

Stability of casein micelles in milk

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Casein micelles in milk are proteinaceous colloidal particles and are essential for the production of flocculated and gelled products such as yogurt, cheese, and ice-cream. The colloidal stability of casein micelles is described here by a calculation of the pair potential, containing the essential contributions of brush repulsion, electrostatic repulsion, and van der Waals attraction. The parameters required are taken from the literature. The results are expressed by the second osmotic virial coefficient and are quite consistent with experimental findings. It appears that the stability is mainly attributable to a steric layer of κ -casein, which can be described as a salted polyelectrolyte brush. © 2002 American Institute of Physics. [DOI: 10.1063/1.1484379]

INTRODUCTION

For millennia, man has known that milk flocculates and gels when it is acidified, as in yogurt production. The acidification is caused by lactic acid bacteria, which convert milk sugar into lactic acid. Near pH 4.8 the onset of a macroscopic flocculation of the casein micelles in milk is observed. The colloidal stability of biological, i.e., waterborne, systems such as milk and blood is of great scientific interest and yet has hardly been described in a quantitative way. In order to understand the properties of casein micelles as present in milk it is necessary to appreciate their colloidal character. Nowadays the “hairy casein micelle” model of Holt¹ is generally accepted and it helps the understanding of several dairy technological aspects of milk products. In this model the casein micelle is regarded as a colloidal particle, which is an associate of about a thousand small nanoclusters. These nanoclusters are the building block of the self-assembling casein micelle.^{1–3} The physiological function of casein micelles is to transport calcium phosphate to the neonate.⁴ To prevent calcination of the mammary gland, small calcium phosphate nuclei are covered by caseins, which are ampholytic proteins. The nanoclusters so formed (20 nm)⁵ associate further into casein micelles (200 nm); also see de Kruif and Holt.⁶ The casein micelle is further characterized by the presence of κ -caseins at the surface which protrude into the solvent. Actually, 63 of the 169 amino acids are on the outside of the casein micelles, while the other 106, which are more hydrophobic, are “inside” the casein micelle.⁶ The “exterior” part of the κ -caseins provides the steric stabilization of the casein micelles. In the “exterior” part there are 15 charged groups with an effective dissociation constant, pK_a , of 4.9.⁷ Casein micelles are polydisperse in size and can be characterized by a number-averaged radius of 100 nm.⁸ Their size distribution is well described by a log-normal distribution,⁸ for instance giving a weight-averaged radius of

140 nm, which is in agreement with light-scattering experiments by Bauer *et al.*⁹ Further, at neutral pH casein micelles behave effectively as hard spheres, as can be derived from the concentration dependence of their self- and collective diffusion¹⁰ and their rheological behavior.¹¹ When skim milk is acidified the transport properties become those of adhesive hard spheres.^{10,12} This means that the interactions between casein micelles change from hard-sphere-like to effectively attractive during a pH decrease. In cheese-making the steric stabilization (the “hairy” layer) is removed enzymically and that induces gelation into the cheese curd.⁶ Here we show that the stability of casein micelles in milk upon acidification and renneting can be described quantitatively using current knowledge of the pair potential. Our approach may also serve as an example for a quantitative understanding of the stability of complex colloidal dispersions in aqueous solutions.

In the second half of the last century the understanding of the stability of colloidal dispersions increased enormously. Of paramount importance was the development of the Derjaguin–Landau–Verwey–Overbeek (DLVO) theory (see Refs. 13 and 14 or an overview in Ref. 15). Payens,^{16,17} and Walstra and Jenness,⁴ discussed the application of these theories to describe the colloidal stability of a dispersion of casein micelles in milk. From their work it follows that the electrostatic repulsion is not strong enough to compensate the van der Waals attraction and prevent flocculation. So, according to the DLVO theory casein micelles are not stable when present in milk. Paradoxically, they are in fact stable and adding kitchen salt does not lead to flocculation. During the 1970s it was realized that steric stabilization of colloids was present in many systems,¹⁸ and Holt¹⁹ noticed that this was also relevant for the description of the interaction between casein micelles. Subsequently, in the last decade it became evident that κ -casein can be regarded as a brush of a grafted polymer at the surface of the casein micelles. Strictly speaking, the κ -caseins have to be regarded as block copolymers, with a block absorbed in the micelle and a nonadsorbing block sticking into the solution. De Kruif and Zhulina²⁰ modeled κ -casein as a polyelectrolyte brush, and their calcu-

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lations explain many features of the stability of casein micelles during renneting. From the foregoing it follows that the knowledge of and insights into casein micelles and their interactions have increased significantly in recent years. Therefore it is intriguing to try to answer the question raised by Holt and Horne:² “Can we quantify the strengths of these micellar interactions?” Here we calculate the pair potential between casein micelles and subsequently the second osmotic virial coefficient, B_2 . We calculate B_2 since this parameter can be used to calculate both equilibrium and transport properties, and is an excellent parameter to characterize the colloidal stability.

Theoretical section

The interaction potential contains many contributions, of which only a few are probably relevant. The three relevant types of interaction that play the most important roles in the stability of casein micelles are van der Waals attraction, electrostatic repulsion, and polymer brush repulsion. Van der Waals dispersion forces arise through mutually fluctuation-induced polarization of the electrons in atoms. For the van der Waals attraction, $W_{\text{vdw}}(h)$, between two spheres separated by a distance h , where $h = r - 2a$, a is the sphere radius and r is the sphere center to center distance, one finds²¹

$$\frac{W_{\text{vdw}}(h)}{kT} = -\frac{A}{6} \left(\frac{2a^2}{(h+2a)^2 - 4a^2} + \frac{2a^2}{(h+2a)^2} + \ln \left[\frac{(h+2a)^2 - 4a^2}{(h+2a)^2} \right] \right), \quad (1)$$

where A is the Hamaker constant. We may use results of the Hamaker constant as found for proteins, which are of the order 1–3 kT.^{22,23} Recently, Schaink and Smit²⁴ calculated the Hamaker constant of β -lactoglobulin as ~ 5 kT near the iso-electric point. Gripon *et al.*²⁵ did scattering experiments on lysozyme solutions from which they derived a Hamaker constant of ~ 7 kT at 25 °C. The protein density of casein micelles is about 6 times less than that of β -lactoglobulin and lysozyme and therefore we estimated A as 1.0 kT. The gel strengths of β -lactoglobulin and lysozyme gels are much greater than that of a casein micelle gel, which is consistent with the much smaller Hamaker constant of casein micelles.

At the physiological and near-neutral pH of 6.7 as in milk the caseins carry charges from dissociated acid and basic protein groups. Charge distributions in solution are usually described by a Boltzmann distribution. The range of electrostatic interaction is expressed by the Debye length κ^{-1} :

$$\kappa^{-1} = \sqrt{\frac{\epsilon_0 \epsilon_r RT}{F^2 I}}, \quad (2)$$

where ϵ_0 is the permittivity in vacuum, ϵ_r is the relative permittivity, F is Faraday's constant, and I is the ionic strength, which is 0.08 M in milk,⁴ corresponding to a Debye length of the order of 1 nm. The electrostatic repulsion between casein micelles in milk is therefore short-ranged. The expression for the interaction potential between two spheres

due to electrostatic repulsion $W_{\text{er}}(h)$ can be derived from a linear approximation to the Boltzmann charge distribution around two colloids:²⁶

$$\frac{W_{\text{er}}(h)}{kT} = -2\pi a \epsilon_0 \epsilon_r \Psi^2 \ln(1 + \exp(\kappa h)), \quad (3)$$

where Ψ is the surface potential. The surface potential cannot be measured directly but the zeta potential, which approximates the potential at a certain distance from the particle surface, can be measured electrokinetically, and was found to be close to -8 mV at neutral pH.²⁷ On decreasing the pH, Schmidt and Poll measured a zeta-potential that approached a value of 0 mV.²⁶ When the casein micelles become unstable, it is hard to measure the zeta-potential properly, but, considering the point of zero charge (PZC) values of the caseins in the micelles, it is expected that the zeta-potential will be zero around pH 4.8 and that it will become positive at lower pH values.

To calculate the steric (repulsive) interaction due to brush repulsion we will first evaluate the relation between the brush height and the pH for κ -caseins in skim milk and will follow the theory of grafted weak polyacids proposed by Israels *et al.*²⁸ As discussed by De Kruif and Zhulina,²⁰ κ -caseins can be described as charged brushes in the (“salted brush”) regime. In this regime the salt concentration is such that it penetrates the brush and screens the electrostatic interactions between the charged polyacid groups; $\kappa^{-1} \ll H$, where H is the brush height. Then the polyacid brush is quasi-neutral; its characteristics are identical to those of neutral brushes. Consequently, in the strong stretching approximation, the brush height then reads^{28,29} as

$$H = Nb \left(\frac{8\nu^{\text{eff}} \theta}{\pi^2} \right)^{1/3}, \quad (4)$$

where N is the number of segments of the brush, each having a length b . The quantity θ is the grafting density: the fraction of “sites” occupied by the brushes at the surface. The parameter σ (m^{-2}) is the grafting density θ divided by the surface area occupied by a brush. The effective excluded volume is denoted by ν^{eff} and is defined as

$$\nu^{\text{eff}} = \nu + \frac{\alpha^2}{\phi_s}, \quad (5)$$

where ν is the (dimensionless) excluded volume per segment (normalized with the segment volume), $1 - 2\chi$ (with χ the Flory–Huggins interaction parameter), and ϕ_s is the salt volume fraction. We take a very simple model for the dissociation of the polyacid brush in which we assume that the various dissociating groups do not affect one another, which leads to the following relation between the pH and the degree of dissociation α :

$$\alpha = \frac{K_a}{K_a + [\text{H}^+]}, \quad (6)$$

where $[\text{H}^+]$ is the proton concentration ($\equiv 10^{-\text{pH}}$).

The above offers us a simplified model for the brush height as a function of pH. It is realized that Eq. (4) loses its

applicability at low pH since then it is less stretched. It is known that a κ -casein brush collapses at low pH .²⁰ Therefore for χ 's close to $\frac{1}{2}$ Eq. (4) still yields a proper qualitative description of the brush height at low pH ; it predicts the collapse. For the interaction potential between hard parallel plates at a distance h carrying brushes we use the Alexander–de Gennes theory,^{30,31} which leads to the following expression for the force between two parallel flat plates:

$$\frac{K_{\text{brush}}(h)}{kT} = \sigma^{3/2} \left[\left(\frac{2H}{h} \right)^{9/4} - \left(\frac{h}{2H} \right)^{3/4} \right], \quad \text{for } h < 2H, \quad (7)$$

$$= 0, \quad \text{for } h > 2H,$$

where the first term between the square brackets represents the increase in the osmotic pressure (leading to repulsion) due to the increase of the brush concentration between the plates and the second term represents the change in elastic free energy of the brush upon compression. The interaction potential between two flat surfaces can be obtained by integrating $K_{\text{brush}}(h)$ over the plate separation distance. By subsequently applying the Derjaguin approximation³² to the potential between two flat surfaces, the following expression for the interaction potential between two spheres, with radius a , is obtained:^{33,34}

$$\frac{W_{\text{brush}}(h)}{kT} = \infty \quad \text{for } h < 0$$

$$= \frac{16\pi a H^2 \sigma^{3/2}}{35} \left[\left(28 \left(\frac{2H}{h} \right)^{1/4} - 1 \right) \right. \\ \left. + \frac{20}{11} \left(1 - \left(\frac{h}{2H} \right)^{11/4} \right) + 12 \left(\frac{h}{2H} - 1 \right) \right], \\ \text{for } 0 < h < 2H \quad (8)$$

$$= 0 \quad \text{for } h > 2H.$$

The Derjaguin approximation is quite reasonable as long as the range of the interaction potential is much smaller than the sphere radius, which is the case for casein micelles in milk. In our model calculations we describe the brush part of the κ -casein as a salted brush containing 15 weakly charged groups that have a pK_a of 4.9. The part of the κ -casein that protrudes into the solvent contains 63 amino acids, each having a length of approximately 0.32 (± 0.04) nm (calculated using the computer program CHARMM). The segment length used in our model [b in Eq. (4)] should thus be at least 0.32 nm. The grafting density σ can be estimated by a calculation of the surface occupied by the κ -caseins at the casein micelle surface. The total amount of surface of casein micelles A_{CM} in the system per unit volume equals

$$A_{\text{CM}} = 6\phi \frac{\int_0^\infty \Psi(D) D^2 dD}{\int_0^\infty \Psi(D) D^3 dD}, \quad (9)$$

where $\Psi(D)$ is the size distribution of the casein micelles, each having a diameter D . De Kruijff⁸ analyzed this size distribution and found that it can be described by a log-normal distribution. In Eq. (9), ϕ is the volume fraction of casein

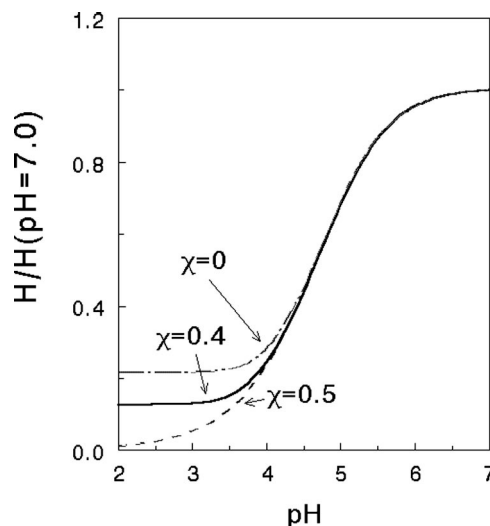


FIG. 1. Relative root-mean-square brush height of κ -caseins at the surface of casein micelles as a function of pH for two solvent qualities for the brush, $\chi=0$, and 0.4, as calculated from Eqs. (4)–(6).

micelles, which equals 0.13 in unheated skim milk.¹¹ This yields a total surface per volume A_{CM} of $1.76 \times 10^3 \text{ m}^2/\text{L}$ skim milk. In milk one has 3.3 g κ -casein/L, which corresponds to a brush density of $1.88 \text{ mg}/\text{m}^2$. The molar mass of κ -casein is 19032 g/mol, the brush density thus corresponds to approximately 7500 chains per casein micelle, and the average distance between the chains is 4 nm. The area occupied by one chain at the surface is at least the squared length of an amino acid group in the κ -casein brush, where the length equals about 0.32 nm. We therefore estimate θ as 0.006. The salt volume fraction is estimated as 0.01 (salt concentration ≈ 7.7 times salt volume fraction²⁸). We set the segment length at 0.60 nm in order to attain a brush height of 7.0 nm, which is in fair agreement with dynamic light-scattering experiments.¹⁰

RESULTS AND DISCUSSION

In Fig. 1 we plot the relative brush height $H/H(pH7)$, where $H(pH7)$ is the brush height at pH 7.0 for three solvent qualities of the brush; $\chi=0$, 0.4, and 0.5. The brush height H is calculated from Eqs. (4)–(6) using the parameters given above. We observe that the relative brush height starts to decrease gradually below pH 6, and decreases very rapidly with decreasing pH below pH 5.3. The curves are hardly affected by the solvent quality above pH 4.2, indicating that the decrease in relative brush height is not sensitive to the solvent quality in the relevant pH range where flocculation occurs. In the rest of this paper we use the results for $\chi=0.5$, since that correlates with a collapse of the brush at low pH . We can now use the pH -brush height dependence to calculate its effect on the interaction between the casein micelles using the Alexander–de Gennes approximation.

In Figure 2 we plot the various contributions to the total interaction potential. Plotted are the van der Waals attraction [Eq. (1) using $a=100$ nm as the radius of a casein micelle⁸], electrostatic repulsion [using Eq. (2) with +20 mV as the surface potential at pH 6.7 which can be estimated from the

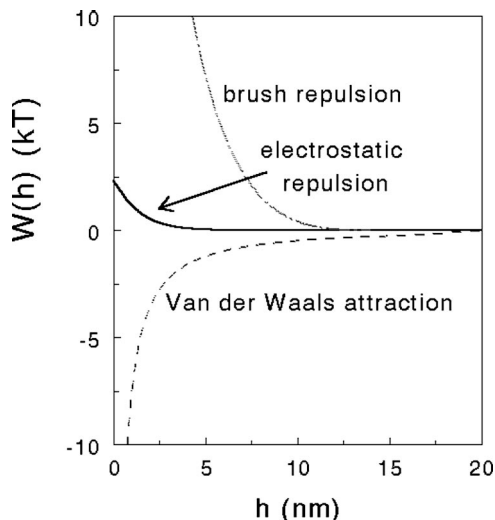


FIG. 2. Contributions to the interaction potential between casein micelles at pH 6.7 due to brush and repulsion, electrostatics, and van der Waals attraction.

zeta-potential measured by Schmidt and Poll²⁷], and brush repulsion [Eq. (8)] at pH 6.7. On lowering the pH it approaches the PZC and one even expects a smaller contribution of the electrostatic repulsion. Close to the flocculation pH (4.8) hardly any net charge remains. We approximated the surface potential ψ to: $\psi \sim (\text{PZC} - \text{pH})$ to take this effect into account. This gives a reasonable description of the zeta-potential measurements of Schmidt and Poll. From the potentials plotted in Fig. 2 it becomes clear that we can neglect electrostatic repulsion since brush repulsion overwhelms it. We note that inserting electrostatic repulsion hardly affects the results.

The total interaction potential is now taken as the sum of Eqs. (1) and (8), and is plotted for a few pH values in Fig. 3. The attraction becomes significant on lowering the pH below 5. This is due to the significant reduction of the brush height. Below pH 5 the brush repulsion becomes significantly weaker, while the casein micelles will probably become slightly positively charged. In this pH the repulsion is overwhelmed by the van der Waals attraction, which leads to a strong net attraction. Given the total interaction potential, the equilibrium (and transport) properties of the system can be evaluated using statistical mechanics.³⁵ From statistical mechanics we can calculate the second osmotic virial coefficient B_2 from $W_{\text{tot}}(r)$, with $r = h + 2a$:

$$B_2 = \frac{2\pi}{V_c} \int_0^\infty r^2 \left[1 - \exp\left(-\frac{W_{\text{tot}}(r)}{kT}\right) \right] dr, \quad (10)$$

where $W_{\text{tot}}(h)$ is the total interaction potential between the casein micelles. For any interaction potential this then yields B_2 , which becomes negative if the net attraction is sufficiently strong. In the calculations $h_0 = 0.5$ nm was taken as a cut-off length, that value of h below which $W_{\text{tot}}(h)$ is defined as ∞ , since it would be impossible to compress the spheres to $h = 0$. A reasonable estimate for the value of B_2 where colloidal suspensions become unstable is $B_2 \approx -6^{36}$. In Fig. 4 the second osmotic virial coefficient is given as a function of

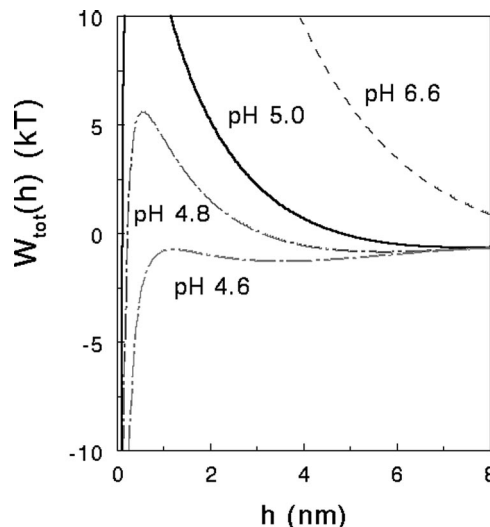


FIG. 3. Calculated total interaction potential between casein micelles for various pH values, as indicated.

the pH. At pH 7 B_2 has a value of 4.4, which is very close to 4, the value for hard spheres.³⁵ Upon decreasing the pH B_2 decreases very gradually up to pH 4.6, after which it drops very rapidly. It is clear that the instability region is reached quite suddenly, and at pH 4.5 one expects the casein micelles to become unstable, as estimated from the value where B_2 becomes -6 . This pH value of 4.5 is close to the onset of flocculation, which is usually referred to as pH 4.7–4.8.⁴ It is also known that decreasing the amount of κ -casein brushes, as occurs during renneting,^{4,37} the first stage in the cheese-making process, increases the flocculation pH.^{8,10} In Fig. 4 we therefore also added the results for the situation that 20%, 50%, and 60% of κ -casein at the casein micelle surface is cleaved off. It is clear that the sudden decrease of the second osmotic virial coefficient now takes place at a higher pH than 4.9: 4.7 for 20% κ -casein cleavage, 5.1 for 50% cleavage, and 5.7 for 60% of cleavage. This effect thus corresponds to experimental findings.^{8,10}

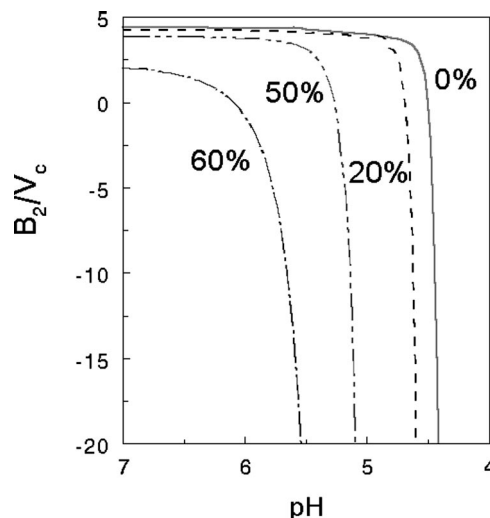


FIG. 4. Second osmotic virial coefficient of casein micelles as calculated from Eqs. (10) and (11) as a function of the pH (full curve). The dashed curves refer to the calculated values of B_2 for casein micelles of which a certain fraction of brushes is cleaved off.

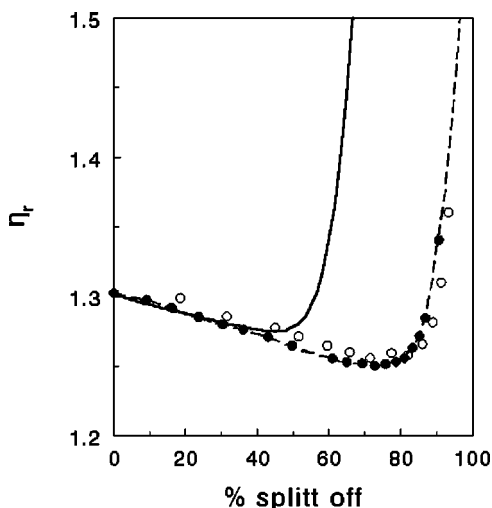


FIG. 5. Viscosity of a skim milk dispersion as a function of the percentage of κ -casein cleaved off during renneting. The experimental data are given as the data points from Ref. 40 and the full curve is calculated using the model proposed here. The dashed curve represents the prediction if the cleaved-off concentrations where the divergence of the viscosity is predicted matches.

Since the potential is short-ranged it is justifiable to refer to the adhesive hard sphere theory of Baxter,³⁸ which is often used to describe equilibrium and transport properties. The effective attraction in this model is expressed by the Baxter parameter τ_B , which relates to $B_2 = 4 - 1/\tau_B$. For the relative viscosity Cichocki and Felderhof³⁹ derived the following equation for adhesive hard spheres:

$$\eta_r = 1 + \frac{5}{2} \phi + \left(5.9 + \frac{1.9}{\tau_B} \right) \phi^2. \quad (11)$$

In Fig. 5 we have plotted the experimental data of De Kruif *et al.*,⁴⁰ who measured the relative viscosity of a skim milk dispersion with a volume fraction of 0.11 during renneting. The rennet time was translated to the percentage of κ -casein cleaved off by using their data. Our data are given in this plot as well. Both experimental data and our theoretical prediction exhibit identical behavior. After the decrease of relative viscosity (due to a decrease of the radii of the micelles and thus of the effective volume fraction) there is a sudden sharp upturn leading to a strong increase in the relative viscosity. There is, however, a shift in the percentage of cleavage where the upturn appears. Whereas our theory predicts that this happens at 60%, experimental data show that this point lies around 80%. In our analysis we assumed that all κ -casein can be cut off and also that there are no other brushes present. There is evidence⁶ that there is also a small fraction of β -casein brushes that are not cut off. They may enhance the stability, which may explain the difference we find.

CONCLUSION

The generally accepted and used models presented adequately describe the behavior and properties of casein micelles as found in milk. Using parameters derived from independent experimental data we were able to describe the stability of casein micelles during the initial stages of the

yogurt and cheese-making processes without using additional adjustable parameters. The results show that the steric stabilization generated by a relatively sparse brush is the most important stabilizing factor. Although our model is quite simple and contains several assumptions, inevitable for such a complex biological association colloid as a casein micelle, it gives a good description of the stability of casein micelles on lowering the pH and this understanding should be helpful for dairy technological developments.

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