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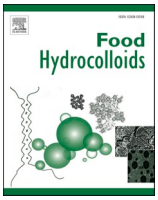

Effect of temperature, pH and calcium phosphate concentration on the properties of reassembled casein micelles

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ABSTRACT

Reassembled casein micelles (RCMs) can be made by reassembling sodium caseinate with calcium and phosphate as well as other ions under controlled conditions into casein micelle-like structures. During the reassembly, the changes in processing parameters lead to differences in the properties of RCMs such as size, composition and structure. Understanding the effect of processing parameters on RCM properties is essential for their potential application in food products. However, the effect of process parameters has not been studied systematically. Therefore, the objective of this study was to evaluate the effect of temperature, pH and calcium phosphate concentration on the properties of reassembled casein micelles. The effect of pH on the size and structure of RCMs both during and after their formation was studied by dynamic light scattering and small-angle X-ray scattering. We found that pH affects both the size and internal structure of RCMs. We could also modulate the size and composition of RCMs by changing the temperature and calcium phosphate concentration. The insights of this study not only can be used to modulate the composition and structure of RCMs, but also help us to understand how processing parameters will influence the assembly of RCMs from novel sources, such as recombinant caseins.

1. Introduction

Caseins are the most abundant dairy proteins in bovine milk. Around 80% of bovine proteins are caseins, which play an essential role in dairy products from both a nutritional and functional perspective. Caseins in cow's milk are assembled into casein micelles in the mammary gland. These casein micelles are unique structures in animal milk, consisting of thousands of casein molecules with calcium and phosphate salts. The structure of casein micelles is essential for many dairy products such as cheese and yoghurt because they play a critical role in the gelation of milk. Meanwhile, society is looking for ways to replace animal-based dairy proteins with animal-free alternatives. A possible transition path is to use recombinant, animal-free milk proteins that are produced from microorganisms (Hettinga & Bijl, 2022). Several studies have shown the possibility to use bacteria or yeasts to produce recombinant κ-, β-, and α_s-caseins (Goda et al., 2000; Hansson et al., 1993; Kang & Richardson, 1988; Kim et al., 1997, 1999). However, these recombinant caseins do not have the same supramolecular structure as caseins in bovine milk, as no assembly process is taking place during or after recombinant expression and secretion. Although there is no assembly method available for the production of casein micelles from recombinant caseins, a method to assemble purified bovine caseins into casein micelles was introduced by Schmidt et al. (1977, pp. 328–341). The casein micelles were assembled by mixing purified caseins with calcium and phosphate salts at pH 6.7 and 37 °C. These types of casein micelles were called artificial casein micelles (ACMs) by Schmidt. In this study, the ACMs are referred to as reassembled casein micelles (RCMs) considering this process involves the reassembly of caseins into a casein micelle structure. Several researchers used this method to create casein micelles in their studies. In 1979, Knoop studied RCMs by electron microscopy (Knoop et al., 1979) and found that RCMs resembled bovine casein micelles. RCMs were also used to study the role of milk salts in casein

Abbreviations: ACMs, artificial casein micelles; DLS, dynamic light scattering; pH_{AA}, reassembled casein micelles adjusted to different pHs after assembly; pH_{DA}, reassembled casein micelles adjusted to different pHs during assembly; RCMs, reassembled casein micelles; SAXS, small-angle X-ray scattering; SMUF, simulated milk ultrafiltrate.

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micelles (Aoki et al., 1987; Zhang et al., 1996) and used as nano-vehicles for encapsulation of fatty acid and vitamins (Loewen et al., 2018; Zimet et al., 2011). The RCMs were reassembled under a variety of processing conditions. For example, in these four studies, the calcium phosphate concentration ranged from 10 mM to 40 mM and temperatures of 4 °C, 25 °C, 37 °C and 73 °C were applied. It is, however, not yet known whether these processing parameters have an impact on the composition and structure of RCMs. Furthermore, most studies were performed at pH 6.7, whereas the pH used to produce dairy-derived products varies across a range of values (approximately 4–8). It is therefore also of interest to study the effect of pH on the properties of RCMs. Having a thorough understanding of the assembly process would help us to modulate the behavior of RCM by changing processing parameters. An in-depth understanding of the assembly process of purified bovine caseins will also help to delineate the processing parameters that likely impact the production of casein micelle-like structure from recombinant caseins, which is an essential step to prepare animal-free dairy products.

The effect of temperature, pH and calcium phosphate concentration on RCM composition and structure has not yet been studied, but their impact on bovine casein micelles was investigated and will be discussed in the following paragraph. Temperature affects both hydrophobic interactions and the solubility of calcium phosphate, both of which are essential to retain the structure of the casein micelles. The effect of heating on casein micelles is often studied using milk as the starting material, which includes whey proteins in addition to caseins. Fewer studies focused on systems without whey proteins. Whey proteins can denature at higher temperatures and interact with casein micelles thereby influencing the properties of casein micelles. Since whey proteins are (virtually) absent in the purified bovine casein fractions that were used to prepare RCMs, we will discuss studies performed at relatively low temperatures (less than 50 °C) here, at which whey proteins do not denature. It was reported that the hydrodynamic diameter (d_H) of casein micelles increased with increasing temperature from 20 °C to 50 °C, when diluted in simulated milk ultrafiltrate (SMUF) or water at the same dilution ratio (Beliciu & Moraru, 2009). Another research also reported a similar trend in d_H in the temperature range of 20 °C–40 °C (Liu et al., 2013). However, the effect of temperature on casein micelles in a whey protein free system in the range of 50 °C–100 °C is lacking. Besides affecting casein micelle size, temperature also influences the solubility of calcium phosphate. As milk is supersaturated with calcium phosphate, changes in temperature lead to solubilization or precipitation of calcium phosphate. The effect of heating has been studied for both milk ultrafiltrate in the absence or presence of caseins. Fine precipitation has been observed in milk ultrafiltrate after it was heated above 30 °C (Brule & Sol, 1978). Unlike milk ultrafiltrate, milk caseins prevented the precipitation of calcium phosphate because phosphorylated caseins can stabilize calcium phosphate (Holt & Sawyer, 1988). Researchers found no significant change in serum calcium and phosphate concentration when milk was heated and then cooled to room temperature (Poulliot et al., 1989; Wang & Ma, 2020).

Prior studies also have noted the importance of pH on casein micelle size, charge, and composition. It was reported that the d_H of casein micelles increased from pH 5.5 to 7.5 (Sinaga et al., 2017). A similar trend has been reported by Foroutanparsa et al. (2021) by measuring the size of casein micelles with super-resolution microscopy. The change in casein micelle size was explained by the increase of electrostatic force between caseins with increasing pH (Sinaga et al., 2017). Next to the change in casein micelle size, pH affects the casein and calcium phosphate distribution between milk serum and casein micelle phase. It was found that during the acidification of milk, calcium, phosphate as well as caseins were released from the casein micelle (Dalgleish & Law, 1988, 1989). When milk is at alkaline pH, caseins are more negatively charged and calcium phosphate is less soluble, which could cause both precipitation of calcium phosphate and the increase in the repulsion between caseins, disrupting casein micelles. As pH has impact on both calcium phosphate solubility and casein charges, consequently, it could also impact the internal structure of casein micelles. Small-angle X-ray scattering (SAXS) is a suitable technique to investigate the internal structure of casein micelles. Several studies described that caseins are rather homogeneously distributed in casein micelles with inhomogeneities at a length scale of 2 nm (Ingham et al., 2015, 2016; Kruif, 2014; Kruif et al., 2012). These studies focused on the structure of casein micelles at their native pH (6.7), and currently, no study has focused on the effect of pH on the internal structure of casein micelles. Furthermore, in studies performed on milk, pH could only be adjusted after the casein micelles had formed and had been secreted into the milk. When making RCMs, pH can be adjusted both during and after the reassembly process, allowing us to study the effect of pH during the formation of casein micelles, which is not possible in mammalian casein synthesis.

The objective of this study was to investigate how temperature, pH and calcium phosphate concentration affect the properties of RCMs. We performed SAXS measurement on the absolute scale to evaluate the effect of pH on the structure of casein micelles both during and after the assembly process. In addition, we also investigated how temperature, pH, and calcium phosphate concentration affect the properties of RCMs. RCMs were heated from 37 °C to 83 °C. The pH of RCMs was adjusted to 5.5 to 8.5, and 60%–120% of calcium phosphate concentrations were used. The size as well as the ions and casein distribution between micelles and serum was measured. This study can lead to better understanding on the behavior of bovine casein micelles. Furthermore, the obtained knowledge can also be further transferred to the assembly process using recombinant caseins.

2. Materials and methods

The effect of temperature, calcium phosphate concentration, and pH on the properties of RCMs was studied. RCMs made at 25 °C, pH 6.7, and with 30 mM Ca^{2+} and 20 mM PO_4^{3-} were compared with bovine casein micelles. Heat treatments from 37 to 83 °C were applied to RCMs. A storage test of RCMs was performed at room temperature and 4 °C to test the stability. The pH was adjusted from 5.5 to 8.5 both during and after reassembly. This pH range was selected because casein micelles were expected to be stable and not form a gel. Finally, different calcium phosphate concentrations (60–120% of the 30 mM Ca^{2+} and 20 mM PO_4^{3-}) were applied to make RCMs. All samples were prepared in duplicate.

2.1. Making reassembled casein micelles (RCMs)

The method of making RCMs was modified from the method of Schmidt et al. (1977, pp. 328–341), with two minor modifications. The first modification in our study was the use of $K_3C_6H_5O_7{\cdot}H_2O$ instead of citric acid. Second, instead of adding citrate and caseinate solution with pumps, they were mixed in a beaker with water before the addition of salt with pumps. A short summary of both stock solution preparation and RCM assembly is provided here. First, 6.3 g of sodium caseinate was added to approximately 75 ml of MilliQ water and stirred at 4 °C overnight to dissolve the power. The caseinate solution was then adjusted with MilliQ water to a final volume of 100 ml in a volumetric flask. Three types of salt solutions were made. First, 6.31 g of $CaCl_2{\cdot}2H_2O$ and 1.18 g of $MgCl_2{\cdot}6\ H_2O$ were weighed in a beaker and mixed with about 75 ml of MilliQ water. The final volume of the calcium and magnesium mixture was adjusted to 100 ml by using a volumetric flask. Second, a solution with 2.39 g KH_2PO_4, and 3.13 g $Na_2HPO_4{\cdot}2H_2O$ was prepared following the same procedure of calcium and magnesium solution. Last, a solution with 4.82 g of $K_3C_6H_5O_7{\cdot}H_2O$ was prepared as the previous two solutions. All chemicals were purchased from Sigma-Aldrich (Germany) with a purity larger than 99 %.

To make RCMs, first, 11 ml of MilliQ water, 2 ml of $K_3C_6H_5O_7{\cdot}H_2O$ and 12 ml of sodium caseinate solution were mixed in a 50 ml beaker, resulting in approximately 2.3 % of the final casein concentration in

RCM solution. Further increasing the casein concentration was difficult as the caseinate stock solution had already reached its maximum solubility in water at 4 °C. The pH of this solution was adjusted to 6.7 by slowly adding 1 M HCl with intense stirring. After that, 2 ml of $CaCl_2{\cdot}2H_2O$ and $MgCl_2{\cdot}6\ H_2O$ solution, as well as 2 ml of KH_2PO_4, and $Na_2HPO_4{\cdot}2H_2O$ solution were slowly added to that 50 ml beaker using syringe pumps (New Era, USA) with intense stirring to create a vortex. The speed of the syringe pump was set to 2 mL/h. During the addition of salt solutions, the pH was dropping constantly. Therefore, the pH of the RCM solution was maintained at 6.7 by slowly adding 0.5 M NaOH during the reassembly process. After finishing the salt addition, the RCM solution was stirred for 30 min, during which the pH was continuously adjusted to 6.7 by adding 0.5 M NaOH.

For testing the effect of temperature, RCMs were first made at room temperature RCMs which was about 22 °C. However, because the magnetic stirrer also slightly heated the sample, the actual sample temperature was 25 ± 1 °C at the end. Then RCMs were heated to 37, 52, 67, and 83 °C for 1h. After cooling to room temperature, RCM solutions were ultracentrifuged after which the serum and pellet were separated. All heated samples were cooled down to room temperature before measurement. Two procedures were used to study the effect of pH on RCMs. First, RCMs were assembled at a certain pH within a pH range spanning from 5.5 to 8.5. Second, RCMs were first made at pH 6.7, after which the pH was adjusted to the same final pH values (i.e., ranging from 5.5 to 8.5) and subsequently stored for 1.5h. RCMs made at different pH values are defined as pH_{DA} (pH during assembly) samples and RCMs adjusted to a different pH after assembly are defined as pH_{AA} (pH after assembly) samples. Last, different calcium phosphate concentrations were applied to make RCMs, ranging from 60% to 120% of the original calcium and phosphate concentration (30 mM Ca^{2+}, 20 mM PO_4^{3-}).

2.2. Small-angle X-ray scattering

Small-angle X-ray scattering (SAXS) was performed on our in-house instrument called SAXSLAB GANESHA 300XL equipped with a GeniX 3D Ultra Low Divergence microfocus sealed tube source with $\lambda = 1.54$ Å X-ray wavelength and $1*10^8$ ph/2 flux. The scattered X-rays were collected with a Pilatus 300K silicon pixel detector with 487*619 pixels of 172 μm*172 μm in size. The investigate q range was 0.07–7 nm^{-1}. Absolute intensities were obtained by the scattering of Milli-Q water as a reference.

The intensity of the scattered X-ray is given as a function of the modulus of the scattering vector

$$q = \frac{4\pi}{\lambda} sin\ \theta \tag{1}$$

where λ is the wavelength of the incident radiation and 2θ is the scattering angle. For samples containing particles, the total measured intensity can be written as the contribution of the normalized form factor $P(q)$ and the structure factor $S(q)$. The form factor is determined by the size and the shape of the particles, and the structure factor $S(q)$ arises from interference between the X-rays scattered by different particles in the sample. The scattered intensity is expressed as

$$I(q) = N/V\Delta\rho^2V^2P(q)S(q) \tag{2}$$

where N/V is the number density of particles.

For the data modelling the recently published model of Pedersen et al. (2022) was used, describing the small-angle X-ray scattering of casein micelles on the absolute intensity scale given in cm^{-1}. The fitting procedure was performed in our own-written Python script using 'lmfit' module. Since the fit was performed on the absolute scale, for the proper normalization we used the concentration of calcium phosphate clusters (see supplementary material). The mass density of calcium phosphate was taken to be 2.31 g/cm^3 (Lie-Piang et al., 2021). For the calculation of the protein contribution, the concentration of our samples (0.027 g/mL) and the specific volume of proteins (0.71 cm^3/g) was used (Pedersen et al., 2022).

In contrast to the cited paper, we used Gaussian distribution to describe the polydispersity of the casein micelle and the intermediate fluctuations and the second term of the partial structure factor of calcium phosphate clusters was not multiplied by the empirical factor of 4. The fitting parameters were total radius of the micelle (R_{total}), the polydispersity of the micelle (σ_{total}), the radius, the polydispersity and the number concentration of the intermediate fluctuation ($R_{intermediate}$, $\sigma_{intermediate}$, $n_{intermediate}$). For the description of protein heterogeneities, a multi-arm polymer expression was used taking into account the cross section of the arms. The protein contribution was optimized by the length, the cross section and the number of arms (L, $R_{cross\ section}$, n_a). Furthermore, the molar mass of the protein heterogeneities was also fitted ($M_{protein}$). To describe the contribution of the calcium phosphate clusters, an ellipsoid of revolution term was used with the equatorial radius and ellipticity. To avoid numerical instability, these parameters were fixed with the same value as in the reference ($R_{small} = 2.5$ nm, $\varepsilon = 0.4$). Further fitted parameters were the number of substructures (n_{sub}) and the volume fraction of, and the interaction radius of, protein heterogenties (η_{PP}, $R_{HS}(PP)$), the cross term volume fraction of and the interaction radius of protein heterogeneities – calcium phosphate contribution (η_{CP}, $R_{HS}(CP)$). The interaction of calcium phosphate clusters with each other was neglected. From the optimized parameters we calculated the number of calcium phosphate clusters and protein heterogenities per micelle (n_{CCP}/micelle and n_P/micelle). For the detailed description of the model see the cited paper.

2.3. Dynamic light scattering (DLS)

The apparent hydrodynamic diameter (d_H), of RCMs was determined by dynamic light scattering (DLS) performed on a Zetasizer Ultra (Malvern, UK) in backscattering geometry at a fixed scattering angle of 175°. Samples were kept in DTS0012 disposable plastic cells held at a fixed temperature of 25 °C. RCMs solutions were diluted 20-fold with MilliQ water immediately before the measurement to avoid multiple scattering. All samples were measured in triplicate at 25 °C. The sample viscosity was set to 10^{-3} Pa s. The general-purpose mode was used to calculate the apparent hydrodynamic diameters.

2.4. Ultracentrifugation

Ultracentrifugation was performed for all RCM samples to separate micelles and serum by using a Beckman Coulter ultracentrifuge (Optima XE, USA). Fifteen milliliters of RCM solution were transferred into a polycarbonate centrifuge tube (Beckman Coulter, USA), and centrifuged at 100,000g for 60 min at 20 °C. For the sample stored at 4 °C, the ultracentrifugation was conducted at 4 °C. The supernatant (serum) was collected and stored at −20 °C for further analysis.

2.5. Ions and casein distribution analysis

The cations of RCM solution and RCM serum were measured by an Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES). Before the measurement, samples were chemically digested to remove organic matter. For this, the microwave-assisted wet digestion method (ISO, 2010) was used with modification, as extra HCl was added to improve the digestion efficiency. First, 0.5 ml sample was mixed with 1.5 ml 65% HNO_3 (Sigma-Aldrich), 7.5 ml 37% HCl (Sigma-Aldrich) and 1 ml 30% H_2O_2 (Sigma-Aldrich) in a Polytetrafluoroethylene (PTFE) tube (Milestone, Italy). Then the samples were digested with a Milestone ethos easy (Milestone, Italy) advanced microwave digestion system. The digested sample was transferred to a 100 ml volumetric flask and the final volume was reached with MilliQ water. The concentrations of calcium (Ca^{2+}), magnesium (Mg^{2+}), phosphorus (P^{5+}), potassium (K^+),

Table 1
Comparison between the average composition of the reassembled casein micelles solution and skim milk. Data from Kruif and Huppertz (2012) for the size measurement and Bijl et al. (2013) for the ion concentrations. The concentrations of Ca^{2+}, Mg^{2+}, P^{5+}, and $PO4^{3-}$ from Bijl et al. (2013) and Schmidt (1977, pp. 328–341) were converted to mM by assuming the density of the solution was 1 g/ml. The total casein concentration from Schmidt (1977, pp. 328–341) was converted by multiplying the total nitrogen concentration with a conversion factor of 6.38.

Parameters	Mean of skim milk	Mean of RCM solution	Mean of RCMs made with whole casein
	Kruif and Huppertz (2012) Bijl et al. (2013)		Schmidt (1977)
Average size (nm)	154 to 230	150.60 ± 5.23	–
Total casein concentration (%)	2.88 ± 0.07 [a]	2.23 ± 0.23 [b]	2.50
Serum casein concentration (%)	–	0.21 ± 0.04	0.21
Total Ca^{2+} (mM)	33.37 ± 0.34 [a]	30.57 ± 0.91 [a]	31.19
Serum Ca^{2+} (mM)	9.17 ± 0.64 [a]	9.13 ± 0.25 [a]	10.72
Total Mg^{2+} (mM)	4.92 ± 0.06 [a]	3.99 ± 0.03 [b]	4.27
Serum Mg^{2+} (mM)	3.19 ± 0.18 [a]	2.84 ± 0.01 [a]	2.88
Total P^{5+} (mM)	32.70 ± 0.06 [a]	31.13 ± 0.60 [a]	–
Serum P^{5+} (mM)	13.74 ± 1.03 [a]	14.14 ± 0.82 [a]	–
Total PO_4^{3-} (mM)	21.47 ± 0.37 [a]	21.09 ± 0.17 [a]	26.80
Serum PO_4^{3-} (mM)	10.10 ± 0.62 [a]	11.84 ± 0.46 [a]	16.46
Total Cl^- (mM)	25.55 ± 1.07 [a]	64.51 ± 1.08 [b]	–
Serum Cl^- (mM)	–	64.75 ± 2.84	–
Total Na^+ (mM)	17.95 ± 0.55 [a]	55.33 ± 0.15 [b]	64.81
Serum Na^+ (mM)	–	55.33 ± 0.11	–
Total K^+ (mM)	42.05 ± 0.61 [a]	41.95 ± 0.54 [a]	46.03
Serum K^+ (mM)	–	40.41 ± 1.24	–
Total citrate (mM)	9.15 ± 0.42 [a]	10.06 ± 0.10 [a]	–
Serum citrate (mM)	8.33 ± 0.41 [a]	9.15 ± 0.41 [a]	–

Table 2
Parameters of reassembled casein micelles that were stored at 4 °C and room temperature for 24 h, numbers with the same character indicate no significant change after 24 h of storage.

Parameters	4 °C		Room temperature	
	T0	T24	T0	T24
Average size (nm)	156.50 ± 3.50 [a]	155.83 ± 1.07 [a]	153.33 ± 1.49 [a]	157.83 ± 3.13 [a]
Serum casein (%)	0.16 ± 0.03 [a]	0.26 ± 0 [b]	0.19 ± 0 [a]	0.19 ± 0 [a]
Serum β-casein (mAU*min)	38.27 ± 1.26 [a]	64.68 ± 0.52 [b]	50.82 ± 0.90 [a]	52.53 ± 0.48 [a]
Serum Ca^{2+} (mM)	8.95 ± 0.15 [a]	9.34 ± 0.03 [b]	8.91 ± 0.12 [a]	8.58 ± 0.03 [a]
Serum P^{5+} (mM)	13.33 ± 0.16 [a]	13.70 ± 0.20 [a]	12.89 ± 0.15 [a]	12.71 ± 0.16 [a]
Serum PO_4^{3-} (mM)	14.25 ± 0.65 [a]	14.06 ± 0.19 [a]	13.33 ± 0.11 [a]	13.56 ± 0.56 [a]

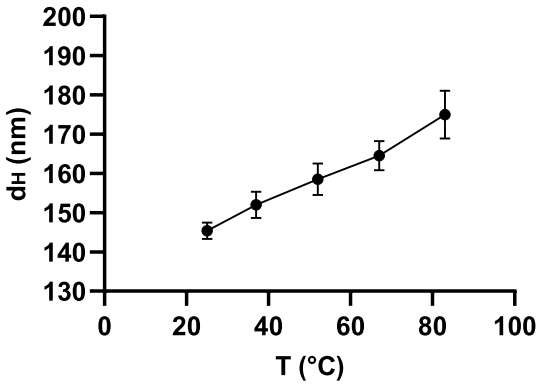

Fig. 1. Hydrodynamic diameter of reassembled casein micelles heated at different temperatures. The lines between data points are drawn to guide the eye.

and sodium (Na^+) were then measured with an Avio 500 ICP-OES system (PerkinElmer, USA). The anions, including citrate' ($C_6H_5O_7^{3-}$), phosphate (PO_4^{3-}) and chloride (Cl^-), were measured by anion-exchange chromatography (IonPac AS19 column, 4 × 250 mm, Dionex; Thermo Scientific, Sunnyvale). The elution steps followed the method from Gaucheron et al. (Gaucheron et al., 1996). RCM samples were diluted 500-fold with MilliQ water and then shaken overnight. All diluted samples were filtrated with a 0.2 μm syringe filter. The casein content was measured with the DUMAS method using an ISO (2002) standard. The concentration of ions and caseins in the micelle phase was determined by subtracting the content in the serum phase from the total content in the RCM solution.

2.6. RP-HPLC

The composition of four types of caseins (β-casein, κ-casein, α_{s1}-casein and α_{s2}-casein) were measured with Reversed-phase High-performance Liquid Chromatography (RP-HPLC) with a WIDEPORE XB-C18 column in an UltiMate 3000 HPLC system (Thermo Scientific, USA) (Bonfatti et al., 2008). The gradient elution was done by mixing two eluents, which are eluent A containing 0.1% Trifluoroacetic acid (Sigma-Aldrich) in MilliQ water and eluent B containing 0.1% Trifluoroacetic acid (Sigma-Aldrich) in acetonitrile. Two buffers have been prepared for sample pre-treatment. Buffer A contained 0.1 M Bis-Tris, 8 M urea, 5.37 mM sodium citrate and 19.5 mM DTT and was adjusted pH to 7 with 6 M HCl. Buffer B contained 6 M Urea, 0.1% Trifluoroacetic acid at pH 2. The RCM solutions were first diluted with buffer A with a ratio sample to buffer equal to 1:3 (v:v), samples were then vortexed for 10 s and incubated at room temperature for 1 h. After incubation, these solutions were diluted with buffer B at the same ratio of 1:3. The final solutions were filtrated with 0.2 μm filters before analysis. The serum samples followed the same protocol as RCM samples except that the ratio was 1:1 with both buffer A and buffer B.

2.7. Micelle stability test

RCMs (25 °C, pH 6.7, 30 mM Ca^{2+} and 20 mM PO_4^{3-}) were made, after which 0.5 % of sodium azide was added to prevent microbial growth. They were stored at room temperature or at 4 °C for 24h. The size, serum Ca^{2+} and PO_4^{3-}, and serum caseins of RCMs after different storage times were measured. These samples have also been frozen at −20 °C and then thawed to evaluate their stability.

2.8. Data processing

The statistical analyses were performed in IBM SPSS statistics (version 28.0.1.1), using one-way ANOVA analysis. The data with the

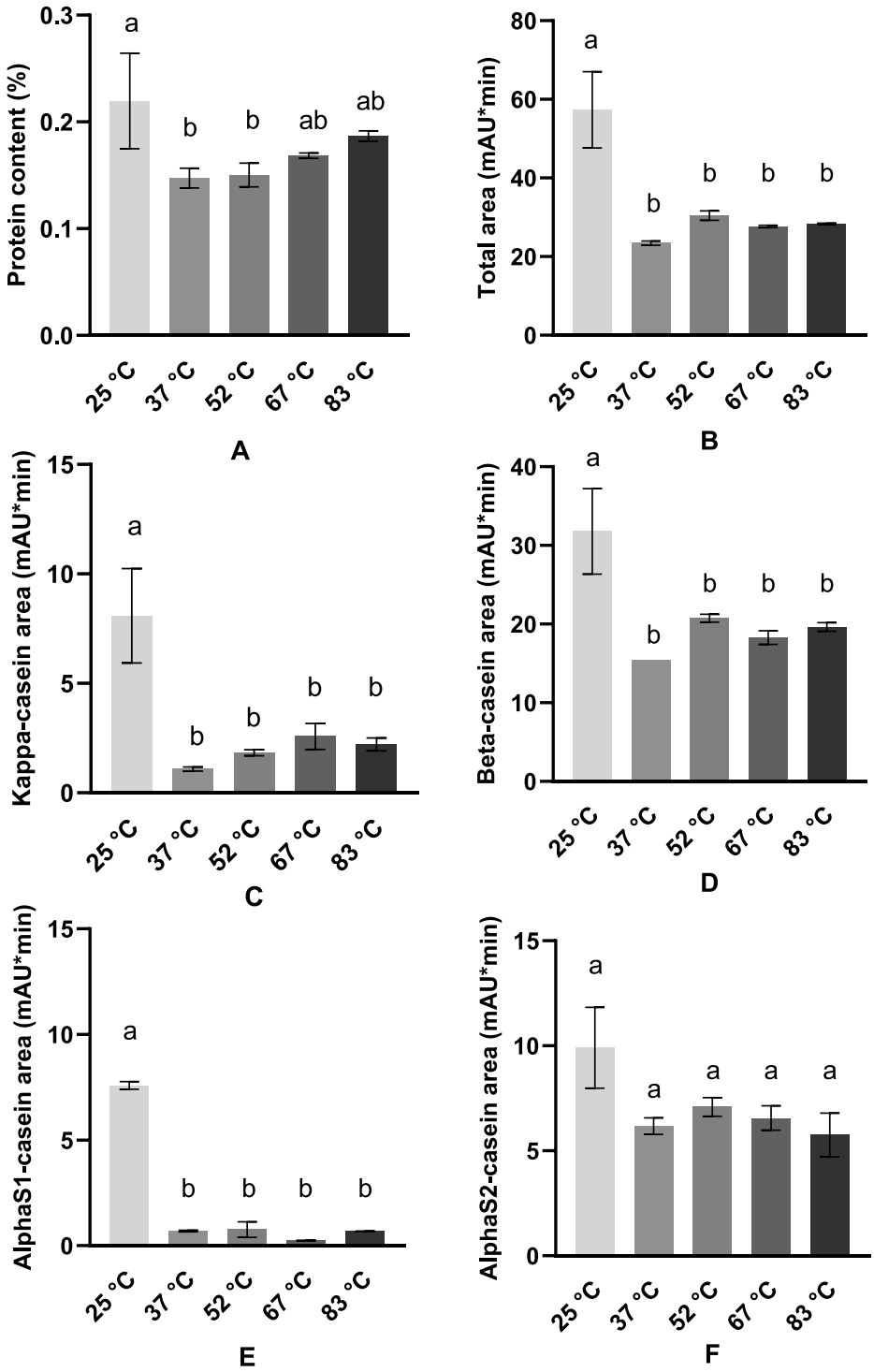

Fig. 2. Casein content in the serum of reassembled casein micelles that heated at different temperatures. A: total protein content measured with DUMAS. B: total area of four types of caseins, measured with HPLC. C: area of k-casein. D: area of β-casein. E: area of a_{S1}-casein. F: area of a_{S2}-casein. The data with the same character indicates no significant difference between them ($p > 0.05$).

same character indicates no significant difference ($p > 0.05$)

3. Result and discussion

3.1. RCM solution vs bovine milk: ions distribution, size distribution and stability

To investigate the similarities and differences between RCMs and bovine casein micelles, the size, calcium, phosphate, as well as other ion concentrations in serum and micellar phase of RCMs were measured. RCMs were assembled at 25 °C at pH 6.7 and had no adjustment on calcium phosphate concentration. The obtained data were compared with the previous work on bovine milk from Kruif and Huppertz (2012), and Bijl et al. (2013). We also compared our results with the RCMs made by Schmidt et al. (1977, pp. 328–341) to check if similar RCMs were obtained.

As shown in Table 1, the average d_H of the RCMs was approximately 150 nm, the composition of RCMs that we made was similar to that

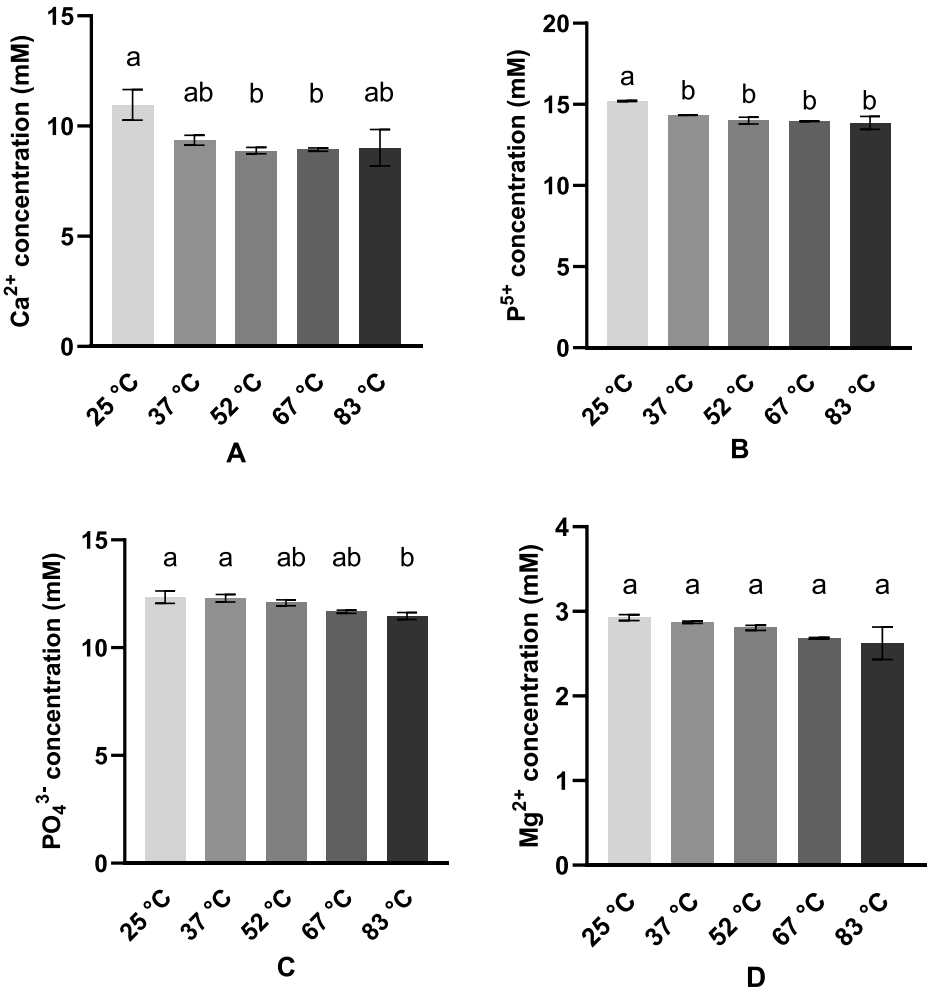

Fig. 3. Ion concentrations in the serum of reassembled casein micelles that heated at different temperatures. A: serum concentration of calcium. B: serum concentration of phosphorus. C: serum concentration of phosphate. D: serum concentration of magnesium. The data with the same character indicates no significant difference between them ($p > 0.05$).

reported by Schmidt et al. (1977, pp. 328–341). The total concentration and distribution of Ca^{2+}, P^{5+}, Mg^{2+}, and PO_4^{3-} were similar between RCM solution and bovine milk. They both had in total around 30 mM of Ca^{2+} and P^{5+} and 20 mM of PO_4^{3-}. In terms of distribution, about two-thirds of Ca^{2+}, half of PO_4^{3-}, and half of P^{5+} were present in the micellar phase. The similarity in the distribution of those ions indicates that RCMs have a similar capacity to stabilize calcium phosphate. Monovalent ions such as K^+, Na^+, and Cl^- are hardly interacting with casein micelles and almost all of them were present in serum (Table 1). Compared with bovine milk, Na^+ and Cl^- content in RCM solution was significantly higher. The higher Na^+ content is caused by three factors: first, the protein source sodium caseinate contains sodium; second, the Na_2HPO4 was added when making RCMs; last, NaOH was added to maintain the pH at 6.7 during the assembly of RCMs. This Na^+ concentration (55 mM) can be calculated back by adding Na^+ from sodium caseinate (1.2 % Na^+ in sodium caseinate, contributed 12 mM Na^+ to final RCM solution), 0.5 M NaOH solution (contributed 19 mM Na^+), and Na_2HPO_4 (contributed 24 mM Na^+). The high Cl^- content could be explained by the fact that $CaCl_2$ (contributed 50 mM Cl^-) and $MgCl_2$ (contributed 8 mM Cl^-) were used in RCM assembly, thereby adding an additional amount of Cl^- to the RCM solution. The additional NaCl was reported to solubilize the calcium phosphate in casein micelles due to the exchange between Na^+ and Ca^{2+} (Zhao & Corredig, 2015). However, the effect was only significant when the NaCl was higher than 100 mM. In our study, the Na^+ concentration is approximately 37 mM higher compared to the average Na^+ content of bovine milk, therefore, the slight increase in Na^+ content should not have a significant influence on the distribution of the Ca^{2+}, P^{5+} and PO_4^{3-}. Overall, the RCMs (25 °C, pH 6.7, 30 mM Ca^{2+} and 20 mM PO_4^{3-}) have similar size and ion distribution compared with bovine casein micelles.

3.2. Stability of RCMs

RCMs were stored at 4 °C and room temperature for 24 h, and they were frozen at −20 °C and thawed at 4 °C to evaluate their stability. The main parameters of RCMs have been displayed in Table 2. The average size of RCMs had no significant difference between 4 °C and room temperature, and their size were similar after being stored for 24 h. When stored at 4 °C, serum β-casein concentration increased by almost 69 %, which is similar to regular bovine milk (Downey & Murphy, 1970; Pierre & Brule, 1981, pp. 417–428). These studies also reported that β-casein was released from bovine casein micelles when stored at 4 °C or 5 °C because of weaker hydrophobic interactions at a lower temperature. The RCMs stored at room temperature had no significant change in serum β-casein concentration and the serum Ca^{2+} and PO_4^{3+} concentration was stable after storage. From those results, we can conclude that the RCMs were stable in size, casein and ion distribution at room temperature. We can suggest that if RCMs solution cannot be analyzed on the same day they were made, it will be still safe to analyze them after 24 h, at room temperature. In contrast, the RCM solution formed a protein network when stored at −20 °C and then thawed at 4 °C. Gelation was not observed upon thawing after −20 °C storage of a

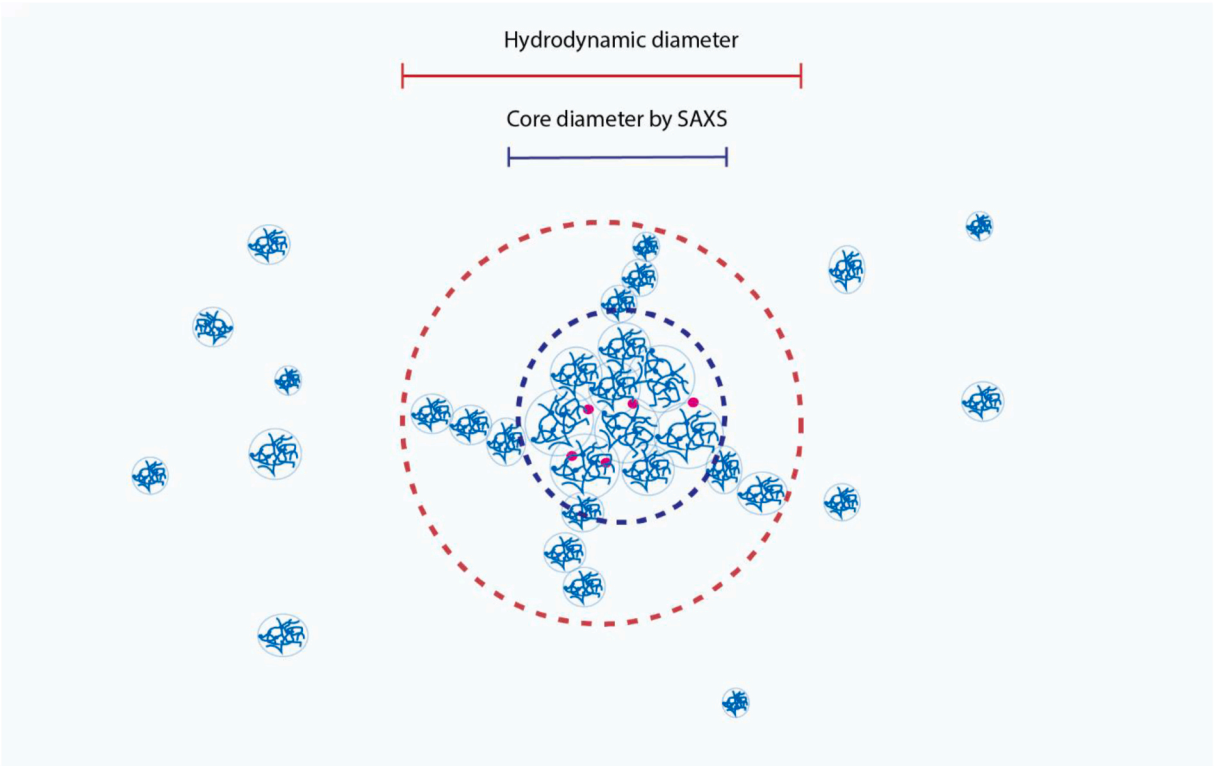

Fig. 4. A schematic representation of reassembled casein micelles and the various structural features experimentally probed by DLS and SAXS. The hydrodynamic diameter, d_H (dashed red circle), is determined by DLS. SAXS offers insight into the core size, d_{total} (dashed blue circle), and the number of protein heterogeneities (encircled in grey) and calcium phosphate nanoclusters (red dots) within the core of the RCMs. The d_H is much larger than d_{total} for those RCMs in which a portion of caseins are loosely attached. The grey circle with blue lines around casein micelles are serum caseins.

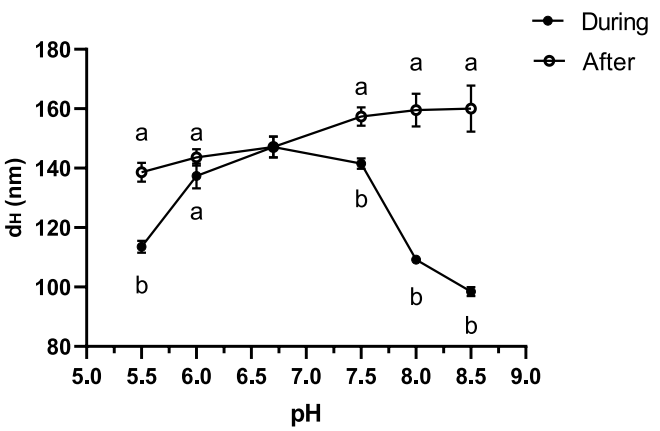

Fig. 5. The hydrodynamic diameter of reassembled casein micelles as a function of pH. During: pH adjusted during the assembly. After: pH adjusted after finishing assembly. The lines between data points are drawn to guide the eye. The data with the same character indicates no significant difference between RCMs with the same pH that was adjusted during or after the assembly ($p > 0.05$).

bovine milk solution. This means that the RCMs are less stable than bovine casein micelles in terms of freezing and thawing. This difference could be related to a different organization of individual caseins within the casein micelle.

3.3. Effect of temperature

One factor that could affect properties of RCMs is temperature. To study this, RCMs were heated at different temperatures. The heating could influence the solubility of calcium phosphate and affect the hydrophobic interactions, thereby influencing the properties of RCMs. As displayed in Fig. 1, the hydrodynamic diameter of RCMs was strongly correlated with the temperature. The d_H of RCMs increases with increasing temperature. The increase in d_H could be either due to more serum caseins joining the micellar phase or micelles aggregated. Those two mechanisms would result in different serum casein concentrations. The serum casein concentrations were therefore measured and casein compositions were analyzed with HPLC (Fig. 2). The total serum casein concentration as well as individual serum casein concentrations were significantly higher at 25 °C. This finding showed that from 25 °C to 37 °C, caseins from serum move to the micellar phase, which may contribute to the increase in the size of individual RCMs. However, with further increase in temperature, although the size of RCMs was increasing, no further transfer of serum caseins to RCMs was noted. A possible explanation for the growth of RCMs at temperatures higher than 37 °C is that smaller micelles complex into casein micelle aggregates. Heating accelerates the diffusion of small micelles and increases their meeting frequency. Also, smaller micelles have a larger total surface area and total κ-casein may not have been sufficient to cover all the surface of small micelles. Furthermore, hydrophobic interactions increase with heating, which causes micelles to aggregate more easily. These reasons together could explain why the average size of RCMs increased with increasing temperature.

In addition, the ion concentrations in RCM serum were measured at different temperatures as this could provide insight into the mechanism of the growth of RCMs (Fig. 3). The Ca^{2+} concentration was significantly higher (approximately 2 mM) at 25 °C compared to the concentration at 52 °C and 67 °C. Similarly, the P^{5+}, PO_4^{3-} and Mg^{2+} serum concentrations also decreased with increasing temperature. However, no significant difference was observed in Ca^{2+} content from the temperature of 37 °C–83 °C. This result seems counterintuitive, because the solubility of

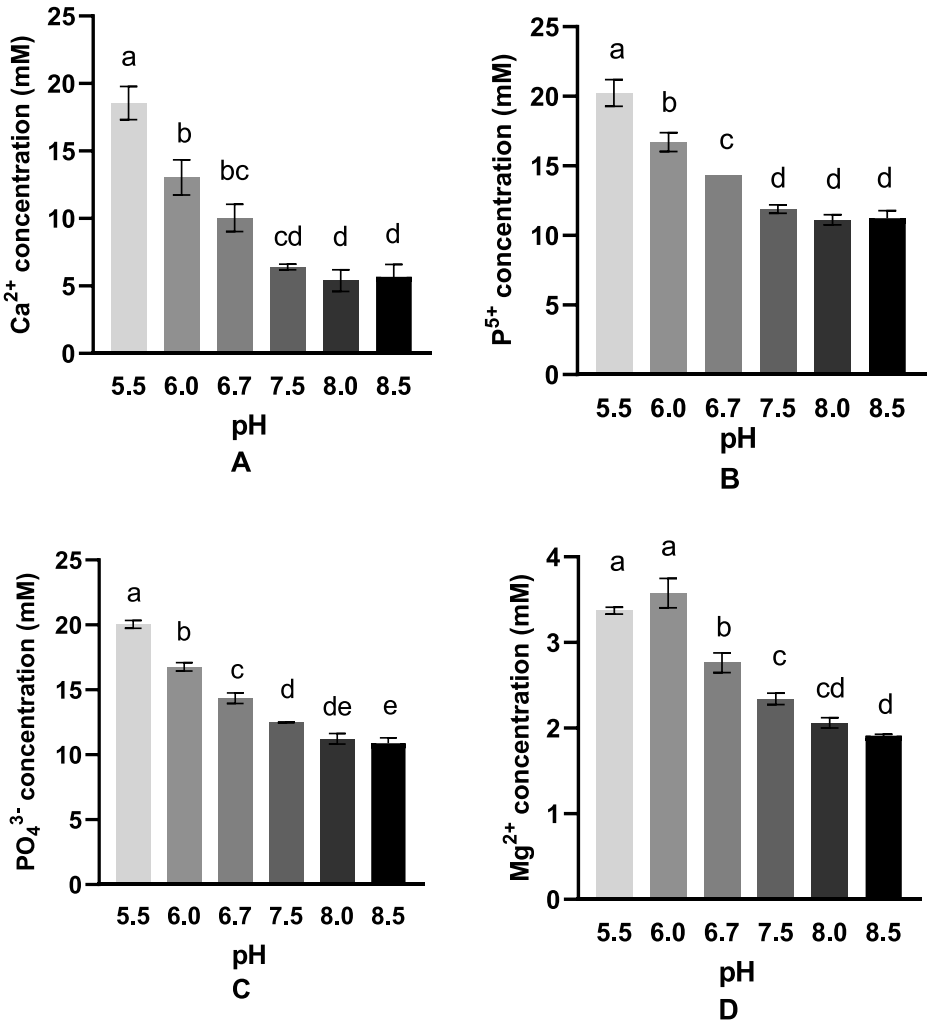

Fig. 6. Ion concentrations of reassembled casein micelle serum that pH adjusted after assembly. A: serum concentration of calcium. B: serum concentration of phosphorus. C: serum concentration of phosphate. D: serum concentration of magnesium. The data with the same character indicates no significant difference between them ($p > 0.05$).

calcium phosphate decreases with increasing temperatures. Poulliot et al. (1989) studied the heat-induced ion balance by collecting milk serum at corresponding temperatures. In that study, serum Ca^{2+} and PO_4^{3-} concentrations decreased with increasing temperature. They also observed that this effect was reversible by collecting milk serum after cooling down. Another study (Wang & Ma, 2020) on the effect of heat treatment on milk salt equilibria also showed that the Ca^{2+} and PO_4^{3-} concentrations did not have significant differences after being heated to 80 °C for 15 min and then cooled down to room temperature. Similarly, the RCM serum was collected after cooling down, in which the precipitated calcium phosphate could resolubilize, therefore, no significant difference in Ca^{2+} and PO_4^{3-} concentrations were found at temperatures higher than 37 °C. Because 37 °C is close to the body temperature of a cow, it is interesting to compare RCMs that were made at this temperature to those at 25 °C. The RCMs heated at 37 °C have slightly less serum casein and slightly larger hydrodynamic diameters. However, no major differences were observed between them. The results from serum ion concentrations together with serum casein concentrations indicate that the growth of casein micelles, especially from 37 °C to 83 °C, is primarily caused by aggregation among micelles and not caused by the growth of individual RCMs. This is essential for the production of dairy products that are made by RCMs in future, as the size of casein micelles e.g. affects their gelation; it was reported that smaller micelles form a firmer gel (Glantz et al., 2010).

3.4. Effect of pH on RCM properties

In this section, we focused on the effect of pH during and after making RCMs. The latter process will be compared to studies on the effect of pH on bovine casein micelles. The effect of pH during the formation of casein micelles had not been studied yet. It was important to fill this gap as the timing of adjusting pH may influence casein micelle properties. First, the effect of pH on the size of RCMs has been discussed in this section. In addition, the composition of RCMs at different pH were analyzed. Furthermore, the internal structure of casein micelles has been examined by SAXS to investigate the structural changes of RCMs when altering the pH.

3.4.1. Effect of pH on the size and composition of RCMs

Two size parameters of RCMs were measured by DLS and SAXS: the hydrodynamic diameter and the core diameter (i.e., $2 \times R_{total}$, refers to d_{total} in the following parts), respectively. The apparent hydrodynamic diameter of RCMs prepared at pH 6.7 was approximately 150 nm (Fig. 5). Interestingly, this is slightly larger than the diameter determined by SAXS (approximately 130 nm, Fig. 10A). This difference could relate to the existence of a fluffy layer of κ-casein on the surface of RCMs. From these results, the thickness of the κ-casein layer was approximately 10 nm for RCMs. This number was close to the thickness of κ-casein layer on native casein micelles, which was reported to be 7 nm (Holt & Horne,

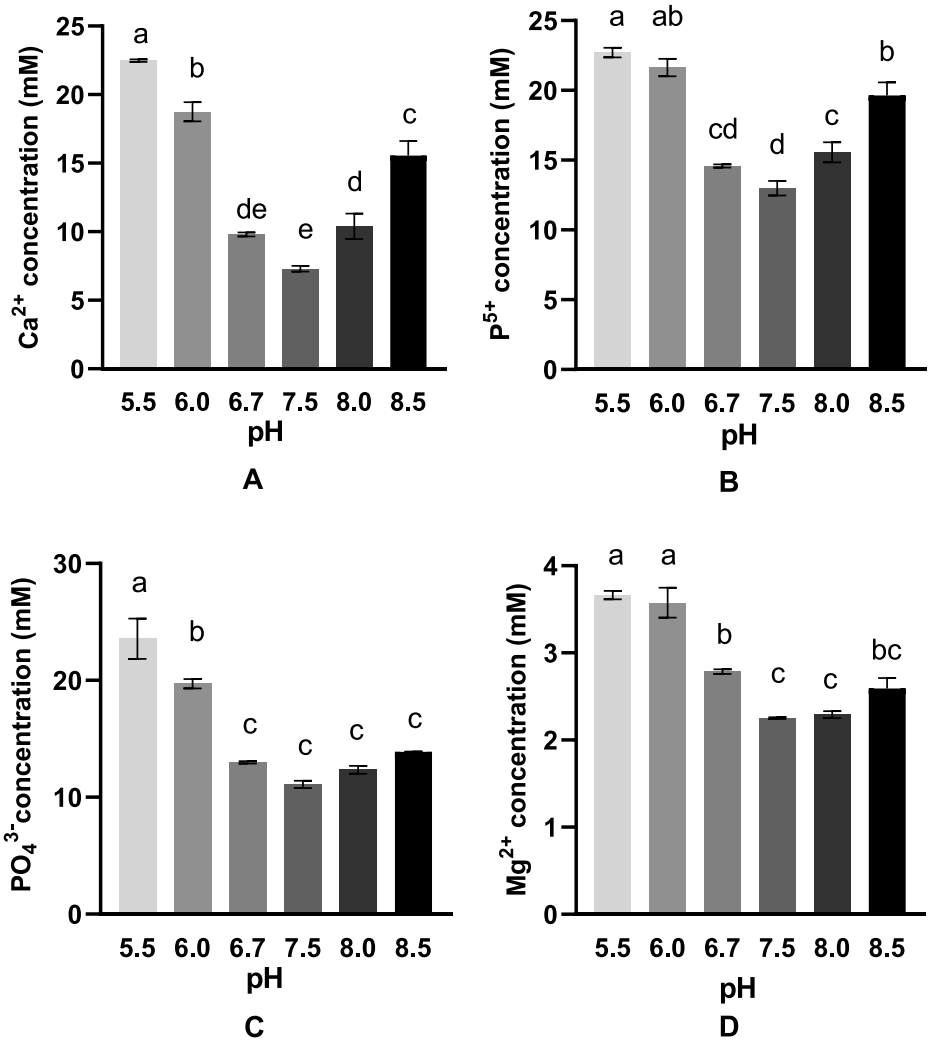

Fig. 7. The ion concentrations of pH_{DA} serum sample at different pH. A: serum concentration of calcium. B: serum concentration of phosphorus. C: serum concentration of phosphate. D: serum concentration of magnesium. The data with the same character indicates no significant difference between them ($p > 0.05$).

1996). These results showed that DLS and SAXS probe size differently (demonstrated in Fig. 4). Therefore, it is also interesting to compare the size measured by these two techniques at different pH values.

For those samples that were pH adjusted after the formation of RCMs, the effect of pH on them was similar to the effect of pH on bovine casein micelles. The hydrodynamic diameter of the pH_{AA} sample increased with increasing pH (Fig. 5). The SAXS result for pH_{AA} samples also showed an increasing trend in size (Fig. 10A). Similar behavior has been reported by Sinaga et al. (2017) for casein micelle size in pasteurized milk. They found that the size of casein micelles decreased when reducing pH to 6.0 and increased when increasing pH from 6.6 to 7.5. This behavior can be explained by pH-induced changes in casein charge and calcium phosphate solubility. First, caseins are more negatively charged with increasing pH. The internal electrostatic repulsion of the casein matrix increases with increasing pH. It was reported that the self-assembled casein matrix has larger size at higher pH (Liu & Guo, 2008). As a result, the casein micelles may swell at higher pH and shrink at lower pH. Another factor that affects the size of casein micelles is calcium phosphate solubility. Similar to bovine milk, calcium phosphate is solubilized at pH lower than 6.7. At pH 5.5, around 72 % of Ca^{2+} ended up in serum, which means almost half of micellar calcium was moved to serum phase (Fig. 6). A similar trend was observed in P^{5+} and PO_4^{3-} (Fig. 6). As calcium phosphate nanoclusters act as "bridges" to connect the casein matrix in casein micelles, higher solubility of calcium phosphate can cause casein micelles to be partially dissociated. This also matched the fact that serum casein increased at pH 5.5 (Fig. 8). Therefore, the decrease in size from pH 6.7 to 5.5 is likely due to the combination of shrinkage and dissociation of casein micelles, whereas the increase in RCM size at pH values higher than 6.7 is likely due to the increase of the charge of caseins at alkaline pH that causes swelling of casein micelles. Although caseins were also released at alkaline pH, it seems that the swelling of RCMs has a greater effect on RCM size compared with the release of caseins.

For those RCMs where the pH was adjusted during the reassembly, the effect of pH on hydrodynamic diameter had similar trend on the acid side of pH_{AA} samples but different on the alkali side. The d_H first increased and then decreased with increasing pH. At pH 5.5, more than 78% of Ca^{2+} was present in serum compared to approximately 30% of Ca^{2+} at pH 6.7. A similar trend was found for serum PO_4^{3-} and P^{5+} (Fig. 7). The serum casein concentration at pH 5.5 in pH_{DA} sample was significantly higher compared to pH_{AA} serum at pH 5.5 (Fig. 8). Combining all these results, we found that for pH_{DA} samples at pH 5.5, significant amount of caseins and calcium phosphates were not incorporated in RCMs. As more casein was not incorporated with casein micelles in pH_{DA} samples compared to the released caseins from pH_{AA} samples at pH 5.5 (Fig. 8), it seems logical that pH_{DA} RCMs have a smaller size than pH_{AA} RCMs at that pH (Fig. 5). However, the size measured from SAXS showed no significant difference between pH_{AA} and pH_{DA} samples at pH 5.5. We should keep in mind that DLS and SAXS probe size based on different physical phenomena. DLS measures the

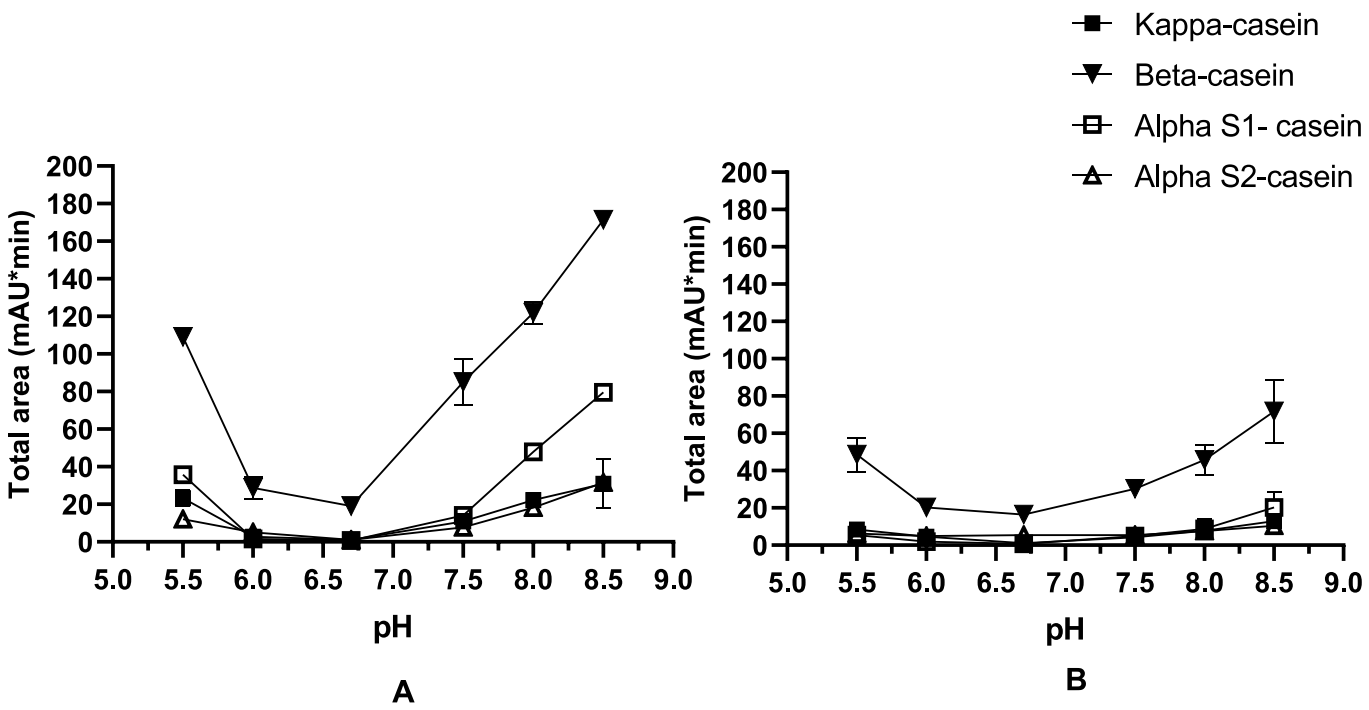

Fig. 8. Composition of the different caseins in the serum depending on the pH either during assembly (pH_{DA}) or after assembly (pH_{AA}). A: serum casein composition in pH_{DA} sample. B: serum casein composition in pH_{AA} sample. The lines between data points are drawn to guide the eye.

hydrodynamic diameter whereas SAXS measures the particle volume having different electron density than that of the medium. The differences between DLS and SAXS results indicate that even though the volume of the protein particles of some pH_{AA} and pH_{DA} samples might be similar, their hydration layer might be larger in pH_{AA} samples, which can be attributed to a less electron dense fluffy protein shell (Fig. 4). From the previous result about the effect of pH on pH_{AA} sample, we know that some caseins dissociated from casein micelles at acid pH. The fluffy layer of pH_{AA} samples could be caseins that had dissociated from the core structure of RCMs, but still loosely attached to them. The pH_{DA} sample at pH 6 showed precipitation during preparation. Therefore, the d_H at this pH was not representing all the particles due to the sample not being homogeneous. The reason why casein started to precipitate at this particular pH is not clear yet. Decreasing the pH closer to the isoelectric point of caseins indeed decreases the electrostatic forces between them and could cause aggregation. However, we did not observe any aggregation at pH 5.5, where casein has even less electrostatic repulsion. This indicates that the aggregation at pH 6 is not only due to the decrease of the repulsion between casein micelles.

At pH higher than 6.7, the hydrodynamic diameter of pH_{DA} RCMs was decreased with increasing pH. Similar in SAXS, a significant drop in size at pH 8 was observed (Fig. 10). When adjusting pH away from the isoelectric point of caseins, they are more negatively charged. The increase in electrostatic repulsion between caseins could prevent caseins from forming casein micelles, therefore, casein micelles with fewer caseins were formed. Casein micelles with fewer caseins could have a smaller size. Those caseins that did not participate in the formation of casein micelles cannot be completely sedimented by ultracentrifugation, therefore, more caseins ended up in serum (Fig. 8). This was also reflected in the content of P^{5+}. At pH 8.5, the P^{5+} content in serum was significantly higher than the P^{5+} content at pH 6.7 (Fig. 7), which was caused by 58% of caseins ending up in serum and most caseins being phosphorylated.

Combining the result from pH_{DA} and pH_{AA} samples, we can conclude that adjusting pH during the assembly of RCMs had a larger impact on RCMs size, ion and casein equilibria compared to adjusting pH after assembly. The pH_{AA} casein micelles were rather stable to pH changes. From those results, we found that once the RCMs were formed in an ideal condition (pH at 6.7), there could be some internal forces against the dissociation of casein micelles.

3.4.2. *Small-angle X-ray scattering at different pH*

Aiming to investigate the effect of pH on the structure of RCMs, SAXS measurements were conducted on both pH_{AA} and pH_{DA} samples (Fig. 9). All scattering data showed rapid decrease at low-q, followed by a well-pronounced shoulder at intermediate-q and moderate decrease at high-q. The similar features of the scattering spectra suggest that the structure of the casein micelles does not change significantly in the accessible length scale (<100 nm). At pH = 5.5, the scattering curves just slightly deviate from each other. Further increasing the pH to 7.5 and 8, we do encounter significant differences compared to data at low pH. The bump at intermediate-q is diminished by the elevated scattering of the local minima. In terms of preparation protocol, the data coincides with each other quite well at intermediate and high-q, consequently significant structural change seems to be absent. One exception is at pH = 6, where the samples showed significant differences. At intermediate and low-q we observed increased intensity for the sample adjusted after. This behavior suggests an increased number of substructures within the micelles. Interestingly, further increasing the pH to 7.5, 8 and 8.5 we do not encounter significant differences between the scattering curves at intermediate and high-q. In contrast, we observed deviation of the low-q behavior for samples, which suggests the change of the size of the whole micelle. Since on the scattering data of the samples adjusted during preparation, the beginning of the Guinier region already gives rise, we assume that the size of the casein micelles adjusted during preparation is smaller than that of the samples adjusted after preparation. If we examine the scattering data with the same preparation method with increasing pH, we can observe that the position of the intermediate shoulder is not affected, and we could conclude that the distance between the substructure did not change significantly. At the same time, the shoulder is getting less pronounced, which suggests the occurrence of an intermediate structure.

To get deeper insight into the features of the SAXS data we performed non-linear least square fitting using Python's lmfit module. For the

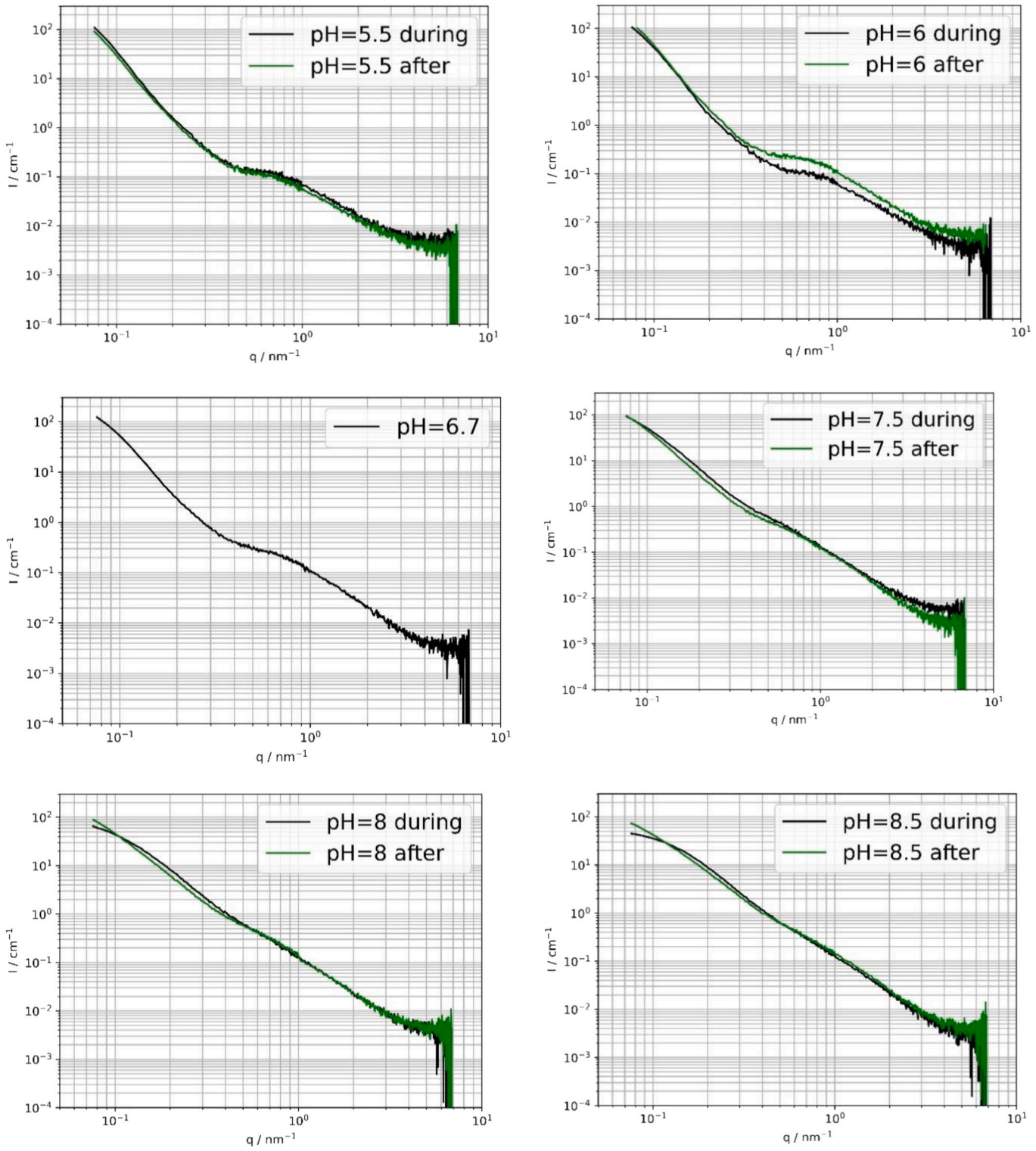

Fig. 9. SAXS curves of reassembled casein micelles at different pH adjusted during (black) and after (green) the sample preparation. Only one line is displayed at pH 6.7, at this pH, reassembled casein micelles prepared during and after assembly are the same sample.

fitting procedure we used the model developed by Pedersen et al. (2022) for casein micelles. The fits are presented in Fig. S1 (see supplementary document) and the optimized parameters are collected in Tables S1 and S2 (see supplementary document). The fitting was performed on the absolute scale, which means that with proper normalization described in the cited work, we could quantify the changes of the internal structure of the RCMs. From the best fits, we determine the radius of the micelle (R_{total}), the molar mass of the protein heterogeneities, the number of calcium phosphate and protein heterogeneities per micelle as a function of pH (Fig. 10). The pH_{DA} samples had practically the same size as pH_{AA} samples, except at pH 8.0, where the observed size is significantly smaller (Fig. 10A).The mass of the protein heterogeneities displayed quite similar increasing behavior for both sample series; namely, increases with increasing the pH and reaching a plateau at alkaline pH.

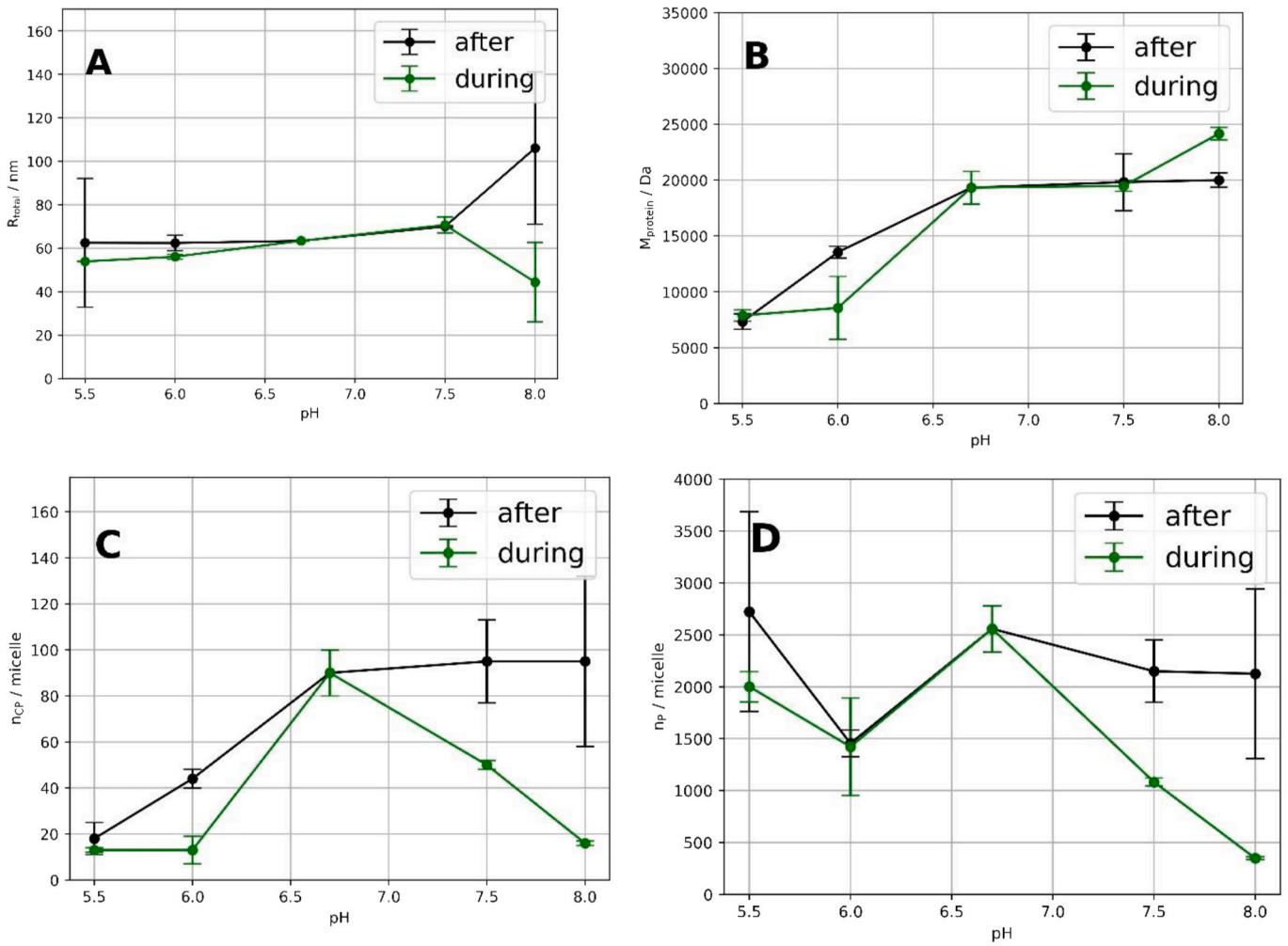

Fig. 10. Structural parameters obtained by model fitting. A: the size of micelles, B: mass of protein heterogeneities, C: number of calcium phosphate clusters per micelle and D: number of protein heterogeneities per micelle. Samples were pH-adjusted after and during the preparation with black and green respectively. The lines are guide to the eye.

However, the number of calcium clusters and protein heterogeneities per micelle showed a different trend. The number of calcium clusters per micelle increases up to pH 6.7 which is in agreement with the fact that calcium phosphate solubility decreases with increasing pH. The number of calcium phosphate clusters reaches a plateau when adjusting the pH after the preparation, however when adjusting the pH during the preparation it starts to decrease again. Note that, in the model, the volume of the calcium clusters was kept constant. Thus, the decrease in their number concentration cannot be interpreted as cluster growth. Instead, it is related to a smaller number of calcium clusters per micelle, which is due to a decrease in RCM size at pH 8. The number of protein heterogeneities shows slightly oscillating behavior below pH 6.7. Above that pH, the value reaches a plateau for the samples adjusted after reassembly, however it decreases for the sample series adjusted during reassembly, which is again due to the decrease of overall size. As we have seen previously, the mass of the protein heterogeneities increases at acid pH for both series, which suggests the growth of the protein clusters happens when increasing the pH until 6.7. These results together show that by varying the pH of the RCMs their internal structure is affected due to differences in casein charge and calcium phosphate solubility. The SAXS profiles of the pH 8.5 samples could not be fitted with our model. Tentatively, we attribute this to a coexistence between casein micelles and larger aggregates, which the model does not take into account.

3.5. *Effect of calcium phosphate concentration*

As discussed in the introduction, and some previous results, calcium phosphate concentration has a significant impact on the formation of RCMs. To evaluate the effect of calcium phosphate concentration on RCMs, RCMs were prepared with different calcium phosphate concentrations at pH 6.7 and 25 °C. As displayed in Fig. 11A, the d_H of RCMs was reduced significantly with decreasing salt concentration. The serum Ca^{2+} and PO_4^{3-} concentration was similar for different samples, while the micellar Ca^{2+} and PO_4^{3-} concentration decreased significantly (Fig. 11). As the solubility of calcium phosphate did not change much at 25 °C, the reduction of total calcium phosphate concentration led to less micellar calcium phosphate. Therefore, less calcium phosphate was available as a building material to form casein micelles, which could limit the size and total number of casein micelles. In the end, less casein was connected by micellar calcium phosphate to form casein micelles, which increased serum casein concentration. When increasing the calcium phosphate concentration to 120 % of the original concentration (30 mM Ca^{2+}, 20 mM PO_4^{3-}), precipitation was observed. This is probably because of the excess amount of calcium phosphate that could not be sequestered by enough caseins to prevent their precipitation (Holt et al., 2013). These results show that calcium phosphate concentration, especially micellar calcium phosphate concentration could be a size-limiting factor at pH 6.7. The precipitation at 120% of the original calcium phosphate concentration (30 mM Ca^{2+}, 20 mM PO_4^{3-}) indicates

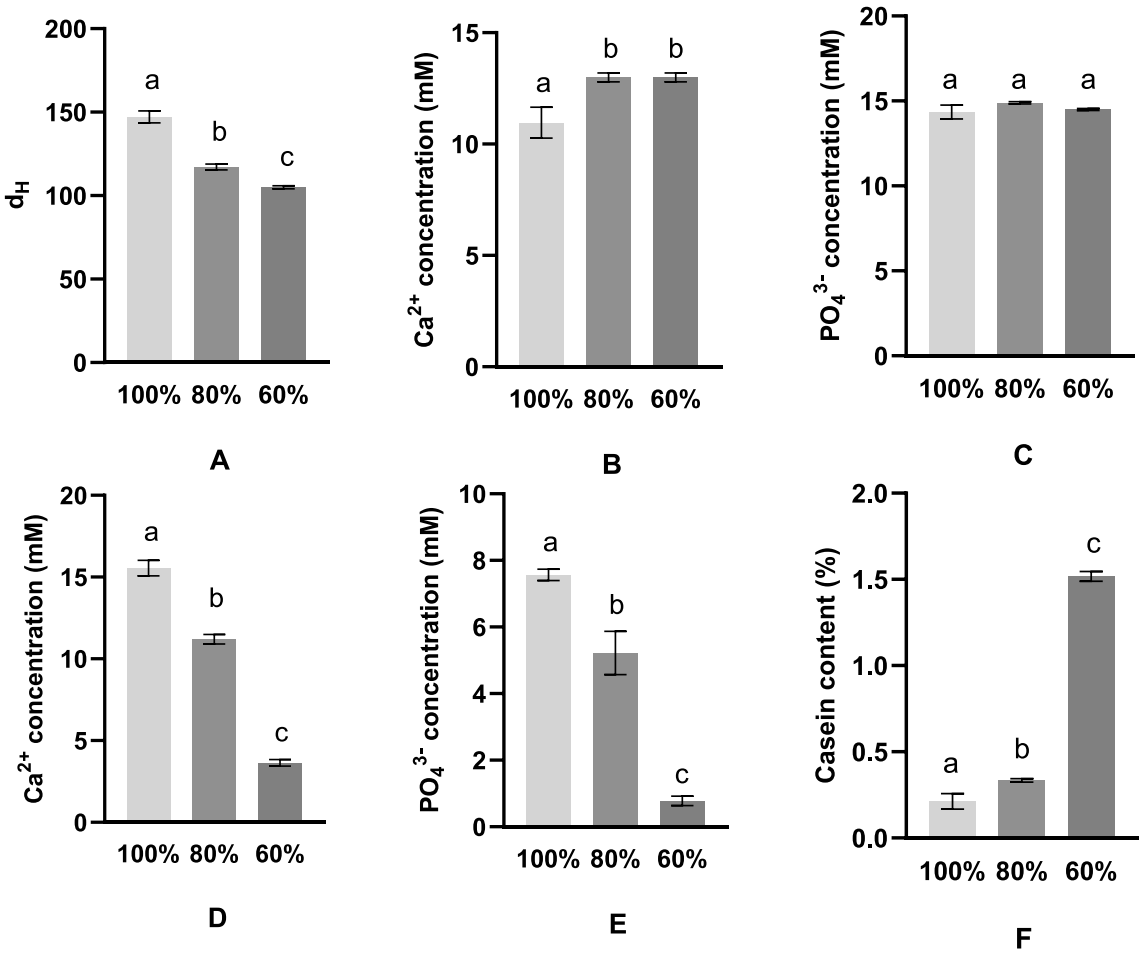

Fig. 11. A: hydrodynamic diameter (d_H) of RCMs made with different calcium phosphate concentrations. B: serum calcium concentration. C serum phosphate concentration. D: micellar calcium concentration. E: micellar phosphate concentration. F: serum casein concentration.

that there is a maximum amount of calcium phosphate that casein micelles can stabilize. Overall, there seems to be an optimal calcium phosphate concentration when making RCMs. This concentration could be connected with the total casein concentration as well as the phosphorylation level of caseins because they play an essential role in stabilizing calcium phosphate.

3.6. Combined discussion of the effect of processing parameters on RCM properties

In the above, we have studied the properties of reassembled casein micelles (RCMs) prepared under different processing conditions and compared their properties to those of bovine casein micelles. We found that the size, ion and casein composition of RCMs made at pH 6.7, 25 °C and an ion concentration of ~30 mM Ca^{2+}, 20 mM PO_4^{3-} (i.e., similar to that of the average bovine milk) are similar to the size, ion and casein composition of bovine casein micelles. Despite these compositional and structural similarities, the freeze/thaw stabilities differed. RCM dispersions gelled upon freezing and thawing, whereas dispersions of bovine casein micelles did not. To elucidate the as-yet unknown mechanistic origin and functional consequences of this difference in stability, additional RCMs properties should be evaluated, such as their renneting tendency, water holding capacity, and the location of individual caseins within the casein micelle.

We also found that variations in temperature, pH and ion concentration have a significant impact on the size and composition of RCMs. Adjusting the pH during reassembly has a larger effect on RCMs than pH adjustments after the reassembly process. DLS revealed that RCMs were significantly smaller when pH was adjusted during the assembly, while SAXS showed that the size only differed at a pH higher than 7.5. DLS SAXS results together helped us to understand how the timing of adjusting pH affects RCMs differently. For adjusting pH after the formation of RCM, at pH 5.5, higher Ca^{2+} and PO_4^{3-} concentrations were found in the serum, indicating calcium phosphate nanoclusters dissolved, causing RCMs to partially dissociate. The dissociated caseins could be partly loosely attached to RCMs and partly moved to the serum. Therefore, the d_H could be larger than the core diameter (d_{total}). Compared to pH_{AA} samples, higher serum casein concentration was found in pH_{DA} sample, indicating less caseins were incorporated with casein micelles and likely, no casein loosely attached with RCMs. Therefore, the d_H and d_{total} were similar. From the SAXS result, pH_{DA} and pH_{AA} RCMs had similar core diameters. Those results together explained why the pH_{AA} RCMs had much larger d_H at acid pH compared with the pH_{DA} RCMs. At higher pH, the internal repulsion among caseins inside of the formed RCMs resulted in the swelling of RCMs. This was confirmed by the increased size of pH_{AA} samples that were measured by DLS and SAXS. On the other hand, during the formation of casein micelles, the increased repulsion between caseins limited the formation of casein micelles, therefore, smaller micelles with fewer caseins were formed. These processes are schematically demonstrated in Fig. 12.

In this research, we investigated the effect of processing parameters on RCMs. We considered that there are several building materials for casein micelles (casein matrix and calcium phosphate). The change in processing parameters has impacts on the building materials themselves and the interactions between them, therefore, affecting the overall properties of casein micelles. This study provides us with essential

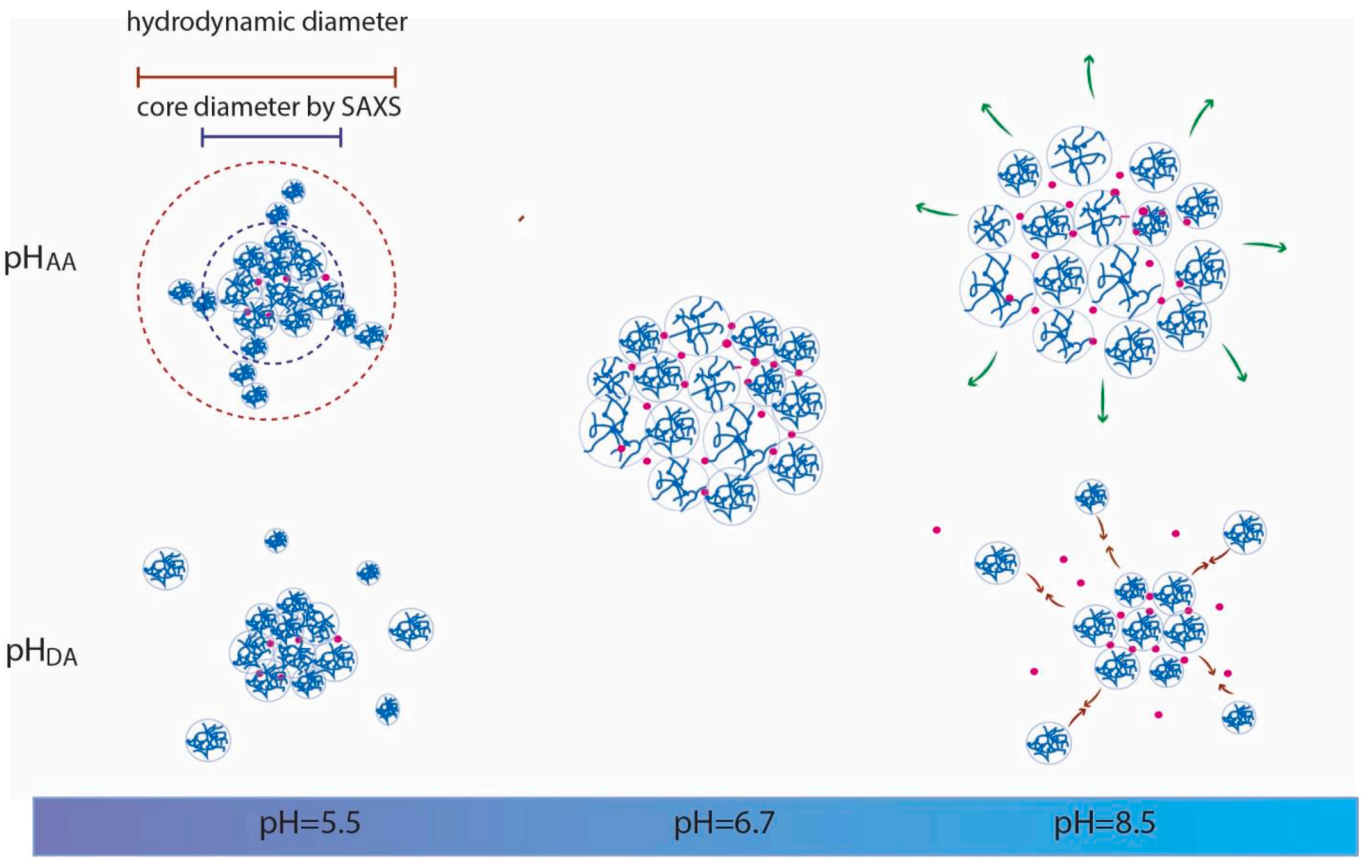

Fig. 12. A schematic representation of the effect of pH on RCMs with pH adapted during the assembly process and after the assembly process. pH_{AA}: reassembled casein micelles adjusted to different pHs after assembly. pH_{DA}: reassembled casein micelles adjusted to different pHs during assembly. Blue lines with grey circles indicate protein inhomogeneities. Red dots: calcium phosphate nanoclusters. Red dash line: hydrodynamic diameter from DLS measurement. Blue dash line: core diameter (d_{total}) from SAXS. Green arrows: the swelling of reassembled casein micelles. Red arrows: the electrostatic repulsion between caseins.

knowledge on casein micelles formation and can be transferred for future studies. In nature, caseins can have variations in the levels of their modifications such as glycosylation and phosphorylation, which have impacts on their hydrophobic interactions and their capacity to stabilize calcium phosphate nanoclusters. Moreover, casein micelles may have different casein ratios, which could affect the overall phosphorylation level of the whole micelle as caseins are differently phosphorylated. Besides bovine caseins, the recombinant caseins could have different types of post-translational modifications. All those variations on caseins make it difficult to assemble casein micelles without adapting any processing parameters. In this study, we obtained a deeper understanding of what parameters and interactions are essential during the formation of casein micelles. Therefore, it points a direction in future on how to adapt processing parameters if we use caseins that are differently modified. Understanding how those factors affect the structure and properties of casein micelles is also essential prior knowledge to assemble recombinant caseins into stable casein micelles in future. For example, reducing calcium phosphate concentrations when assembling caseins that are less phosphorylated, or increasing the temperature during the assembly when recombinant caseins have less hydrophobic region, could be used for improved assembly of recombinant caseins.

4. Conclusion

This study focused on the effect of processing parameters on the properties of RCMs. Our data showed the processing parameters have effects on the size, ion and casein distribution as well as the structure of RCMs. We found that to assemble RCMs that are close to bovine casein micelles, it is required to assemble them at pH 6.7 with an average cow's milk salt concentration (approximately 30 mM of Ca^{2+} and 20 mM of PO_4^{3-}) at 25 or 37 °C. If so desired, temperature can be used as a factor to adjust the size of RCMs. The internal structure of RCMs can be modulated by adjusting the pH during or after the reassembly. By studying the effect of temperature, pH and salt on RCMs, we obtained knowledge on critical aspects of casein micelles formation. We noticed the importance of hydrophobic interaction, electrostatic repulsions, and solubility of calcium phosphate in the formation process of casein micelles. That knowledge can be transferred as an important foundation to assemble RCMs by using recombinant caseins.

Authorship contribution statement

Zekun Fan: Conceptualization, Methodology, Formal analysis, Visualization, Writing – original draft, review & editing. Bence Fehér: Conceptualization, Methodology, Writing – review & editing, Visualization. Kasper Hettinga: Conceptualization, Supervision, Writing – review & editing. Ilja K. Voets: Conceptualization, Writing – review & editing. Etske Bijl: Project administration, Conceptualization, Supervision, Writing – review & editing.

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 paper.

Data availability

Data will be made available on request.

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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.2023.109592.

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