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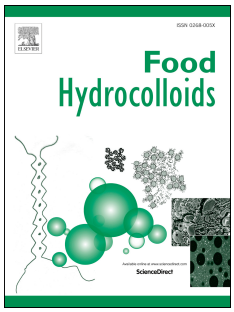

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Author statement

Kilian Daffner: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Original Draft

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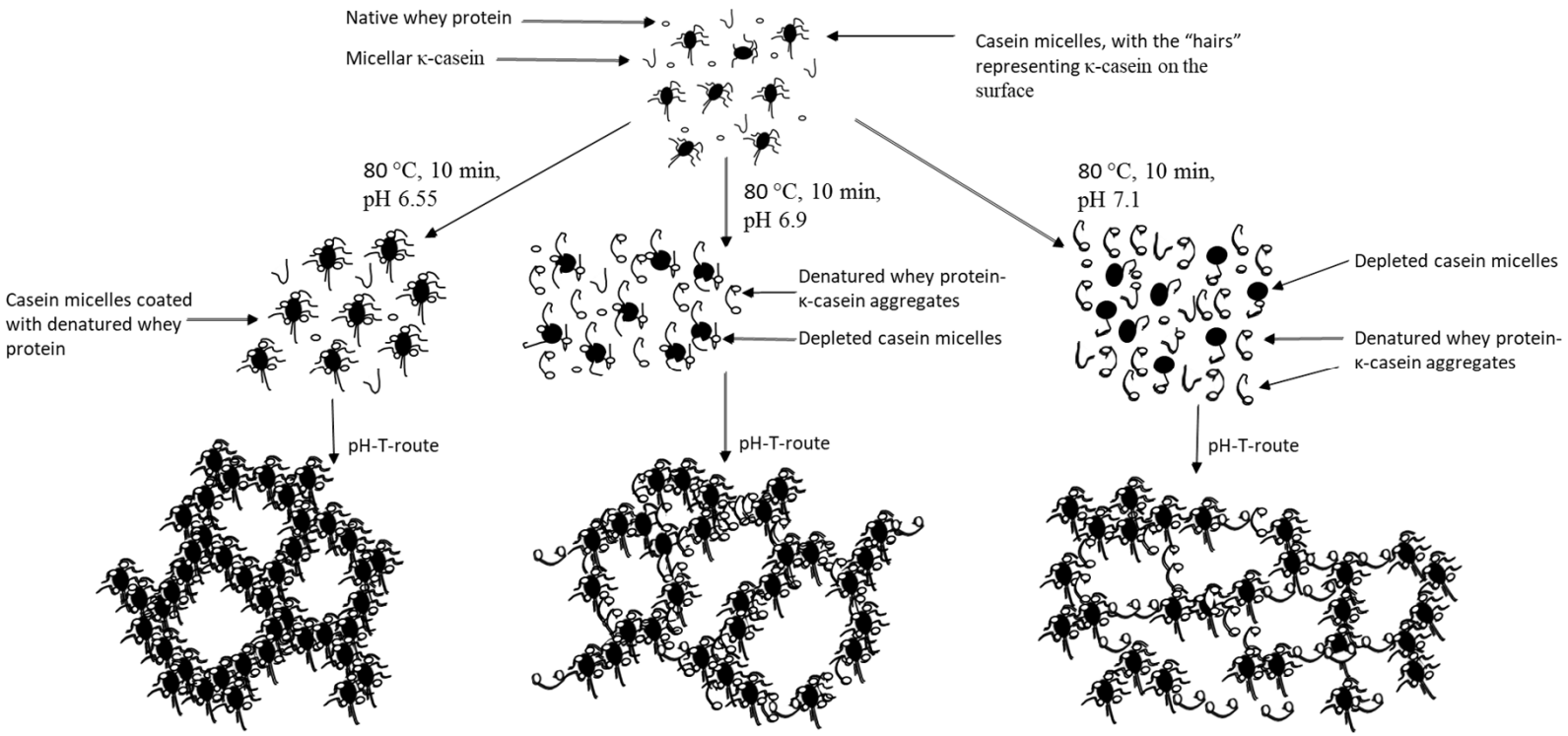
Native whey protein
Micellar κ-casein
Casein micelles, with the "hairs" representing κ-casein on the surface
80 °C, 10 min, pH 6.55
80 °C, 10 min, pH 6.9
80 °C, 10 min, pH 7.1
Casein micelles coated with denatured whey protein
Denatured whey protein-κ-casein aggregates
Depleted casein micelles
Depleted casein micelles
Denatured whey protein-κ-casein aggregates
pH-T-route
pH-T-route
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Journal of Food Hydrocolloids

Design and characterization of casein-whey protein suspensions via the pH-temperature-route for application in extrusion-based 3D-Printing

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A B S T R A C T

The current interest in individualized food through additive manufacturing has identified a need for more information on the formulation and printability of potential ingredients. Here, the effect of formulation parameters of casein-whey protein suspensions like the pH (4.8-5.4) as well as the casein content (8.0-12.0% (w/w)) mixed with whey protein (2.0-3.0% (w/w)) and the effect of pre-processing parameters including the denaturation of whey proteins (80 °C, 10 min; adjusted pH 6.55, 6.9 and 7.1) on the gel formation via the pH-temperature (T)-route was studied. Rheological measurements showed that the sol-gel transition temperature (G' = 1 Pa) decreased and the aggregation rate of the casein-whey protein suspensions increased with increasing heating pH value. The aggregation rate was considered to be a key parameter predicting the printability of formulations. By exceeding a certain aggregation rate (250 Pa/10 K), casein-whey protein suspensions were found to be printable resulting in firm and stable gels.

1 Introduction

3D-Printing is a new trend in the food sector that is receiving increasing attention. In addition to the traditional additive manufacturing process with plastic or metal as feed material, 3D-Printing can be conducted as food layered manufacturing (FLM). In general, 3D-Printing is an additive manufacturing technology with a layer by layer deposition of the material to form an object that cannot be created with conventional techniques. Technologies like controlled fusion and controlled deposition are amongst the most popular for FLM (Wegrzyn, Golding, & Archer, 2012). Although most FLM technologies are still being researched, different food materials have successfully been printed. The complex microstructure of food and the sol-gel transition of food ingredients (e.g. crystallization of cocoa butter from liquid to solid to manufacture chocolate or protein-based gelation for dairy products) are just two of the challenges that must be addressed to implement food for 3D-Printing, which could result in highly customized products for individuals (Ross, Kelly, & Crowley, 2019). The design of edible, printable and individualized casein-whey protein suspensions and the assessment of suspension printability is therefore a step towards the printing of more types of food.

Recent studies have shown that dairy-based materials can be used for 3D-Printing, with the first being processed cheese (Le Tohic et al., 2018). A significant decrease (49 %) of the hardness for melted and printed cheese was found compared to untreated cheese. Moreover, sodium caseinate, which showed reversible gelation characteristics, was used for extrusion-based printing (Schutyser, Houlder, de Wit, Buijsse, & Alting, 2018). The addition of pectin, sucrose and starch facilitated the printing process. Nöbel et al. (Nöbel, Seifert, Schäfer, Daffner, & Hinrichs, 2018) used cold acidified concentrates from milk microfiltration differing in pH (4.8-5.4) and protein content (8-12% (w/w)) which were heated in an extrusion-based 3D-Printer to induce a sol-gel transition. At pH 4.8, firm and homogeneous milk gels were printed, while milk gels at pH 5.0 were not mechanically stable after printing. For fur-

ther information about 3D-printed dairy-based materials, a review (Voon, An, Wong, Zhang, & Chua, 2019) is recommended.

Bovine milk contains about 34 g L^{-1} proteins in the form of casein and whey protein. Casein represents around 80 % of the protein content and consists of four main types (α_{s1}- (40%), α_{s2}- (10%), β- (40%) and κ-casein (10%)) which together with colloidal calcium phosphate form complexes called casein micelles (CM). The hydrodynamic diameter of the CM is about 200 nm and the zeta-potential is about -19 mV at the native pH of 6.7 (Anema & Klostermeyer, 1996). Three main interactions, negative charge, steric repulsion and surface hydration between the layers of κ-casein around the micelles, stabilise the CM against aggregation (Heertje, Visser, & Smits, 1985; Horne, 1986).

The whey proteins are globular proteins with defined secondary and tertiary structure. At temperatures around 70 °C, they denature and interact or undergo aggregation reactions with κ-casein (Anema, 2008a), mainly via thiol-disulfide exchange reactions. Depending on the heating pH, different types of interaction between denatured whey proteins and CM occur (Anema, Lee, Lowe, & Klostermeyer, 2004a; Anema, Lowe, & Lee, 2004b). When heating at pH 6.5, around 70% of the denatured whey proteins were attached to the surface of the CM, increasing its diameter up to 30-35 nm. With increased heating at pH 6.7, the level of association decreased with around 30% whey proteins covering the CM (Anema & Li, 2003a). This interaction depended on the location of κ-casein, which dissociated from the CM with increasing heating pH (Singh & Fox, 1985; Anema & Klostermeyer, 1997). It is unclear why dissociation occurred, although κ-casein dissociated into the serum at temperatures lower than required for whey protein denaturation (Anema & Klostermeyer, 1997; Anema, 2008a).

During a traditional fermentation process for dairy products at constant temperature, gelation of milk occurs due to a reduction of the pH value by lactic acid bacteria and following the decrease of the net charge of the CM. While the fermentation process (T-pH route) takes sev-

eral hours under steady conditions, the alternative pH-Temperature (T)-route can be used for immediate solidification during heat up of milk concentrates that have been pre-acidified in the cold. For the pH-T route, the two steps of acidification and gelation, which normally overlap in the fermentation processes (T-pH), occur separately, showing great potential for 3D-Printing under dynamic conditions (Nöbel et al., 2018). Cold pre-acidification of milk up to pH 4.6 at less than 4 °C reduced the hydrophobic interactions and helped to maintain solution (sol)-characteristics. Subsequent heating of the acidified material caused collision and aggregation of the CM and gelation occurred (Roefs, 1986; Schäfer et al., 2018). Vasbinder et al. (Vasbinder, Rollema, Bot, & De Kruif, 2003a) also found that firmer gels were obtained via the pH-T-route compared to the T-pH-route at the same concentration. Recent studies (Silva, Balakrishnan, Schmitt, Chassenieux, & Nicolai, 2018; Kharlamova, Nicolai, & Chassenieux, 2019) showed the effect of adding native as well as denatured whey protein on the gelation behaviour of casein micelles via the pH-T route. Kharlamova et al. (2019) showed that the addition of fractal whey protein isolate aggregates to aqueous suspensions of micellar casein lowered the temperature of gelation (at a fixed micellar casein concentration) and increased the Storage Modulus G' of the milk gels. A further decrease of the gelation temperature was found, if the pH was decreased, the protein concentration increased or $CaCl_2$ added.

The success of 3D-Printing as a method to produce highly individualized and tailored nutrition for specific requirements will strongly depend on the food materials and the printability of the recipes. To the best of our knowledge, no prior studies have investigated the usage of micellar casein combined with whey protein for extrusion-based 3D-Printing via the pH-T route, inclusive a tailored sol-gel transition. Casein-based microgels can be induced with mechanical processing, heat treatment, changes in the environmental conditions (e.g. pH) or in surface properties (interaction with whey proteins) (Loewen, Nöbel, & Hinrichs, 2017). We hypothesized that by altering one of the pre-processing parameters, e.g. the pH during heat

treatment, tailoring of the surface characteristics of the CM causes changes in the sol-gel transition temperature and thus, increases the aggregation rate. Either weaker or firmer printable gels for tailored nutrition can potentially be provided by this method. Four parameters, the protein content, casein-whey protein ratio, heating - and acidification pH of the casein-whey protein suspensions were adjusted to investigate the material characteristics and to correlate these properties with printability.

2 Material and methods

2.1 Material

Micellar casein concentrate (MCC 85) was provided by Sachsenmilch Milk & Whey Ingredients (Sachsenmilch Leppersdorf GmbH, Wachau, Germany). As specified by the manufacturer, MCC 85 was composed of 85% (w/w) protein in dry matter, with a 9:1 ratio of casein to whey protein. This specific batch of MCC 85 contained 87.60% (w/w) protein in dry matter, 1.45% (w/w) fat, 2.48% (w/w) lactose and 7.44% (w/w) ash. GermanProt 9000 - Whey protein isolate (WPI) was provided by the same manufacturer. This specific batch was composed of 93.74% (w/w) protein in dry matter, 0.23% (w/w) fat, 0.61% (w/w) of lactose and 3.16% (w/w) of ash. Citric acid (1M) (Sigma Aldrich, UK) was prepared by mixing with Milli-Q water (Elix® 5 distillation apparatus, Millipore®, USA) and sodium hydroxide (1M) was bought from Sigma Aldrich (UK) and used for pH adjustment.

2.2 Sample preparation

Solutions of micellar casein were prepared using deionized water and agitated at a constant speed for 5 h at 40 °C to disperse the powder. The suspensions were cooled to room temperature and stored at 4 °C overnight to allow hydration of the caseins, with starting pH values of 6.7 ± 0.05. Three different casein contents (8.0-, 10.0- or 12.0% (w/w)) were tested. To adjust the casein to whey protein ratio to 4:1 and to keep impurities (high lactose content, for example in skim milk powder) as low as possible, powdered whey protein isolate was added to all

three micellar casein contents. A total amount of 2.0-, 2.5- or 3.0% (w/w) of whey protein was mixed with casein, dispersed in deionized water and heated at 40 °C for 5 h. A high pressure homogenizer (Panda NS1001L-2K, Gea Niro Soavi, Parma, Italy) was used to ensure a homogenous distribution of the particles (one pass, 500 bar). The influence of the denaturation of whey proteins on the sol-gel transition temperature and the aggregation rate was tested as follows. The pH of the casein-whey protein suspension was adjusted to 6.55 (1M citric acid) or to 6.9/7.1 (1M NaOH) before heat treatment. Bottles containing the suspensions were indirectly heated (80 °C, 10 min) in a water bath on a stirring plate to ensure denaturation (degree of denaturation$_{\beta\text{-LG}} \geq 80\%$; as estimated from Kessler, 2002) of the whey proteins. After heating, samples were filled in a double-walled beaker connected to a water bath at 2 °C. Cold acidification was performed dropwise with citric acid under agitation. After decreasing the pH (5.4-4.8), the solution was equilibrated for 30 min and the pH was readjusted, if changes occurred (Schäfer et al., 2018; Nöbel et al., 2018).

2.3 Rheology

Rheological measurements were conducted by a Kinexus Pro rheometer (Malvern Instruments, UK) with a cup (D = 27.17 mm, depth = 63.5)-vane (d = 61 mm, height = 25 mm)-geometry. For dynamic oscillatory measurements, temperature sweeps were performed from 2-60°C with a heating rate of 1.0 K min^{-1}. The samples were pre-sheared with a shear rate of 100 s^{-1}, followed by an equilibration time for 300 s with no shearing. The oscillation step was performed with a deformation of 0.3 % and a frequency of 1 rad s^{-1} to ensure a non-destructive measurement in the linear viscoelastic region of the final gel. During measurements the Storage Modulus (G'), the Loss Modulus (G''), the Phase Angle ($tan\ \delta$) and the temperature were monitored. The sol-gel transition temperature was determined when G' reached a value of 1 Pa (Schäfer et al., 2018, Nöbel et al., 2018).

2.4 Zeta-potential and particle size measurements

The particle size and the zeta (ζ)-potential were determined using a Zetasizer (Malvern Instruments, UK). For particle size measurements, samples were prepared at native pH (6.7) by diluting them with deionized water and measured at 20 °C. Casein-whey protein suspensions were analysed in the exact same way after the denaturation step at different pH values (6.55, 6.9, 7.1). Refraction indices of 1.33 (water) and 1.57 (casein) were adjusted for the volume-based particle size distribution (Griffin and Griffin, 1985), calculated via the Mie theory. Averaged particle sizes were presented by the intensity weighted mean hydrodynamic diameter z-average.

After a stepwise (0.2 units) decrease of the pH to lower values than the native pH (6.7) by means of 1M citric acid, the ζ-potential was measured and plotted against the solution pH. Before every experiment, samples were diluted with deionized water. The temperature of each sample, the water (diluent) and within the zetasizer was adjusted to 2 °C to ensure no pre-gelation or precipitation of the caseins during measurements.

2.5 SDS-PAGE

2.5.1 Preparation of the samples via centrifugation

After heating (80 °C, 10 min) at different pH values (6.55, 6.9 and 7.1) with continuous rocking, an amount of 1.5 ml for each sample was placed in small plastic tubes. Serum proteins were defined as the particles that did not sediment from the sample during a centrifugation step (Type 5424, Eppendorf, North Ryde, Australia) at 21.000g at 20 °C for 1 h (Anema, 2007). The supernatants were analysed on the same day.

2.5.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed using precast Bis-Tris 12% polyacrylamide gels and an electrophoresis system (Invitrogen, Mount Waverley, Australia). Centrifugal supernatants were diluted (1:25) with deionized water and mixed (10 µl) with 5 µl of LDS (Sigma Aldrich, UK), 2 µl of reducing agent (Sigma Aldrich, UK) and 5 µl of β-mercaptoethanol (Bio-Rad Laborato-

ries Ltd., UK). Each of the non-heated reference samples was diluted in a similar way and the same amount of reagents was added. All samples were heated at 100 °C for 3 min prior to loading. The gels were run at 115 V for 90 min and a solution containing 0.025% w/v Coomassie (Bio-Rad Laboratories Ltd., Watford, UK), 40% w/w methanol and 7.5% w/w acetic acid was used for staining for 90 min and a 7.5% w/w acetic acid solution was used for destaining the gels overnight. Gels were visualized using a Fuji Film Intelligent Dark Box II with Fuji Film LAS-3000 V2.2 software (Brookvale, Australia). The proteins were identified by comparison to the molecular weight (Mw 10-250 kDa) of protein standards (Precision Plus, Kaleidoscope, BioRad, Australia).

2.6 Set-up of a customized 3D-Printer

A customized printer was created to print dairy materials via the pH-T route, including a sol-gel transition, similar to Nöbel et al. (2018). A commercially available plastic printer (Creality Ender 3 Printer; Creality, Shenzhen, China) was retrofitted. For better control of the additional weight of the new parts, the motor and the syringe with the double walled cooling jacket were fixed on the top of the printer. The stepper motor, connected to a screw plunger, controlled and powered the syringe to perform the extrusion-based printing. Two stainless steel bars held all the parts in line and several computer-aided design (CAD)-printed parts supported the straight attachment of the motor.

Before the printing process, the syringe was loaded with 60 ml of the cold acidified casein-whey protein suspension. To maintain sol-characteristics, a temperature of 2 °C was maintained within the syringe cooling jacket, connected to a water bath. Materials were transported via a pipe to the copper nozzle (plastic dye at the end, 1.15 mm in diameter), heated with the heating element and a sol-gel transition was induced. The software used to control the printer was Repetier. To create a printed object, a CAD model (25 x 25 x 3 mm rectangle slice) of the object was created in the software. The printing line speed was set to 20 mm s^{-1}. The layer

height was adjusted to 1 mm for all three layers, which were printed above each other. This resulted in an overall height of around 3mm for every printed rectangle. Before each print, the bed level had to be calibrated manually. Printing was performed on a hydrophobic printing paper (10 x 10 cm; Legamaster International B.V., The Netherlands) to prevent spreading of the first layer.

2.7 Statistics

The data plotted in the publication includes the average of at least three measurements accompanied by error bars that consist of the standard deviation of the mean. In the case where mean values of an observation are compared between samples the data have been subjected to analysis of variance (ANOVA) in order to determine significant differences. Data analysis was conducted with Sigma Plot 12.5 (Systat Software Inc., San Jose, CA, USA). Individual samples were compared with Student's t-test and a level of significance of $p < 0.05$ was chosen.

3 Results and discussion

3.1 Physico-chemical characterization of the sol-state

3.1.1 Particle size

The rheological characteristics of colloidal gels formed from milk proteins depend on intrinsic - (e.g. size, shape, availability, protein content) and extrinsic parameters (e.g. temperature, ionic strength, pH) (Dickinson, 2016). The size of the CM was considered to be a crucial parameter influencing the printing characteristics of the starting material, due to the requirement of a fast and irreversible sol-gel transition from sol to gel during the printing process (Nöbel et al., 2018). Smaller particles move faster due to Brownian motion, which can result in a faster aggregation process and be expected to provide denser and hence, firmer gels during printing. The volume-based distribution of the casein-whey protein suspensions is shown in Fig. 2, with protein particles ranging between 50-650 nm. After a homogenization step, per-

formed to avoid any aggregates that may have formed during hydration overnight, the average intensity weighted diameter (z-average) of the protein particles in all formulations, independent of the heating pH, was around 230-240 nm. Anema et al. (2004b) measured the size of CM in reconstituted skim milk and found a similar value of around 215 nm for unheated milk at pH 6.5. Heating at the same pH for 30 min at 80 °C, 90 °C and 100 °C resulted in an increase of the size of 15-, 30- and 40 nm respectively. This small increase in the size of the CM was explained by the interaction of the denatured whey proteins, especially β-lactoglobulin, with κ-casein on the surface of the CM via thiol-disulfide bond exchange reactions as well as hydrophobic and ionic interactions. Due to higher temperatures and a longer heating time, it is proposed that Anema et al. (2004b) were able to show a significant decrease in the size of the CM at higher heating pH compared to results in this study. The range of sizes of particles was confirmed with SEM - (showing the raspberry structure of the CM) and cryo-EM images (see Supplementary Fig 1).

3.1.2 Zeta-Potential

The ζ-potential as a function of the pH of pure, non-heated micellar casein - and heated (pH 6.55, 6.9, 7.1) casein-whey protein suspensions is shown in Fig. 3. The ζ-potential of each formulation was averaged for all the protein particles captured within this sample. Independent of the type of formulation, an almost linear decrease of the ζ-potential with decreasing acidification pH was found. A significant downshift of up to 5 mV of the ζ-potential of heated casein-whey protein suspensions over the whole range of pH values was evident compared to the non-heated sample. Heating resulted in physical and chemical changes, e.g. precipitation of calcium phosphate and desposohorylation of caseins, which altered the ζ-potential of the CM (Fox, 1981; Singh & Creamer, 1992). No significant changes in the ζ-potential over all acidification pH values were found for any of the pH adjusted conditions performed before the heating step (6.55, 6.9, 7.1). Anema & Klostermeyer (1996) found that CM heated at pH 6.6 showed a higher (more negative) ζ-potential than CM heated at pH 7.1, although only

very small differences were found below pH 6.0 and no measure of variation was provided in their publication.

Darling and Dickinson (1979) reported the same almost linear decrease in the ζ-potential of CM when the pH was reduced, although significantly lower values were found. At pH 4.75, Anema & Klostermeyer (1996) found a ζ-potential of -13.5 mV (30 °C) for heated milk samples (120 °C, 6 min) which was very similar compared -13.1 mV ± 1.3 mV at pH 4.8 for non-heated, pure casein suspensions in our study. This was not in accordance with the results of Darling & Dickson (1979) who found a value of 0 mV at the IEP of casein (4.6). It has been suggested that an increasing ζ-potential was caused by a dissociation of colloidal calcium phosphate, which increased with decreasing pH up to a complete solubilisation at around pH 5.3 (Dalgleish & Law, 1988). As a result, ionic calcium binds to the surface of the CM, thereby reducing the electrophoretic mobility and decreasing the ζ-potential of CM (Dalgleish, 1984). Anema & Klostermeyer (1996) proposed that this binding of the calcium to the CM shields their negative charges, resulting in a decrease of the ζ-potential.

3.1.3 SDS-PAGE

Fig 4 shows the SDS-PAGE analysis of the supernatants obtained from non-heated (6.55, 6.9, 7.1) - and heated (6.55, 6.9, 7.1) casein-whey protein suspensions following centrifugation, with a main focus on the disintegration of the casein micelle. Heat treatment at pH 6.55 resulted in a significantly lower amount of whey proteins in the supernatant, confirming other results with whey protein (mainly β-lactoglobulin) covering the surface and being bound to the CM at this pH (Anema et al., 2004a; Anema, 2007). Increasing the heating pH to 6.9 and 7.1 caused increasing amounts of κ-casein dissociating from the CM into the serum, resulting in additional free κ-casein-whey protein complexes in the serum and decreased levels of CM covered with whey proteins, evidenced by the same amount of whey proteins in the supernatant. Our results are consistent with Anema (2008a), who demonstrated the pH dependent

dissociation of κ-casein on heating. It is estimated that a less significant difference in κ-casein dissociation between the lower and the higher heating pH values compared to other results (Anema, 2007; Anema, 2008a; Anema, 2008b) was due to a lower heating temperature during our denaturation process, but could also be explained with a lower protein concentration used in the studies of Anema (2008a). Singh & Creamer (1991) found that the dissociation of κ-casein from the CM depended on two parameters, the pH and the total solids content of the sample before a heating process. Both parameters, higher heating pH values (6.5-7.1) and higher proteins concentration, caused an increase in the extent of dissociation out of the CM.

3.2 Rheological characterization of the sol-gel transition

The gelation behaviour of casein-based milk concentrates at different acidification pH-protein combinations showed promising results for extrusion-based 3D-Printing (Nöbel et al., 2018). The sol-gel transition temperatures were obtained by temperature sweeps from the point when the Storage Modulus G' equalled 1 Pa (Schäfer et al., 2018, Nöbel et al., 2018). This value could be further used to adjust the temperature in the nozzle during the printing process to intentionally induce a sol-gel transition. In Fig. 5 (B-D) the sol-gel transition temperatures of cold acidified casein-whey protein suspensions after a heating step at different pH values (6.55, 6.9, 7.1) are illustrated. For comparison, the result of a pure micellar casein sample (no addition of whey protein, no heating step) is provided in each subfigure and discussed first. Fig 5 (A) depicts the sol-gel transition temperature of micellar casein suspensions differing in the protein content as function of the acidification pH, which was chosen according to previous results (Nöbel et al., 2018).

For casein suspensions a linear relationship between the acidification pH and the sol-gel transition temperature (G' = 1 Pa) was found. As shown in Fig 5 (A), the CM stayed in colloidal solution below the coagulation line and direct acidification followed by heating resulted in gelation of the CM. Above the coagulation line, spontaneous coagulation occurred during

acidification and the casein precipitated (Hammelehle, Schkoda, & Kessler, 1997; Schäfer et al., 2018). The coagulation line depends on the composition (pH, protein content) and the pre-treatment (e.g. heating time, -temperature and - pH). At all protein contents, sol-gel transition temperatures decreased significantly with decreasing acidification pH, as observed elsewhere for microfiltrated skim milk retentates (Schäfer et al., 2018), rehydrated phosphocaseinate powders (Thomar & Nicolai, 2016), reconstituted pasteurised skim milk (Vasbinder & De Kruif, 2003b) and micellar casein suspensions with added whey protein isolate (Kharlamova et al., 2019).

The sol-gel transition temperatures for heated (pH 6.55) casein-whey protein suspensions also decreased with decreasing acidification pH (Fig. 5, B). Suspensions at the highest protein content showed significantly lower sol-gel transition temperatures at pH 5.0, 5.2 and 5.4 compared to lower protein concentrations, while pre-gelation was found at pH 4.8 (G' > 1 Pa). This significant decrease of the sol-gel transition temperature at higher protein contents was proposed to appear with an increased amount of particles per unit volume, actively contributing to the gelation process. The mixed casein (8-10% (w/w)) - whey protein (2-2.5% (w/w)) suspension that was heated at pH 6.55 showed a similar coagulation line during acidification when compared to the non-heated casein sample.

For all casein-whey protein suspensions after a heating pH of 6.55, independent of the acidification pH, no sol-gel transition temperatures were obtained at the highest protein concentration (12.0% (w/w) CS and 3.0% (w/w) WPI). At an acidification pH of 5.3, CM started to collide and aggregate, resulting in bigger particles, which caused unintended pre-gelation. This was evidenced by an increase of the Storage Modulus (G' > 1 Pa) prior to heating, preventing any sol-gel transition. For the highest heating pH (7.1), all sol-gel transition temperatures of the formulations with the medium protein content were found below the coagulation line of pure micellar casein, indicating the dependency of the gelation point on the protein

content during acidification. Fig. 5 (C) and (D) clearly showed that heating at a higher pH (6.9, 7.1) caused a shift of the coagulation line towards higher acidification pH values, explained with earlier acid induced coagulation of the surface-modified protein particles, confirming results of Hammelehle (1994).

3.3 Influence of the aggregation rate on printability

The aggregation rate (Pa/ 10 K), represented by the evolution of the Storage Moduli G' during linear temperature ramps, was chosen to be an important parameter to characterize the formulations differing in product - (pH and protein content) and pre-process parameters (heating pH) regarding their printability (three layers printed). The aggregation rate was the parameter which was analysed when comparing the increase of the Storage Modulus G' from the sol-gel transition temperature (G' =1 Pa) until the next 10 K, as demonstrated with a casein-whey protein suspension in Fig. 6. This specific value of ten degrees above the sol-gel transition temperature was chosen to compare how fast and how strong a three-dimensional network was built after the sol-gel transition temperature was reached. A fast aggregation rate of the CM was considered as a prerequisite for a sol-gel transition from sol to gel to obtain a firm gel network, supporting its own weight during an extrusion-based layer by layer printing process.

In a previous study (Nöbel et al., 2018), pure casein-based suspensions were found to be printable at an acidification pH of 4.8, showing firm and homogeneous characteristics, while milk gels at pH 5.0 were not mechanically stable after the extrusion-based printing process. Within our study, only the aggregation rate of a non-heated, pure micellar casein-based suspensions (Fig. 7) was shown for comparison with casein-whey protein suspensions, but no printing was performed for these samples. An increase in the casein content from lowest - (8.0% (w/w)) to highest values (12.0% (w/w)) resulted in significantly firmer gels, regardless of the acidification pH value.

The aggregation rate of casein-whey protein suspensions after a heating step (at pH 6.55, 6.9, 7.1) is illustrated in Fig. 8-10 and related to the printability by means of inset pictures. The aggregation rate of the casein-whey protein suspensions, that significantly depended on the protein content, the acidification - and the heating pH values, was compared to the 3D-printed images (25 x 25 x 3 mm, 3 layers). At the lowest heating pH (6.55), the aggregation rate increased with decreasing pH and increasing protein content, apart from the highest protein content where the aggregation rate was not determined because of pre-gelation behaviour (Fig. 8). All casein-whey protein suspensions at an acidification pH value of 4.8 showed firm and stable gels after the printing process, while higher acidification pH values ($\geq$ 5.0) resulted in spreading of the material over the printing bed and no stable three-layered printing process.

An almost linear increase in the aggregation rate with decreasing acidification pH and increasing protein content was found after a heating step at pH 6.9 and pH 7.1 (Fig. 9 and Fig. 10). All casein-whey protein suspensions with an aggregation rate higher than a chosen threshold of about 250 Pa/ 10 K showed firm gels after the printing process and maintained their shape. For the most promising formulation (pH 4.8, 10% CS, 2.5% WP, heated pH 6.9; compare Fig. 9) regarding the fastest aggregation from oscillatory measurements (315.5 ± 27.2 Pa/ 10K), pre-gelation occurred during real printing processes. The clogged nozzle did not allow printing due to a low sol-gel transition temperature of 7.6 ± 0.6 °C (Fig 5, (C)) and high absolute protein content of the sample. Slight fluctuations in the temperature during conveying may have induced this unintended gelation.

The aggregation rate of the casein-whey protein suspensions after a heating pH of 7.1 increased with decreasing acidification pH, showing significantly lower values at the higher acidification pH values (5.2, 5.4) and lower protein content (Fig 10). The increase in the aggregation rate at the fixed lower protein content with decreasing pH was in accordance with the results of formulations heated at pH 6.9 (Fig. 9) although a tendency to a faster aggrega-

tion at heating pH 7.1 was found. Similarly to formulations after a heating pH of 6.9, unintended pre-gelation occurred at an acidification pH of 4.8 during printing (low sol-gel transition temperature of 8.5 °C; Fig. 5, (D)) and no printing could be conducted, independent of the protein content.

3.4 Tailored casein micelle surface characteristics for printability

For the aggregation rate, a value of 250 Pa/ 10 K was proposed to predict the printability the best for formulations in the concentration and temperature range investigated in this study. Images of printed gels are shown in Fig. 8-10. We found that formulations with an acidification pH of 5.0 were printable only by addition of whey protein and a heating step at higher pH values (≥ 6.9). Standardisation of such formulations to a micellar casein to whey protein ratio of 4:1 with the addition of whey protein isolate and a small change in the pH value (6.9, 7.1) before heating resulted in depleted CM with less steric repulsion forces and more κ-casein-whey protein complexes in the serum. This intended tailoring of the surface characteristics of the CM and the increased number of particles per unit volume allowed us to print casein-whey protein suspensions at both acidification pH values, 4.8 and 5.0, with the latter found not to be printable for non-heated, pure casein-based systems (Nöbel et al., 2018).

The pH sensitive CM are the main particles contributing to a gelation process of dairy gels, if they coagulate. An intended modification of the surface characteristics (depleted CM, heating pH value ≥ 6.9) resulted in suitable casein-whey protein suspensions for extrusion-based 3D-Printing. After a heating step, denatured whey proteins contributed to the three-dimensional network, decreased the sol-gel transition temperature (≥ heating pH 6.9) and increased the aggregation rate (≤ acidification pH values of 5.0) of milk gels. In this study, the aggregation rate (Fig. 8) of casein-whey protein suspensions after heating at pH 6.55 increased only for formulations examined at an acidification pH of 4.8, while the sol-gel transition temperature (Fig. 5, B) did not change compared to pure non-heated, micellar casein-based samples. The

results from the literature and this study suggest that the number of particles contributing to the aggregation did not increase during a heating step at pH 6.55. At this pH, Vasbinder et al. (2003b) proposed that almost all the denatured whey proteins covered the surface of the CM while Anema et al. (2004b) found values between 55-85% of whey protein covering the surface of the CM for a heating pH of 6.5, depending on the heating temperature.

As the heating pH was increased (6.9, 7.1), Anema & Li (2003b) and Anema (2007) found a decreased amount of denatured whey protein on the surface of the CM, changing the composition and the surface characteristics of the CM. Results of SDS-PAGE in our study confirmed a dissociation of κ-casein from the CM into the serum at higher heating pH values (Fig. 4). At higher heating pH, denatured β-lactoglobulin and κ-casein formed complexes in the serum, apart from covering the CM, reducing the concentration of free κ-casein in the serum phase. The dissociation of κ-casein into the serum suggests that the density of the hairy layer covering the surface of the CM was reduced, which facilitated the collapse of the remaining κ-casein, if the acidification pH was lowered. Therefore, aggregation of the CM at lower temperatures may occur due to less steric repulsion forces provided by a weaker hairy layer of κ-casein, while electrostatic repulsion forces did not change after heating at different pH values (Fig. 3). A similar explanation was provided by Lakemond & Van Vliet (2008), who heated skim milk at different pH values and proposed that CM heated at higher pH (6.9) collided and aggregated faster than CM heated at lower pH (6.2), due to a higher steric hindrance occurring at a lower heating pH. These results showed the effect of small changes in the pH before the heat treatment on the type of casein-whey protein complexes and κ-casein dissociation out of the CM and how these changes resulted in more promising material characteristics for a 3D-Printing process of casein-whey protein suspensions. A simple schematic model is proposed in Fig. 11 based on microstructural observations of proteins in this study, which illustrates the differences of gel structures after the 3D-Printing process.

4 Conclusion

Casein-whey protein suspensions differing in product - and pre-process parameters were investigated regarding their potential use for extrusion-based 3D-Printing for tailored nutrition. This study demonstrated the influence of different pH values during heat treatment and the use of the acidification pH to manipulate the sol-gel transition temperature and the aggregation rate of casein-whey protein suspensions, if gelation was applied via the pH-T-route. Sol characteristics at cold temperatures were confirmed with rheological - and zeta-potential measurements, providing a liquid feed material for a layer by layer printing process inclusive a full sol-gel transition from sol to gel.

Depending on the location of κ-casein, denatured whey protein was either mainly bound to the surface of the CM (pH 6.55) or mostly found as complexes with κ-casein in the serum phase (pH 6.9, 7.1) which significantly modified the surface characteristics of the CM. These higher heating pH values, causing depleted CM, decreased the sol-gel transition temperature and increased the aggregation rate. The latter property was proposed as a good indicator for the printability of the formulations used within this study, if values of 250 Pa/ 10 K for the aggregation rate (represented by the Storage Modulus G') were recorded during a linear temperature ramp. The aggregation rate will be a good prospective indicator, but has to be investigated for each material and related to printing characteristics, as currently no quantitative and analytical method is available to objectively predict printability.

Lower acidification pH values and higher protein concentrations of the cold acidified casein-whey protein suspensions resulted in an increase in the aggregation rate. Extrusion-based 3D-Printing showed firm and stable gels at acidification pH values of 4.8 and 5.0, with more suitable formulations found after higher heating pH values. Future studies focused on more complex systems including different calcium/protein concentrations, cream or lactose will provide

459 more options for tailored and individualized nutrition via printing and will allow to design and
460 understand more formulations.

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Figure captions

Fig. 1. a) Set-up of the customized 3D-Food Printer after the retrofitting process including 1) syringe in double walled cooling jacket, 2) insulated pipe for transport of feed material to nozzle, 3) thermometer, thermocouples (TC) and software, 4) firmware of Ender 3 printer, 5) nozzle with die at the end, 6) heating element for nozzle and 7) stepper motor. b) Heating element in contact with the copper pipe. c) Nozzle with dye and thermocouples at the end. The small inset image in (a) shows a simplified schematic of the set-up with the flow direction of the feed material in red.

Fig. 2. Particle size distribution for non-heated CS (○/green), heated (pH 6.55) CS + WP (●/blue), heated (pH 6.9) CS + WP (▲ /orange) and heated (pH 7.1) CS + WP (Δ/yellow) casein-whey protein suspensions.

Fig. 3. Changes in the ζ-potential with decreasing pH of non-heated micellar CS (○), heated (pH 6.55) CS + WP (●), heated (pH 7.1) CS + WP (Δ) and heated (pH 6.9) CS + WP (▲) suspensions (casein to whey protein ratio of 4:1). Measurements were performed at a temperature of 3 °C for the sample, the diluent (deionized water) and within the Zetasizer.

Fig. 4. SDS-PAGE analysis of serum phase proteins with - and without thermal treatment (80 °C, 10 min). Lane I-III: non-heated, pH 6.55, 6.9 and 7.1 (from left to right). Lane IV-V: heated, pH 6.55; Lane VI-VII, heated, pH 6.9 and Lane VIII-IX, heated, pH 7.1.

Fig. 5. Sol-gel transition temperatures of cold acidified micellar casein - (9:1 ratio of CS to WP, (A)) and casein-whey protein suspensions (4:1 ratio of CS to WP, B-D) at a heating rate of 1 K min^{-1}. With no heating step (A) and a heating step at 80 °C for 10 min at pH 6.55 (B), pH 6.9 (C) and pH 7.1 (D). The dashed line in all images represents the coagulation line for pure micellar casein from (A) and is shown for comparison.

Fig. 6. Example of a temperature sweep to illustrate how the aggregation rate (Pa/ 10 K) was defined. A casein-whey protein suspension (4:1 ratio, 8.0% (w/w) CS and 2.0% (w/w) WP) was heated at pH 6.9, cold acidifed at 2 °C to pH 5.0 and heated in a rheometer (heating rate of 1 K min^{-1}). A sol-gel transition temperature (G' = 1 Pa) was obtained at 11.92 °C. The aggregation rate was calculated from the moduli increase between 11.92 °C to 21.92 °C.

Fig. 7. Aggregation rate (Pa/10K) for non-heated, micellar casein suspensions at different pH values (4.8-5.4) and casein contents (8.0-12.0% (w/w)) at 10 °C after/higher than sol-gel transition temperature obtained by temperature sweeps (heating rate of 1 K min^{-1}). The dotted line indicates the threshold where above 250 Pa/ 10 K the gels produced were stable after the printing process.

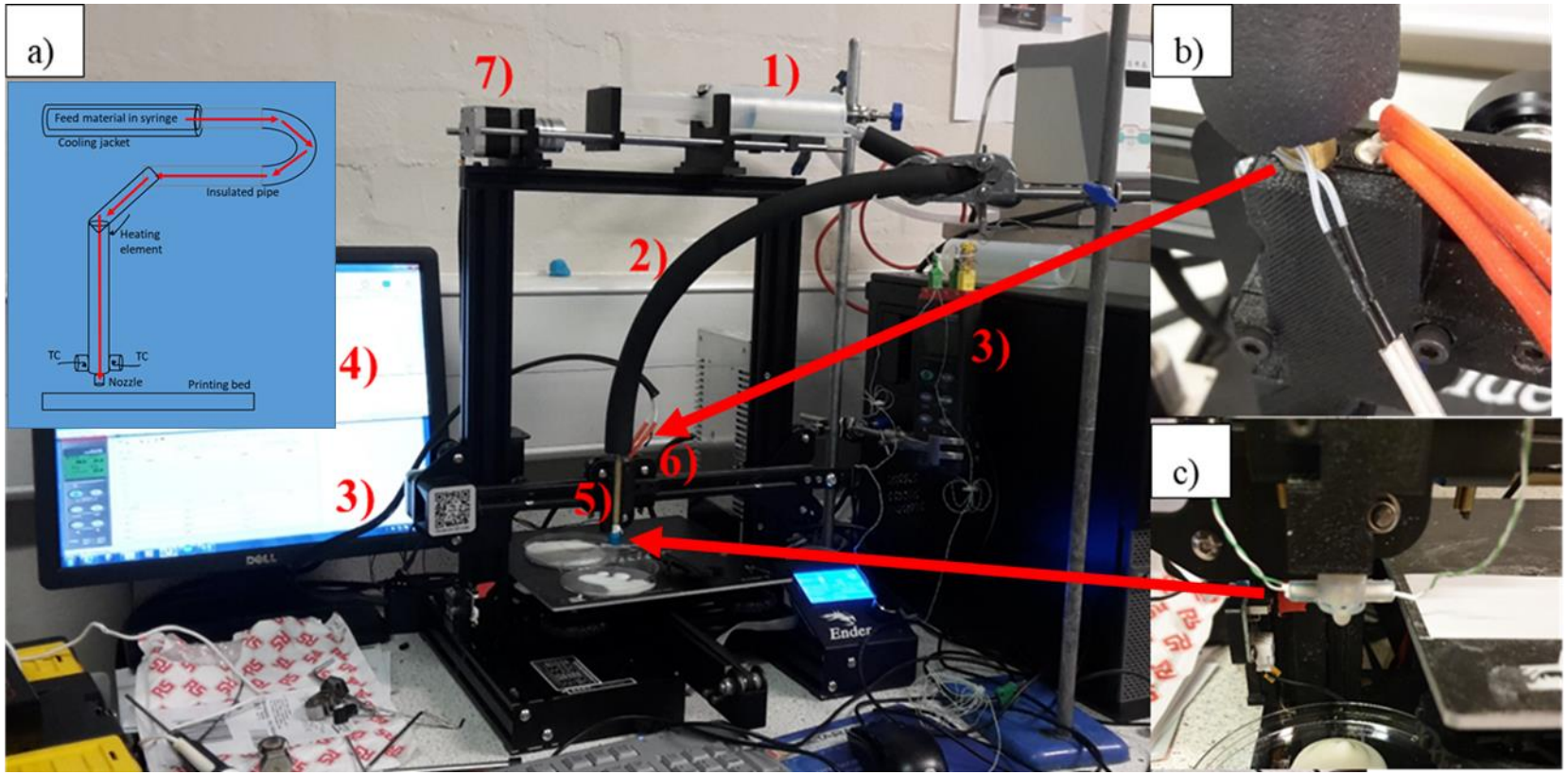
Fig. 8. Aggregation rate (Pa/10K) at different pH values (4.8-5.4) for casein-whey protein suspensions after a heating step (80 °C, 10 min) at pH 6.55 at 10 °C after/higher than the sol-gel transition temperatures obtained by temperature sweeps (heating rate of 1 K min^{-1}). Printed images are shown to relate the aggregation rate to printability. The dotted line indicates the threshold where above 250 Pa/ 10 K the gels produced were stable after the printing process.

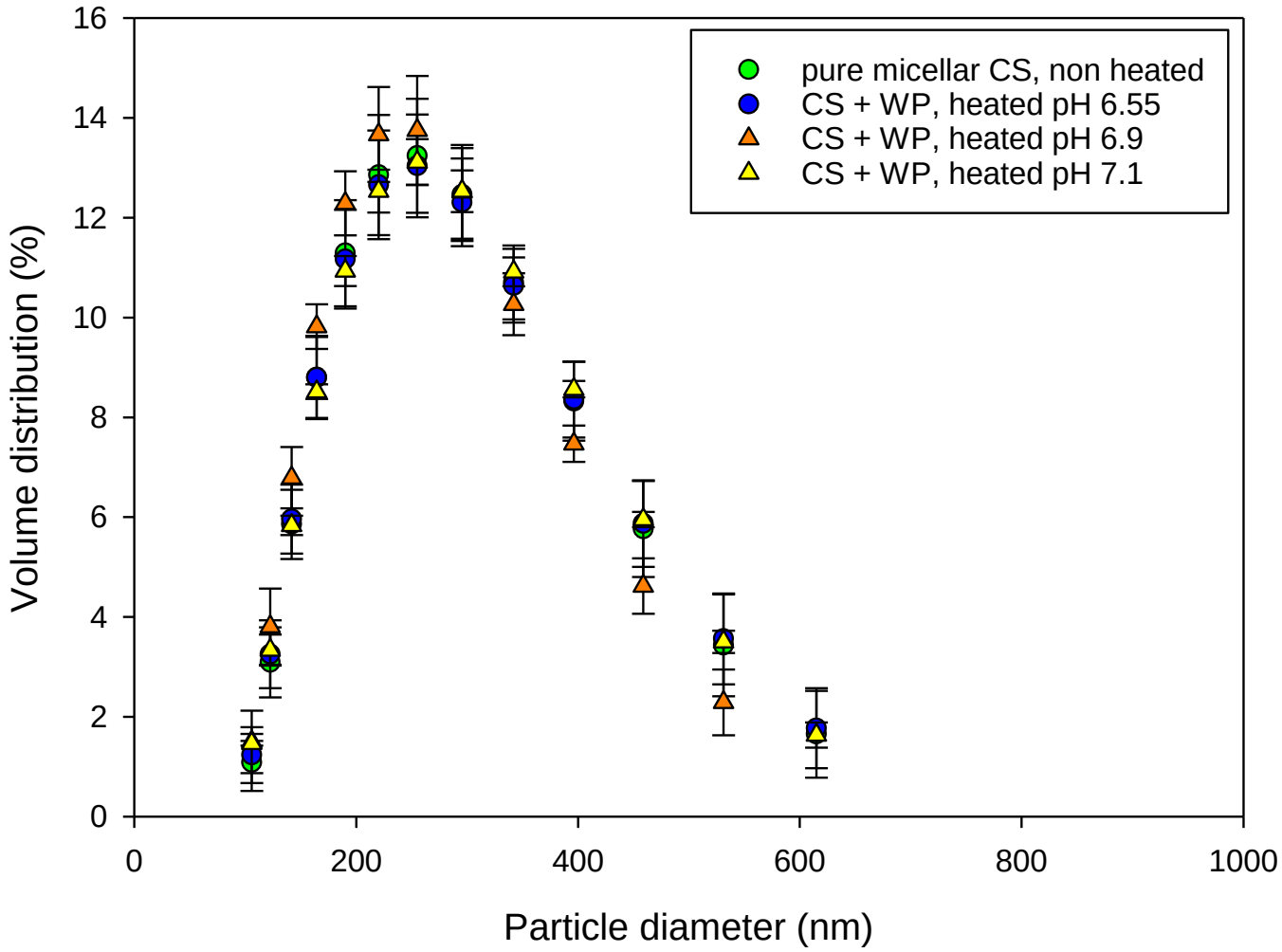
Fig. 9. Aggregation rate (Pa/10K) at different pH values (4.8-5.4) for casein-whey protein suspensions after a heating step (80 °C, 10 min) at pH 6.9 at 10 °C after/higher than the sol-gel transition temperatures obtained by temperature sweeps (heating rate of 1 K min^{-1}). Printed images are shown to relate the aggregation rate to printability. The dotted line indicates the threshold where above 250 Pa/ 10 K the gels produced were stable after the printing process.

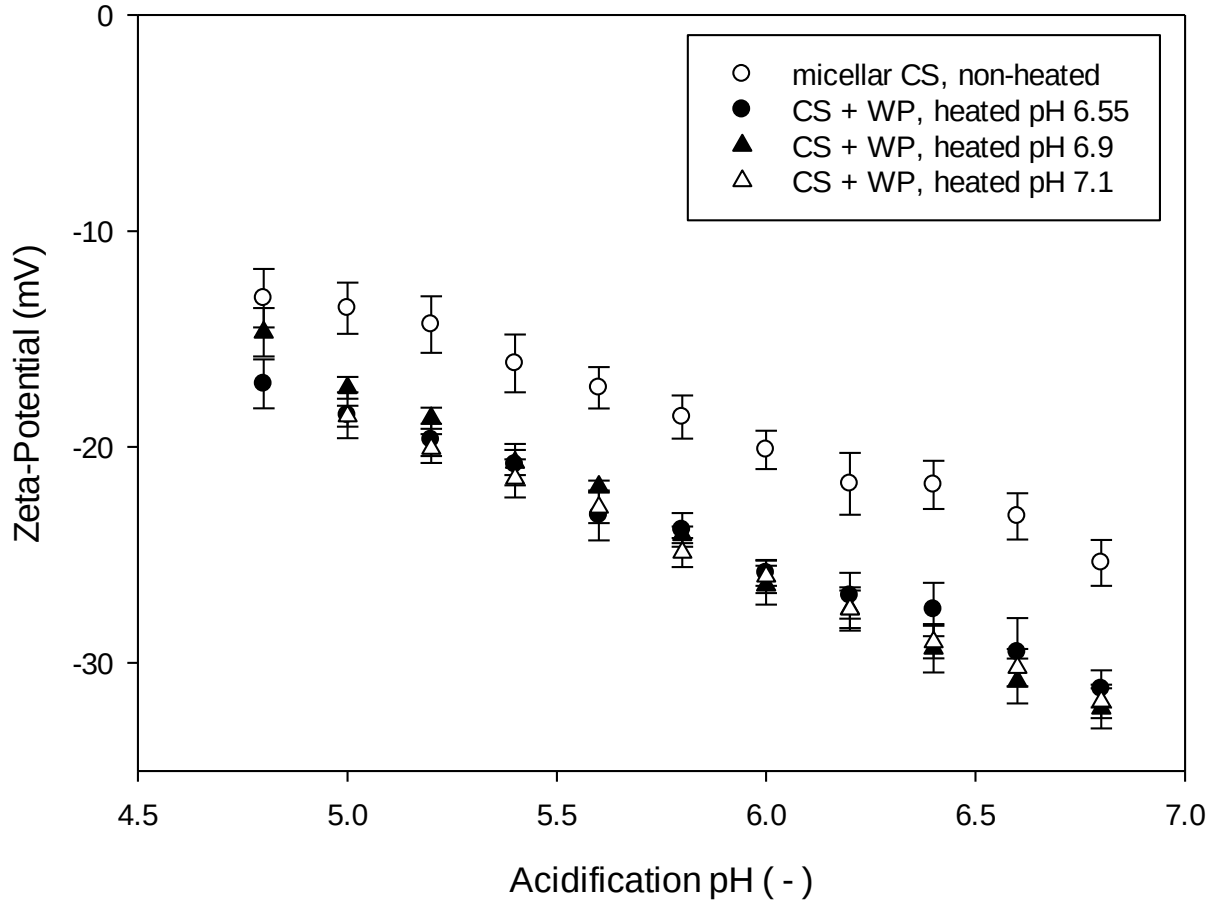
Fig. 10. Aggregation rate (Pa/10K) at different pH values (4.8-5.4) for casein-whey protein suspensions after a heating step (80 °C, 10 min) at pH 7.1 at 10 °C after/higher than the sol-gel transition temperatures obtained by temperature sweeps (heating rate of 1 K min^{-1}). Printed images are shown to relate the aggregation rate to printability. The dotted line indicates the threshold where above 250 Pa/ 10 K the gels produced were stable after the printing process.

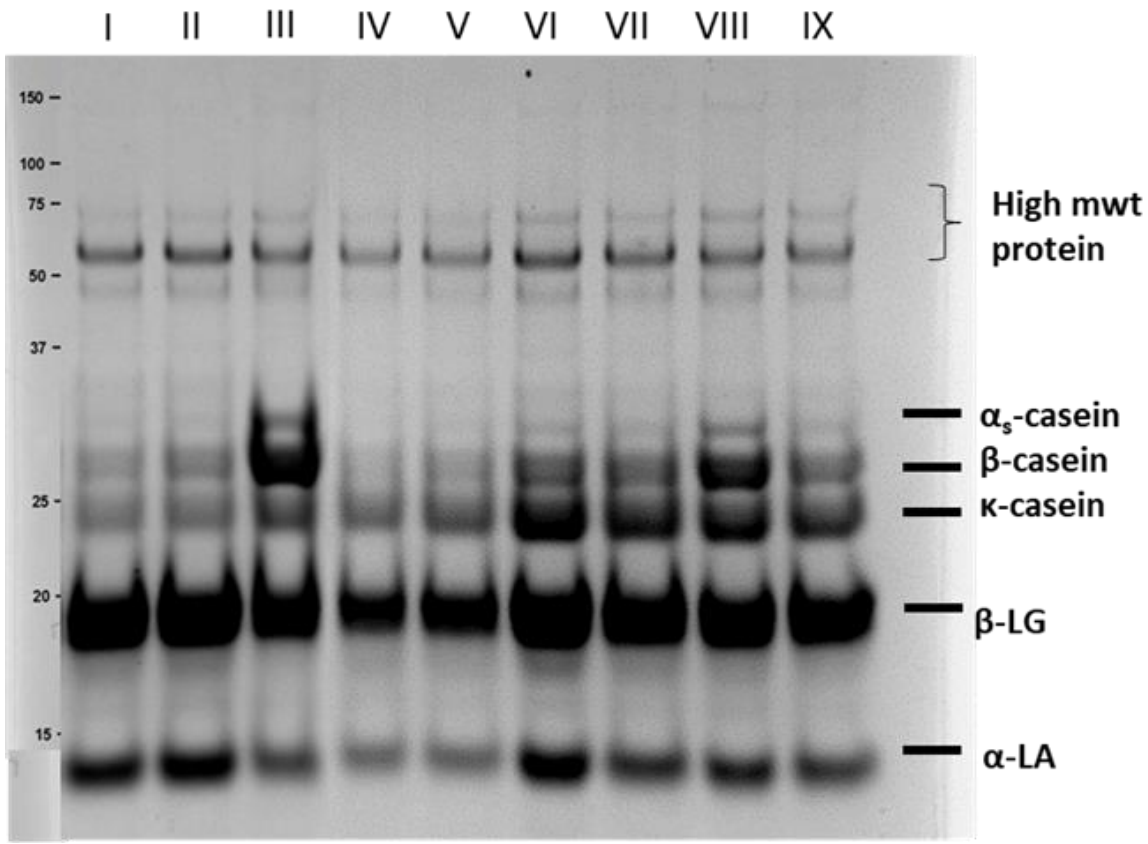
Fig. 11. Schematic representation depicting the interactions of casein-whey protein formulations depending on the pH value during the heating step and explaining the differences of gels after a printing process.

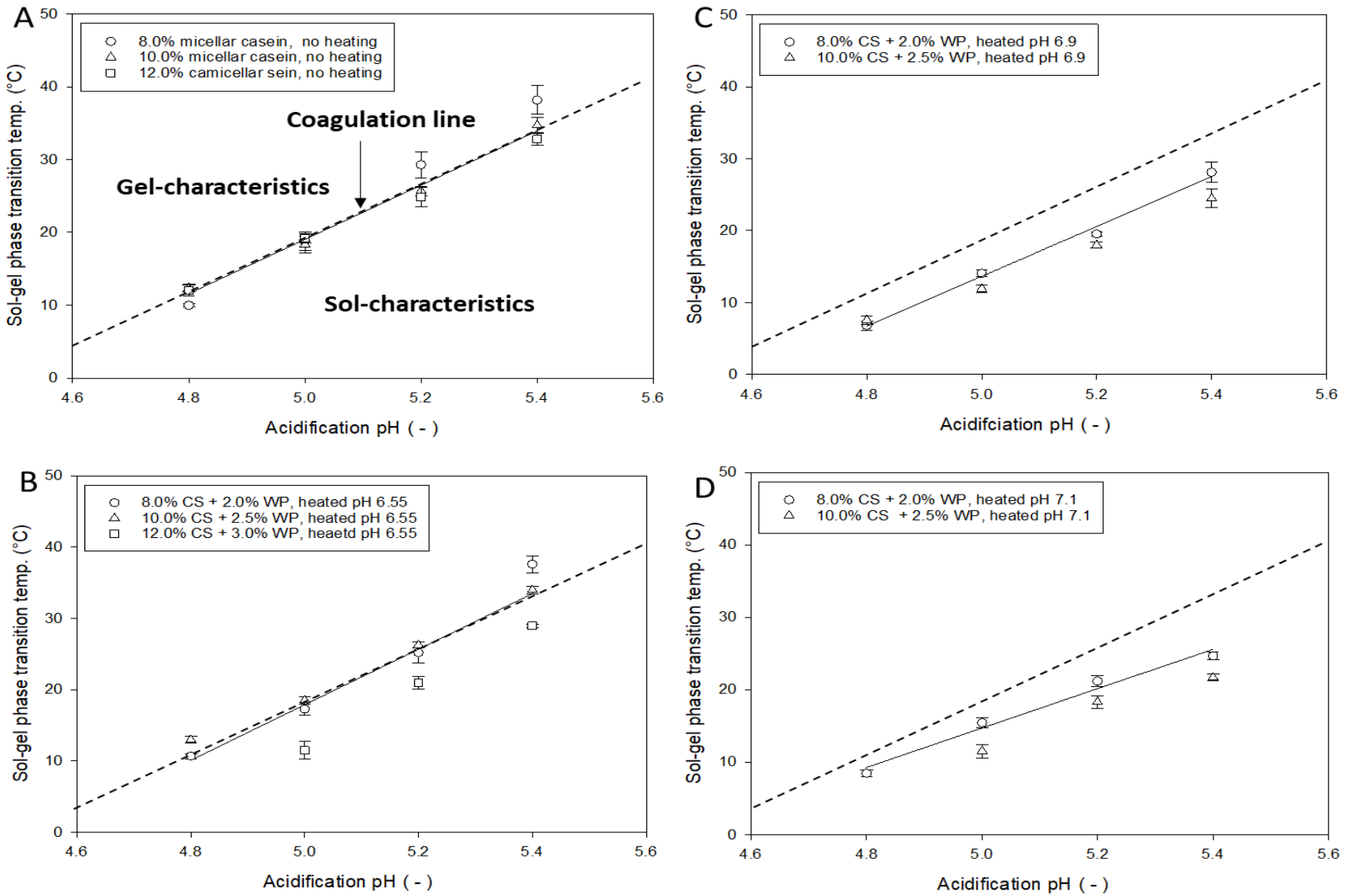
Supplementary Fig. 1. Electron micrograph of only casein micelles in pure micellar casein - (top, left) and casein-whey protein suspension (heated pH 6.9, 10 min, 80°C; top right), made using scanning electron microscopy and micrograph of casein micelles in casein-whey protein suspension (heated pH 6.55, 10 min, 80°C) on the bottom (left and right), made using cryo-EM.

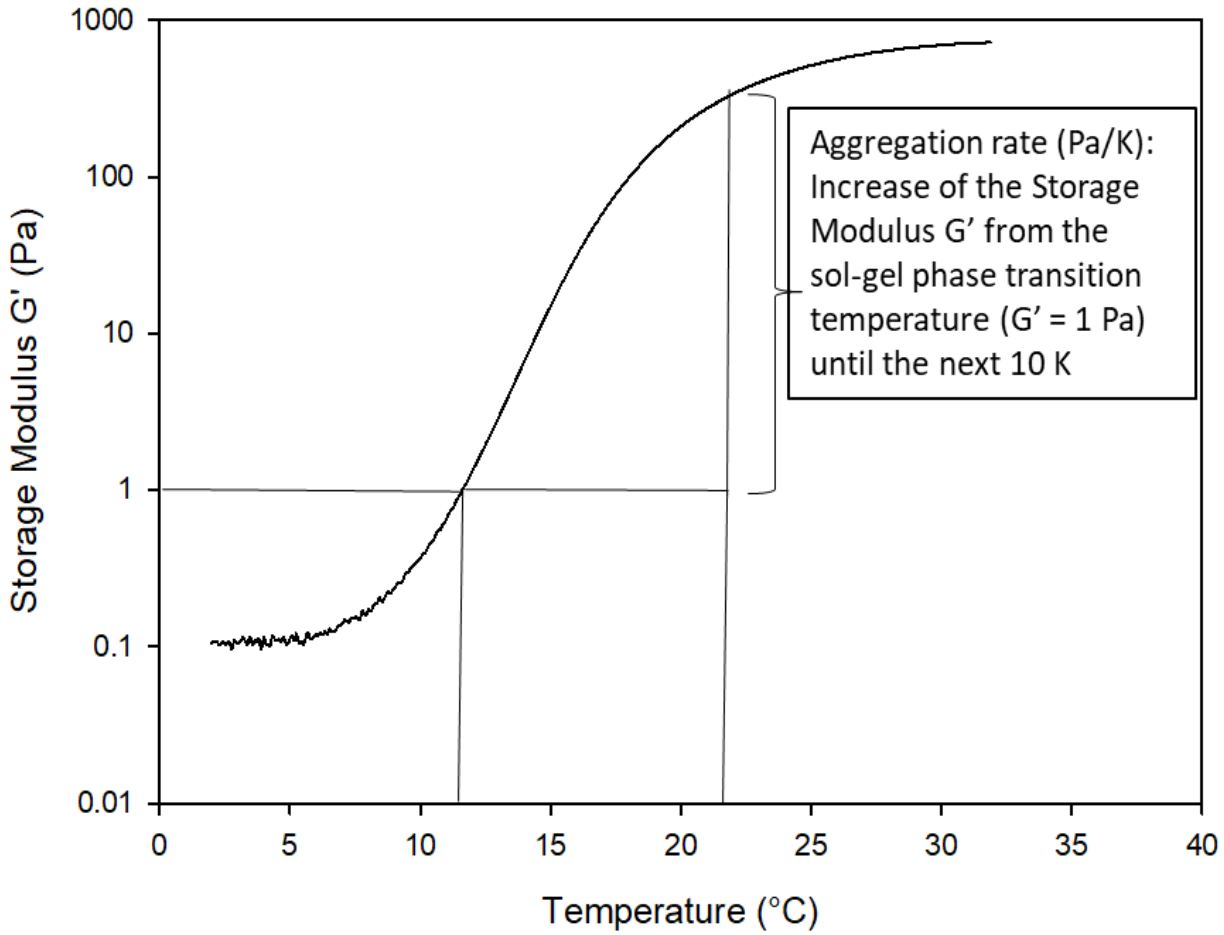

a)
b)
c)
Feed material in syringe
Cooling jacket
Insulated pipe
Heating element
TC
TC
Nozzle
Printing bed
1)
2)
3)
4)
5)
6)
7)
Ender

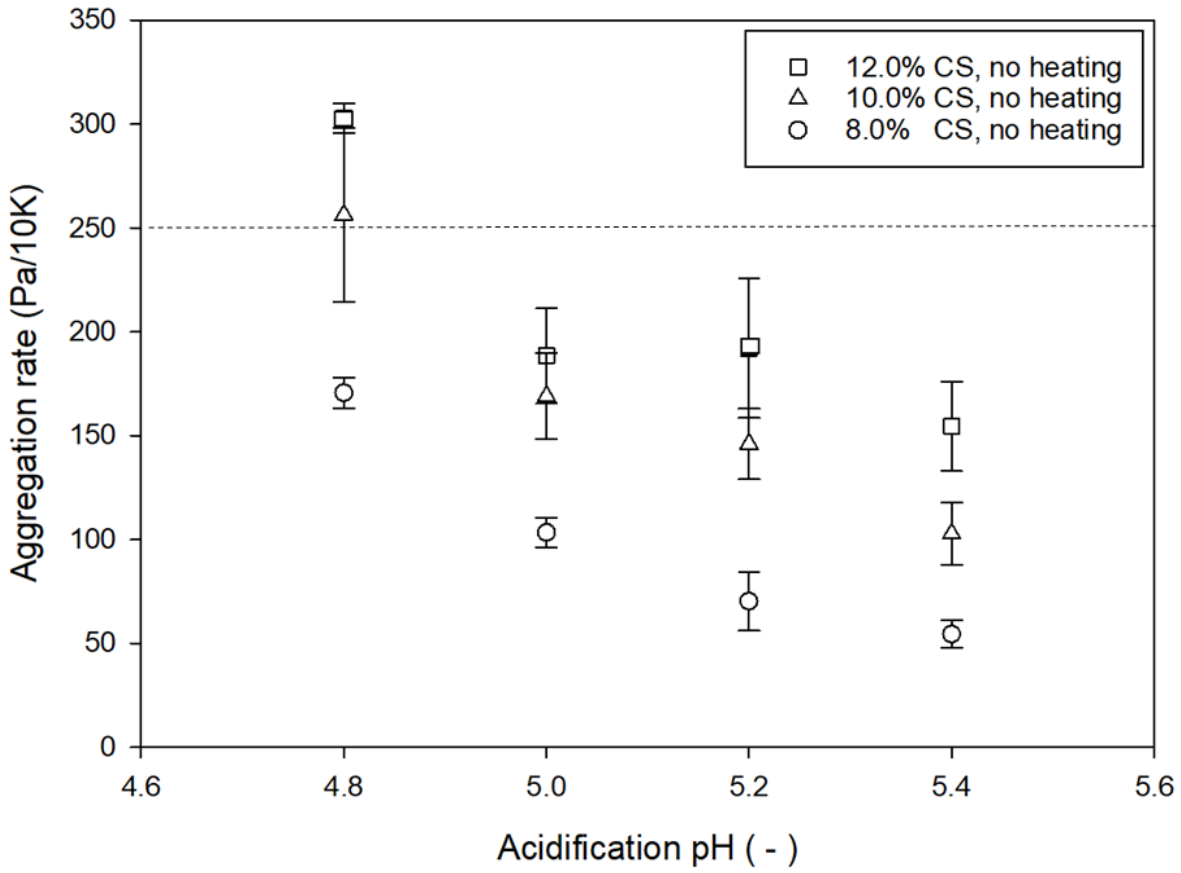

pure micellar CS, non heated
CS + WP, heated pH 6.55
CS + WP, heated pH 6.9
CS + WP, heated pH 7.1
Volume distribution (%)
Particle diameter (nm)
0
2
4
6
8
10
12
14
16
0
200
400
600
800
1000

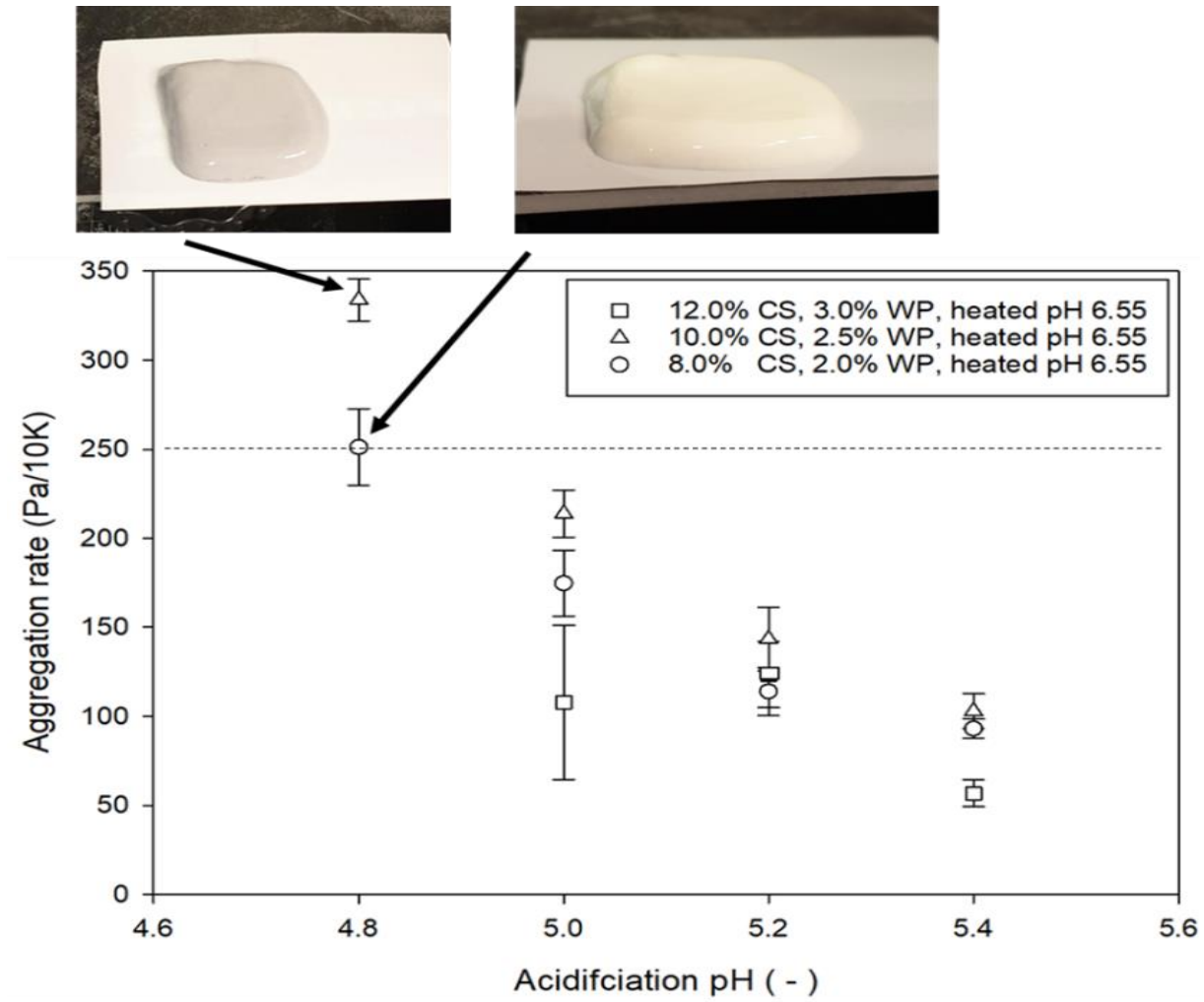

0
-10
-20
-30
Zeta-Potential (mV)
4.5
5.0
5.5
6.0
6.5
7.0
Acidification pH (-)
micellar CS, non-heated
CS + WP, heated pH 6.55
CS + WP, heated pH 6.9
CS + WP, heated pH 7.1

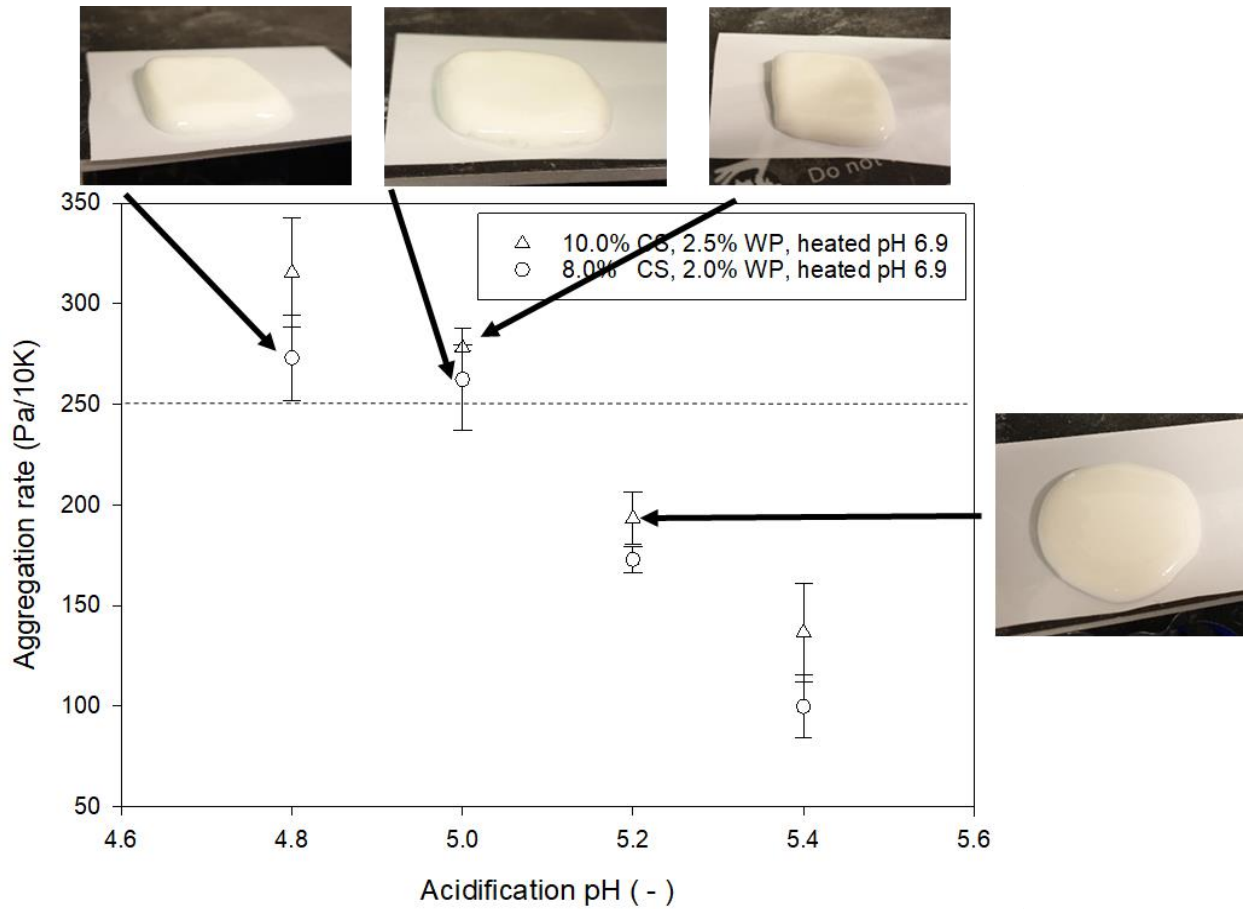

I
II
III
IV
V
VI
VII
VIII
IX
150
100
75
50
37
25
20
15
High mwt protein
α_s-casein
β-casein
κ-casein
β-LG
α-LA

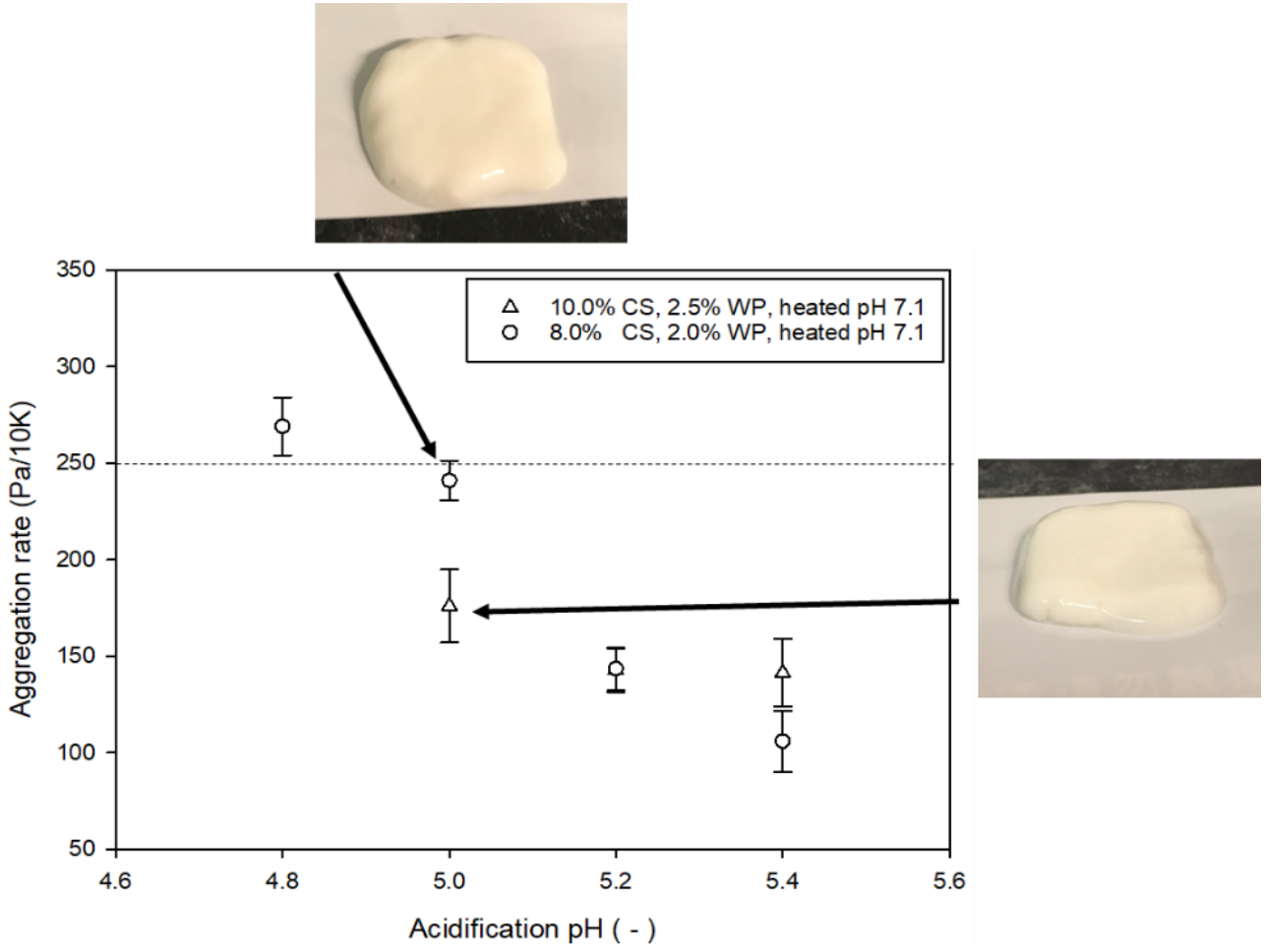

A
8.0% micellar casein, no heating
10.0% micellar casein, no heating
12.0% camicellar sein, no heating
Coagulation line
Gel-characteristics
Sol-characteristics
Sol-gel phase transition temp. (°C)
Acidification pH (-)
B
8.0% CS + 2.0% WP, heated pH 6.55
10.0% CS + 2.5% WP, heated pH 6.55
12.0% CS + 3.0% WP, heaetd pH 6.55
Sol-gel phase transition temp. (°C)
Acidification pH (-)
C
8.0% CS + 2.0% WP, heated pH 6.9
10.0% CS + 2.5% WP, heated pH 6.9
Sol-gel phase transition temp. (°C)
Acidifciation pH (-)
D
8.0% CS + 2.0% WP, heated pH 7.1
10.0% CS + 2.5% WP, heated pH 7.1
Sol-gel phase transition temp. (°C)
Acidification pH (-)

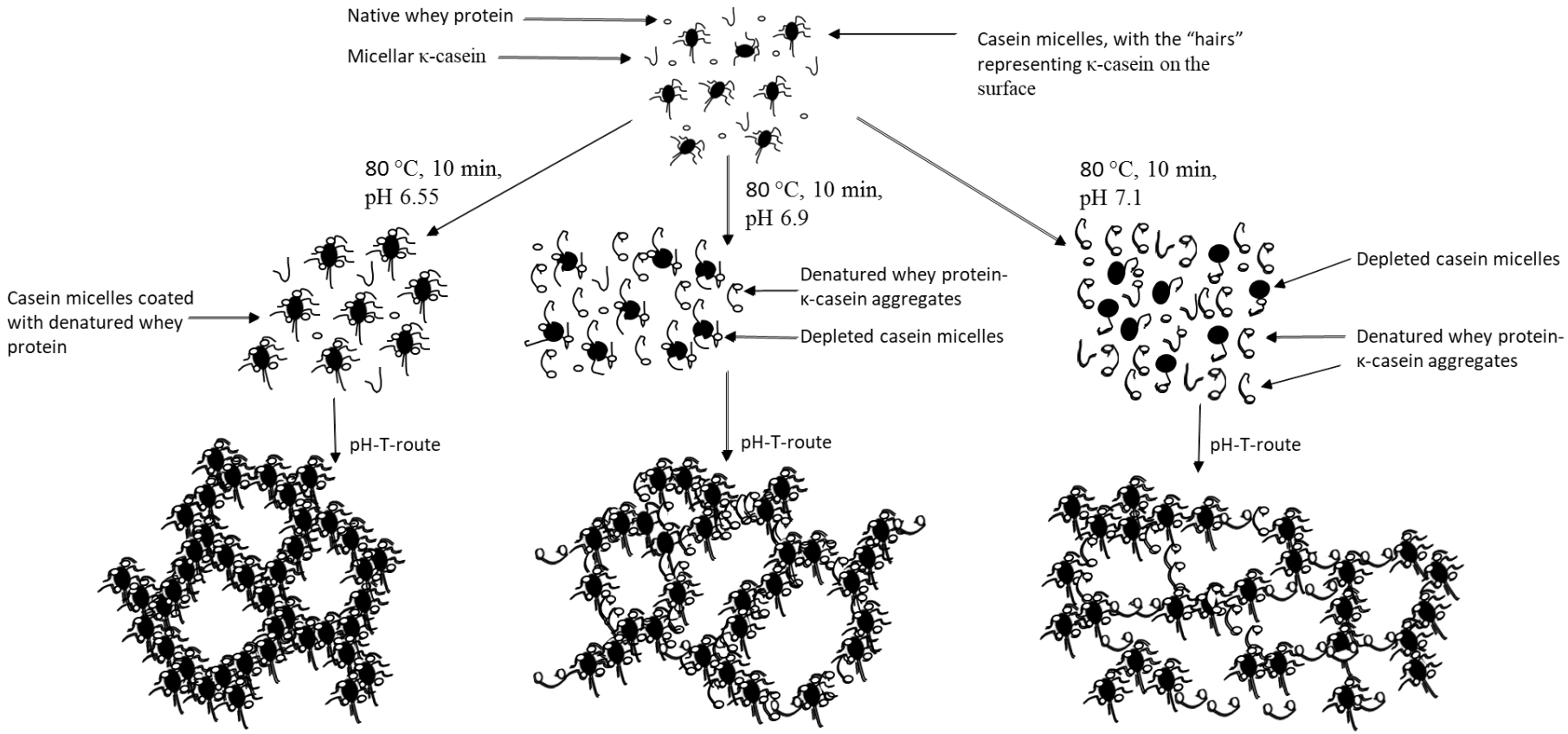

1000
100
10
1
0.1
0.01
Storage Modulus G' (Pa)
0
5
10
15
20
25
30
35
40
Temperature (°C)
Aggregation rate (Pa/K):
Increase of the Storage
Modulus G' from the
sol-gel phase transition
temperature (G' = 1 Pa)
until the next 10 K


12.0% CS, no heating
10.0% CS, no heating
8.0% CS, no heating
Aggregation rate (Pa/10K)
Acidification pH (-)
0
50
100
150
200
250
300
350
4.6
4.8
5.0
5.2
5.4
5.6


12.0% CS, 3.0% WP, heated pH 6.55
10.0% CS, 2.5% WP, heated pH 6.55
8.0% CS, 2.0% WP, heated pH 6.55
Aggregation rate (Pa/10K)
0
50
100
150
200
250
300
350
4.6
4.8
5.0
5.2
5.4
5.6
Acidifciation pH (-)


10.0% CS, 2.5% WP, heated pH 6.9
8.0% CS, 2.0% WP, heated pH 6.9
Aggregation rate (Pa/10K)
Acidification pH (-)
350
300
250
200
150
100
50
4.6
4.8
5.0
5.2
5.4
5.6


10.0% CS, 2.5% WP, heated pH 7.1
8.0% CS, 2.0% WP, heated pH 7.1
Aggregation rate (Pa/10K)
350
300
250
200
150
100
50
4.6
4.8
5.0
5.2
5.4
5.6
Acidification pH (-)


Native whey protein
Micellar κ-casein
Casein micelles, with the "hairs" representing κ-casein on the surface
80 °C, 10 min, pH 6.55
80 °C, 10 min, pH 6.9
80 °C, 10 min, pH 7.1
Casein micelles coated with denatured whey protein
Denatured whey protein-κ-casein aggregates
Depleted casein micelles
Depleted casein micelles
Denatured whey protein-κ-casein aggregates
pH-T-route
pH-T-route
pH-T-route

Highlights

- Casein-whey protein suspensions were tested for 3D-Printing via the pH-T-route

- Size, zeta-potential, microscopy, electrophoresis and rheology were characterised

- Phase transition temperature decreased with increasing heating pH

- Printability was related to aggregation rate

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: