Investigating the effect of diluents and fat globules on the size measurement of casein micelles by dynamic light scattering

Teng Wang*, Hao Li, Quenten Denon, Chak Ming To, Paul Van der Meeren

Particle and Interfacial Technology Group (PaInT), Department of Green Chemistry and Technology, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, 9000, Gent, Belgium

*Corresponding author

E-mail address: Teng.Wang@ugent.be (T. Wang)

ABSTRACT

The hydrodynamic size of casein micelles was examined using dynamic light scattering (DLS). Four types of diluents [ultrafiltration (UF) permeate, lactose-free simulated milk ultrafiltrate (SMUF), calcium imidazole buffer, and deionised water] were compared in their effect on the stability of casein micelles. Results of dilution series and kinetic size measurements revealed that both UF permeate and lactose-free SMUF enable size measurements that are hardly affected by the required dilution step, whereas a calcium imidazole buffer containing 10 mM $CaCl_2$ only could provide accurate sizing results within a short time after dilution. The contribution of residual fat globules to the particle size distribution was negligible in skim milk. For semi-skimmed milk, the size of the casein micelles could be estimated by subtracting the contribution of the fat droplets (obtained upon dilution in a casein dissolving solution) from the raw DLS data (as obtained upon dilution in milk ultrafiltrate).

1. Introduction

In bovine milk, caseins (i.e., α_{S1}-, α_{S2}-, β-, and κ-casein) make up about 80% of the total proteins, and these caseins are combined with amorphous calcium phosphate nanoclusters and divalent cations (i.e., mainly Ca^{2+} and Mg^{2+}), known as casein micelles (Holt, 2021). These micelles are highly hydrated, with a voluminosity of 4.1 mL g^{-1} at 20 °C (Nöbel, Weidendorfer, & Hinrichs, 2012). In addition, the polydisperse nature of casein micelles is pronounced since their hydrodynamic diameter may range from 50 to 600 nm in native milk (Horne & Dalgleish, 1985) and their average diameters are from 150 to 200 nm (Dalgleish & Corredig, 2012). The complicated structure of casein micelles, which are roughly spherical aggregates composed of casein fractions (α_{S1}-, α_{S2}-, β- and κ-casein) and amorphous calcium phosphate, is closely related with their functionality in dairy products. Hereby, the monitoring of the hydrodynamic size (distribution) of casein micelles during milk processing may help to understand the structural integrity or/and stability of casein micelles, as well as provide useful insights into the processing of dairy products.

In the past few decades, the particle size of casein micelles has been investigated via several techniques, including scanning electron microscopy (SEM) (Sandra & Dalgleish, 2005; Silva et al., 2013) as well as transmission electron microscopy (TEM) (Karlsson, Ipsen, & Ardö, 2007; McMahon & McManus, 1998; Trejo, Dokland, Jurat-Fuentes, & Harte, 2011), atomic force microscopy (AFM) (Ouanezar, Guyomarc, & Bouchoux, 2012), and dynamic light scattering (DLS) (Choi & Zhong, 2020; De Kruif

& Huppertz, 2012; Liu & Guo, 2008). Additionally, more advanced techniques, such as small-angle X-ray scattering (SAXS) and small-angle neutron scattering (SANS) can provide information related to the size and internal structure of casein micelles (Adams et al., 2019; Ingham et al., 2015, 2016). Compared with electron microscopy, AFM, or small-angle X-ray scattering, dynamic light scattering (DLS) is a more accessible technique that has its own benefits for characterising the size distribution of casein micelles, as it is non-invasive, rapid, and requires less extensive sample pretreatment. More importantly, the structural integrity of casein micelles can be better preserved during the measurements as DLS does not require any previous dehydration (Bhattacharjee, 2016). Another advantage of DLS is that it enables to achieve statistics on the particle size distribution and the average diameter as the primary data result from the simultaneous light scattering by a very large number of particles. However, the low light transmission of milk means that a sufficiently high degree of dilution is required to enable light scattering experiments that do not suffer from multiple light scattering (Bhattacharjee, 2016).

In a recent study considering 48 Holstein-Friesian cows, Nieuwenhuijse & Huppertz (2022) reported that milk contains an average of 31.4 mM of total calcium concentration, of which 9.6 mM is soluble and the remaining 22.7 mM is colloidal. These reported calcium levels are comparable with those described by Lewis (2011), where the ionic calcium content was also reported, typically ranging from about 2 to 4 mM. Considering that the ionic equilibrium affects the structure and stability of casein micelles, a particular concern is to select a suitable diluent that can retain the original

state of the casein micelles during the sample preparation and subsequent measurements.

Lactose-free SMUF is a complex electrolyte solution that is meant to mimic the composition of the serum phase of native milk as obtained via ultrafiltration (Jenness & Koops, 1962). It was used to dilute skim milk in investigating the effect of chymosin on the hydrodynamic size of casein micelles (Walstra, Bloomfield, Wei, & Jenness, 1981), as well as to reconstitute milk protein concentrates (Ranadheera et al., 2019) and freeze-dried milk protein (Schorsch, Wilkins, Jones, & Norton, 2001). To prevent the precipitation of Ca-phosphate in preparing lactose-free SMUF, phosphopeptides can be introduced to stabilise the buffer and extend its storage time (Holt, Davies, & Law, 1986; Zhang, Fuji, & Aoki, 1996).

According to prior work (Holt, 1982), to preserve the colloidal calcium the diluent should contain the same ion activity product of calcium phosphate as in milk. Hence, the calcium ion concentration in the calcium phosphate buffer can be altered by a compensating change in the phosphate concentration (Holt et al., 1986). In the current study, phosphate-free buffers, also called calcium imidazole buffers in some other light scattering studies (Sandra & Dalgleish, 2005; Tran Le, Saveyn, Hoa, & Van der Meeren, 2008), were also employed to evaluate the dissociation degree of casein micelles they induced after long-time incubation. Moreover, the complexity of the lactose-free SMUF can be reduced considerably by eliminating most salts other than calcium, phosphate and background electrolyte. Thus, Little and Holt (2004) used a diluent free of magnesium, citrate and all of the minor salts in the lactose-free SMUF. In addition, water was applied as a diluent to reconstitute native phosphocaseinate powder; the time

dependent turbidity measurements showed that the micelle dissociation degree is related to its original casein concentration (Thomar & Nicolai, 2015).

As milk processing steps, such as acidification, thermal changes or the addition of electrolytes, induce a variation in the mineral and caseins partitioning between the colloidal and serum phase compared with the native state in milk (Broyard & Gaucheron, 2015), it is clear that one single universal diluent that enables dilution without affecting the ionic composition of the serum or altering the casein concentration does not exist. Instead, for any diluent, compromises will have to be made, considering a minimal impact on the casein micelle characteristics. In this respect, it is important to consider that micelle dissociation and adaptation to the environment are processes that take days to come to an equilibrium. As it is difficult to design one specific dilution buffer for each system, several researchers preferred to obtain the UF permeate of their own system as dilution liquid for DLS measurements (De Kort, Minor, Snoeren, van Hooijdonk, & van der Linden, 2011; Liu et al., 2017; Liu, Weeks, Dunstan, & Martin, 2013; Wang, Jin, Guo, Zhao, & Ren, 2015).

The interference of fat globules in milk during the size measurement of casein micelles is another important aspect. According to Horne and Dalgleish (1985), this is especially crucial when the detection angle is low, as the larger fat globules have a higher contribution to the scattered intensity under these conditions. The interference of residual fat globules was estimated by particle size analysis of a skim milk sample with and without adding a casein-dissolving agent by Beliciu and Moraru (2009), but no method was provided to only obtain the contribution of the casein micelles from a mixed

sample.

The objective of this study was to optimise the particle size measurement procedure of casein micelles by DLS in terms of the type of diluent, dilution degree and detection angle. Hereby, the absorbance values were measured at the same wavelength to provide a simple indication for the appropriate dilution degree. Additionally, the interference of residual fat globules was considered. Hereby, a mathematical approach was introduced to calculate the corrected autocorrelation function by subtracting the contribution of fat globules and hence to evaluate the contribution of casein micelles only.

2. Materials and methods

2.1. Materials

Pasteurised skim milk (3.8%, w/w, protein; less than 0.1% residual fat), semi-skimmed milk (3.4% protein; 1.9% fat), and ultrafiltration (UF) permeate were obtained from Milcobel Dairy Corporation (Kallo, Beveren, Belgium). The UF permeate was obtained by membrane filtration with a nominal cutoff of 20 kDa at a temperature of 10 °C; its pH value was 6.7 at 25 °C. Additionally, two different diluting agents were prepared for the DLS measurements, including calcium imidazole buffer, and lactose-free SMUF buffer. The calcium imidazole buffer contained 20 mM imidazole, 5 mM $CaCl_2 \cdot H_2O$, 30 mM NaCl, and 1.5 mM NaN_3 (Tran Le et al., 2008). Its pH was adjusted

to 6.65 using 1 M HCl. Additionally, 10 and 20 mM $CaCl_2 \cdot H_2O$ containing imidazole buffer was also made. The lactose-free SMUF buffer was prepared using analytical grade mineral salts according to Beliciu and Moraru (2009). To avoid precipitation, all ingredients were separated into two groups to make two stock solutions: solution A was composed of KH_2PO_4, K_3citrate$\cdot H_2O$, Na_3citrate$\cdot 2H_2O$, K_2SO_4 and K_2CO_3, while solution B contained $CaCl_2 \cdot 2H_2O$, $MgCl_2 \cdot 6H_2O$ as well as KCl. Solution A and B were mixed in a 1/1 volumetric ratio and adjusted to pH 6.65 by a 1 M KOH solution prior to use. Casein-dissolving solution was prepared by adding 35 mM disodium EDTA to distilled water.

2.2. Particle size measurement

The measurements were performed in triplicate at 15, 25, and 35 °C during 120 s with a Spectrometer PCS100-M (Malvern Instruments, Malvern, UK) containing a 15 mW He–Ne laser. The samples and dilution liquids were incubated in a water bath at 15, 25, and 35 ± 0.1 °C prior to dilution to eliminate the interference of temperature effects, and the analyses were completed within 20 min after dilution to avoid time effects at high dilution degrees, unless stated differently. To enable a direct comparison of the scattered light intensities, the attenuation aperture diameter was fixed at 100 μm and the scattering angle was either 90° or 150°.

The apparent hydrodynamic particle diameter was derived from cumulant analysis by the Automeasure software (Malvern Instruments, Malvern, UK). Whereas most

commercial DLS software indicates the latter as the so-called z-average diameter, it has to be taken into account that this is only true provided that the particle size is smaller than the inverse of the scattering vector, which is mostly not the case (Jin, Jarand, Brader, & Reed, 2022). In the latter case, the average diameter derived from cumulant analysis is actually the intensity-weighted harmonic mean diameter (ISO 22412).

Multimodal data-analysis was selected to obtain the intensity-weighted particle size distribution. The viscosity and refractive index (RI) of the diluents were determined using a capillary Ubbelohde viscometer (K=0.005 $mm^2\ s^{-2}$, Schott AG, Mainz, Germany) and an Abbe refractometer (Officine Galileo, Florence, Italy), respectively. The results were summarised in Table 1.

In DLS, the scattered light intensity autocorrelation function (ACF), which is also indicated as the second order ACF, is measured. The latter is converted to the normalised electric field correlation function $g^{(1)}$, which is also indicated as the normalised first order ACF using the Siegert relationship. Provided that a sample containing two components (A and B) and its two components are measured with the same photomultiplier aperture, its ACF can be written as (de Vleeschauwer & Van der Meeren, 1998):

$$I_{\mathrm{A+B}} * {g^{(1)}}_{\mathrm{A+B}}(\tau) = I_{\mathrm{A}} * {g^{(1)}}_{\mathrm{A}}(\tau) + I_{\mathrm{B}} * {g^{(1)}}_{\mathrm{B}}(\tau) \quad (1)$$

$$I_{\mathrm{A+B}} = I_{\mathrm{A}} + I_{\mathrm{B}} \quad (2)$$

Where $I_{\mathrm{A+B}}$ is the count rate due to components A and B, I_{A} represents the count rate of component A, whereas I_{B} corresponds to the count rate of component B. It follows that both the count rate and the autocorrelation function (ACF) of one of the components (i.e.,

casein micelles in this specific case) can be estimated, provided that the count rate and the ACF of the combined sample (i.e., casein micelles and fat globules) and of one of the separated components (i.e., fat globules) can be determined experimentally.

$$g^{(1)}{}_{\mathrm{B}}(\tau) = \frac{I_{\mathrm{A+B}}}{I_{\mathrm{B}}}\left[g^{(1)}{}_{\mathrm{A+B}}(\tau) - \left(\frac{I_{\mathrm{A}}}{I_{\mathrm{A+B}}}\right) * g^{(1)}{}_{\mathrm{A}}(\tau)\right] \quad (3)$$

To obtain the ACF due to casein micelles only in a sample that also contains fat globules, the intensity and ACF of this sample are measured upon dilution in a dilution liquid that does not affect the casein micelles (giving rise to scattering by both casein micelles and fat globules), as well as upon dilution (to the same degree) in a casein dissolving solution (giving rise to scattering by the residual fat globules only). Hereby, both measurements should be done with the same aperture, as well as he same fundamental sampling time.

2.3. Absorbance at 632 nm

To estimate the turbidity, the optical density (also known as the absorbance) of all samples was measured at 632 nm with a UV-VIS spectrophotometer (UV-1600PC, VWR) using disposable cuvettes with a path length of 1 cm. Deionised water was used to set the zero value prior to the measurements. As dilution may give rise to gradual effects as a function of storage time, all measurements were done within 20 min.

2.4. Sample preparation

2.4.1. Skim milk

A dilution series of skim milk was obtained upon sequential 1/1 (v/v) dilutions (from 1/1 to 1/16384) using UF permeate, deionised water, Ca-imidazole buffer, lactose-free SMUF buffer and casein-dissolving solution (i.e., 35 mM EDTA solution), respectively.

2.4.2. Homogenised semi-skimmed milk

The semi-skimmed milk was homogenised using a Microfluidizer 110 S (Microfluidics Corporation, Newton, MA, USA) at 55 °C and a compressed air pressure of 0.1 MPa (corresponding to a liquid pressure of 14 MPa) for one pass. Subsequently, the homogenised semi-skimmed milk was diluted with the UF permeate and 35 mM EDTA solution (1/256) for particle size measurement.

2.4.3. Lab-scale skim milk

The unprocessed semi-skimmed milk was centrifuged using a Sigma 3-16P centrifuge at 3000 × *g* and ambient temperature for 20 min to separate the fat layer. Afterwards, the thick fat layer was removed by a spatula and the residual milk was collected as skim milk for size measurement. To correct for a possible effect of microfluidisation on the casein micelle characteristics, this lab-scale skimmed milk was subsequently subjected to the same microfluidisation process as described in section

237 2.4.2.

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239 *2.5. Data analysis*

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241 Data are presented as the mean ± standard deviation of at least three replicates.
242 The difference between samples was evaluated using one-way ANOVA. Besides, power
243 law fitting and linear regression fitting were performed using GraphPad Prism 8.0
244 software (San Diego, CA, USA).

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246 **3. Results and discussion**

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248 *3.1. Effect of degree of dilution of skim milk on the DLS measurements*

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250 The optical density of diluted skim milk was determined as a first and simple
251 estimation of the count rate during DLS measurements. As dilution may give rise to
252 gradual effects as a function of storage time, all measurements were done within 20 min.
253 Both the absorbance values and the dilution degree are shown on a logarithmic scale in
254 Fig. 1, where the points should yield a straight line (with slope equal to –1) in the
255 absence of multiple scattering and of dilution-induced structural changes, based on the
256 inverse relationship between OD and dilution degree (DD):

257 $$OD = OD_{\text{undiluted}}/DD$$

258 $$\log(OD) = \log(OD_{\text{undiluted}}) - \log(DD)$$

The linear part of the data points of each sample diluted by different media was selected to fit a power law model. Statistical analysis revealed that the estimated power was not significantly different from the theoretically expected value of –1 for four out of the five dilution liquids, whereas for water as diluent it was (Supplementary material Table S1). This already indicated that dilution with water induced a more pronounced decrease in OD than expected by dilution only, and hence points to the fact that the scattering particles became affected by the dilution with distilled water. Especially, all OD values were highly similar (except for dilution with EDTA) when the dilution degree was below 2^5, which is due to the fact that the dissociation of the proteins is also concentration-dependent. As the undiluted sample has a dilution degree of 1 (i.e., 2^0 on the X axis), the constant of the power law model (Supplementary material Table S1) indicates the extrapolated contribution of particles in the ideal undiluted situation (i.e., without multiple scattering). For the samples diluted with UF permeate, Ca-imidazole buffer, and lactose-free SMUF, the constant values referred to the contribution from casein micelles and fat globules, whereas for the sample diluted with 35 mM EDTA, the constant only reflected the contribution of fat globules. Besides, the OD value from the sample diluted with water was lower compared with the other three buffers from a dilution degree of 2^5 onwards, sustaining the hypothesis that the intensity not only decreased by dilution, but also by the disintegration of some of the scattering particles (i.e., the casein micelles).

As shown in Fig. 2b, the apparent hydrodynamic diameter of the skim milk sample diluted by all the diluents except for the casein dissolving (EDTA) solution continuously increased as the dilution degree increased from 2^1 to 2^4, which was due to

281 the multiple scattering of the milk sample at high concentrations. Meanwhile, the merit
282 value was below 50% during this stage (as shown in Supplementary material Fig. S1b).
283 Fig. 2a indicates that OD measurements can be used as a quick quality control: OD
284 values above 1.0 caused pronounced multiple scattering during DLS.
285 Likewise, there is a minimum photon count rate required to achieve a good
286 signal-to-noise ratio. Otherwise, large variations in apparent hydrodynamic diameter
287 between three replicate measurements were observed, as shown in Fig. 2b when the
288 dilution degree was over 2^{10} (using UF permeate, Ca-imidazole buffer, and lactose-free
289 SMUF buffer). Fig. 2a indicates that an OD of at least 0.008 is needed to ensure at least
290 10 counts ms^{-1} in the experimental conditions used; the latter seems a useful rule of
291 thumb since the reproducibility of the estimated size became negatively affected at
292 lower values. At high dilution degrees, weak scattering by the dilution liquid may
293 interfere with the scattering by the particles of interest. In this respect, it can be
294 mentioned that the scattered intensity of water, Ca-imidazole buffer, EDTA-solution,
295 lactose-free SMUF and UF permeate was 0.5, 0.8, 0.5, 1 and 2 counts ms^{-1}, respectively.
296 For completeness, it should be mentioned that the count rate of each buffer as measured
297 over a period of 8 days remained constant for the Ca-imidazole buffer, whereas it started
298 to increase after 16 h and 2 days for lactose-free SMUF and UF permeate, respectively.
299 (Supplementary material Fig. S2). This increase in count rate indicated (slow) calcium
300 phosphate precipitation, which could be prevented in the lactose-free SMUF by
301 phosphopeptide addition during preparation (Holt et al., 1986; Zhang et al., 1996).
302 When the dilution degree ranged from 2^5 to 2^9, the apparent hydrodynamic

diameter of the samples diluted by UF permeate, Ca-imidazole buffer and lactose-free SMUF did not change significantly with the dilution degree (Supplementary material Table S2). Moreover, within that range of dilution degrees, the coefficient of variation (i.e., standard deviation relative to the apparent hydrodynamic diameter) was only 1–2% and the merit value was at least 50%. Hence, accurate measurements could be performed when the dilution degree was 2^5 to 2^9 for skim milk diluted with UF permeate, Ca-imidazole buffer or lactose-free SMUF buffer and measured at a scattering angle of 90°. For completeness, it should be mentioned that the absence of a dilution effect on the hydrodynamic size does not necessarily indicate the absence of internal structural changes within the casein micelles. More advanced techniques, such as SAXS or SANS, are needed to study this into more detail.

To study the influence of the scattering angle used, the size of the casein micelles upon dilution by UF permeate was measured at both 90° (which is commonly used in dynamic light scattering set-ups), and 150°, the largest scattering angle that was accessible with the equipment used. The apparent hydrodynamic diameters estimated at these two scattering angles were significantly different (Supplementary material Fig. S3a): the apparent hydrodynamic diameter of the skim milk sample was about 166 nm at 150°, whereas it was around 174 nm at 90°. This observation was fully in line with the fact that larger particles scatter preferentially in the forward direction, and hence become more represented when the scattering angle is decreased (Tran Le et al., 2008). On the other hand, the appropriate dilution degree ranged from 2^5 (to reach a merit of about 50%) to 2^{10} (to avoid excessive standard deviations upon more pronounced dilution)

when a scattering angle of 150° was applied, which was quite in line with the findings at a scattering angle of 90 degrees. For further analyses, a dilution degree of 2^7 at 90° and 2^8 at a scattering angle of 150° was selected.

3.2. Effect of the diluents on the DLS measurement kinetics

Casein micelle dissociation (i.e., either by loss of calcium caseinate or by dissociation of the casein complexes with calcium phosphate nanoclusters) upon dilution is time-dependent as well as casein concentration-dependent. If it is by means of the dissociation of casein complexes with calcium phosphate nanoclusters, dilution can change not just the mass of the micelles but also the shape and average radius of the size distribution because of the dynamic nature of protein-protein interactions by intrinsically disordered proteins. In this respect, a suitable diluent should stabilise the structure of the dispersed particles (i.e., casein micelles in skim milk) for a sufficiently long time even at a high dilution degree. Especially, no time effects should be observed during sample preparation and subsequent sample analysis. All the samples were first held in the temperature-controlled measuring cell for 10 min to ensure thermal equilibration during DLS measurements. Fig. 3a shows the kinetic changes in particle count rate from the skim milk samples diluted with UF permeate, lactose-free SMUF buffer, Ca-imidazole buffer (5 mM $CaCl_2$) and deionised water. It is seen that the particle count rate of the skim milk sample diluted by deionised water dropped vigorously during the consecutive measurements over a period of 2 h. For completeness, it has to be mentioned that despite

of the huge effect on the scattered light intensity, the effect on the estimated particle size was much less pronounced. This phenomenon was probably due to the fact that the contribution of fat globules to the size measurements became pronounced, and thus the size reduction was compromised. Fig. 3a shows a decrease in scattered light intensity for the milk sample diluted with the Ca-imidazole buffer (5 mM $CaCl_2$) during 2 h of measurement. More importantly, a similar effect is observed in the apparent hydrodynamic diameter (Fig. 3b). As the slope of the apparent hydrodynamic diameter versus time was significantly different from zero (Supplementary material Table S3), it follows that Ca-imidazole (5 mM $CaCl_2$) was clearly a better dilution liquid than water, but still induced some obvious alterations when the time frame became longer than 30 min. Regarding the sample diluted with UF permeate and lactose-free SMUF, statistical analysis indicated that the slope for the apparent hydrodynamic diameter versus time was not significantly different from zero during 2 h measurement (Supplementary material Table S3). This result further confirmed that UF permeate and lactose-free SMUF enabled to maintain the size characteristics of casein micelles, even at a high dilution degree (i.e., 2^7) and upon long incubation times (up to 2 h), which is mainly ascribed to their similar ionic components as in milk.

To further investigate the effect of the Ca^{2+} concentration, the effect of the calcium concentrations (i.e., 5, 10, or 20 mM $CaCl_2$) in Ca-imidazole buffer was evaluated (at 15, 25, and 35 °C). Whereas the calcium level hardly affected the viscosity and refractive index of the dilution liquid (Supplementary material Table S4), it had an obvious effect on the count rate as well as particle size evolution as a function of time

after dilution: the count rate and apparent hydrodynamic diameter of the casein micelles dropped continuously as a function of incubation time in the presence of 5 mM $CaCl_2$, whereas the count rate increased gradually when diluted with the buffer containing 20 mM $CaCl_2$. The intermediate calcium concentration (10 mM), on the other hand, exhibited an excellent ability to stabilise the casein micellar count rate and size upon dilution over 2 hours. Highly similar trends were observed also at 15, and 35 °C (Supplementary material Fig. S4): also under these conditions, the 10 mM $CaCl_2$ containing imidazole buffer had the lowest effect on both count rate and estimated size as a function of incubation time. This experimentally observed reduced dissociation rate of casein micelles upon increasing the calcium content (to 10 mM) in the Ca-imidazole buffer was in line with prior observations where 6 mM calcium addition was found to delay the dissociation rate of casein micelles to a larger extent than 3 mM Ca (Holt et al., 1986). For completeness, it can be mentioned that casein micelle dissociation may result from either calcium caseinate dissociation or colloidal calcium phosphate dissociation. Moreover, according to Thomar and Nicolai (2015), the dissociation rate of casein micelles also depends on the casein concentration in the diluted system, which is due to the concentration-dependent dissociation of the proteins to establish an equilibrium distribution of calcium caseinate between the micelles and the continuous phase. As a further consequence, an ideal diluent should contain the necessary salt concentrations as well as caseins. However, the scattered light due to the casein monomers/complexes in this diluent may interfere during light scattering measurements and hence negatively influence the accuracy of size measurements. When it comes to the Ca-imidazole buffer

(10 mM) employed in this investigation, reliable particle size determinations could only be obtained within a short time after dilution since this calcium buffer itself did not contain caseins and did not match the ionic activity product of calcium phosphate in milk.

3.3. Evaluation of the interference of fat globules on the size measurements of casein micelles

Whereas other, more complicated DLS data-analysis tools (such as the CONTIN analysis) are available which (in theory should) enable to recover multimodal particle size distributions, only one broad particle size distribution was obtained that did not enable to resolve the casein micelles from the fat globules for skimmed as well as semi-skimmed milk. In fact, this limited resolution issue of DLS has also been described before (Anderson, Kozak, Coleman, Jämting, & Trau, 2013; Caputo et al., 2021).

To overcome this limitation, a method to (mathematically) eliminate the interference of fat globules during particle size analysis of casein micelles was developed. To investigate its usefulness, semi-skimmed milk was also analysed to blow up the contribution of the interfering fat globules. A homogenisation step was included to reduce the size of the fat droplets in this sample to the submicron range, which was indicated as homogenised semi-skimmed milk. To verify the mathematical calculation, semi-skimmed milk was also subjected to centrifugation for obtaining lab-scale skim milk.

As shown in Table 2, the count rate of the casein micelles in the diluted homogenised semi-skimmed milk was estimated from the count rate of homogenised semi-skimmed milk upon 2^8 dilution in UF permeate diminished by the count rate at the same dilution degree in casein dissolving solution. On the basis of the corrected autocorrelation function, the estimated apparent hydrodynamic diameter of the casein micelles was 168.7±3.0 nm. By comparison with lab-scale skim milk, the count rate of homogenised lab-scale skim milk (i.e., 161.6±2.6 counts ms^{-1}) and its apparent hydrodynamic diameter were not significantly different (P >0.05), which suggested that the homogenisation process had no significant effect on the size of the casein micelles. In addition, the estimated apparent hydrodynamic diameter of the casein micelles in homogenised semi-skimmed milk (i.e., 168.7±3.0 nm) was not significantly different (P >0.05) from that of homogenised skim milk (i.e., 167.2±1.6 nm) and skim milk (i.e., 164.3±1.9 nm). The similar characteristics of the casein micelles in semi-skimmed milk and lab-scale skim milk can also be clearly seen from the fact that the curves of the corrected ACF of semi-skimmed milk and the ACF of the lab-scale skim milk partially overlapped in Fig. 5. Hence, both Table 2 and Fig. 5 indicate that it was feasible to estimate the average size of the casein micelles in semi-skimmed milk by subtracting the contribution of the fat droplets. Considering the limited contribution of the fat globules in the light scattering by skimmed milk (Supplementary material Table S6), the interference of these residual fat globules in casein micelle size estimation was limited (Supplementary material Fig. S5), and hence the mathematical elimination of its contribution had no significant effect.

Based on the results obtained for semi-skimmed milk, the proposed method to mathematically eliminate the interference of fat droplets was preferred over the centrifugation approach to physically accomplish this effect, as the former is expected to have less effect on the casein micelle characteristics. This will be especially true for commercial (homogenised) semi-skimmed milk whereby a higher centrifugal force will be needed to accomplish complete fat droplet removal, leading to an increased probability of selectively removing the larger casein micelles, and hence underestimating the average casein micelle size.

4. Conclusions

We investigated the effect of the type of diluent, the dilution degree, and the time effect on the hydrodynamic size of casein micelles to reveal some pitfalls during DLS particle size analysis. The findings showed that both milk UF permeate and lactose-free SMUF buffer were suitable diluents for native casein micelles in skim milk at 25 °C because they contain similar ionic components. Whereas dilution in calcium imidazole buffer may provide useful particle size results provided that the analyses are done within a short time after dilution, still the former two diluents are preferable whenever possible. Deionised water cannot be used as the dilution medium as it induces dissociation of casein micelles. Additionally, optical density can be used as a quick quality control to estimate the suitable dilution degree. The appropriate dilution degree to obtain reliable and reproducible particle size data by DLS ranged from 2^5 to 2^9 for skim milk.

In the established method, the interference of fat globules on DLS measurements of casein micelles was assessed. When the residual fat content was below 0.1%, the contribution of fat globules to the size estimation of casein micelles could be ignored (as seen in supplementary information). On the other hand, the situation in semi-skimmed milk was more complex, whereby the average particle size was affected by both the casein micelles and fat droplets present. Based on the corrected autocorrelation function, accurate casein micelle size estimation was only possible upon mathematical elimination of the contribution of fat droplets in homogenised semi-skimmed milk.

This research provided a clear and systematic protocol for establishing a method for determining the particle size of casein micelles by dynamic light scattering. Though it is a convenient, non-invasive technique, it has to be kept in mind that DLS is not sensitive to the perturbing effect of diluents on the internal structure of casein micelles as it is merely a particle sizing technique. Therefore, the presence or absence of perturbation mentioned in this study only reflected the size characteristics. More detailed information (i.e., internal structure characteristics) can only be obtained by alternative techniques, such as SAXS or SANS.

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Figure legends

Fig. 1. Optical density (OD) of skim milk at 632 nm upon dilution by different diluents as a function of dilution degree at 25 °C.

Fig. 2. Relation between count rate and OD value (a), as well as the apparent hydrodynamic diameter (D_h) (b) of skim milk diluted by different diluents (●, —, UF permeate; ▲, —, calcium-imidazole buffer; ◆, —, SMUF; ▼, —, deionised water; ■, —, EDTA) as a function of dilution degree (2^1–2^{14}), measured at a scattering angle of 90° at 25 °C.

Fig. 3. The change of count rate (a) and apparent hydrodynamic diameter (D_h) (b) during dynamic light scattering measurements at a scattering angle of 90° for skim milk that was diluted 2^7 times with four different diluents at 25 °C (, UF permeate; , calcium-imidazole buffer; , SMUF; , deionised water; solid lines refer to linear regression analysis).

Fig. 4. The change of count rate (a) and apparent hydrodynamic diameter (D_h) (b) during dynamic light scattering measurements at a scattering angle of 90° at 25 °C for skim milk that was diluted 2^7 times with a calcium imidazole buffer containing either 5 ( ), 10 ( ), or 20 ( ) mM $CaCl_2$. Solid lines refer to linear regression analysis (Supplementary material Table S5).

Fig. 5. Normalised first-order autocorrelation function (ACF) of homogenised semi-skimmed milk (□) and the corresponding fat globules (○), as well as the corrected autocorrelation

function of the casein micelles only (△), as obtained at a scattering angle of 150° at 25 °C. The ACF of lab-scale skim milk is also shown (□). The dilution degree is 2^8, and the data are presented as average ± standard deviation based on three replicates.

Table 1

Viscosity and refractive index of the diluents as determined at 25 °C. [a]

Diluent	Viscosity (mPa s)	Refractive index (-)
Deionised water	0.890±0.002	1.331±0.001
Calcium imidazole buffer		
5 mM $CaCl_2$	0.898±0.001	1.333±0.000
10 mM $CaCl_2$	0.901±0.001	1.333±0.000
20 mM $CaCl_2$	0.903±0.002	1.333±0.000
Lactose-free SMUF buffer	0.904±0.003	1.333±0.000
35 mM EDTA solution	0.928±0.002	1.335±0.001
UF permeate	1.035±0.001	1.342±0.001

[a] Values are the average and standard deviation, based on three repetitions.

Table 2

Count rate and cumulant analysis results of the (corrected) ACF of homogenised semi-skimmed milk, the fat droplets and casein micelles in the homogenised semi-skimmed milk, and of the lab-scale skim milk. [a]

Parameter	Homogenised semi-skimmed milk	Fat droplets	Casein micelles	Lab-scale skim milk	Homogenised lab-scale skim milk
Count rate (m s^{-1})	227.4±1.1	72.4±0.4	154.9±0.8	156.3±2.2	161.6±2.6
R^2 (-)	0.999±0.000	0.999±0.000	0.999±0.000	0.999±0.000	0.999±0.000
Merit (%)	62.8±1.2	58.1±2.5	64.5±1.8	76.2±0.5	74.7±0.6
Apparent hydrodynamic diameter (nm)	202.9±5.6	354.1±26.3	168.7±3.0	164.3±1.9	167.2±1.6
Polydispersity index	0.186±0.033	0.149±0.113	0.125±0.057	0.052±0.005	0.057±0.008

[a] All data are presented as average ± standard deviation of three replicates. The dilution degree of all the samples was 256× and measurements were performed at a scattering angle of 150°. The lab-scale skim milk was obtained from semi-skimmed milk.

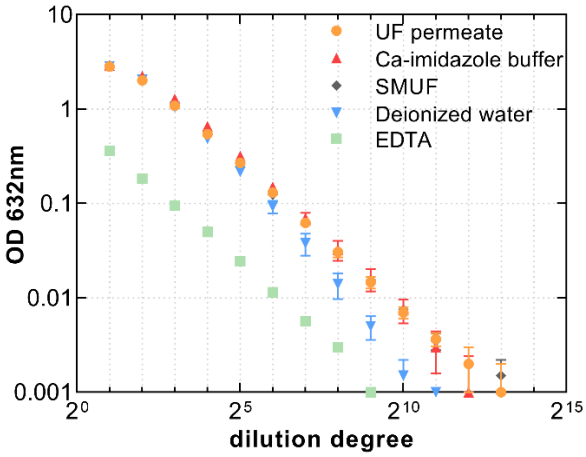
10
1
0.1
0.01
0.001
OD 632nm
2^0
2^5
2^{10}
2^{15}
dilution degree
UF permeate
Ca-imidazole buffer
SMUF
Deionized water
EDTA

Figure 1

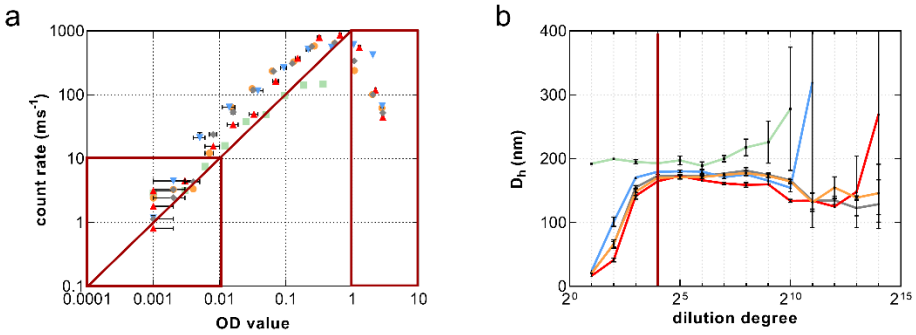

Figure 2

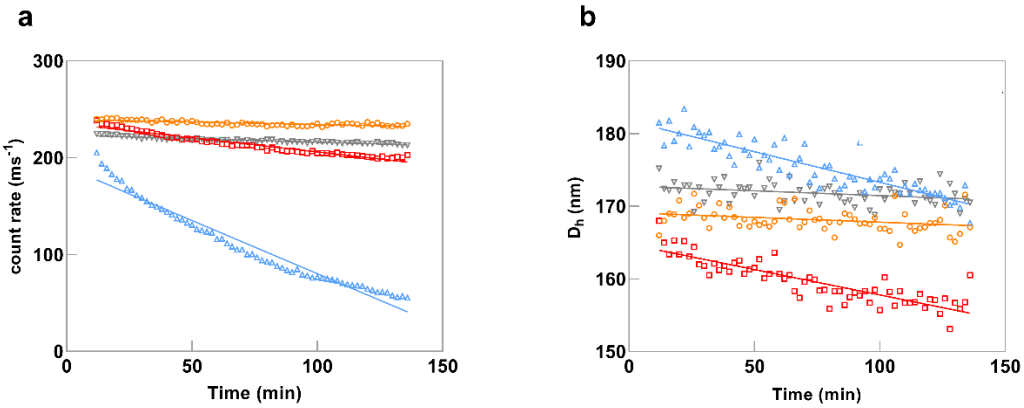
a
300
200
100
0
count rate (ms^{-1})
0
50
100
150
Time (min)
b
190
180
170
160
150
D_h (nm)
0
50
100
150
Time (min)

Figure 3

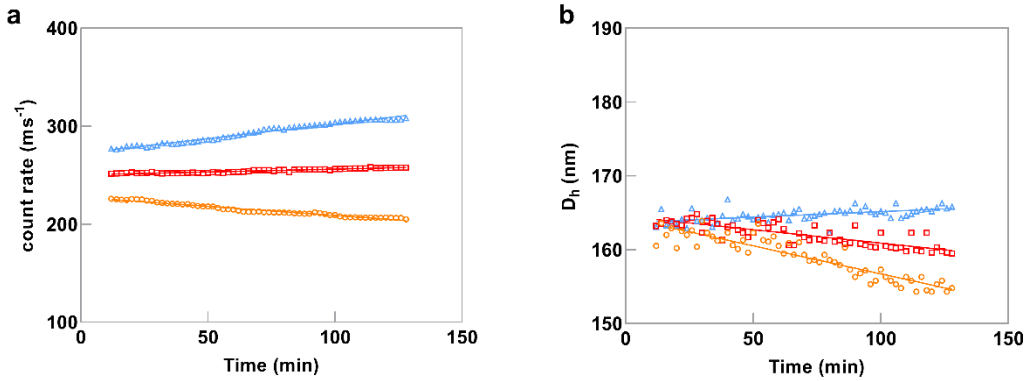
a
400
300
200
100
count rate (ms^{-1})
0
50
100
150
Time (min)
b
190
180
170
160
150
D_h (nm)
0
50
100
150
Time (min)

Figure 4

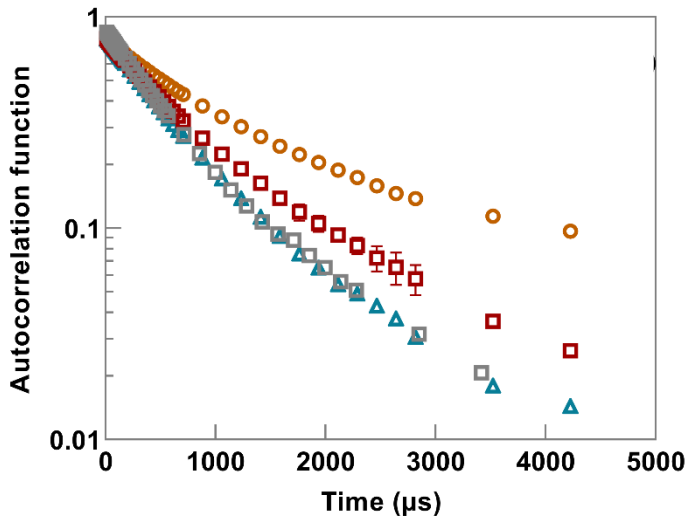
Autocorrelation function
1
0.1
0.01
0
1000
2000
3000
4000
5000
Time (μs)

Figure 5