



Tuning the structure and coagulation behaviour of artificial casein micelles by varying the casein composition

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ABSTRACT

The casein composition of milk varies significantly within and between mammalian species, but its effect on the structure and (functional) properties of casein micelles is poorly understood. The preparation of artificial casein micelles (ACM) allows systematic variation of the casein composition, potentially enabling the design of functional ACM from a selection of casein fractions with customised properties. We prepared ACM from α_s - and κ -casein and β - and κ -casein in different ratios, as well as ACM from α_s -, β -, and κ -casein, and analysed their composition, size, hydration, and rennet coagulation behaviour. Our results demonstrate that ACM properties and coagulation vary significantly with casein composition. α_s -Caseins form dense and compact micelles yielding firm gels upon renneting, while β -casein forms loose, highly hydrated micelles resulting in softer gels. Varying the relative proportions of caseins affects micelle size and hydration and influences the gel strength. Higher proportions of κ -casein produce smaller ACM and firmer curds, while higher proportions of β -casein increase micelle hydration and decrease curd firmness. These findings enable the design of ACM with tailored properties from blends of casein fractions, providing valuable insights for food manufacturers and the precision fermentation industry to streamline the production of animal-free dairy alternatives.

1. Introduction

Caseins constitute the major fraction of the protein in most mammalian milks (Jenness, 1974). They self-assemble into micelles through interactions between the caseins and interactions of the caseins with calcium phosphate nanoclusters. Caseins interact with these nanoclusters through short, phosphorylated moieties (phosphate centres), of which α_{s1} -casein has two, α_{s2} - has three, β - has one, and κ -casein has none (de Kruif & Holt, 2003). Due to the presence of multiple phosphate centres, α_s -caseins can cross-link multiple nanoclusters, as well as being cross-linked by the nanoclusters (Holt, 1992). By contrast, β -casein binds to the nanoclusters but cannot form cross-links since it has only a single phosphate centre. In bovine milk, about 70% of the calcium-sensitive casein is directly bound to calcium phosphate nanoclusters (Bijl, Huppertz, van Valenberg, & Holt, 2019). This comprises most of the α_s -caseins and part of the β -casein (Aoki, 1989; Aoki, Yamada, Tomita, Kako, & Imamura, 1987). The binding of the negatively-charged caseins to the nanoclusters reduces the net negative charge of the caseins, which enables them to interact with other casein-stabilised nanoclusters and 'free' caseins (mainly the other part of

the β -casein and the κ -caseins) through a multitude of weak attractive interactions (Holt & Carver, 2022). The binding of free caseins, and especially the calcium-insensitive κ -casein, prevents the casein-stabilised nanocluster complexes from precipitation, stabilising the micelles.

The four major casein families – α_{s1} -, α_{s2} -, β -, and κ -casein – occur in bovine milk in an approximate ratio of 4:1:3½:1½, respectively. However, the casein composition of milk varies within species and shows especially large variation between species (Alichanidis, Moatsou, & Polychroniadou, 2016). For instance, human milk contains little α_{s1} - and no α_{s2} -casein and, as a consequence, casein micelles in human milk are mainly composed of β - and κ -caseins (Guo, 2014; Meng, Uniacke-Lowe, Ryan, & Kelly, 2021). Likewise, α_{s1} -casein is only a minor protein fraction in goat milk, if present at all (Breunig, Crooijmans, Bovenhuis, Hettinga, & Bijl, 2024), and none of the α_s -caseins are present in elephant milk (Madende et al., 2015). Remarkably, equine milk contains little to no κ -casein (Miranda, Mahé, Leroux, & Martin, 2004; Ochir-khuyag, Chobert, Dalgalarondo, & Haertlé, 2000). The evolutionary reasons underlying this interspecific variation are often obscure, as is our understanding of the influence of varying casein compositions on the

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properties and functionality of casein micelles.

Studies on fractionated bulk milk or milk of individual cows have attempted to relate the casein composition of casein micelles to, amongst others, their size, hydration, heat stability, and rennet coagulation. This has yielded valuable insights into, for example, the inverse relationship between the concentration of (glycosylated) κ -casein and casein micelle size (Bijl, de Vries, van Valenberg, Huppertz, & van Hooijdonk, 2014; Davies & Law, 1983). However, the relationship between casein composition and rennet coagulation has been elusive (Amalfitano et al., 2019; Bonfatti, Cecchinato, Gallo, Blasco, & Carnier, 2011; Clark & Sherbon, 2000; Frederiksen et al., 2011; Jöudu, Henno, Kaart, Püssa, & Kärt, 2008; Ketto et al., 2017; Nilsson et al., 2020). While there is consensus that higher κ -casein proportions improve the coagulation properties of casein micelles (i.e. decreased rennet coagulation time and increased curd firmness), the effects of α_s -caseins or β -casein on rennet coagulation are less clear. This ambiguity may stem from the concurrent effect of multiple variables, complicating the interpretation of the results. Furthermore, these types of studies are limited by the bandwidth of natural intra- and interspecific variation in casein composition.

Artificial casein micelles (ACM) prepared from purified casein fractions present an elegant opportunity to get a clearer view on the effect of casein composition on micelle properties and functionality. ACM preparation provides direct control over casein composition and allows variance beyond the constraints of natural variation. It also enables control over the concentrations of ionic species, the relative amounts of casein genetic variants, and the presence of other components (e.g. whey proteins, milk fat, lactose), simplifying the interpretation of the results. Despite the potential, studies on ACM in which the casein composition is systematically varied are surprisingly scarce. Schmidt, Koops, and Westerbeek (1977) determined the effect of casein composition on casein micelle size, Schmidt and Koops (1977) investigated how this impacts the stability of micelles towards ethanol, dialysis, pressure, and heat, while Pearce, Linklater, Hall, and Mackinlay (1986) provided insight into the effects on rennet coagulation time and curd syneresis. There is still a need to improve our understanding of the role of each casein in the casein micelle by studying ACM of varying composition.

Better establishing the impact of casein composition on micelle properties and coagulation is also relevant in light of the emerging production of animal-free caseins through precision fermentation. These recombinant caseins could be used to produce animal-free alternatives to yoghurt and cheese either directly or by first assembling them into casein micelles artificially (Antuma, Braitmaier, et al., 2024). Given the current challenges and costs associated with recombinant protein production, it is relevant to investigate whether functional ACM can be created from combinations of two or three caseins instead of all four. Prior studies on β -casein-deficient mice (Kumar et al., 1994) and ACM with different casein compositions (Azuma, Kaminogawa, & Yamauchi, 1985; Noble & Waugh, 1965; Raynes et al., 2024; Schmidt et al., 1977; Sood, Erickson, & Slattery, 2002; Yang et al., 2024), as well as the natural occurrence of milk lacking specific caseins, suggest that this is possible. However, the properties and especially the functionality of such micelles are understudied and there is little scientific guidance on the optimal casein composition to achieve the desired casein micelle functionality for application in future dairy alternatives.

We here aim to elucidate the effects of casein composition on the properties and rennet coagulation behaviour of casein micelles by assembling ACM with specific casein fractions. We hypothesise that ACM can be created from combinations of only one calcium-sensitive casein fraction and κ -casein and that the properties and coagulation behaviour of these micelles will vary greatly based on the specific caseins used and their relative proportions. We assume that α_s -caseins will form ACM with an interconnected internal network of casein-stabilised nanoclusters, forming strong gels upon coagulation, whereas ACM with β -casein have a loose internal structure forming weaker gels. To this end, we prepared ACM from α_s - and κ -casein and β - and κ -casein in different

ratios and analysed their composition, size, hydration, and coagulation upon renneting.

2. Materials and methods

2.1. Materials

Bovine α -casein (C6780, 91.1% protein (analysed according to Section 2.2.1), of which 90% α_s -caseins and 10% β -casein), calcium chloride (C1016), magnesium chloride (M8266), potassium phosphate monobasic (P5379), sodium phosphate dibasic (S7907), sodium citrate tribasic dihydrate (S4641), potassium hydroxide (1.05033), sodium hydroxide (221465), potassium chloride (1.04936), potassium carbonate (1.04928), potassium sulphate (1.05153), magnesium citrate tribasic nonahydrate (63067), hydrochloric acid (1.13386), nitric acid (1.00456), hydrogen peroxide (1.07209), guanidine hydrochloride (50950), L-dithiothreitol (D0632), and lactic acid solution (252476) were all purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Hydrochloric acid solution (7647-01-0) and acetonitrile ULC-MS (75-05-8) were purchased from Actua-All (Actua-All Chemicals B.V., Oss, Netherlands). Tripotassium citrate monohydrate (6100-05-6) was bought from VWR (VWR International bvba, Leuven, Belgium). Trifluoroacetic acid (44630) was purchased from Alfa Aesar (Thermo Fisher Scientific, Kandel, Germany). Recombinantly produced chymosin (CHY-MAX Plus, batch no. 3634543) was obtained from Chr. Hansen Holding A/S (Hørsholm, Denmark). Ultrapure water (MilliQ system, Merck KGaA, Darmstadt, Germany) was used for all experiments unless indicated otherwise.

2.2. Methods

2.2.1. Purification of β - and κ -casein

Raw bovine milk from the research station Meiereihof (University of Hohenheim, Stuttgart, Germany) was heated to 55 °C and separated into skim milk and a cream phase. The skim milk was pasteurised at 74 °C for 30 s. β -Casein was isolated from the skim milk according to Schäfer, Schubert, and Atamer (2019) with minor modifications. The skim milk was concentrated by microfiltration (MF) and then subjected to microfiltration in diafiltration mode (MF-DF) to reduce the whey protein content. The retentate (micellar casein concentrate) was diluted 4x with demineralised water and stored overnight at 5 °C. The micellar casein concentrate was then subjected to cold MF-DF (mass reduction ratio (MRR) = 2.0), which resulted in a β -casein-rich permeate and β -casein-depleted retentate, the latter of which was diluted 2x with demineralised water and stored at 5 °C for 2 h. The cold MF-DF and subsequent dilution of the retentate were repeated three times, each time using the diluted retentate of the previous filtration step as feed. The β -casein-rich permeates from these filtration steps were mixed, tempered, concentrated by ultrafiltration (UF) at 30 °C, and then spray dried, all according to Schäfer et al. (2019). This yielded a β -casein powder (97.3% β -casein and 2.7% κ -casein with a protein content of 91.9%) of which the protein composition was determined in duplicate as described in Section 2.2.4 and the protein content was determined in triplicate with the Dumas nitrogen combustion method (Rapid N exceed®, Elementar Analysensysteme GmbH, Langenselbold, Germany) with a protein conversion factor of 6.38.

The β -casein-depleted retentates were mixed, further concentrated by ultrafiltration (MRR = 3.3), and then enriched in κ -casein by calcium precipitation of the calcium-sensitive caseins and subsequent decanter centrifugation according to Schubert, Ergin, Panetta, Hinrichs, and Atamer (2021; separation S1). The κ -casein-rich supernatant was then purified and concentrated through ultrafiltration, followed by UF-DF (6 repetitive DF stages using 20 kg water) and another UF step (MRR = 2.0). The retentate of this ultrafiltration step was then spray dried according to Schäfer et al. (2019). This yielded a κ -casein powder with a protein content of 82.3% composed of 67.8% κ -casein, 23.3% β -casein,

and 8.9% α_s -caseins (analyses were carried out as aforementioned).

2.2.2. Preparation of artificial casein micelles

Purified caseins were mixed in the following combinations: α_s -, β -, and κ -casein, α_s - and κ -casein in various ratios, and β - and κ -casein in various ratios. The casein mixtures were dissolved in 22.5 mM sodium citrate in water to a total casein concentration of 64.1 g L⁻¹ by stirring at 60 °C for 30 min. Two salt solutions were prepared: solution I was composed of CaCl₂ and MgCl₂ and was adjusted to pH 6.70 with 0.1M HCl and solution II was composed of KH₂PO₄ and Na₂HPO₄. The concentrations of the salts were adapted to the content of ionic species of the casein powders to ensure that the final composition of the ACM samples with regard to ionic species was approximately 30 mM calcium, 22 mM phosphate, 9 mM citrate, and 5 mM magnesium. ACM from β - and κ -casein were prepared in triplicate through the controlled addition of 60 mL of the casein solutions and 10 mL of each salt solution to an initial volume of 67 mL water. ACM from the other casein combinations were prepared in singlicate through the controlled addition of 40 mL of the casein solutions and 6.67 mL of each salt solution to an initial volume of 45 mL water. The solutions were added to a jacketed glass vessel at 37 °C for 60 min through syringe pumps (Harvard PHD 2000, Harvard Apparatus, Holliston, MA, USA and ProSense NE-1600, ProSense B.V., Oosterhout, Netherlands). During addition, the pH of the mixture was maintained at pH 6.70 by titration (877 Titrimo Plus, Metrohm AG, Barendrecht, Netherlands) with 1M NaOH. Titration was continued for 20 min after reaching the final composition to equilibrate the pH. Both during mixing and pH equilibration, the mixture was vigorously stirred through a magnetic stirrer. The solutions were brought to the desired final volume of 150 mL (ACM from β - and κ -casein) or 100 mL (ACM from the other casein combinations) with water. This yielded ACM samples with a final approximate composition of 25.6 g L⁻¹ casein and the aforementioned concentrations of ionic species. Samples were stored at 4 °C for at least 12 h before analysis.

2.2.3. Ultracentrifugation and determination of apparent casein micelle hydration

ACM samples were equilibrated at room temperature and ultracentrifuged in duplicate for 1 h at 100 000 g. The apparent casein micelle hydration was then determined through analysis of the moisture content of the pellets by means of oven drying, all according to Antuma, Steiner, Garamus, Boom, and Keppler (2023).

2.2.4. Analysis of casein composition and partitioning

ACM samples were diluted at least tenfold and supernatants at least twofold in a buffer solution of 6 M guanidine hydrochloride, 20 mM dithiothreitol, and 5 mM sodium citrate. Likewise, casein powders were dissolved in the buffer solution at protein concentrations ranging from 1.5 to 3.5 g L⁻¹. The casein composition and the casein content of all casein powders, as well as the casein content and casein composition of ACM samples and their ultracentrifugal supernatants, were determined by high-performance liquid chromatography (HPLC) with an adapted method of Schubert, Meric, Boom, Hinrichs, and Atamer (2018) as described in Antuma, Stadler, Garamus, Boom, and Keppler (2024). Micellar casein was defined and calculated as the difference between the total and supernatant casein.

2.2.5. Quantification of anions and cations

The content of cations (calcium, phosphorus, magnesium, sodium, and potassium) of ACM samples and their ultracentrifugal supernatants was analysed by inductively coupled plasma optical emission spectrometry (ICP-OES). First, samples were prepared by microwave-assisted wet digestion. Approximately 0.5 mL solution was mixed with 10 mL aqua regia (7.5 mL HCl + 2.5 mL HNO₃) and 1 mL H₂O₂. Subsequently, the mixture was subjected to a temperature program in an ETHOS EASY microwave digestion system (Milestone Srl, Sorisole, Italy) according to Guimarães, Gremmen, Wijffels, Barbosa, and D'Adamo

(2021). The digested material was washed and diluted with water to a total volume of 100 mL. Samples were prepared in duplicate and analysed in triplicate with the operational conditions as specified in Guimarães et al. (2021). Elements were detected in radial view, unless stated otherwise, at the following wavelengths: Ca 393.366 nm, P 178.221 nm (axial), Mg 279.553 nm, Na 589.592 nm, K 766.490 nm.

The content of anions (chloride, phosphate, and citrate) of ACM samples and their ultracentrifugal supernatants was analysed by ion chromatography (IC). ACM samples were diluted 500-fold and supernatants were diluted 200-fold in water. Solutions were then analysed on a Dionex ICS-6000 liquid chromatography system equipped with a 2 mm standard bore Dionex IonPac AS17-C column for anion analysis (Thermo Fisher Scientific B.V., Breda, Netherlands) at 30 °C with a flow rate of 0.25 mL min⁻¹ and injection volume of 5 μ L. Gradient elution was conducted with KOH, first set to 5 mM for 10 min, followed by a linear increase to 40 mM within 15 min, an isocratic elution at 40 mM for 6 min, and a linear decrease to 5 mM in 5 min. A conductivity detector was used for peak detection. Samples were analysed in duplicate.

Concentrations of ionic species in the supernatants were corrected for the excluded volume with a correction factor K calculated according to Pierre and Brulé (1981):

$$K = \frac{1000 - P(1 + W)}{1000} \quad (1)$$

where P is the measured micellar protein content in g L⁻¹ and W is the measured apparent micelle hydration in g water g⁻¹ dry matter. The micellar concentration of the ionic species was then calculated as the difference between the total concentration and the corrected supernatant concentration.

2.2.6. Particle size analysis

ACM samples were diluted 100-fold in simulated milk ultrafiltrate (SMUF; produced according to Dümpler, Kieferle, Wohlschläger, and Kulozik (2017)) after which the hydrodynamic diameter and polydispersity of the micelles were analysed by means of dynamic light scattering according to Antuma, Stadler, et al. (2024). The refractive index of the dispersant (SMUF) was set at 1.33, its viscosity at 0.8872 mPa s, and the refractive index of the casein micelles at 1.57 (Griffin & Griffin, 1985).

2.2.7. Rheological characterisation of rennet-induced coagulation

The pH of ACM samples was adjusted to 6.3 through acidification below 10 °C with an 8.5% lactic acid solution in water. Samples were left to equilibrate overnight at 4 °C, after which the pH was readjusted to 6.3 before analysis. Dilution due to addition of the lactic acid solution was considered negligible. Subsequently, 0.04% (v/w) calcium chloride was added by means of a 4% (w/v) calcium chloride solution and the samples were heated to 30 °C while stirring. Samples were then renneted by adding 0.02% (v/w) chymosin and immediately transferred to a rheometer (MCR 302, Anton Paar, Graz, Austria) equipped with a double-gap device (DG26.7) to monitor the rennet-induced coagulation through oscillatory rheometry. A strain amplitude of 0.001 and a frequency of 1 Hz were applied at a fixed temperature of 30 °C (controlled by a Peltier element). The storage modulus G' of the samples was recorded for 60 min. Samples were analysed in duplicate.

2.2.8. Statistical data analysis

Statistical analysis of the data obtained from analyses on samples prepared in triplicate was performed with OriginPro (version 2022, OriginLab Corporation, Northampton, MA, USA). Significance levels were assessed by one-way analysis of variance followed by Tukey's Honest Significant Difference post hoc test at a 95% confidence interval. Results are expressed as mean \pm standard deviation of independent triplicates. Technical replicates were averaged before statistical analysis. Results from analyses on samples prepared in singlicate are

expressed as mean \pm standard deviation of duplicate measurements. Trendlines in figures are only shown if the slope is statistically different ($p < 0.05$) from zero (the standard error was scaled with the square root of reduced Chi-square).

3. Results and discussion

3.1. Micelle compositions

ACM composed of predominantly α_s - and κ -casein (hereinafter referred to as ACM $\alpha:\kappa$) or β - and κ -casein (hereinafter referred to as ACM $\beta:\kappa$) in different ratios were prepared. Their casein composition is presented in Fig. 1. The fractions of α_s - and κ -casein of the total casein content in ACM $\alpha:\kappa$ ranged from $60.9 \pm 0.8\%$ and $25.5 \pm 0.1\%$ to $79.5 \pm 0.4\%$ and $11.6 \pm 0.5\%$, respectively, with a maximum of $13.5 \pm 0.8\%$ β -casein. ACM $\beta:\kappa$ had compositions ranging from $62.8 \pm 0.1\%$ β -casein and $30.8 \pm 0.2\%$ κ -casein to $78.2 \pm 0.3\%$ and $18.6 \pm 0.3\%$, respectively, and contained a maximum of $6.4 \pm 0.1\%$ α_s -caseins. An extra sample composed of $87.7 \pm 0.4\%$ β -casein and $10.6 \pm 0.2\%$ κ -casein was unstable (i.e. visible casein aggregation and sedimentation) and was therefore excluded from analysis. Additionally, ACM with a bovine milk-like casein composition of $48.8 \pm <0.1\%$ α_s -caseins, $32.0 \pm 0.2\%$ β -casein, and $19.2 \pm 0.2\%$ κ -casein (hereinafter referred to as ACM $\alpha:\beta:\kappa$) were prepared to serve as a reference.

In systems with bovine milk-like casein compositions, generally about 90% of the casein assembles into micelles, both for natural and artificial casein micelles (Antuma, Braitmaier, et al., 2024; Davies & Law, 1983). ACM $\alpha:\beta:\kappa$ showed a similar level of micellar casein of $88.6 \pm <0.1\%$ (Table 1). ACM $\alpha:\kappa$ showed higher levels of micellar casein of about 95–96%, whereas lower levels (86.5 ± 0.9 to $89.1 \pm 0.3\%$) were observed for ACM $\beta:\kappa$. All or almost all of the α_s -caseins were present in the micellar phase of the prepared ACM. By contrast, about 90% of the total β -casein present in ACM $\beta:\kappa$ and ACM $\alpha:\kappa$ assembled into micelles. Moreover, about 75% of the total κ -casein was present in the micellar phase of ACM $\alpha:\beta:\kappa$ and ACM $\beta:\kappa$, compared to about 83% in ACM $\alpha:\kappa$. In bovine milk, β - and κ -casein are generally the major non-micellar caseins (Davies & Law, 1983). This is a direct result of the degree to which they interact with calcium phosphate nanoclusters, which is higher for α_s -caseins than for β -casein and zero for κ -casein (Aoki, 1989). Part of the β -casein and all of the κ -casein is instead associated with the micelle through weaker interactions (e.g. hydrophobic interactions; Creamer, Berry, & Mills, 1977; Dalgleish, 2011). As a result, ACM samples with higher proportions of β -casein and κ -casein showed a lower overall

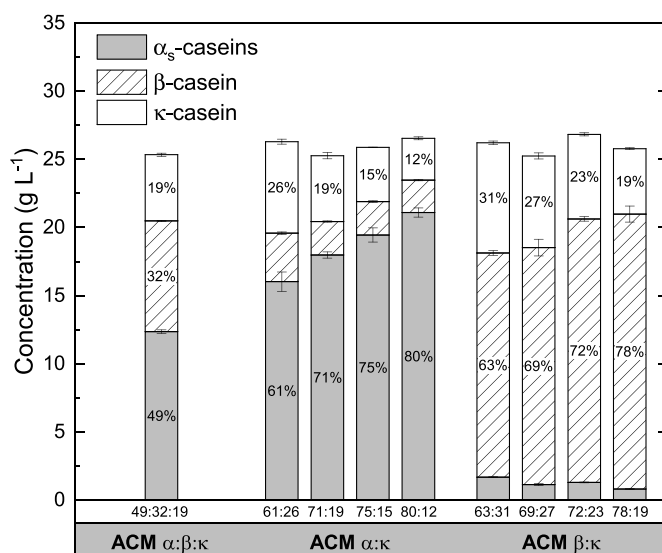


Fig. 1. Casein compositions of the prepared samples.

Table 1

Total micellar casein as a percentage of the total casein concentration, the micellar fraction of the total concentration of each casein, and the molar ratio of micellar calcium-sensitive caseins to micellar κ -casein of ACM $\alpha:\beta:\kappa$, ACM $\alpha:\kappa$, and ACM $\beta:\kappa$. Different superscript letters indicate significantly different results ($p < 0.05$) within columns.

Sample	Micellar casein (%)				$\frac{\alpha_m + \beta_m}{\kappa_m}$
	Total	α_s -CN	β -CN	κ -CN	
ACM $\alpha:\beta:\kappa$ 49:32:19	$88.6 \pm <0.1$	$99.2 \pm <0.1$	80.5 ± 0.1	$75.0 \pm <0.1$	4.1 ± 0.1
ACM $\alpha:\kappa$ 61:26	94.7 ± 0.1	99.2 ± 0.1	94.0 ± 0.1	84.5 ± 0.1	$2.7 \pm <0.1$
ACM $\alpha:\kappa$ 71:19	$95.7 \pm <0.1$	$99.6 \pm <0.1$	92.2 ± 0.1	83.0 ± 0.7	4.0 ± 0.3
ACM $\alpha:\kappa$ 75:15	96.3 ± 0.1	$99.5 \pm <0.1$	92.9 ± 0.2	82.5 ± 0.2	5.3 ± 0.1
ACM $\alpha:\kappa$ 80:12	96.4 ± 0.1	98.9 ± 0.1	92.4 ± 0.1	82.2 ± 1.2	7.3 ± 0.5
ACM $\beta:\kappa$ 63:31	86.5 ± 0.9^a	100.0 ± 0.0	89.1 ± 0.8^c	78.2 ± 1.1^a	$2.0 \pm <0.1^d$
ACM $\beta:\kappa$ 69:27	86.6 ± 0.8^b	100.0 ± 0.0	90.1 ± 0.7^{bc}	75.5 ± 1.3^b	2.6 ± 0.1^c
ACM $\beta:\kappa$ 72:23	89.1 ± 0.3^a	100.0 ± 0.0	92.0 ± 0.3^a	77.7 ± 0.6^{ab}	$3.1 \pm <0.1^b$
ACM $\beta:\kappa$ 78:19	87.6 ± 0.2^b	100.0 ± 0.0	91.0 ± 0.3^{ab}	71.3 ± 0.2^c	4.4 ± 0.1^a

micellar casein content (Table 1).

Nevertheless, the micellar fraction of the total κ -casein was remarkably constant given the overall decrease in the κ -casein fraction and only decreased from 84.5 ± 0.1 to $82.2 \pm 1.2\%$ for ACM $\alpha:\kappa$ and from 78.2 ± 1.1 to $71.3 \pm 0.2\%$ for ACM $\beta:\kappa$, while the overall κ -casein content decreased from 25.5 ± 0.1 to $11.6 \pm 0.5\%$ and from 30.8 ± 0.2 to $18.6 \pm 0.3\%$, respectively. The molar ratio of micellar calcium-sensitive casein to micellar κ -casein consequently increased by a factor 2–3 (Table 1). Thus, the micellar fraction of the total κ -casein was relatively independent of the overall casein composition. Instead, it correlated with the overall level of micellar casein, which was previously observed by Huppertz, Heck, Bijl, Poulsen, and Larsen (2021) in milk of individual cows. This was likely facilitated by the reduced negative charge of nanocluster-bound caseins, which allowed attractive interactions with other caseins to dominate (e.g. via hydrophobic interactions; Horne, 1998). Since the molar ratio of micellar calcium-sensitive casein to micellar κ -casein in the prepared ACM was at least 2 (Table 1), this created ample opportunity for κ -casein to interact with calcium-sensitive caseins, thereby migrating to the micellar phase.

The overall composition of the prepared samples with regard to the ionic species is presented in Table 2. The total concentrations of calcium, magnesium, and citrate showed minimal variation and were similar to those found in bovine milk (Gaucheron, 2010). The concentration of inorganic phosphorus, however, was slightly higher in ACM $\alpha:\kappa$ and ACM $\beta:\kappa$ than the average concentration in bovine milk of 21 mM. Furthermore, the samples contained consistently more sodium, less potassium, and less chloride than bovine milk, except for ACM $\beta:\kappa$, which contained more chloride than bovine milk (Gaucheron, 2010). These differences originated from the use of these ions in the purification of the caseins and the resulting content of these ions in the protein powders. The use of sodium hydroxide to maintain the pH during ACM assembly further contributed to the inflated sodium concentrations. However, the differences in composition between the samples were relatively small. We therefore expect that the much larger variation in casein composition allows for a relevant comparison of the properties and functionality of the micelles independent of their ion composition.

In the prepared ACM, about 70% of the total calcium was present in the micellar phase (Table 3). No obvious differences were observed between ACM $\alpha:\kappa$ and ACM $\beta:\kappa$. ACM $\beta:\kappa$ contained a considerably larger fraction of the inorganic phosphorus than ACM $\alpha:\kappa$ and ACM $\alpha:\beta:\kappa$ (Table 3), which is likely related to the decreased levels of organic

Table 2

Total concentrations of calcium (Ca), inorganic phosphorus (P_i), magnesium (Mg), citrate (Cit), sodium (Na), potassium (K), and chloride (Cl) in the prepared samples. Different superscript letters indicate significantly different results ($p < 0.05$) within columns.

Sample	Ca (mM)	P _i (mM)	Mg (mM)	Cit (mM)	Na (mM)	K (mM)	Cl (mM)
ACM α : β : κ 49:32:19	28.7 \pm 0.2	26.4 \pm 0.3	4.7 \pm <0.1	8.4 \pm 0.1	81.0 \pm 0.6	14.2 \pm 0.9	11.6 \pm 0.1
ACM α : κ 61:26	30.0 \pm <0.1	25.1 \pm <0.1	4.8 \pm <0.1	8.1 \pm <0.1	86.5 \pm 0.8	14.3 \pm 1.0	12.9 \pm <0.1
ACM α : κ 71:19	29.4 \pm 0.1	25.2 \pm 0.6	4.7 \pm <0.1	8.3 \pm 0.2	86.0 \pm 0.4	13.4 \pm 0.3	12.8 \pm 0.2
ACM α : κ 75:15	28.5 \pm <0.1	24.3 \pm 0.1	4.6 \pm 0.1	8.0 \pm <0.1	87.4 \pm 1.2	13.3 \pm 0.4	12.3 \pm <0.1
ACM α : κ 80:12	28.4 \pm 0.5	24.8 \pm 0.1	4.7 \pm <0.1	8.0 \pm <0.1	87.7 \pm 0.6	13.7 \pm 0.2	12.5 \pm <0.1
ACM β : κ 63:31	29.1 \pm 0.1 ^a	25.9 \pm 0.3 ^{bc}	4.7 \pm <0.1 ^b	10.5 \pm 0.1 ^{ab}	63.6 \pm 0.7 ^a	11.6 \pm 0.2 ^a	45.8 \pm 1.2 ^a
ACM β : κ 69:27	29.0 \pm 0.3 ^a	26.5 \pm 0.3 ^c	4.6 \pm <0.1 ^c	10.3 \pm 0.1 ^b	62.3 \pm 0.1 ^b	11.7 \pm 0.2 ^a	45.9 \pm 0.5 ^a
ACM β : κ 72:23	29.2 \pm 0.2 ^a	26.2 \pm 0.2 ^{ab}	4.8 \pm <0.1 ^a	10.6 \pm <0.1 ^a	63.8 \pm 0.1 ^a	12.0 \pm 0.4 ^a	46.5 \pm 0.3 ^a
ACM β : κ 78:19	29.0 \pm 0.2 ^a	27.1 \pm 0.2 ^a	4.7 \pm <0.1 ^b	10.6 \pm 0.1 ^a	62.3 \pm 0.2 ^b	11.7 \pm 0.2 ^a	47.2 \pm 0.1 ^a

Table 3

Proportion of the total concentrations of ionic species present in the micellar phase of ACM with various casein compositions. Different superscript letters indicate significantly different results ($p < 0.05$) within columns.

Sample	Ca (%)	P _i (%)	Mg (%)	Cit (%)
ACM α : β : κ 49:32:19	72.5 \pm 0.4	41.1 \pm 0.7	39.7 \pm 1.1	9.5 \pm 0.9
ACM α : κ 61:26	75.0 \pm 0.1	42.6 \pm 0.3	42.2 \pm 0.6	9.7 \pm 0.3
ACM α : κ 71:19	73.7 \pm 0.2	42.4 \pm 3.1	41.9 \pm 0.1	10.6 \pm 4.6
ACM α : κ 75:15	73.8 \pm 0.2	38.8 \pm 2.5	42.1 \pm 0.8	8.9 \pm 4.1
ACM α : κ 80:12	73.7 \pm 0.5	36.2 \pm 1.6	42.5 \pm 0.2	5.7 \pm 3.2
ACM β : κ 63:31	74.8 \pm 0.5 ^a	60.0 \pm 1.0 ^a	42.0 \pm 1.5 ^a	25.3 \pm 2.2 ^a
ACM β : κ 69:27	72.8 \pm 0.2 ^b	57.5 \pm 0.6 ^b	39.3 \pm 0.7 ^b	24.3 \pm 1.6 ^a
ACM β : κ 72:23	75.8 \pm 0.3 ^a	59.6 \pm <0.1 ^a	41.8 \pm 0.6 ^a	23.6 \pm 0.4 ^a
ACM β : κ 78:19	72.7 \pm 0.4 ^b	57.5 \pm 0.7 ^b	38.1 \pm 0.3 ^b	24.0 \pm 1.3 ^a

phosphorus in these micelles originating from the lower level of phosphorylation of β -casein. The substitution of organic phosphorus with inorganic phosphorus has also been observed upon dephosphorylation of the caseins (Antuma et al., 2023). This observation perhaps also explains the seemingly lower levels of inorganic phosphorus upon increasing proportions of α _s-caseins and β -casein in ACM α : κ and ACM β : κ , respectively (Table 3). The micellar proportion of magnesium showed little variation between the samples. Lastly, micelles prepared with β -casein contained considerably more micellar citrate than those prepared with α _s-caseins. Overall, the mineralisation of the prepared ACM generally corresponded well to the mineralisation of casein micelles in bovine milk and ACM prepared from sodium caseinate (Antuma et al., 2023; Bijl, van Valenberg, Huppertz, & van Hooijdonk, 2013).

3.2. Micellar properties

The changes in the ratio of calcium-sensitive casein to κ -casein were expected to affect the size of the ACM, as the inverse relation between the proportion of κ -casein and micelle size is well-documented in literature (Bijl et al., 2014; Dalgleish, Horne, & Law, 1989; Davies & Law, 1983; Schmidt et al., 1977; Yoshikawa, Takeuchi, Sasaki, & Chiba, 1982). This relationship was indeed observed for ACM α : κ and ACM β : κ (Fig. 2A), although to varying degrees. At the tested casein compositions, β - and κ -casein formed larger micelles than α _s- and κ -casein. As the ratio increased by a unit (indicating a decreasing proportion of κ -casein), the average size of ACM α : κ increased linearly ($R^2 = 0.978$) by approximately 16 nm and that of ACM β : κ linearly ($R^2 = 0.948$) by approximately 59 nm. Extrapolation of the linear fits to lower ratios suggests that ACM α : κ would have been larger than ACM β : κ at ratios lower than 1.7. An increase in the proportion of β -casein at the expense of α _s-caseins (i.e. ACM α : β : κ) only increased the size by 1 nm compared to the predicted size of ACM α : κ at the same ratio. Given that both ACM α : β : κ and ACM α : κ were prepared in singlicate, this increase is insignificant.

Because the size of the ACM increased upon an increasing ratio, the average volume of a single micelle also increased (for ACM α : κ by a factor 3.9 and for ACM β : κ by a factor 10.8; Table 4). However, the overall apparent hydration of the micellar phase decreased upon an increasing ratio (Fig. 2B). The apparent hydration of ACM α : κ was lower than that of ACM β : κ at the tested casein compositions. As the ratio increased by a unit, the overall apparent hydration of ACM α : κ decreased linearly ($R^2 = 0.925$) by 0.03 g water g⁻¹ micellar casein and that of ACM β : κ linearly ($R^2 = 0.941$) by 0.43 g water g⁻¹ micellar casein. Similar to the effect on micelle size, an increase in the proportion of β -casein at the expense of α _s-caseins at a fixed ratio of calcium-sensitive

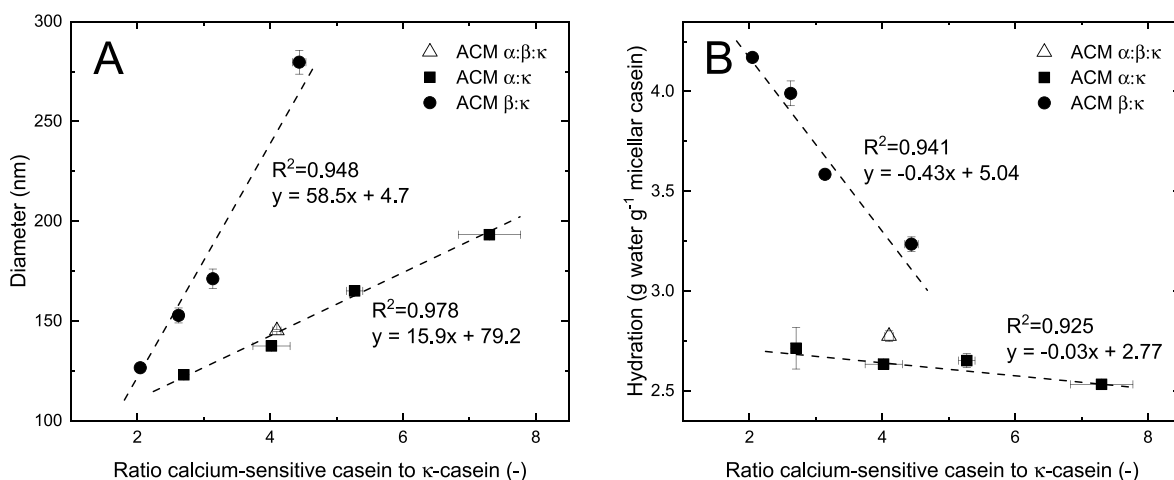


Fig. 2. (A) Hydrodynamic diameter and (B) apparent hydration of ACM α : β : κ , ACM α : κ , and ACM β : κ plotted against the molar ratio of micellar calcium-sensitive caseins to micellar κ -casein.

Table 4

An approximation of the volume fraction, average volume and surface of a single micelle, number of micelles, total micelle surface area, and number of micellar κ -casein residues per litre and per 100 nm² available micelle surface of ACM with various casein compositions.

Sample	Micelle volume fraction (–)	Average micelle volume (L)	Average micelle surface (nm ²)	Number of micelles L ⁻¹ (–)	Total micelle surface (m ² L ⁻¹)	Micellar κ -casein monomers L ⁻¹ (–)	Micellar κ -casein monomers per 100 nm ² available surface (–)
ACM α : β : κ 49:32:19	0.062	1.60E-18	6.6E+04	3.9E+16	2573	1.2E+20	4.5
ACM α : κ 61:26	0.068	9.76E-19	4.8E+04	6.9E+16	3297	1.8E+20	5.4
ACM α : κ 71:19	0.064	1.36E-18	5.9E+04	4.7E+16	2781	1.3E+20	4.6
ACM α : κ 75:15	0.066	2.36E-18	8.6E+04	2.8E+16	2401	1.0E+20	4.3
ACM α : κ 80:12	0.065	3.78E-18	1.2E+05	1.7E+16	2013	8.0E+19	4.0
ACM β : κ 63:31	0.095	1.06E-18	5.0E+04	8.9E+16	4480	2.0E+20	4.5
ACM β : κ 69:27	0.087	1.87E-18	7.3E+04	4.7E+16	3428	1.6E+20	4.7
ACM β : κ 72:23	0.086	2.63E-18	9.2E+04	3.3E+16	3004	1.5E+20	5.1
ACM β : κ 78:19	0.073	1.15E-17	2.5E+05	6.4E+15	1569	1.1E+20	6.9

casein to κ -casein increased the hydration towards that of ACM β : κ . The resulting apparent hydration of ACM α : β : κ was 2.77 ± 0.03 g water g⁻¹ micellar casein, which is in a similar range as earlier reported apparent hydration of both bovine and artificial casein micelles (Antuma, Braitmaier, et al., 2024; Huppertz et al., 2017).

Since the average volume of a micelle increased upon an increasing ratio of calcium-sensitive casein to κ -casein while the overall hydration of the micellar phase decreased, it would be logical to conclude that the total number of micelles decreased. Combining the gathered data on the size and hydration of the prepared ACM allowed for an approximation of the number of micelles and their total surface area (Table 4). The calculations and the assumptions underlying them are presented and discussed in Supplementary material A. Based on these calculations, the number of micelles in ACM α : κ samples is estimated to have decreased by a factor of 4 and that in ACM β : κ samples by a factor of 14 upon increasing ratios of calcium-sensitive casein to κ -casein (Table 4). The total surface area generated by the ACM thereby decreased with a factor 1.6 and 2.9, respectively.

These calculations also allow for an approximation of the number of micellar κ -casein molecules per unit available micellar surface area. For ACM α : κ , the number of micellar κ -casein molecules per 100 nm² available micelle surface area decreased upon increasing ratios (i.e. decreasing proportions of κ -casein; Fig. 3) and the corresponding increase in micelle size (Fig. 2A). However, ACM β : κ showed the opposite effect with a linear increase in the number of micellar κ -casein molecules per 100 nm² available micelle surface upon an increasing ratio and a corresponding increase in micelle size. This originated from a larger

decrease in the total micelle surface than the decrease in the number of micellar κ -casein molecules (Table 4).

If we were to assume that all micellar κ -casein was present on the micelle surface, this would suggest that the surface coverage of κ -casein increased for ACM β : κ of increasing size, even though the overall concentration of κ -casein decreased. The location of κ -casein within the casein micelle is currently subject to scientific debate (Holt & Carver, 2022; Horne, 2020). Studies that evaluated casein micelle size as a function of the casein composition (Dagleish et al., 1989; Davies & Law, 1983) suggest that the casein micelle surface is enriched in κ -casein but only slightly depleted in the other caseins, which is in agreement with electron microscopy evidence from Schmidt and Both (1982) and has been adopted in the most recent micelle model of Holt and Carver (2022). *Vice versa*, κ -casein is thought not to be exclusively present on the surface of casein micelles, but also in their interior. Therefore, it is more likely that a larger proportion of the micellar κ -casein was incorporated in the interior of ACM β : κ with increasing ratios of calcium-sensitive casein to κ -casein.

The presented observations can be explained by outlining the structures formed by α - and κ -casein and β - and κ -casein in different ratios. Let us first consider the structure of ACM formed by α - and κ -casein as a function of the ratio of micellar α -caseins (α_m) to micellar κ -casein (κ_m). Due to the presence of multiple phosphate centres, α -caseins interact strongly with calcium phosphate nanoclusters and can cross-link multiple nanoclusters (Holt, 1992). This interaction also reduces the negative charge on the α -caseins. The charge of α_{s1} -casein was calculated to shift from -15.9 to -1.4 and that of α_{s2} -casein from -4.3 to $+18.5$ (Bijl et al., 2019). The reduction in charge of α_{s1} -casein and the positive charge of α_{s2} -casein permits attractive interactions with other caseins to dominate so that α -caseins can simultaneously engage in casein-casein interactions.

At low $\alpha_m : \kappa_m$ ratios, it can be expected that all the α -caseins are involved in the stabilisation of the nanoclusters by which a limited number of nanocluster cross-links will be formed. Next to that, multiple casein-stabilised nanoclusters can associate and κ -casein may be incorporated into this network through casein-casein interactions. Further growth of this network is prohibited at locations where κ -casein binds because it cannot facilitate interactions with multiple caseins, nor can it interact with calcium phosphate nanoclusters (Horne, 1998). Logically, κ -casein is predominantly present on the surface of these micelles. At higher $\alpha_m : \kappa_m$ ratios, the α -caseins are increasingly in excess and these free α -caseins could interact with the nanocluster-bound caseins in a similar fashion as κ -casein. Thus, we postulate that, at low proportions of α -caseins and high proportions of κ -casein (i.e. low $\alpha_m : \kappa_m$), the α -caseins form a dense network of a relatively small number of cross-linked casein-stabilised nanoclusters, which is covered by the abundant κ -casein. *Vice versa*, at high $\alpha_m : \kappa_m$ ratios, the high proportion of α -caseins forms a larger network due to the cross-linking of a larger number of casein-stabilised nanoclusters enabled by the lower proportion of κ -casein.

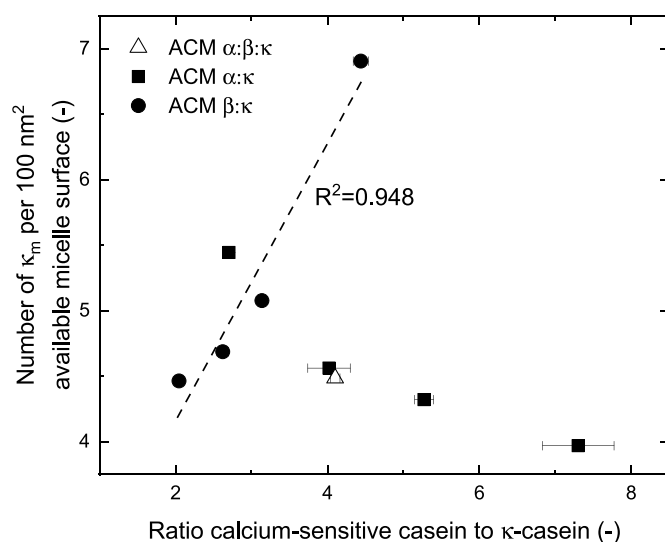


Fig. 3. An approximation of the number of micellar κ -casein (κ_m) residues per 100 nm² available micelle surface of ACM with various casein compositions.

By contrast, β -casein only has one phosphate centre and is therefore unable to cross-link calcium phosphate nanoclusters. Therefore, in the absence of α_s -caseins, β -casein forms a corona around a single calcium phosphate nanocluster (a typical nanocluster can bind approximately five casein monomers; Holt & Hukins, 1991), with the phosphate centres of the proteins in the interior and the parts of the protein able to engage in casein-casein interactions on the outside. This reduces the negative charge of β -casein from -9.5 to -1.8 (Bijl et al., 2019), enabling the self-association of multiple casein-stabilised nanoclusters and interactions with κ -casein.

At low ratios of micellar β -casein (β_m) to micellar κ -casein (κ_m), it is likely that all the β -casein is required for stabilisation of the calcium phosphate nanoclusters, whereas κ -casein is present at such high levels that it can stabilise single casein-stabilised nanoclusters or a self-associated aggregate of a few of them. Complexes of a single nanocluster stabilised by hydrophilic β -casein peptides (the first 25 residues including its phosphate centre) are approximately 8 nm in diameter (Holt, Timmins, Errington, & Leaver, 1998). Extrapolation of our results to a $\beta_m : \kappa_m$ ratio approaching zero predicts the diameter of single nanoclusters stabilised by intact β -casein to be 4.7 ± 19.4 nm (Fig. 2A). The relatively large error can be attributed to the limited number of datapoints and large extrapolation of the fit. It is unlikely that such complexes can be formed exclusively by intact β -casein and, if possible, they are more likely larger than those formed by β -casein peptides (e.g. 15–20 nm in diameter). Extrapolation of our data thus approaches the hypothetical size of these complexes but slightly underestimates it.

At high $\beta_m : \kappa_m$ ratios, the low proportion of κ -casein presumably allowed more of the casein-stabilised nanoclusters to associate, while it competed for the available casein-casein interaction sites with the excess β -casein. Due to the absence of nanocluster cross-links in this scenario, the interaction of free β -casein and κ -casein with nanocluster-bound casein could disrupt the formation of a continuous network of casein-stabilised nanoclusters. The unneutralised charges on the free β -casein and the κ -casein would repel other casein-stabilised nanoclusters partially covered with free β -casein and κ -casein. It has been postulated that this causes the formation of water channels in the interior of casein micelles, which are stabilised by free β -casein as well as κ -casein (Dalgleish, 2011).

Thus, ACM $\beta:\kappa$ at higher $\beta_m : \kappa_m$ ratios have a loose, increasingly porous structure comprising strands of β -casein-stabilised nanoclusters in which the water channels are stabilised by free β -casein and κ -casein. This explains the larger size and volume of ACM $\beta:\kappa$ compared to ACM $\alpha:\kappa$ at ratios larger than 1.7 (Fig. 2A). Furthermore, the earlier interpretation that an increasing fraction of the micellar κ -casein was present in the interior in ACM $\beta:\kappa$ at increasing ratios of calcium-sensitive casein to κ -casein (Fig. 3) is then reasonable, since the κ -casein was increasingly divided between the outer surface of the micelles and the internal surface area generated by the water channels. The reasoning also provides an explanation for the instability of the extra ACM $\beta:\kappa$ sample prepared with $87.7 \pm 0.4\%$ β -casein and $10.6 \pm 0.2\%$ κ -casein that was excluded from further analysis. In this sample, the level of κ -casein was likely not sufficient to stabilise both the outer surface of the micelles as well as the water channels in their interior, resulting in casein aggregation and precipitation.

ACM $\alpha:\kappa$ and ACM $\beta:\kappa$ represent extreme cases in which the molar ratio of micellar α_s -caseins (α_m) to micellar β -casein (β_m) is constant and approaches infinity and zero, respectively. Most mammalian milks, however, contain considerable amounts of both α_s -caseins and β -casein. Let us therefore apply the above hypotheses to describe the structure of casein micelles formed from all casein fractions (i.e. ACM $\alpha:\beta:\kappa$). This time, we will consider a fixed ratio of calcium-sensitive casein to κ -casein and a varying $\alpha_m : \beta_m$ ratio.

Independent of the $\alpha_m : \beta_m$ ratio, most of the α_s -caseins and part of the β -casein can be expected to stabilise calcium phosphate nanoclusters based on the number of phosphate centres they possess. We envision that, at a high $\alpha_m : \beta_m$ ratio (e.g. ≈ 3), the nanoclusters are almost

exclusively stabilised by α_s -caseins. This results in a highly cross-linked network of casein-stabilised nanoclusters, similar to ACM $\alpha:\kappa$, which cannot be disrupted by free β -casein or κ -casein. Upon decreasing the $\alpha_m : \beta_m$ ratio, progressively more β -casein supplements the smaller amount of α_s -caseins interacting with the nanoclusters, which decreases the level of nanocluster cross-linking. At the same time, the concentration of free β -casein is increased. In this case, the lower interconnectivity of the network of casein-stabilised nanoclusters allows the free β -casein and the κ -casein to disrupt it and give rise to water channels in the micelle interior, thereby increasing the size and hydration of the micelles.

As predicted by the percolation theory (Harmon, Holehouse, Rosen, & Pappu, 2017; Sahimi, 1994), this effect of free β -casein would be much larger at higher proportions of β -casein than at lower proportions, because the connectivity of a percolated network increases with a power law dependency (exponent < 1) on the concentration of cross-linking agent (α_s -caseins in this case). This explains the relatively small increase in size of 1 nm from the predicted size of ACM $\alpha:\kappa$ to ACM $\alpha:\beta:\kappa$ ($\alpha_m : \beta_m = 1.9$) at an equal ratio of calcium-sensitive casein to κ -casein (Fig. 2A). Further increases in the proportion of β -casein at the expense of α_s -caseins have a more pronounced effect, which is reflected by the size of ACM prepared from sodium caseinate ($\alpha:\beta:\kappa = 33:47:20$, $(\alpha_m + \beta_m)/\kappa_m = 3.3$, $\alpha_m : \beta_m = 0.8$) in our earlier study (Antuma, Stadler, et al., 2024). These were 156 nm in diameter, which translates to an increase of 25 nm compared to the predicted size of ACM $\alpha:\kappa$ at the same ratio of micellar calcium-sensitive casein to κ -casein (Fig. 2A).

Overall, these hypotheses align well with the casein micelle models of Dalgleish (2011) and Bouchoux, Gésan-Guiziou, Pérez, and Cabane (2010) that include voids or cavities in the micelle interior and with electron microscopy evidence that supports the existence of such voids (Trejo, Dokland, Jurat-Fuentes, & Harte, 2011). The hypotheses also agree with the observations of Schmidt, van der Spek, Buchheim, and Hinz (1974) that ACM from β - and κ -casein were fewer in number, looser, and more irregularly shaped compared to ACM formed of all four casein types. The trend that we observed for the sizes of the ACM aligns well with the results of Schmidt et al. (1977), who evaluated the size of ACM of different casein compositions.

Furthermore, our hypotheses can be reconciled with studies in which up to 60% of the total β -casein was removed from casein micelles by cooling and centrifuging (Yahimi Yazdi, Corredig, & Dalgleish, 2014; Zhang et al., 2018), upon which the size of the micelles was hardly affected or increased slightly. In this case, the cooling only removed part of the free β -casein stabilising the water channels, while the β -casein involved in stabilising calcium phosphate nanoclusters probably remained in place. Upon removal of part of the free β -casein, the remaining free β -casein and the κ -casein were apparently sufficient to maintain the stability of the water channels and the interconnectivity of the internal casein network was unaffected. By contrast, if these micelles were prepared with 60% less β -casein, we would expect much less β -casein to interact with nanoclusters, resulting in a higher interconnectivity of the casein network. In turn, this would allow for less disruption of the network by free β -casein, resulting in a decreased micelle size.

3.3. Coagulation behaviour

The coagulation behaviour of the prepared ACM was monitored by rheometry to study the effect of different casein compositions on casein micelle coagulation. Coagulation of ACM $\alpha:\kappa$ commenced after about 6 min of incubation with chymosin independent of the casein composition (Fig. 4). After 60 min of incubation, the ACM attained storage moduli ranging from 162.3 ± 3.4 Pa for ACM $\alpha:\kappa$ 71:19 to 127.4 ± 3.8 Pa for ACM $\alpha:\kappa$ 80:12. The rennet coagulation time of ACM $\beta:\kappa$ was slightly shorter at about 4 min, which could be related to the hypothesis that a larger part of the κ -casein is present in the interior of these micelles as compared to ACM $\alpha:\kappa$. ACM $\beta:\kappa$ would then be destabilised at lower

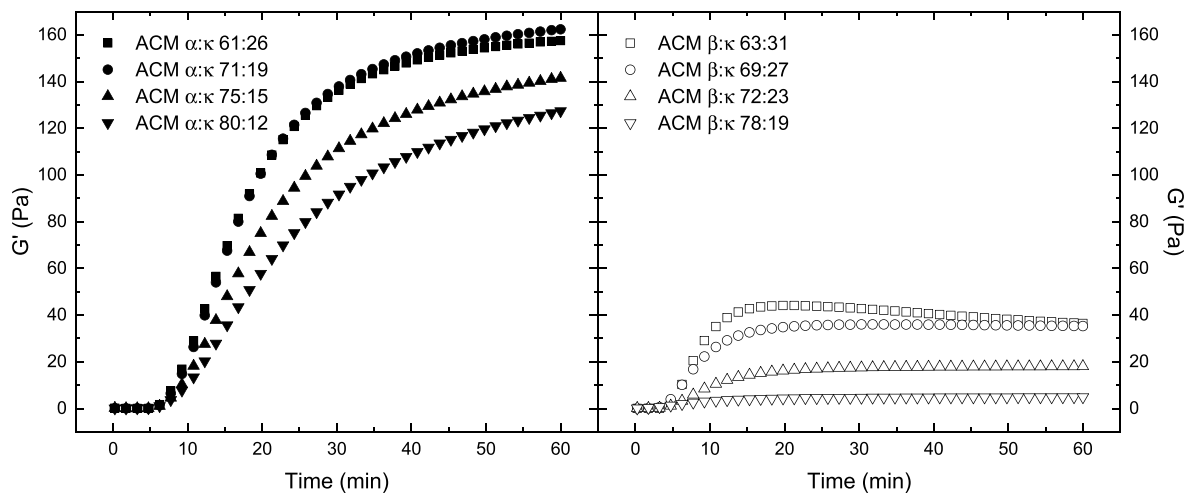


Fig. 4. Development of the storage modulus (G') over time during incubation with chymosin of ACM α : κ and ACM β : κ with different casein compositions.

levels of κ -casein proteolysis than ACM α : κ . This is in agreement with studies by Bonfatti et al. (2011), Clark and Sherbon (2000), and Pearse et al. (1986) that reported that the α_{s1} -casein content correlated positively and the β -casein content correlated negatively with the rennet coagulation time.

Compared to ACM α : κ , ACM β : κ attained much lower storage moduli during an hour of incubation with chymosin (Fig. 4). This can be attributed to two factors. Firstly, we reasoned that ACM α : κ comprised stronger internal casein networks than ACM β : κ and they can therefore be expected to form a stronger gel network upon coagulation. This aligns well with studies that suggested that α_{s1} -casein is the backbone of cheddar and Emmental cheese (Creamer & Olson, 1982; Gagnaire, Trotel, Le Graët, & Léonil, 2002). Secondly, ACM α : κ were smaller than ACM β : κ at the tested casein compositions (Fig. 2A) and smaller micelles have previously been shown to produce firmer curds upon renneting (Amalfitano et al., 2019; Bonfatti et al., 2011; Ketto et al., 2017). Bonfatti et al. (2011) hypothesised that this is related to the increased surface area generated by smaller micelles, which allows for a more compact arrangement of the micelles and an increased number of intermicellar bonds.

The maximum G' attained by ACM α : β : κ was 123.8 ± 1.3 Pa, which is comparable to earlier findings on ACM prepared from sodium caseinate with a similar method and coagulated under similar conditions (Antuma et al., 2023; Antuma, Stadler, et al., 2024) and about 40 Pa lower than that attained by ACM α : κ with a similar ratio of calcium-sensitive casein to κ -casein (Fig. 5). This aligns well with the finding of O'Mahony, McSweeney, and Lucey (2009) that casein micelles with higher $\alpha_m : \beta_m$ ratios produce firmer curds upon renneting and is likely a result of the less interconnected casein network of ACM α : β : κ compared to ACM α : κ at a similar ratio of calcium-sensitive casein to κ -casein.

The effect of casein micelle size on curd firmness also partly explains the decreased curd firmness of ACM with increasing ratios of calcium-sensitive casein to κ -casein (Fig. 5), which first increases and then decreases semi-linearly ($R^2 = 0.896$) for ACM α : κ and decreases linearly ($R^2 = 0.940$) for ACM β : κ . The approximation of the total surface area of these ACM suggests a decrease of the total surface area of the micelles by a factor 1.6 for ACM α : κ and a factor 2.9 for ACM β : κ (Table 4). The larger surface area decrease for ACM β : κ also explains the more pronounced decrease of the maximum G' (i.e. more negative slope) for these micelles as compared to ACM α : κ . For ACM α : κ with lower ratios (e.g. <3), the decreasing level of α_s -caseins likely counteracted the positive effect of an increased surface area on the curd firmness. In that case, the protein fraction that cross-links the gel network is progressively substituted by a protein fraction that does not form cross-links, thereby decreasing the strength of the gel network. Therefore, the maximum G'

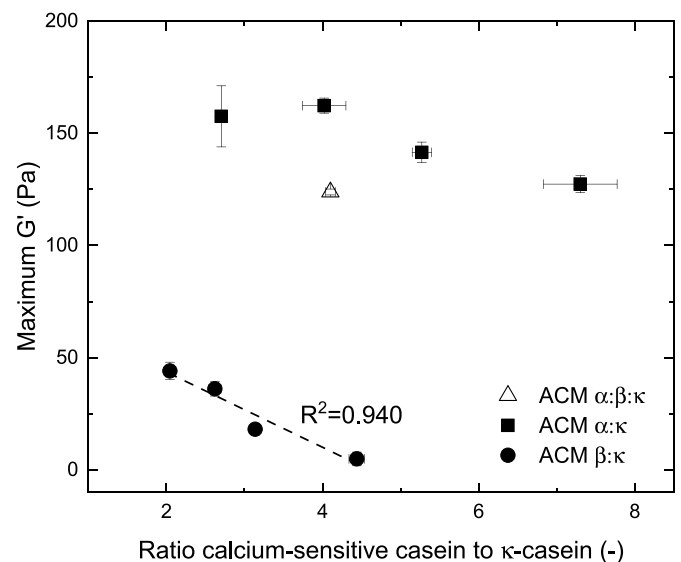


Fig. 5. The maximum recorded storage modulus (G'), as a measure of curd firmness, of ACM α : β : κ , ACM α : κ , and ACM β : κ with different molar ratios of micellar calcium-sensitive caseins to micellar κ -casein during 1 h of incubation with chymosin.

attained by ACM α : κ did not increase upon decreasing the ratio of calcium-sensitive casein to κ -casein from 4.0 ± 0.3 to $2.7 \pm <0.1$, but rather levelled off or even decreased (Fig. 5). By contrast, the gel firmness of ACM β : κ would probably continuously increase at decreasing ratios. In that case both the β -casein and κ -casein are unable to form cross-links and contribute to the firmness equally, leaving only the influence of the increasing surface area generated by the micelles.

Lastly, the storage modulus of the curd formed by ACM β : κ 63:31 was observed to decrease after reaching its maximum during incubation with chymosin (Fig. 4). This can point towards rearrangement of the gel network facilitated by the lack of cross-links in these micelles, which may ultimately lead to contraction of the gel network and expulsion of water (syneresis; Walstra, 1993). Both may result in a decrease of the measured storage modulus during renneting. ACM with increased proportions of β -casein have previously been shown to exhibit increased syneresis upon renneting (Pearse et al., 1986). Similarly, goat milk, which contains a larger proportion of β -casein than bovine milk at a similar casein content (Breunig et al., 2024), exhibits greater syneresis than bovine milk (Storry, Grandison, Millard, Owen, & Ford, 1983).

4. Conclusions

Artificial casein micelles can be formed from different casein fractions. Their properties and rennet coagulation behaviour vary greatly depending on the casein fractions used. α_s -Caseins can cross-link calcium phosphate nanoclusters through their multiple phosphate centres and be cross-linked by them. Consequently, artificial casein micelles predominantly composed of α_s -caseins and κ -casein are dense and compact and form firm gels upon renneting. By contrast, β -casein, with its single phosphate centre, cannot cross-link nanoclusters but can only be cross-linked by them. Therefore, it forms loose and highly hydrated casein micelles in combination with κ -casein, resulting in weak gels upon renneting.

In compositions with considerable proportions of both α_s -caseins and β -casein, free β -casein reduces the percolation of a continuous internal casein network, giving rise to water channels in the micelle interior. This results in larger, more hydrated casein micelles that form softer curds. Varying the relative proportions of the caseins in the composition directly impacts the properties and coagulation of the micelles. An increasing molar ratio of micellar calcium-sensitive casein to micellar κ -casein (i.e. decreasing proportions of κ -casein) yields larger micelles and decreases the overall hydration of the micellar phase, resulting in weaker curds upon renneting.

Thus, α_s -caseins are required to confer strength to casein micelles and the gel networks they form upon coagulation. β -Casein moderates their strength and enhances casein micelle hydration. κ -Casein stabilises the micelles and offers an additional means to control micelle size and thereby their coagulation behaviour. These findings highlight the possibility of designing artificial casein micelles from specific casein fractions and customising their properties and functionality by varying the relative proportions of the fractions.

CRedit authorship contribution statement

Laurens J. Antuma: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Remko M. Boom:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Julia K. Keppler:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this research. The authors are cited as inventors on the published patent application WO 2024/134 610 and provisional patent application AU2024901933 which are both based on part of the results presented in the present publication, but the application and all intellectual property rights to this work are held by Formo Bio GmbH (Berlin, Germany). The latter was part of the consortium that received funding from the European Union's Horizon 2020 research and innovation programme in which Wageningen University & Research participated. None of the authors have any pecuniary interest in the intellectual property rights nor do they benefit financially from this arrangement or the commercial exploitation of this intellectual property.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodhyd.2025.111136>.

Data availability

Data will be made available on request.

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