Biocompatible Micro-Gel Particles from Cross-Linked Casein Micelles

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The stability of internally cross-linked casein micelles against disruption by urea (which disrupts hydrogen bonds and hydrophobic interactions) and trisodium citrate (which sequesters micellar calcium phosphate) was investigated. Addition of urea (0−6 mol L^{-1}) and/or citrate (0−50 mmol L^{-1}) progressively reduced the turbidity of a suspension of casein micelles cross-linked by transglutaminase and increased particle size (determined by dynamic and static light scattering and small-angle neutron scattering), which was attributed to swelling of the micelles. Furthermore, model calculations, assuming a completely stable casein network, were performed to describe the decreases in turbidity on addition of urea and citrate. Measured and described turbidity values are in agreement, indicating that cross-linking of casein micelles with transglutaminase results in a covalently bound protein network, which is entirely stable to disruption by urea and/or citrate. This may offer potential applications for the use of cross-linked casein micelles as biocompatible protein micro-gel particles.

1. Introduction

Bovine milk contains ∼30−35 g L^{-1} protein, of which the major fraction (∼80%) is a class of four pH 4.6-insoluble phosphoproteins, i.e., α_{s1}-, α_{s2}-, β- and κ-casein.[1] Most casein in milk exists in the form of casein micelles, which are highly hydrated (∼3.4 g H_2O g^{-1} protein[2]) and sterically stabilized by a polyelectrolyte brush.[3] The dry matter of bovine casein micelles consists of ∼94% protein and 6% inorganic material, referred to as micellar calcium phosphate (MCP).[1] MCP, consisting primarily of calcium and phosphate, as well as smaller amounts of other mineral constituents, is predominantly present in the form of small amorphous nanoclusters, which are stabilized by a shell of α_{s1}-, α_{s2}- and β-caseins, all of which contain centers of phosphorylation, i.e., at least three phosphorylated serine-residues in close proximity.[4] Since α_{s1}- and α_{s2}- contain more than one center of phosphorylation, these proteins can cross-link nanoclusters, thereby facilitating creation of aggregates of nanoclusters.[3] Aggregation also occurs through attractive van der Waals and hydrophobic interactions and hydrogen bonding between the caseins associated with the nanoclusters, ultimately leading to the formation of particles of colloidal dimensions, i.e., casein micelles.[3] The casein micelles are sterically stabilized by a polyelectrolyte brush consisting primarily of the glycosylated N-terminal part of κ-casein.[5,6]

The inter-micellar stability refers to the stability of the casein micelles against aggregation and is governed primarily by the steric repulsion provided by the polyelectrolyte brush.[6] As for most colloids stabilized in this manner, intermicellar stability may be reduced by removal (enzymatic cleavage) or collapse (acid- or ethanol-induced) destabilisation of the brush.[7] The intramicellar stability refers to the ability of the casein micelle to retain its structural integrity under adverse environmental conditions. Removal of MCP (through calcium chelation) or disruption of hydrogen bonding and hydrophobic interaction (by urea) results in near-complete disruption of casein micelles. However, initial observations by O'Sullivan et al.[8] and a more extensive recent study by Smiddy et al.[9] indicated that intramicellar stability was enhanced considerably by covalent intramicellar cross-linking of caseins by the enzyme transglutaminase (TGase), which catalyzes an acyl-transfer reaction between the carboxyl group of a peptide-bound glutamine residue and the α-amino group of a lysine residue. Micellar stability increased with degree of cross-linking and results suggested that extensive cross-linking could make micelles very stable against disruption by almost any means.[9]

The ability to completely stabilize casein micelles against disruption is of significant interest as this would enable the production of protein micro-gel particles from caseins. We draw a parallel with the poly acrylamide micro-gel particles studied by Stieger and Richtering.[10] The application of cross-linked casein micelles as biocompatible micro-gel particles makes extremely high demands on the stability of the micelles. In the studies presented in this communication, the stability of cross-linked casein micelles on the solubilization of MCP and/or the disruption of hydrogen bonds and hydrophobic interactions was investigated with the aim of.

2. Theoretical Considerations

2.1. Light Scattering. In a nonabsorbing dispersion, turbidity (τ) is the attenuation of a light beam by scattering and turbidity is related to scattering by[11]

$$\tau = 2\pi \int_0^{\pi} R(Q) \sin\theta \, d\theta \qquad (2.1)$$

with

$$Q = (4\pi n/\lambda_0) \sin(\theta/2) \qquad (2.2)$$

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where $R(Q)$ is the so-called Rayleigh ratio for unpolarized light, Q the scattering wave vector, θ the scattering angle, n the refractive index of the sample, and λ_0 the wavelength in vacuo. For monodisperse particle size distribution, it follows from the Rayleigh-Gans-Debeye theory of light scattering that (see[12,13])

$$R(Q) = Q^*cMP(Q)S(Q)(1 + \cos^2\theta) \quad (2.3)$$

where c and M are the weight concentration and molar mass of the scattering particles, $P(Q)$ is the form factor which accounts for the interference of light scattered from different parts within one particle, $S(Q)$ is the structure factor, which accounts for the interference of light scattered from different particles and

$$Q^* = 2\pi^2 n^2 (\mathrm{d}n/\mathrm{d}c)^2 / N_A \lambda_0^4 \quad (2.4)$$

where N_A is Avogadro's number and dn/dc is the refractive index increment. At low concentrations, as used in the experiments presented in this communication, $S(Q)$ becomes 1, so

$$R(Q) = Q^*cMP(Q)(1 + \cos^2\theta) \quad (2.5)$$

For turbidity measurements, eq 2.5 can be expressed as[14]

$$\tau = HcM\xi(d/\lambda_0) \quad (2.6)$$

where $\xi(d/\lambda_0)$ is the turbidimetric equivalent of the form factor, d is the diameter of the particle, and H equals (16/3) πK*:

$$H = \frac{32\pi^3 n_0^2 (\mathrm{d}n/\mathrm{d}c)^2}{3N_A \lambda_0^4} \quad (2.7)$$

with n_0 being the refractive index of the solvent. At small values of $\xi(d/\lambda_0)$, turbidity thus depends on concentration, molar mass and refractive index increment. The $\xi(d/\lambda_0)$ function is the integral of the form-factor between $\theta = 0$ and $\theta = \pi$. The function $\xi(\mathrm{d}/\lambda_0)$ decreases exponentially with $1/\lambda_0$, just as a form factor decreases with wavevector. However, the proportionality factor H increases with $(1/\lambda_0^4)$. Turbidity (the amount of scattered light) decreases with $1/\lambda_0$. With all things being equal, therefore, turbidity will slightly increase if a casein micelle (microgel) expands because $\xi(d/\lambda_0)$ will be slightly larger. The concentration and molecular mass dependence of scattering (and turbidity) derives from the fact that scattering scales in the number density and volume of the particles squared. Therefore at a constant wavelength, turbidity may be scaled according to:

$$\tau \propto M^2 n_0^2 (\mathrm{d}n/\mathrm{d}c)^2 \xi(d/\lambda_0) \quad (2.8)$$

The refractive index (or index of refraction) with a magnetic permeability equal to that of vacuum (μ_0) of a material is defined as

$$n = \sqrt{\epsilon_r \mu_r} \quad (2.9)$$

where ϵ_r is the material's relative permittivity and $\mu_r = 1$ (here) is the material's relative permeability.[15] For a multicomponent system, such as a casein micelle, which consist of casein and MCP and are highly hydrated with milk serum, n can be expressed by

$$n = \sum m_{\mathrm{casein}} n_{\mathrm{casein}} + m_{\mathrm{MCP}} n_{\mathrm{MCP}} + (1 - m_{\mathrm{casein}} - m_{\mathrm{MCP}}) n_{\mathrm{serum}} \quad (2.10)$$

where m is the mass fraction of the compounds in the casein micelle. For completely cross-linked casein micelles, casein cannot be removed from the micelles but MCP can be removed; furthermore, the micelles can shrink or swell as a result of altered hydration and n_{serum} can be altered by addition or removal of components. Combining casein and MCP into a single fraction denoted micellar solids simplifies eq 2.10 to

$$n = \sum m_{\mathrm{micellar-solids}} n_{\mathrm{micellar-solids}} + (1 - m_{\mathrm{micellar-solids}}) n_{\mathrm{serum}} \quad (2.11)$$

The refractive index increment, dn/dc is the change in n with solute concentration; the specific refractive index increment, υ, is given by

$$\upsilon = \left.\frac{\mathrm{d}n}{\mathrm{d}c}\right|_{c=0} = \lim_{c\to 0}\left(\frac{n - n_0}{c}\right) \quad (2.12)$$

Thus dn/dc depends on the difference in refractive index between solute and solvent:

$$\mathrm{d}n/\mathrm{d}c \propto n - n_0 \quad (2.13)$$

Combination of eqs 2.11 and 2.13 yields

$$\mathrm{d}n/\mathrm{d}c \propto m_{\mathrm{micellar-solids}}(n_{\mathrm{micellar-solids}} - n_{\mathrm{serum}}) \quad (2.14)$$

2.2. Small-Angle Neutron Scattering. Excellent treatments on the scattering of cold thermal neutrons by colloidal matter are given by Jacrot[16] and Hayter.[17] When the neutrons are viewed as electromagnetic waves ($\lambda_0 \sim 1$ nm), it is understandable that the resulting scattering equations strongly parallel those in the Rayleigh−Gans−Debye theory of light scattering. In small-angle neutron scattering (SANS) experiments, the normalized scattering intensity [$I(Q)$] is measured as a function of the scattering wave vector (Q). For a dispersion of monodisperse particles, $I(Q)$ is related to the interparticle structure factor [$S(Q)$], the particle scattering factor [$P(Q)$] and the volume fraction of particles (ϕ) according to

$$I(Q) \propto \phi P(Q) S(Q) \quad (2.15)$$

The proportionality constant depends on the scattering contrast, i.e., the difference between scattering length densities of the particle and the solvent. For homogeneous spherical colloids of radius R, the particle scattering factor, $P(Q,R)$ is given by

$$P(Q,R) = \left\{3\left(\frac{\sin(QR) - QR\cos(QR)}{(QR)^3}\right)\right\}^2 \quad (2.16)$$

For $Q \cdot R_g < 1$, eq. 2.16 is approximated by

$$P(Q) = \exp\left(\frac{Q^2 R_g^2}{3}\right) \quad (2.17)$$

3. Materials and Methods

3.1. Sample Preparation. Serum protein-free (SPF) skimmed milk powder was prepared by exhaustive diafiltration (0.1 μm pore diameter) of thermised skimmed bovine milk with permeate obtained from ultrafiltration (10 kDa molecular weight cutoff) of skimmed bovine milk, followed by spray-drying under conditions traditionally applied for low-heat skim milk powder. The powder obtained as such contains all milk constituents at the normal proportion, with the notable exception of whey proteins, whose concentration was reduced by >95%. SPF milk powder was resuspended in demineralised water, containing 0.5 g L^{-1} sodium azide, at a level of 100 g L^{-1} and stirred for 24 h at 5 °C to

ensure complete hydration of casein micelles and equilibration of the mineral balance. To remove fat from the samples, casein micelles were pelleted by ultracentrifugation at 100 000*g* for 90 min at 20 °C. Pelleted casein micelles were subsequently resuspended in milk serum, which was prepared by dialyzing a 50 g L^{-1} solution of lactose against 2 × 20 volumes of reconstituted (90 g L^{-1}) low heat skimmed milk powder (SMP; Irish Dairy Board, Dublin, Ireland), containing 0.5 g L^{-1} sodium azide, for 48 h at 5 °C, using a tissue homogenizer. Resuspended casein micelles were stirred for 24 h at 20 °C and subsequently centrifuged at 500*g* for 20 min at 5 °C to remove any undissolved material. The nitrogen 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 25 g L^{-1} casein by addition of milk serum.

3.2. Cross-Linking of Casein Micelles. The casein micelle suspension was incubated with 0.5 g L^{-1} TGase (Ajinomoto, Hamburg, Germany) for 24 h at 30 °C, followed by inactivation of the enzyme by heating at 70 °C for 10 min and rapid cooling to 20 °C in ice−water. The extent of TGase-induced cross-linking was estimated by determination of the levels of monomeric casein by SDS-PAGE, as outlined by Smiddy et al.[9] MCP-free cross-linked casein micelles were prepared by acidification to pH 4.6 at 5 °C, followed by dialysis against 2 × 20 volumes of bulk milk at 5 °C for 48 h.

3.3. Turbidity Measurement. Milk serum containing 0, 2.22, 4.44, or 6.67 mol L^{-1} urea was prepared by dialyzing demineralised water against 2 × 25 volumes of reconstituted (90 g L^{-1}) low-heat skim milk powder (Irish Dairy Board, Dublin, Ireland) containing 0, 2.22, 4.44, or 6.67 mol L^{-1} urea, respectively. Trisodium citrate was added to subsamples of each serum to a final concentration of 0.0−55.6 mmol L^{-1}, at 5.56 mmol L^{-1} intervals. One volume of cross-linked casein micelle suspension was subsequently mixed with 9 volumes of milk serum, to yield final concentration of 2.5 g L^{-1} casein, 0.0, 2.0, 4.0, or 6.0 mol L^{-1} urea and 0.0−50.0 mmol L^{-1} citrate, at 5.0 mmol L^{-1} intervals. After mixing and incubation at 25 °C for 60 min, the absorbance of the sample was determined at 600 nm in a 10 mm path-length cuvette using a Perkin-Elmer Lambda 35 UV/vis Spectrophotometer. Absorbance (A) values were converted to transmission (Tr) and turbidity (τ) values according to Lambert−Beer's law:

$$Tr = \frac{1}{10^A} \tag{3.1}$$

and

$$\tau = -\ln(Tr) \tag{3.2}$$

and normalized according to

$$\tau_{\text{normalised}} = \frac{(\tau_{\text{sample}} - \tau_{\text{serum}})}{(\tau_{\text{control}} - \tau_{\text{serum}})} \tag{3.3}$$

where τ_{serum} and τ_{control} are the turbidity of the serum and sample without added urea or citrate.

3.4. Refractive Index Measurement. The refractive index increment of cross-linked casein micelles and micellar calcium phosphate-free cross-linked casein micelles was determined from a dilution series of casein micelles suspensions in milk serum. Refractive index measurements were carried out in triplicate, at 25 °C, using an ABBE refractometer the refractive index increment, dn/dc, was calculated using a least-squares fit. The refractive index of milk sera containing urea and/or citrate was determined under similar conditions.

3.5. Particle Size Measurement. Particle size was determined by dynamic light scattering (DLS) and static light scattering (SLS) using an ALV Compact Goniometer System (ALV-Laser Vertriebsgesellschaft m-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) was used, operating with vertically polarized light with a wavelength, λ, of 532.0 nm. Measurements were performed at 16 scattering wave vectors in the range $4.7 \times 10^{-3} < Q < 3.0 \times 10^{-2}$ nm^{-1} ($Q = 4\pi \cdot n_0 \cdot \sin(\theta/2)/\lambda$), where n_0 is the refractive index of the medium and θ is the angle of observation). The cuvette holder was controlled at 25.0 ± 0.1 °C. Casein micelle suspensions were diluted 100 times with milk serum containing 0.00, 2.02, 4.04 or 6.06 mol L^{-1} urea and 0.0, 20.2, or 50.5 mmol L^{-1} trisodium citrate, filtered through a 5.0 μm filter and centrifuged at 1000*g* for 5 min. DLS and SLS measurements were performed until at least 10^7 photons were collected at each angle. For DLS, the diffusion coefficient (D) was calculated from each auto-correlation function and the hydrodynamic radius (R_h) was calculated using the Stokes−Einstein equation:

$$D = \frac{k_B T}{6\pi\eta R_h} \tag{3.4}$$

where k_B is the Boltzmann constant, T is the absolute temperature of the fluid and η is the viscosity of the medium.

3.6. Small-Angle Neutron Scattering. For SANS measurements, 25 g L^{-1} suspensions of cross-linked casein micelles were prepared as described in section 3.1, with the exception that serum protein-free milk powder was reconstituted in D_2O, rather than H_2O, and that the pelleted micelles were resuspended in its ultracentrifugal supernatant, which was first clarified of serum protein and residual fat globules by filtration through a membrane with a 10 kDa MWCO. Samples were mixed with appropriate levels of clarified ultracentrifugal supernatant, urea and/or trisodium citrate to yield samples containing 10 g L^{-1} casein, 0−50 mmol L^{-1} citrate and 0−6 mol L^{-1} urea. Samples were placed in quartz cells with a path-length of 2 mm and analyzed 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 ∼0.08−2.5 nm^{-1}. All SANS measurements were performed at 25 °C.

4. Results and Discussion

As described in the introduction, casein micelles are association colloids consisting of proteins (caseins) and nanoclusters of MCP. Treatment of a casein micelle suspension with TGase results in intramicellar covalent protein cross-linking, the extent of which increases with treatment time;[9] for the conditions employed in this study no residual monomeric casein remained after incubation, as determined by SDS-PAGE (data not shown), which indicated that all micellar casein is covalently cross-linked. As such, completely cross-linked casein micelles thus consist of a soft-material, highly hydrated (∼75%), cross-linked polymer network of 100−300 nm in diameter and can thus be classified as swollen microgel particles.

Addition of increasing levels of urea (0−6 mol L^{-1}) progressively reduced the turbidity (τ) of a cross-linked casein micelle suspension by 40% (Figure 1). Urea affects protein interactions through disruption of hydrophobic and hydrogen bonds.[18,19] In native casein micelles, addition of urea leads to the extensive micellar disruption[20,21] but does not affect the calcium-phosphate bonding of caseins to MCP;[22] hence, urea probably disrupts native casein micelles into nanoclusters or small aggregates of nanoclusters cross-linked by caseins containing more than one center of phosphorylation.[3,20,22] However, for cross-linked casein micelles, covalent linking of proteins prevents disruption of casein micelles. This is supported by the data in Figure 2, which indicate that addition of urea to milk does not reduce particle size. Instead, small increases in micelle size are observed; these are assumed to be a result of swelling of the micelles, rather than aggregation since the slope of R_h as a function of Q^2 is not altered on adding urea (Figure 2A), suggesting no difference in the polydispersity of the micelle suspension. Urea-induced

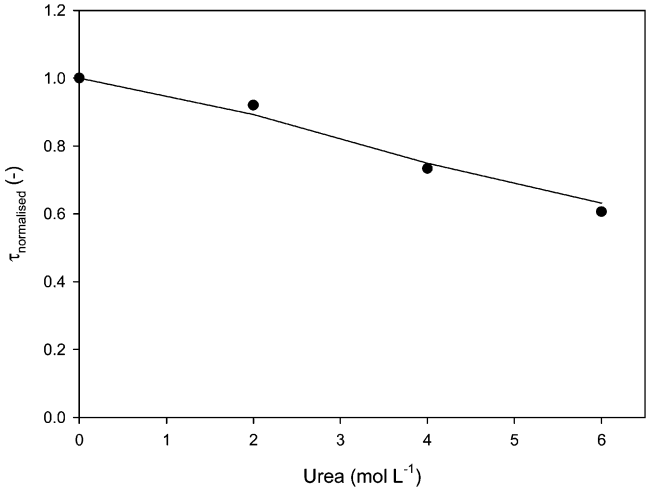

Figure 1. Comparison of data obtained experimentally (●) or by predictive calculation (—; for explanation, see text) on the influence of added urea (0−6 mol L^{-1}) on the turbidity of a suspension (2.5 g L^{-1}) of cross-linked casein micelles in milk serum. Data points are means of experiments on three individual milk samples; the coefficient of variation was <5% for each data point.

swelling of casein micelles may also be deduced from SLS (Figure 2B) and SANS (Figure 2C) measurements. For both measurement techniques, the slope of a plot of ln(I[Q]) versus Q for cross-linked micelles increased with increasing concentration of urea, which, according to eq 2.17 suggests an increase in R_g. The fact that $I(Q)$ is not affected by urea concentration for $Q \rightarrow 0$ suggests that the molecular mass of the cross-linked micelles is not altered by addition of urea. Urea-induced swelling was previously observed for various other gel particles, e.g., polyampholytic hydrogels,[23] cross-linked gelatin gels,[24] and poly(ethylenimine) gels.[25] Urea-induced swelling of gels is probably due to its ability to diminish hydrophobic interactions; as a result, the polymer chains undergo hydrophobic hydration, leading to swelling of gel particles.

Assuming that addition of urea to cross-linked casein micelles does not result in dissociation of caseins or MCP from the micelle, the molar mass (M) of the casein micelle does not change through shrinking or swelling. Therefore, the $Q(Q=0)$ value is not influenced by swelling but only the shape of the scattering function as was shown by Stieger and Richtering[10] for polyacrylamide micro-gels. However, n_0 increases on addition of urea,[26] as a result of which dn/dc changes, since ($n - n_0$), i.e., the contrast, is decreased. Swelling of the micelle influences turbidity only slightly, for $\xi(d/\lambda_0)$ is small. Thus, if only contrast is changed, turbidity is proportional to

$$\tau \propto n_0^2 (n - n_0)^2 \qquad (4.1)$$

From a dilution curve, $n_{\text{micellar solids}}$ was determined to be 1.570, in agreement with previous data,[27] whereas n_0 was 1.341, 1.356, 1.377, or 1.395 for milk serum containing 0, 2, 4, or 6 mol L^{-1} urea, so

$$n_0(C_{\text{urea}}) = 1.341 + 0.009C_{\text{urea}} \qquad (4.2)$$

where C_{urea} is the concentration of urea (mol L^{-1}). Figure 1 shows good correlation between values calculated using eqs 4.1 and 4.2 with experimental data. The data in Figure 1 thus prove that cross-linked casein micelles are completely resistant against urea-induced disruption and that the changes in light scattering observed on addition of urea are a result of the reduced contrast

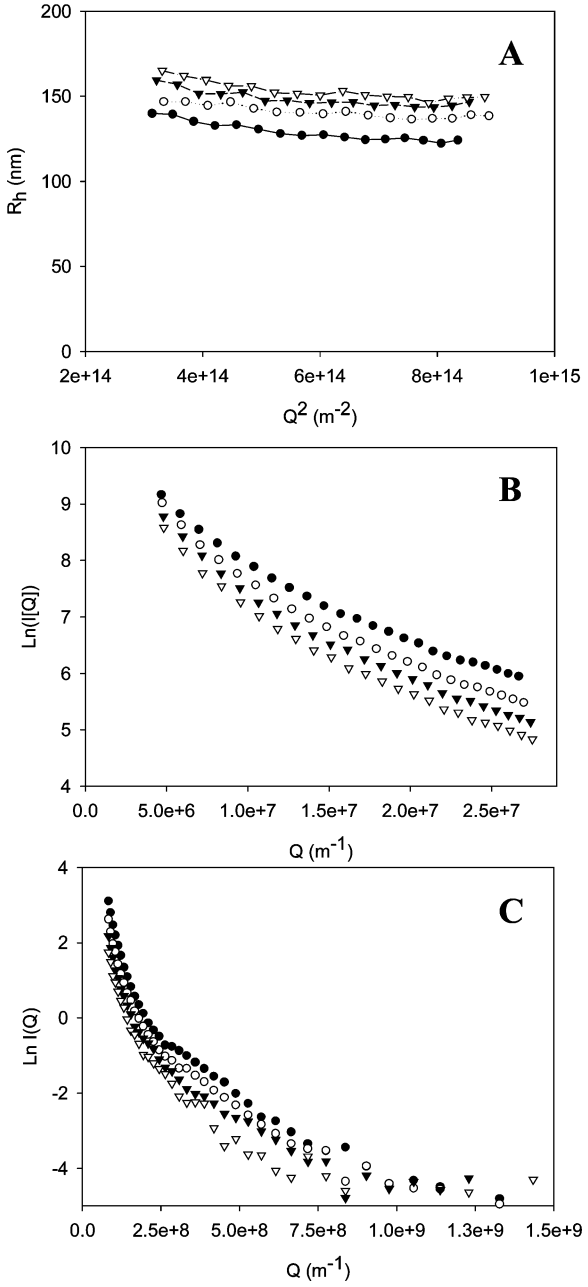

Figure 2. (A) Hydrodynamic radius (nm), (B) SLS scattering intensity, or (C) SANS scattering intensity as a function of Q for cross-linked casein micelles in the presence of 0 (●), 2 (○), 4 (▼) or 6 (▽) mol L^{-1} urea. Data points are means of experiments on three individual milk samples; the coefficient of variation was <5% for each data point.

(i.e., $n - n_0$) through the increase in refractive index of the milk serum (n_0) caused by adding urea.

Of course if the molar mass of the micro-gel particles is changed, without changing the number of micro-gel particles, both c and M change in proportion and turbidity scales as

$$\tau \propto M^2 \qquad (4.3)$$

This is the case for the addition of citrate to a suspension of cross-linked casein micelles. Citrate is a strong calcium-chelating agent; for native casein micelles, addition of a sufficiently high level of citrate results in the complete disruption of casein micelles[3,9,28] as a result of the disruption of the MCP nanoclusters. For cross-linked casein micelles, the covalent protein framework is expected to prevent micellar disruption, but MCP can be removed from the micelles, thereby reducing M. Addition

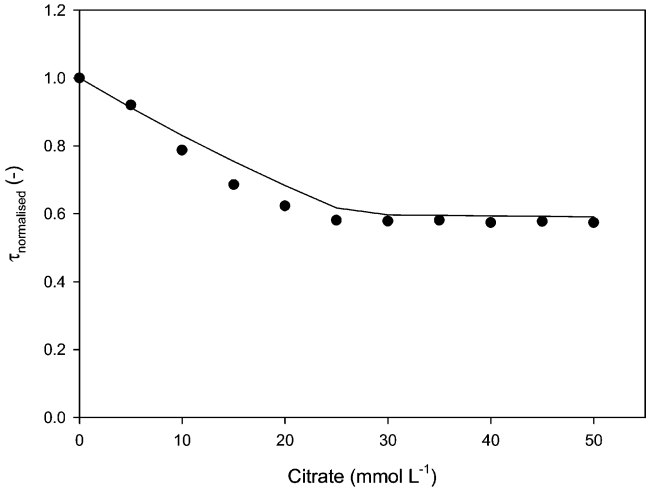

Figure 3. Comparison of data obtained experimentally (●) or by predictive calculation (—; for explanation see text) on the influence of added citrate (0−50 mmol L^{-1}) on the turbidity of a suspension (2.5 g L^{-1}) of cross-linked casein micelles in milk serum. Data points are means of experiments on three individual milk samples; the coefficient of variation was <5% for each data point.

of citrate at a level up to 25 mmol L^{-1} progressively reduced τ, with little further changes at higher concentration of citrate (30−50 mmol L^{-1}; Figure 3). The stability of cross-linked micelles against disruption by calcium-chelation is evident from Figure 4, which shows that adding citrate causes swelling, rather than disruption of cross-linked casein micelles (Figure 4A−C), even though citrate-induced increases in $I(Q)$ at first sight may suggest otherwise (Figure 4C); however, it should be realized in this respect that for the SANS measurements, the casein micelles have a match point at a ~40:60 ratio of D_2O:H_2O. The scattering length density of the MCP nanoclusters is slightly smaller than that of the solvent. Removing the MCP will change contrast but also the scattering mass and therewith the radius.[29] Citrate-induced swelling of casein micelles is probably due to the fact that removal of MCP increases protein charge, due to the exposure of charged phosphoserine groups. Increased charge in cross-linked micelles is likely to lead to swelling due to increased repulsion, as well as the osmotic contribution of an increased level of counterions. In SANS we thus observe an increase in size as the radius of gyration is determined by the caseins

The refractive index of MCP free casein micelles was found as 1.540, compared to 1.570 for native casein micelles; thus, if α is the proportion of MCP solubilized:

$$n_{CM}(\alpha) = 1.570(1 - \alpha) + 1.540\alpha \qquad (4.4)$$

Due to the solubilization of the MCP, the serum refractive index increases from 1.341 to 1.345; furthermore, adding 50 mmol L^{-1} trisodium citrate increases n_0 by 0.003.[30] Hence, assuming that refractive index increments are additive:

$$n_0(\alpha, C_{cit}) = 1.341(1 - \alpha) + 1.345\alpha + 0.06C_{cit} \qquad (4.5)$$

where C_{cit} is the concentration of added citrate (mol L^{-1}). Assuming that molar mass of the casein micelles is only influenced by the solubilization of the MCP we have

$$M(\alpha) = M_0(1 - 0.06\alpha) \qquad (4.6)$$

where M_0 is the molar mass of a cross-linked casein micelles at $\alpha = 0$. We will work on the assumption that all MCP is

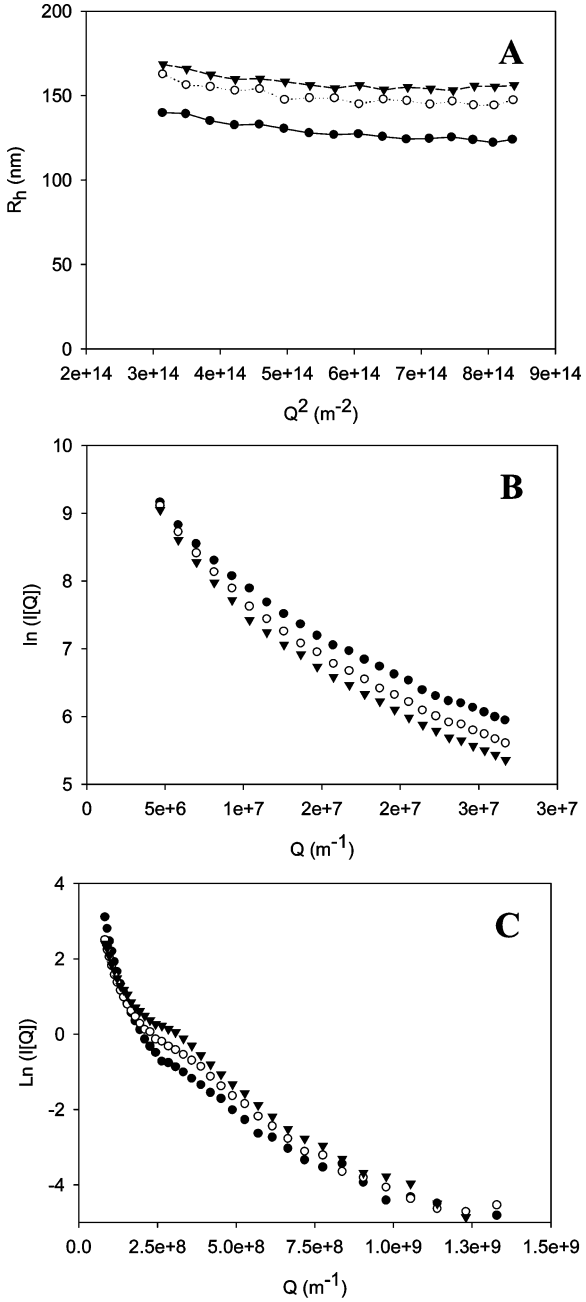

Figure 4. Hydrodynamic radius (nm), (B) SLS scattering intensity, or (C) SANS scattering intensity as a function of Q for cross-linked casein micelles in the presence of 0 (●), 20 (○), or 50 (▼) mmol L^{-1} citrate. Data points are means of experiments on three individual milk samples; the coefficient of variation was <5% for each data point.

solubilized on addition of 25 mmol L^{-1} MCP; this assumption is justified since a 25 g L^{-1} casein micelle suspension contains ~25 mmol L^{-1} calcium (Fox, 2003) and calcium chelation by citrate occurs in equimolar amounts. Hence, $\alpha = (40 \times M_{cit})$ for $0 \le C_{cit} \le 0.025$ and $\alpha = 1$ for $C_{cit} > 0.025$. By inserting the appropriate values for α and C_{cit}, τ can be calculated. Figure 3 shows that data calculated as such show good correlation with experimental data for the influence of added citrate on the turbidity light scattering by cross-linked casein micelles. This further stresses that the covalent protein network in cross-linked casein micelles is completely resistant against citrate-induced solubilization of MCP.

Figure 5 indicates that the combined effect of urea and citrate on the light scattering by casein micelles, which appears to be almost equal to the sum of the individual effects of the addition

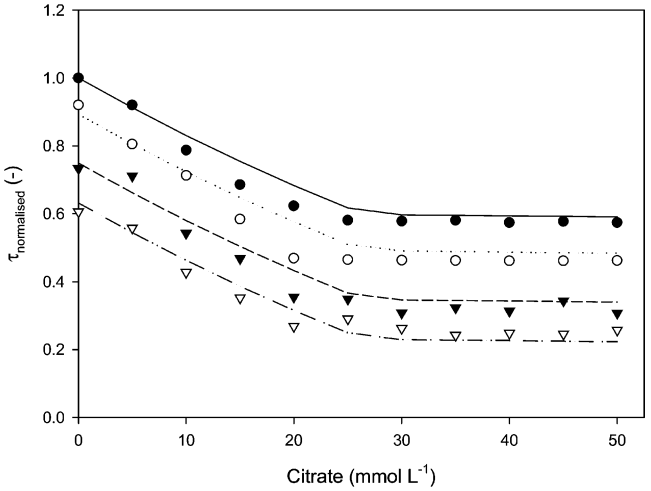

Figure 5. Comparison of data obtained experimentally (data points) or by predictive calculation (lines; for explanation see text) on the influence of added citrate (0−50 mmol L^{-1}) on the turbidity of a suspension (2.5 g L^{-1}) of cross-linked casein micelles in milk serum containing 0 (●, —), 2 (○, ···), 4 (▼, ---) or 6 (▽, -···-) mol L^{-1} urea. Data points are means of experiments on three individual milk samples; the coefficient of variation was <5% for each data point.

of urea and citrate on the light scattering; indeed, these turbidity values can once again be excellently predicted (Figure 5) by simply combining equations [4.2] and [4.5], which yields

$$n_0(\alpha, C_{cit}, C_{urea}) = 1.341(1 - \alpha) + 1.345\alpha + 0.06C_{cit} + 0.009C_{urea} \quad (4.7)$$

These data further stress the extreme stability of cross-linked casein micelles against disruption. The combined addition of 6 mol L^{-1} urea and 50 mmol L^{-1} caused a slight increase in R_h compared to casein micelles suspended in milk serum containing 6 mol L^{-1} urea or 50 mmol L^{-1} citrate (data not shown); this stresses further swelling of casein micelles. Hence, further justification for the classification of cross-linked casein micelles as microgel particles is provided.

5. Conclusions

Cross-linking of caseins by the enzyme transglutaminase creates a covalent protein network which is not disrupted by the addition of urea. MCP may be removed from this network by addition of trisodium citrate, but this does not affect the stability of the micelle. On addition of urea or citrate, cross-linked micelles displayed swelling behavior. Changes in turbidity can be readily accounted for. From the data presented in this communication, it is apparent that by enzymatic cross-linking, casein micelles can be transferred from association colloids to microgel particles. Microgel particles have been of interest in a number of applications involving the uptake and release of biologically active compounds; the use of biocompatible cross-linked casein micelles provides a very interesting and promising addition to this spectrum. A more detailed study of the swelling behavior of cross-linked casein micelles seems warranted for investigation of the full potential of these casein microgel particles.

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