International Dairy Journal 99 (2019) 104542

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

The effect of dephosphorylation and succinylation on the properties of casein proteins adsorbed to the surface of latex particles

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ARTICLE INFO

Article history:
Received 17 April 2019
Received in revised form
27 June 2019
Accepted 27 June 2019
Available online 7 August 2019

ABSTRACT

Dephosphorylation decreased the absolute zeta potential and diameters of α_{S1}-casein-coated latex particles. Similar effects were observed for α_{S1}-casein dephosphorylated prior to or after adsorption. If α_{S1}-casein adopts a loop arrangement at hydrophobic surfaces, then it appears that dephosphorylation causes the loop to move closer to the surface. Dephosphorylation caused a larger change for particles coated with α_{S1}-casein than β-casein, whereas small changes were observed for κ-casein, consistent with the phosphorylation levels of these caseins. Succinylation increased the absolute value of zeta potential and the diameters of α_{S1}-casein-coated latex particles. A higher level of succinylated α_{S1}-casein was required to saturate the particle surface. It is proposed that succinylated α_{S1}-casein has the C-terminal region adsorbed to the particle surface and N-terminal region extending from the surface as a hair. Results of this study help explain the effect of dephosphorylation and succinylation on the functional properties of the caseins, particularly in stabilising emulsions.

1. Introduction

Casein has four gene products: α_{S1}-casein, α_{S2}-casein, β-casein and κ-casein, and they account for approximately 35%, 15%, 35% and 15% of whole casein, respectively (Farrell et al., 2004; Swaisgood, 2003). All the casein proteins have high levels of the amino acid proline and this amino acid is relatively uniformly distributed along the protein chains, which decreases the flexibility of the protein chain and diminishes the ability of the casein proteins to form large regions of α-helix and β-sheet structures (Fox, 2003; Swaisgood, 2003). Each of the caseins also has an amphipathic nature due to the non-uniform distribution of its hydrophobic and hydrophilic/charged amino acid residues (Farrell et al., 2004; Horne, 1998; Swaisgood, 2003).

Sodium caseinate (the sodium salt of whole casein) and the sodium salts of the individual caseins have exceptional functional properties, especially as foaming and emulsifying agents, and for this reason casein proteins are widely used in various food formulations (Dickinson, 2003; Kinsella, 1984). The properties of the caseins can be modified through physical, chemical and enzymatic processes, which also influence their functional properties (Broyard & Gaucheron, 2015; Smithers, Bradford, Regester, & Pearce, 1991).

One type of enzymatic modification of casein is dephosphorylation, where the phosphate groups are removed from the serine amino acid residues using phosphatase enzymes. Acid phosphatase has shown residual proteolytic activity that can result in the formation of peptides (Darewicz, Dziuba, & Minkiewicz, 2005; McCarthy, Kelly, O'Mahony, & Fenelon, 2013). In contrast, no proteolysis was observed with alkaline phosphatase, therefore, alkaline phosphatase has been commonly used for the dephosphorylation of milk proteins (Darewicz, Dziuba, Caessens, & Gruppen, 2000; Darewicz et al., 2005; Koudelka, Hoffmann, & Carver, 2009).

The phosphate groups carry a negative charge and therefore dephosphorylation can decrease the net negative charge and increase the isoelectric pH of the caseins (Bingham, 1976; Darewicz et al., 2005; McCarthy et al., 2013). This change in the charge distribution of the caseins also affects their self-association behaviour

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https://doi.org/10.1016/j.idairyj.2019.104542
0958-6946/

(Pepper & Thompson, 1963; Yoshikawa, Tamaki, Sugimoto, & Chiba, 1974). As the phosphoserine residues can bind high levels of calcium, dephosphorylation markedly decreases the calcium ion binding ability of the caseins, as well as increasing their relative hydrophobicity and solubility at acidic pH (Bingham, 1976; McCarthy et al., 2013).

Consequently, dephosphorylation can change the stability of emulsions prepared using the casein proteins, although contrasting effects have been reported (Husband, Wilde, Mackie, & Garrood, 1997; Lorenzen & Reimerdes, 1992; McCarthy et al., 2013). In one study, emulsions prepared from completely dephosphorylated α_S-casein or β-casein had substantially better creaming stability compared with emulsions prepared using the native proteins (Lorenzen & Reimerdes, 1992). In contrast, Darewicz et al. (2000) reported that dephosphorylation of β-casein did not significantly affect the emulsifying properties of the protein, whereas Husband et al. (1997) reported a decrease in stability for emulsions prepared from dephosphorylated β-casein when compared with the native protein. Darewicz et al. (2005) reported that the volume and stability of foams formed from native and dephosphorylated β-casein were similar whereas Husband et al. (1997) reported that the foaming ability of β-casein was improved by dephosphorylation although the foam stability was unchanged.

A common chemical modification of food proteins is succinylation using succinic anhydride, which converts the lysine amino acid residues into succinyl-lysine groups. This modification removes one positive charge and adds one negative charge for each lysine that is converted to a succinyl-lysine group (Hoagland, 1966; Means & Feeney, 1998; Schwenke & Rauschal, 1980; Strange, Holsinger, & Kleyn, 1993). As a consequence, succinylated casein has an increased net negative charge and there is a shift of the isoelectric point to lower pH values (Vidal, Marchesseau, Lagaude, & Cuq, 1998).

Shilpashree Arora, Chawla, Vakkalagadda, and Sharma (2015) reported that succinylation decreased the surface hydrophobicity of sodium caseinate. Yang et al. (2014) and Shilpashree, Arora, Chawla, Vakkalagadda, and Sharma (2015) showed that succinylation increased the solubility, viscosity and emulsifying activity of sodium caseinate, whereas the ability of sodium caseinate to stabilise foams was reported to be unchanged (Shilpashree et al., 2015), decreased (Yang et al., 2014) or increased (Kim, Choi, & Hong, 1997) on succinylation. Succinylated caseins were more soluble especially at acidic pH than native caseins (Kim et al., 1997; Shilpashree et al., 2015). In addition, succinylated (and acylated) caseins were less sensitive to calcium ions (Hoagland, 1966,1968).

The dephosphorylation and succinylation of casein proteins affect their functional properties, especially their properties when adsorbed to oil droplet interfaces when stabilising emulsions. The modified charge of casein proteins may affect the thickness of the adsorbed layers, the surface charge (zeta potential) of adsorbed layers and/or the level of protein adsorbed at the interface. Therefore, to understand the effect of dephosphorylation and succinylation on the protein layers at an interface, the properties of latex particles with dephosphorylated or succinylated casein adsorbed to the surface were investigated. Most experiments were conducted with α_{S1}-casein, although a few of the dephosphorylation experiments were also done using β-casein and κ-casein. The experiments were conducted in two ways: in one set of experiments, the casein was first dephosphorylated or succinylated and then progressively bound to the latex particles, with the size change and zeta potential monitored during adsorption. In the second set of experiments, the casein was first bound to the latex particles and the dephosphorylation or succinylation was performed on the coated particles, with the size change and zeta potentials monitored during dephosphorylation or after succinylation. From the changes in size and zeta potential, some information on the level of protein adsorbed and the nature of the adsorbed layers was determined.

2. Materials and methods

2.1. Latex particles solution

Polystyrene latex particles with nominal diameters of 60 and 100 nm were obtained from the Duke Scientific Corp. (3060A and 3100A, Nanospheres™, Palo Alto, CA, USA). The polystyrene latex particles were supplied as aqueous solutions of 1% total solids. Latex particle solutions were prepared by adding 50 μL of the 60 or 100 nm latex particles to 5 mL of imidazole buffer (20 mM imidazole, pH 7.0) to give latex particle solutions with a concentration of 0.01%.

2.2. Casein protein solutions

Bovine α_S-casein (C6780), β-casein (C6905) and κ-casein (C0406) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Electrophoresis indicated that the α_S-casein was predominantly α_{S1}-casein with low levels of α_{S2}-casein, the β-casein was essentially pure, and the κ-casein had low levels (<5% combined) of α_{S1}-casein and β-casein present (results not shown). For the purpose of this study and to simplify the discussion, the α_S-casein will be referred to as α_{S1}-casein as this was the predominant casein in this sample. The casein proteins were dissolved in Milli-Q water to ~10 mg mL^{-1} and the pH was adjusted to ~ pH 6.55. Each protein solution was filtered (0.4 μm syringe filters) and the protein concentration determined using UV absorption at 280 nm and the known extinction coefficients for each casein (Farrell et al., 2004). Working solutions were prepared by mixing the casein solutions with imidazole buffer (20 mM, pH 7) at a 1:1 ratio.

2.3. Preparation of latex particles coated with native caseins

The α_{S1}-casein, β-casein or κ-casein solutions were titrated into the 0.01% latex solutions (5 mL) and the level of casein added was monitored. After each aliquot of casein was added, the sample was gently mixed and then the size and zeta potential were measured at 20 °C before the next aliquot of casein was added.

2.4. Preparation of latex particles coated with dephosphorylated casein

The effect of dephosphorylation on the casein adsorbed to latex particles was studied in two ways. The caseins were either dephosphorylated prior to adsorption onto the latex particles (α_{S1}-casein only), or the native casein was adsorbed to the latex particles and then dephosphorylated in-situ (α_{S1}-casein, β-casein, and κ-casein).

The alkaline phosphatase used was from bovine intestinal mucosa and supplied by Sigma–Aldrich. The completely dephosphorylated α_{S1}-casein was prepared by adding alkaline phosphatase to the α_{S1}-casein solution at a level of 0.25 U of phosphatase per mg α_{S1}-casein, and then incubated at 37 °C for 24 h. The latex particle solutions (5 mL) were titrated with fully dephosphorylated α_{S1}-casein at 20 °C. After each aliquot of fully dephosphorylated α_{S1}-casein was added, the sample was gently mixed and then the size and zeta potential were measured at 20 °C before the next aliquot of casein was added.

For the in situ dephosphorylation of the casein-coated latex particles, the latex particle solutions were first fully coated with native α_{S1}-casein, β-casein, or κ-casein. A centrifugation and re-

suspension procedure was used to remove the excess native casein from the latex particle solutions. The casein-coated particles were centrifuged at ~21,000 X *g* at 25 °C for 20 min. After centrifugation, the supernatant was removed and the sedimented latex particles were re-suspended in imidazole buffer to the same volume as originally present. This centrifugation and re-suspension step was repeated three times. After the centrifugation and re-suspension procedure, the casein-coated latex particle solutions (5 mL) were incubated with different levels of alkaline phosphatase at 37 °C for up to 12 h and the change in size during the in situ dephosphorylation was monitored.

2.5. Preparation of latex particles coated with succinylated α_{S1}-casein

The latex particles were coated with succinylated α_{S1}-casein in two ways. The α_{S1}-casein was either fully succinylated prior to adsorption onto the latex particles, or the native α_{S1}-casein was adsorbed to the latex particles and then the protein was succinylated in-situ.

Fully succinylated α_{S1}-casein was prepared by adding succinic anhydride (Sigma–Aldrich) to the α_{S1}-casein solution at a level of 0.5 mg succinic anhydride per mg of α_{S1}-casein, and then mixed at 20 °C for 3 h. The fully succinylated α_{S1}-casein was titrated into the latex particle solutions (5 mL) at 20 °C. After each aliquot of fully succinylated α_{S1}-casein was added, the sample was gently mixed and then the size and zeta potential were measured at 20 °C before the next aliquot of casein was added.

For the in situ succinylation of the α_{S1}-casein-coated latex particles, the latex particle solutions were first fully coated with native α_{S1}-casein. The excess native α_{S1}-casein was removed from the latex particles using the centrifugation/re-suspension method described above. After the centrifugation and re-suspension procedure, the succinic anhydride (0.4 mg of succinic anhydride per mg α_{S1}-casein, based on the level of casein added to fully coat the latex, which was an excess of that required to fully succinylate all lysine groups) was added into the coated latex particle solutions and the change in size and zeta potential after the in situ succinylation was monitored.

2.6. Particle size and zeta potential measurements

The size of the bare and casein-coated latex particles were measured by dynamic light scattering (DLS) using a Malvern Zetasizer Nano-ZS (Malvern Instruments Ltd, Malvern, Worchestershire, UK). The measurements of the dynamics of the scattered light were collected at a scattering angle of 173°. The temperature of the samples was maintained at 20 °C (or 37 °C for the in situ dephosphorylation experiments) for the duration of the experiments. The dispersant (imidazole buffer) was considered to have the properties of water with a refractive index of 1.330 and viscosity of 1.0031 cP at 20 °C (Anema & Klostermeyer, 1997). The average diffusion coefficients were determined using the cumulants method and converted to average particle diameters using the Stokes–Einstein relationship for spheres (Anema & Li, 2003).

The zeta potential of the latex particles were also determined using the Zetasizer Nano-ZS instrument using the disposable folded capillary cells and the techniques described previously (Anema & de Kruif, 2012; Anema & Klostermeyer, 1996). An applied voltage of 50 V was used and the temperature was maintained at 20 °C.

3. Results

Latex particles are negatively charged and have hydrophobic surfaces. Several studies have used latex particle surfaces as a model for the emulsion interface to study protein adsorption onto hydrophobic surfaces. Proteins such as the caseins will spontaneously adsorb onto the surface of latex particles and the proteins are orientated with their hydrophobic regions associating with the particle surface and the hydrophilic regions protruding into the serum phase (Anema, 1997; Brooksbank, Davidson, Horne, & Leaver, 1993; Dalgleish, 1990,1993; Horne & Leaver, 1995; Leaver, Brooksbank, & Horne, 1994; Leaver & Horne, 1996; Lee, Martic, & Tan, 1989; Nakanishi, Sakiyama, & Imamura, 2001; Young & Shoemaker, 1990).

A study comparing the trypsin-induced hydrolysis pattern of individual caseins adsorbed to latex particles and those in casein stabilised oil-in-water emulsion systems produced almost identical hydrolysis patterns, and when additional casein was added to the hydrolysed system, an almost identical increase in adsorbed layers was observed. This indicates that the adsorption behaviour of the caseins to latex particles was similar to those at the surface of casein stabilised emulsions (Dalgleish, 1993). A monolayer of casein adsorbs to the surface of the latex particles (Mackie, Mingins, & North, 1991), as is observed when casein proteins adsorb at the oil/water interface in emulsion systems (Chen, Dickinson, & Iveson, 1993; Dalgleish, 1996; Dickinson, 1994; Fang & Dalgleish, 1993). Thus, latex particles offer a stable hydrophobic surface to study the effects of dephosphorylation and succinylation on the adsorbed layers of caseins at interfaces.

To ensure changes were due to adsorbed layers and not to other effects (such as a bridging flocculation of some particles) latex particles of different diameters were used as similar effects should be observed regardless of the latex particle diameter. The sizes of the bare 60 nm and 100 nm latex particles as measured by DLS were 64 ± 1 nm and 105 ± 1 nm respectively, which is larger than the certified diameters. This is a consequence of the DLS measuring hydrodynamic size which is larger than the certified size that is obtained by electron microscopy. Due to the different sized latex particles the results will be presented as a change in diameter.

3.1. Adsorption of native casein proteins on latex particles

Samples of α_{S1}-casein, β-casein and κ-casein were titrated into the latex particle solutions and the change in particle size was monitored during the titration (Fig. 1). For each casein protein, the diameters of the latex particles increased with increasing levels of added casein, and then the change in the diameter plateaued when the amount of added caseins was sufficient to fully cover the surface of the latex particles. A similar behaviour was observed for both the 60 nm and 100 nm diameter latex particles. A lower level of β-casein was required for saturation coverage than κ-casein or α_{S1}-casein, whereas, κ-casein and α_{S1}-casein had similar saturation coverage levels (Fig. 1). This is consistent with the fact that β-casein has a higher hydrophobicity than κ-casein and α_{S1}-casein due to its large hydrophobic C-terminal region (Kumosinski, Brown, & Farrell, 1993; Swaisgood, 2003), thus, a lower level of β-casein would be required to provide sufficient hydrophobic regions that fully cover the latex surface compared with κ-casein and α_{S1}-casein.

In both 60 and 100 nm diameter latex samples, at the saturation point the addition of α_{S1}-casein, β-casein, and κ-casein were found to increase the diameter of the particles by about 21 nm, 25 nm, and 23 nm respectively (Fig. 1). This is consistent with previous studies as it has been reported that the addition of α_{S1}-casein to latex particles increased their diameter by about 21 nm (Dalgleish, 1990), β-casein increased their diameters by about 26–30 nm (Brooksbank et al., 1993; Dalgleish, 1990,1993), and κ-casein

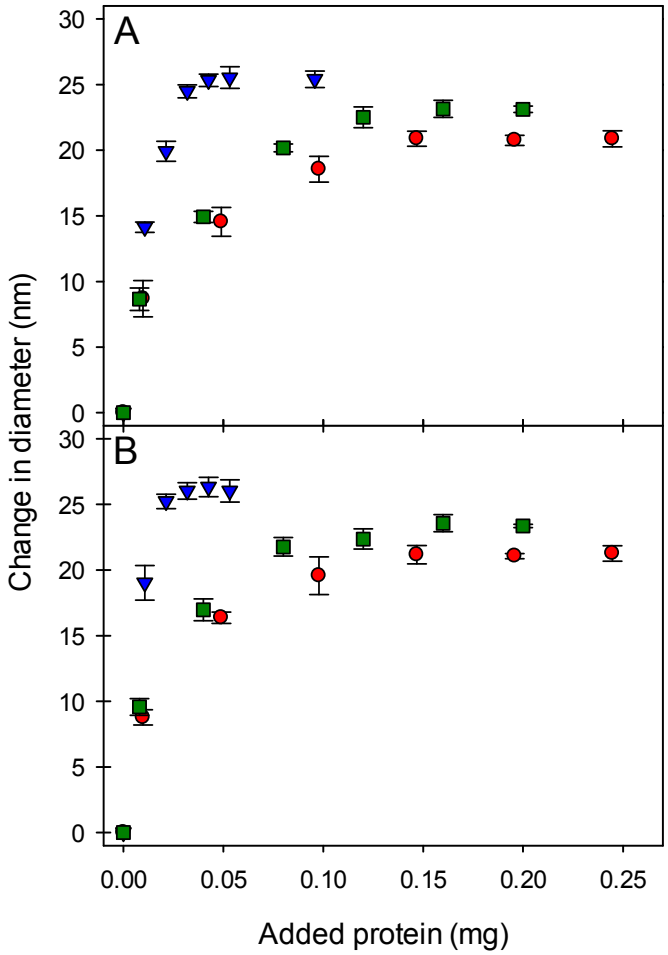

Fig. 1. The change in the diameters of 60 nm (A) and 100 nm (B) latex particles with added native α_{S1}-casein (●), β-casein (▼), and κ-casein (■). The addition levels are reported as mg of casein per 5 mL of 0.01% latex solution.

increased their diameters by about 25 nm (Anema, 1997; Leaver et al., 1994; Leaver & Horne, 1996).

3.2. Adsorption of fully dephosphorylated α_{S1}-casein on latex particles

Fully dephosphorylated α_{S1}-casein was prepared by adding alkaline phosphatase to the casein solution and allowing the reaction to go to completion. The degree of dephosphorylation was confirmed by electrophoresis and mass spectrometry (results not shown). For both the 60 and 100 nm latex samples, the addition of dephosphorylated α_{S1}-casein increased the diameter of latex particles and at saturation, the diameter increased by about 15–16 nm, which was less than the ~20 nm increase in size observed when native α_{S1}-casein was adsorbed onto the latex particles (Fig. 2A,B). The point at which saturation occurred was similar for both the native and dephosphorylated α_{S1}-casein indicating that similar levels adsorbed to the latex surface. These results show that, at saturation, dephosphorylation produced a thinner protein layer (the diameter decreased by about 4–6 nm, thus the layer was 2–3 nm) on the latex surface.

The 60 and 100 nm latex particles had zeta potentials of −52 and −56 mV, respectively (Fig. 2C,D). When native and dephosphorylated α_{S1}-casein were titrated into the latex particle solutions, the absolute value of the zeta potential decreased. For native α_{S1}-casein, the zeta potential plateaued at about −30 mV, whereas a greater decrease in absolute zeta potential was observed for the dephosphorylated α_{S1}-casein, with the charge plateauing at about −20 mV, which is about a 10 mV difference compared with the native α_{S1}-casein (Fig. 2C,D). This is consistent with dephosphorylation decreasing the net charge of the α_{S1}-casein.

3.3. Dephosphorylation of casein-coated latex particles

Different levels of alkaline phosphatase was added to α_{S1}-casein-coated latex particles and the size of the particles was monitored as the dephosphorylation reaction proceeded (Fig. 3). In these experiments, the size measurements were carried out at

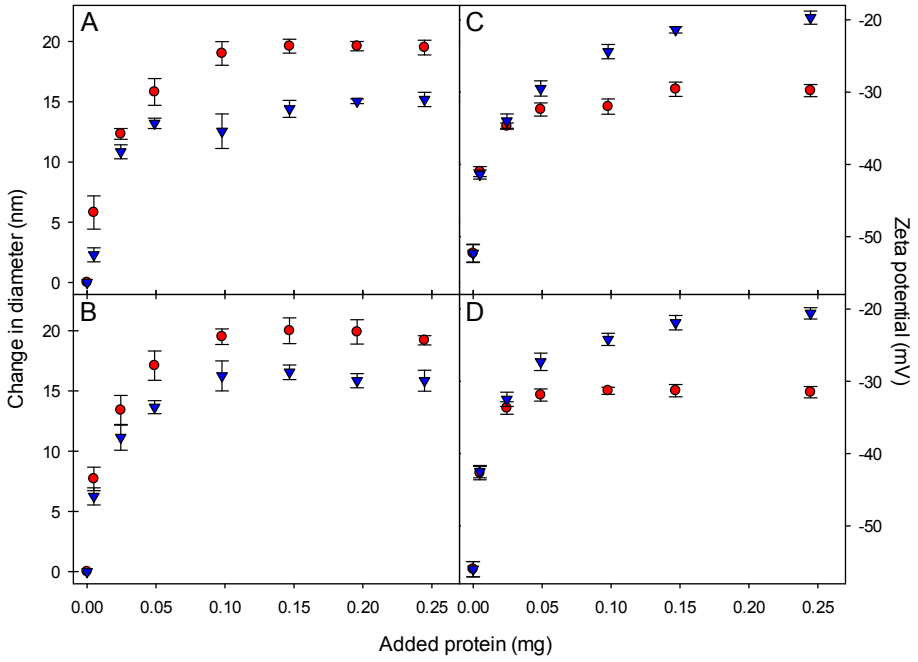

Fig. 2. The change in the diameters (A, B) and the zeta potentials (C, D) of 60 nm (A, C) and 100 nm (B, D) latex particles with added native α_{S1}-casein (●) and fully dephosphorylated α_{S1}-casein (▼). Each data point is an average of two replicates. The addition levels are reported as mg of α_{S1}-casein per 5 mL of 0.01% latex solution. Error bars represent the standard deviation of the mean of the replicates.

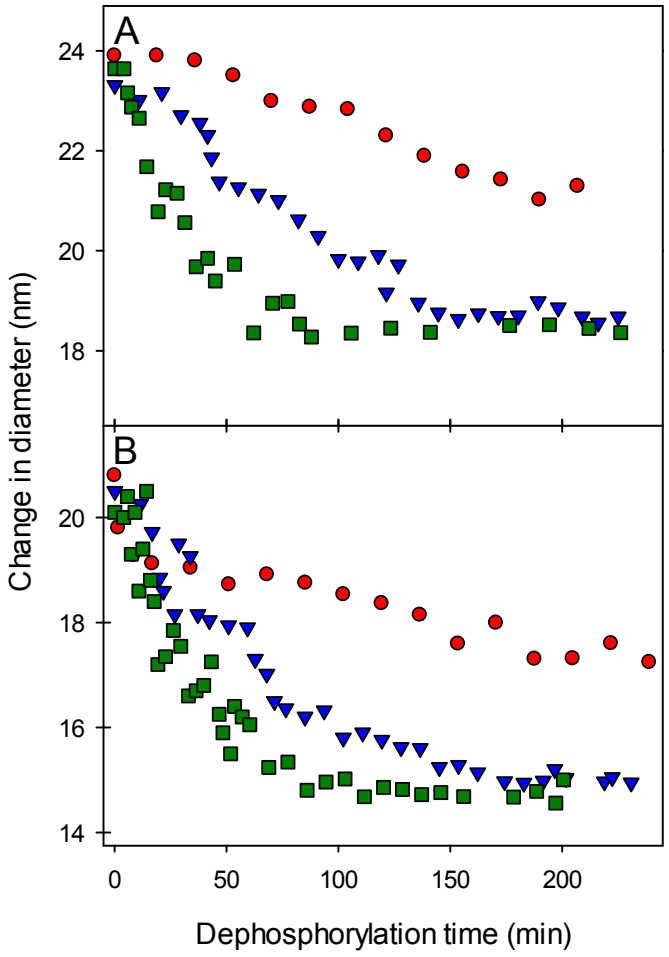

Fig. 3. The changes in the diameters of 60 nm (A) and 100 nm (B) latex particles coated in native α_{S1}-casein prior to incubation for different times with 2.1U (●), 12.6U (▼) and 25.2U (■) of alkaline phosphatase per mL of α_{S1}-casein-coated latex particle solution.

37 °C as this is close to the optimum temperature for the enzyme reaction (Lorient & Linden, 1976), and high levels of alkaline phosphatase were added so that the reaction would proceed within a reasonable time. At the start of the interaction, the addition of native α_{S1}-casein increased the diameter of 60 nm latex particles by about 24 nm (Fig. 3A), which is slightly higher than the 21 nm increase observed when the experiments were conducted at 20 °C (Figs. 1 and 2). This effect was not due to the different measurement temperatures (results not shown). The changes in size on dephosphorylation were similar between the two samples, suggesting that the size of the original bare latex particles may have been underestimated.

The diameters of α_{S1}-casein-coated latex particles decreased with time due to the gradual dephosphorylation of the α_{S1}-casein (Fig. 3). The highest level of added phosphatase (25.2 U of phosphatase per mL of α_{S1}-casein-coated latex particle solution) resulted in the fastest rate in decreasing the diameters of α_{S1}-casein-coated latex particles and the lowest level of phosphatase (2.1 U of phosphatase per mL of α_{S1}-casein-coated latex particle solution) had the slowest rate of size decrease. When the α_{S1}-casein was fully dephosphorylated, the diameters of α_{S1}-casein-coated latex particles remained unchanged with further increases in incubation time. This occurred after about 100 min for the sample with 25.2 U of added phosphatase, and after about 150 min for the sample with 12.6 U of added phosphatase. However, for the sample with 2.1 U of added phosphatase, the size was still decreasing after 200 min, which was the longest reaction time used, indicating that the α_{S1}-casein was only partially dephosphorylated (Fig. 3). For both the 60 and 100 nm latex samples, the addition of 12.6 U and 25.2 U of phosphatase decreased the diameter of α_{S1}-casein-coated latex particles by about 5 nm after a reaction time of about 200 min. This is a similar difference in size when native and fully dephosphorylated were adsorbed to the latex particles (Fig. 2).

The different casein proteins have different degrees of phosphorylation. α_{S1}-Casein has eight or nine phosphate groups, although the species with eight is predominant. β-Casein has four or five phosphate groups, with the five-phosphate species predominant, whereas κ-casein has one or two phosphate groups with the one-phosphate species predominant (Farrell et al., 2004; Swaisgood, 2003). To confirm that the decrease in size was due to the dephosphorylation, latex particles fully coated in α_{S1}-casein, β-casein or κ-casein were dephosphorylated in situ using the high level of phosphatase (25.2 U of phosphatase per mL of α_{S1}-casein-coated latex particle solution) and the change in size was monitored with incubation time (Fig. 4).

The α_{S1}-casein-coated latex particles decreased in size by about 6 nm when fully dephosphorylated, which is consistent with the earlier experiment (compare Fig. 3 with Fig. 4). For β-casein, the

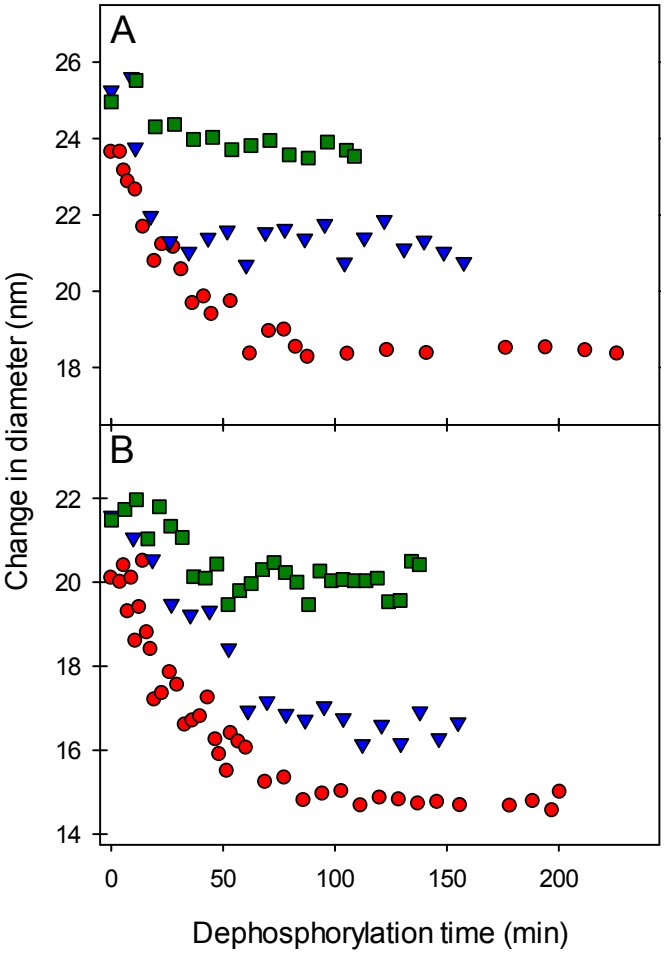

Fig. 4. The changes in diameters of 60 nm (A) and 100 nm (B) latex particles with native α_{S1}-casein (●), β-casein (▼) and κ-casein (■) adsorbed prior to incubation for different times with 25.2U of alkaline phosphatase per mL of α_{S1}-casein-coated latex particle solution.

size decreased by about 4–5 nm, and for κ-casein the size decreased by about 1 nm. Thus, the lower the number of phosphoserine residues on the casein, the smaller the decrease in the diameter of the casein-coated latex particles on dephosphorylation. For β-casein and κ-casein, a 1 nm decrease in diameter was observed for each potential phosphorylation, whereas for α_{S1}-casein, the decrease was less than 1 nm for each phosphorylation.

3.4. Adsorption of fully succinylated α_{S1}-casein on latex particles

Fully succinylated α_{S1}-casein was prepared by adding an excess of succinic anhydride to the casein solution and allowing the reaction to go to completion. The degree of succinylation was confirmed by a *o*-phthaldialdehyde (OPA) colorimetric assay, electrophoresis and mass spectrometry (results not shown). In contrast to the decreased charge on dephosphorylation, succinylation increases the net charge of proteins by converting the lysine amino acid residues into succinyl lysine residues that contain a carboxylate group, thereby increasing the negative charge (Broyard & Gaucheron, 2015; Ma et al., 2009; Strange et al., 1993). If dephosphorylation causes a decrease in the diameter of latex particles coated in α_{S1}-casein due to the decrease in charge repulsions, then succinylation would be expected to increase the diameter of the latex particles due to the increased charge repulsions.

When native α_{S1}-casein was titrated into the 60 and 100 nm latex particle solutions, the particle size increased and then plateaued when about 0.5 mg of the casein had been added, and the size had increased by about 21 nm, which is consistent with the previous experiments (compare Fig. 5 with Figs. 1–3). In contrast, when fully succinylated α_{S1}-casein was titrated into the latex particles, a much higher level (about 2 mg) was required to reach the plateau, and the size increase was much larger at about 31 nm (Fig. 5, Table 1).

When succinylated α_{S1}-casein was adsorbed to the latex particles, the absolute zeta potential was about 6–7 mV greater when compared with latex particles coated in native α_{S1}-casein (Table 1). This indicates that the succinylated α_{S1}-casein has an increased negative charge compared with native α_{S1}-casein, and the negative charged regions protrude from the latex surface thus increasing the absolute zeta potential of the coated latex particles.

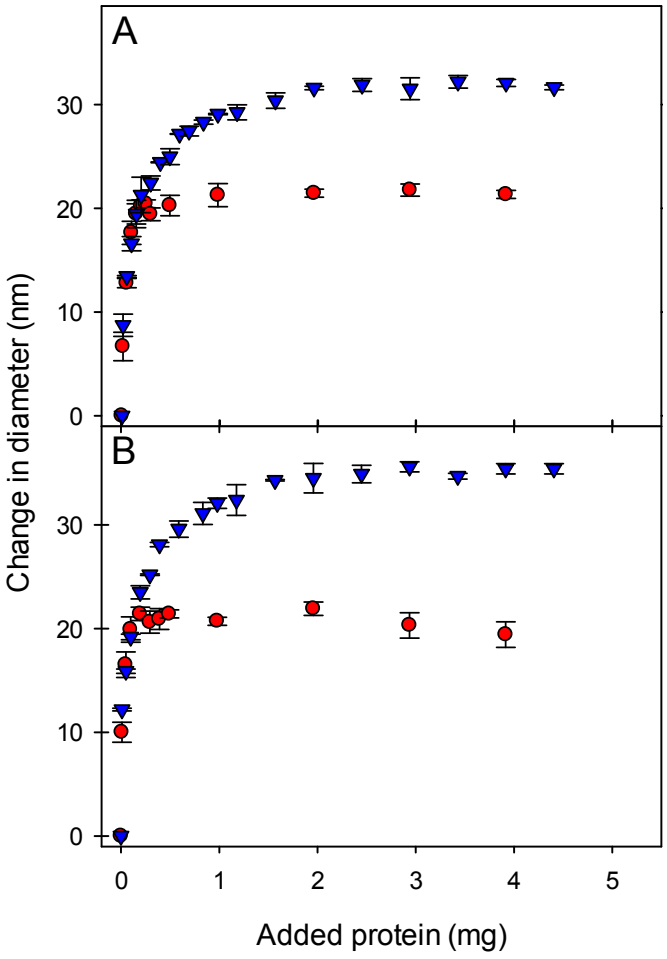

Fig. 5. The change in the diameters of 60 nm (A) and 100 nm (B) latex particles with added native α_{S1}-casein (●) and fully succinylated α_{S1}-casein (▼). The addition levels are reported as mg of α_{S1}-casein per 5 mL of 0.01% latex solution. Each data point is an average of two replicates. Error bars represent the standard deviation of the mean of the replicates.

3.5. Succinylation of α_{S1}-casein-coated latex particles

The in situ succinylation of the α_{S1}-casein-coated latex particles is more difficult than the in situ dephosphorylation reaction as it is difficult to titrate in different levels of the succinic anhydride in the very small volumes. Therefore, for the in situ experiments, one level of succinic anhydride was added, which, from previous experiments, was considered sufficient to fully succinylate all available lysine residues.

Addition of the α_{S1}-casein to the latex particles increased their diameters by about 19 and 21 nm for the 100 nm and 60 nm diameter latex particles respectively. Succinylation of the α_{S1}-casein-coated latex particles led to a 5 and 3 nm increase in the diameters for the 60 and 100 nm latex particles, respectively (Table 2). This increase in the diameters of the α_{S1}-casein-coated latex particles on succinylation was much less than when the α_{S1}-casein was succinylated before adsorbing onto the latex particles (compare Table 1 with Table 2). The in situ succinylation of α_{S1}-casein-coated latex particles led to an increase in the absolute zeta potential of the latex particles (Table 2). However, the change of 2 mV was less than that observed when fully succinylated α_{S1}-casein was adsorbed to the latex particles (Table 1).

Table 1
The zeta potential and size of native α_{S1}-casein and fully succinylated α_{S1}-casein-coated 60 nm and 100 nm latex particles; the α_{S1}-casein was succinylated prior to adsorption on the latex particles.

Latex particle (nm)	Zeta potential (mV)/diameter (nm) of α_{S1}-casein-coated latex particles		
	Native α_{S1}-casein	Succinylated α_{S1}-casein	Change on succinylation
60	−30.6 ± 1.5/85.5 ± 0.6	−36.6 ± 1.9/95.9 ± 0.6	−6.0/10.1
100	−37.1 ± 2.0/125.6 ± 0.9	−43.7 ± 2.5/140.3 ± 0.9	−6.6/14.7

Table 2
The zeta potential and size of native α_{S1}-casein and fully succinylated α_{S1}-casein-coated 60 nm and 100 nm latex particles; the α_{S1}-casein was succinylated in situ after adsorption to the latex particles.

Latex particle (nm)	Zeta potential (mV)/diameter (nm) of α_{S1}-casein-coated latex particles		
	Native α_{S1}-casein	Succinylated α_{S1}-casein	Change on succinylation
60	−34.7 ± 1.8/85.3 ± 0.4	−37.2 ± 1.9/90.8 ± 0.8	−2.5/5.5
100	−37.6 ± 1.3/123.2 ± 1.3	−39.7 ± 1.1/125.9 ± 0.9	−2.1/2.7

4. Discussion

The primary sequences of the individual caseins, highlighting the positions of the phosphorylated serine residues and the lysine residues that would be modified on dephosphorylation and succinylation respectively, have been reported (Farrell et al., 2004; Swaisgood, 2003). α_S-Casein, β-casein and κ-casein can adsorb to the surface of latex particles (Fig. 1; Anema, 1997; Brooksbank et al., 1993; Dalgleish, 1990,1993; Leaver et al., 1994; Leaver & Horne, 1996). Although not unequivocally established, it has been suggested that the α_{S1}-casein adsorbs on the latex surface via the hydrophobic regions at the N-terminus and C-terminus, with the hydrophilic region that contains the phosphoserine residues stretching out from the surface as a loop structure (Fig. 6A). In contrast, both β-casein (Fig. 6B) and κ-casein (Fig. 6C) are reported to have the hydrophobic regions associated with the latex surface and the hydrophilic, negatively charged and phosphorylated region protruding into the serum as a hair (Dickinson, Horne, Pinfield, & Leermakers, 1997a; Dickinson, Pinfield, Horne, & Leermakers, 1997b). These orientations will be used to discuss the results in this manuscript.

Dephosphorylated α_{S1}-casein-coated latex particles have a lower absolute zeta potential than with particles coated with the native protein (Fig. 2C,D). When the charge of dephosphorylated α_{S1}-casein was decreased, the electrostatic repulsion within the loop structure and between the loop and the latex particles surface will be diminished, allowing the loop to be closer to the surface of the latex particles (Fig. 2A,B). This is depicted schematically in Fig. 6A.

The change in size between native and dephosphorylated α_{S1}-casein was similar regardless of whether the casein was dephosphorylated first and then adsorbed to the latex particles (Fig. 2) or dephosphorylated after adsorption to the latex particles (Fig. 3). This indicates that the level and orientation of the adsorbed protein was similar for both the native and dephosphorylated α_{S1}-casein, with just the distance the loop extends being decreased by dephosphorylation (Fig. 6A).

Overall the changes in size on dephosphorylation of the casein-coated latex particles (Fig. 4) were consistent with the phosphorylation levels of the caseins. α_{S1}-Casein has a high level of phosphoserine residues (8–9) and showed the largest decrease in size on dephosphorylation, whereas κ-casein has a low level of phosphoserine residues (1–2) and showed a small change in size on dephosphorylation. β-Casein is intermediate between α_{S1}-casein and κ-casein in the level of phosphoserine residues (4–5) and the change in size on dephosphorylation was between that of the α_{S1}-casein-coated and the κ-casein-coated particles (Fig. 4).

The β-casein and κ-casein-coated latex particles showed about 1 nm decrease in diameter for each phosphate removed from the casein on dephosphorylation, whereas for α_{S1}-casein, the decrease was smaller at about 0.6 nm for each phosphate group removed. This difference may be due to the orientation of the proteins at the latex particle surface. The loop orientation of α_{S1}-casein may limit the extent to which the protein can extend or contract from the surface and thus account for the smaller decrease in size on dephosphorylation. In contrast the extended hair of β-casein and κ-casein may contract more on dephosphorylation, and thus a greater size decrease is observed for each phosphate removed. This is depicted schematically in Fig. 6A–C for α_{S1}-casein, β-casein and κ-casein respectively.

A higher level of succinylated α_{S1}-casein was required to saturate the latex particle surface and a much larger increase in size was observed when compared with native α_{S1}-casein (Fig. 5, Table 1). Adsorption of succinylated α_{S1}-casein onto the latex particles resulted in a higher absolute zeta potential of the casein coated latex particles when compared with particles coated with the native protein (Table 1), which is consistent with the conversion of positively lysine residues to negatively charged succinyl lysine groups. α_{S1}-Casein has a hydrophobic region at the N terminus consisting of about the first 40 amino acids. This is followed by a highly negatively charged region, from about amino acids 41 to 80, that contains the phosphoserine residues. The remainder of the protein, from amino acid 81–199 has no net charge and is distinctly hydrophobic (Horne, 1998; Swaisgood, 2003). There are 14 lysine residues in α_{S1}-casein, four within the hydrophobic region at the N terminus and three within the negatively charged hydrophilic region. Six of the remaining seven lysine residues are found in the region from amino acid 81 to 132, with only one lysine near the C-terminus (Farrell et al., 2004; Swaisgood, 2003).

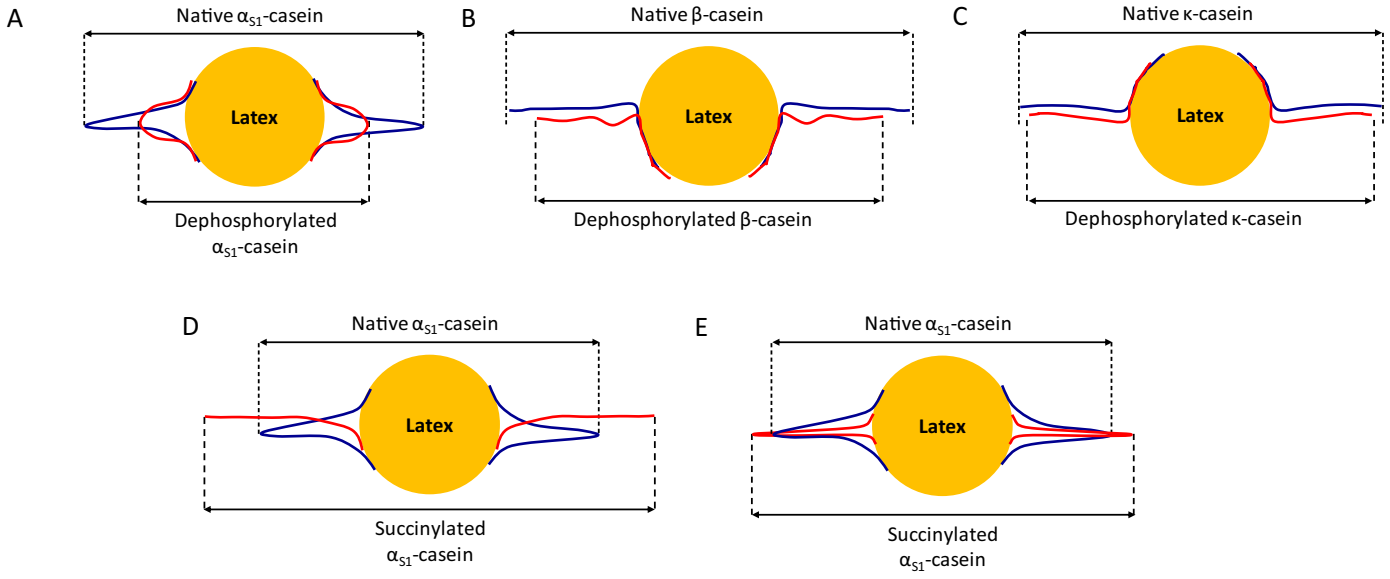

Fig. 6. A, B, C: The proposed structure of native (blue line) and dephosphorylated (red line) α_{S1}-casein (A), β-casein (B) or κ-casein (C) on the surface of a latex particles. The proposed structure of native and succinylated α_{S1}-casein on the latex surface C, D: The proposed structure of native (blue line) and succinylated (red line) α_{S1}-casein on the surface of latex particles either as an extended chain (D) or an expended loop (E). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

On succinylation, the region from amino acid 1 to 132 gains 13 negative charges, whereas the region from amino acid 133 to 199 gains a single negative charge. Thus, it is proposed that succinylated α_{S1}-casein added to latex particles adsorbs to the latex particle via the hydrophobic region at the C-terminus (from amino acids 133 to 199) with the N-terminus region projecting as a flexible hair. This orientation would account for the higher levels of succinylated α_{S1}-casein binding to the latex particles and the larger increase in diameter on binding when compared with native α_{S1}-casein (Fig. 5, Table 1). This is represented schematically in Fig. 6D.

When succinylation was performed in situ, there was a smaller increase in size and a smaller increase in absolute zeta potential (Table 2). One possible explanation is that the lysine residues on the N-terminal hydrophobic regions of α_{S1}-casein when adsorbed to the latex particles may be less accessible for succinylation as they will be close to the surface of the latex particles. Therefore, succinylation may only occur on the lysine residues that are located on the hydrophilic region of the α_{S1}-casein that is extended as a loop from the particle surface, and this increase in negative charge may result in the hydrophilic loop extending further from the surface of latex particles (depicted schematically in Fig. 6E).

A more likely explanation for the smaller increase in size on the in situ succinylation is that there is a lower level of native α_{S1}-casein to be succinylated for the in situ experiments. About 0.1–0.2 mg of native α_{S1}-casein was required to saturate the surface of the latex particles (Figs. 1, 2 and 5), and the addition of 0.2 mg succinylated α_{S1}-casein to the latex particles increased the diameter by about 5 nm (Fig. 5), which is a similar increase in diameter to that observed for the in situ succinylation of the α_{S1}-casein-coated latex particles (Table 2). Thus, it seems likely that during in situ succinylation, the N-terminal hydrophobic region of α_{S1}-casein detaches from the surface of the latex particle. This would result in a similar size change to that observed when a similar level of succinylated α_{S1}-casein was added to the latex particles. This lower level of succinylated α_{S1}-casein at the surface is also consistent with the smaller change in the zeta potential for the in situ succinylated latex particles when compared with those where fully succinylated α_{S1}-casein was adsorbed to the particles to saturation (compare zeta potentials presented in Tables 1 and 2).

The effect of dephosphorylation or succinylation on the properties of caseins when adsorbed to latex particles may be useful in helping understand the effect of these modifications on the stability of emulsions prepared from the caseins. For dephosphorylation, the decreased zeta potential along with the thinner adsorbed layer would be expected to result in a decreased emulsion stability. This is consistent with the results reported by Husband et al. (1997) for β-casein stabilised emulsions where a decrease in stability was observed for the dephosphorylated protein. However, other studies have reported either minimal change in emulsion stability (Darewicz et al., 2000, 2005) or improved emulsion stability (Lorenzen & Reimerdes, 1992) for the dephosphorylated protein when compared with the native protein. For succinylation, the increased zeta potential and the thicker adsorbed layer would be expected to improve the stability of emulsions prepared from the caseins, and this is consistent with the few studies on the emulsifying properties of succinylated caseins when compared with the native proteins (Shilpashree et al., 2015; Yang et al., 2014).

5. Conclusions

Dephosphorylation decreased the absolute value of zeta potential and the diameters of α_{S1}-casein-coated latex particles. α_S-Casein adopts a loop arrangement at the latex particle surface with the phosphoserine residues in the loop that extends from the latex surface. It is proposed that dephosphorylation decreases the charge within this loop, which causes the loop to move closer to the latex surface. On dephosphorylation, there was a larger change in the diameter of α_{S1}-casein-coated latex particles than β-casein-coated particles, whereas the κ-casein-coated particles showed only a very small change in size. This was in accord with the level of phosphorylation of the individual casein proteins.

Succinylation increased the net charge of α_{S1}-casein, and consequently led to an increase in the absolute value of zeta potential and the diameters of α_{S1}-casein-coated latex. In addition, a higher level of succinylated α_{S1}-casein was required to fully saturate the latex particle surface. It is proposed that the succinylated α_{S1}-casein adopts a different orientation at the latex surface. The hydrophobic C-terminal region (from amino acids 133 to 199) of α_{S1}-casein is relatively unaffected by succinylation and would adsorb to the latex surface. However, the remaining N-terminal region, and especially the hydrophobic region from amino acids 1 to 80 gains a significant negative charge that may prevent this region from adsorbing to the latex surface. Thus, the region from amino acids 1 to 133 extends from the latex as a hair. This orientation allows a greater level of α_{S1}-casein to adsorb to the latex particles and also allows the α_{S1}-casein to extend further into the serum, increasing the thickness of the adsorbed layer.

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