Effects of urea addition on the structural and material properties of caseinate solutions and emulsions

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Abstract

The effects of urea addition on the self-assembly of caseinate sub-micelles and subsequent impact on the structural and material properties of caseinate solutions and caseinate stabilised oil-in-water emulsions (30 vol% oil) were studied.

Sub-micellar particle size and distribution were measured using dynamic light scattering. Sodium caseinate in solution showed a bimodal particle size distribution centred at 20 and 120 nm (radius). The increasing addition of urea (1.1 to 6.6 M) was seen to cause a reduction in the size of caseinate sub-micelle, which was attributed to a dissociation of the sub-micellar fraction arising from a reduction in hydrophobic interactions.

For sodium caseinate solutions above estimated sub-micellar close-packing phase volumes (＞10 wt% protein concentration), increasing addition of urea was seen to significantly lower solution viscosity with more pronounced changes observed with increasing protein concentration. All the urea-treated solutions were Newtonian.

For sodium caseinate stabilised emulsions, where protein concentrations were above the critical threshold for onset of depletion flocculation, the addition of urea was seen to result in a reduction in depletion free energy of the emulsions. The effects of reduced depletion interaction on emulsion stability, structural and rheological properties were found to be dependent not only on the concentration of added urea, but also on the protein concentration in the emulsions. For 2 and 4 wt% caseinate concentrations, depleted droplets reduced with increasing urea concentration and the emulsion stability increased as well. For 6 wt% protein concentration, the stability decreased with the addition of 1.1 and 3.3 M urea, and it was re-established when the urea concentration was up to 6.6 M. This was attributed to the synergic effects on the depletion potential and continuous phase viscosity which were induced by urea, and it was supported by the results of creaming profiles, rheological properties and microstructure. Likewise, urea had similar effects on the emulsions stabilised by potassium caseinate.

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1. Introduction

Caseinates are widely used in food industry, being derived from the native milk caseins with the removal of colloidal calcium phosphate by alkali (Mulvihill & Ennis, 2003). Sodium caseinate, particularly, is a high value commercial food ingredient as it provides a pleasing flavour, a nutritional source of protein, desirable temperature stability and excellent emulsifying properties in the application of various food products (Kinsella & Morr, 1984). Sodium caseinate is a common food ingredient used in beverage, dairy products, meat products and ice cream.

In solution, sodium caseinate undergoes self-association into nano-particles, commonly termed sub-micelles (Kumosinski, Pessen, Farrell, & Brumberger, 1988). The association of caseinate into sub-micelles is contributory to the high viscosity of caseinate solutions at phase volumes above sub-micellar close packing (Farrer & Lips, 1999; Loveday, Rao, Creamer, & Singh, 2010). As a result of this, more energy is required during processing of the high viscosity solution and the protein concentration in spray dry stage of caseinate manufacture is limited (Fichtali, van de Voort, & Doyon, 1993; Fox, 2003). Furthermore, sodium caseinate induces creaming instability in oil-in-water emulsions at sufficiently high concentrations, which is attributed to depletion flocculation arising from an excess of nonadsorbed sub-micelles (Dickinson & Golding, 1997a; Dickinson & Golding, 1998a; Huck-Iriart, Álvarez-Cerimedo, Candal, & Herrera, 2011; Srinivasan, Singh, & Munro, 2001).

Therefore, there is scope for improving the physico-chemical and functional properties of solutions and emulsions containing sodium caseinate from the self-assembly behaviour. This study will aim to show how urea disruption of hydrophobic interactions leads to a dissociation of the sub-micellar structure, and how the sub-micellar dissociation affects the properties of solutions and emulsions containing sodium caseinate. The modifications to solution rheological properties and emulsion stability, structural and material properties in the systems with the addition of urea are

of interest here. In comparison with sodium caseinate, the effects of urea on emulsions stabilised by potassium caseinate are also studied to determine whether sub-micellar properties are influenced by the change in counterion.

Findings from this study notably, that urea addition reduced the particle size of caseinate sub-micelles, indicates the dissociation of the self-assembly caseinate aggregates. Moreover, it was found that urea addition substantially reduced the viscosity of caseinate solutions above close packing (>10 wt% caseinate concentration), and that viscosity decreased correlating with increasing urea concentration. The dissociation effect of urea on solution viscosity was also sensitive to temperature.

The effects of sub-micellar dissociation on the properties of caseinate-stabilised emulsions with excess nonadsorbed proteins were studied as well. Urea addition enhanced the emulsion stability by lowering the depletion free energy for 2 and 4 wt% protein concentrations. At 6 wt% protein concentration, the emulsion stability was adjusted through the synergic effects of urea on lowering of depletion potential and the reduction of continuous phase viscosity, resulting in transition stability behaviour which was dependent on urea concentration. No notable differences were observed in the effects of urea on the relatively stability of emulsions stabilised with potassium and sodium caseinates, although some differences in particle size distribution were observed.

This fundamental research tends to probe a better understanding of using urea to dissociate the aggregates induced by self-assembly of caseinates. This approach may provide understanding of the role of caseinate self-association on ingredient and product properties during manufacture of both caseinate as a raw material and emulsion based food products formulated with caseinate. Whilst the concentrations of urea used in this study are not applicable in food systems, other (food grade) means of inducing sub-micellar dissociation may produce corresponding structural and material effects, and this research provides knowledge on the structural outcomes achievable by this approach.

2 Literature Review

2.1 General introduction of milk protein

Milk is widely regarded as an ideal food for its excellent nutritional, physiological and organoleptic functions. It plays a significant role in growth and development during infancy and also provides important nourishment for adults (Varnam & Sutherland, 2001). The main components in milk are water, proteins, lactose, lipids, vitamins, enzymes, minerals, hormones and et al., with water being the major component, ranging from 80~90% (Kailasapathy, 2008). The work of Jennes, Dickinson and Stainsby (as cited in Morris, 2002) has shown that bovine milk typically comprises 3-4% protein, with caseins and whey proteins being the main classes. Casein can be derived from milk by acid precipitation, while whey protein remains in the liquid (Fox, 2003). As well as providing nutritional value, the two protein type are recognized for their wide ranging (technical) functional properties in manufactured food systems, which derived from their particular structural properties .

2.2 Casein

2.2.1 Characteristics of casein

Casein is the main component, comprising approximately 80% of the protein in milk (Loveday et al., 2010). Fox (2003) provided a comprehensive background regarding the characteristics of casein. It is now well established that casein in milk is present in the form of spherical complexes, arising from hydrophobic and electrostatic interaction between individual casein molecules and colloidal calcium phosphate (CCP). These complexes are called casein micelles. Casein micelles are mixtures of α_{s1}, α_{s2}, β and κ-casein. The colloidal dimensions of casein micelles scatter light, which contributes to the white colour of milk. This opacity decreases when the structure of casein micelles is perturbed. According to Fox (2003), the particle size of the casein micelle ranges from 50 to 500 nm, with an average value of 150 nm, and mass of 10^6 to 3×10^9 Da, with

an average value of 10^8 Da. The number of micelles in milk is between 10^{14} and 10^{16} per ml.

Casein micelles have remarkable stability under a range of conditions encountered during food processing (Fox, 2003). In terms of temperature, casein micelles remain intact during high temperature pasteurization and can withstand heat treatment at 100℃ for 24 h at natural milk pH. Casein micelles are stable under commercial homogenisation, and can be redispersed in solution by mild agitation after drying or ultracentrifugation. They also show good tolerance towards Ca^{2+}, with the addition of 200 mM Ca^{2+} and temperature being up to 50℃. However, there are some treatments leading to the aggregation of casein micelles (which are essential for the manufacture of certain dairy food). For example, micelle aggregation and precipitation occur when the pH of milk is adjusted to casein isoelectric point (pH 4.6). Addition of rennet and ethanol also result in aggregate formation and precipitation. Long-time exposure to high temperatures (such as UHT treatment) also has a similar effect.

2.2.2 Individual caseins

Four major types of individual caseins constitute the casein micelle, namely: α_{s1}, α_{s2}, β and κ-casein, with the weight ratio of 3: 1: 3: 1 in bovine milk (Schmidt, 1980). The work of McKenzie and Murphy (as cited in Morris, 2002) suggested that the caseins were conjugated proteins, and Swaisgood (as cited in Morris, 2002) argued that caseins were characterised by the presence of phosphorylated serine group(Swaisgood, 2003), which contribute to calcium binding(Ho & Waugh, 1965; Ono, Kaminogawa, Odagiri, & Yamauchi, 1976; Ono, Yahagi, & Odagiri, 1980), and the presence of significant numbers of proline residues (Farrell, 1973; Kumosinski, Brown, & Farrell, 1991a, 1991b) ,which contribute to the disordered structure of the casein molecules (Guo, Fox, Flynn, & Kindstedt, 1995). Some specific structural aspects of the individual caseins are provided in Table 2.1.

Table 2.1 The characteristics of individual caseins

Casein individual	Molecular weight (g/mol)	Structure	Calcium sensitive	Self-association
α_{s1}-casein	23000	amphiphilic	√	$(\alpha_{s1}\text{-casein})_n + \alpha_{s1}\text{-casein} \rightarrow (\alpha_{s1}\text{-casein})_{n+1}$
α_{s2}-casein	25000	amphiphilic, the most hydrophilic casein	√	$(\alpha_{s2}\text{-casein})_n + \alpha_{s2}\text{-casein} \rightarrow (\alpha_{s2}\text{-casein})_{n+1}$
β-casein	24000	very amphiphilic	√	n (β-casein) → $(\beta\text{-casein})_n$
κ-casein	19000	amphiphilic, facilitates the stabilisation of micelle against calcium	–	n (κ-casein) → $(\kappa\text{-casein})_n$

(Adopted from Morris, 2002)

2.2.3 Casein micelle structure

The structure of the casein micelle is closely involved in protein structuring as part of the manufacture of many dairy-based food products, for example cheese, sweetened-condensed milk and yogurt (Fox, 2003).

Extensive research has been done on the structure of the casein micelles in the last several decades, and several models have been proposed. One of the proposed theories is based on a sub-micellar structural model, with casein micelles consisting of sub-micellar unities linked together by CCP (Figure 2.1, (A)to (F)). This theory is based on the self-assembly behaviour of caseins.

Morr (1967) is the first one to propose the model of sub-micelles. As it can be seen in Figure 2.1 (A), a spherical rosette structure is introduced by Waugh, Creamer, Slattery, and Dresdner (1970), which is close to the surfactant micelle structure proposed by Shinoda, Nakagawa, Tamamushi and Isemura (as cited in Waugh et al., 1970). According to Waugh, the polar ends of α_{s1}, α_{s2} and β-casein are arranged outward to reduce electrostatic free energy in the core polymers (sub-micelles). Each sub-micelle is covered by one molecular layer of κ-casein, and the whole micelle is surrounded by a coat of κ-casein. However, the author did not mention the role of CCP in the development of the structure.

Figure 2.1 (B) to (D) shows the model proposed by Schmidt (1982), who suggested that a small group of casein molecules (15~20) formed the sub units through hydrophobic bonds, and the units vary in κ-casein content. The sub-micelles which are sufficient in κ-casein locate on the surface, creating a κ-casein-rich surface for the micelle, while the κ-casein deficient sub-micelles reside in the interior of the micelle and they are glued by CCP.

Schmidt's model is futher developed by Walstra (1990, 1999). As it can be seen in Figure 2.1(E), (F), sub-micelles are formed by the casein molecules linked togther through the hydrophobic and electrostatic bonds, and they are aggregations of roughly spherical shape. These sub units are glued by CCP to form the micelles and the mobility of the molecules are restrited. Two kinds of sub-micelles exist in micelles, depending on the content of κ-casein, poor or rich. It is proposed by Walstra that the outside of the micelles is dominantly covered by κ-casein, and the hydrophilic part (C-terminal end) of the κ-casein protrudes from the micelle surface to the solvent. These hairs of κ-casein can provide steric and electrostatic repulsions, which retard flocculation of the micelles .

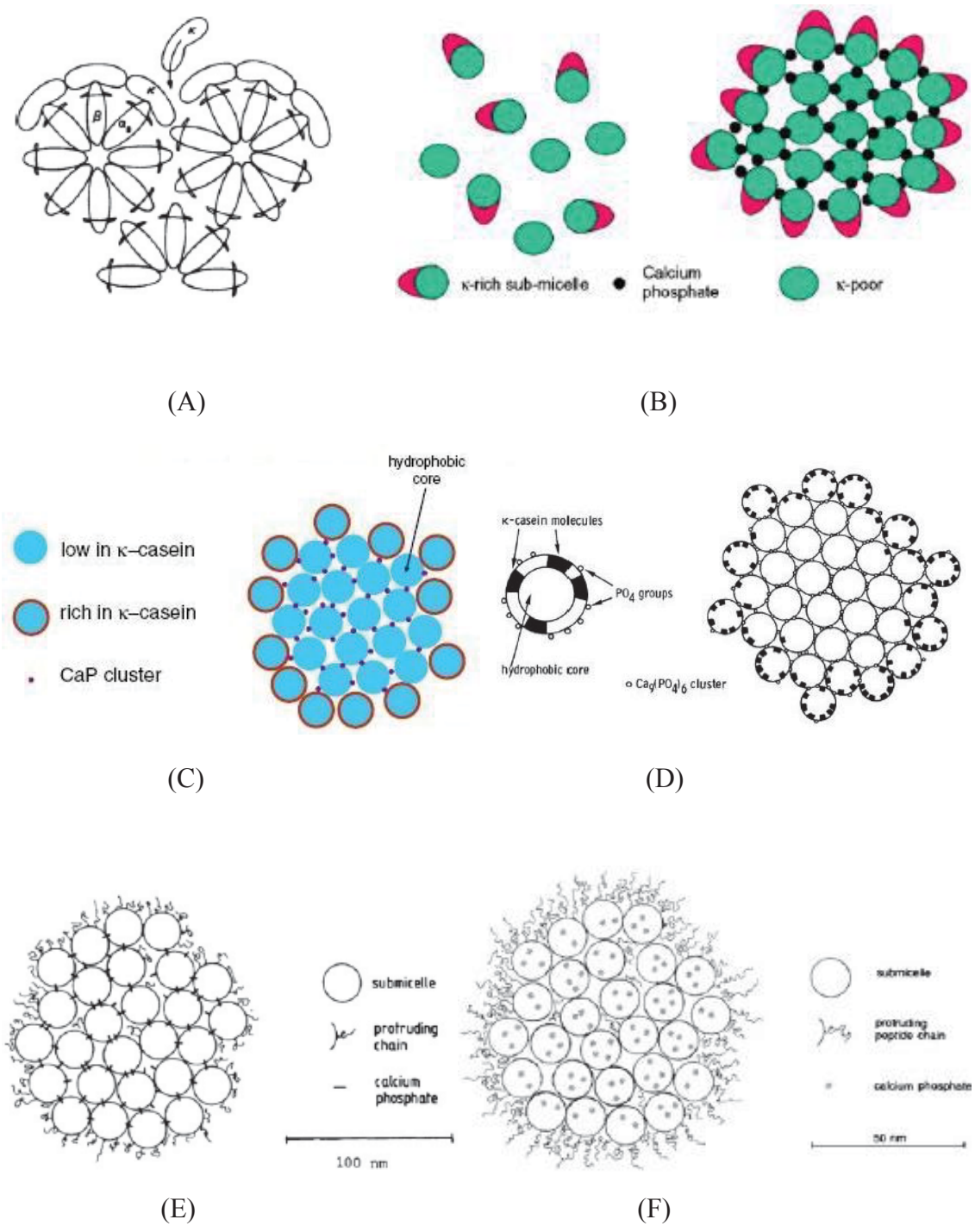


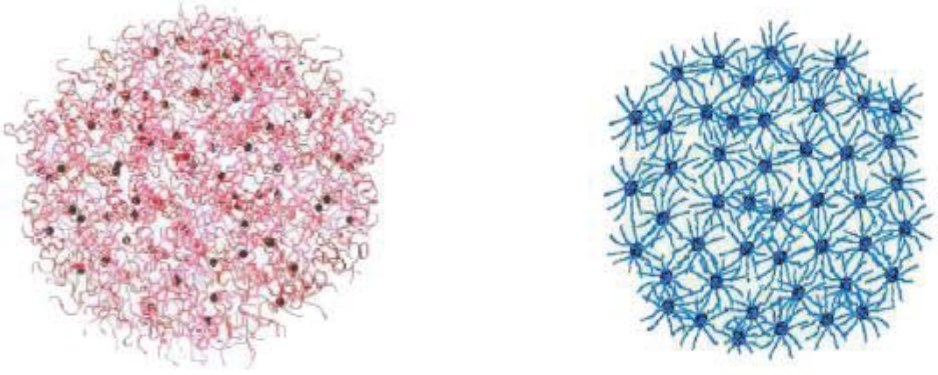
Figure 2.1 Models of casein - part A.

(A) Model of casein micelles proposed by Waugh; (B) Interpretation of Schmidt's model; (C), (D) Representations of the model of casein micelles proposed by Schmidt; (E), (F) Representations of the model of casein micelles proposed by Walstra. (Adopted from de Kruif, Huppertz, Urban, & Petukhov, 2012; Schmidt, 1982)

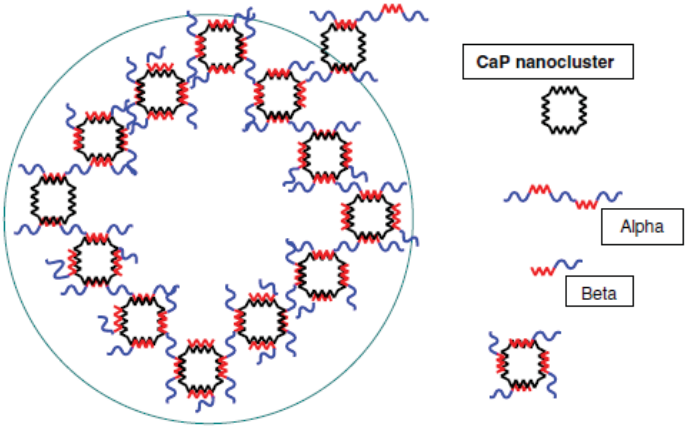
Meanwhile, there are two additional models which refute the sub-micellar structure. As demonstrated in Figure 2. 2(A) to (C), the nanocluster model of Holt and Kruif is mainly a homogeneous protein gel matrix structure without subunits, and it is open and highly hydrated (Holt, 1992; Kruif & Holt, 2003). It is suggested that the calcium phosphate nanoclusters (CaP nanoclusters) are the core of the structure, with calcium phosphate embedding inside. Some of the peptides from α -casein cross-link the CaP nanoclusters and others form loops. These aggregates are bounded by the phosphopeptide of β -casein and give rise to the 3-dimensional network. They argued that the extension of the colloidal dimensions might be affected by the formation of loop or nanoclusters, as loop closure or the termination of the nanoclusters development limited the growth of the network. Horne (2006) also proposed an illustration of the nanocluster model to assist in interpretation of the model.

A dual-binding model is proposed by Horne (1998) and is shown in Figure 2.2(D) and (E). It is based on the self-consistent-field theory which Dickinson, Pinfield, Horne, and Leermakers (1997b) used to predict the conformation of α_{s1}-casein and β -casein, in which α_{s1}-casein can be defined as a train–loop-train structure, while β -casein is a tail-train structure. The hydrophobic ends of caseins bind together to form clusters, while the hydrophilic regions of the calcium sensitive caseins form salt bridging with the colloidal calcium phosphate. Further growth of the network is limited by κ -casein, and in doing so, distributes on the surface of the structure (Horne, 1998).

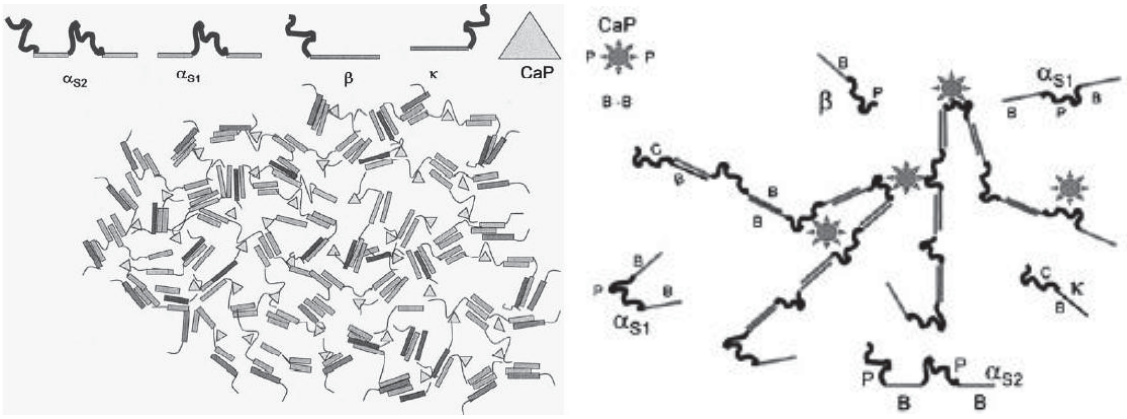
Although there are some differences among these models, there is a general agreement on κ -casein forming the surface layer which induces steric and electrostatic balance in casein solution. However, the location of κ -casein is still somewhat vague in Holt's model, as there is no substantive role for it (Horne, 2006).



(A) (B)



(C)



(D) (E)

Figure 2.2 Models of casein - part B.

(A), (B) Representations of the model of casein micelles proposed by Holt; (C) Illustration of network formation in the Holt model; (D), (E) Representations of the dual binding model proposed by Horne. (Adopted from de Kruif et al., 2012; Horne, 1998, 2006)

2.2.4 Casein manufacture

The processes involved in casein manufacture are shown in Figure 2.3 (Mulvihill & Ennis, 2003). The first step in casein manufacture is to remove fat from milk by centrifugation. Two mechanisms are able to isolate the casein from the skim milk, depending on the type of product being manufactured. The skim milk can be destabilised by isoelectric precipitation, which includes mineral acid, ion exchange resin or lactic starter, used in the manufacture of acid casein. The isolation of the casein can also be achieved by proteolytic coagulation through calf rennet or substitute, which produces rennet casein and which is the basis for cheese manufacture. Then the curd is cooked after the precipitation/coagulation. The following step is dewheying, where the curd is separated from the whey as the casein becomes insoluble and the whey remains in the solution. During the washing stage, the residuals from dewheyed curd, eg. lactose, whey protein, salts and extra acid will be removed by washing and diffusion. The washed curd is dewatered to reduce the quantity of water prior to drying. The moisture of casein is dried to < 12% to ensure a stable condition. It is tempered, blended and ground to produce a suitable particle size, and selected according to the particle size range by screen.

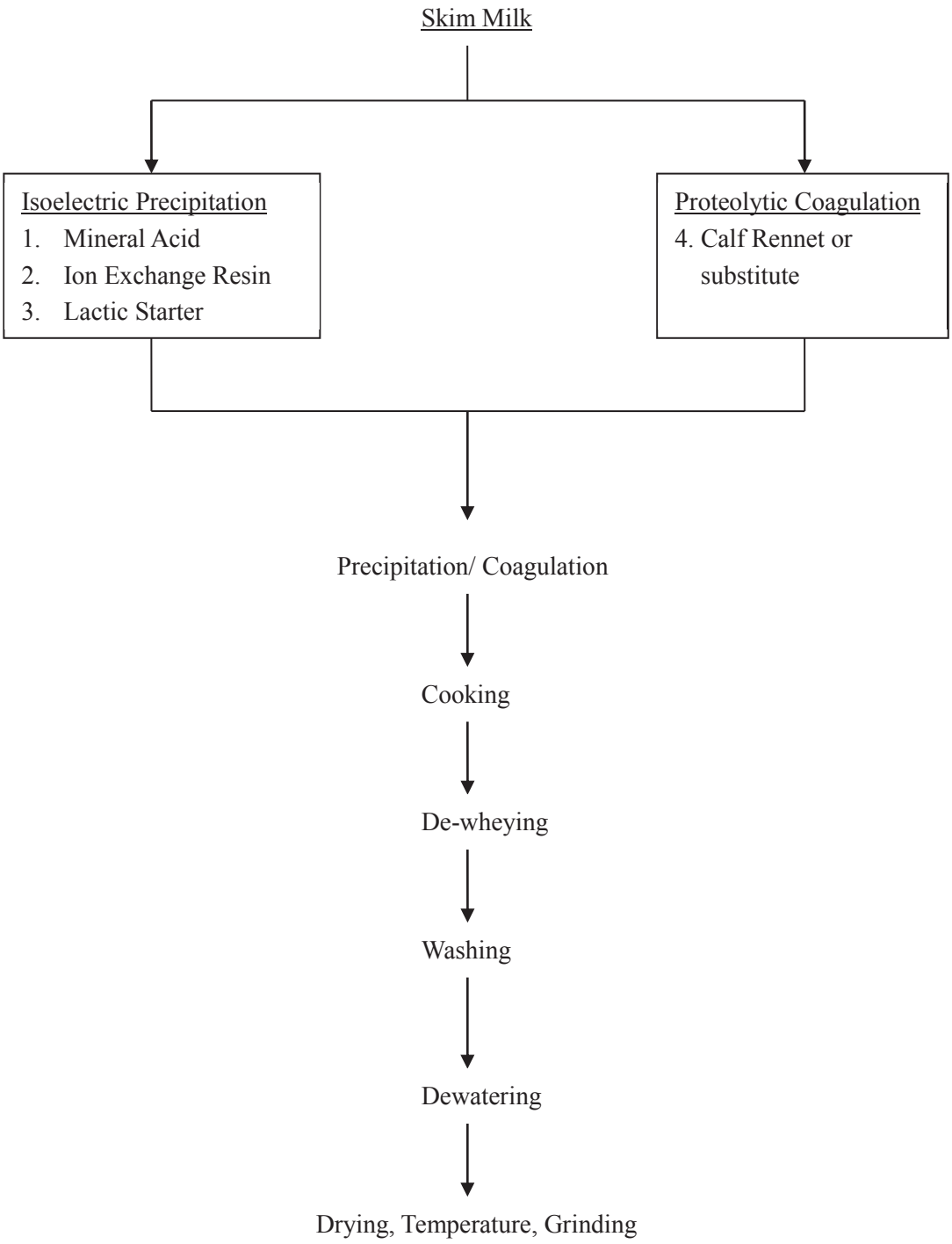


Figure 2.3 Processes for the manufacture of caseins.

Reprinted with permission of Advanced Dairy Chemistry (Springer) (Mulvihill & Ennis, 2003)

2.3 Caseinate

Caseinate is obtained through the removal of the colloidal calcium phosphate from casein by adding alkali (typically a hydroxide) to previously prepared acid casein (Mulvihill & Ennis, 2003). As the structure of native caseins is altered by the addition of acid first and then alkali during the processing, caseinate possesses a markedly different structure relative to the casein micelle in milk (Kinsella & Morr, 1984). Generally, Morr (as cited in Kinsella & Morr, 1984) found that the particle size of caseinate was smaller than the casein micelles (hence solutions of caseinate appear transparent rather than opaque, with the exception of calcium caseinate), and it was more susceptible to the conditions in the solution, such as pH and ionic strength. The types of caseinate depend on the associated cations, for instance, sodium, potassium, calcium and ammonium caseinate. Among these casein derivatives, sodium caseinate is widely used in food industry with its desirable functionalities, including heat stability and capacities of water-holding, fat binding, thickening, gelling, foaming, emulsifying and stabilising, according to Mulvihill (as cited in Perrechil & Cunha, 2013).

2.3.1 Characteristics of caseinate

Several authors have probed the characteristics of sodium caseinate, ranging from the composition, weight-average molar mass (M_w), particle size, rheological properties and self-assembly behaviour (Chu, Zhou, Wu, & Farrell, 1995; Dickinson et al., 2001; Farrer & Lips, 1999; HadjSadok, Pitkowski, Nicolai, Benyahia, & Moulai-Mostefa, 2008; Kinsella & Morr, 1984; Loveday et al., 2010; Lucey, Srinivasan, Singh, & Munro, 2000; Nash, Pinder, Hemar, & Singh, 2002; Panouille, Benyahia, Durand, & Nicolai, 2005; Pitkowski, Durand, & Nicolai, 2008). Being derived from native casein, sodium caseinate has the same major proteins as caseins, which are α_{s1}, α_{s2}, β and κ-casein, but the chemical composition could vary within batches from different manufacturing processes (Dalgleish & Law, 1988). Lucey et al. (2000) stated that sodium caseinate solution was a mixture of casein monomers, casein complexes (sub-micelles) and lager particle size aggregates. The M_w value of sodium caseinate were studied using multiangle laser light scattering (MALLS) and size-exclusion chromatography (SEC) as

well. With MALLS alone, the value of weight-average molar mass range of sodium caseinate was determined as 30~757 and 1200~4700 kDa, with and without ultracentrifugation. Meanwhile, according to the results from MALLS and SEC, bimodal distribution was found with M_w of 420~750 and 39~69 kDa for two peaks, respectively.

Several studies have been done on the particle size of sodium caseinate. Chu et al. (1995) used dynamic light scattering (DLS) to identify two distinct populations of sub-micelles and larger aggregates in sodium caseinate solutions, with hydrodynamic radii of approximately 10 nm and 80 nm, respectively. A similar result was found by Nash et al. (2002) using the same technique. However, a value of ~100 nm was found by Dickinson et al. (2001) , which was the average hydrodynamic radii of the protein (aggregates) in the caseinate solution. The use of multiangle laser light scattering (MALLS) and size-exclusion chromatography (SEC) applied by Lucey and co-workers determined that the R_g value varied between 22~49 and 50~120 nm for the samples with or without ultracentrifuge treatment, respectively (Lucey et al., 2000). It should be noted that the variations in measurements could be related to variations caseinate composition and the ionic environment in which measurements were made.

Previous studies have investigated the rheological properties of sodium caseinate solutions. Hermansson (1975) showed that at low protein concentration (c ＜12 wt%) caseinate solutions were Newtonian, yet at higher protein concentration (c ＞12 wt%) they became increasingly pseudoplastic. The apparent viscosity increased with elevated caseinate concentration, and a dramatic increase was observed when the concentration increased above 12 wt%. A similar trend was observed as the rheological behaviour of caseinate solution changed from Newtonian to pseudoplastic with increasing protein concentration (Pitkowski et al., 2008; Roeper & Winter, 1982; Towler, 1974). The concentration dependence of the zero-shear rate viscosity of the solutions was also measured at different pH and temperatures by Pitkowski and coauthors (Pitkowski et al., 2008). They noted that the zero-shear rate viscosity of all the samples increased with elevated protein concentration, with a strong increase with protein concentration above

100 g/L and that the zero-shear rate was temperature dependent at high caseinate concentration. For solution with 200 g/L caseinate, the viscosity decreased with increasing temperature, with a reduction of four orders of magnitude from 5 to 90℃. The rheological behaviour of highly concentrated sodium caseinate solutions has been further studied by Loveday et al. (2010). Shear-thinning was observed for samples at elevated protein concentrations ($c \geq 25$ wt%) ,which was in line with the data of Farrer and Lips (1999). In the frequency sweep experiment, colloidal glass behaviour was observed for the solutions with higher concentration ($c \geq 23$ wt%), and solid-like behaviour increased with rising concentration. At 40.1 wt% caseinate concentration, the solution displayed solid-like behaviour over the entire frequency range. Moreover, reversible G' and G'' values were obtained from heating and cooling scans, and the raise in gelation and softening temperatures was found to be linear with the protein concentration.

2.3.2 Self-assembly behaviour in caseinate solution

As indicated by particle size measurements, sodium caseinate has been demonstrated to self-assemble in solution (Chu et al., 1995; HadjSadok et al., 2008; Nash et al., 2002). These sub-micellar aggregates were determined as containing between 5 and 15 casein monomers (Farrer & Lips, 1999; Pitkowski, Nicolai, & Durand, 2009). There were two separate structural regimes in the solution, at sub-micellar phase volume below and above close packing (Farrer & Lips, 1999). It was proposed that the onset protein concentration of sub-micellar close packing was 10 wt%. Previous rheological studies provide similar evidence to support this observation (Pitkowski et al., 2008). The viscosity of sodium caseinate solution was found to increase markedly above a critical concentration ($c \approx 100$ g/L), which was attributed to transition above the close-packing limits for sub-micellar aggregates. It was also found that this jamming behaviour was determined by the effective volume fraction of sub-micelles, as the viscosity increased either with increasing protein concentration or decreasing temperature.

The mechanism of the self-assembly of individual casein components is governed by the balance of hydrophobic attraction and electrostatic repulsion, and self-association occurs when the attractive hydrophobic interaction overcomes the electrostatic repulsion (Horne, 1998). It is proposed that the mechanism for the individual caseins with self-association probably can be applied to the prevailing casein products in the industry (Horne, 1998), and therefore it was plausible to adopt this hypothesis to the self-assembly of sodium caseinate in aqueous solution system. This balance is affected by many factors, including calcium ion concentration, ionic strength, pH and temperature (Dickinson, 2006). Chu et al. (1995) studied the calcium-induced aggregates in caseinate solution, which suggested that the threshold concentration of calcium ion inducing self-association was 10 mM. The formation of the calcium-induced aggregates is attributed to the reduction of the electrostatic repulsion caused by the binding of calcium sensitive caseins with calcium (Harold E. Swaisgood, 1993). In terms of ionic strength, when the ionic strength is low in the system, the electrostatic interaction is strong enough to keep the casein molecules from association. Nevertheless, at high ionic strength, the hydrophobic parts of the casein have a strong tendency to associate with the electrostatic interaction being screened (HadjSadok et al., 2008). It was suggested that caseinate remained in the form of individual molecules at low ionic strength (3 mM) and displayed self-association at high ionic strength (>100 mM). Additionally, the aggregation number increases with elevated temperature, while decreases with decreasing pH (HadjSadok et al., 2008).

The self-assembly behaviour of caseinate in aqueous solution has a negative impact on the manufacture and application of caseinate. It was found that the activation energy needed for flow increased with increasing protein concentration, which indicated that more energy was required during shearing (Fichtali et al., 1993). Moreover, due to the high viscosity, protein concentration in the spray dried process is limited to around 20 wt%, which increases the cost of the drying process (Fox, 2003), and with high moisture content, more energy is needed to dry the casein curds. The dissolving time is also affected as a result of the high viscosity (Fichtali et al., 1993).

Apart from the sub-micelles induced by self-association, there is a small weight fraction of larger aggregates existing in the system, with a radius of around 80 nm (Chu et al., 1995). Even though they are a relative small population, light scattering technique is sensitive to these supra-molecular assemblies. They are not residual native casein micelles, for they do not precipitate by ultracentrifuging. The nature of the larger particles is still a paradox, but it is inferred that fat and proteins may constitute the particles with a density close to that of water (HadjSadok et al., 2008).

2.3.3 Functional properties of caseinates

The following section provides a short summary of the technical and functional properties of caseinates.

Heat stability

Sodium, potassium and ammonium caseinates exhibit excellent heat stability, while calcium caseinate is less stable. Sodium caseinate remains stable under heat treatment at 140℃ at pH 7 for 60 min, whereas gelation happens when calcium caseinate is heated at 50~60℃ (Mulvihill, 1992). Meanwhile, the heat stability of caseinates is affected by the other factors, for instance, calcium ions. Sodium and potassium caseinates have a better heat stability than calcium caseinate when calcium presents in the system (Kinsella & Morr, 1984).

Solubility

The solubility of sodium, potassium and ammonium caseinate is desirable, as they are readily soluble. However, calcium caseinate forms a colloidal solution. Within pH 3~5 (near the pI), the solubility of caseinates is poor (Kinsella & Morr, 1984).

Hydration properties

In terms of hydration properties, 1g caseinate can bind or entrap up to 3.8 g water, it is temperature dependent, as the water binding of sodium caseinate drops from 3 g/g to around 0 when the temperature increases from 25 to 80℃ (Mulvihill, 1992). The water

binding capacity of caseinate is also related to the relative humidity as well. (Mulvihill, 1992).

Viscosity

The viscosity of caseinate solutions depends on the condition of the solutions, such as pH, temperature, calcium ions and protein concentration (Mulvihill, 1992). For sodium caseinate solution, the viscosity is highly pH dependent, with a minimum value at neutral pH. The viscosity of sodium caseinate solution is proportional to log concentration, and the log viscosity is a linear function of the reciprocal of absolute temperature. Calcium concentration has an impact on the viscosity of caseinate solution, since with low levels of Ca^{2+} the viscosity increases in alkaline system and reduces with the decline of pH.

Surface active properties

The surface activity of sodium caseinate is better than whey protein, soy protein or gelatin, as it depresses the interfacial tension effectively, diffuses and adsorbs quickly to the interface (Mulvihill, 1992).

Emulsifying and foaming properties

The emulsifying and foaming properties of caseinate are due to the amphipathic nature of the casein proteins, and the foaming and emulsifying abilities would be influenced by the ratio of the individual caseins (Hunt & Dalgleish, 1994). Accordingly, caseinates are able to adsorb at the interfaces (air/water and oil/water) and stabilise the system through electrostatic and steric repulsion. The mechanical energy for emulsion and foam formation is reduced, which is attributed to the decrease of the surface and interfacial tension (Mohanty, Mulvihill, & Fox, 1988). According to Schut, Tornberg and Lundh (as cited in Kinsella & Morr, 1984) , sodium and potassium caseinates have good emulsifying capacity while calcium caseinate has poor emulsifying functionality (a further discussion of caseinate-stabilised emulsions will be seen in section 2.4).

Several studies have focused on the foaming capacity of sodium caseinate (Carrera Sanchez & Rodriguez Patino, 2005; Corzo-Martínez, Carrera-Sánchez, Villamiel, Rodríguez-Patino, & Moreno, 2012; Marinova et al., 2009; Mohanty et al., 1988; Sceni & Wagner, 2007). Compared to whey protein concentrates and egg white, caseinates can produce foams with higher overrun but shorter break-down time (Mulvihill & Ennis, 2003).

Gelation

Gelation occurs when sodium caseinate solutions or emulsions undertake various treatments, such as heating, acidification and high-pressure process. The emulsion gelation will be further affected when proteins interact with other ingredients in the system, for example, calcium ions, alcohol, sugar, surfactants and hydrocolloids (Dickinson, 2006).

Thermoplastic and film forming properties

Several studies have reported the film forming properties of sodium caseinate (Arrieta, Peltzer, Garrigós, & Jiménez, 2013; Caprioli, O'Sullivan, & Monahan, 2009; Matsakidou, Biliaderis, & Kiosseoglou, 2013; Pereda, Aranguren, & Marcovich, 2008; Schou et al., 2005). Caseinates are considered as good materials for edible films for food package as they provide appropriate technical properties, such as network formation, plasticity and elasticity, transparency, high barrier for oxygen, carbon dioxide and aromas, good nutritional quality and biodegradability (Caprioli et al., 2009; Pereda et al., 2008). These edible films are obtained from caseinate solution (Arvanitoyannis, Psomiadou, & Nakayama, 1996).

2.3.4 General methods of caseinate manufacture

The methods of caseinates manufacture used in food industry are shown in Figure 2.4 and 2.5, including the procedures for sodium, potassium, ammonium, calcium and citrated caseinates (Mulvihill & Ennis, 2003).

Sodium caseinate

As it can be seen in Figure 2.4, the production of sodium caseinate involves the reaction of acid casein and NaOH. It starts with the acid casein curd or dry acid casein, and it is minced and mixed with water to get a solids content of approximately 25% prior to passing through a colloid mill. The resulting casein slurry is reacted with NaOH under 45℃ to get a final pH of 6.6~6.8, then the mixture is vigorously agitated and heated in a vat and recirculated and/ or transferred to the next vat to complete the solubilisation process at around 75℃. NaOH solution will be added to adjust the pH if it is necessary. The temperature of the curd is then raised to around 95℃ in the heat exchanger and passed to the balance tank. Finally the solution is spray dried into sodium caseinate powder in the spray drier when the viscosity of the solution is set to a desired level.

There are some additional ways to produce sodium caseinate. By using the roller-drier, the casein curd with 50~65% moisture is mixed with alkaline sodium salt (Na_2CO_3 or $NaHCO_3$) to produce the roller-dried sodium caseinate. In order to increase the bulk density and enhance the dispersibility of caseinate, granular caseinate is produced by mixing acid casein curd (< 40% moisture) and Na_2CO_3 together and is agitated for 1 h and dried in a fluidized bed drier or a pneumatic ring drier. There is also another way to raise the bulk density. Acid casein curd and Na_2CO_3 are dried in the attrition drier to manufacture a spray-dried like sodium caseinate. The extrusion techniques can also be adopted in caseinate manufacture.

Potassium and ammonium caseinates

The methods for producing potassium and ammonium caseinates are similar to the one used for sodium caseinate, except for KOH and NH_4OH are used in the manufacture, respectively. Ammonium caseinate can be produced by the reaction of dry acid casein and the ammonia gas, and then degas the extra ammonia from the final product.

Calcium caseinate

The particle size of the acid casein curd is mixed to be uniform in a mixer where there is 25% total solids in the mixture. The mixture is transferred to a colloid mill and the temperature is set to 35~40℃. The slurry is mixed with 10 vol% $Ca(OH)_2$ and agitation and recirculation occurs in the conversion tank for more than 10 min at a low temperature. The solution is heated at 70℃ prior to spray drying.

Citrated caseinate

Citrated caseinate is produced in a manner which is analogous to the spray-dried sodium caseinate, as NaOH is replaced by trisodium citrate and tripotassium citrate.

2.3.5 Applications in food industry

Based on the multiple functionalities, caseinates are widely used in food industry, and the applications are summarized in Table 2.2.

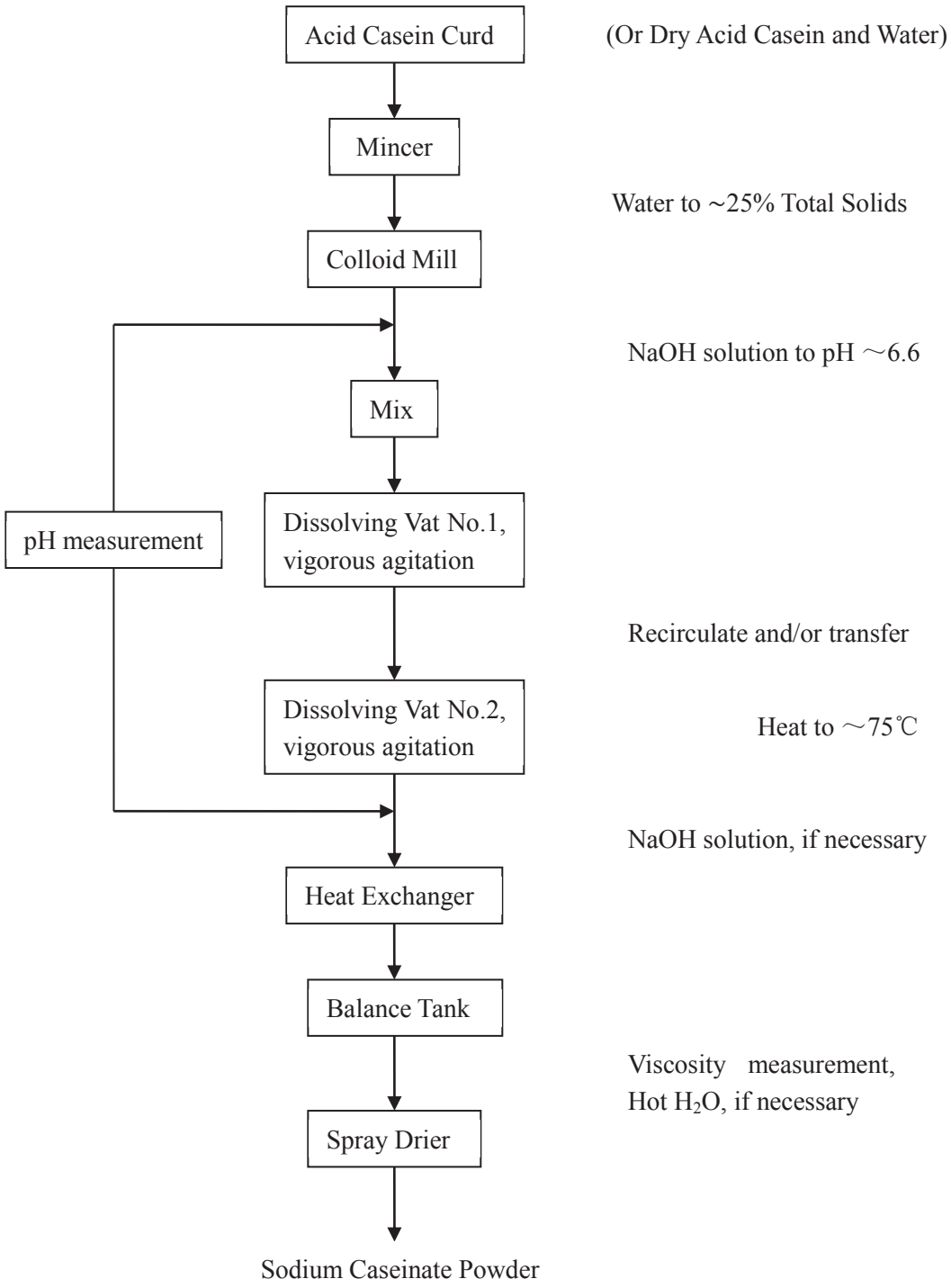


Figure 2.4 Method for the manufacture of sodium caseinate.

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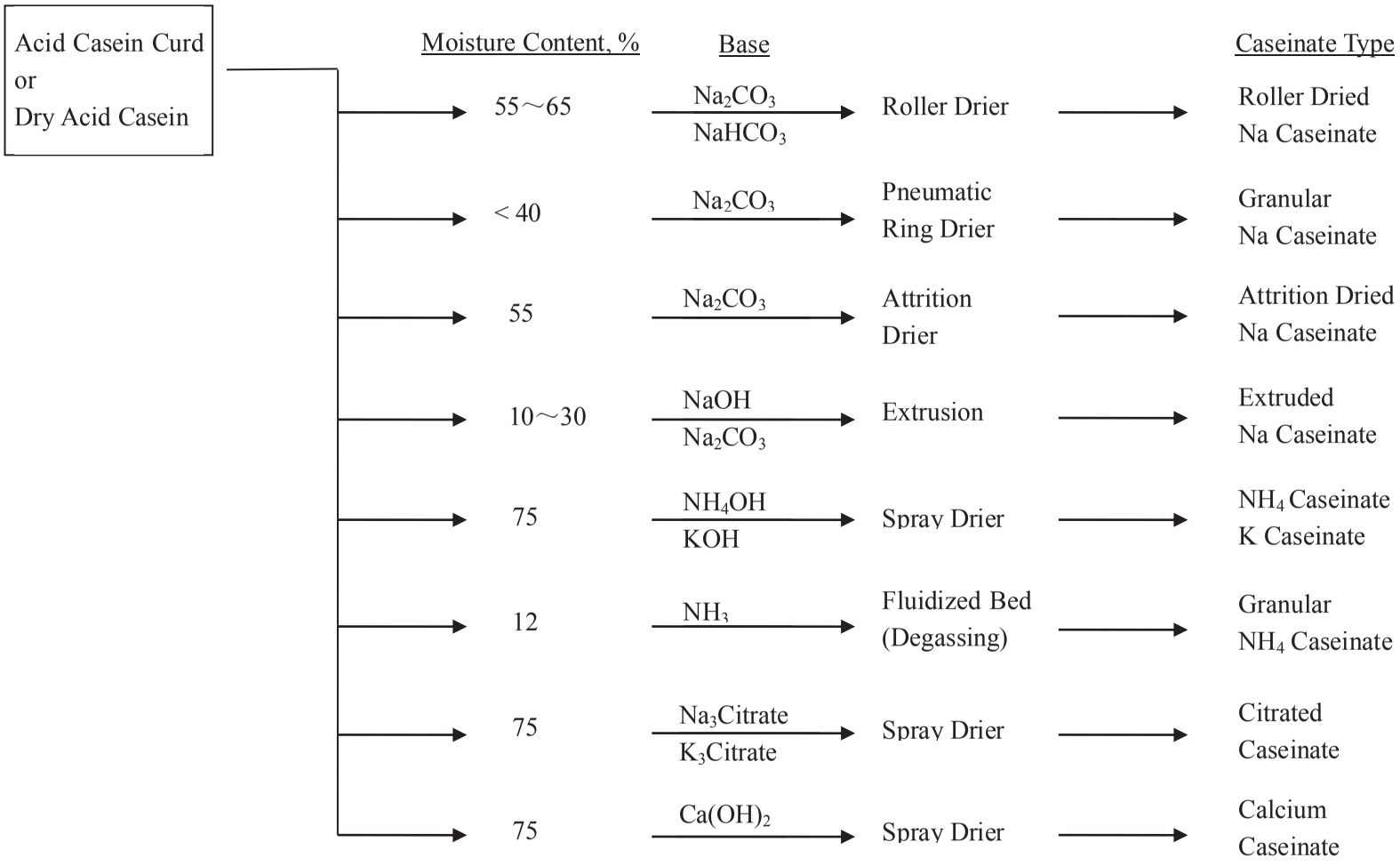

Figure 2.5 Methods for the manufacture of different caseinate types.

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Table 2.2 Applications of caseinates in food industry*

Category	Application	Effect
Dairy products	imitation cheese	fat and water binding, texture enhancing, melting properties, stringiness and shredding properties
	coffee creamer	emulsifier, whitener, gives body and texture, promotes resistance to feathering, sensory properties
	cultured milk products, e.g. yoghurt	increase gel firmness, reduces syneresis
	milk beverages, imitation milk, liquid milk fortification, milk shakes	nutritional, emulsifier, foaming properties
	high fat powders, shortening	emulsifier, encapsulating agent
Beverages	cream liqueurs	emulsifier
	wine apertifs	fines removal
Dessert type products	ice cream, frozen desserts	whipping properties, body and texture
	mousses, instant pudding, whipped topping	whipping properties, film former, emulsifier, imparts body and flavor
Bakery products	bread, biscuits/cookies, breakfast cereals, cake mixes, pastries, frozen cakes and pastries, pastry glaze	nutritional, sensory, emulsifier, dough consistency, texture, volume/yield
Confectionary	toffee, caramel, fudges	confers firm, resilient, chewy texture; water binding, emulsifier
Pasta products	macaroni, pasta, imitation pasta	nutritional, texture, freeze-thaw stability, microwaveable
Meat products	comminuted meat products	emulsifier, water binding, improves consistency, release meat proteins for gel formation and water binding
Pharmaceutical products	infant foods	low-lactose infant formulae, specific mineral balance infant foods
Convenience foods	gravy mixes, canned cream soups and sauces, dehydrated cream soups and sauces, convenience food	whitening agents, emulsifying agents, viscosity controllers, skim milk powders replacement
Texture products	protein-enriched snack-type products	texture
Edible film	Edible film	control tensile, strength, stretchability and opacity, moisture barrier, sensory properties

* The caseinates here refer to caseinates, the co-precipitates of caseinates and caseins or caseinate-whey protein blend in food industry.(Adopted from Mulvihill & Ennis, 2003)

2.4 Emulsions

A detailed introduction to the field of food emulsions, their formation, stabilisation and destabilisation is provided by Dickinson (1992b). An emulsion is defined as a dispersion of a liquid phase in another immiscible liquid continuous medium. There are two principal types of emulsions, oil-in-water emulsion and water-in-oil emulsion, although there are additional classes of emulsions, such as double and nano-emulsions. It is noted by Dickinson that emulsion formation is not spontaneous, usually requiring a considerable amount of mechanical energy supplied through intense agitation. High-pressure homogenisation is widely used in food industry in producing fine emulsions. There are also some other ways to generate vigorous agitation, such as laboratory blenders and mixers, colloid mills and ultrasonication.

Emulsions made by mechanical stirring are kinetically rather than thermodynamically stabilised (Dickinson, 1992b). Relative stability is influenced by particle size and the adsorption of emulsifiers to the oil-water interface. Surfactants, phospholipids, proteins and polysaccharides are all emulsifiers which are commonly used in food industry (Garti, 1999; Krog & Sparso, 2004). Proteins, which are widely used, are able to adsorb to the surface of the droplets with their amphiphilic structure, lowering the interfacial tension and establishing a mechanically robust film. Moreover, proteins stabilise the emulsions through a combination of steric and electrostatic repulsion which keep neighbouring droplet separated (Dickinson, 1997, 1999).

2.5 Emulsion stability

Apart from lipids and water, commercial emulsion food products are usually mixed systems comprising a range of food ingredients, such as proteins, polysaccharides, small-molecule surfactants, salts, sugars, alcohol, preservatives, colours and flavours (Dickinson, 1992b). Shelf-life and product stability is dependent on controlling the

various mechanisms of instability that occur in these complex systems, including the role of creaming, flocculation, coalescence, Ostwald ripening and phase inversion.

2.5.1 Creaming

Creaming is the movement of droplets under gravity to form a cream layer at the top of the oil-in-water emulsion system. Oil droplets are usually less dense than the surrounding aqueous phase, and are accordingly driven upwards through gravity or centrifugal forces without necessarily changing the droplet size distribution (Dickinson, 1992b). Creaming in the absence of coalescence or droplet interactions is reversible, as gentle shaking can turn the system to be the original stage. The creaming behaviour of the emulsion can be expressed in Stokes' Law (Dickinson, 1992b):

$$v_s = 2a^2(\rho_0 - \rho)g/9\eta_0$$

Where v_s is the settling speed of the droplet, a is the radius of the droplet, ρ_0 is the density of the continuous phase, ρ is the density of the droplet, g is the local acceleration of free fall and η_0 is the Newtonian shear viscosity of the continuous phase. This equation assumes that the droplet is isolated, rigid, uncharged. Creaming behaviour can be interpreted by Stokes' Law when there is no flocculation in the system. According to Stokes' Law, there are three ways to retard creaming, which are the reduction of the density difference between phases, the diminution of the average droplet size and the increase of the disperse phase viscosity (or the introduction of a yield stress). Creaming is considered to be negligible when v_s is less than 1mm a day (Dickinson, 1992b).

2.5.2 Coalescence

Coalescence is the movement of two or more droplets coming close together to form a larger droplet and is an irreversible process (Dickinson, 1992b). It is suggested that the breakage of the liquid film between droplets gives rise to coalescence. Accordingly, coalescence might happen when droplets are in close contact, such as within a cream layer or flocculated state, and the thickness of the intervening film has thinned to a

certain point. Two stages are involved in coalescence, which are film thinning and film rupture (Dickinson, 1992b). Coalescence happens when a thin liquid film is formed between two droplets and this intervening film (lamella) is ruptured under random surface fluctuations.

2.5.3 Flocculation

Emulsions tend to flocculate when the interparticle pair potential between droplets is appreciably negative (Dickinson, 1992b). Droplet flocculation can affect the destabilization mechanisms in emulsion systems, such as creaming and coalescence (Robins, 2000; Tcholakova, Denkov, Ivanov, & Campbell, 2006). For sodium caseinate stabilised emulsions, protein concentration plays a marked role in inducing flocculation (Dickinson & Golding, 1997a, 1997b; Dickinson, Golding, & Povey, 1997a). At low protein concentrations, bridging flocculation occurs when there is insufficient protein present to saturate fully the droplet surfaces. Conversely, at high protein concentration, excess non-adsorbed proteins cause depletion flocculation. Moreover, flocculation will also arise from the perturbations reducing the protein adsorption at the interface, like thermal treatment, changing of pH, addition of calcium ions and low-molecular-weight surfactants (Dickinson, 1992b). Addition of polysaccharides can also induce flocculation as well through either bridging or depletion mechanisms. The degree and the reversibility of the flocculation depend on the interaction and permanency of bonds formed between droplets (Cao, Dickinson, & Wedlock, 1990; Dickinson, 2008; Dickinson, Semenova, Antipova, & Pelan, 1998; Hemar, Tamehana, Munro, & Singh, 2001; Moschakis, Murray, & Dickinson, 2005; Perrechil & Cunha, 2013).

2.5.4 Ostwald ripening

Ostwald ripening happens when larger droplets grow at the expense of smaller ones, as the dispersed phase in the smaller droplets dissolves into the continuous phase and transports to the larger one (Dickinson, 1992b). It is not a commonly encountered mechanism of instability in food emulsions.

2.5.5 Phase inversion

Emulsion phase inversion is the phenomenon that the disperse phase converses into the continuous phase, which is a conversion of an O/W emulsion into a W/O emulsion or in an opposite way. Phase inversion is usually unlikely to be spontaneous (except for changing the temperature in some emulsion systems), as it requires a considerable amount of mechanical energy (Dickinson, 1992b).

As the stability of emulsions is closed related to the quality of the food, like shelf-life, texture and mouthfeel, it is crucial to control the factors affecting the stability and rheological properties of emulsions. The physical factors which are generally important in influencing the quality of the emulsion system are listed in Table 2.3.

Table 2.3 Key physical factors affecting the stability and rheology of emulsions

	Mechanisms			
	Creaming	**Flocculation**	**Coalescence**	**Rheology**
Factors	droplet size; droplet size distribution; droplet volume fraction; density difference between phases; rheology of continuous phase	droplet volume fraction; rheology of continuous phase; electrostatic interactions; steric (polymeric) interactions	droplet volume fraction; rheology of adsorbed layer; thickness of adsorbed layer; fat crystallisation	droplet volume fraction; rheology of continuous phase; fat crystallisation

(Adopted from Dickinson, 1992b)

2.6 Caseinate-stabilised oil-in-water emulsions

Sodium caseinate is widely used as an emulsifier in food industry, as it provides a rapid adsorption to the surface of droplets through the amphiphilic structure of the component caseins (especially β -casein), lowering the interfacial tension and creating a good stabilization of the emulsion. This remarkable emulsifying functionality is attributed to the combination of electrostatic and steric interparticle repulsion (Dickinson, 1989, 1992a; Robson & Dalgleish, 1987).

2.6.1 Self-assembly in caseinate-stabilised emulsions

As indicated earlier, for emulsions stabilised with caseinate, one particular destabilization scenario observed at high protein concentration is depletion flocculation caused by non-adsorbed self-assembled caseinate sub-micelles in the continuous phase. According to the study of Asakura and Oosawa (as cited in Dickinson, 1992b) the mechanism of depletion flocculation is postulated to be caused by the osmotic pressure gradient generated from the exclusion of micelles between a pair of droplets. Based on this theory, when two droplets get close together and the distance between the two surfaces is less than the micelle diameter ($<2R_g$), non-adsorbing micelles are excluded from the intervening space. The increase of the concentration of excess unbound entities around the droplets leads to an osmotic pressure gradient, and pushes the droplets into contact through attractive interaction (Figure 2.6). Depletion layer thickness (Δ) is an important indicator for evaluating the range of the depletion force. Sperry (as cited in Radford & Dickinson, 2004) suggested that the depletion force was more long-ranged when the value of depletion layer thickness increases with larger non-adsorbed caseinate, and consequently the volume fraction of the non-adsorbed species needed to induce flocculation was lower. However, according to Vrij (as cited in Radford & Dickinson, 2004) when the depletion layer thickness became smaller, the depletion interaction was diminished.

Based on the research of McClements (as cited in Dickinson & Golding, 1997b) a thermodynamic model is used to quantitatively estimate the free energy required for inducing depletion flocculation in the emulsion system. The non-adsorbed micelles in the model are assumed to be spherical, with a single molecular weight, and have athermal behaviour in the continuous phase. The equations are as follow.

$$\Delta G_{dep} = -2\pi r_m^2 P_{osm}\left(r_d + \frac{2r_m}{3}\right) \qquad [1]$$

$$P_{osm} = \frac{CRT}{M}\left(1 + \frac{2C}{\rho}\right) \qquad [2]$$

Where r_m and r_d are the radii of the micelles and droplets, respectively; P_{osm} is the osmotic pressure arises from the exclusion of the micelles in the solution; C is the micelle concentration in the continuous phase, M is the average molecular weight of a micelle, and ρ is the micelle density. It is plausible that the model and equations can also be adopted for the depletion flocculation occurring in caseinate emulsions. Based on these equations, the depletion interaction free energy change $|\Delta G_{dep}|$ in caseinate-based emulsion should be more than a few kT (Dickinson, 1998). However, equation [1] can only be considered valid when $r_d \geq r_m$, as depletion flocculation is induced only when the particle size of non-adsorbed entities is more than 3∼4 times smaller than the droplet size, otherwise self-flocculation of both emulsion and the non-adsorbed entities would occur instead of depletion flocculation (Dickinson & Golding, 1998b).

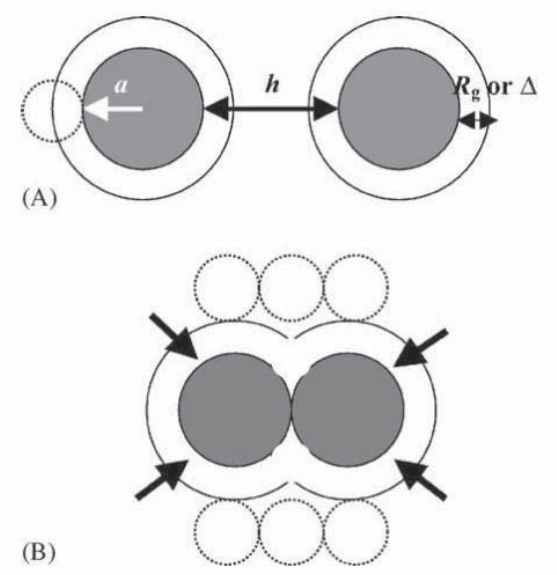


Figure 2.6 The mechanism of depletion flocculation of emulsion droplets by micelles.

(A) Two large spheres (radius, a) separated by a surface−surface distance, h, possess a depletion layer (Δ) of a thickness of the order of the radius Rg of the non-adsorbed species (dashed circle). (B) Osmotic pressure of the external medium pushes the large spheres together in the directions of the arrows. For clarity, this diagram is not to scale: the size ratio of large spheres to non-adsorbed species is typically considerably greater (Radford & Dickinson, 2004). Reprinted with permission of the Colloids and Surfaces A: Physicochemical and Engineering Aspects (Elsevier).

The optimum size of sub-micelles causing depletion flocculation in emulsion system has been studied as well (Radford & Dickinson, 2004). It is suggested that, the optimum size of the non-adsorbed caseinate sub-micelles for inducing depletion flocculation is approximately 20 nm in radius, as calculated theoretically. Asakura & Oosawa (as cited in Radford & Dickinson, 2004) noted that the optimum particle size obtained here was based on the assumption that caseinate sub-micelles are monodisperse and spherical, so in this case the value might be smaller if the sub-micelle was present in a rod-like conformation. Moreover, for the aggregates formed by self-assembled caseinate, the optimum size of the polymer was interpreted in size ratio (a/r = 10, where a is the radius of protein nano-particle, and r is the effective droplet radius). The depletion flocculation induced by these non-adsorbed self-associating aggregates will decline when the size ratio deviates from the optimum value (Radford & Dickinson, 2004) (Figure 2.7).

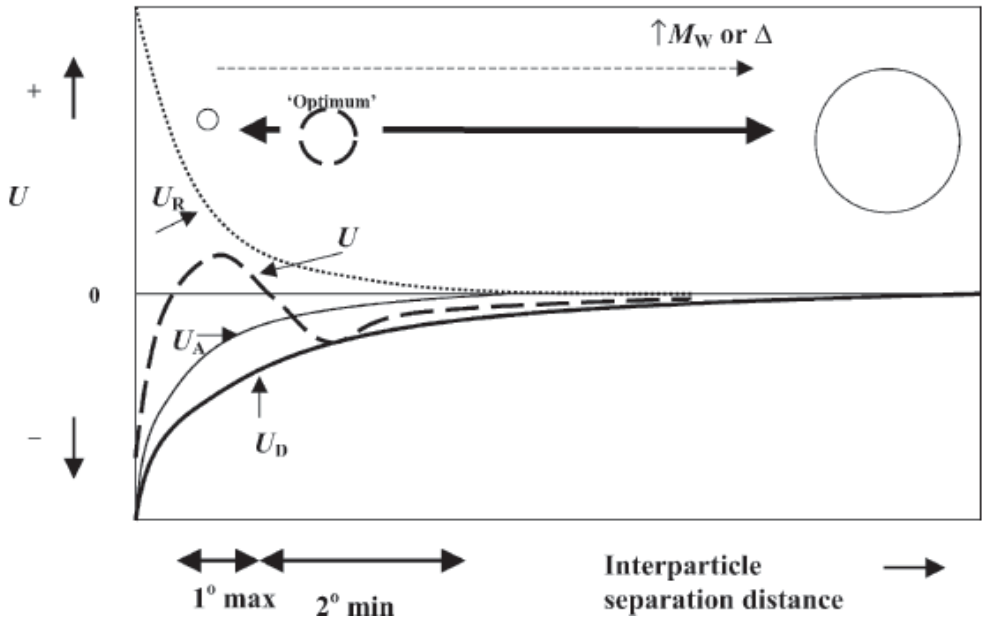


Figure 2.7 Schematic diagram showing the interplay between the various interparticle energies.

U_R (dotted line), U_A (thin solid line), U_D (thick solid line), and U (thick dashed line), and the mass/size of the excluded non-adsorbed species (M_w, Δ), in determining the optimum size for maximising depletion interactions. The primary maximum (1°) and secondary minimum (2°) regions are indicated (Radford & Dickinson, 2004). Reprinted with permission of the Colloids and Surfaces A: Physicochemical and Engineering Aspects (Elsevier).

2.6.2 Factors affecting the creaming stability of caseinate-stabilised emulsions

Protein concentration

Previous studied show that the stability of caseinate-stabilised emulsion was close related to the protein/oil ratio, which can be classified into three stages (Dickinson & Golding, 1997a; Dickinson et al., 1997a). According to the results, when the protein concentration was relative low, there was insufficient protein to saturate the interface (approximately less than half of the amount of saturation monolayer surface coverage), so proteins molecules were shared between adjacent droplets and bridging flocculation occurred. Bridging flocculation was irreversible, and it can accelerate creaming at low oil volume fractions while inhibited creaming with the formation of a network structure at high volume fractions. Bridging can also accelerate coalescence. At 2 wt% caseinate concentration, the droplet surface was nearly fully covered by the adsorbed protein layer and the emulsion remained stable for a period of time. When there were moderate excess non-adsorbed protein in the system ($c>c^*$, where $c^*$was the protein concentration for the onset of flocculation), phase separation occurred due to depletion flocculation. Depletion flocculation was reversible, as there was no change in the droplet size distribution. At high protein content ($c\geq c^*$), the flocculated droplets developed a strong gel network which retarded creaming and re-stabilised the emulsion system.

Temperature

It is suggested that emulsions stabilised with sodium caseinate (2 wt% protein) remain stable when they are heated at 90℃ for 30 min or 121℃ for 15 min (Hunt & Dalgleish, 1995). The mechanism for the stability of emulsion treated at sterilization temperature (121℃ for 15min) has also been reported (Srinivasan, Singh, & Munro, 2002) . However, under some certain circumstances, sodium caseinate stabilised emulsions are susceptible to temperature. Under controlled pH and calcium ions, the concentrated caseinate-based emulsion exhibits heat-induced thermoreversible gelation (Dickinson & Casanova, 1999; Dickinson & Eliot, 2003; Eliot & Dickinson, 2003).

pH

When the pH deviates from the isoelectric point of caseinate, the droplets with adsorbed caseinate become negatively or positively charged (above or below pI), and sufficient repulsive forces can retard the aggregation of droplets and improve the emulsion stability (Perrechil & Cunha, 2013). Meanwhile, McClements (as cited in Perrechil & Cunha, 2013) showed that flocculation occurs when the pH was close to pI, which was due to the droplet-droplet net attraction.

Adjusting the pH of the emulsion system can lead to acid-induced gelation. This is due to the shrink and collapse of the protecting layer on the surface of the droplets (Horne & Leaver, 1995). Lowering the pH towards the sodium caseinate isoelectric point (pH4.6) neutralizes the carboxylic groups leading to a minimization of charge repulsion both between proteins adsorbed at the interface, and the adsorbed layers of adjacent droplets (Horne & Leaver, 1995). The pH for sol-gel transition ranges from 3.2 to 5.8 (Chen, Dickinson, & Edwards, 1999).

Calcium ions

The calcium concentration has a great effect on the stability of caseinate-stabilised emulsions. According to Dickinson, Whyman and Dalgleish (as cited in Dickinson & Golding, 1998b) high calcium ion concentration (15 mM) induced electrostatic flocculation. Whereas, for a relative low calcium ion concentration (5 or 8 mM), the addition of Ca^{2+} enhanced the emulsion stability against depletion flocculation (Dickinson & Golding, 1998b). Here, the depletion attraction was found to decrease when the concentration of Ca^{2+} is between 5~10 mM (Dickinson et al., 2001). However, it should be noted that order of addition can influence stability. For emulsions in which ionic calcium was introduced before homogenisation, the system stayed stable when calcium concentration was no more than 6 mM. In contrast, destabilisation occurred when more than 10 mM ionic calcium was added after emulsification (Dickinson & Davies, 1999). The mechanism of the calcium ions inhibiting depletion flocculation has been studied as well (Horne & Leaver, 1995).

A further study demonstrated the synergistic effect of ionic calcium and non-ionic surfactant on the stability of emulsions (Dickinson, Radford, & Golding, 2003). It is suggested that when the calcium/caseinate molar ratio R was in the range of 2~4 accompanying with 0.25~0.75 wt% Tween 20, the emulsion was stabilised from a flocculated system. In this case, the adsorbed proteins were competitively displaced by Tween 20, and the calcium was released along with the proteins from the droplets, bound to the non-adsorbed proteins in the bulk and formed large sub-micelles aggregates. Consequently, the osmotic pressure decreased and weakened the depletion flocculation. However, the emulsion became unstable again when Tween 20 concentration increased.

The synergic effects of calcium ions and ethanol has also been studied, and a cooperative window for retarding depletion flocculation (calcium/caseinate molar ratio R = 0~8, 25~0 wt% ethanol) was found (Radford, Dickinson, & Golding, 2004).

Ionic strength

Emulsion stability is sensitive to the ionic strength in the aqueous phase. Srinivasan, Singh, and Munro (2000) studied the formation and stability of emulsions stabilised by sodium caseinate with the addition of NaCl. At low protein concentration (1 wt%), due to the increase in surface coverage and/or the rearrangement of the surface composition, the presence of NaCl slightly enhanced the stability of the emulsion. When the protein concentration increased (>2 wt%), the addition of NaCl led to a remarkable improvement in the creaming stability. The concentration of non-adsorbed protein in the continuous phase declined with increasing surface protein coverage, which inhibited depletion flocculation. Meanwhile, with the presence of NaCl the protein concentration required for inducing depletion flocculation was increased accordingly, compared to the emulsion without NaCl. There was no notable difference in the droplet size of the caseinate emulsion when NaCl was added before or after homogenisation. The study of Hunt and Dalgleish (1996) showed no significant change in the particle size distribution of caseinate emulsions with the KCl addition to 200 mM at pH 7, however the

adsorption behaviour of the individual caseins in the caseinate was seen to be affected with the KCl concentrations in excess of 25 mM .

Alcohol

For caseinate-stabilised emulsions, a high concentration of alcohol (30 ～40 wt%) resulted in emulsion phase separation (Agboola & Dalgleish, 1996; Burgaud & Dickinson, 1990). Here, the addition of alcohol reduced solvent quality, which favored caseinate precipitation and droplet flocculation (Herskovits, Gadegbeku, & Jaillet, 1970). In contrast, alcohol at a low concentration improved creaming stability, which was due to a reduction in the interfacial tension allowing smaller droplets to be produced (Dickinson & Woskett, 1988).

At protein concentrations above the saturation coverage for droplets, it has been shown that a relative high concentration of alcohol (25 vol%) can reduce depletion flocculation, compared to the emulsion with low alcohol concentration (≤10 vol%) or without alcohol, most likely due to increased droplet surface area which reduced the relative concentration of non-adsorbed protein. Alcohol can slow down flocculation, but it has limited effect on the creaming stability for a long period, which was attributed to Ostwald ripening and a subsequent reduction in surface area over time (Dickinson & Golding, 1998a).

Polysaccharides

Combination of protein and polysaccharides can influence structure, stability and the properties of emulsions (Moschakis et al., 2005). Polysaccharides are generally highly hydrophilic and thus not surface active (Dickinson, 2003). Addition of polysaccharide can modify the stability of caseinate-stabilised emulsions through two different types of mechanisms, depending on the interaction between protein and polysaccharide. For the caseinate-based emulsion with addition of non-interacting polysaccharide, low concentrations of polysaccharide can lead to a decrease in creaming stability due to depletion flocculation. However, when the polysaccharide concentration increases,

creaming is retarded and emulsion stability is enhanced due to increasing continuous phase viscosity, which restricts the mobility of the droplets and prevent them from flocculating or creaming (Hemar et al., 2001). Polysaccharides can also undergo attraction to the adsorbed protein layer, for example where protein-polysaccharide bonds are formed as a consequence of opposing electrostatic charges between the two species, or alternatively where the two species carry a like charge which is then bridged via multivalent counterion (Dickinson, 2008). In these emulsions systems, low polysaccharide concentration can lead to bridging flocculation. Increasing concentration can lead to complete coverage of the protein layer with the polysaccharide, stabilising the emulsion, whilst increasing concentration further can cause instability due to the presence of non-adsorbed polysaccharide inducing depletion effects(Dickinson, 2008).

2.6.3 Creaming profiles of caseinate-stabilised emulsions

At low to moderate protein concentration (above the critical concentration c^*), rapid phase separation occurs with the elevated caseinate concentration by depletion flocculation, yet at high protein concentration, creaming rate is slowed down (Dickinson & Golding, 1997a; Dickinson et al., 1997a; Liang et al., 2014). According to Stokes' Law, the creaming of the individual floc is not affected by other flocs in the system, but in practice, especially in concentrated caseinate-stabilised emulsion, the creaming mechanism cannot be simply described by Stokes' Law, it is a combination effect of the gravitational force and the impediment from the continuous phase (Liang et al., 2014). McClements (as cited in Liang et al., 2014) introduced an equation for the calculation of the creaming behaviour.

$$\nu = \frac{2a^2 \Delta\rho g}{9\eta_s}(1 - \phi/\phi_{max})^{8\phi_{max}}$$

Where ν is the creaming velocity, a is the droplet size (radius), $\Delta\rho$ is the density difference between the two phases, g is the acceleration of free fall, η_s is the continuous phase viscosity, and ϕ is the volume fraction of the continuous phase. According to this

equation, the propensity of creaming for aggregated or flocculated droplets will be enhanced with a larger size relative to the individual droplets (Dickinson, 2010), and creaming would be hindered by high continuous phase viscosity or a firm droplet network, as the diffusion of the droplets is restricted (McClements, 1999). Therefore, the continuous phase viscosity and the microstructure of the emulsion system play an important role in the creaming stability.

2.6.4 Rheological properties of caseinate-stabilised emulsions

A number of studies have been published detailing the rheological properties of sodium caseinate stabilised emulsion (Dickinson & Golding, 1997b; Dickinson & Golding, 1998b; Dickinson et al., 2003; Dickinson & Woskett, 1988; Liang et al., 2014; Radford et al., 2004; Tan & McGrath, 2013). For emulsions undergoing bridging flocculation, shear-thinning behaviour was observed (Dickinson & Golding, 1997b). This is due to the fact that emulsion viscosity increased as a certain amount of continuous phase was trapped in the flocculated aggregates, so when the emulsion underwent high shear stresses, with the breakage of the flocs, the viscosity reduced as some of the trapped continuous phase was released. According to their findings, for the emulsion with sufficient protein to cover the surface of droplets, Newtonian behaviour was observed. With the onset of depletion flocculation at increasing protein levels, shear thinning behaviour was again observed. Shear thinning was attributed to the formation of the flocculated network, and showed time dependent behaviour. Time dependent rheology observing the formation of the droplet network after pre-shearing showed an increase in apparent viscosity after the removal of the pre-shear step. Subsequent time dependent reduction in viscosity was believed to be due to structural consolidation, as flocs became more closely packed and some of the trapped continuous phase was released from the network. Eventually, the viscosity increased steadily, which was attributed to the reinforcement of the network structure and probably the slow restructure of the non-adsorbed self-assembled caseins in the continuous phase.

Liang et al. (2014) discussed the effects of the non-adsorbed proteins in the aqueous phase on the stability of the emulsions, noting that at elevated caseinate concentrations

emulsion stability was enhanced as a consequence of increasing continuous phase viscosity. Structural and rheological observations indicated that as continuous phase viscosity was increased, rate of network formation was decreased, and thus stability was not governed by the formation of a percolating network.

2.6.5 Microstructure of caseinate-stabilised emulsions

The changes in microstructure vary as a function of the protein concentration (Dickinson et al., 1997a). Discrete individual clusters were observed when the caseinate-based emulsion underwent depletion flocculation at a relative low protein concentration. With the increase of the caseinate concentration, the droplet network became more expansive and a gel-like network was formed through the interconnected flocs. The structural evolution of caseinate-stabilised emulsion during a long timescale has been studied as well (Liang et al., 2014). For 2, 4 and 5 wt% protein concentration, open network was observed and the emulsion compacted during aging. At 6 wt% protein concentration, flocculated clusters were present and an open network was formed after 4 and 6 h, respectively. However, for 8 wt% protein concentration, there was no discernable droplet network forming in the first 6 h. The development of the droplet network was retarded as there was a slow rearrangement of the clusters under high viscosity, although the depletion attraction was strengthened by the increasing protein concentration.

2.7 The dissociation of caseins aggregates

Addition of urea, SDS, κ- or β-casein, adjustment of pH and the removal of colloidal calcium phosphate are known to lead to the dissociation of casein micelles (Fox, 2003; Kruif & Holt, 2003). Urea has been widely used for the dissociation of proteins. It is found that urea has the capability to alter the hydrophobic and hydrogen bonds in proteins and reduces the viscosity with the breakage of the hydrophobic aggregates, while the linkages of calcium phosphate remain intact (Aoki, Kako, & Imamura, 1986; Aoki, Yamada, Tomita, Kako, & Imamura, 1987; Towler, Creamer, & Southward, 1981). Casein components can be fractionated from casein dues to the difference in

solubility in urea aqueous solutions (Hipp, Groves, Custer, & McMeekin, 1952). The addition of 6 M urea in skim milk would lead to a significant diminishment of the white appearance, and the average size of the micelles become smaller than the native ones, falling below the size threshold for light scattering (McGann & Fox, 1974). It is also reported that caseinate dissolves completely in 6.6 M urea solution (Haller & Pallansch, 1960). In the paper coating industry likewise, according to Salzberg & Marino (as cited in Towler et al., 1981), urea is applied to reduce the casein solution viscosity. It is suggested that urea (e.g. 6 M) might be able to be used to deliberately reduce the nano-particle size of caseinate by breaking the hydrophobic bonds in the caseinate association, which assists to predict dissociation effects of urea on the depletion flocculation in emulsions with non-adsorbed caseinate (Radford & Dickinson, 2004).

Therefore, it is plausible that urea can be applied in sodium caseinate solutions or emulsions to dissociate the aggregates induced by self-assembly. Self-assembly of caseinate leads to high viscosity in solution, and it also induces depletion flocculation in emulsion at high protein concentration, hence it is speculated that dissociation of caseinate aggregates might cause reduction in apparent viscosity in solution and impede depletion interaction in emulsion.

2.8 Research questions

So far there have been extensive studies about the application of sodium caseinate in solutions and emulsions. The researchers have been concerned about the particle size distribution and rheological properties of the caseinate solutions and the emulsifying functionality of caseinate in the emulsions, and some of them have shown great interest in the effects on the structure, rheological properties and functionalities of the caseinate-based solutions and emulsions caused by the self-assembly behaviour of caseinate.

It is suggested that the self-assembly of caseinate leads to some negative effects in the application of caseinate in solutions, which are long dissolving time and limitation of the protein concentration in spray drying. Meanwhile, for caseinate-stabilised emulsions, depletion flocculation and creaming are known to occur due to caseinate

self-association. However, there are few studies about the dissociation of caseinate aggregates induced by self-assembly behaviour in solutions and emulsions containing caseinate. Hence, the influences of urea on the self-assembled caseinate aggregates in caseinate solutions and emulsions are of interest in here. The hypothesis here is that as urea has been proved to be able to dissociate caseins, the dissociation of self-assembled caseinate will improve the functionalities of caseinate in solutions and emulsions.

In this study, the particle size and rheological properties of sodium caseinate solutions with the addition of urea are investigated, and the urea effects on the creaming profiles, rheological properties and microstructure of the oil-in-water emulsions stabilised with sodium caseinate are studied as well. How urea affects the emulsions stabilised by potassium caseinate, particularly regarding any possible effects of the potassium counterions on depletion effects, are also studied for comparison.

3 Materials and Methods

3.1 Materials

Sodium caseinate and potassium caseinate powders were obtained from Tatua Co-operative Dairy Company Limited (New Zealand), and the product analysis can be seen in Table 3.1. Soybean oil was used in oil-in-water emulsions, which was obtained from James Gilmour & Co. Ltd. (New Zealand), containing 100% soyabean oil, antifoam and antioxidants (no sodium or potassium). 2 vol% methylene blue solution (20 vol% ethanol, 78 vol% water) was prepared as the stain for visualizing the creaming profiles in emulsions. All the chemicals used were of chemical grade.

Table 3.1 Product analysis of caseinates

Parameter	Analysis	
	sodium caseinate (wt%)	**potassium caseinate (wt%)**
Protein (Dry basis)	97.4	96.5
Fat	0.6	0.8
Moisture	4.4	4.5
Ash	3.5	4.6

3.2 Sample preparation

3.2.1 Preparation of caseinate solutions

Sodium caseinate powder was dissolved under shear in R.O. water or urea solutions at 60 °C at a range of protein concentrations. Sodium azide powder (0.02 wt%) was added into the solutions as a bacteriostatic agent. The concentration of urea in solutions was 1.1, 3.3 and 6.6 M.

Potassium caseinate powder was dissolved under shear in R.O. water or 6.6 M urea solution at 60 °C at 20 wt% protein concentration. Again, 0.02 wt% sodium azide was added as a bacteriostatic agent.

3.2.2 Preparation of caseinate-stabilised emulsions

Sodium caseinate stabilised emulsions were prepared in the following steps. Sodium caseinate powder was dissolved in R.O. water at 50 °C at concentrations of 2, 4 and 6 wt%. Soybean oil (30 vol%) was blended with sodium caseinate solutions and pre-homogenised at 10,000 rpm for 1 min using a high speed mixer (D500 series, Labserv, Germany) to form coarse emulsions. The coarse emulsions were then homogenised at ambient temperature using a two-stage homogeniser (APV-2000, SPX, US) at 200 bar (stage one). Sodium azide powder (0.02 wt%) was added into the emulsions as a bacteriostatic agent. Urea powder was then added to the emulsions at concentrations of 1.1- 6.6 M relative to the continuous phase. The pH of the emulsions was normalised at 6.8 by adding addition of 0.1 M HCl.

Potassium caseinate stabilised emulsions were prepared in the same procedure as described above.

3.3 Analyses of the samples

3.3.1 Particle size of caseinates

Particle size data of sodium caseinate and potassium caseinate were obtained at 25 °C with dynamic light scattering (DLS) (Zetasizer Nano ZS, Malvern Instruments Ltd UK), operating at 173° backscatter. Replicates of the measurements (6 times) were taken to yield consistent results.

For the sodium caseinate solutions used in the particle size analysis, sodium caseinate powder was dissolved in R.O. water or urea solutions at ambient temperature, to achieve a concentration of 1 wt%. The concentration of urea solution was 1.1, 3.3 and 6.6 M, respectively. EDTA disodium salt was added into the solutions at a range of concentrations (50, 100, 200 mM). The pH of the samples with EDTA was normalised at 6.6 by addition of 1 M NaOH.

For the potassium caseinate solutions likewise, potassium caseinate powder was dissolved in R.O. water or 6.6 M urea solution at ambient temperature at 1 wt% protein concentration. EDTA disodium salt was added into the solutions at concentration of 200

mM. The pH of the samples with EDTA was normalised at 6.6 by addition of 1 M NaOH.

According to the size quality report, the samples were too polydisperse for cumulant analysis, and thus the z-average radius was not considered reliable. Accordingly, size distribution analysis was used to evaluate the particle size of the components in caseinate solutions. The results of size distribution were displayed in two ways, which were size distribution by intensity and by number. According to the theory of DLS, the size distribution by intensity is what is measured by DLS, and the size distribution by number is calculated from the results of intensity. The size distribution by number shows the size at which particles are present in greatest number. The results were the mean values of the multiple measurements. General purpose (normal resolution) was used as the analysis mode.

3.3.2 Droplet size of caseinate-stabilised emulsions

The droplet size of the caseinate emulsions was determined by a laser diffraction instrument (Mastersizer 2000, Malvern Instruments Ltd., Worcestershire, UK). The refractive index of the emulsion droplets was 1.47 and the one of dispersant was 1.33. The absorbance value of the emulsion droplets was 0.001. The size of the droplets was determined as the surface-weighted mean diameter $d_{3,2}$ (μm), the volume-weighted mean diameter $d_{4,3}$ (μm), the specific surface area (m^2/g) and droplet size distribution. The droplet size was reported as the average of two measurements.

3.3.3 Rheological measurements of caseinate solutions

For shear rate dependence experiments, measurements of caseinate solutions were carried out at 25 °C with a TA Instruments AR-G2 rheometer using cup and bob geometry (15mm stator inner radius, 14 mm rotor outer radius, 42 mm cylinder immersed height, 5920 μm gap). Shear rates in the measurement were from 0.01 to 100 s^{-1} in logarithm mode, and 6 measuring data points were collected for each decade. Foam was carefully removed and samples equilibrated for 2 min before sampling.

For the remainder of the rheological experiments, the viscosity of caseinate solutions was measured with the same geometry. The shear rate was fixed to 10 s^{-1} and the

temperature was set to 25 °C, except for the temperature dependence experiment. Since for 20 wt% sodium caseinate solution without urea, the solution viscosity at 5℃ was out of the range of measurement at the shear rate of 10 s^{-1}, and thus 0.5 s^{-1} was used. Foam was carefully removed before testing and 2 min for equilibration was used. For all the rheological experiments, triplicate measurements were performed to yield stable results, and the mean values were obtained.

3.3.4 Rheological measurements of caseinate-stabilised emulsions

The rheological behaviour of the emulsions was measured at 25 °C with a TA Instruments AR-G2 rheometer using double gap geometry (17.50 mm rotor outer radius, 16.00 mm rotor inner radius, 15.10 mm stator inner radius, 53.00 mm cylinder immersed height, 2000 μm gap). A logarithmic setting of shear rates was applied from 1 to 100 s^{-1}, with 6 measuring data points collected for each decade. Foam was carefully removed and samples equilibrated for 2 min before sampling. Measurements were carried out twice to yield stable results for each sample, and mean values were obtained.

For time dependence experiments, emulsions were measured at 25 °C with a TA Instruments AR-G2 rheometer using cup and bob geometry (15mm stator inner radius, 14 mm rotor outer radius, 42 mm cylinder immersed height, 5920 μm gap). Foam was carefully removed from the sample and a layer of mineral oil was added to minimize water evaporation during the measurement. Samples equilibrated for 2 min before pre-shearing. Pre-shear was performed at the shear rate of 300 s^{-1} for 1 min to break up any existing flocs. An oscillation test was conducted at a constant frequency of 1Hz and shear strain of 0.1 (which was in the linear viscoelastic region).

3.3.5 Creaming stability of caseinate-stabilised emulsions

Emulsion stability was studied using a Turbiscan Classic MA 2000 (Formulaction, Toulouse, France) at room temperature. Details of methodology were previously outlined by Liang et al. (Liang, Patel, Matia-Merino, Ye, & Golding, 2013). Visual creaming profiles of the emulsions were studied as well. Emulsions were transferred into flat-bottom screw-top tubes (15 mm x 100 mm), and then 20 μL of 2 vol% methylene blue solution was added to 10 mL emulsion as a contrast agent for visual

determination of creaming. As methylene blue is a water-soluble dye, most of the dye will be taken up by the continuous phase when phase separation occurs in the emulsion. The colour and the turbidity of the two phases can be used to determine the extent of the creaming behaviour. This was fully mixed for 1 min with a vortex mixer. Samples were stored at room temperature.

3.3.6 Microstructure of caseinate-stabilised emulsions

Microstructure of emulsions was visualised using a confocal laser scanning microscopy (CLSM) (Leica SP5 DM6000B, Leica Microsystems, Heidelberg, Germany). 20 µl of 0.1 wt% Nile Red and 0.1 wt% Fast Green were used as fluorescent stains for fat and protein respectively, added to 0.5 mL of sample and mixed thoroughly in a centrifuge tube. Each sample was then further mixed for 1 min with a vortex mixer (PV-1, Grant-bio, England) and allowed to rest for 45 min at room temperature before scanning. A 5µL of sample was place on a microscope slide and then covered with a cover slip. Samples were examined at λ= 488 nm for Nile Red and λ = 633 nm for Fast Green. A magnification lens at 63x was used.

4 Effects of urea on sodium caseinate solutions

4.1 Particle size of sodium caseinate

4.1.1 Sample preparation and particle size of sodium caseinate

Particle size distribution for 1 wt% sodium caseinate solutions in the absence of urea, but containing increasing concentration of EDTA are shown in Figure 4.1 (A). For the sample without EDTA, a unimodal distribution was observed, with the radius of the model distribution centred at 120 nm. However, on addition of EDTA, in size distribution by intensity, a bimodal distribution was observed (Figure 4.1 (B) to (D)). The first modal distribution of particles centred between 14 and 21 nm, and the second one centred at ~120 nm. As the EDTA concentration increased from 50 to 200 mM, the relative peak intensity of the first modal rose from 27% to 45%. On the other hand, as it can be seen from Figure 4.2, in size distribution by number, the second modal vanished with the addition of 200 mM EDTA. Similar results were observed from the samples with the addition of 50 and 100 mM EDTA (data not shown).

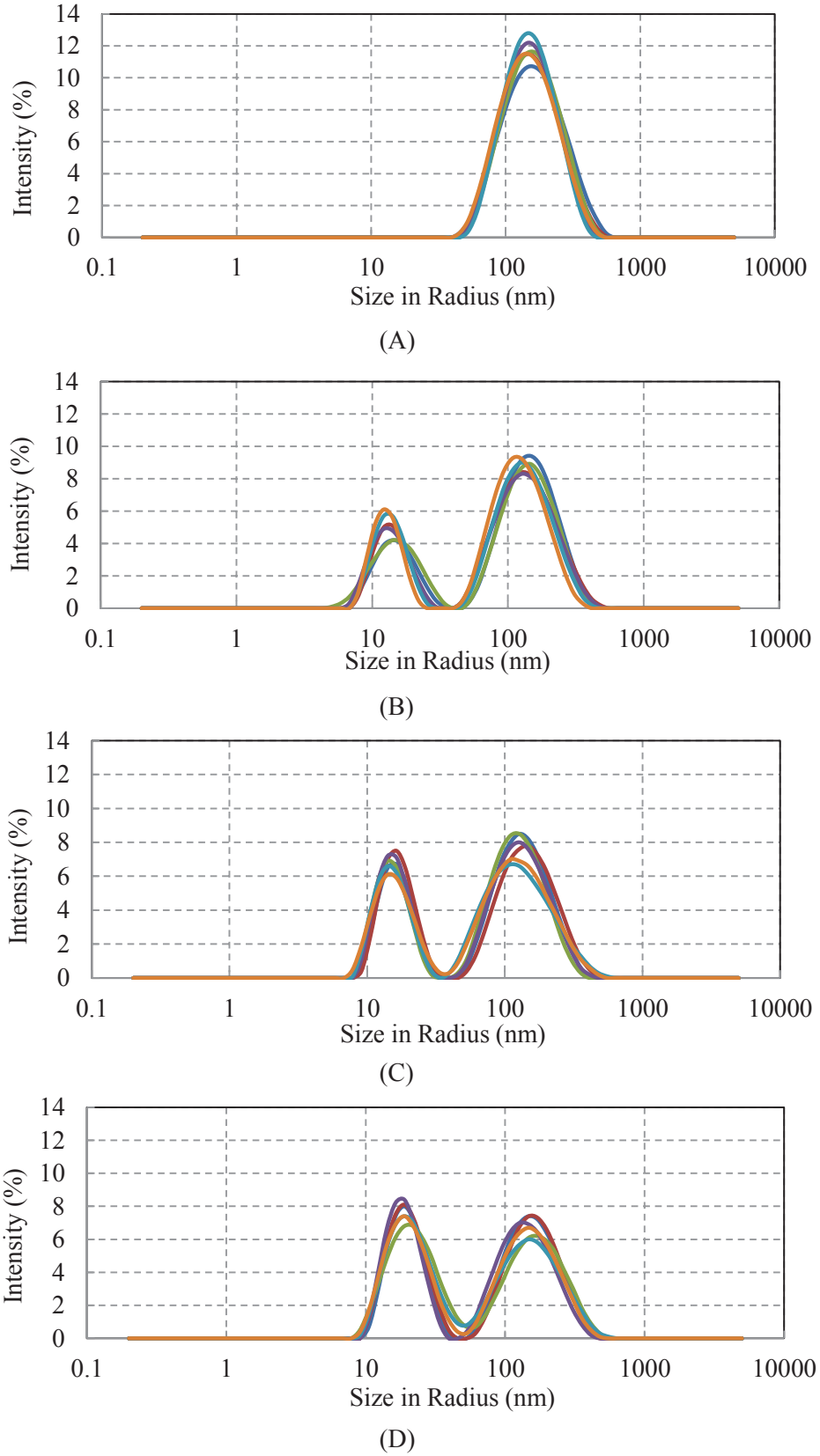


Figure 4.1 Size distributions by intensity of 1 wt% sodium caseinate solutions with EDTA.

(A) control (EDTA-free) (B) 50 mM EDTA (C) 100 mM EDTA (D) 200 mM EDTA

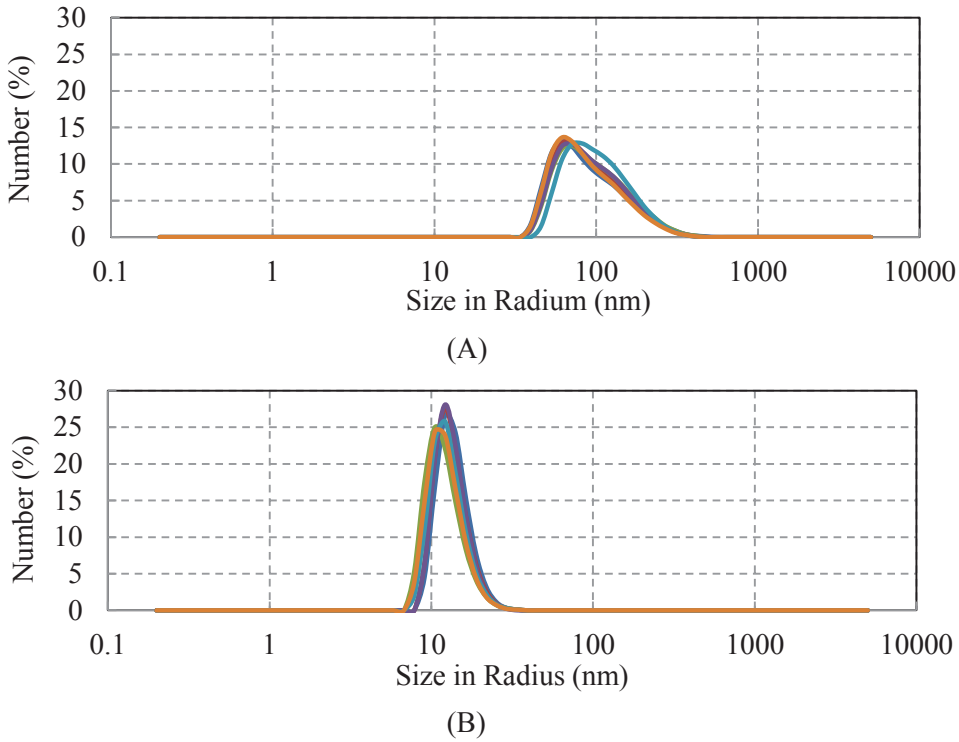
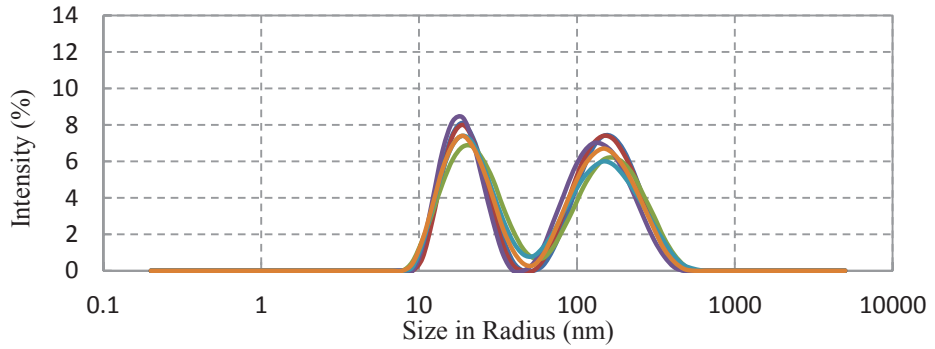


Figure 4.2 Size distributions by number of 1 wt% sodium caseinate solutions with EDTA.

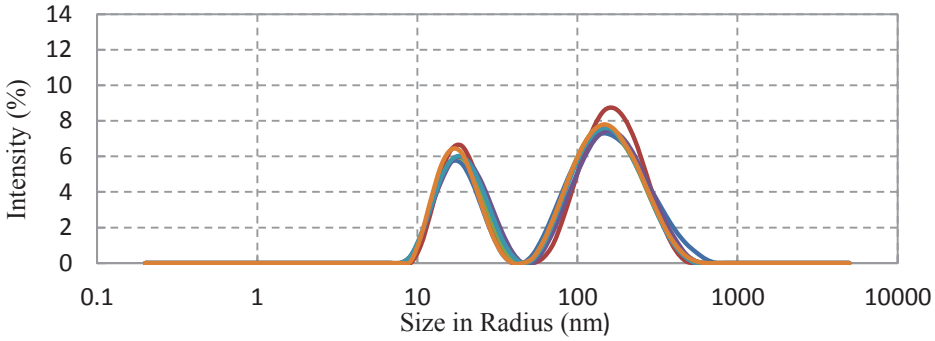
(A) control (EDTA-free) (B) 200 mM EDTA

4.1.2 Effects of urea on the self-assembly in sodium caseinate solutions

The urea effect on the size distribution by intensity and by number of sodium caseinate solution was shown in Figure 4.3 and 4.4. All DLS experiments were performed on samples containing 200 mM EDTA. According to Figure 4.3, in the absence of added urea, a bimodal distribution was observed, with the radius of the first modal distribution centred at ~20 nm and a second modal distribution of particles centred at radius of ~120 nm. With increasing addition of urea, a reduction in the relative peak intensity of the first modal distribution was observed, decreasing from 45% (urea absent) to 34%, 14% and 4% at urea concentrations of 1.1, 3.3 and 6.6 M respectively. The addition of urea was seen to have markedly less effect on the relative peak intensity of the second modal distribution. In Figure 4.4, the size distribution by number also showed the size of caseinate sub-micelles gradually decreased with the increase of urea concentration. For the samples with 3.3 and 6.6 M urea, the size became diverse. There was no notable change in the number of sub-micelles.



(A)



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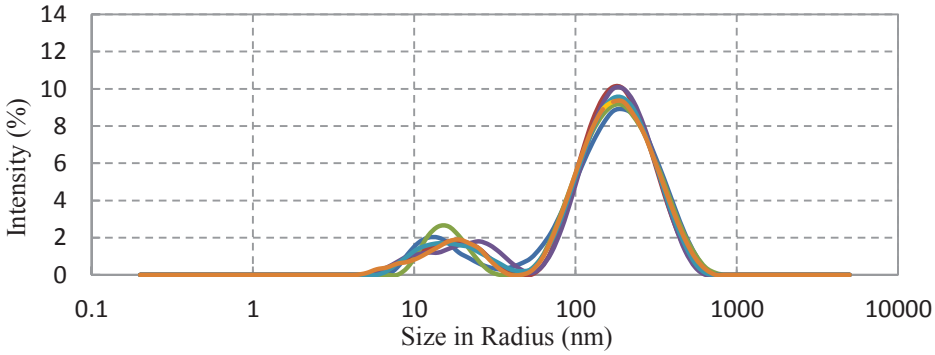
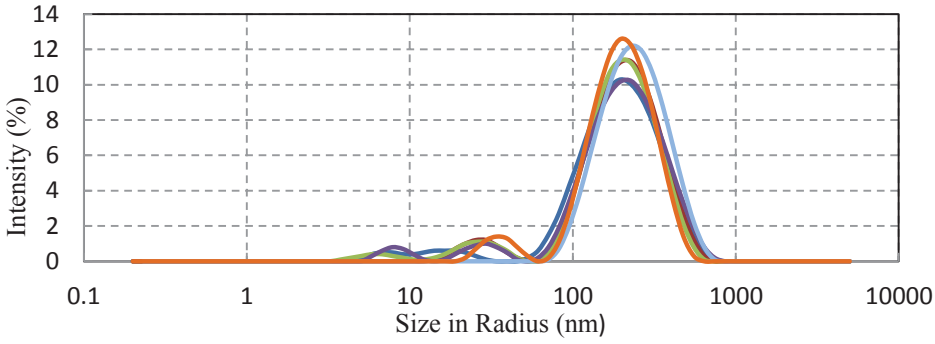


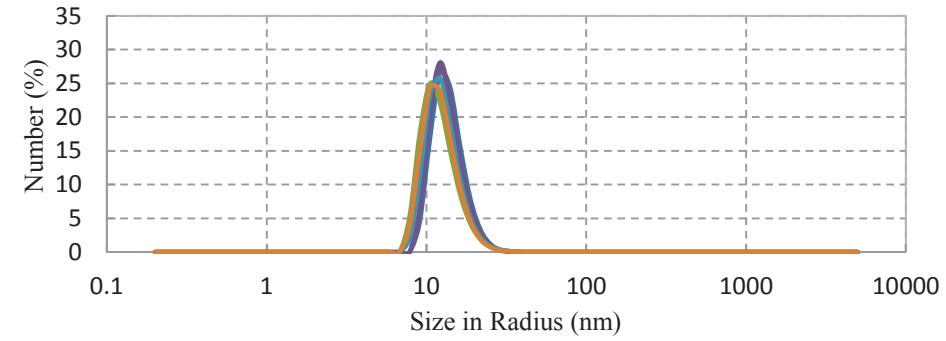
(C)



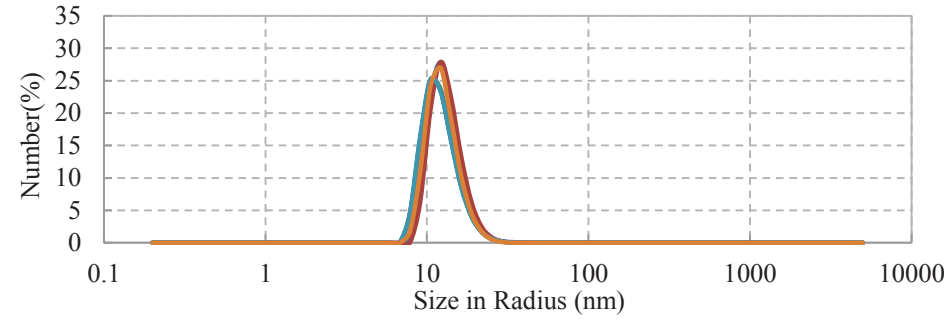
(D)

Figure 4.3 Size distributions by intensity of 1 wt% sodium caseinate solutions made with 200 mM EDTA and urea.

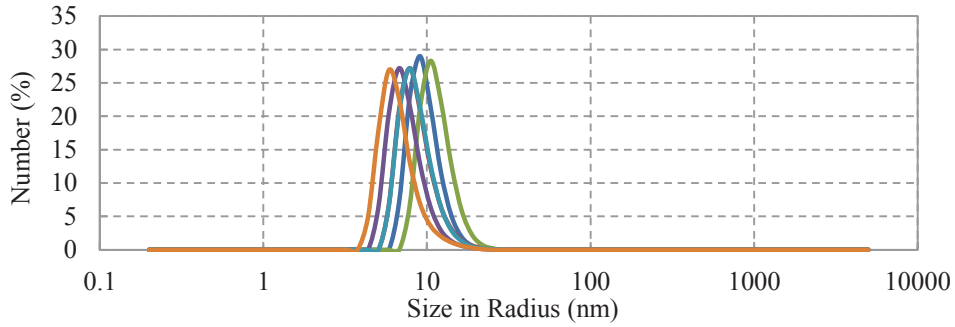
(A) control (urea-free) (B) 1.1 M urea (C) 3.3 M urea (D) 6.6 M urea



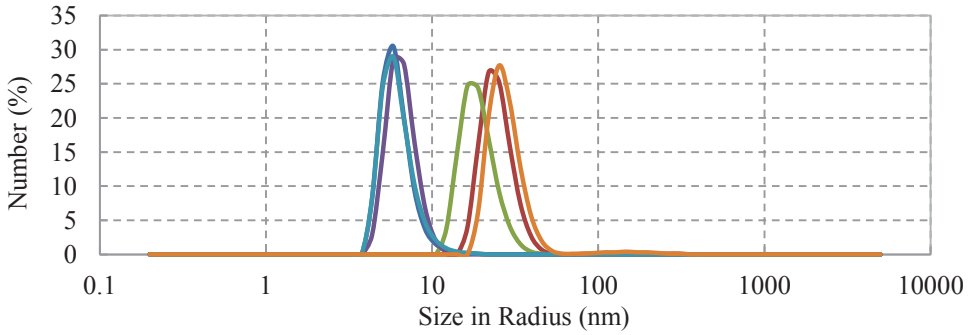
(A)



(B)



(C)



(D)

Figure 4.4 Size distributions by number of 1 wt% sodium caseinate solutions made with 200 mM EDTA and urea.

(A) control (urea-free) (B) 1.1 M urea (C) 3.3 M urea (D) 6.6 M urea

4.2 Rheological properties of sodium caseinate solutions with urea

4.2.1 Shear rate dependence

The shear rate dependence of sodium caseinate solutions with urea was plotted in Figure 4.5. With the exception of 20 wt% caseinate with no added urea, which was shown to be marginally pseudoplastic, all samples were seen to be Newtonian under the applied shear rate conditions. Figure 4.5(A) to (D) showed the viscosity of caseinate solutions scaling with protein concentration, rising from <0.01 Pa.s at 5 wt% caseinate up to 100 Pa.s at 20 wt% (in the absence of urea). Increasing addition of urea was shown to progressively reduce solution viscosity at protein concentrations of 15 and 20 wt% (with 1.1, 3.3 and 6.6 M urea addition resulting in log reductions of viscosity at 20 wt% caseinate). At protein concentrations of 5 and 10 wt%, the addition of urea was seen to have less effect on solution viscosity.

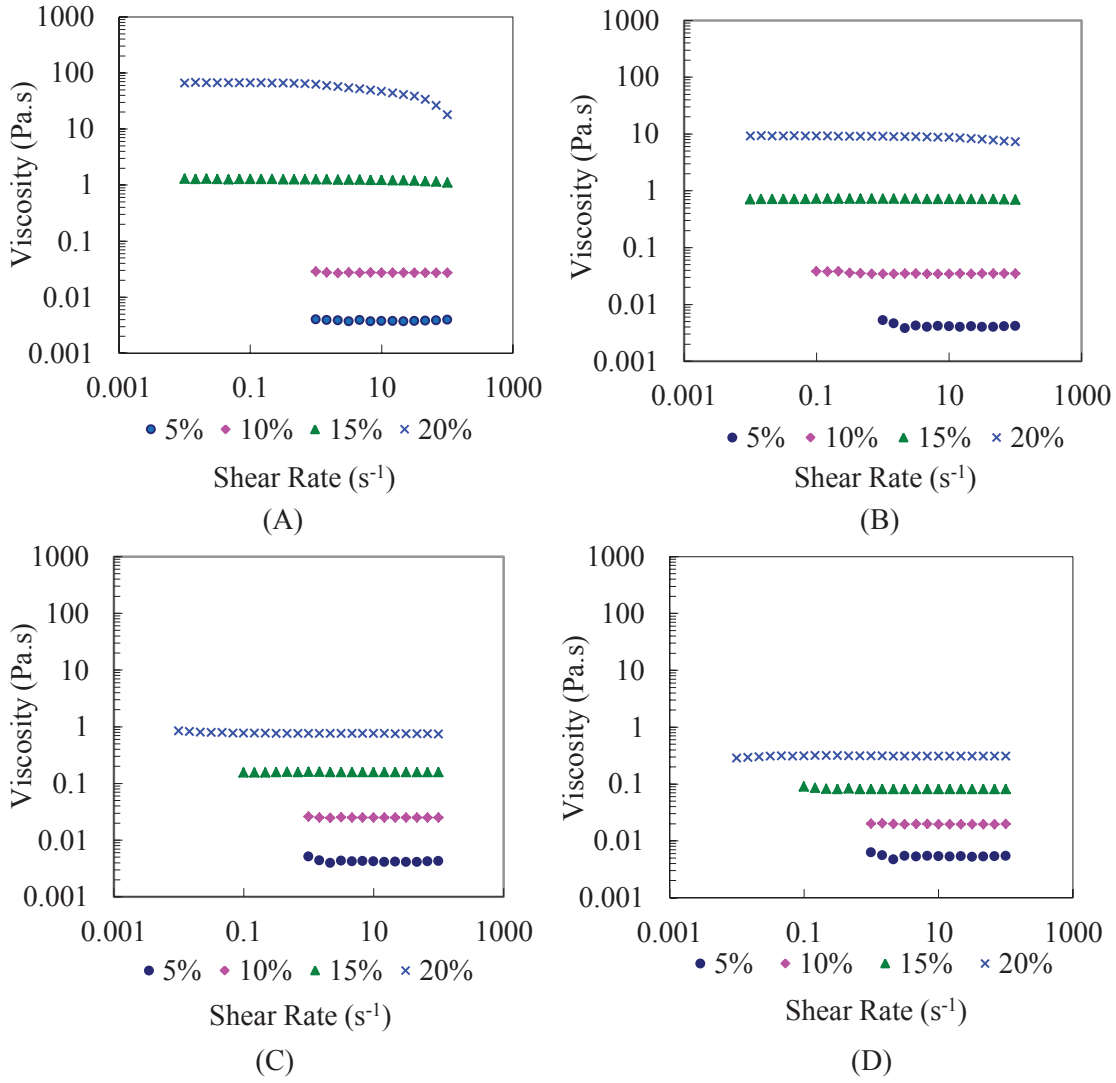


Figure 4.5 Shear rate dependence of the apparent viscosity of sodium caseinate solutions from 5 to 20 wt% with urea (T = 25 ℃).

(A) control (urea-free) (B) 1.1 M urea (C) 3.3 M urea (D) 6.6 M urea

4.2.2 Concentration dependence

As the viscosity of sodium caseinate solution was shear rate independent, a fixed shear rate (10 s^{-1}) was chosen in order to better compare the effects of urea addition on the viscous properties of caseinate solutions at variable concentration. As more clearly shown in Figure 4.6, at lower sodium caseinate concentrations (5, 10 wt%), the addition of urea had a negligible effect on solution viscosity. At elevated caseinate concentrations

(c>10 wt%), increasing concentrations of urea addition resulted in a progressive decrease in solution viscosity, with the magnitude of the viscosity reduction being more pronounced at 20 wt% caseinate relative to 15 wt% caseinate.

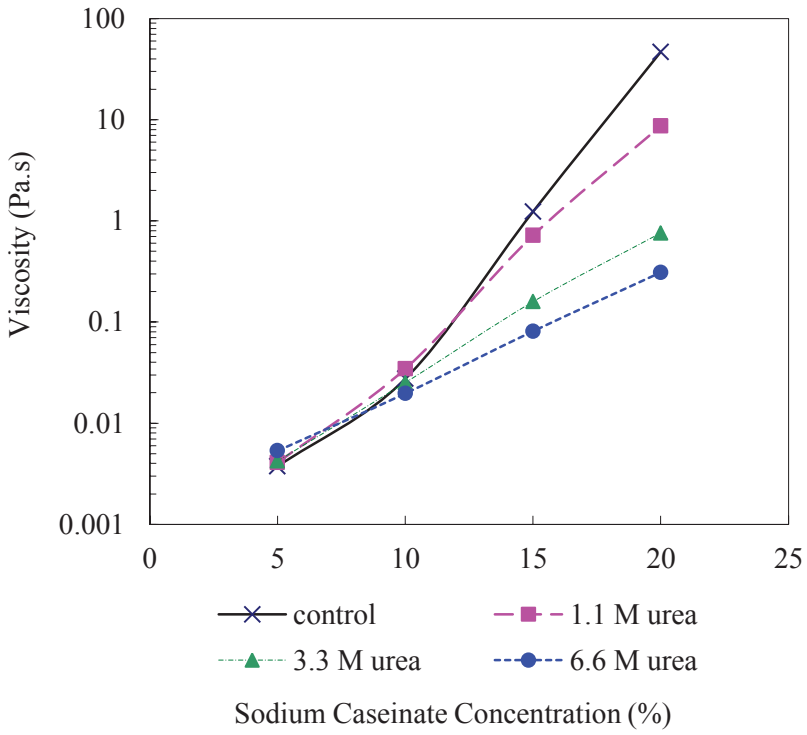


Figure 4.6 Apparent viscosity of sodium caseinate solutions from 5 to 20 wt% with and without urea (T = 25 ℃, shear rate = 10 s^{-1}).

4.2.3 Temperature dependence

The temperature dependence of the viscosity of sodium caseinate solutions with urea absent and urea present is shown in Figure 4.7. A substantive decrease in the viscosity of all the solutions was observed as temperature was increased. At 10 wt% the addition of urea had minimal effect on solution viscosity over the range of temperatures studied. However, at elevated protein concentration addition of urea resulted in reductions to viscosity over the temperature range studied, with urea concentration having the most significant effect for highest protein concentration (20 wt%) and lowest temperature.

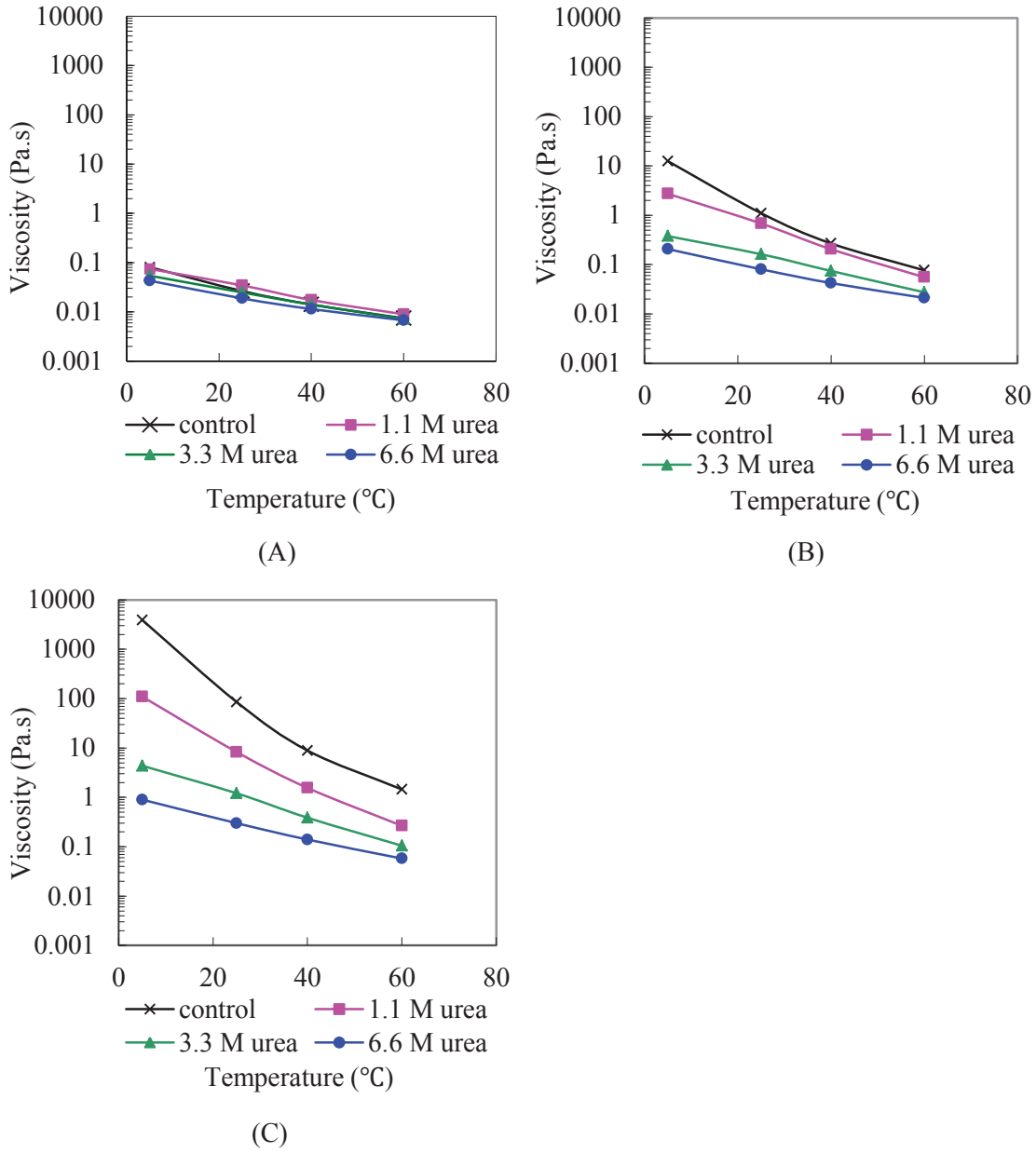


Figure 4.7 Temperature dependence of apparent viscosity of sodium caseinate solutions with and without urea (shear rate = 0.1 s^{-1}).

(A) 10 wt% sodium caseinate (B) 15 wt% sodium caseinate (C) 20 wt% sodium caseinate.

4.2.4 Highly concentrated sodium caseinate solutions

The viscosity of highly concentrated sodium caseinate solutions with different urea concentrations measuring at a shear rate of 10 s^{-1} is shown in Table 4.1. The more urea was added, the higher sodium caseinate concentration can be achieved. The maximum concentration of caseinate solutions was 40 wt% caseinate with 6.6 M urea.

Table 4.1 Apparent viscosity of highly concentrated sodium caseinate solutions with urea

Sodium caseinate concentration (%)	Viscosity at $10s^{-1}$, 25°C (Pa.s)		
	Urea concentration		
	1.1 M	3.3 M	6.6 M
20	8.66	0.76	0.31
25	53.37	4.83	1.17
30	281.03	24.39	4.76
35	/	88.49	21.28
40	/	/	89.09

Symbol "/" means the solutions can hardly be prepared.

4.3 Discussion

4.3.1 Sample preparation

It has been reported that caseinate solution is a coexisting system of casein monomers, casein complexes (sub-micelles) and larger aggregates (Lucey et al., 2000). Even at very low volume fractions these large-sized particles scatter light quite strongly, which invariably influences the DLS result when attempting to get an accurate measurement of the sub-micellar fraction. Additionally, a good preparation step is critical to remove these large aggregates or higher order structures. Unfortunately, no complete purification has been achieved so far (HadjSadok et al., 2008). Treatment with EDTA has been shown to increase the relative peak intensity of the first modal distribution in sodium caseinate solution, as it could reduce some of the larger aggregates by disrupting the Ca bridges between phosphorylated caseins in them (Semenova, Belyakova, Polikarpov, Antipova, & Dickinson, 2009). Here EDTA was added to sequester any residual calcium in the samples, although clearly a large population of aggregates remains.

As it can be seen in Figure 4.1 (A), there was only unimodal distribution in the result, as lager-size material in sodium caseinate solution scattered most of the light, and sub-micelle became hard to detect. However, in Figure 4.1(B) to (D), with the addition of EDTA the size of sub-micelles can be observed readily, and the first modal indicated the existence of the sub-micellar fractions. The separation of peaks for sub-micelles and

larger aggregates was good as well. Within the EDTA concentration range investigated, the relative peak intensity of the first modal distribution reached the highest value when 200 mM EDTA was added. It is the ideal EDTA concentration for sample preparation while measuring the particle size of sub-micelle in sodium caseinate solution. On the other hand, for the size distribution by number, the single peak shows the particles which are quantitatively dominant in the solution. Based on the results of Figure 4.2, it is suggested that larger particles were greatly reduced and sub-micelles became the dominant fraction in the solution with the addition of EDTA, and thus the caseinate sub-micelles could be clearly detected in sodium caseinate solution. Therefore, from the results of size distribution by intensity and by number, it is proven that EDTA could, to a degree, diminish the influence of large aggregates, thereby enabling the detection of sub-micelles in DLS measurement, which is consistent with the result of Semenova (Semenova et al., 2009).

In considering the nature and structure of aggregates comprising the second model distribution in sodium caseinate solution, further understanding was obtained through the addition of EDTA and urea. Optically, caseinate solutions were transparent, even at elevated protein levels (albeit with some haziness). Solution transparency provided an indication that the fractions of larger particles - which were of sufficient size to scatter light, and thereby impart opacity - comprised only a small fraction of the particulates present. For dynamic light scattering analysis of particle size, EDTA was added to the caseinate solutions to sequester the residual calcium. It is indicated that there was more than one component in larger aggregates in the system. Although the larger micellar assemblies were partly dissociated which might be due to the casein formed through calcium bridging, the larger particles were still observed after addition of EDTA (HadjSadok et al argues that these larger entities are complexes of protein and fat (HadjSadok et al., 2008), and according to Table 3.1, there is 0.6 wt% fat in the sodium caseinate powder). The second modal distribution also appeared relatively unaffected by addition of urea (Figure 4. 3(B) to (D)), indicating that these structures may not be hydrophobically assembled casein particles.

4.3.2 Particle size of sodium caseinate

It should be noticed that there are differences in the particle size values presented in this study in comparison to those reported previous studies. The size of sodium caseinate sub-micelles obtained here (18~20nm, by intensity) was lower than the value (100 nm) reported by Dickinson et al. (2001), but higher than the one (8nm) observed by Chu et al. (1995). Two factors are probably responsible for the differences. First, it could be the varieties in sample preparation. Addition of EDTA was used in this study and ultracentrifuge was used in Lucey's method, while some chemical and physical steps were introduced in Chu's. Though the samples have been purified to remove part of the large-sized particles, the residuals might still have some influence on the results. Additionally, the equipment for measuring the samples might bring in differences.

4.3.3 Disassociation effects of urea on sodium caseinate solutions

Results in Figure 4.3 and 4.4 have shown that urea exhibits a dissociating effect on the self-assembly behaviour in sodium caseinate solutions, as the size of sub-micelles decreased with the increase of urea concentration. When increased urea addition, a reduction of the first modal distribution was observed, with almost complete absence of the first modal at 6.6 M urea. This is attributed to a combination of reduced hydrophobic interactions and intermolecular electrostatic repulsion (since α_{s1}, α_{s2}, β, κ-casein all carry an appreciable negative charge at neutral pH), causing progressive dissociation of the sub-micellar fraction. Furthermore, it was revealed that there was no critical point for the full dissociation of the self-assembly of sodium caseinate, as the area of the first modal distribution decreased gradually with increasing urea concentration. In addition, according to Figure 4.4, there was no significant change in the number of sub-micelles with the increase of urea concentration, so urea might cause more effects on the size rather than the number of sub-micelles.

This apparent sub-micellar dissociation was seen to have a profound effect on the viscous properties of caseinate solutions at elevated protein concentration (Figures 4.5 and 4.6). Farrer and Lips (1999) described caseinate sub-micelles as soft particles, with particle close packing being observed at >10 wt% caseinate (φ - ~0.4 – 0.5).

Accordingly solution viscosity showed a gradual increase with concentration up to close packing limits, and then increased more steeply due to jamming and immobilization of the sub-micellar aggregates (Loveday et al., 2010; Pitkowski et al., 2008). For protein concentrations lower than close packing limits, urea dissociation of sub-micelles did not noticeably affect solution viscosity (Figure 4.6), due to the fact that viscous properties were not influenced by the impedance in sub-micellar mobility at these levels. However at protein concentrations >10 wt%, increasing addition of urea progressively and markedly decreased solution viscosity. Based on these observations, it was presumed that sub-micellar dissociation reduced the effective phase volume of particles, increasing the molecular mobility of the protein in solution. At 6.6 M urea, the viscosity of 15 and 20 wt% caseinate solution showed a linear concentration dependency, which was similar to 5 and 10 wt% caseinate samples without urea, presumably indicating that sub-micellar volume fraction at these higher protein concentrations with 6.6 M urea remained below the close packing threshold. Caseinate solution viscosity was also dependent on temperature, with viscosity increasing as temperature was decreased (Loveday et al., 2010; Pitkowski et al., 2008). Whilst hydrophobic interactions were lessened at lower temperatures, the elevated viscosities observed for protein concentrations appreciably above close packing (Figure 4.7) indicated that any such reduction in hydrophobic interactions was not sufficient to cause sub-micellar dissociation.

4.3.4 Highly concentrated sodium caseinate solutions

In sodium caseinate solution, self-assembly of caseinate leads to high viscosity, which means the more concentrated the solution is, the higher viscosity the solution has. Viscosity limits the concentration of protein that can readily be solubilised in solution. With the addition of urea, obtaining a higher concentration of sodium caseinate solution become feasible, as urea could effectively reduce the viscosity of the solution, enabling solutions with protein concentration possibly in excess of 40 wt% to be prepared, which was not achievable in the absence of urea. It is speculated that similar effect could be

achieved by other mechanisms which are capable of disrupting the hydrophobic interactions considered responsible for caseinate self-assembly.

5 Effects of urea on sodium caseinate stabilised emulsions

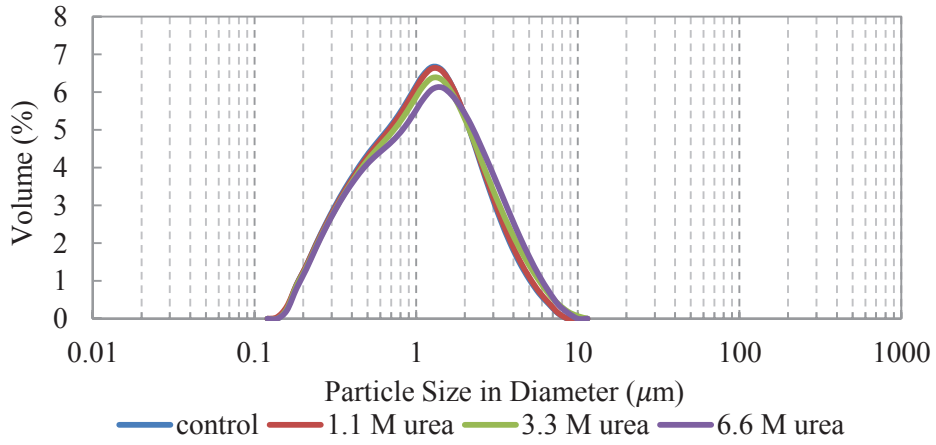
5.1 Droplet size of sodium caseinate stabilised emulsions

The droplet size of sodium caseinate emulsions under condition of varying protein and urea concentration is seen in Table 5.1. The droplet size distribution is shown in Figure 5.1.

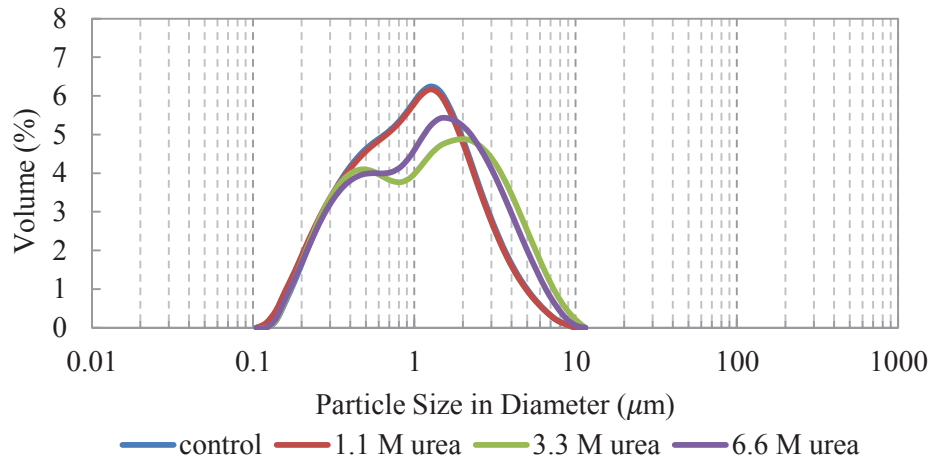
As it can be seen from Table 5.1, in terms of D[3,2] and D[4,3], generally the droplet size of emulsions with the same protein concentration showed a slight upward trend with increasing urea concentration. In Figure 5.1(A) to (C), for 2 wt% sodium caseinate emulsions, all samples displayed monomodal distributions. At 4 wt% caseinate, the urea-free and 1.1 M urea added samples showed monomodal distributions, while the ones with 3.3 and 6.6 M urea presented bimodal distributions. Similar results can be observed from 6 wt% sodium caseinate stabilised emulsions.

Table 5.1 Droplet size of sodium caseinate oil-in-water emulsions (30 vol% oil) with urea

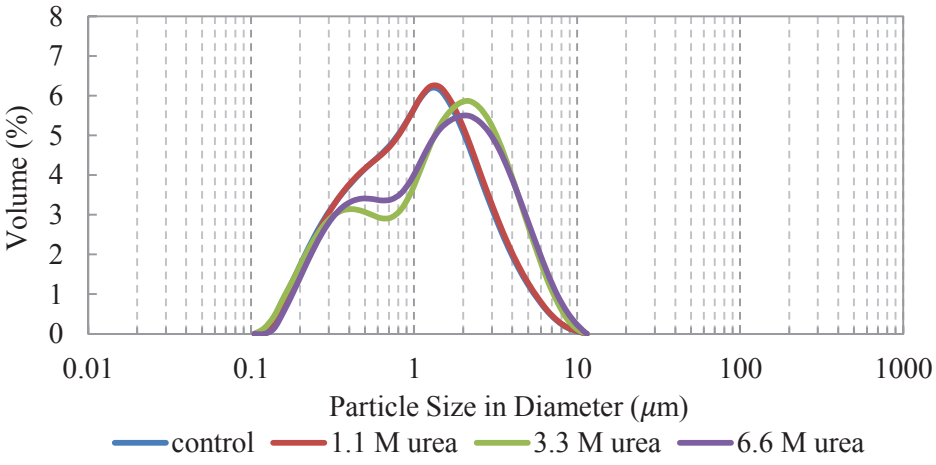
Sample	D[3,2] Surface weighted mean (μm)	D[4,3] Volume weighted mean (μm)	Specific surface area (m^2/g)
2 wt% sodium caseinate emulsion control	0.700	1.300	8.58
2 wt% sodium caseinate emulsion 1.1 M urea	0.702	1.316	8.55
2 wt% sodium caseinate emulsion 3.3 M urea	0.721	1.415	8.32
2 wt% sodium caseinate emulsion 6.6 M urea	0.738	1.472	8.13
4 wt% sodium caseinate emulsion control	0.634	1.243	9.47
4 wt% sodium caseinate emulsion 1.1 M urea	0.605	1.218	9.91
4 wt% sodium caseinate emulsion 3.3 M urea	0.682	1.672	8.80
4 wt% sodium caseinate emulsion 6.6 M urea	0.677	1.538	8.86
6 wt% sodium caseinate emulsion control	0.640	1.333	9.38
6 wt% sodium caseinate emulsion 1.1 M urea	0.666	1.362	9.00
6 wt% sodium caseinate emulsion 3.3 M urea	0.724	1.785	8.29
6 wt% sodium caseinate emulsion 6.6 M urea	0.764	1.820	7.86



(A)



(B)



(C)

Figure 5.1 Droplet size distributions of oil-in-water emulsions (30 vol% oil) stabilised with sodium caseinate.

(A) 2 wt% sodium caseinate (B) 4 wt% sodium caseinate (C) 6 wt% sodium caseinate

5.2 Creaming stability of sodium caseinate stabilised emulsions with urea

5.2.1 Visual creaming profiles

Creaming of emulsions with 2, 4 and 6 wt% caseinate and with various urea concentrations was visually observed during storage at room temperature over a 48 h period (Figure 5.2). For visual observations the blue colouration of the emulsion provides a clearer indication of cream layer formation.

For 2 wt% caseinate emulsion, in the absence of urea, extensive emulsion separation was seen to have taken place in over the first 24 h of storage, with some additional compaction of the cream layer observed at 48 h. Addition of urea was seen to reduce cream layer thickness with increasing urea concentration, with 6.6 M urea addition producing a considerably more stable emulsion over the storage period.

At 4 wt% caseinate, emulsions showed a thicker cream layer relative to 2 wt% urea-free emulsion, and at 1.1 M urea addition. The serum layer also showed a greater degree of optical clarity in comparison with the emulsion prepared with 2 wt% caseinate. At 3.3 M urea, the cream layer thickness was reduced, and the serum layer was seen to be more turbid. Some minimal creaming was observed at 6.6 M urea after 24 h, showed a further partial increase after 48 h.

The urea-free emulsion stabilised with 6 wt% caseinate showed good stability after 24 h storage, with some marginal serum separation after 48 h. Addition of 1.1 and 3.3 M urea caused a reduction in creaming stability with extensive serum separation observed after 24 and 48 h. Stability was improved with addition of 6.6 M urea, although some cream separation was observed during storage period (although there was no noticeable change in the visual creaming profile after 24 h, some creaming separation was observed from the result of Turbiscan). The appearance of the serum layer was observed to be appreciably more turbid than 1.1 and 3.3 M urea addition.

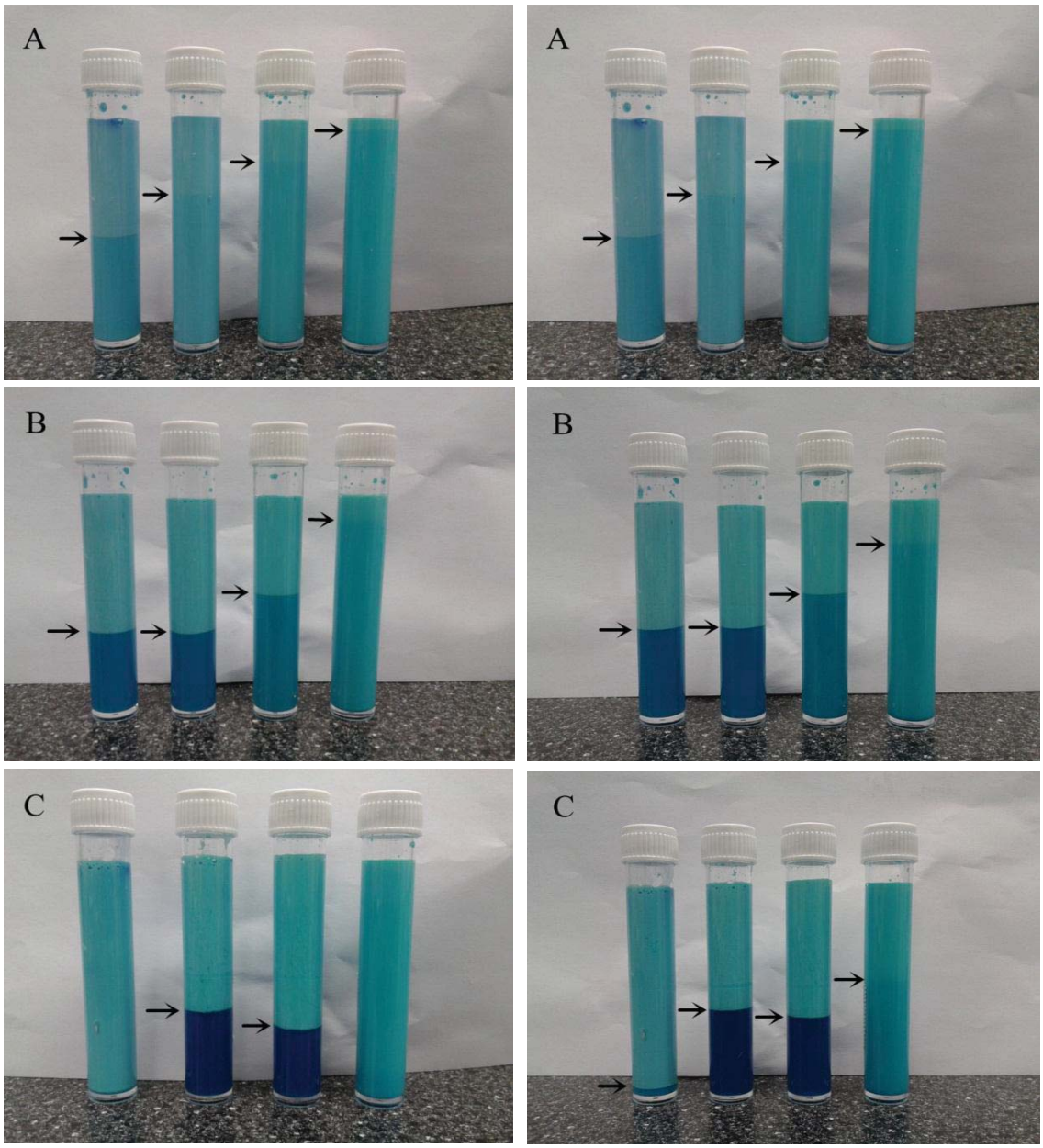


Figure 5.2 Visual creaming profiles (after 24, 48 h of storage at room temperature) of oil-in-water emulsions (30 vol% oil) made with sodium caseinate and urea.

(A) 2 wt% sodium caseinate (B) 4 wt% sodium caseinate (C) 6 wt% sodium caseinate

Tubes start from left to right (non-urea, 1.1, 3.3, 6.6 M urea); Cream-serum boundary has been marked by arrow.

5.2.2 Turbiscan backscattering

More precise creaming profiles of sodium caseinate emulsions were obtained by Turbiscan backscattering. The distribution of droplet in terms of the height of the tube can be determined in Figures 5.3, 5.4 and 5.5. The backscattered light intensity is inversely related to the amount of droplets in the solution, so a decrease in the backscattered light intensity means an increase of volume fraction of creaming. As it can be seen from the results, the creaming behaviour of samples right after preparation, 24 and 48 h of storage was generally consistent with the results of Figure 5.2, except for the 4 wt% caseinate emulsion without urea. Figures 5.6, 5.7 and 5.8 show the creaming rate of the emulsions during 24 h of storage.

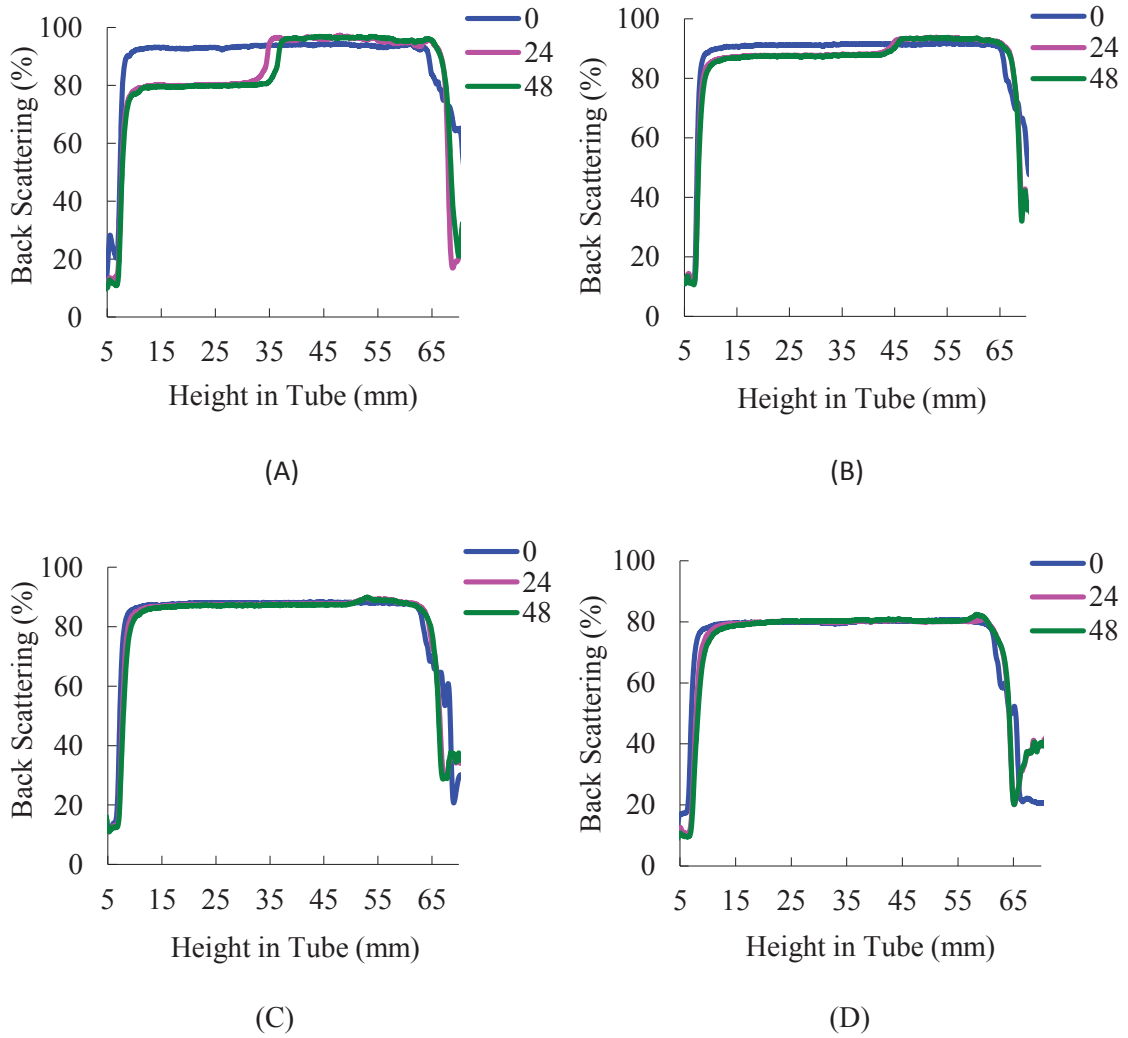


Figure 5.3 Creaming profiles (Turbiscan) of oil-in-water emulsions (30 vol% oil) made with 2 wt% sodium caseinate and urea after preparation, 24 and 48 h of storage.

(A) urea-free (B) 1.1 M urea (C) 3.3 M urea (D) 6.6 M urea

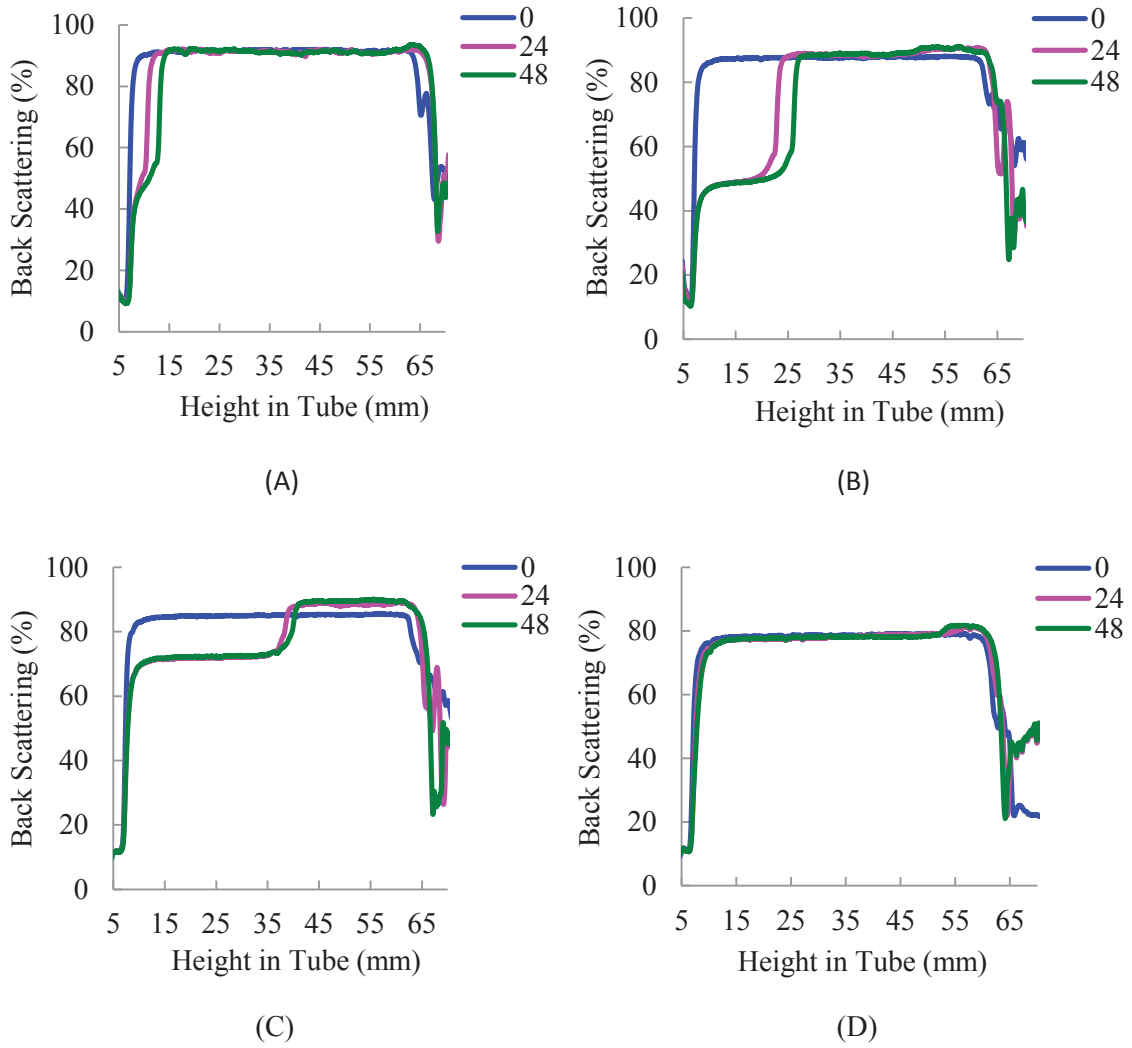


Figure 5.4 Creaming profiles (Turbiscan) of oil-in-water emulsions (30 vol% oil) made with 4 wt% sodium caseinate and urea after preparation, 24 and 48 h of storage.

(A) urea-free (B) 1.1 M urea (C) 3.3 M urea (D) 6.6 M urea

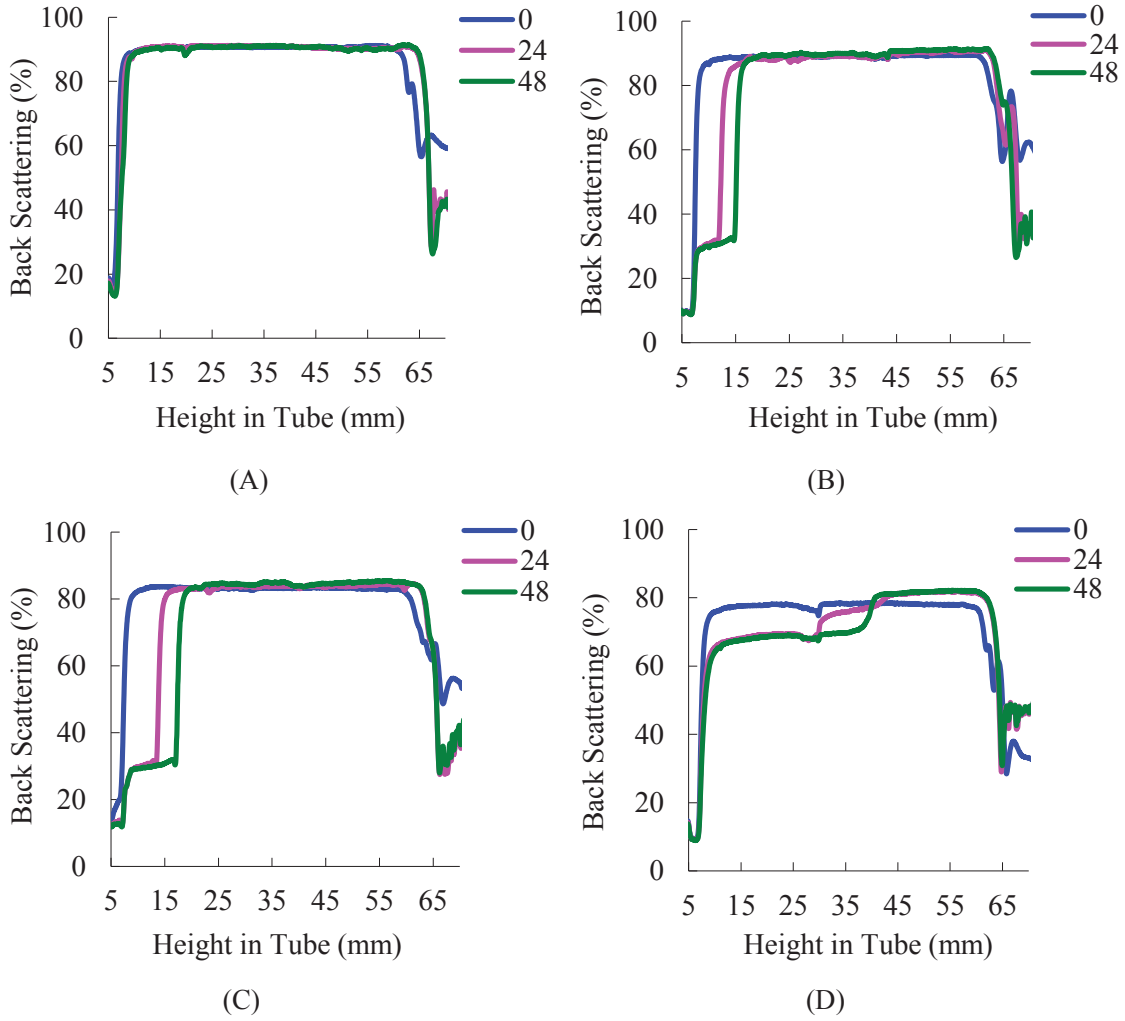


Figure 5.5 Creaming profiles (Turbiscan) of oil-in-water emulsions (30 vol% oil) made with 6 wt% sodium caseinate and urea after preparation, 24 and 48 h of storage.

(A) urea-free (B) 1.1 M urea (C) 3.3 M urea (D) 6.6 M urea

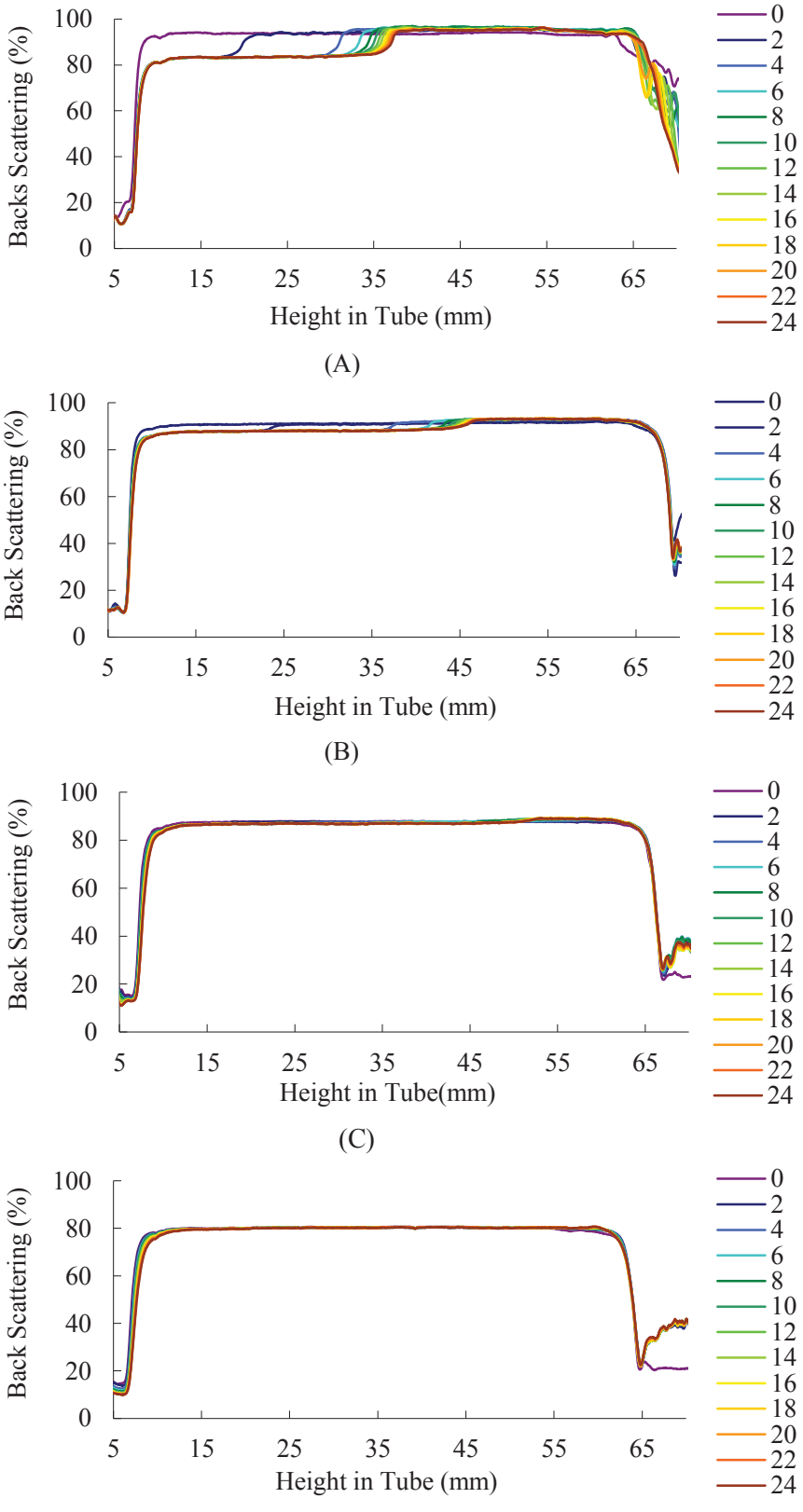


Figure 5.6 Dynamic creaming profiles (Turbiscan) of oil-in-water emulsions (30 vol% oil) made with 2 wt% sodium caseinate and urea in 24 h of storage.

(A) urea-free (B) 1.1 M urea (C) 3.3 M urea (D) 6.6 M urea

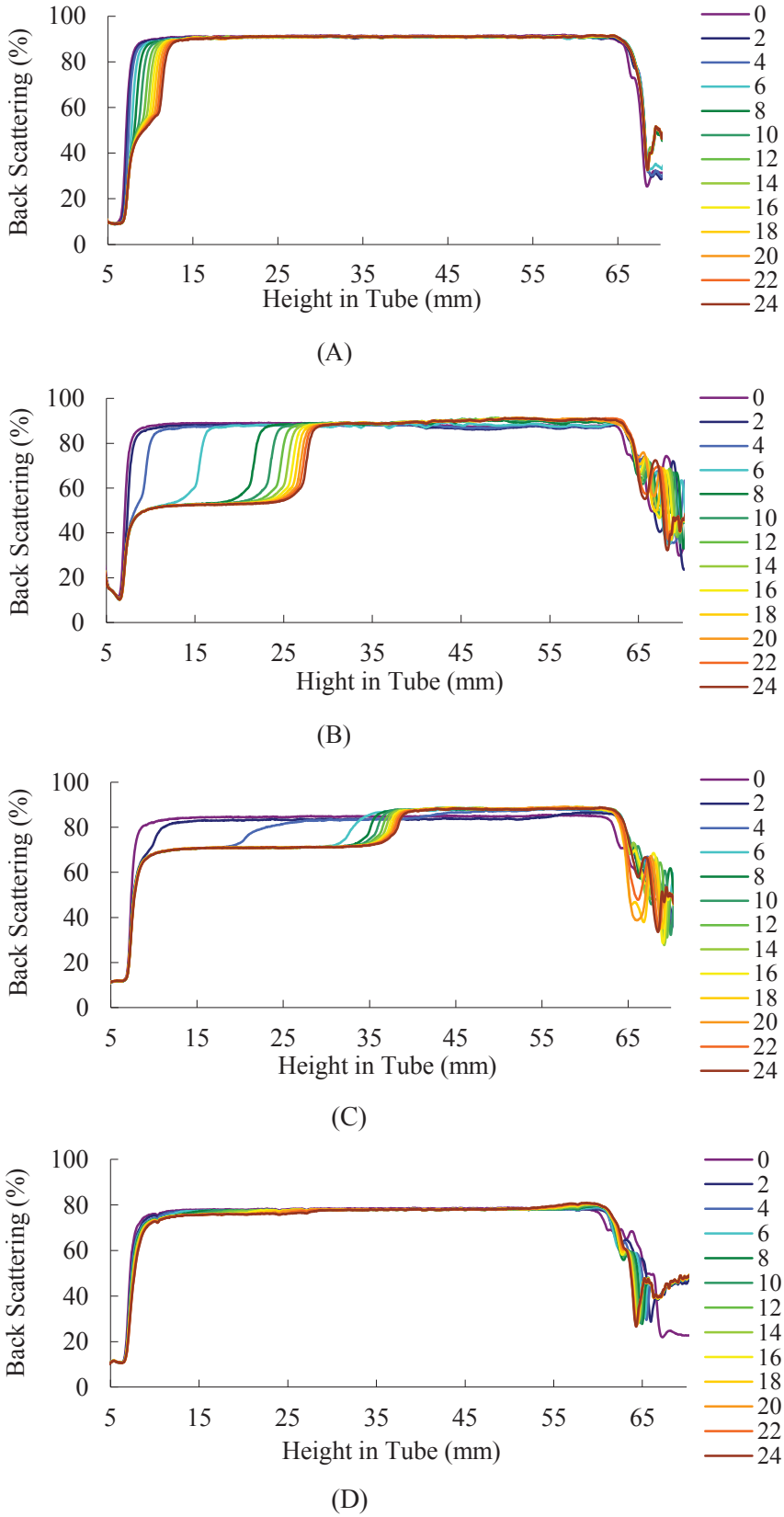


Figure 5.7 Dynamic creaming profiles (Turbiscan) of oil-in-water emulsions (30 vol% oil) made with 4 wt% sodium caseinate and urea in 24 h of storage.

(A) urea-free (B) 1.1 M urea (C) 3.3 M urea (D) 6.6 M urea

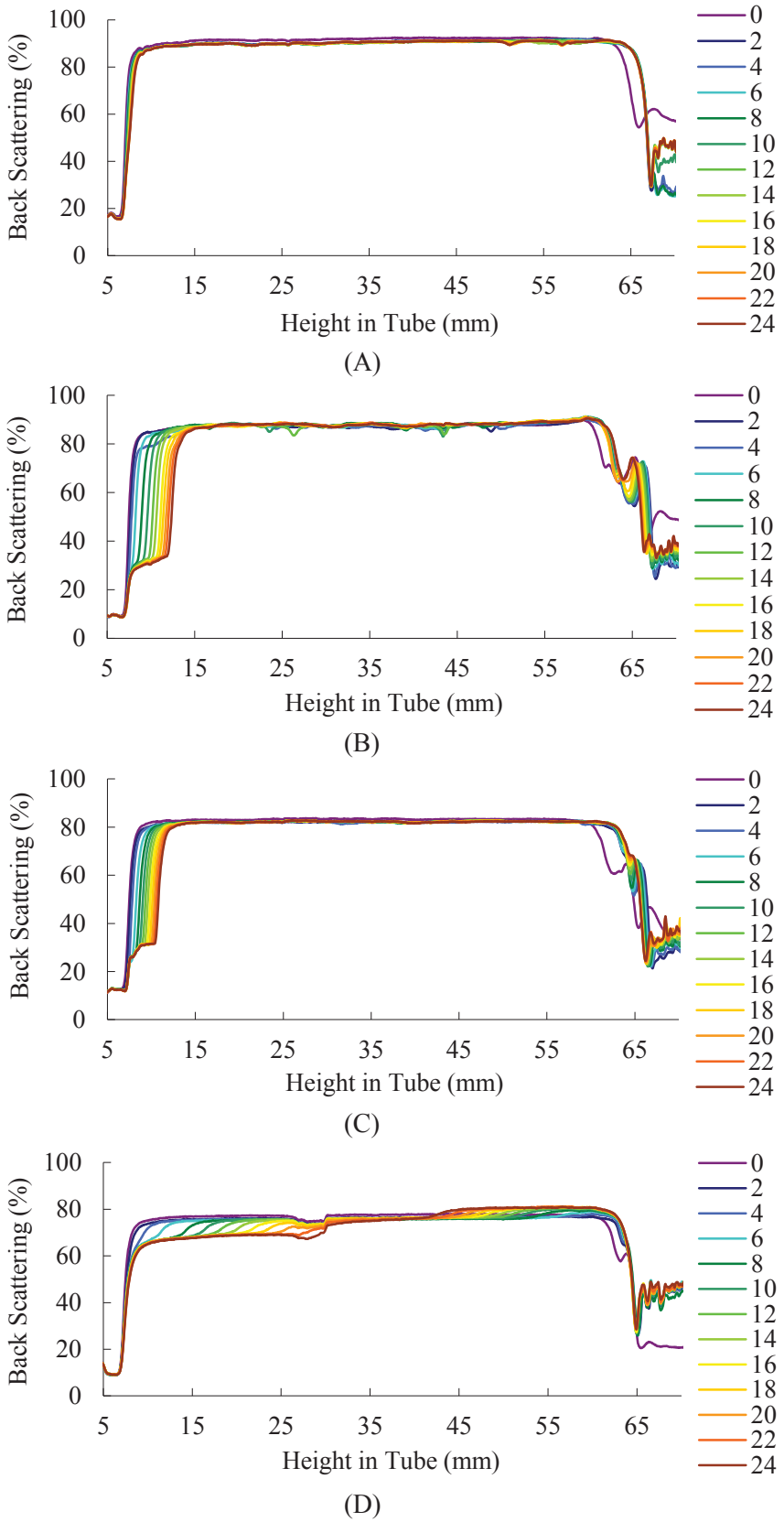


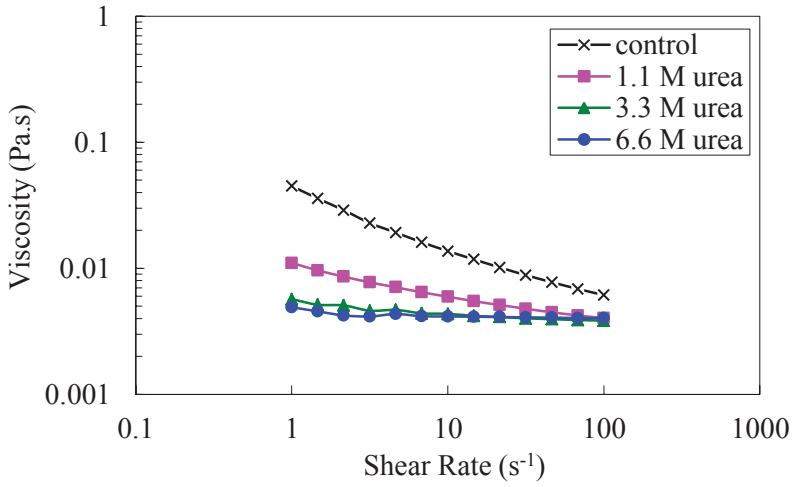
Figure 5.8 Dynamic creaming profiles (Turbiscan) of oil-in-water emulsions (30 vol% oil) made with 6 wt% sodium caseinate and urea in 24 h of storage.

(A) urea-free (B) 1.1 M urea (C) 3.3 M urea (D) 6.6 M urea

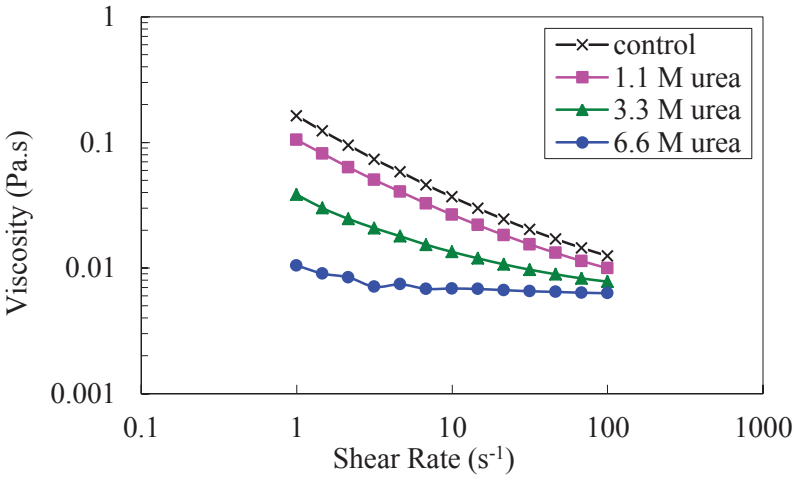
5.3 Rheological properties of sodium caseinate stabilised emulsions with urea

5.3.1 Shear rate dependence

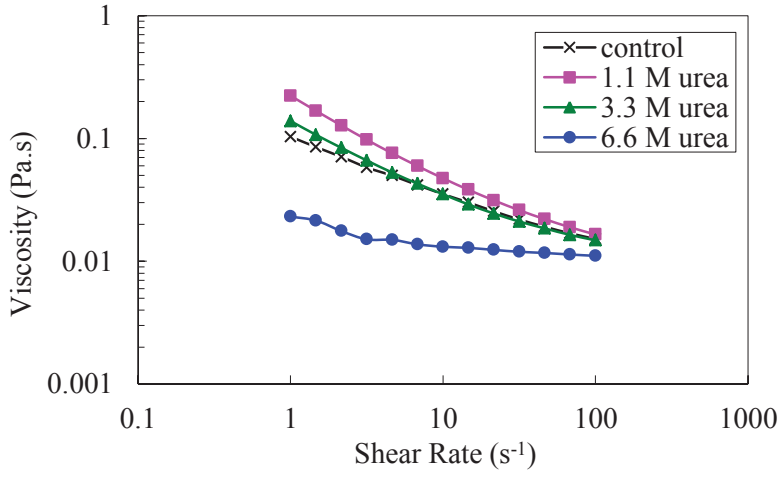
Figure 5.9 showed the apparent viscosity data for emulsions stabilised with 2, 4 and 6 wt% caseinate, and with 0 - 6.6 M urea added. In the absence of urea, emulsions at all protein concentrations were shown to be pseudoplastic, with low shear viscosity observed to be highest at 4 wt% caseinate (although it is acknowledged that wall slip may be occurring at low shear rates in these systems). At 2 and 4 wt% protein, addition of urea resulted in a lowering of apparent viscosity, most noticeably under low shear conditions, and a reduction in pseudoplastic behaviour, particularly at 6.6 M urea. At 6 wt% caseinate, addition of urea at 1.1 and 3.3 M resulted in a slight increase in low shear viscosity and pseudoplasticity, compared to the urea-free sample, with a reduction in low shear viscosity and pseudoplasticity observed at 6.6 M urea.



(A)



(B)



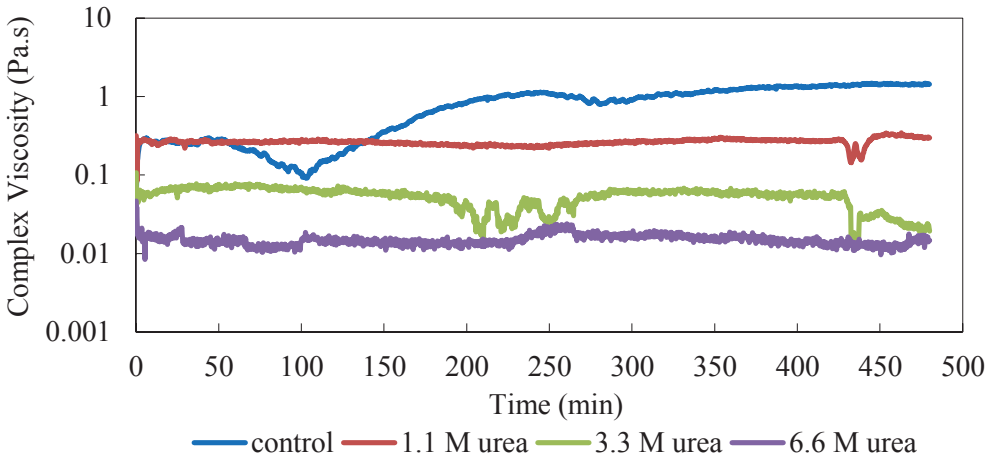
(C)

Figure 5.9 Shear rate dependence of apparent viscosity of oil-in-water emulsions (30 vol% oil) made with sodium caseinate and urea.

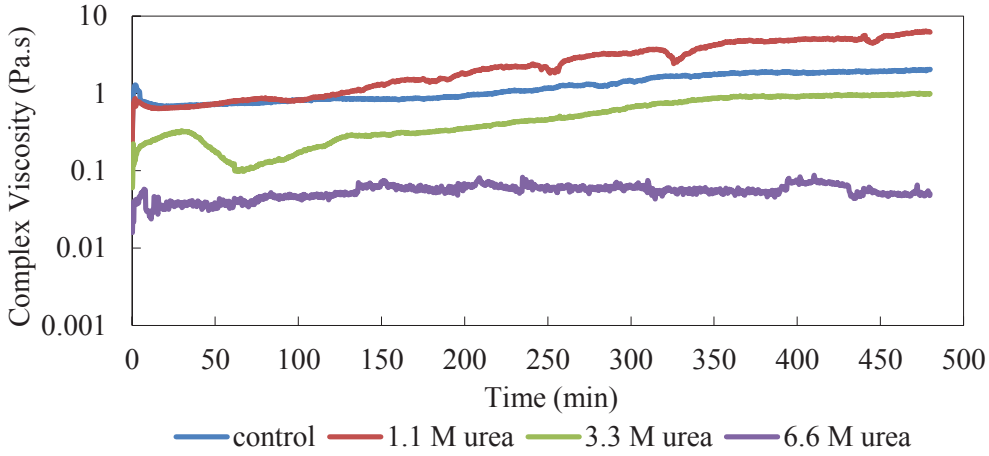
(A) 2 wt% sodium caseinate (B) 4 wt% sodium caseinate (C) 6 wt% sodium caseinate

5.3.2 Time dependence

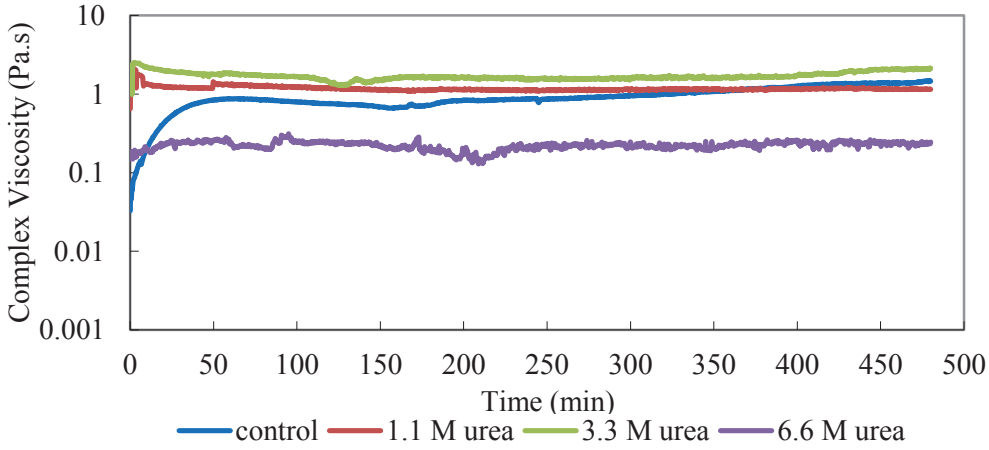
The development of depletion-induced network in sodium caseinate stabilised emulsion can be seen from Figure 5.11. The initial increase in the complex viscosity reflected the onset of the droplet network formation in the emulsions. Moreover, the velocity of forming network was related to the depletion potential, as a weak network was more likely to develop in a lower depletion potential. On the other hand, the structural change of the emulsions during flocs development was observed in Figure 5.10. For 2 wt% caseinate emulsion, an upward trend was observed during the entire experiment, except for a significant drop in the early stage. However, it was fairly unchanged in complex viscosity for the emulsions with urea (1.1, 3.3 and 6.6 M) over the course of the measurement, along with some slight fluctuations. For 4 wt% caseinate emulsion (urea-free and 1.1 M urea), the complex viscosity gradually increased during the whole experiment, while for the emulsion with 3.3 M urea demonstrated a similar trend as the 2 wt% caseinate sample. At 6.6 M urea, emulsion did not display time-dependent rheological behaviour in the process. The complex viscosity of 6 wt% caseinate emulsions with urea all remained stable during the time investigated, while the control sample initially showed a tendency to increase and remained constant for the rest of the time.



(A)



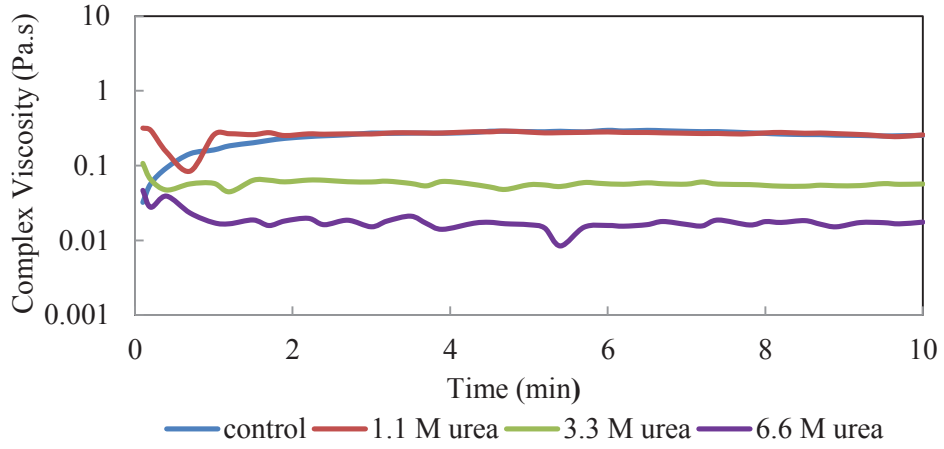
(B)



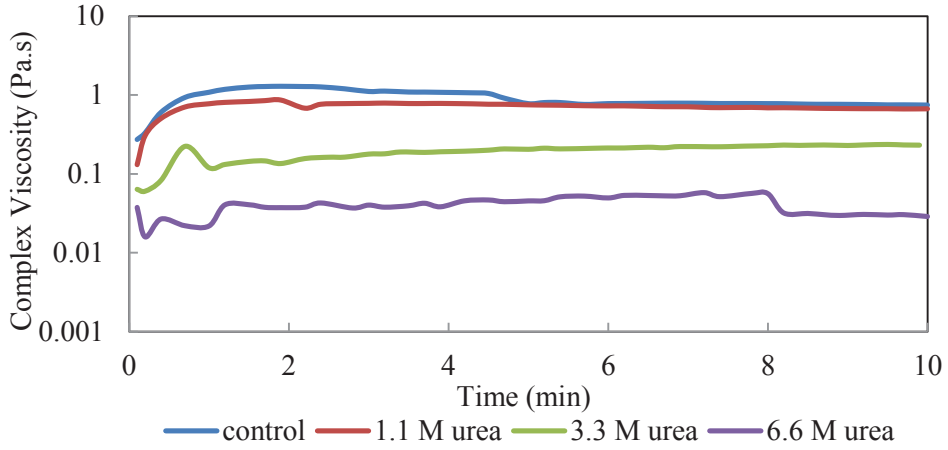
(C)

Figure 5.10 Time dependence of complex viscosity of oil-in-water emulsions (30 vol% oil) made with sodium caseinate and urea.

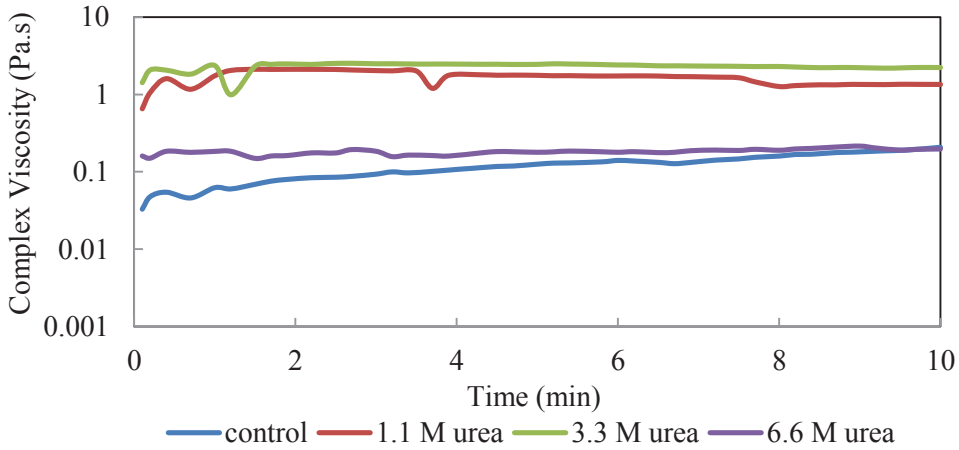
(A) 2 wt% sodium caseinate (B) 4 wt% sodium caseinate (C) 6 wt% sodium caseinate



(A)



(B)



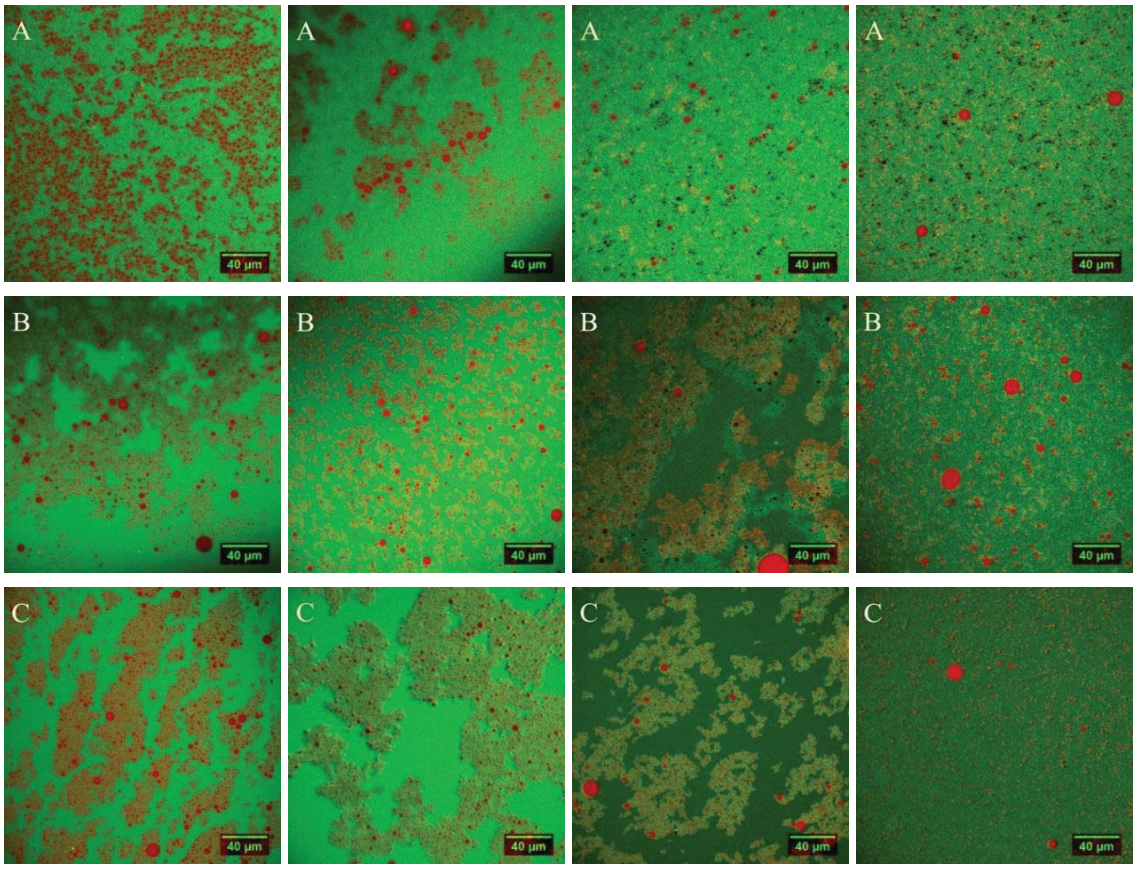
(C)

Figure 5.11 A close up of the first 10 min of the time dependence rheological properties of Figure 5.10.

(A) 2 wt% sodium caseinate (B) 4 wt% sodium caseinate (C) 6 wt% sodium caseinate

5.4 Microstructure of sodium caseinate stabilised emulsions with urea

The microstructure of emulsions was shown by confocal micrographs in Figure 5.12. For the emulsions with 2 and 4 wt% sodium caseinate, an interconnected network was observed in the absence of urea. The addition of urea was seen to have a dissociative effect on the network structure, with higher urea concentrations required for network disruption at 4 wt% protein. For emulsions with 6 wt% protein, dense droplet domains were observed, although these were not seen to result in formation of a continuous droplet network. Addition of urea at concentrations of 1.1 and 3.3 M resulted in more extensive network formation, with disruption of the droplet network again observed at 6.6 M urea. Generally, with urea concentration increased, droplets clusters were smaller and fewer, and the system became increasingly homogeneous.



Control **1.1 M urea** **3.3 M urea** **6.6 M urea**

Figure 5.12 Confocal micrographs of oil-in-water emulsions (30 vol% oil) made with sodium caseinate and urea (scale bar is 40µm).

(A) 2 wt% sodium caseinate (B) 4 wt% sodium caseinate (C) 6 wt% sodium caseinate

5.5 Discussion

It is now well established that emulsions stabilised with sodium caseinate undergo depletion flocculation due to exclusion of sub-micelles from the intervening space between emulsion droplets, arising from an osmotic pressure gradient (Dickinson & Golding, 1997a, 1997b; Dickinson et al., 1997a; Huck-Iriart et al., 2011; Srinivasan et al., 2001). McClements (as cited in Dickinson & Golding, 1997b) introduced the equations for calculating the depletion free energy:

$$\Delta G_{dep} = -2\pi r_m^2 P_{osm}\left(r_d + \frac{2r_m}{3}\right) \quad [1]$$

$$P_{osm} = \frac{CRT}{M}\left(1 + \frac{2C}{\rho}\right) \quad [2]$$

Where r_m and r_d are the radii of the micelles and droplets, respectively; P_{osm} is the osmotic pressure arises from the exclusion of the micelles in the solution; C is the micelle concentration in the continuous phase, M is the average molecular weight of a micelle, and ρ is the micelle density.

Accordingly, depletion potential can be seen to scale with the size of the droplets, the size of the depleting entities and the concentration of protein, whilst being inversely related to the molecular weight of the micellar component. Meanwhile, urea is considered as having a disruptive effect on the hydrophobic interactions responsible for the self-assembly of sodium caseinate sub-micelles. Based on the results presented here, with the addition of urea, the droplet size of the emulsions slightly increased when the particle size of sub-micelles decreased. Referring to equations [1] and [2], the size of sub-micelle is more influential than the droplet size of the emulsion on depletion free energy. Although the droplet size of emulsions slightly increased with the addition of urea, depletion interaction of the sodium caseinate stabilised emulsions was reduced with elevated urea concentration, as the particle size of sub-micelles decreased. Therefore, the hypothesis here is that depletion flocculation in sodium caseinate stabilised emulsions can be weakened by the addition of urea and the stability of the

emulsions would be improved, which is attributed to the decline in depletion potential of the emulsions.

The effects of urea on adjusting the stability and the structure of the sodium caseinate based emulsions were demonstrated in the combination of macroscopic and microscopic measurements, including creaming profile, rheology and microstructure. The creaming stability of the emulsions was shown by visual creaming profile and Turbiscan. In terms of the rheological behaviours, flocculated emulsions would display an increase in low-shear viscosity and pseudoplastic behaviour with increasing high shear rate (Campanella, Dorward, & Singh, 1995); The development of depletion-induced network in caseinate-stabilised emulsion can be implied from the time-dependent evolution of complex viscosity (Dickinson & Golding, 1997b). The dissociation of droplet network in the emulsions by the addition of urea was shown in the confocal micrographs.

Visual observations and Turbiscan analysis of 2 and 4 wt% protein stabilised emulsions without added urea (Figure 5.2, 5.3 and 5.4) showed rapid separation after 24 h into a cream rich upper phase and a fat depleted lower phase. The higher turbidity of the serum phase in the 2 wt% caseinate-stabilised emulsion was attributed to the fact that the depletion potential at this protein concentration was insufficient to cause smaller fat droplets in this serum layer to flocculate (Dickinson & Golding, 1998a). At 4 wt% protein, the higher depletion potential caused the entire droplet distribution to participate in flocculation; accordingly the lower serum phase was optically transparent.

The observed improvement in creaming stability at 6 wt% protein (Figure 5.2 and 5.5) has previously been hypothesised as being due to a reinforcement of the flocculated droplet network arising from a strengthening of particle interactions at higher concentrations (Dickinson et al., 1997a; Manoj, Watson, Hibberd, Fillery-Travis, & Robins, 1998b; Parker, Gunning, Ng, & Robins, 1995; Starrs, Poon, Hibberd, & Robins, 2002). However, it was interesting to note that the low shear viscosity of the 6 wt% protein stabilised emulsions appeared marginally lower than that of the 4 wt% protein sample (Figure 5.9). Additionally, at 6 wt% protein, confocal microscopy (Figure 5.12)

showed an absence of a percolating droplet structure, with dense, localised aggregates observed. A second hypothesis, in which the increasing viscosity of the continuous phase reduced the rate of flocculation, was considered appropriate for explaining the enhanced creaming stability at higher protein concentrations (Liang et al., 2014; Manoj, Fillery-Travis, Watson, Hibberd, & Robins, 1998a). This hypothesis was also in line with the result of apparent viscosity, as more continuous phase was trapped in the network of 4 wt% caseinate emulsion than the 6 wt% caseinate sample without network structure, which led to a higher apparent viscosity for the 4 wt% protein sample.

Dissociation of sub-micelles through the addition of urea was seen to reduce cream layer thickness of the 2 wt% caseinate-stabilised emulsion, due to a progressive decrease in depletion potential with increasing urea concentration. This was reflected in the apparent viscosity data, in which the pseudoplasticity of the emulsions decreased with increasing urea concentration, and further evidenced through confocal microscopy which showed a clear dissociation of network structures towards higher urea concentrations.

Similar behaviour was observed at 4 wt% protein, although it was observed that emulsion properties (in terms of creaming stability, rheology and structure) for 1.1 M urea addition were comparable to the emulsion system in the absence of urea, indicating that the depletion potential was not sufficiently reduced so as to inhibit flocculation, and implying only limited sub-micellar dissociation at this urea concentration. With further increases in urea concentration, changes to stability and structure arising from reductions in depletion potential became more apparent.

Curiously, at 6 wt% protein, the addition of 1.1 and 3.3 M urea was found to reduce creaming stability over 48 h compared to the sample without urea. It is speculated that the addition of urea at these concentrations resulted in a reduction to the sub-micellar volume fraction; however, this decrease appeared insufficient to reduce depletion interactions below the critical potential for onset of flocculation. Moreover, the apparent reduction in sub-micelle phase volume reduced the continuous phase viscosity, thereby increasing the mobility of droplets in forming flocculated networks (Liang et al., 2014;

Manoj et al., 1998a; Manoj et al., 1998b). These weakly aggregated structures rapidly collapsed under gravity, leading to formation of the serum layer through syneresis. The increase in low shear viscosity and pseudoplasticity of the emulsions containing 1.1 and 3.3 M urea, relative to the urea-free sample reinforced this observation, as did the confocal micrographs, where more percolating fat droplets structures were observed at 1.1 and 3.3 M urea compared to the urea-free sample. At 6.6 M urea, the improvement in emulsion creaming stability was considered a consequence of extensive sub-micelle dissociation, resulting in a significant reduction in depletion potential. Some creaming was observed over time, which may have been due to larger fat droplets being subject to depletion effects. The high turbidity of the serum phase observed visually, and using the Turbiscan indicated that a significant population of emulsions droplets remain unflocculated (presumably those small enough to be below the threshold depletion potential required to cause flocculation). Again, rheological and microscopic analyses supported this hypothesis.

6 Effects of urea on potassium caseinate stabilised emulsions

6.1 Characteristics of potassium caseinate

6.1.1 Particle size of potassium caseinate

The particle size of potassium caseinate with urea present and urea absent is show in Table 6.1, in comparison to sodium caseinate. Regarding to the particle size distribution, there is no noticeable difference between potassium caseinate and sodium caseinate, with or without urea treatment.

Table 6.1 Particle size of caseinates

Sample	Peak 1 Mean (nm)	Peak 2 Mean (nm)	Peak 1 Area (%)	Peak 2 Area (%)
potassium caseinate	177.2	20.62	55.9	44.1
sodium caseinate	168.1	20.82	54.5	45.5
potassium caseinate + urea	217.7	16.96	95%	<4%
sodium caseinate + urea	226.3	16.11	96%	<4%

* Particle size measurements were operated on 1 wt% caseinate solutions with 200 mM EDTA. The urea concentration was 6.6 M. The particle size was measured in radius. Peak 1 represented the aggregates with larger size in solution while peak 2 stood for caseinate sub-micelles. As there were additional components existing in the system with a trace amount, the total percentage of the peak area in the Table might not be 100%.

6.1.2 Viscosity of potassium caseinate solutions

The apparent viscosity of the potassium caseinate solution was measured under different shear rates. Measurement was made for a single protein concentration of 20 wt% and the result was compared to the equivalent concentration of sodium caseinate. As it can be seen in Figure 6.1, potassium caseinate solution had a similar apparent viscosity as sodium caseinate solution across the entire shear rates investigated. Likewise, the apparent viscosity of the potassium caseinate solution with addition of

urea was studied as well. 6.6 M urea was added into the solution, as a higher urea concentration was more effective in dissociation. It was observed that the apparent viscosity of potassium and sodium caseinate solutions with urea added was analogous.

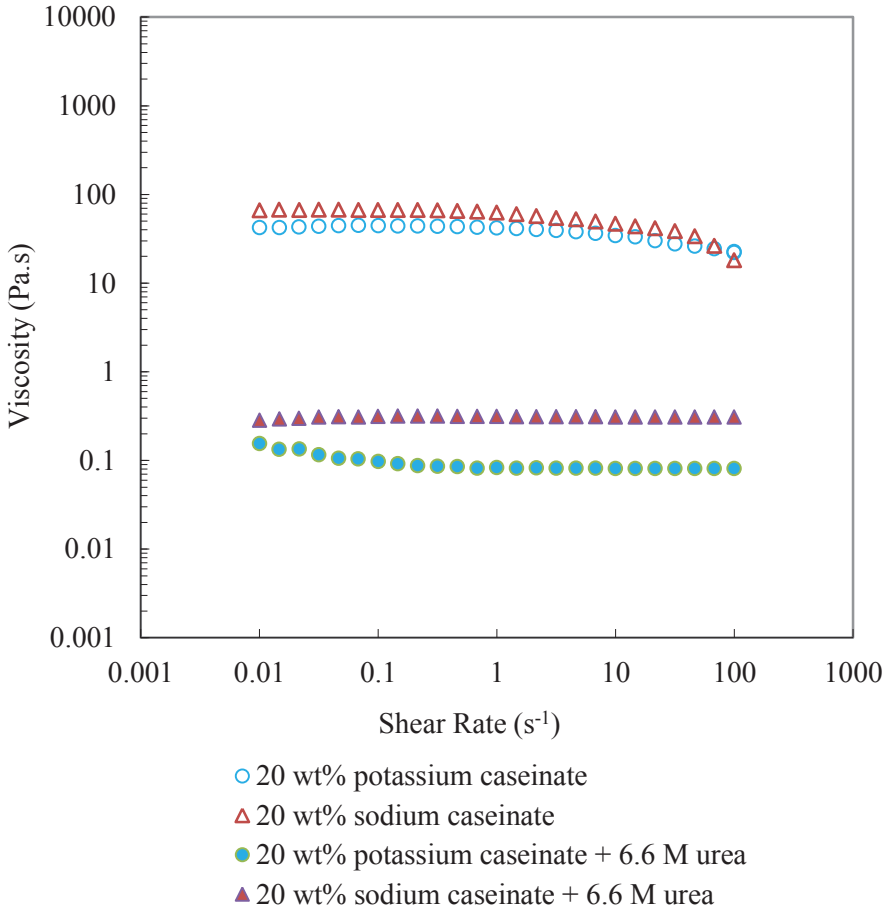


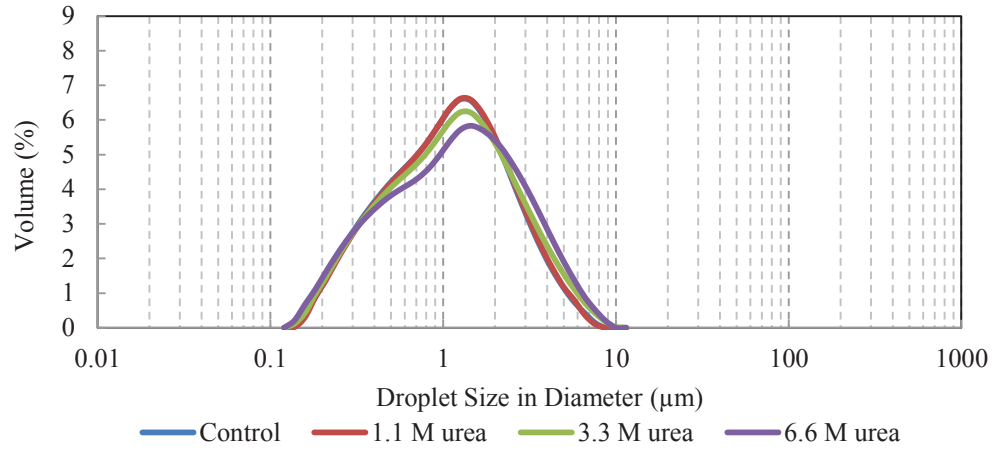
Figure 6.1 Apparent viscosity of 20 wt% potassium and sodium caseinate solutions.

6.1.3 Droplet size of potassium caseinate stabilised emulsions

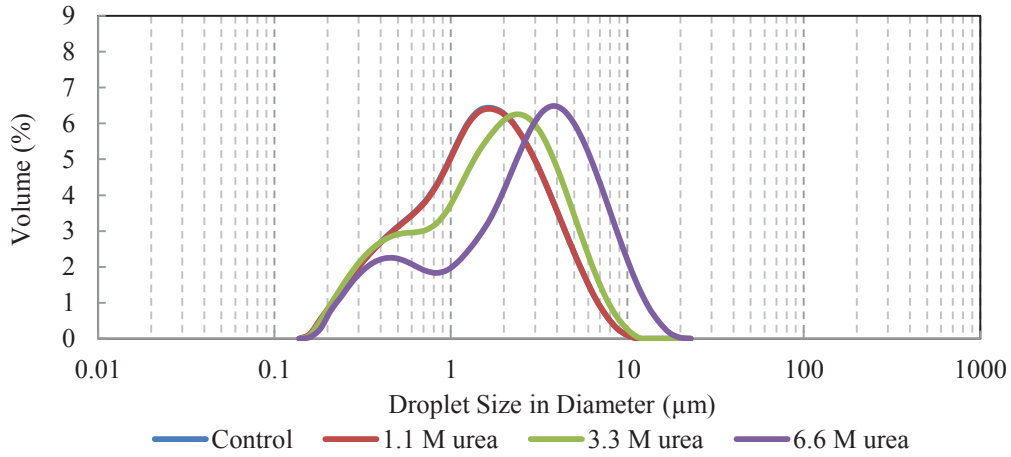
The droplet size distribution of potassium caseinate stabilised emulsions with and without the addition of urea can be seen from Table 6.2 and Figure 6.2. From the results of Table 6.2, the droplet size of the emulsions above a certain protein concentration increased with increasing urea concentration. Potassium caseinate emulsions all displayed monomodal distribution, except for the 4 and 6 wt% caseinate emulsions with 3.3 and 6.6 M urea.

Table 6.2 Droplet size of potassium caseinate stabilised oil-in-water emulsions (30 vol% oil) with urea

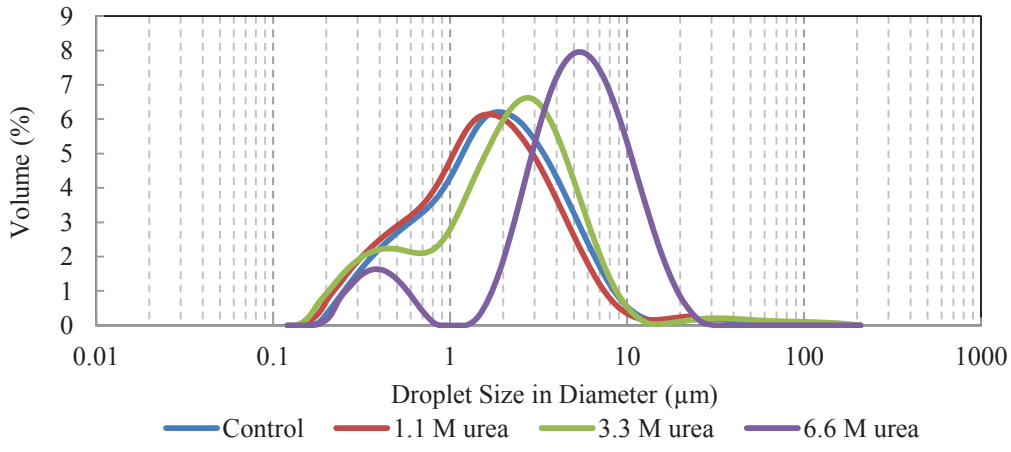
Sample	D[3,2] Surface weighted mean (μm)	D[4,3] Volume weighted mean (μm)	Specific surface area (m^2/g)
2 wt% potassium caseinate emulsion control	0.704	1.331	8.52
2 wt% potassium caseinate emulsion 1.1 M urea	0.714	1.341	8.40
2 wt% potassium caseinate emulsion 3.3 M urea	0.715	1.444	8.40
2 wt% potassium caseinate emulsion 6.6 M urea	0.713	1.534	8.41
4 wt% potassium caseinate emulsion control	0.887	1.761	6.76
4 wt% potassium caseinate emulsion 1.1 M urea	0.877	1.760	6.84
4 wt% potassium caseinate emulsion 3.3 M urea	0.933	2.053	6.43
4 wt% potassium caseinate emulsion 6.6 M urea	1.204	3.325	4.98
6 wt% potassium caseinate emulsion control	0.923	2.142	6.50
6 wt% potassium caseinate emulsion 1.1 M urea	0.938	2.423	6.40
6 wt% potassium caseinate emulsion 3.3 M urea	1.033	3.550	5.81
6 wt% potassium caseinate emulsion 6.6 M urea	1.977	5.579	3.03



(A)



(B)



(C)

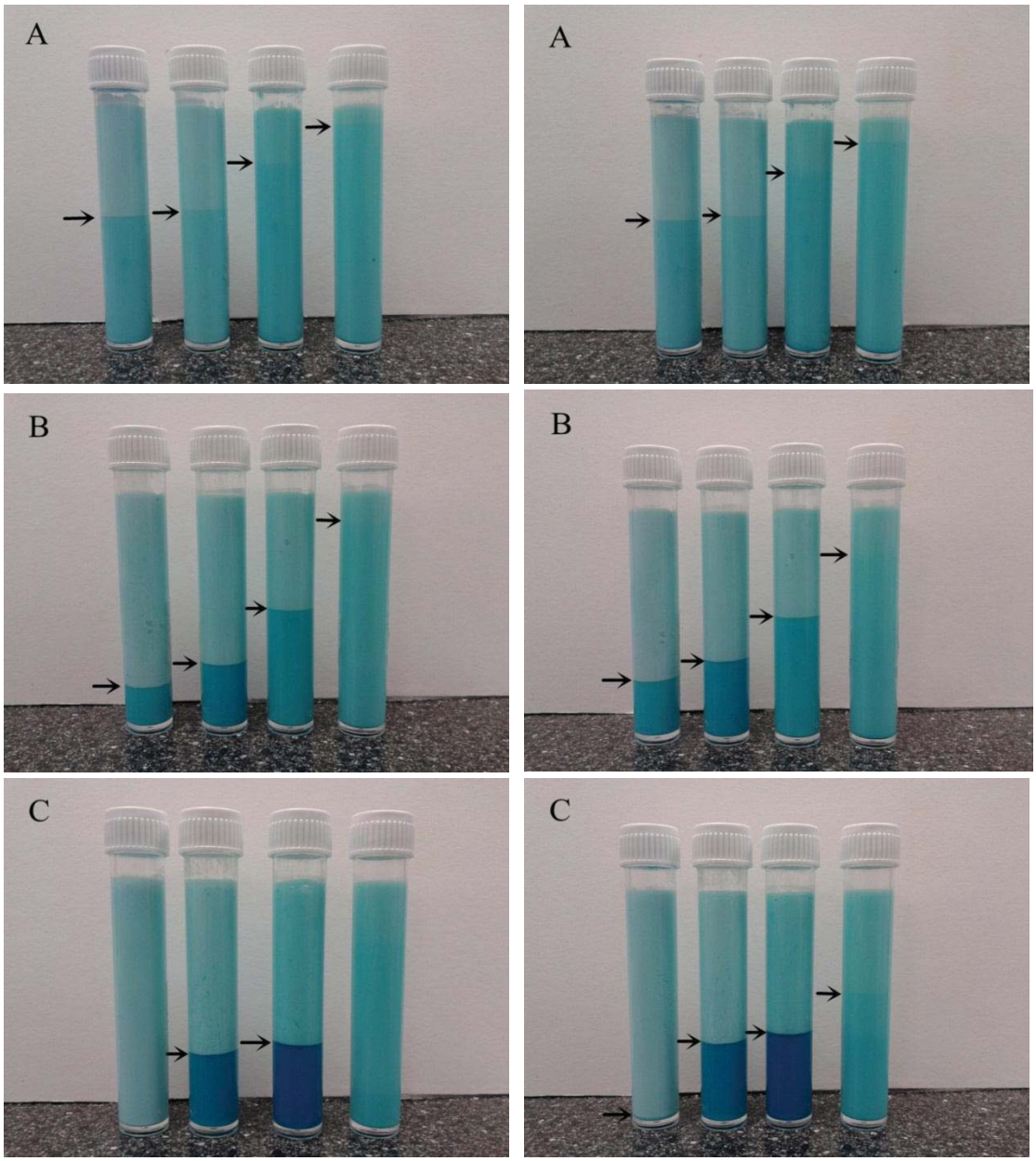
Figure 6.2 Droplet size distributions of oil-in-water emulsions (30 vol% oil) stabilised with potassium caseinate.

(A) 2 wt% potassium caseinate (B) 4 wt% potassium caseinate (C) 6 wt% potassium caseinate

6.2 Creaming stability of potassium caseinate stabilised emulsions with urea

6.2.1 Visual creaming profiles

Creaming behaviour of emulsions stabilised with 2, 4 and 6 wt% caseinate in the presence of various urea concentrations was visually observed during storage at room temperature over a 48 h period (Figure 6.3). For 2 and 4 wt% caseinate emulsions, the stability of the emulsions was improved with increasing urea concentration, as the volume fraction of the creaming phase was observed to be decreased and the turbidity of the serum phase increased. At 6 wt% caseinate, the control sample stayed relatively stable during 48 h of storage, while for the emulsions with 1.1 and 3.3 M urea, phase separation occurred and the opacity of the serum phase increased. The emulsion became re-stabilised again with 6.6 M urea.



24h **48h**

Figure 6.3 Visual creaming profiles (after 24, 48 h of storage at room temperature) of oil-in-water emulsions (30 vol% oil) made with potassium caseinate and urea.

(A) 2 wt% potassium caseinate (B) 4 wt% potassium caseinate (C) 6 wt% potassium caseinate.

Tubes start from left to right (non-urea, 1.1, 3.3, 6.6 M urea); Cream-serum boundary has been marked by arrow.

6.2.2 Turbiscan backscattering

The demixing profile determined by Turbiscan of potassium caseinate emulsions is showed in Figure 6.4, 6.5 and 6.6, which is in line with the results of Figure 6.3. Meanwhile, as it can be seen in Figure 6.7, 6.8 and 6.9, the creaming rate and turbidity of 2 wt% potassium caseinate emulsions decreased with increasing urea concentration. For 4 wt% caseinate emulsions with 1.1 and 3.3 M urea, flocculation proceed faster than the control one, with higher initial rate in the first 8 h, while the rate of emulsion demixing kept stable with further urea addition (6.6 M). The system flocculated steadily for all the 6 wt% caseinate emulsions during the whole measurement.

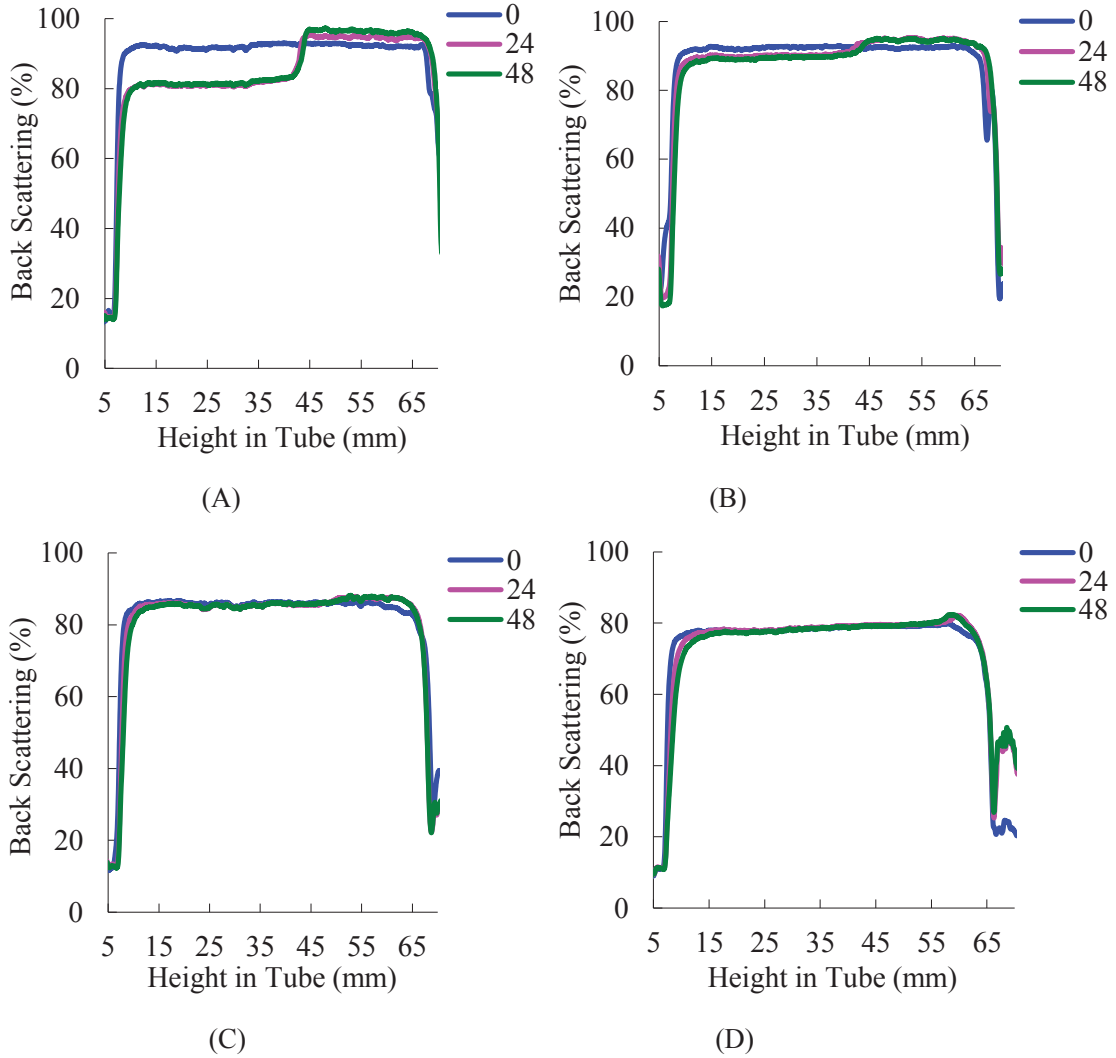


Figure 6.4 Creaming profiles (Turbiscan) of oil-in-water emulsions (30 vol% oil) made with 2 wt% potassium caseinate and urea after preparation, 24 and 48 h of storage.

(A) urea-free (B) 1.1 M urea (C) 3.3 M urea (D) 6.6 M urea

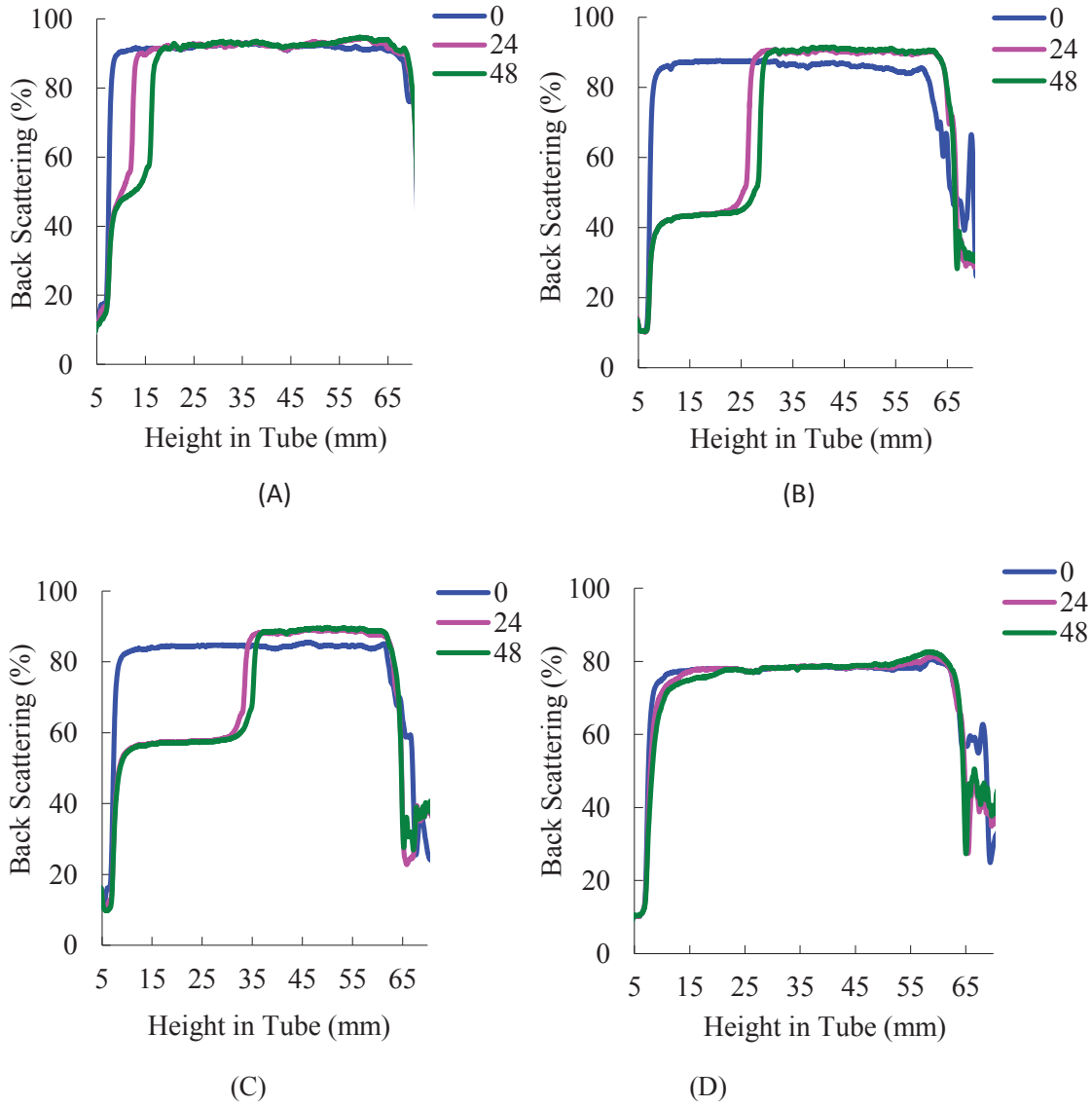


Figure 6.5 Creaming profiles (Turbiscan) of oil-in-water emulsions (30 vol% oil) made with 4 wt% potassium caseinate and urea after preparation, 24 and 48 h of storage.

(A) urea-free (B) 1.1 M urea (C) 3.3 M urea (D) 6.6 M urea

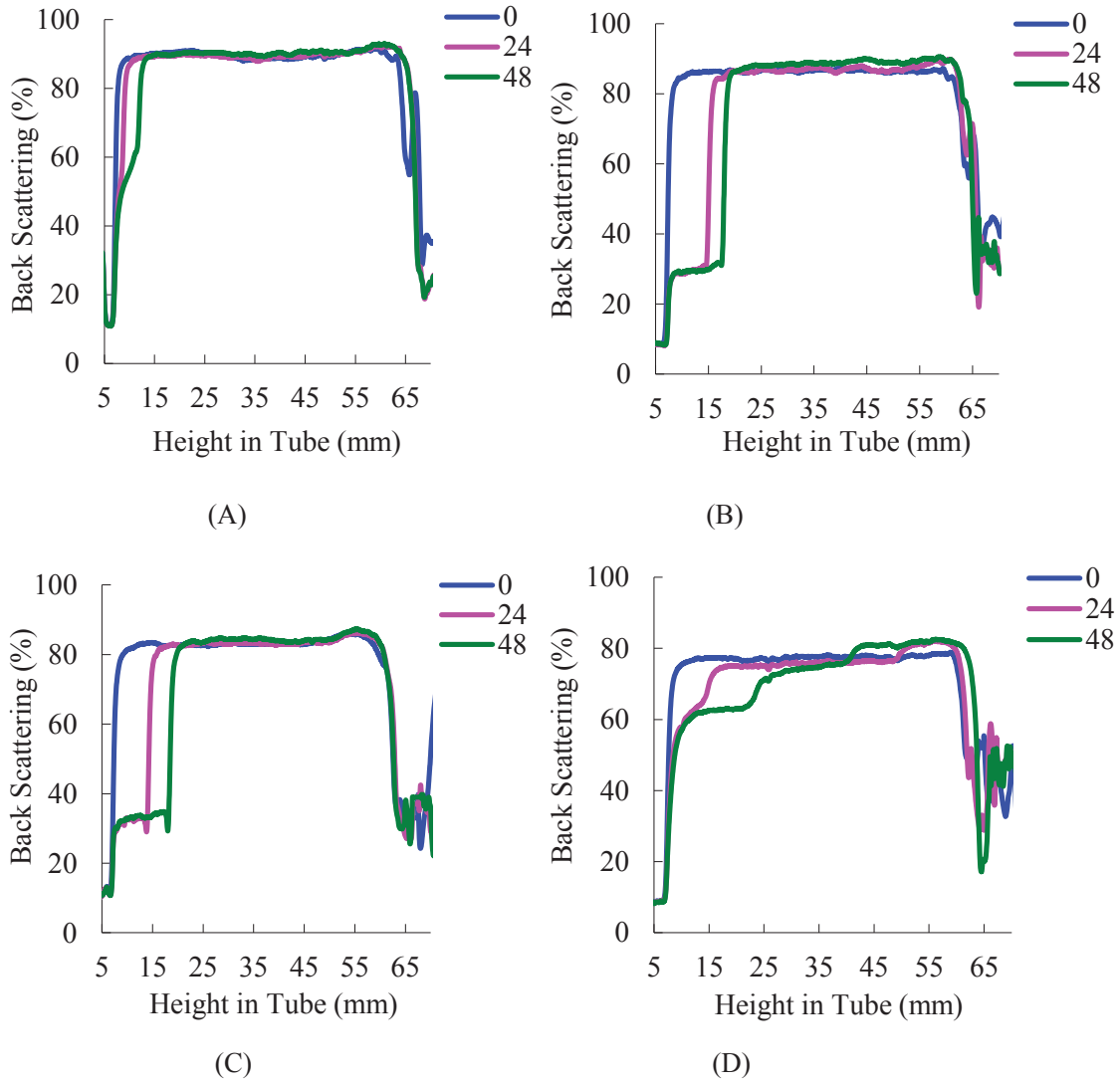


Figure 6.6 Creaming profiles (Turbiscan) of oil-in-water emulsions (30 vol% oil) made with 6 wt% potassium caseinate and urea after preparation, 24 and 48 h of storage.

(A) urea-free (B) 1.1 M urea (C) 3.3 M urea (D) 6.6 M urea

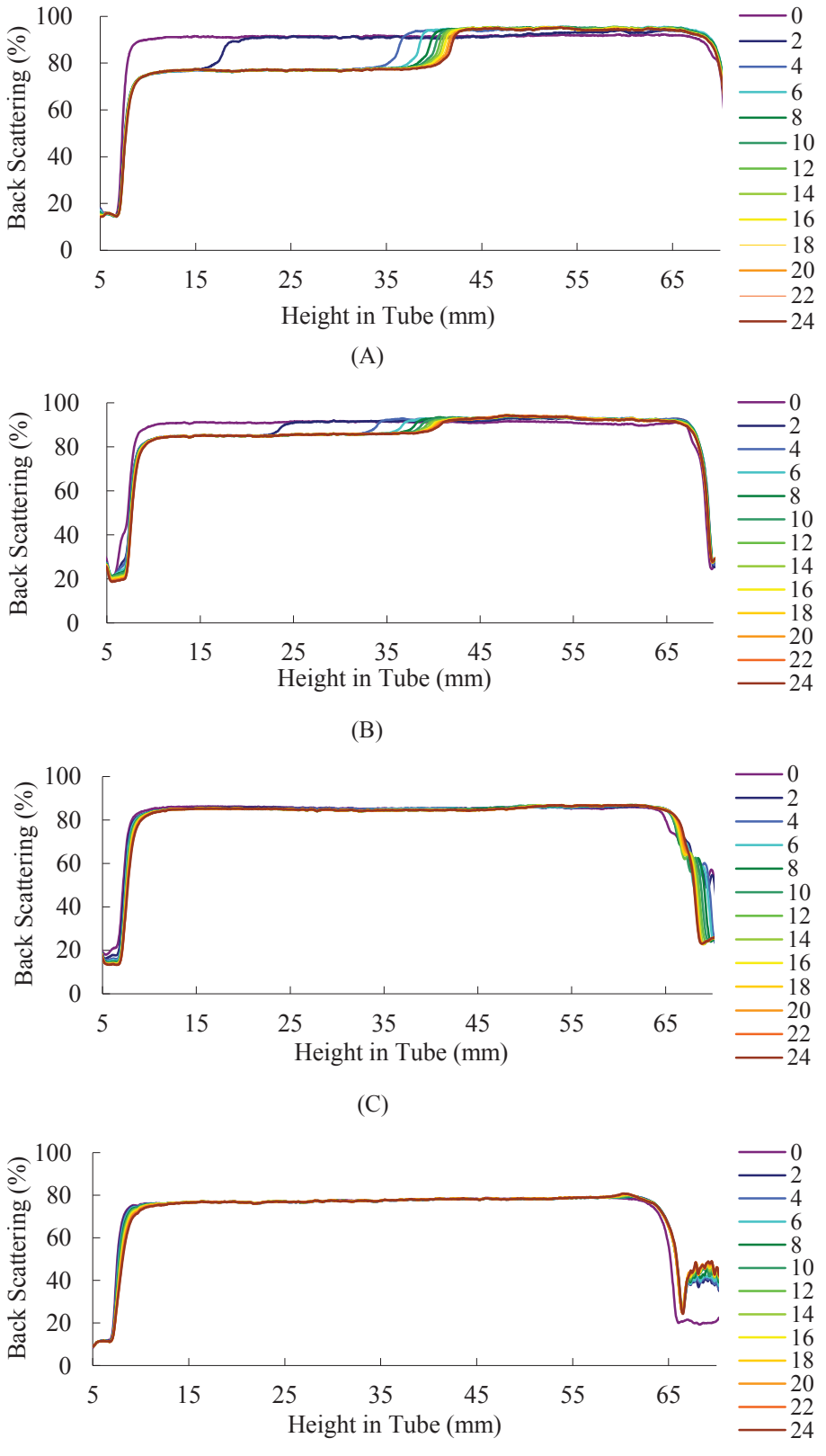


Figure 6.7 Dynamic creaming profiles (Turbiscan) of oil-in-water emulsions (30 vol% oil) made with 2 wt% potassium caseinate and urea in 24 h of storage.

(A) urea-free (B) 1.1 M urea (C) 3.3 M urea (D) 6.6 M urea

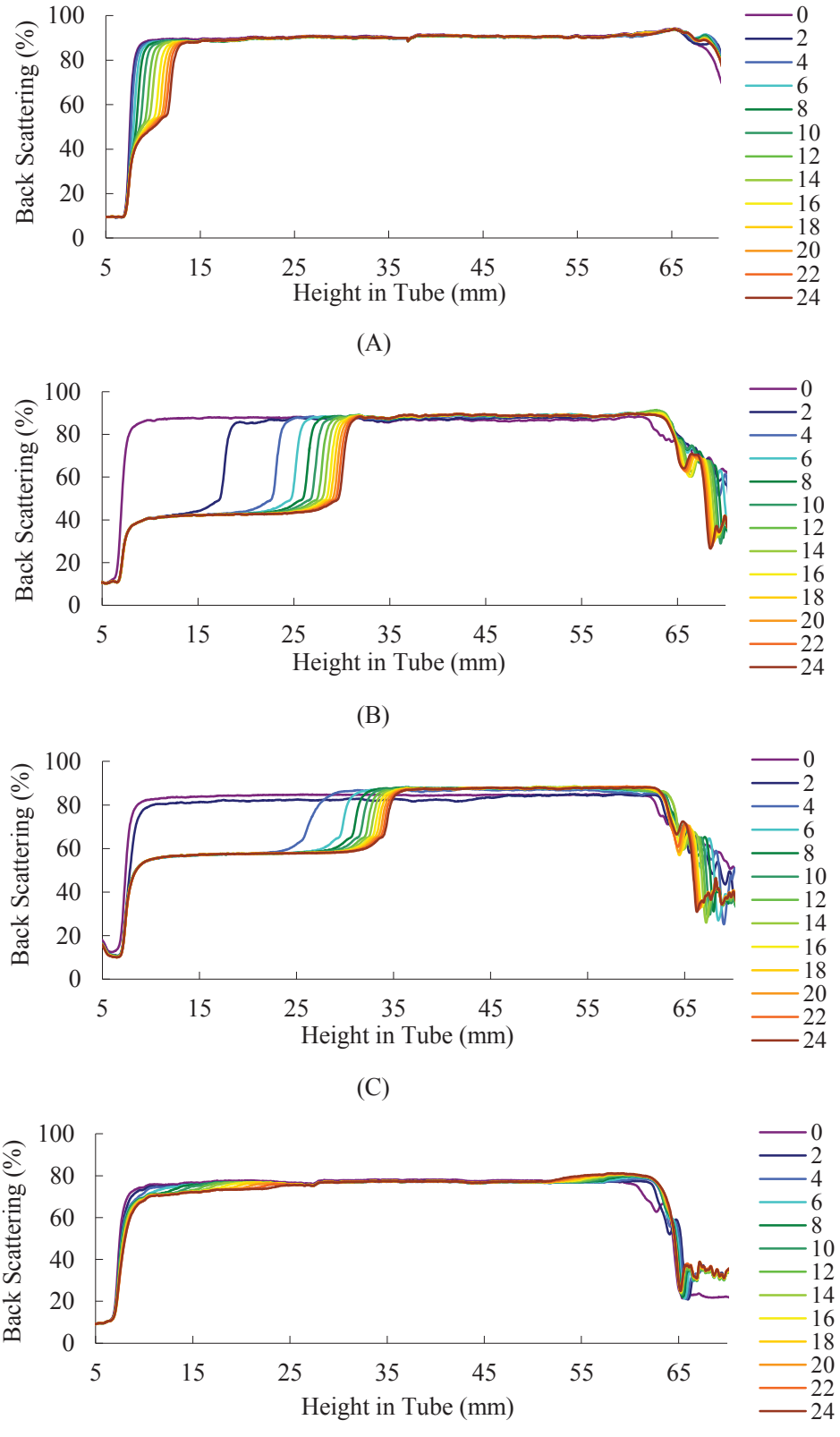


Figure 6.8 Dynamic creaming profiles (Turbiscan) of oil-in-water emulsions (30 vol% oil) made with 4 wt% potassium caseinate and urea in 24 h of storage.

(A) urea-free (B) 1.1 M urea (C) 3.3 M urea (D) 6.6 M urea

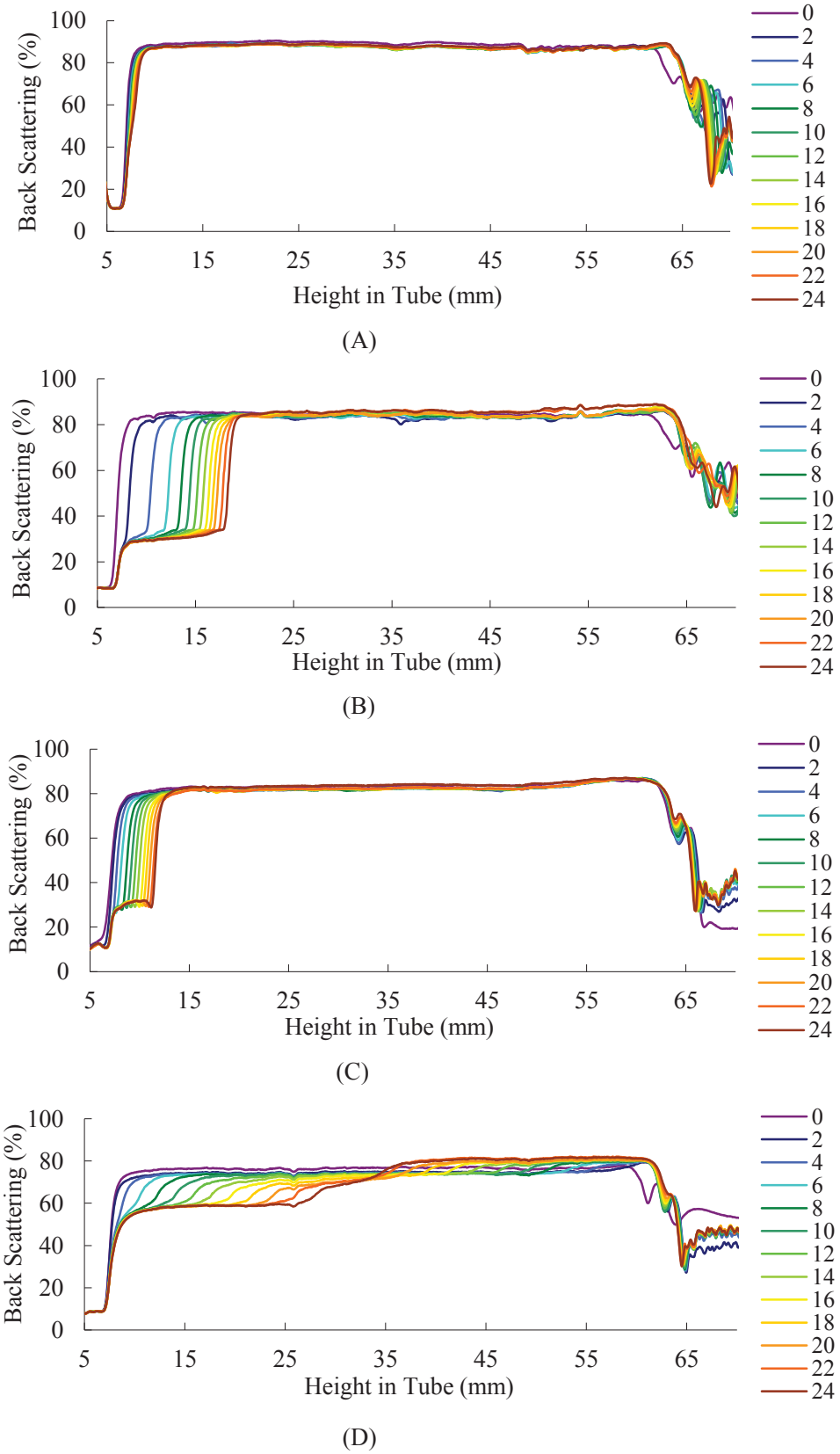


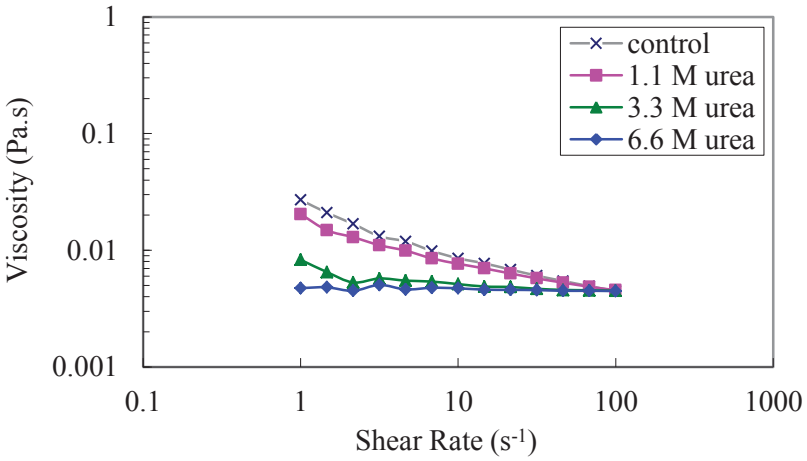
Figure 6.9 Dynamic creaming profiles (Turbiscan) of oil-in-water emulsions (30 vol% oil) made with 6 wt% potassium caseinate and urea in 24 h of storage.

(A) urea-free (B) 1.1 M urea (C) 3.3 M urea (D) 6.6 M urea

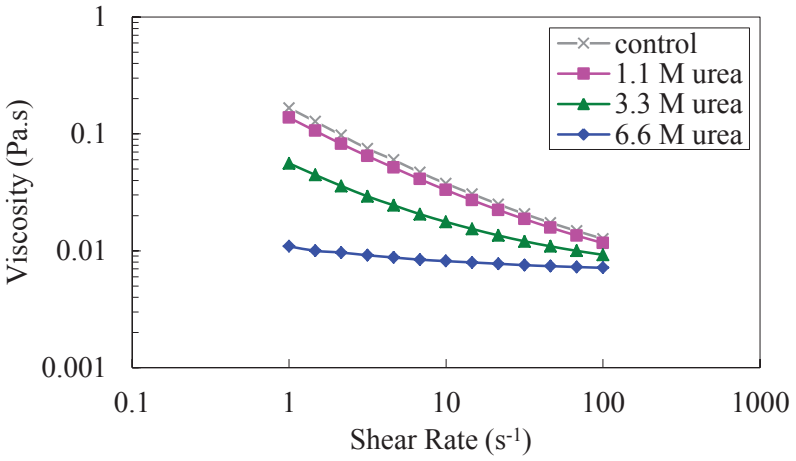
6.3 Rheological properties of potassium caseinate stabilised emulsions with urea

6.3.1 Shear rate dependence

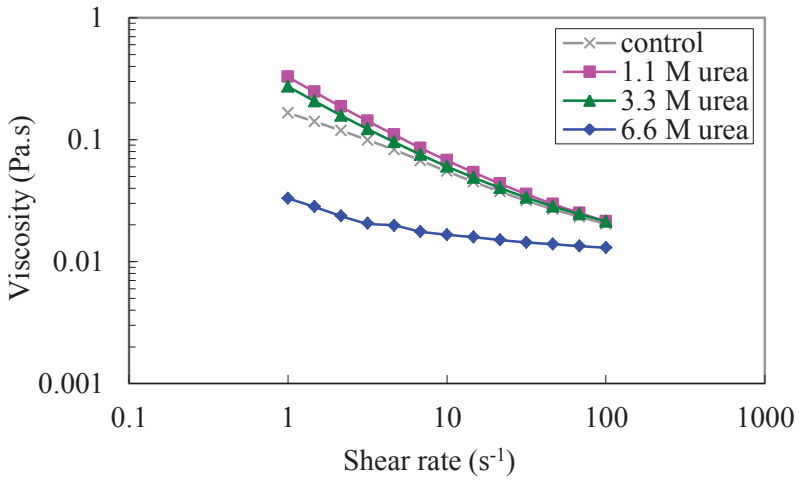
The apparent viscosity of potassium caseinate stabilised emulsions as a function of shear rate can be observed in Figure 6.10. With addition of urea, there was a reduction in low shear viscosity and pseudoplasticity behaviour in 2 wt% caseinate emulsions. A similar trend can be observed for 4 wt% caseinate emulsions. At 6 wt% caseinate, the addition of 1.1 and 3.3 M urea both induced a slight increase in pseudoplasticity and the low shear viscosity was relatively higher than the control sample. Meanwhile, low shear viscosity went down and pseudoplasticity behaviour was weakened with 6.6 M urea added.



(A)



(B)



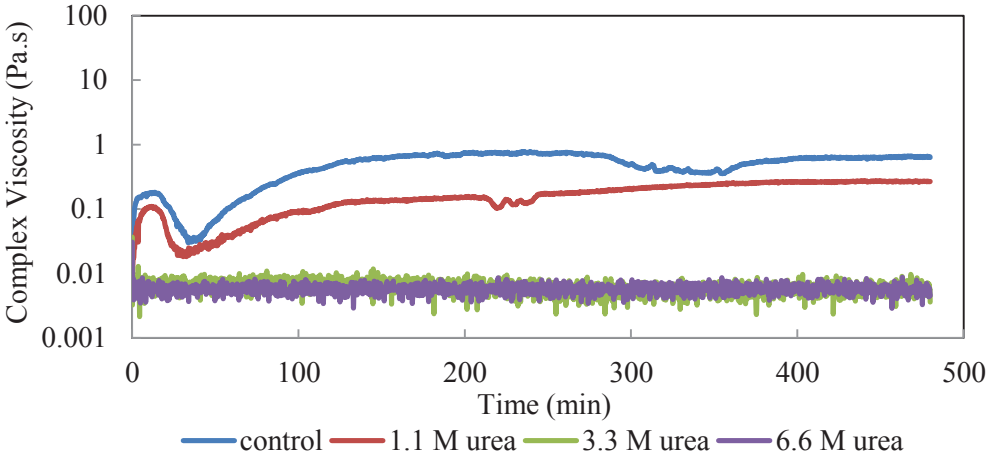
(C)

Figure 6.10 Shear rate dependence of apparent viscosity of oil-in-water emulsions (30 vol% oil) made with potassium caseinate and urea.

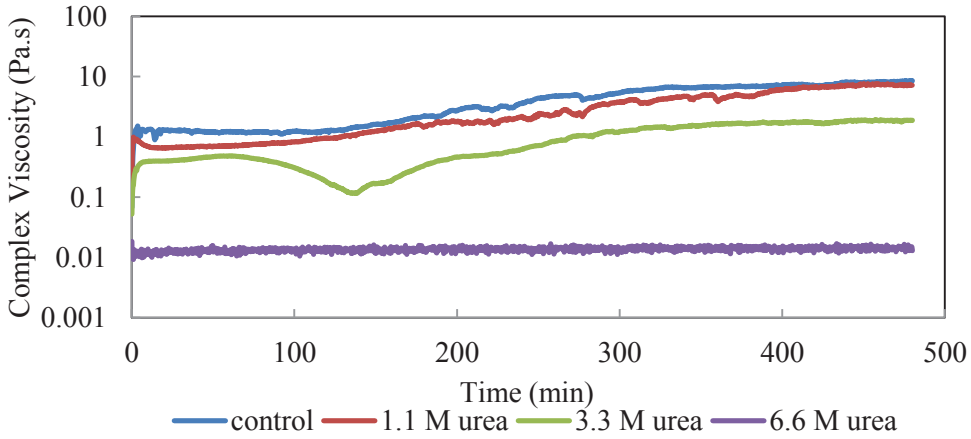
(A) 2 wt% potassium caseinate (B) 4 wt% potassium caseinate (C) 6 wt% potassium caseinate

6.3.2 Time dependence

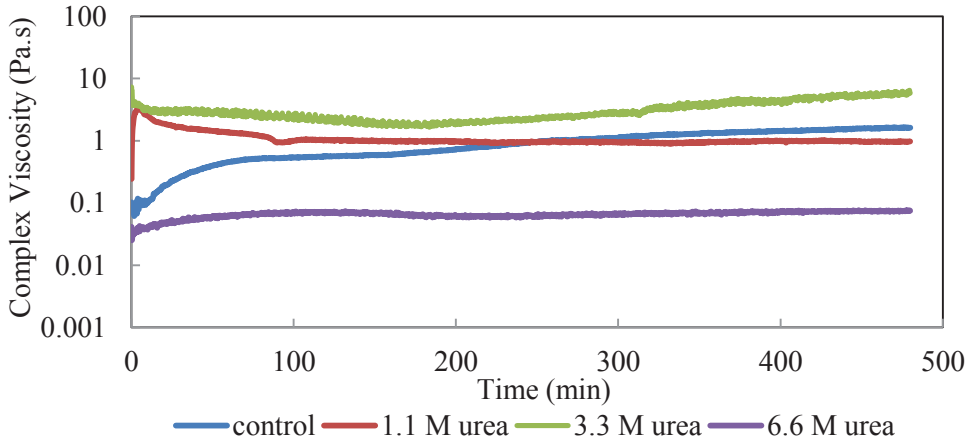
The depletion-induced network development in emulsions is shown in Figure 6.12, as the initial increase in complex viscosity value implies the onset of the formation of the weak droplet network. The evolution of the structural change in potassium caseinate stabilised emulsions can be observed in Figure 6.11. For all the emulsions with 6.6 M urea, the complex viscosity was found to be fairly constant within the experimental period, so did the 2 wt% caseinate emulsion with 3.3 M urea. In the meantime, for the rest of the samples, reorganisation and restructuring in the emulsion system can be observed during the whole process.



(A)



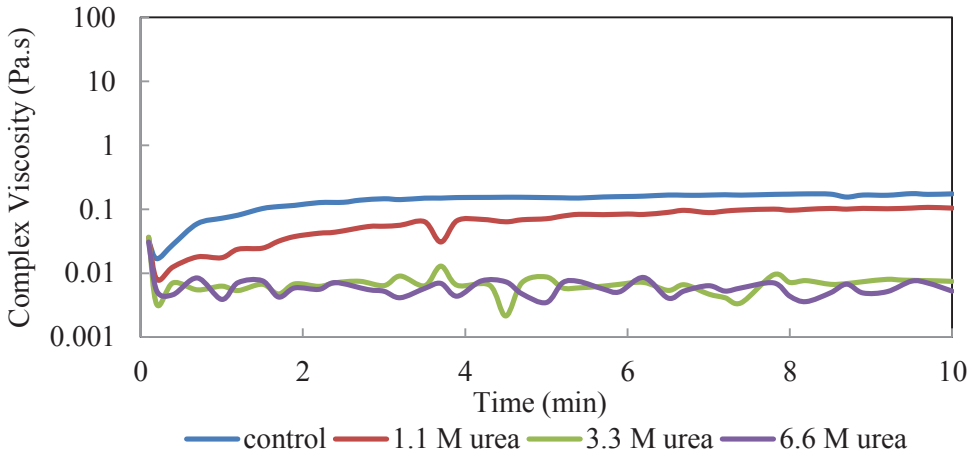
(B)



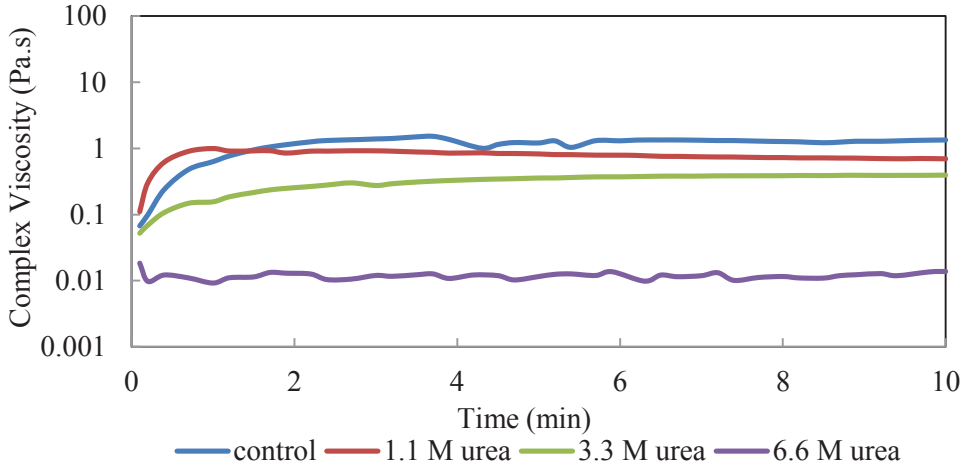
(C)

Figure 6.11 Time dependence of complex viscosity of oil-in-water emulsions (30 vol% oil) made with potassium caseinate and urea.

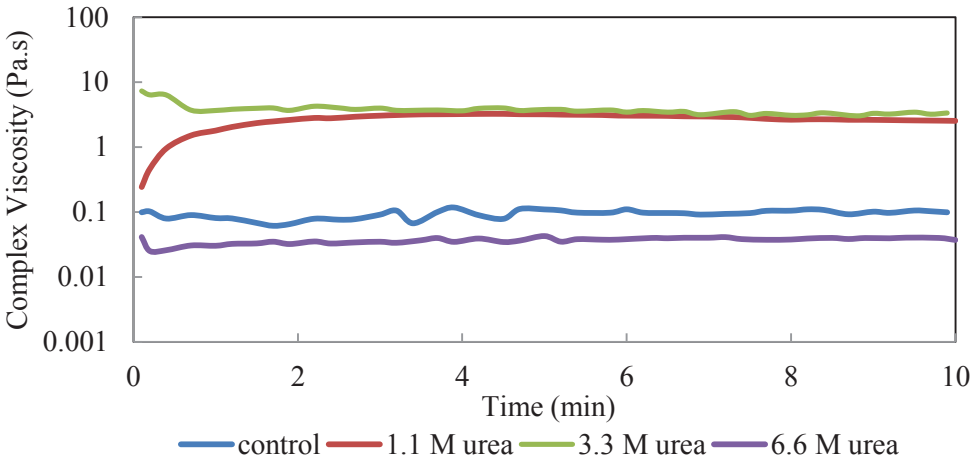
(A) 2 wt% potassium caseinate (B) 4 wt% potassium caseinate (C) 6 wt% potassium caseinate



(A)



(B)



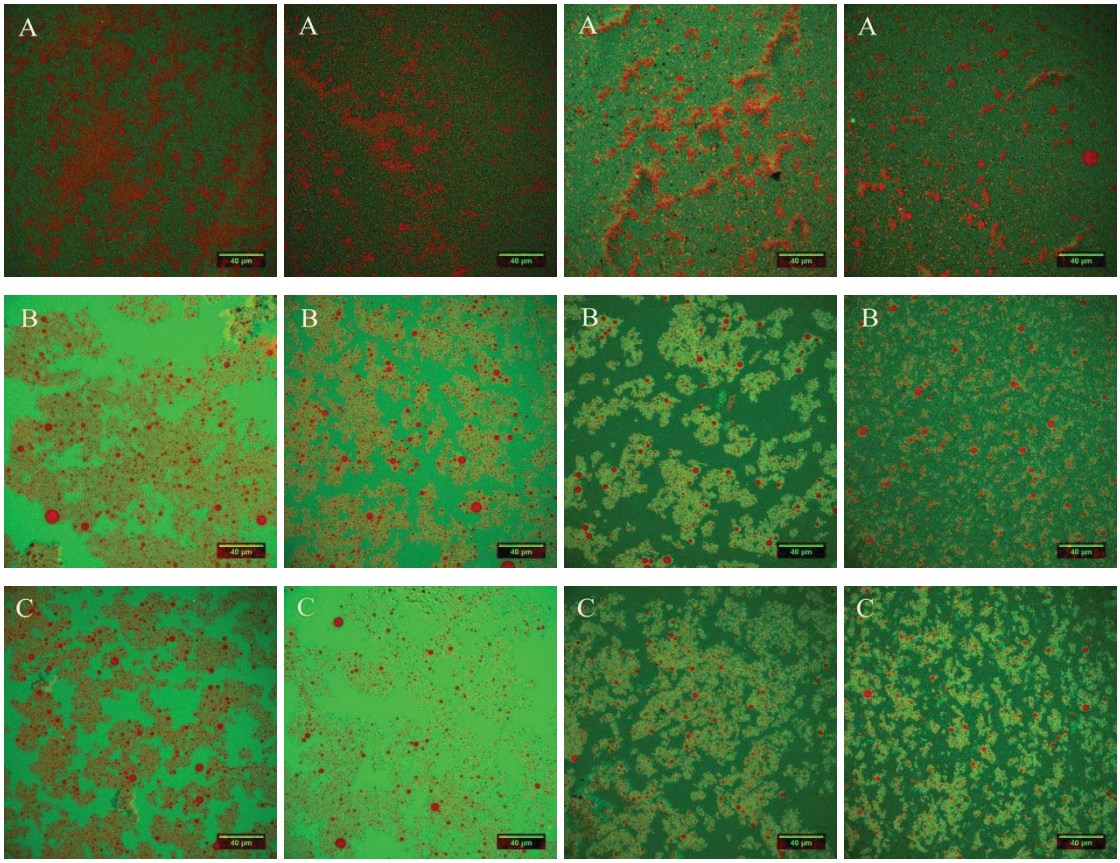
(C)

Figure 6.12 A close up of the first 10 min of the time dependence rheological properties of Figure 6.11.

(A) 2 wt% potassium caseinate (B) 4 wt% potassium caseinate (C) 6 wt% potassium caseinate

6.4 Microstructure of potassium caseinate stabilised emulsions with urea

As it can be seen from Figure 6.13, there was interconnected droplet network observed in 2 and 4 wt% urea-free caseinate emulsions, with more extensive network formed in higher protein concentration. With increasing urea concentration, there was a gradually weakened in droplet flocculation and network formation. The depletion-induced network was completely disturbed in 6.6 M urea for 2 and 4 wt% caseinate emulsions, with fewer flocculated droplets and a more homogenous system. For 6 wt% emulsion, there was no network but depleted droplet clusters observed, and with the addition of urea, open network developed in a urea concentration of 1.1 and 3.3 M. The emulsion became homogenous and network broke down again when urea concentration went up to 6.6 M.



Control **1.1 M urea** **3.3 M urea** **6.6 M urea**

Figure 6.13 Confocal micrographs of oil-in-water emulsions (30 vol% oil) made with potassium caseinate and urea (scale bar is 40μm).

(A) 2% potassium caseinate (B) 4% potassium caseinate (C) 6% potassium caseinate

6.5 Discussion

According to the aforementioned study in chapter 5, urea addition had a marked influence on the creaming stability of sodium caseinate emulsions, arising from dissociation of caseinate sub-micelles. It was assumed that urea had a similar effect on the potassium caseinate emulsions as it did in improving the stabilisation of sodium caseinate emulsions.

Based on the results in this chapter, no notable difference was found between the effects of urea on potassium caseinate and sodium caseinate emulsions, which was supported by the observations of creaming behaviours, rheological properties and microstructure. Whereas, it should be noted that for the visual creaming profile, the colour and the turbidity of the potassium caseinate emulsions were slightly different from those of the sodium caseinate samples, which might be due to the effect of the stain. The observations obtained here are in accordance with the hypothesis that urea modifies the stability of the potassium caseinate emulsions by declining the depletion potential and correspondingly reducing the continuous phase viscosity.

On the other hand, there are two elements which could also be the possible explanations for the analogy between the stability of potassium caseinate and sodium caseinate emulsions with the addition of urea. First of all, as it can be seen in Table 6.1, the difference regarding to the particle size and the size distribution of potassium and sodium caseinate sub-micelles is negligible, and hence with r_m being a vital variable in determining depletion free energy, there is no notable difference in the depletion potential of the caseinate emulsions. Moreover, the viscosity of potassium caseinate solutions is comparable with sodium caseinate solutions (Figure 6.1); therefore, as expected, the continuous phase viscosity of both types of the caseinate emulsions is analogous. However, it is noted that the droplet size and droplet size distribution of potassium caseinate are slightly different from those of sodium caseinate, and as there is no sufficient data or theory to explain these observations, further experiments are needed in the future. Accordingly, the variation in cation type between the two caseinate

compositions appears to have little impact on solution and emulsion properties, although some subtle differences are observed.

7 Overall discussions

The addition of urea has been shown to have a remarkable dissociating effect on the sub-micelles in sodium caseinate solutions. Urea alters the hydrophobic interactions in sub-micelles, which leads to the dissociation of the aggregates induced by self-assembly. This is evidenced by the results of particle size and rheological properties of sodium caseinate solutions. Based on the results of particle size measurements by intensity and number, the size of caseinate sub-micelles in urea-treated samples decreased with increasing urea concentration, indicating urea induced dissociation of sub-micelles. In terms of rheology, there was a significant reduction in the apparent viscosity of the samples with the addition of urea when the protein concentrations were above the close packed level. Meanwhile, it was indicated that the addition of urea led to progressive dissociation of the sub-micelles, as the apparent viscosity deceased without a particular turning point.

Meanwhile, the dissociation effect of urea is temperature-sensitive. It is suggested that the hydrophobic interactions are susceptive to the temperature, and it increases with elevated temperature (Thompson & Farrell, 1973). In this case, the dissociation effect of urea is supposed to be stronger in low temperature, as the hydrophobic bonds are easier to break. On the other hand, the viscosity of the caseinate solution without urea reduces with increasing temperature, which is due to the reduction of the effective volume fraction of sub-micelles, caused by the decline in inter-particle repulsive interaction (Pitkowski et al., 2008). In the study here, the apparent viscosity of the samples with urea was observed to drop with increasing temperature, implying that the effect of temperature on the hydrophobic forces are negligible compared to the one on the repulsive forces. Moreover, the dissociation of sub-micelles was concentration dependent, as the effect of urea became more significant in protein concentrations above the close packing regime (10 wt%).

Likewise, sub-micellar dissociation arising from the addition of urea has been shown to have a pronounced effect on the stability of caseinate-stabilised emulsions undergoing depletion flocculation. For the addition of urea on the caseinate-stabilised emulsions with excess non-adsorbed proteins, two specific effects are noteworthy, including the weakening of depletion interaction and the corresponding reduction in continuous phase viscosity. (1) Urea reduces the particle size of nano-particles, weakens the depletion potential and improves the stability of the emulsion. This is the case for 2 and 4 wt% caseinate samples. (2) For the casein-rich samples (6 wt% protein), while depletion potential was somewhat reduced with 1.1 and 3.3 M urea, the concurrent decrease in continuous phase viscosity increased the droplet mobility and accelerated the network development. Hence, instead of being more stable, phase separation occurred as the weak network collapsed under gravity. When urea concentration was up to 6.6 M, the emulsion was re-stabilised as the depletion potential had been effectively reduced and it was not sufficient for depleting droplets.

In terms of the effect of cations in the caseinates, there was no notable difference in the effects of urea on the emulsions stabilised with potassium and sodium caseinate, including creaming stability, rheological properties and microstructure. As the particle size of potassium caseinate was closely analogous to the one of sodium caseinate and the trend of the change of the droplet size was similar, it was postulated that the depletion potential of the emulsions with either of these caseinates was similar. Therefore the cations in these two caseinates might not play an important role in the dissociation effects of urea on the emulsions.

8 Conclusions and Recommendations

8.1 Effects of urea on caseinate solutions

Hydrophobic self-association of sodium caseinate into putative sub-micellar moieties are considered contributory to the high viscous properties of caseinate solutions above sub-micellar close packing limits. Addition of urea, which has been shown to have a disruptive effect on sub-micelle association by diminishing hydrophobic interactions, substantially reduced the viscosity of caseinate solutions for sub-micelle phase volumes above close packing. The viscosity of the solutions decreased with increasing urea concentration, with 6.6 M urea having a significant dissociation effect on the caseinate sub-micelles. The viscosity of the solutions with urea present is temperature-sensitive, as the viscosity decreased with higher temperature. With addition of urea, the preparation of concentrated caseinate solutions becomes attainable, and the protein concentration can go up to 40 wt%.

8.2 Effects of urea on emulsions stabilised by caseinates

Urea also influences the physico-chemical properties in caseinate-stabilised emulsions which undergo depletion flocculation. Urea impacts the emulsion system through two effects, as it is capable to reduce depletion potential and decrease the continuous phase viscosity as well. For 2 and 4 wt% caseinate emulsions, the addition of urea improved the creaming stability, while at 6 wt% protein concentration, phase separation occurred when 1.1 and 3.3 M urea were added in the system, and the stability was re-established when urea concentration went up to 6.6 M. According to the microstructure of caseinate emulsions, depletion flocculation was nearly fully disintegrated by 6.6 M urea for all the samples with various protein concentrations, and homogeneous droplets were observed. Generally, urea alters the emulsion stability mainly by reducing the depletion potential, accompanying with the corresponding effect of adjusting continuous phase viscosity.

Additionally, there was no conspicuous difference in the creaming stability, rheological properties and microstructure of the emulsions with different caseinates (Na and K) with the addition of urea, so it was implied that the dissociation effect of urea would not be influenced by the cations in these caseinates.

8.3 Applications of findings to industry

Findings in this study have implications for improving efficiency in the manufacture of caseinate powders by reducing solution viscosity at high solids, as well as potentially improving the stability and material properties in caseinate protein enriched emulsion formulations.

8.4 Recommendations for future work

Based on the study here, there are some topics can be further developed in the future work. It is recommended that:

The possibility of adopting alternative ways to dissociate the caseinate sub-micelles is of interest, especially for the food grade reagents with the ability to alter the hydrophobic interactions in caseinate sub-micelles. This would help to eliminate the negative effects caused by the self-assembly of caseinate in solutions and emulsions, and improve the application of caseinates in food industry.

The sequence of urea addition on the dissociation effect on the self-assembly of caseinate might be examined, which involves the differences in the physico-chemical and functional properties in the system when urea is added before or after the addition of caseinates (for solutions) and homogenisation (for emulsions).

As emulsion is a complex system with different food ingredients co-existing, how these ingredients interact with urea and they influence the dissociation effect of urea might be worth to determine.

The changes in the composition and microstructure of the sub-micelles and the larger aggregates in the urea-treated caseinate solutions might be clarified, which would help to establish a better understanding about the mechanism of the dissociation effect of urea on caseinates.

The effects of urea on different caseinate products, such as ammonium caseinate and caseinate hydrolysate could be investigated.

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