induced coagulation

As illustrated in Fig. 3, the colloidal stability of cross-linked casein micelles differs considerably from that of native casein micelles. Acid-induced flocculation of casein micelles, as illustrated by rapid increase in normalised relaxation times ($\tau_{1/2,\text{normalised}}$), occurred at a considerably higher pH for cross-linked casein micelles than for native casein micelles (Fig. 3A). Similar results were reported by Schorsch, Carrie, and Norton (2000), while others (Anema, Lauber, Lee, Henle, & Klostermeyer, 2005; Jaros, Patzold, Schwarzenbolz, & Rohm, 2006b; Lauber, Henle, & Klostermeyer, 2000; Lorenzen, Mautner, & Schlimme, 1999) have observed that pre-treatment of milk with TGase yields firmer acid milk gels. The mechanism for the TGase-induced increase in acid-flocculation pH has not been elucidated thus far. It is our hypothesis that cross-linking of the κ-casein brush on the micellar surface restricts its freedom of movement and thus reduces its conformational entropy. A reduction in conformational entropy reduces the steric stabilisation provided by the brush and hence increases the susceptibility of the micelles to acid-induced flocculation (Tuinier & de Kruif, 2002).

3.2.2. Heat-induced coagulation

The stability of casein nanogel particles to heat-induced coagulation also differs considerably from that of native micelles, both in the presence (Fig. 3B) and absence (Fig. 3C) of whey proteins. Control milk displayed a typical so-called type-A pH-HCT profile, with a maximum

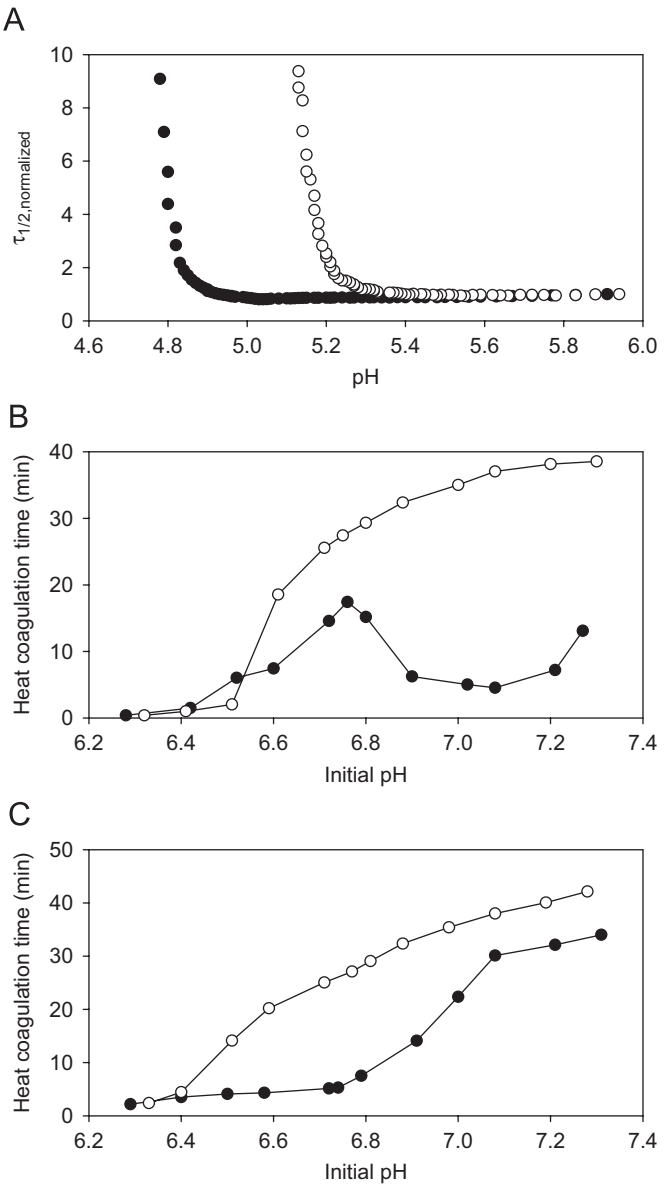

Fig. 3. Physicochemical properties of native casein micelles (●) and casein nanogel particles (○): (A) Normalised relaxation time as function of pH during acidification with 2.5% (w/v) glucono-delta-lactone at 32 °C, (B) heat coagulation time at 140 °C as a function of initial pH for unconcentrated milk and (C) heat coagulation time at 140 °C as a function of initial pH for serum protein-free milk.

at pH ~6.7 and a minimum at pH ~7.1. Enzymatic cross-linking reduces HCT at pH < 6.6, but increases it at higher pH values, and transforms the pH-HCT profile into one where HCT increases with pH (Fig. 3B). Comparable TGase-induced changes in the pH-HCT profile of milk were observed by O'Sullivan, Kelly, and Fox (2002a), O'Sullivan et al. (2001) and Mounsey, O'Kennedy, and Kelly (2005). The increase in HCT at pH > 6.6 (Fig. 5B) is probably because enzymatic cross-linking reduces the extent of heat-induced dissociation of κ-casein (O'Sullivan et al., 2002a), which is a major contributor to heat-induced coagulation of milk in this pH region (O'Connell & Fox,

2003; Singh, 2004). Therefore it seems that the anchoring of the κ-casein to the surface extends HCT, by maintaining the steric stabilisation of κ-casein.

In the absence of heat-induced dissociation of κ-CN, other factors cause heat-induced coagulation of casein nanogel particles, e.g., heat-induced acidification and heat-induced precipitation of calcium phosphate from the milk serum onto the micelles (Singh, 2004; Van Boekel, Nieuwenhuijse, & Walstra, 1989). Because the solubility of calcium phosphate decreases with increasing pH, whereas HCT of casein nanogel particles increases with increasing pH (Fig. 4B and C) and heat-induced precipitation of calcium phosphate is a very rapid process (<5 min; Van Boekel et al., 1989), heat-induced acidification, rather than heat-induced precipitation of calcium phosphate, is likely to be the major contributor to thermal instability of casein nanogel particles. This implies that the steric stabilisation of the nanogel particles diminishes when the pH of milk is reduced below a critical value so heat stability becomes a function of the time required to induce a sufficient degree of acidification. Fox (1981) estimated that milk pH decreases from ~6.7 to ~5.7 on heating milk up to 140 °C and further decreased progressively with increasing holding time to ~5.0 after 20 min at 140 °C. Such data are in good agreement with those previously reported by Rose and Tessier (1959). At 32 °C, acid-induced flocculation of casein nanogel particles commenced at pH ~5.1 (Fig. 3A), a value which according to Fox (1981) would be reached after heating ~18 min. The value compares well with a HCT of casein nanogel particles of 20–25 min at an original pH of 6.7 (Figs. 3B and 3C) so it appears that heat-induced coagulation of casein nanogel particles, at least at natural pH, can be predicted reasonably well as a result of heat-induced acidification of milk, particularly considering that a wide variety of other factors will also contribute to the final HCT (Fox, 1981; O'Connell & Fox, 2003; Singh, 2004; Van Boekel et al., 1989). As the time required to reduce pH to a certain value increases with increasing original pH (Van Boekel et al., 1989), HCT of casein nanogel particles will increase with pH. The very low HCT of casein nanogel suspensions at pH <6.4 is probably related to the initial reduction in pH on heating the samples to 140 °C, combined with the elevated concentration of ionic calcium at low pH, which is sufficient to induce near-instantaneous coagulation. For samples that 'survive' this warm up period, HCT will then depend on the time required to induce sufficient acidification to cause instability.

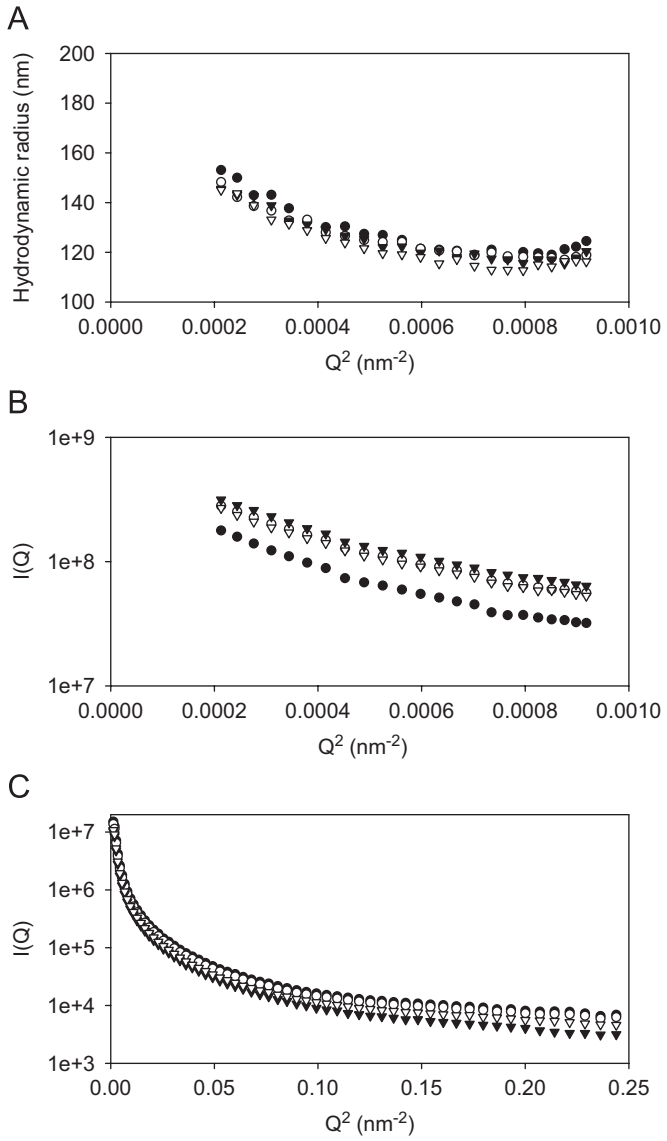

Fig. 4. Hydrodynamic radius from dynamic light scattering (A) or scattering intensity from static light scattering (B) or small-angle X-ray scattering (C) as a function of scattering wave vector squared (Q^2) of casein nanogel particles containing 0% (●), 60% (○), 100% (▼) or 150% (▽) of their original content of micellar calcium phosphate.

3.3. *Properties of casein nanogel particles with altered MCP content*

By changing the amount of MCP of casein nanogel particles, we investigated its influence on the colloidal stability of the particles. Using the method outlined in Section 2.3, casein nanogel particles containing 0–150% of their original MCP content were prepared (Table 1). Comparable adjustments to the MCP content of native casein micelles were reported by Black, Huppertz, Fitzgerald, and Kelly (2007). Ionic calcium activity in the serum phase of suspensions of casein nanogel particles was not influenced by MCP content of the particles (Table 1).

Adjustment of MCP content did not affect the size of the nanogel particles, as measured by DLS (Fig. 4A) and SLS (Fig. 4B). The similarity of the SAXS spectra of casein nanogel particles of differing MCP content (Fig. 4C) further highlights the similarity of the particles in terms of size and substructure. Unfortunately, SAXS measurement did not provide information on the nature of the additional MCP in casein nanogel particles with increased MCP content. However, the fact that the zeta-potential of casein

Table 1
Influence of pH adjustment of casein nanogel suspensions, followed by exhaustive dialysis against bulk milk, on some physicochemical properties of the nanogel suspensions[a]

Milk pH prior to dialysis	Micellar calcium (% of control)	Calcium in solution (mmol L^{-1})	Zeta-potential (mV)	Ethanol stability at pH 6.6 (%, v/v)
4.5	0.7±0.4	1.9±0.1	−16.2±1.6	93.8±1.3
5.5	58.8±2.4	1.9±0.2	−16.4±1.6	95.0±1.6
6.0	85.8±3.6	1.9±0.1	−16.5±1.7	92.5±2.1
6.6 (control)	100.0±0.0	1.8±0.1	−16.4±1.6	93.8±1.3
8.0	127.9±2.6	1.8±0.2	−16.8±1.6	91.3±3.8
9.0	153.4±4.3	1.9±0.1	−16.7±1.4	92.0±2.5

[a]Values are means of experiments on three individual milk samples±S.D.

nanogel particles was not affected significantly by MCP content (Table 1) suggests that the additional MCP in nanogel particles with increased MCP content is unlikely to be associated with the κ-casein brush on the micellar surface. The zeta-potential of native casein micelles was −16.8±1.9 mV, which is comparable to casein nanogel particles (Table 1), indicating that the enzymatic cross-linking process has no significant influence on the zeta-potential of the micelles.

3.4. Influence of MCP content on the colloidal stability of casein nanogel particles

3.4.1. Ethanol-induced coagulation

The high ethanol stability of casein nanogel particles (Table 1) is in agreement with the report of Huppertz and de Kruif (2007a) that cross-linking of caseins increases the ethanol stability of milk, probably by converting the associated electrolyte brush of κ-casein on the micellar surface into a grafted and tethered brush. The results in Table 1 show that crosslinking makes the ethanol stability independent of the amount of MCP content. Ethanol-induced flocculation of milk is a result of the collapse of the salted brush because of reduced solvent quality (de Kruif, 1999; Horne, 2003) and as such primarily a surface phenomenon. Ethanol-induced flocculation of milk is strongly dependent on the mineral content and composition of the serum phase of milk (Horne, 2003), but the data in Table 1 clearly show that the concentration of MCP in the nanogel particles does not influence their ethanol stability.

3.4.2. Acid-induced coagulation

The pH at which acid-induced flocculation of the casein nanogel particles commenced increased with increasing MCP content of the particles (Fig. 5A). Acid-induced flocculation of casein micelles, and presumably also casein nanogel particles, is a result of a collapse of the κ-casein brush because of reduced solvency due to reduced dissociation of glutamic acid residues in the C-terminus of κ-casein (de Kruif & Zhulina, 1996). At first sight, it is tempting to attribute the increase in flocculation pH with increasing MCP content (Fig. 5A) to the fact that micellar calcium is solubilised on acidification and reduces the charge on the C-terminus of κ-casein through interactions with glutamic acid residues. This would reduce the degree of acidification required to induce collapse of the brush and thereby acid-induced flocculation. This mechanism may indeed contribute to MCP-free casein nanogel particles commencing acid-induced flocculation at a lower pH than

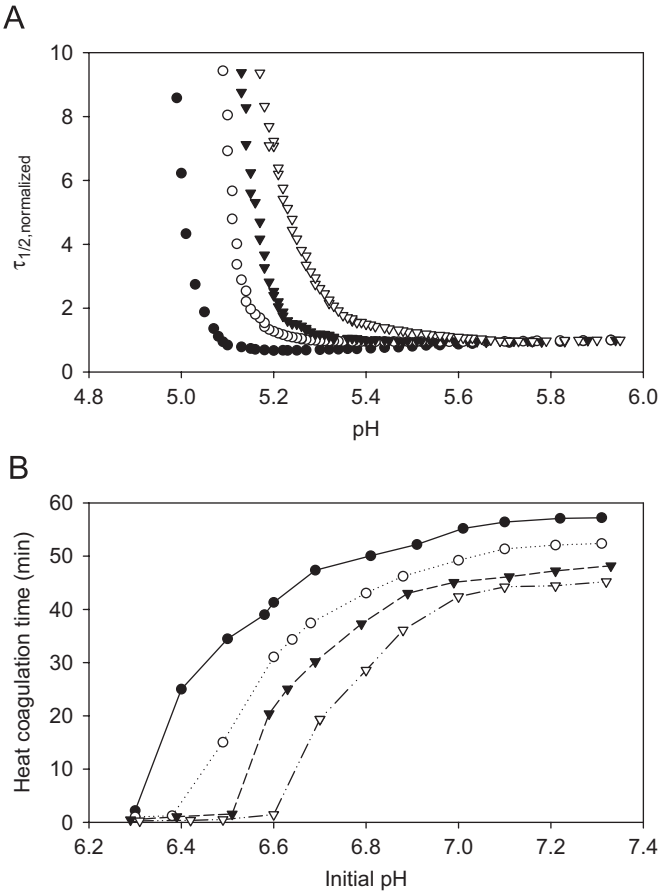

Fig. 5. Colloidal stability of casein nanogel particles containing 0% (●), 60% (○), 100% (▼) or 150% (▽) of their original content of micellar calcium phosphate: (A) DWS traces as a function of pH during acidification with 2.5% (w/v) glucono-delta-lactone at 32 °C and (B) heat coagulation time at 140 °C as a function of initial pH.

nanogel particles containing ⩾60% of their original MCP content (Fig. 5A).

However, for casein nanogel particles containing 60–150% of their original MCP content, acid-induced flocculation commenced at pH ~5.45, 5.55 and 5.75, respectively (Fig. 5A) whereas 60% of MCP in milk is solubilised only at pH ~5.3 (Dalgleish & Law, 1989; Dalgleish, Verespej, Alexander, & Corredig, 2005). Hence, the salt composition of the serum of suspensions of casein nanogel particles originally containing 60%, 100% or 150% of their original level of MCP will be comparable at a pH > 5.3 and significant amounts of MCP are expected in the nanogel particles at the point of acid-induced flocculation. It thus appears that the amount of residual micellar MCP, rather than the amount of MCP solubilised, plays a major role in the stability of casein nanogel particles to acid-induced flocculation. The exact mechanism through which MCP influences acid-induced flocculation of casein nanogel particles is unknown at this stage, and is of interest for further research.

3.4.3. Heat stability of MCP-adjusted casein nanogel particles

The susceptibility of casein nanogel particles to heat-induced coagulation increased with increasing MCP content; i.e., at all pH values studied, HCT increased with decreasing MCP content (Fig. 5B). As outlined in Section 3.2.2, we propose that heat-induced coagulation of casein nanogel particles is primarily a result of heat-induced acidification of the suspension, as a result of which the stabilizing κ-casein brush will collapse at a critical value. Heat-induced acidification is not influenced by MCP content of the casein nanogel suspension (Table 2). The increased susceptibility of casein nanogel particles to acid-induced coagulation with increasing MCP content (see Section 3.4.2.) is likely to be the main contributor to the influence of MCP on heat stability of casein nanogel particles. The influence of MCP on the heat stability of casein nanogel particles is unlikely to be attributable to differences in heat-induced dephosphorylation, since dephosphorylation of casein in the absence of MCP, e.g., in the form of sodium caseinate, is more extensive than in skim milk (Belec & Jenness, 1962a, b).

Table 2
The pH of suspensions of casein microgel particles containing 0%, 60%, 100% or 150% of their original MCP content measured at 20 °C immediately after heating at 130 °C for up to 20 min[a]

Heating time (min)	MCP content (% of original)			
	0	60	100	150
Prior to heating	6.71±0.02	6.70±0.03	6.68±0.03	6.70±0.04
5	6.52±0.03	6.54±0.04	6.51±0.04	6.52±0.05
10	6.39±0.03	6.40±0.04	6.37±0.03	6.37±0.03
15	6.31±0.04	6.32±0.03	6.28±0.02	6.30±0.05
20	6.18±0.03	6.21±0.04	6.17±0.03	6.18±0.04

[a]Values are means of experiment on three individual milk samples ± S.D.

4. Conclusions

Cross-linking casein micelles internally with TGase leads to well defined and stable nanogel particles. Casein nanogel particles can be used potentially for a number of encapsulation and protection technologies, for instance to transport minerals, vitamins, and pharmaceuticals. Furthermore, it would be of interest to study the use of calcium depleted casein nanogel particles as a replacement of caseinate in its various food and non-food applications. A second type of application for casein nanogel particles is in fundamental research perspective, where they allow estimation of changes in the mineral balance in milk which have proved extremely difficult to measure otherwise. They allowed Huppertz and de Kruif (2007b) to calculate the extent of solubilisation of MCP using in situ light transmission at pressures up to 300 MPa. Several other applications in this area are possible, where one can alter milk serum composition without inducing changes in micelle structure, due to the fact that cross-linking creates nanogel particles with a covalently linked protein network.

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