**Formation of lactoferrin/sodium caseinate complexes and their adsorption
behaviour at the air/water interface**

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Abstract

This research investigated the **complexation** behaviour between lactoferrin (Lf) and sodium caseinate (**NaCas**) before and after heat treatment. The results showed that heating facilitated their interaction and different complexes were formed at different **Lf/NaCas ratios**. The presence of low concentrations of **NaCas** resulted in the rapid precipitation of Lf, while no precipitation was observed at the NaCas concentrations higher than Lf/NaCas ratio of 2:1. The formed complexes at the ratio of 2:1 have an average diameter of 194 ± 9.0 nm and they exhibited a great capacity in lowering the air/water interfacial tension. Further increase of NaCas concentration to ratios of 1:1 and 1:2 resulted in the formation of smaller complexes with average diameters of 60 ± 2.5 nm. The complexes formed at these two ratios showed similar adsorption behaviour at the air/water interface and they exhibited lower capacity in decreasing the interfacial tension than the ratio of 2:1.

Keywords

Sodium caseinate; Lactoferrin; Heat stability; **Interfacial tension**; Turbidity; Zeta potential

1. Introduction

The formation of complexes between oppositely charged polymers is of both theoretical and practical interest. The combination between polymers is mainly through electrostatic attraction, although other forces, such as hydrophobic interaction, disulphide bond and van der Waals force, also contribute to the complexation (De Kruif, Weinbreck, & De Vries, 2004). The formed complexes can be regarded as new colloidal entities and they can be soluble or precipitated, depending on the environmental conditions, such as charge density of the polymers, pH, ionic strength and heat treatment (Liu, Wang, Ma, & Gao, 2016). The electrostatic interaction between the polymers is of practical relevance for both the food and pharmaceutical industries. For instance, the microspheres formed between carboxymethyl-chitosan and alginate have been successfully used to encapsulate bovine serum albumin (BSA) to control its release (Zhang, Guo, Peng, & Li, 2004); addition of pectin, which is able to combine with positively charged milk proteins, can improve the stability of yogurt drinks (Tromp, de Kruif, van Eljk, & Rolin, 2008); the interaction between chitosan and whey proteins is able to protect against heat-induced denaturation and aggregation of whey protein molecules (Hong & McClements, 2007; Zhao & Xiao, 2016).

Bovine native lactoferrin (Lf) is a cationic protein with a high PI (pH 8.9) and a molecular weight of 88 kDa (Yamniuk, Burling, & Vogel, 2009). Due to its special iron-binding capacity, FeLf has been widely used as a food supplement for iron fortification. In addition, Lf has various biological functionalities, including antibacterial, antiviral, antifungal, antiparasitic, anti-inflammatory, anticarcinogenic and antitumor activities (Ward, Paz, & Conneely, 2005; Tomita, Wakabayash, Shin, Yamauchi, Yaeshima, & Iwatsuki, 2009). Lf can also be used as emulsifier to produce o/w emulsions (Ye & Singh, 2007; Ye, Lo, & Singh, 2012). Since the stability of the

produced emulsions decreases with increasing pH (Acero-Lopez, Schell, Corredig, & Alexander, 2010), anionic polysaccharides are usually added to interact with Lf at the o/w interface to improve the stability of emulsion (De Lorenzis, Semeraro, De Blasi, Mita, & Poltronieri, 2008; Su, He, Guo, Jian, Gao, & Yuan, 2015). Moreover, Lf can interact with the negatively charged proteins. In milk, about half of the Lf is combined with negatively charged casein micelles through electrostatic attraction (Croguennec, Li, Phelebon, Garnier-Lambrouin, & Gésan-Guiziou, 2012), higher levels of which is bound to smaller casein micelles, indicating that Lf is mainly bonded to the surface κ -casein layer (Anema & De Kruif, 2013). After adding Lf to milk, the attachment of Lf on the surface of casein micelles initially increased the size and turbidity of casein micelles, followed by the disintegration of casein micelles (Anema & De Kruif, 2012a).

One universal processing procedure during food production is heat treatment, which is known to denature the Lf molecules, particularly at neutral pH (7.0). The denaturation of Lf is related to the degree of iron saturation. Iron-saturated Lf (holo-Lf, iron saturation>90%) has high thermal stability with a T_m value of $90\pm1^\circ\text{C}$ (Bokkhim, Bansal, Grondahl, & Bhandari, 2013). In contrast, when Lf is in its native (iron saturation 15%-20%) or apo (iron depleted) forms, heating at temperatures higher than 70°C resulted in the irreversible changes of structure. It was reported that when increasing the temperature from 25 to 80°C , the α -helix content of native Lf decreased from 23.5% to 21.2% and more hydrophobic residues were exposed (Stănciuc, Aprodu, Râpeanu, van der Plancken, Bahrim, & Hendrickx, 2013). Through the hydrophobic interaction and disulphide bond, the denatured Lf molecules aggregate with each other resulting in the formation of high-molecular-weight aggregates and precipitates (De Kruif et al., 2004). Meanwhile, the denatured Lf molecules lose the iron-holding capacity. Therefore, to increase the application in food industry, the thermal stability of Lf has to be improved. It was reported that the formations

of Lf/pectin or Lf/soluble soybean polysaccharide complexes were able to protect Lf against heat-induced denaturation and to maintain its iron-binding ability (Bengoechea, Jones, Guerrero, & McClements, 2011; Hiroshi et al., 2012). The polyphenol compounds, which could conjugate with Lf, were shown having the ability to prevent Lf from thermal aggregation by increasing the steric hindrance and electrostatic repulsion (Liu et al., 2016).

Sodium caseinate (NaCas), the sodium salt of caseins, has a random-coiled structure, and is negatively charged and heat stable. Based on the properties of NaCas, it could be a qualified candidate to improve the heat stability of Lf by the formation of stable complexes and interacting with the exposed hydrophobic groups to prevent the aggregation of Lf molecules during heating. Although previous research found that the interaction between NaCas and Lf is weak as NaCas solution creates high ionic environment (Anema & de Kruif, 2012b), no research about the complexation between Lf and NaCas after heat treatment can be found.

In this research, we hypothesized that the weak complexation interaction between NaCas and native Lf is able to protect Lf against heat-induced aggregation and precipitation. To test our hypothesis, the Lf and NaCas solution were mixed at different ratios (4:1, 3:1, 2:1, 1:1 and 1:2) and then subjected to heat treatment (90°C, 10min). The change of the turbidity was used to determine the stability of different solutions while zeta potential and size were used to characterize the complexation between NaCas and Lf. In addition, the drop tensiometry was used to investigate the adsorption behaviour of formed complexes at air/water interface.

2. Materials and Methods

2.1. Solution preparation and heat treatment

The native bovine Lf (>90% purity; Fonterra Cooperative Group, New Zealand) and NaCas (Sigma-Aldrich, Shanghai, China) solutions (1%, w/w) were prepared by dissolving in distilled water with stirring at room temperature for 2 h. Sodium azide (0.02%) was added to prevent the growth of bacteria. The protein solutions were then stored in 4 °C fridge overnight to achieve the complete hydration. After that, the solutions were filtered through a 0.45 µm filter (Nylon Millipore, Fisher Sci. Mississauga, ON) to remove insoluble particles and the filtered solutions were stored at room temperature (22°C) for at least one hour before mixing. The pH of each protein solutions were adjusted to pH 7.0 using 1 mol L⁻¹ NaOH or HCl.

The Lf and NaCas solution were mixed to achieve different Lf/NaCas ratios (w/w) of 4:1, 3:1, 2:1, 1:1 and 1:2. All the mixtures have a fixed total protein content of 1%. The mixed solutions were placed at room temperature (22°C) with stirring for one hour and transferred to 5 ml capped glass vials for heating. After heating at 90°C for 10 min in a thermostatically-controlled water bath, the vials were immediately cooled in ice water and stored at 22 °C.

2.2. Turbidity measurements

The turbidity was determined by the absorbance at the wavelength of 600 nm using a Hitachi UV-1100 spectrophotometer at room temperature (22 °C). 10 mm x10 mm polystyrene cuvettes (Sigma–Aldrich, St. Louis, MO, USA) were used.

2.3. Particle size measurements

The apparent hydrodynamic radius of Lf-NaCas complexes was measured using dynamic light scattering (Zetasizer Nano, Malvern Instruments, Worcestershire, UK) at 25 °C. Radius values were reported as intensity-based average size using cumulants analysis. A backscattering mode

(detection angle=173°) was used and the particle size distribution was determined on the basis of volume frequency.

2.4. Zeta potential measurements

Zeta potential indicates the net charge on the surface of a particle, which depends on not only the charge on the particles, but also the charge carried by any associated ions that move along with the particles in an electric field (Surh, Decker, & McClements, 2006). In the present work, the zeta potential was measured by a laser Doppler electrophoresis using the Nano-S Zetasizer with the DTS1060 capillary cell. The Smoluchowski model was used to calculate zeta potential from the mobility values. Samples were determined 200 times and the results were expressed in absolute values (mV).

2.5. Drop shape tensiometry

The interfacial tension (γ) and elastic modulus (E) of the mixed solutions for both before and after heat treatment were measured using drop shape tensiometry (Tracker, IT Concept, Longessaigne, France) at room temperature. The solutions were diluted 10 times using milli-Q water to reach a protein concentration of 0.1% (w/w) before transferring to an optical glass cuvette for analysis. A J-shaped syringe needle was immersed in the test sample and then a 5 μ L air bubble was formed at the tip of the needle. The volume of the drop was consistently controlled using a volume control regulation program. The changes of interfacial tension (γ) were calculated by analyzing profiles every 0.5 s in accordance with the Laplace equation (Gülseren & Corredig, 2012). The determination of dilatational viscoelasticity was conducted after an equilibration period of 10 min. After that, a sinusoidal oscillatory changes of volume/surface area of the air were performed with the strain amplitude kept constant at 10% ($\Delta A/A=0.1$, where A is the droplet surface area). The surface dilatational modulus was

calculated from the change in interfacial tension ($d\gamma$) relative to the change of air surface area (dA), as described in the following equation (Gülseren & Corredig, 2012).

$$E = d\gamma / (d \ln A) \quad (\text{Eq 1})$$

2.6. Statistical analysis

The reported values in this study are means of at least three replicates. ANOVA and Tukey HSD were performed on the data using Minitab statistical package release 15 (Minitab Inc., State College, PA, USA).

3. Results and Discussion

3.1. Stability and turbidity before and after heat treatment

Before heat treatment, the Lf/NaCas mixed solutions are transparent and no precipitate could be observed for all samples (Fig. 1). This phenomenon is in agreement with previous finding, which can be explained by the weak interaction between Lf and NaCas (Anema & de Kruif, 2012b). After heating, precipitation was found at the Lf/NaCas ratio of 4:1, while a change from transparent to turbid was observed at Lf/NaCas ratios of 3:1, 2:1 and Lf control. For ratio of 3:1 and Lf, significant precipitates were observed from the bottom after a few hours storage, while this phenomenon was not found for the sample with the ratio of 2:1, indicating the formation of stable complexes. In comparison, solutions with ratios of 1:1 and 1:2 kept transparent after heat treatment, showing that further increasing the amount of NaCas significantly improved the heat stability of Lf molecules.

Table 1 summarizes the changes of turbidity before and after heat treatment. Lf had a turbidity value of 0.31 ± 0.01 before heating and this value increased to 2.76 ± 0.10 after heating as a result of denaturation and aggregation, which were driven by the hydrophobic and intermolecular

thiol/disulphide reactions (Brisson, Britten, & Pouliot, 2007). Addition of small amount of NaCas (Lf/ NaCas ratio 4:1) decreased the turbidity from 0.31 ± 0.01 to 0.11 ± 0.00 . The reason for the decreased turbidity is unclear, but the effect of co-solubility of Lf and NaCas can be excluded as further increase of the NaCas addition has no influence on the turbidity of the system. For the ratio of 3:1, the turbidity value after heating is more than 3.0, which is higher than Lf control indicating the addition of small quantities of NaCas (4:1 and 3:1) accelerated the heat-induced reorganization and aggregation of Lf molecules. Similar phenomenon was also observed when using NaCas to stabilize oil/water droplet, where the presence of insufficient NaCas resulted in the bridging flocculation of emulsion (Dickson, Golding, & Povey, 1997).

At the Lf/NaCas ratio of 2:1, prominent denaturation of Lf molecules also occurred after heating as the turbidity increased to a very high value of 2.34 ± 0.3 . However, no precipitate was observed in this case. Further increasing the NaCas addition to the ratio of 1:1 resulted in the decrease of turbidity to a very low value of 0.12 ± 0.01 , which is similar to the ratio of 1:2. The results demonstrated that the denaturation of Lf molecules can be inhibited in the presence of sufficient NaCas.

3.2. Zeta potential before and after heat treatment

Lf (pI=8.9) is positively charged at pH 7.0 (Fig. 2). The heating exposed more charged groups, leading to the increase of zeta potential after heat treatment, which is in accordance with previous study (Liu et al., 2016). The addition of NaCas changed the zeta potential from positive to negative, as NaCas is a negatively charged protein at pH 7.0 with a zeta potential value of about -31mV. The similar behavior had been observed when soluble soy polysaccharide was mixed with Lf (Hiroshi et al., 2012). The transition from positive to negative suggested that the NaCas molecules prefer to combine to the surface of Lf. Moreover, the negative charge

decreased with increasing NaCas addition, indicating more NaCas molecules interacted with Lf.

The zeta potentials of Lf-NaCas complexes at ratios of 4:1 and 3:1 were only around -6.1 ± 0.2 mV and -9.0 ± 0.3 mV, respectively. The low electrostatic repulsion could result in the increased aggregation and precipitation of Lf molecules. At the Lf/NaCas ratio of 1:2, the formed complexes have a zeta potential of -26.4 ± 0.8 mV, which is close to the value of NaCas (-31 mV), suggesting that a complete coverage of NaCas on the surface of Lf was achieved at this concentration. In addition, no significant change of the zeta potential was detected after heat treatment at this ratio, indicating the strong electrostatic repulsion provided by the NaCas layer on the surface of Lf prevented the approach of complexes to each other.

As a globular protein, Lf maintained its globular structure after covered by NaCas molecules on the surface. Since the heat stable NaCas layer is highly charged and random structured, the electrostatic and steric repulsions from it were believed to be the main reason for the suppressed denaturation of Lf during heat treatment. Similar phenomenon had been observed when soy polysaccharide was used to increase the heat stability of Lf, where it was found that thermal stability of Lf can only be achieved when soy polysaccharide/Lf ratio was higher than 1:1 (Hiroshi et al., 2012). At this ratio, the formed soy polysaccharide-Lf complexes have zeta potential of around -22 mV, which could be used to explain the improved Lf stability.

3.3. Size of Lf-NaCas complexes before and after heat treatment

The average sizes before and after heat treatment are summarized in Fig. 3A. The NaCas used in the research has an average diameter of 230 ± 5.2 nm, and the mixing of Lf with NaCas decreased the average diameter to around 40 nm, indicating the interaction between the molecules. Similar results have been reported in previous publication (Anema & de Kruif, 2012b). Moreover, the increase of NaCas addition from the ratio of 4:1 to 2:1 did not influence

the diameter of the complexes, which suggested that the NaCas was insufficient to complex with all the Lf molecules. Further increasing the amount of NaCas to the ratio of 1:1 significantly increased the size and a much higher diameter value of 126 ± 3.2 nm was determined at the ratio of 1:2. The increased combination between Lf and NaCas at higher NaCas addition was responsible for the increased average size.

Heat treatment significantly influenced the size of the Lf-NaCas complexes. Measuring the diameter at low NaCas concentrations (ratio of 4:1 and 3:1) is impossible, as much larger aggregates were formed which precipitated very quickly. The diameter at the ratio of 2:1 increased from 36.7 ± 0.6 nm to 194.4 ± 9.1 nm, indicating aggregation also happened for this sample. In contrast, no change of the diameter was detected for ratio of 1:1 and the value decreased from 126.0 ± 3.2 nm to 63.2 ± 1.3 nm at the ratio of 1:2 after heat treatment. Furthermore, similar diameters of ~60 nm were determined at ratios of 1:1 and 1:2 after heat treatment, demonstrating that there are sufficient NaCas molecules to complex with Lf and to prevent its denaturation and aggregation. The data above show that heating facilitated the complexation between Lf and NaCas, probably through the increased hydrophobic and disulphide exchange interactions (Brisson, Britten, & Pouliot, 2007).

In order to better characterize the heat-induced Lf-NaCas complexes, the size distributions of samples with stable complexes (ratios of 2:1, 1:1 and 1:2) were determined and displayed in Fig. 3B. In comparison with Lf/NaCas ratios of 1:1 and 1:2, a much larger and wider size distribution was observed for the ratio of 2:1. Significant denaturation and aggregation of Lf molecules were believed to happen at this NaCas concentration, as the sample was opaque and had a very large turbidity value (Fig. 1 and Table 1). In this situation, there were not enough NaCas molecules to interact with the exposed hydrophobic groups, thereby denatured Lf molecules aggregated with

each other to decrease the total surface area and energy of the system. Even so, the steric and electrostatic repulsions provided by the NaCas molecules were strong enough to prevent the precipitation of aggregates. Considering the average diameter of the aggregates, it is obvious that most of them are present in the form of trimers and tetramers. In contrast, very narrow size distributions, with an average diameter of 60 nm, were observed for the samples at ratios of 1:1 and 1:2. The overlapping of the curves for these two ratios indicated that a fully coverage of Lf was achieved at the ratio of 1:1. The complexes probably presented in the form of micelles, where the Lf molecules were surrounded by the adsorbed NaCas molecules, which can fully inhibit the denaturation and aggregation of Lf molecules. Apparently, different Lf-NaCas complexes were formed when different amounts of NaCas were mixed with Lf.

3.4. Adsorption behaviours of different Lf-NaCas complexes on the air/water interface

Determination of interfacial tension is an important tool to determine the emulsion stability and adsorption kinetics (Gülseren & Corredig, 2012). The drop shape tensitometry enables real time measurements of interfacial tension by employing image analysis of the drop or bubble shapes (Faour, Grimaldi, Richou, & Bois, 1996; Pelipenko, Kristl, Rošic, Baumgartner, & Kocbek, 2012). This technique has been successfully adopted to investigate the foaming ability of protein (Wang & Narsimhan, 2005), the emulsion stability (Aske, Orr, & Sjoblom, 2002), and the interaction between bioactive compounds and emulsifiers (Faour et al., 1996; Gülseren & Corredig, 2012). To illustrate our hypothesis that different complexes were formed in the presence of different amounts of NaCas before and after heat treatment, drop shape tensiometry was used to determine their adsorption behaviour at the air/water interface (Fig. 4). Our preliminary results showed that NaCas adsorbed at a much faster speed at first 300 s comparing

268 to **globular** Lf. However, the same surface tension value of 47 ± 1.2 mN.s was obtained at 600s
269 (data was not shown).

270 As indicated in Fig. 4A, the addition of NaCas did not change the adsorption curve of Lf at the
271 air/water interface. All the samples showed similar adsorption behaviour and the final surface
272 tension values fell in the range of 46-48 mN.s. The adsorption of protein on the air/water
273 interface is related to the amount of hydrophobic groups exposed on its structure; hence the
274 change in the adsorption is an indication of reorganization of protein structure (Foegeding, Luck,
275 & Davis, 2006). Since the adsorption behaviour was not influenced by the weak complexation
276 between Lf and NaCas molecules, it can be concluded that Lf and NaCas can maintain their
277 structure. The results are in accordance with the turbidity measurement (Table 1).

278 After heat treatment, significant precipitation was observed for the samples with low NaCas
279 concentration (Lf/NaCas ratio of 4:1 and 3:1), thus only samples with good stability were
280 investigated (Fig. 4B). Surprisingly, the heated sample with a ratio of 2:1 showed a better ability
281 in decreasing the surface tension, while samples with Lf/NaCas ratios of 1:1 and 1:2 exhibited
282 the same adsorption behaviour as NaCas. The results above reinforce that the NaCas molecules
283 present on the surface of Lf and they are the dominating components that influence the
284 adsorption behaviour of the complexes. It can be further concluded that the saturation attachment
285 of NaCas on the surface of Lf was reached at Lf/NaCas ratio of 1:1. It was reported that the
286 exposed hydrophobicity accelerated the adsorption of protein to the air/water interface (De Jongh
287 et al., 2004), hence the faster adsorption for the sample at the ratio of 2:1 indicated the presence
288 of higher amount of hydrophobic patches on the surface of Lf-NaCas aggregates. Under this
289 circumstance, the NaCas was not enough to block all the exposed hydrophobic groups of Lf from
290 heat treatment. Therefore, the capacity of decreasing interfacial tension was improved due to the

increased hydrophobic interaction at the air/water interface, resulting in a much lower surface tension of about 39 ± 0.8 mN.s. As summarized in Fig. 4C, no significant differences were detected for other samples.

Elastic modulus is a **measure** of the resistance to the changes of air bubble surface area and it is related to the structural and emulsifying properties of the adsorbed compound. In this research, a sinusoidal oscillatory procedure was used to measure the elasticity of the air bubble after creating in the samples for 10 mins, and the results **are** shown in Fig. 4D. The Lf showed a high elastic modulus value **of** 36 ± 0.8 mN.s, while a much lower value of 8.0 ± 2.0 mN.s was detected for NaCas solution. The addition of NaCas to Lf rapidly decreased the elastic modulus of ratios 4:1 and 2:1 to 14 ± 2.0 mN.s and 7.0 ± 1.5 mN.s, respectively. Apparently, NaCas played the main role in the magnitude of elasticity. After heat treatment, elastic modulus of the samples with high amount of NaCas (ratios of 1:1 and 1:2) increased to a value of around 17 ± 2.7 mN.s. At the ratio of 2:1, a higher elastic modulus value of 25 ± 2.5 mN.s was determined, suggesting that the presence of large aggregates at the air/water interface increased the elasticity of air bubble by restricting the change of the surface area during oscillation process. The above data confirms the results from size measurement (Fig. 3B). Similar results had been reported by Martin et al. (2002), where they found that the adsorbed surface layer with ordered and larger proteins had higher dilatational modulus.

The aforementioned results obviously proved that the structure of heat-induced Lf-NaCas complexes was influenced by the adding amount of NaCas. Heating facilitated the interaction between Lf and NaCas, not only through the electrostatic repulsion but also by the hydrophobic interaction and disulphide exchange interaction. The NaCas molecule is very soluble and has a random-coiled structure, so it is mainly present on the surface of Lf. Once sufficient NaCas is

attached to Lf, the complexes will form a core-shell structure with Lf as the core and NaCas layer as the shell, which provides strong repulsions to inhibit the approach of complexes. This structure is comparable to that of casein micelles, the most important protein in milk. The casein micelles are described to be heat stable and having a core-shell structure, where the α - and β -caseins are present in the core phase and the most soluble κ -casein located on the surface, providing steric repulsion to keep them away from each other even after severe heat treatment (Dalgleish & Corredig, 2012). Based on the addition amount of NaCas, the heat-induced formation of Lf-NaCas complexes can be divided into 3 stages, as illustrated in Fig. 5. At low concentration of NaCas, substantial denaturation of Lf molecules occurred and the NaCas played a role as bridging agent, which resulted in the formation of big aggregates and rapid precipitation. Increasing the addition of NaCas to a middle concentration, the interaction between Lf and NaCas was promoted but the NaCas was insufficient to block all the exposed hydrophobic groups. In this case, the denatured Lf molecules aggregated with each other and formed oligomers with NaCas layer covered, which was able to keep aggregates stable in the solution. At high concentration of NaCas, the formation of micelle structure ensured the heat stability by the steric and electrostatic repulsions provided by the dangling NaCas hairy layer.

4. Conclusion

The denaturation and aggregation of Lf can be inhibited by forming soluble Lf-NaCas complexes. All the results from turbidity, zeta potential and size distribution demonstrated that NaCas played a role as stabilizer to protect Lf against the heat-induced precipitation. Depending on the adding amount of NaCas, the formed Lf-NaCas complexes have different structural and functional properties. The complexes at medium Lf/NaCas ratio of 2:1 showed better foaming and emulsifying capacities. In contrast, the formation of complexes at high NaCas addition (1:1 and

1:2) significantly prevented the denaturation and aggregation. This research is of both practical and theoretical significance for maintaining the structure and functionalities of Lf during the production of Lf-enriched food products. Further research about the structure and stability of the formed complexes at different ionic strength and pH is necessary.

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Table

Table 1 Changes of turbidity for samples with different Lf/NaCas ratios

Turbidity	Before heat	After heat
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Lf only	0.31±0.01 ^a	2.76±0.10 ^a
Lf/NaCas 4:1	0.11±0.00 ^b	N/A
Lf/NaCas 3:1	0.08±0.00 ^c	>3 ^b
Lf/NaCas 2:1	0.07±0.00 ^c	2.34±0.3 ^a
Lf/NaCas 1:1	0.06±0.01 ^c	0.12±0.01 ^c
Lf/NaCas 1:2	0.06±0.01 ^c	0.08±0.01 ^c

Values are means of triplicate experiments, ± standard deviation. Values in the same column with different superscript letter are significantly different (p<0.05).

Figures

Fig.1. Optical images of Lf-NaCas mixture solutions before and after heat treatment. The numbers at the bottom indicated the Lf/NaCas ratios.

Fig. 2. Zeta potential for different samples before (black bar) and after (grey bar) heat treatment. The values with different letter are significantly different ($p < 0.05$).

Fig.3. (A) Average diameter for different samples before (black bar) and after (grey bar) heat treatment and (B) the size distribution for samples with **Lf/NaCas** ratios of 2:1 (filled circle), 1:1 (empty circle), and 1:2 (filled triangle) after heat treatment. The values with different letter are significantly different ($p < 0.05$).

Fig. 4. Changes of interfacial tension of the air/water interface as a function of time for samples with different Lf/NaCas ratios: 4:1 (filled circle), 3:1 (empty circle), 2:1 (filled triangle), 1:1 (empty triangle) and 1:2 (filled squares) before (A) and after (B) heat treatment. Average interfacial tension at the time of 600 s (C) and elastic modulus (D) for samples before (black bar) and after (grey bar) heat treatment. The values with different letter are significantly different ($p < 0.05$).

Fig.5. A schematic representation of the formation of Lf-NaCas complexes at different concentration levels (low, middle and high) of NaCas. The black circles represent the Lf molecules and the irregular curves represent the NaCas molecules.