

# Formation of Multilayers at the Interface of Oil-in-Water Emulsion via Interactions between Lactoferrin and $\beta$ -Lactoglobulin

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**Abstract** The interactions between negatively charged  $\beta$ -lactoglobulin and the positively charged lactoferrin at the droplet surface to form a multi-protein surface layer were examined. Addition of lactoferrin to the aqueous phase of emulsions formed with  $\beta$ -lactoglobulin at pH 7.0 caused an increase in the  $\zeta$ -potential of emulsion droplets, and the  $\zeta$ -potential became positive as the concentration of added lactoferrin was higher than 1% in the system. It is found that lactoferrin binds to adsorbed  $\beta$ -lactoglobulin at droplet surface probably via electrostatic interactions. The amount of lactoferrin at interface increased with increasing the concentration of added lactoferrin, but it decreased with a decrease in the pH. No lactoferrin was observed at interface at pH 3 and 4. By contrast, when  $\beta$ -lactoglobulin was added in the emulsions formed with lactoferrin at pH 7.0, the  $\zeta$ -potential of emulsions changed from positive to negative as the concentration of added  $\beta$ -lactoglobulin increased. The amount of  $\beta$ -lactoglobulin at surface increased correspondingly with increasing the concentration of added  $\beta$ -lactoglobulin. However, in this case,  $\beta$ -lactoglobulin remained bound at interface even at pH 3 and 4 where both lactoferrin and  $\beta$ -lactoglobulin are positively charged. The association of lactoferrin or  $\beta$ -lactoglobulin with the surface proteins that have oppositely charge is probably mainly through electro-

static interactions between the two proteins. It appears that alternative layers of these proteins could be created at the droplet surface.

**Keywords**  $\beta$ -Lactoglobulin · Lactoferrin emulsion ·  $\zeta$ -Potential · Multi-protein surface

## Introduction

Lactoferrin is a glycoprotein with about 700 amino acid residues and a molecular weight of about 80,000 Da.<sup>1</sup> The polypeptide is folded into two globular lobes, representing its N- and C-terminal halves, commonly referred to as the N-lobe and the C-lobe. These two lobes are linked together by a short  $\alpha$ -helix peptide, and there is some 40% amino acid sequence identity between the N- and C-lobes.<sup>1–3</sup> The most important feature of lactoferrin is its very high affinity for iron; consequently, its biological functions with respect to its strong bacteriostatic properties depend on its iron-binding properties. The surface of the lactoferrin molecule has several regions with high concentrations of positive charge, giving it a high isoelectric point ( $pI \approx 9$ ). This positive charge is one of the features that distinguishes lactoferrin from other milk proteins, such as  $\beta$ -lactoglobulin, which have isoelectric points in the range 4.5–5.5 and are negatively charged at neutral pH. Consequently, lactoferrin has been shown to interact with other milk proteins. This interaction appears to have a certain degree of specificity, as it occurs with  $\beta$ -lactoglobulin but not with  $\alpha$ -lactalbumin.<sup>4</sup>

$\beta$ -Lactoglobulin is one of the major whey proteins, and it has a negative net charge at pH 7.0.  $\beta$ -Lactoglobulin possesses a considerable, ordered secondary and a compact tertiary structure. At neutral pH,  $\beta$ -lactoglobulin exists as a dimer held together by noncovalent interactions.  $\beta$ -

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Lactoglobulin is surface active<sup>5</sup> and has been shown to adsorb at the air–water interface to give a monolayer that is thinner (thickness is about 3 nm) and value of surface coverage is about  $1.7 \text{ mg m}^{-2}$  using neutron reflectivity measurements.<sup>6</sup> The adsorbed globular protein layer can probably be regarded as a pseudo-two-dimensional system of densely packed deformable particle.<sup>7</sup> This adsorbed globular protein layer may further interact with components at the environment by a combination of electrostatic, hydrophobic, and hydrogen bonds to form a multilayer.<sup>8</sup>

The adsorption of lactoferrin onto solid surfaces<sup>8, 9</sup> and at air–water interfaces<sup>10</sup> has been studied. Wahlgren et al.<sup>8</sup> showed that electrostatic interactions between lactoferrin and  $\beta$ -lactoglobulin caused an increase in the amount of protein adsorbed on a hydrophilic silica surface, as compared with single proteins. Neutron reflectivity measurements revealed a strong structural unfolding of the lactoferrin molecule when adsorbed at the air–water interface from a pH 7.0 buffer solution over a wide concentration range.<sup>10</sup> Two distinct regions, a top dense layer of 15–20 Å on the air side and a bottom diffuse layer of some 50 Å into the aqueous phase, characterized the unfolded interfacial layer.

We recently examined the adsorption behavior of lactoferrin in oil-in-water emulsions at pH 3.0 and 7.0.<sup>11</sup> It was shown that lactoferrin, like casein and whey proteins, is an excellent emulsifying agent but that cationic emulsion droplets can be formed with lactoferrin. For emulsions prepared under the same conditions, the droplet sizes in lactoferrin-stabilized emulsions were found to be similar to those in  $\beta$ -lactoglobulin-stabilized emulsions, but the surface protein coverage ( $\text{mg/m}^2$ ) was higher in lactoferrin emulsions, possibly because of the higher molecular weight of lactoferrin. The adsorption of lactoferrin from a binary mixture of lactoferrin and  $\beta$ -lactoglobulin at pH 7.0 was affected by electrostatic interactions, leading to higher amounts of adsorbed proteins at the droplet surface. Competitive adsorption was observed at pH 3.0, where both proteins carry net positive charges:  $\beta$ -Lactoglobulin was adsorbed in preference to lactoferrin in emulsions made using low protein concentrations ( $\leq 1\%$ ), whereas lactoferrin appeared to be adsorbed in preference to  $\beta$ -lactoglobulin in emulsions made using high protein concentrations.

The objectives of this study were to further investigate the interactions between lactoferrin and  $\beta$ -lactoglobulin in oil-in-water emulsions and to explore the possibility of making multilayered emulsions using these two proteins. Multilayered food emulsions, involving proteins, lecithin, and polysaccharides, have been used to improve the stability of oil-in-water emulsions to environmental stresses, such as pH extremes, high mineral contents, thermal processing, freezing, and drying.<sup>12</sup> In addition, it has also been used to engineer novel functional properties

into colloidal dispersions, such as the encapsulation of active ingredients, controlled or triggered release of active agents, controlled enzyme reactions, and so forth.<sup>13, 14</sup>

## Materials and methods

### Materials

Lactoferrin was a gift from Tatura Co-operative Dairy Company Limited, New Zealand. The powder contained 1.1% moisture and 99.7% protein, of which 92% was lactoferrin.  $\beta$ -Lactoglobulin was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Soya oil was purchased from Davis Trading Company, Palmerston North, New Zealand. All the chemicals used were of analytical grade and were obtained from either BDH Chemicals (BDH Ltd., Poole, England) or Sigma Chemical Co. unless specified otherwise.

### Emulsion preparation

Protein solutions (1.0 wt%) were prepared by dispersing lactoferrin and  $\beta$ -lactoglobulin powders into Milli-Q water (water purified by treatment with a Milli-Q apparatus, Millipore Corp., Bedford, MA, USA) and then stirring for 2 h at room temperature to ensure complete dispersion. The pH of the protein solution was adjusted to 7.0 using 1 M NaOH or 1 M HCl. Appropriate quantities of soya oil were then mixed with the protein solution to form the final emulsions containing 30 wt% soya oil and 70 wt% aqueous. The mixture of protein solution and soya oil was heated to 55 °C and was then homogenized in a two-stage homogenizer (APV 2000, Denmark) at a first-stage pressure of 25 MPa and a second-stage pressure of 4 MPa. The emulsions were homogenized twice for more effective mixing of the oil phase. The emulsions were stored at 20 °C. All emulsions were prepared at least in duplicate.

Emulsion samples (50 g) prepared using 1 wt% protein (lactoferrin or  $\beta$ -lactoglobulin) were mixed with 35 g aqueous solution containing different concentrations of protein (lactoferrin or  $\beta$ -lactoglobulin) and gently stirred (100 rpm) using a magnetic stirrer for 2 h at room temperature. The mixtures that contained 20 wt% oil were then adjusted to different pH values using 1 M NaOH or 1 M HCl. The emulsions and mixtures of emulsions and protein solutions were prepared in duplicate for subsequent determinations.

### Determination of average droplet size

A Malvern MasterSizer MSE (Malvern Instruments Ltd., Malvern, Worcestershire, UK) was used to determine the

average diameter of the emulsion droplets. The parameters that were used to analyze the droplet size distribution were defined by the presentation code 2NAD. The relative refractive index ( $N$ ), i.e., the ratio of the refractive index of the emulsion droplets (1.456) to that of the dispersion medium, water (1.33), was 1.095. The absorbance value of the emulsion droplets was 0.001. Droplet size measurements are reported as the Sauter-average diameter,  $d_{32}$  ( $=\sum n_i d_i^3 / \sum n_i d_i^2$  where  $n_i$  is the number of particles with diameter  $d_i$ ). Mean particle diameters were calculated as the average of duplicate measurements.

#### Determination of surface protein concentration and composition

The emulsions were centrifuged at  $45,000\times g$  for 40 min at  $20^\circ\text{C}$  in a temperature-controlled centrifuge (Sorvall RC5C, DuPont Co., Wilmington, DE, USA). The subnatants were carefully removed using a syringe. The cream layer was dispersed in deionized water and recentrifuged at  $45,000\times g$  for 40 min. The subnatant was filtered sequentially through 0.45 and  $0.22\ \mu\text{m}$  filters (Millipore Corp.). The filtrates were analyzed separately for total protein using the Kjeldahl method (1026 Distilling Unit and 1007 Digestor Block, Tecator AB, Hoganas, Sweden). The surface protein concentration ( $\text{mg}/\text{m}^2$ ) was calculated from the surface area of the oil droplets, determined by MasterSizer, and the difference in the amount of protein used to prepare the emulsion and that measured in the subnatant after centrifugation.

The composition of the protein adsorbed at the surface of the emulsion droplets was determined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), as described by Ye and Singh.<sup>15</sup> A certain amount of cream was spread on to a filter paper, and a known amount of dried cream was mixed with SDS buffer (0.5 M Tris, 2% SDS, 0.05% mercaptoethanol, pH 6.8). A portion ( $5\ \mu\text{l}$ ) of this dispersion was applied to the SDS gels previously prepared on a Miniprotean II system (Bio-Rad Laboratories, Richmond, CA, USA). After destaining, the gels were scanned on a laser densitometer (LKB Ultrosan XL, LKB Produkter AB, Bromma, Sweden). The percentage composition of each sample was determined by scanning the areas for lactoferrin and  $\beta$ -lactoglobulin and expressing the individual whey protein peaks as a fraction of the sum total.

#### Electrophoretic mobility and $\zeta$ -potential determination

The electrophoretic mobilities and hence the calculated  $\zeta$ -potentials of emulsion droplets were determined using a Malvern Zetasizer 4 instrument, the associated Malvern Multi-8 64 channel correlator, and the AZ4 standard electrophoresis cell, which incorporates a 4-mm diameter

quartz capillary (Malvern Instruments Ltd.). The temperature of the electrophoresis cell was maintained at  $25\pm 0.5^\circ\text{C}$  using a water jacket that was temperature controlled by the Peltier system. An applied voltage of 80 V (corresponding to a sensed voltage of approximately 60 V across the capillary, i.e., approximately 12 V/cm) and a modulation frequency of 250 Hz were used in all experiments. Emulsion ( $20\ \mu\text{l}$ ) was diluted with Milli-Q water (1 ml), and the pH of diluted sample was checked before determination of the  $\zeta$ -potentials. Mean  $\zeta$ -potentials were calculated as the average of duplicate measurements.

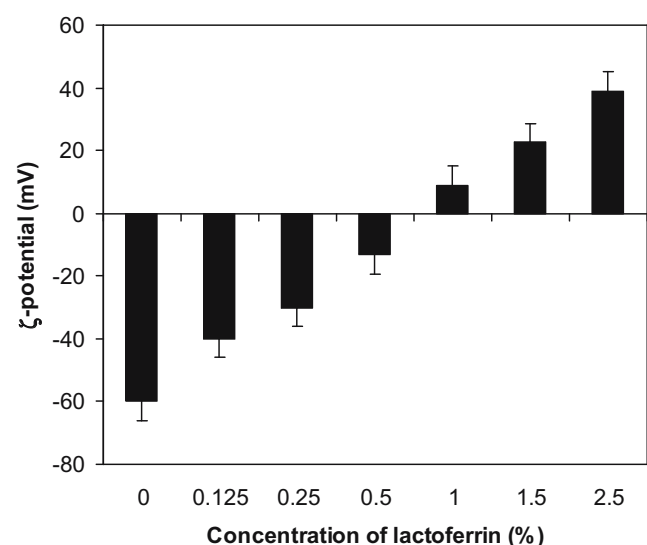
#### Statistical analysis

The results were analyzed statistically using the Minitab 12 for Windows package. Differences were considered to be significant at  $p\leq 0.05$ . Analysis of 12 separate emulsions, made with 1.0 wt% whey proteins and 30% soy oil, showed that the variations were  $\pm 0.02\ \mu\text{m}$  for  $d_{32}$ ,  $\sim 8\%$  for surface protein concentration, and  $\sim 4\%$  for  $\beta$ -lactoglobulin.

## Results and discussion

#### Addition of lactoferrin to $\beta$ -lactoglobulin-stabilized emulsions

The  $\zeta$ -potential values of emulsions ( $d_{32}=\sim 0.5\ \mu\text{m}$ ) formed with 1 wt%  $\beta$ -lactoglobulin at pH 7.0 and then diluted with aqueous phase containing a range of lactoferrin concentrations are shown in Figure 1. In the absence of lactoferrin, the  $\zeta$ -potential of the  $\beta$ -lactoglobulin-stabilized droplets (1 wt% protein, pH 7.0) was about  $-60\ \text{mV}$  (Figure 1),

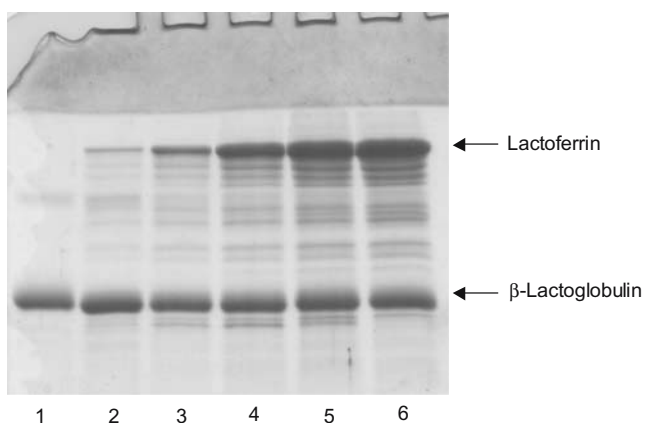


**Fig. 1.** Influence of addition of lactoferrin into emulsions formed with 1 wt%  $\beta$ -lactoglobulin (30 wt% soya oil, pH 7.0) on the  $\zeta$ -potential of emulsion droplets

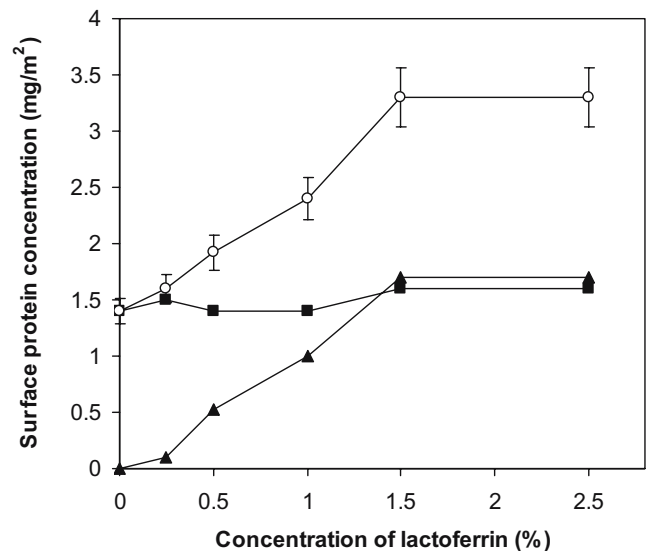
indicating that these emulsion droplets had a relatively high negative charge. The  $\zeta$ -potential became increasingly less negative and eventually changed from a negative value to a positive value, as the lactoferrin concentration in the emulsion was increased. A slightly positive value was obtained when the lactoferrin concentration was about 1.0 wt%; the  $\zeta$ -potential continued to become more positive with further increases in the lactoferrin concentration. This indicates that lactoferrin may adsorb on to the surface of  $\beta$ -lactoglobulin-coated emulsion droplets. The reversal of charge upon the adsorption of charged polymers on the surface of oppositely charged colloidal particles has been shown by many workers.<sup>8, 16</sup> This presumably occurs because only a fraction of the charged groups on the polymer is required to neutralize the oppositely charged groups on the particle surface, and the remaining charged groups may remain on the particle surface or may protrude into the aqueous phase.

Further evidence of lactoferrin adsorption onto the lactoferrin-stabilized droplets was obtained by SDS-PAGE analysis of the emulsion droplets; it was shown that the intensity of the lactoferrin band at the droplet surface increased with increasing lactoferrin concentration in the emulsion, whereas the intensity of  $\beta$ -lactoglobulin band remained essentially constant (Figure 2).

The surface protein coverage ( $\text{mg}/\text{m}^2$ ) of the emulsion droplets increased almost linearly from approximately 1.5 to 3.5  $\text{mg}/\text{m}^2$  with an increase in the lactoferrin concentration up to 1.5 wt%, but reached a plateau thereafter (Figure 3). Quantitative SDS-PAGE analysis of the emulsion droplets showed clearly that, with increasing lactoferrin concentration in the emulsion, the amount of the  $\beta$ -lactoglobulin at the surface remained essentially constant,



**Fig. 2.** SDS-PAGE protein patterns of adsorbed protein; lanes 2–7 show increasing additions of lactoferrin, whereas lane 1 represents emulsion made with 1%  $\beta$ -lactoglobulin alone, lane 2 1%  $\beta$ -lactoglobulin emulsion+0.25% lactoferrin, lane 3 1%  $\beta$ -lactoglobulin emulsion+0.50% lactoferrin, lane 4 1%  $\beta$ -lactoglobulin emulsion+1.0% lactoferrin, lane 5 1%  $\beta$ -lactoglobulin emulsion+1.50% lactoferrin, lane 6 1%  $\beta$ -lactoglobulin emulsion+2.5% lactoferrin)

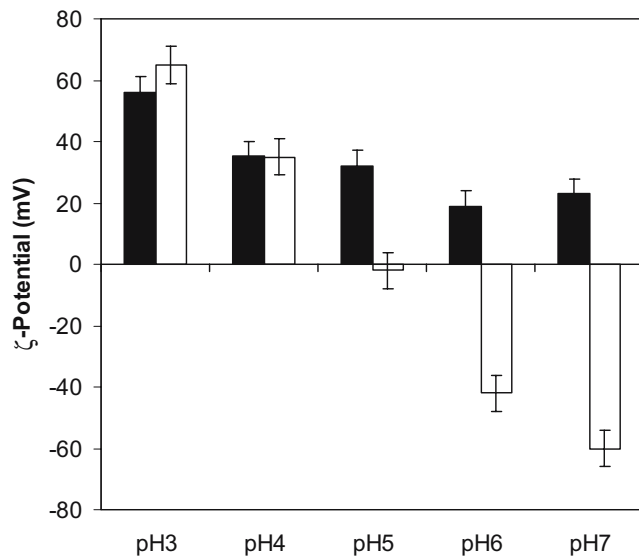


**Fig. 3.** Changes in surface protein coverage ( $\text{mg}/\text{m}^2$ ) of emulsion droplets made with 300 wt% soya oil and 1 wt%  $\beta$ -lactoglobulin (pH 7.0) as a function of added lactoferrin. Total surface protein coverage (open circle), surface  $\beta$ -lactoglobulin concentration (filled square), and surface lactoferrin concentration (filled triangle)

whereas the amount of lactoferrin at the surface became greater with increasing lactoferrin concentration up to 1.5 wt%, which is consistent with the surface protein coverage results (Figure 3). These results suggest that the increase in the total surface protein concentration upon addition of lactoferrin was because of the adsorption of lactoferrin on the droplet surface of  $\beta$ -lactoglobulin-stabilized emulsions. At lactoferrin concentration  $>1.5\%$ , the surface concentrations of lactoferrin were almost equal to the surface concentrations of  $\beta$ -lactoglobulin.

Particle size and creaming stability measurements showed that all emulsions were stable to droplet aggregation, as there appeared to be no significant changes in the  $d_{32}$  values or the creaming stability with increasing lactoferrin concentration in the emulsion (data not shown). It is interesting to note that some emulsions with very low or almost no overall charge were still stable to aggregation, indicating that the stability of these emulsions is not determined by charge, but possibly by steric interactions.

Emulsions were formed with 1%  $\beta$ -lactoglobulin, mixed with 1.5% lactoferrin, and adjusted to a range of pH values (7.0 to 3.0). The  $\zeta$ -potential of the  $\beta$ -lactoglobulin-stabilized emulsion increased from a strong negative (approximately  $-60$  mV) to a positive (approximately  $+20$  mV) value with the addition of 1.5 wt% lactoferrin because of the adsorption of lactoferrin at the surface at pH 7.0. When the pH of emulsions was decreased from pH 7.0 to 3.0, the  $\zeta$ -potentials of emulsions became more positive (Figure 4) as expected. At  $\text{pH} \geq 5.0$ , the overall positive charge of the emulsion droplets was due mainly to the adsorption of the positively charged lactoferrin at the droplet surface. However, at  $\text{pH} < 5.0$  (below the  $\text{pI}$  of  $\beta$ -



**Fig. 4.** Influence of pH on the  $\zeta$ -potential of 1 wt%  $\beta$ -lactoglobulin-stabilized emulsion droplets (*empty*) and emulsions with 1.5 wt% added lactoferrin (*solid*)

lactoglobulin),  $\beta$ -lactoglobulin-coated emulsion droplets had an overall positive charge (Figure 4).

SDS-PAGE of the surface proteins (Figure 5a) showed that the intensity of lactoferrin was lower at the sample of pH 5.0 compared with the samples at pH 6.0 or 7.0. No clear lactoferrin band was observed in samples at pH 4.0 and 3.0. However, the intensity of  $\beta$ -lactoglobulin band remained almost constant in the pH range 3.0 to 7.0. Further quantitative analysis on the SDS-PAGE showed that the concentration of lactoferrin at the droplet surface was higher at pH 6.0 than at pH 7.0 (Figure 5b), but it decreased at pH 5.0, and only a small amount of lactoferrin was observed at the surface of emulsion droplets at pH 4.0. Furthermore, no lactoferrin was able to bind to the surface of  $\beta$ -lactoglobulin stabilized emulsion droplets at pH 3.0 (Figure 5b).

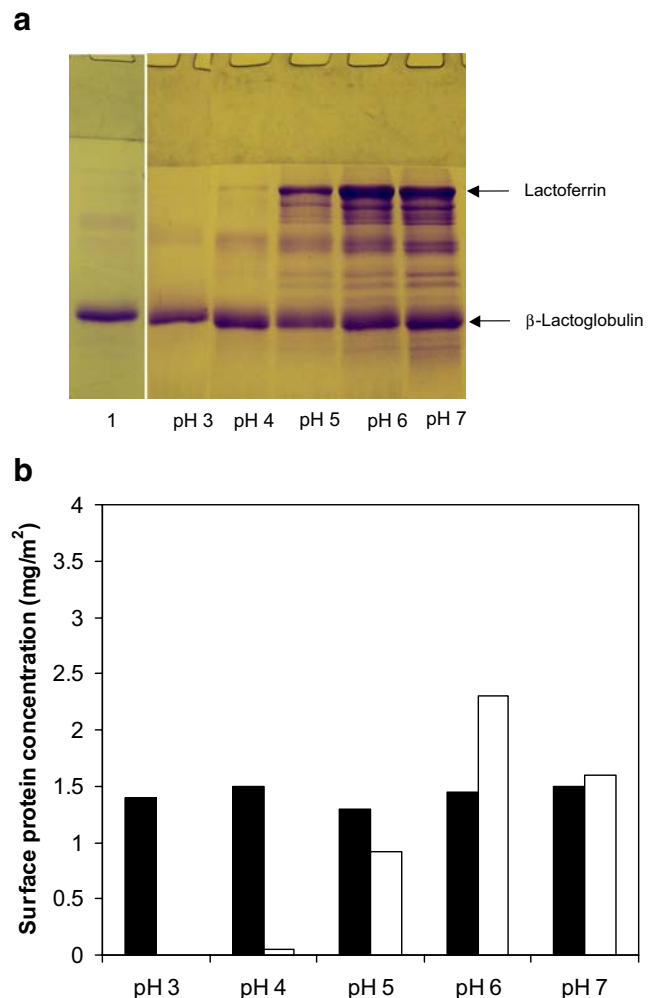
At pH 6.0, lactoferrin has a higher positive charge density than that at pH 7.0 ( $pI \sim 9.0$ ), which may increase its binding with  $\beta$ -lactoglobulin at the droplet surface. However, at pH 5.0, because of a lower negative charge density on  $\beta$ -lactoglobulin, the binding of lactoferrin may be reduced. When pH of emulsions decreased to lower than  $pI$  of  $\beta$ -lactoglobulin, the positively charged lactoferrin could not interact with the positively charged  $\beta$ -lactoglobulin-coated droplet surface because of electrostatic repulsions.

#### Addition of $\beta$ -lactoglobulin to lactoferrin-stabilized emulsions

In the absence of  $\beta$ -lactoglobulin, the  $\zeta$ -potential of the emulsion droplets formed by lactoferrin was around +33 mV (Figure 6), because the lactoferrin used to stabilize the droplets has a net positive charge at pH 7.0, as shown above. The  $\zeta$ -potential became less positive and eventually changed

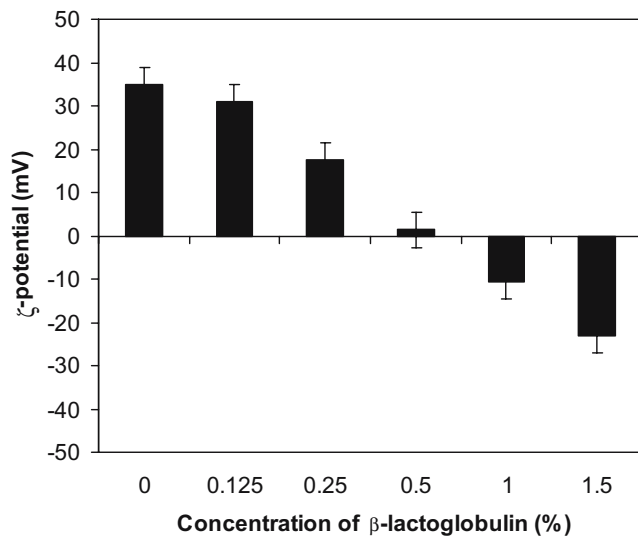
from positive to negative, as the  $\beta$ -lactoglobulin concentration in the emulsion was increased. This change suggests that  $\beta$ -lactoglobulin, which has a net negative charge at pH 7.0, adsorbed to the surface of lactoferrin-coated emulsion droplets. The  $\zeta$ -potential was very close to zero (i.e., no net charge on the droplet surface) at 0.50 wt% addition of  $\beta$ -lactoglobulin. Further increases in the  $\beta$ -lactoglobulin concentration resulted in negative values of the  $\zeta$ -potential.

The changes in  $\zeta$ -potential were also reflected in measurements of the surface protein concentration ( $\text{mg protein/m}^2$ ), which showed an increase in adsorbed protein up to 0.5 wt% added  $\beta$ -lactoglobulin, but no further change above this concentration (Figure 7). Further evidence of  $\beta$ -lactoglobulin adsorption on to the lactoferrin-stabilized droplets was obtained by SDS-PAGE analysis of the



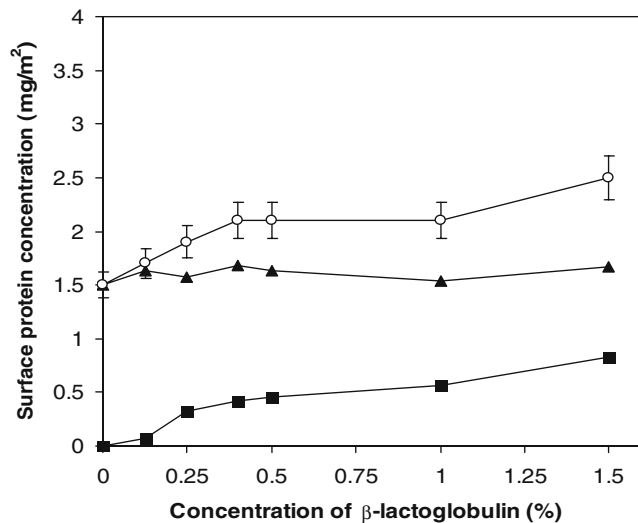
**Fig. 5.** SDS-PAGE protein patterns (a) of adsorbed protein and surface protein concentration ( $\text{mg/m}^2$ ) (b) of the emulsion droplets formed with 1 wt%  $\beta$ -lactoglobulin with 1.5 wt% added lactoferrin at different pH; lane 1 represents emulsion made with 1%  $\beta$ -lactoglobulin alone at pH 7.0. Surface lactoferrin (*empty*) or  $\beta$ -lactoferrin (*solid*) concentrations



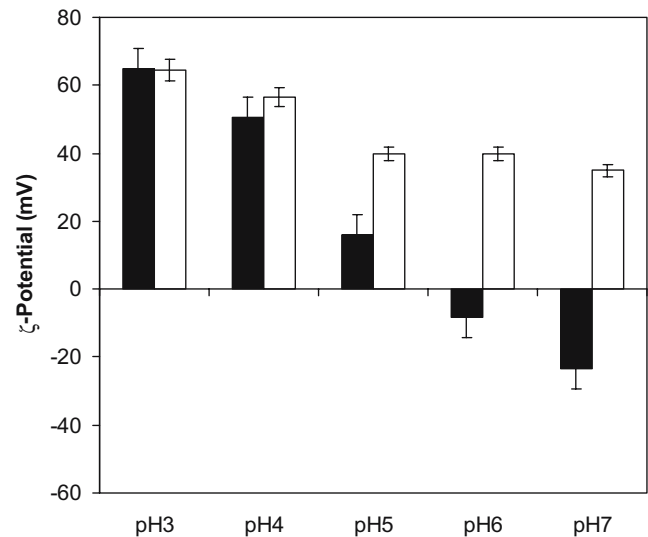


**Fig. 6.** Influence of addition of  $\beta$ -lactoglobulin into emulsions formed with 1 wt% lactoferrin (30 wt% soya oil, pH 7.0) on the  $\zeta$ -potential of emulsion droplets

emulsion droplets; it was shown that the concentration of the  $\beta$ -lactoglobulin at the droplet surface increased with increasing  $\beta$ -lactoglobulin concentration in the emulsion, whereas the concentration of the lactoferrin remained essentially constant (Figure 7). The adsorption of  $\beta$ -lactoglobulin appeared to increase considerably with an increase in the  $\beta$ -lactoglobulin concentration up to 0.5 wt %, with very little change above this concentration. It indicated that the increase in the total surface protein concentration with addition of  $\beta$ -lactoglobulin was because of the adsorption of  $\beta$ -lactoglobulin on the droplet surface of lactoferrin-stabilized emulsions.



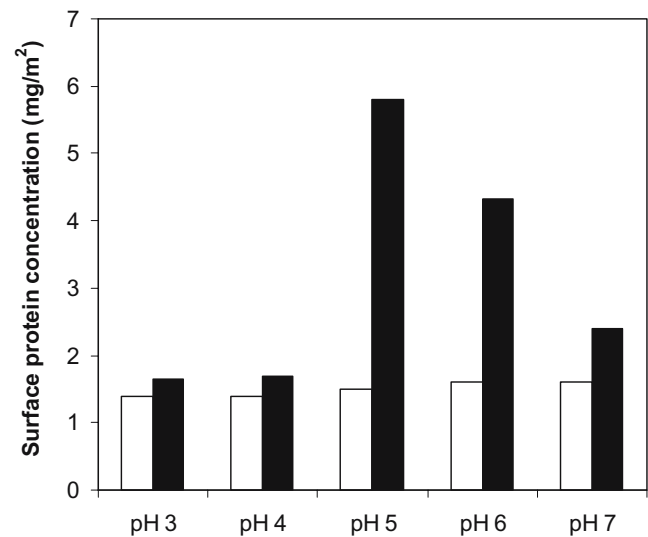
**Fig. 7.** Changes in surface protein coverage (mg/m<sup>2</sup>) of emulsion droplets made with 30 wt% soya oil and 1 wt% lactoferrin (pH 7.0) as a function of added  $\beta$ -lactoglobulin. Total surface protein coverage (open circle), surface  $\beta$ -lactoglobulin concentration (filled square), and surface lactoferrin concentration (filled triangle)



**Fig. 8.** Influence of pH on the  $\zeta$ -potential of 1 wt% lactoferrin-stabilized emulsion droplets (empty) and emulsions with 1.5 wt% added  $\beta$ -lactoglobulin (solid)

There were no significant changes in the  $d_{32}$  values or the creaming stability with an increase in the  $\beta$ -lactoglobulin concentration in the emulsion (data not shown), suggesting that these emulsions remained very stable despite major changes in the net charges of the droplets. There was no evidence of droplet aggregation, even at 0.50 wt%  $\beta$ -lactoglobulin addition, which corresponded to the emulsions where almost complete charge neutralization had occurred ( $\zeta$ -potential was close to zero). This suggests that steric repulsion may play a more important role than the electrostatic interactions in the stabilization of these emulsions.

The  $\zeta$ -potential of the lactoferrin-stabilized emulsion decreased from positive (approximately +30 mV) to



**Fig. 9.** Surface protein concentration (mg/m<sup>2</sup>) of emulsion droplets made with 1 wt% lactoferrin emulsion droplets with 1.5 wt % added  $\beta$ -lactoglobulin at different pH. Surface lactoferrin (empty) or  $\beta$ -lactoglobulin (solid) concentrations

negative (approximately  $-25$  mV) with the addition of  $\beta$ -lactoglobulin because of the adsorption of  $\beta$ -lactoglobulin at the surface at pH 7.0 (Figure 8). When the pH of emulsions gradually decreased from 7.0 to 3.0, the  $\zeta$ -potential increased and became positive at  $\text{pH} \leq 5.0$  (Figure 8). At pH 3 and 4, the  $\zeta$ -potential of the lactoferrin-stabilized emulsions with added  $\beta$ -lactoglobulin were very close to that of emulsions formed with lactoferrin alone at same pH (Figure 8). The changes in the  $\zeta$ -potential with change in the pH of emulsions may suggest that the surface protein composition of the emulsion droplets has been altered with the change in the pH.

The protein composition of droplet surface obtained from the SDS-PAGE analysis showed that the concentration of  $\beta$ -lactoglobulin at the droplet surface increased to reach a maximum with a decrease in pH from 7.0 to 5.0, but its concentration decreased markedly with a further decrease in pH to 3.0 (Figure 9). As stated earlier, the binding of  $\beta$ -lactoglobulin to lactoferrin-coated emulsion droplets at neutral pH occurs because of electrostatic interactions between the positively charged lactoferrin and negatively charged  $\beta$ -lactoglobulin. At pH 5.0 and 6.0, lactoferrin has a higher positive charge density than that at pH 7.0, which may lead to greater binding of  $\beta$ -lactoglobulin onto the droplet surface, although  $\beta$ -lactoglobulin has a lower negative charge density at pH 5 and 6. Furthermore,  $\beta$ -lactoglobulin has been shown to form octamers at  $\text{pH} \sim 5.0$  with a maximum at pH 4.5 (17); this behavior may lead to the binding of  $\beta$ -lactoglobulin octamers at  $\text{pH} \sim 5.0$  and hence largely increase the amount of  $\beta$ -lactoglobulin at the droplet surface.

However, it is not clear how  $\beta$ -lactoglobulin adsorbed to the surface of lactoferrin-coated droplets at pH 3 and 4, where both  $\beta$ -lactoglobulin and lactoferrin carry overall positive charges. A possible explanation is that  $\beta$ -lactoglobulin displaced some of the adsorbed lactoferrin from the surface, but the data shown in Figure 9 indicates no significant changes in adsorbed lactoferrin at low pH. It has been reported that the  $\beta$ -lactoglobulin and lactoferrin competitively adsorbed onto the surface of oil droplet in the different concentrations at pH 3.0.<sup>11</sup> Alternatively, a large conformational change that occurs in lactoferrin<sup>18</sup> at low pH that causes a release of bound iron may expose some negatively charged patches or hydrophobic residues on the droplet surface, allowing further interactions with  $\beta$ -lactoglobulin. However, this effect does not seem to occur when  $\beta$ -lactoglobulin stabilized emulsions are mixed with lactoferrin.

These results show that multilayered emulsions can be produced by interactions of oppositely charged proteins at neutral pH. A primary emulsion containing either anionic droplets, coated with  $\beta$ -lactoglobulin, or cationic droplets, coated with lactoferrin, can be produced. A secondary emulsion can then be made by mixing either  $\beta$ -lactoglobulin solution or lactoferrin solution with the primary

emulsion. The amount of protein required to saturate the droplet surface in these emulsion systems can be estimated, as described by McClements<sup>12</sup>:

$$C_{\text{sat}} = \frac{3\phi\Gamma_{\text{sat}}}{r_{32}}$$

where  $\phi$  is the volume fraction of the particles,  $r_{32}$  is the volume-surface mean radius of the droplets (in meters),  $\Gamma_{\text{sat}}$  is the surface protein coverage at saturation (in  $\text{kg m}^{-2}$ ), and  $C_{\text{sat}}$  is the minimum concentration of protein in the whole system that is required to saturate the surfaces (in  $\text{kg m}^{-3}$ ). This is based on the assumption that, below the saturation concentration ( $C < C_{\text{sat}}$ ), all of the protein added to the system is adsorbed to the droplet surfaces.

Based on this equation, a minimum concentration of  $\approx 8.8 \text{ kg m}^{-3}$  of  $\beta$ -lactoglobulin would be required to saturate the surfaces of the emulsion droplets coated with lactoferrin. Similarly, a minimum concentration of  $\approx 14 \text{ kg m}^{-3}$  of lactoferrin would be needed to saturate the surfaces of the emulsion droplets formed with  $\beta$ -lactoglobulin.

Oil-in-water emulsions containing oil droplets surrounded by multilayered interfacial layers consisting of low molecular weight emulsifiers (SDS or lecithin) and biopolymers (gelatin, chitosan, pectin) have been studied extensively.<sup>13, 19, 20</sup> These systems tend to show extensive bridging flocculation upon charge neutralization, as the biopolymers with an opposite charge from that of the droplets are added to the emulsion. In contrast, the multilayered emulsion droplets formed using  $\beta$ -lactoglobulin and lactoferrin appear to be stable to droplet aggregation even at very low net electrical charges, presumably because of strong steric repulsion associated with protein interfacial layers. The pH of system will be an important factor to influence the formation of multilayered emulsion based on the electrostatic interaction between the opposite charge of  $\beta$ -lactoglobulin and lactoferrin.

Further work is underway to determine the behavior of these emulsions under different environmental conditions, such as temperature and ionic strength.

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