

# Re-association Behavior of Casein Submicelles in Highly Alkaline Environments

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*Dedicated to Professor Wolfgang Beck on the occasion of his 80<sup>th</sup> birthday*

The pH-dependent dissociation and re-association behavior of casein proteins in alkaline environment was studied. In dilute aqueous solutions of pH = 12 and 13, casein was found to exist in the form of submicelles which represent the fundamental structural unit of casein micelles. Morphology and dimension of the casein submicelles were examined by scanning electron microscopy (SEM), transmission electron microscopy (TEM) and atomic force microscopy (AFM), revealing spherical particles of ~ 10–30 nm in diameter. When the pH was increased to 14, unexpected re-association of casein submicelles occurred, resulting in the formation of large network aggregates at pH > 14. Furthermore, calcium phosphate was found to be the main driving force behind the re-association, as verified by comparative experiments employing Ca-depleted casein. Based on an analysis of potential interactions between the proteins, this re-association is ascribed to decreased electrostatic repulsion, strengthened hydrophobic attraction and formation of calcium phosphate linkages between casein proteins.

**Key words:** Casein, Submicelle, Highly Alkaline Environment, Dissociation/Association, Calcium Phosphate Linkage

## Introduction

Casein is a biopolymer obtained by acid precipitation from milk. It is composed of a group of different phosphoproteins and accounts for approximately 80 wt.-% of the total protein content of milk. In general, native casein exists in the form of colloidal particles, so called “micelles”. These micelles are composed of protein molecules and inorganic constituents, mainly calcium phosphate, and their diameter was found to range between 50 and 500 nm (average about 120 nm) [1]. Towards a better understanding of the casein micellar structure, a variety of models have been developed since the 1960s [2–6]. Among them, one of the most commonly accepted was proposed by Walstra [6]. It suggests that the casein micelles are built of roughly spherical submicelles (12–15 nm in diameter) which are held together by hydrophobic interactions between the proteins, and by calcium phosphate linkages.

Casein contains three major protein fractions, which are referred to as the families of  $\alpha$ -,  $\beta$ - and  $\kappa$ -caseins,

each possessing different numbers of amino acids and functional groups due to variations in their amino acid sequences. For instance, the casein proteins can contain different amounts of phosphate groups which are linked to serine through esterification, depending on the type of protein. Specifically,  $\alpha$ -casein possesses 8–10 phosphoserine residues,  $\beta$ -casein five, whereas  $\kappa$ -caseins has only one. In casein micelles, these proteins are linked through their ester phosphate groups and inorganic calcium phosphate, resulting in the formation of micellar calcium phosphate (MCP) clusters [4].

The inorganic constituent calcium phosphate occurs as nano-sized ion clusters in the casein micelles. They function not only as cross-linkers, but also as positively charged neutralizing agents which can bind to negatively charged phosphoserine groups [3]. The importance of calcium phosphate was subject to extensive investigation over the last years, however, the debate still continues. Some earlier research claimed that calcium phosphate is an essential component which holds casein micelles together. Therefore, addition of a calcium-chelating agent can lead to extensive micel-

lar disintegration due to the extraction of the calcium components from the calcium phosphate ion pairs [7]. Opposite to this, Madadlou *et al.* argued that the colloidal calcium phosphate plays only a minor role in the formation and structural features of casein micelles since the calcium content in casein is rather low [8].

Recently, extensive research has been carried out to determine the stability of casein micelles in different environments [9–14]. For example, as mentioned above, in the presence of calcium-chelating agents such as EDTA- $\text{Na}_2$ , calcium and phosphate ions diffuse out of casein micelles, resulting in a reduction of micellar stability [15]. Also, solvent-mediated disintegration of casein micelles can be achieved by heating milk in the presence of ethanol, or by introducing urea to the aqueous solution as a chaotropic agent [16, 17]. Similarly, high hydrostatic pressure (250 to 310 MPa) was reported to promote extensive disruption of the casein micelles [18].

Among the processes leading to disintegration of the casein micelle, the alkaline-induced reduction in casein micelle stability has attracted considerable interest. Vaia *et al.* reported on the disruption of casein micelles at  $\text{pH} > 9$  and proposed a mechanism based on increased casein solubility at alkaline  $\text{pH}$  [19]. They argued that the decreased content of calcium phosphate clusters in alkaline media would increase the solvent quality, leading to reduced cohesive interaction between the hydrophobic regions of the casein proteins and thus increased solubility. However, Liu and Guo describe that at  $\text{pH} = 5.5$ –12 association of casein proteins into micelles occurs [20]. Using the fluorescent technique together with dynamic light scattering (DLS) and turbidity measurements, at low  $\text{pH}$  a more compact casein micellar structure and at high  $\text{pH}$  a looser structure were found. Obviously, these studies contradict each other, and accordingly the actual micellar behavior of casein proteins in alkaline environment is still not clear. Additionally, the  $\text{pH}$  range

investigated so far has been limited to  $< 12$ . This differs from some of the industrial applications such as interior wall paints where casein is applied at a  $\text{pH}$  of  $\sim 13$ . Likewise, in Portland cement-based building materials where casein is used as a superplasticizer to disperse cement, highly alkaline pore solutions with  $\text{pH}$  values of between 13 and 14 may occur. Thus, an understanding of the structure of casein micelles under these highly alkaline conditions will help to understand the working mechanism and performance of casein in such technical applications characterized by high  $\text{pH}$ .

In this work, we studied the alkaline-induced alteration in casein micellar behavior. Compared to previous studies, extremely high  $\text{pH}$  values ( $\text{pH} = 12$  to  $\text{pH} > 14$ ) were selected to correspond to the respective conditions of the industrial applications. Specifically, the dissociation and re-association behavior of casein and of Ca-depleted casein were investigated as a function of the  $\text{pH}$  of the solution. A mechanism underlying this process is proposed based on an analysis of potential interactions between the casein proteins. Furthermore, the role of calcium in the association process is discussed. From the above results, a schematic diagram was developed which summarizes the alteration of the casein micellar structure in highly alkaline environments. Lastly, the morphology of casein submicelles was visualized by scanning electron microscopy (SEM) and atomic force microscopy (AFM).

## Results and Discussion

### *The pH-dependent solubility of casein*

First, the  $\text{pH}$ -dependent solubility of casein in de-ionized water and in alkaline solutions ( $\text{pH} = 8$  to  $\text{pH} > 14$ ) was examined, and optical micrographs were taken (Fig. 1). In de-ionized water and at  $\text{pH} = 8$ , casein appeared rather insoluble, and the powder settled

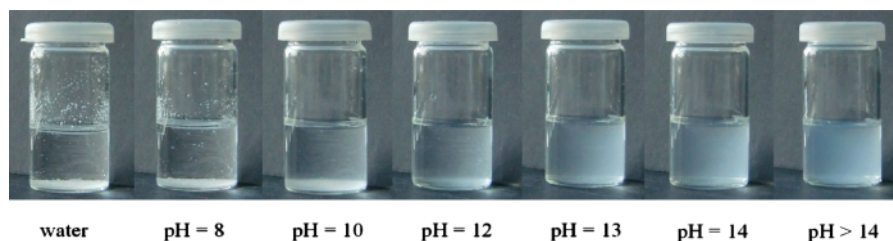


Fig. 1 (color online). Images of casein dispersed in de-ionized water and at  $\text{pH} = 8$  to  $> 14$ .

at the bottom of the vials. At pH = 10, the powder started to dissolve, as indicated by the appearance of a slight turbidity. As the pH was increased further, stable and homogeneous colloidal dispersions were obtained, thus demonstrating an increased solubility of casein in highly alkaline solution, specifically in the pH range of 12 to > 14. Under these conditions, no more precipitate was observed at the bottom of the vial.

To better understand this solubility behavior, pH-dependent turbidity measurements were employed to monitor the disintegration and association behavior of the casein sample under highly alkaline conditions.

#### *Dissociation and re-association of casein in highly alkaline media*

The turbidity of casein solutions at pH = 12 to > 14 was determined. According to Fig. 2, at pH = 12 and 13 the turbidity of the casein solutions was low (transmission 92% of water reference) and constant, thus indicating disruption of original casein micelles into smaller units with increased solubility [19]. At pH = 14, the turbidity first increased slightly and then sharply when the pH became > 14. This behavior is attributed to re-association of subunits which first were released from micelles and now form larger aggregates.

This concept was confirmed by TEM measurements of particles contained in the casein solutions. There, negatively stained casein particles are visible as bright areas in the micrographs (Fig. 3). At pH = 12 and 13,

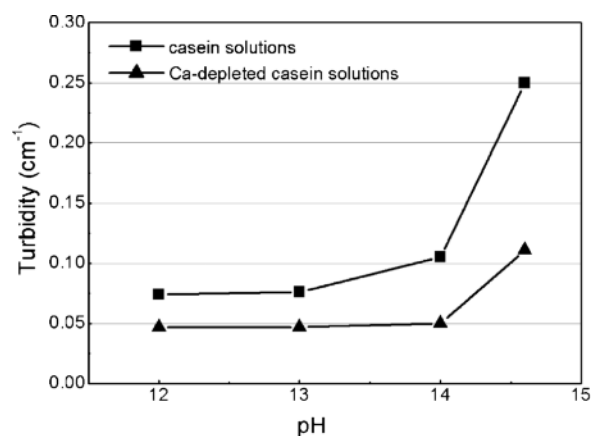


Fig. 2. Turbidity of casein solutions and of Ca-depleted casein solutions as a function of pH ( $c_{\text{casein}} = 1 \text{ g L}^{-1}$ ).

diameters of  $\sim 10 \text{ nm}$  were found for the casein particles. This value is comparable to the size reported for casein submicelles. No evidence for the existence of large casein micelles or aggregates was found, indicating that at pH = 12 and 13, casein micelles are completely dissociated into submicelles. These observations are in agreement with the low turbidity values measured for the corresponding casein solutions (Fig. 2). As the pH increased to 14, beginning aggregation of the submicelles became apparent. Larger particles (diameter  $d \sim 50 \text{ nm}$ ) are observed which explain the slight increase in turbidity seen before. Further increase in pH to > 14 led to strong aggregation of submicelles and resulted in large aggregates ( $d = \text{several hundred nm}$ ) which obviously produce higher turbidity. The TEM images confirm initial alkaline-induced dissociation of the casein micelles at pH = 12–13 which is followed by the re-association of the submicelles at very high pH (14 and higher).

To confirm the re-association process, the anionic charge densities of casein particles existing in alkaline solutions were measured. In alkaline solution, the casein molecule generally is negatively charged due to deprotonation of amino acid residues contained in the proteins. For example,  $=\text{NH}_2^+$  in arginine is neutralized to  $=\text{NH}$ , and the  $-\text{COOH}$  group in glutamic acid is converted to  $-\text{COO}^-$  [21]. At increased pH, it can be

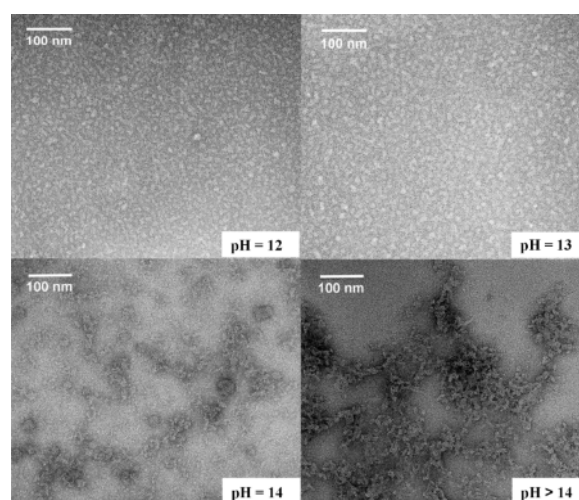


Fig. 3. Transmission electron micrographs of aqueous casein solutions ( $c_{\text{casein}} = 1 \text{ g L}^{-1}$ ) under alkaline conditions; pH = 12 and 13: casein submicelles ( $d \sim 10 \text{ nm}$ ) are dominant species; pH = 14: beginning association of submicelles into larger units ( $d \sim 50 \text{ nm}$ ); pH > 14: formation of large associated aggregates.

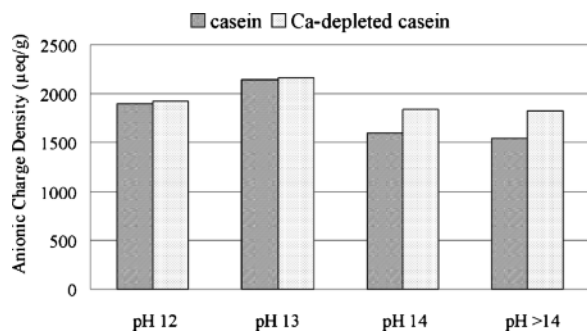


Fig. 4. Anionic charge density of casein particles as a function of solution pH. ( $c_{\text{casein}} = 1 \text{ g L}^{-1}$ ).

expected that the anionic charge of the casein units increases as a result of stronger deprotonation. It is worth mentioning that the detected charges appear at the surface of casein particles rather than at individual protein molecules. Especially when an aggregated structure is formed in solution, the surface charge density of the particles supposedly decreases with increased association. This is because some charged amino acid groups may be embedded in the particle interior, consequently, they are not able to contribute to the overall surface charge of the particle.

As is shown in Fig. 4, at pH = 12 the anionic charge density of the casein particles was  $-1894 \mu\text{eq g}^{-1}$ , indicating a highly anionic particle. As the pH increased to 13, as expected the charge density increased further to attain a value of  $-2141 \mu\text{eq g}^{-1}$ . At this pH, nearly complete deprotonation has been achieved because arginine, the most basic amino acid contained in casein, possesses a  $\text{pK}_a$  value of 12.5 [22], while the other amino acids exhibit  $\text{pK}_a$  values of less than 10.5 [19]. Therefore, the surface charge of casein should remain stable at pH > 13. However, the titration experiments revealed a decrease of the anionic charge density by 25% at pH = 14 to  $\sim 1600 \mu\text{eq g}^{-1}$ , and by 28% to  $\sim 1500 \mu\text{eq g}^{-1}$  at pH > 14. These results support the concept of association of casein proteins into larger aggregates as has been found before by turbidity measurements and TEM analysis.

#### *The influence of ionic strength on the association behavior*

One can argue whether the observed re-association of casein submicelles is induced by the highly alkaline conditions, or is in fact a result of the high ionic

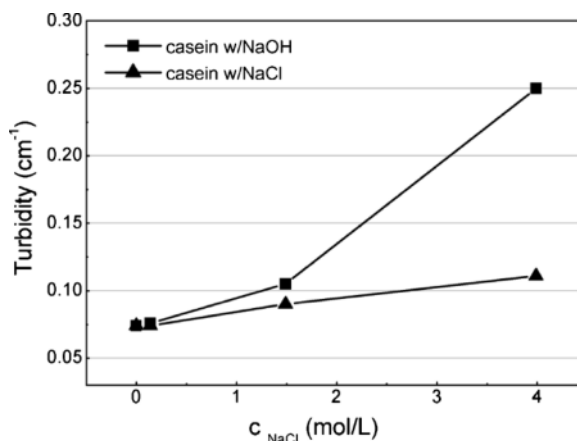


Fig. 5. Turbidity of casein solutions ( $c_{\text{casein}} = 1 \text{ g L}^{-1}$ ) as a function of ionic strength.

strength existing in the alkaline solutions. To clarify this point, casein solutions containing different NaCl concentrations as a replacement for NaOH were prepared. The ionic strength of the NaCl solutions was adjusted to correspond to the NaOH concentrations existing in casein solutions at pH = 13, pH = 14 and pH > 14, respectively. Again, turbidity measurements were performed, and the results are shown in Fig. 5. For comparison, the turbidity of casein solutions at pH = 12 to pH > 14 are displayed.

It was found that the turbidity of the casein solutions containing NaCl increases only slightly with increased ionic strength of the solutions. In comparison, for casein solutions containing NaOH, the turbidity increased dramatically by 278% as the ionic strength increased from 0.01 M to 4 M. The results allow to conclude that association of casein submicelles occurring at pH  $\geq 14$  primarily originates from a rise in solution pH.

#### *The role of calcium phosphate in the submicelle association*

The role of calcium phosphate in the re-association process of casein submicelles was investigated by means of Ca-depleted casein. The removal of calcium was achieved by employing urea and a calcium-chelating agent. The amount of calcium retained in the Ca-depleted casein was  $0.2 \text{ mg L}^{-1}$  only, which is significantly less compared to  $1.6 \text{ mg L}^{-1}$  of calcium contained in the original casein sample.

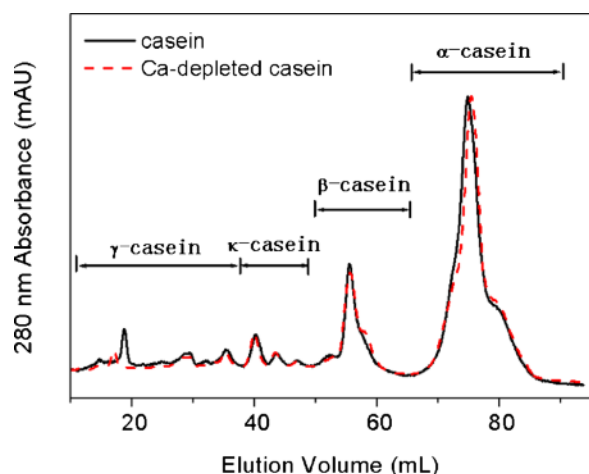


Fig. 6 (color online). FPLC profiles of casein and of Ca-depleted casein.

A chromatographic analysis of the Ca-depleted casein was performed using ion exchange fast protein liquid chromatography (FPLC) (Fig. 6). For comparison, the elution profile of the original casein sample is also displayed. Assignment of the elution peaks to individual casein fractions has been described in an earlier work [23]. As can be seen, the absorbance of Ca-depleted casein is practically identical with that of original casein, indicating that removal of calcium does not have much influence on the structure and composition of the proteins.

Next, the turbidity of Ca-depleted casein at alkaline pH was measured (Fig. 2). The turbidity of Ca-depleted casein increases with increasing pH of the solution, but much less than observed for the original casein. This result suggests that at high pH Ca-depleted casein associates much less than the casein sample as received.

Additionally, the surface charge of Ca-depleted casein was determined, and the results are shown in Fig. 4. At pH = 12 and 13, the anionic charge density of Ca-depleted casein was comparable to that of the original casein sample, whereas at pH = 14 and > 14, the charge density of Ca-depleted casein did not decrease much compared to that of the original casein. Again, this suggests that Ca-depleted casein exhibits a decreased tendency for association into larger units.

These experiments reveal the importance of calcium in the formation of large casein aggregates at extreme alkalinity. In its absence, the association of casein submicelles is severely impeded.

### Interactions between casein proteins

While dissociation of casein in alkaline medium has been reported and explained before [19], the newly observed re-association of casein submicelles occurring under extremely alkaline pH conditions requires a mechanistic explanation. For this purpose, the pH-dependent interactions between the casein proteins were studied. It is widely accepted that the micellar structure of casein is mainly governed by a delicate balance of electrostatic repulsion, hydrophobic attraction as well as by calcium phosphate linkages between casein submicelles [5]. Over the pH range of this study, the electric charge of casein would not change much, due to almost complete deprotonation of the amino acid residues present in the proteins, as discussed before. But increased binding of positively charged calcium phosphate clusters onto phosphoserine groups can partially neutralize the negative charges of the proteins [3], thus facilitating a reduction of the electrostatic repulsion between the proteins. It has been reported that the binding of calcium phosphate to phosphoserine groups increases with increasing pH of the solution, which is ascribed to a higher equilibrium binding constant at higher pH [24]. Consequently, the electrostatic repulsion between casein proteins decreases in highly alkaline environment, which may promote a tendency to associate into larger units.

Hydrophobic effects are among the most general and complicated chemical binding forces which can be influenced by pH, temperature, ionic strength of solutions *etc.* Here, hydrophobic interactions between the casein proteins may be impacted (1) by alteration of the surface charge of the casein particles at highly alkaline pH and (2) by an increase in the ionic strength of the solutions. As mentioned above, the number of negative charges carried by each casein is reduced as more calcium phosphate binds at higher pH, which prompts an increase in the hydrophobic domains present in the proteins. These newly exposed hydrophobic regions presumably enhance hydrophobic interactions between casein units. Also, at increased pH the ionic strength of the casein solutions increases which strengthens hydrophobic interactions between proteins as discussed above. Therefore, association of casein proteins is favored at very high pH.

Based on the above considerations, calcium phosphate plays an important role in the re-association process of casein submicelles. It moderates the in-



teractions between proteins by neutralizing their negative charge. Specifically, electrostatic repulsion is weakened, and hydrophobic interaction is strengthened in highly alkaline environment. Additionally, calcium phosphate can crosslink casein units by forming phosphoserine-calcium phosphate-phosphoserine linkages between proteins [4], which are also referred to as micellar calcium phosphate (MCP). MCP has been reported to build up under highly alkaline conditions as a result of an increased equilibrium constant [24]. All these effects promote the re-association of casein submicelles and the formation of large aggregates.

*Diagram of the dissociation and re-association process*

Summarizing the results from above, a schematic diagram for the pH-dependent dissociation and re-association behavior of casein in alkaline solution was developed (Fig. 7). At pH  $\sim 7$ , casein consists of micelle-like particles which in turn are made up from spherical submicelles held together by hydrophobic interactions and calcium phosphate linkages (Fig. 7a).

When casein is dissolved in alkaline solution,  $\text{OH}^-$  ions diffuse into the micelle due to its loose and porous nature [25]. This results in the deprotonation of amino acid residues and instigates a high negative charge of the proteins. At pH = 12–13, electrostatic repulsion occurring between the proteins exceeds the attractive forces provided by calcium phosphate linkages and hydrophobic interactions. Hence, the casein micelle starts to disrupt, and negatively charged submicelles dissociate (Figs. 7b and 7c). This process is believed to be behind the formation of so called “sodium caseinate” which has been reported to consist of  $\sim 15$  proteins and to possess an average diameter of  $\sim 11$  nm [26]. These submicelles constitute the fundamental structural units after dissociation and present the building blocks for large casein micelles or aggregates at extreme alkalinity.

When the pH is raised to  $\geq 14$ , electrostatic repulsion between the submicelles is diminished, and hydrophobic attraction as well as MCP linkages increase. Consequently, re-association of the casein submicelles occurs, leading to large network aggregates at pH  $> 14$  (Figs. 7d and 7e).

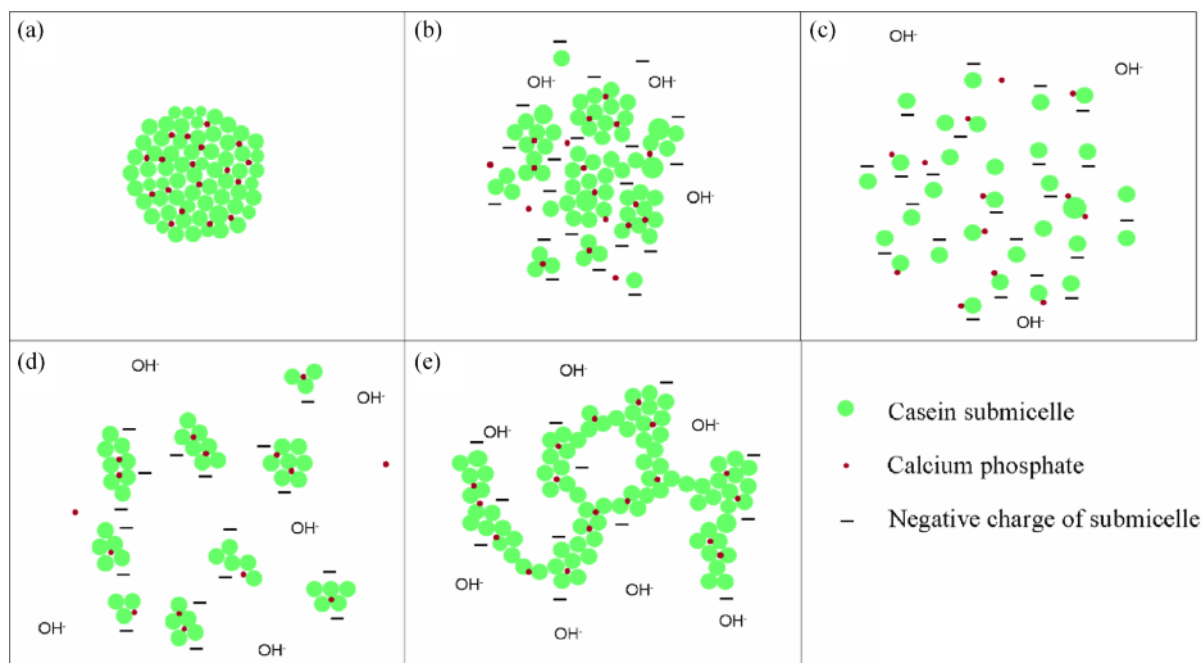


Fig. 7 (color online). Schematic diagram illustrating the dissociation of casein micelles and the re-association of casein submicelles in highly alkaline solutions: (a) casein micelle at pH  $\sim 7$ ; (b) disruption of the micelle at moderate alkalinity; (c) at pH = 12–13, negatively charged submicelles dissociate from the casein micelle; (d) slight re-association of submicelles occurs at pH = 14; (e) formation of network aggregates at pH  $> 14$ .

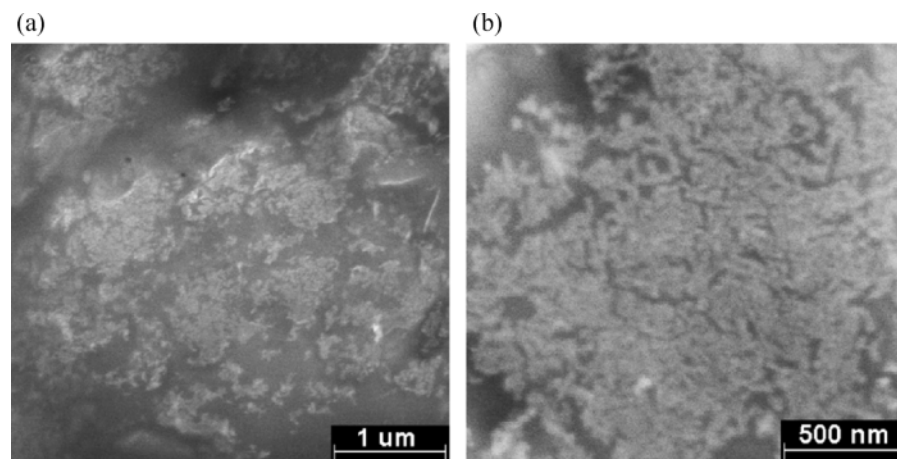


Fig. 8. SEM images of casein submicelles deposited from solution ( $c_{\text{casein}} = 1 \text{ g L}^{-1}$ ,  $\text{pH} = 12$ ).

### Morphology of casein submicelles

SEM and AFM techniques are capable of providing direct visualization of sample topologies, thus they were applied to assess the morphology and particle size distribution of casein particles. The sample was prepared from a casein solution at  $\text{pH} = 12$ , which allows observation of casein submicelles existing under these conditions. Fig. 8 presents SEM images of casein deposits after drying. Closely packed submicelles exhibiting an average diameter of 10–30 nm are visible on the substrate.

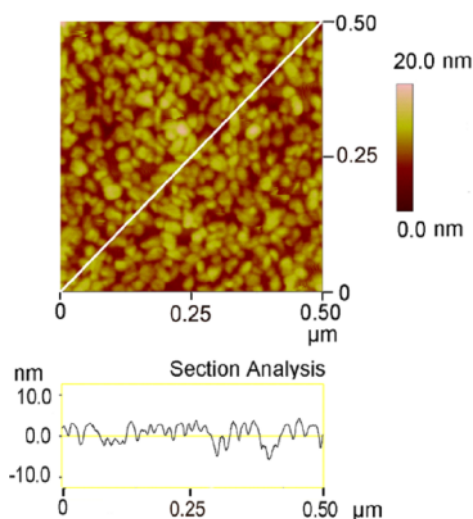


Fig. 9 (color online). Top: AFM topographical image of casein submicelles obtained from dried casein solution ( $c_{\text{casein}} = 1 \text{ g L}^{-1}$ ,  $\text{pH} = 12$ ); bottom: section analysis of the deposit surface along the indicated line.

AFM characterization confirmed the results of the SEM analysis (Fig. 9). The topographical image clearly revealed a spherical appearance of casein submicelles with a diameter of  $\sim 20 \text{ nm}$ . AFM section analysis produced an average height of 11 nm for the submicelles, which was less than the corresponding diameter, but comparable to the diameter obtained by TEM imaging (Fig. 3).

### Conclusion

The micellar behavior of casein under extremely alkaline conditions ( $\text{pH} = 12$  to  $\text{pH} > 14$ ) was investigated. Turbidity experiments along with TEM imaging and surface charge measurements revealed complete dissociation of casein micelles into submicelles to occur at  $\text{pH} = 12$  and 13. While at  $\text{pH} \geq 14$ , an unexpected re-association of the submicelles was observed, resulting in the formation of large, aggregated structures. Calcium phosphate was found to play a critical role in this re-association process. It moderates interactions between proteins by neutralizing their negative charge, and by establishing phosphoserine-calcium phosphate-phosphoserine linkages. The importance of calcium phosphate was confirmed by the behavior of Ca-depleted casein. Using SEM and AFM analyses, spherical submicelles ( $d \sim 10\text{--}30 \text{ nm}$ ) were visualized and identified as the fundamental structural units constructing casein micelles and aggregates in alkaline solutions.

The results suggest that casein, because of its pH-dependent micellar assembly behavior, may become a promising candidate for biodegradable functional

materials which can be widely used in various industries including food, detergent and building products. The understanding of the micellar behavior at high pH presented in this work may help to foster the use of casein in applications where extremely high pH is prevalent, such as in cement.

## Experimental Section

### Materials

#### Casein sample

Technical-grade bovine casein powder provided by Ardex GmbH, Witten/Germany, manufactured by acid (HCl) precipitation from milk and subsequent drum-drying, was used directly without any treatment and purification. Fast protein liquid chromatography (FPLC) analysis produced the protein contents (wt.-%) as follows: 50.6 %  $\alpha$ -casein, 34.1 %  $\beta$ -casein and 5.3 %  $\kappa$ -casein. Elemental analysis of the casein powder showed the composition (wt.-%) as follows: 48.53 % C, 7.34 % H, 13.42 % N, 29.15 % O, 0.61 % S, 0.20 % Ca, and 0.75 % P. The moisture content (measured as gravimetric weight loss) was determined to be 8.16 wt.-%.

#### Colloidal casein solutions

Aqueous dispersions of casein were prepared by simply stirring casein powder in de-ionized water. The casein concentration was  $1 \text{ g L}^{-1}$ . Different pH values were adjusted by adding 40 wt.-% aqueous NaOH solution to the casein suspension. The resulting colloidal solutions were allowed to stand at room temperature for 1 h prior to use. The pH was controlled either using a glass electrode [pH = 8–12; electrode: BlueLine 14 pH (Schott, Mainz/Germany)] or by calculation of  $\text{OH}^-$  activity in the casein solutions (pH = 13 to pH > 14). To study at extreme alkaline condition, casein powder was dispersed in 14 wt.-% aqueous NaOH (4 M), and the resulting solution was designated as “casein pH > 14”.

#### Casein solutions containing NaCl

To simulate the  $\text{Na}^+$  concentrations existing in NaOH solutions at pH = 13, 14 and > 14, 0.08 g, 0.87 g and 2.33 g of NaCl salt were added to 10 mL of  $1 \text{ g L}^{-1}$  casein solutions possessing a pH of 12. This results in solutions which are 0.15 M, 1.5 M and 4 M, relative to NaCl. The volume change of the solutions associated with the addition of NaCl was ignored.

#### Ca-depleted casein

Ca-depleted casein was prepared as follows: 3 g of casein powder was dissolved in 200 mL of 0.8 M aqueous urea which contained 0.3 g of  $\text{EDTA-Na}_2 \cdot 2\text{H}_2\text{O}$ . The resulting solution was stirred for 2 h at room temperature and after-

wards centrifuged for 5 min at 9600 rpm. The supernatant was dialyzed using a cross-flow filtration system (Quixstand; GE Healthcare, Munich/Germany) to remove urea and salt. The protein solutions were pumped in a circuit through a hollow fiber cartridge (UFP-3-C-3X2MA, 3 kDa molecular weight cut-off) under a system pressure between 0.207 and 0.275 MPa. The dialyzed sample was concentrated under vacuum and then lyophilized in a laboratory freeze dryer, finally resulting in the dried Ca-depleted casein.

The same procedure was applied to produce Ca-depleted casein in solutions at pH = 12, 13, 14 and > 14.

### Methods

#### Turbidity

The turbidity of colloidal casein solutions was measured at 600 nm wavelength on a SpectroFlex 6100 photometer (WTW, Alès/France) using 1 cm path-length quartz cuvettes. The turbidity was calculated according to Eq. 1, where  $I$  and  $I_{\text{ref}}$  are the intensities of the transmitted light for the casein dispersions and the de-ionized water reference, respectively, while  $L$  is the path length of the cuvette.

$$\tau = -\frac{1}{L} \ln \frac{I}{I_{\text{ref}}} \quad (1)$$

All measurements were conducted at room temperature.

#### Transmission electron microscopy (TEM)

TEM images were recorded on a JEM 100 CX microscope (Jeol Ltd., Tokyo/Japan) equipped with a tungsten cathode. For sample preparation, casein dispersions were dripped onto a thin carbon support, allowed to rest for 30 s followed by washing and negatively staining for 1 min in 1 wt.-% ammonium molybdate. Imaging was carried out under an accelerating voltage of 100 kV.

#### Atomic absorption spectroscopy (AAS)

The calcium content in casein was determined employing an atomic absorption spectrophotometer (Perkin Elmer 1100 B, Überlingen/Germany). For sample preparation, 6 M urea was added as chaotropic solvent to  $1 \text{ g L}^{-1}$  casein solutions. Additionally, an excessive amount of  $\text{EDTA-Na}_2$  was added to overcome the interference of phosphate bound to calcium in solution.

#### Anionic charge of casein

The anionic charge density of casein particles existing at different pH values was measured using a PCD-03 particle charge detector (BTG Müttek GmbH, Herrsching/Germany). Cationic poly(diallyl dimethyl ammonium chloride) (polydadmac) (BTG Müttek GmbH, Herrsching/Germany) was used as counter polymer for polyelectrolyte titration. In each experiment, 10 mL of the casein solution were filled into the



measuring cell and titrated with 0.001 N polydadmec solution until the isoelectric point was reached. Afterwards, the specific anionic charge density of casein was calculated according to Eq. 2 [27], where  $q$  is the specific anionic charge density ( $\mu\text{eq g}^{-1}$ ),  $V$  is the consumed volume of polydadmec solution (L) during titration,  $c$  is the concentration of polydadmec (here 0.001 N, equivalent to  $0.001 \text{ eq L}^{-1}$ ), and  $w$  is the mass (g) of casein contained in the solution.

$$q = \frac{Vc}{w} \times 10^6 \quad (2)$$

#### Fast protein liquid chromatography (FPLC)

An ÄKTA Explorer (GE Healthcare Munich/Germany) was employed for FPLC. A 1 mL resource Q column (GE Healthcare Munich/Germany) was used as ion exchange medium, and the absorption was monitored at 280 nm on a UV detector. More details, e. g. buffer preparation and chromatographic conditions, can be found in earlier publications [23, 28].

#### Scanning electron microscopy (SEM)

SEM micrographs were taken under high-vacuum conditions ( $10^{-5}$  Torr) on an FEI XL 30 microscope (FEI, Eindhoven/Netherlands) equipped with a large field detector.

For sample preparation, a drop of casein solution ( $c_{\text{casein}} = 1 \text{ g L}^{-1}$ ) possessing a pH of 12 was placed on the carbon tape of the aluminum holder, and allowed to dry under  $\text{N}_2$  flow. Prior to imaging, the sample was sputtered with a thin layer of Au ( $\sim 15 \text{ nm}$ ) to provide conductivity. Afterwards, images were taken at an acceleration voltage of 20 kV.

#### Atomic force microscopy (AFM)

AFM images were acquired using a Nanoscope IIIa scanning probe microscope (Veeco Instruments, Mannheim/Germany). The microscope was operated in tapping mode using a silicon cantilever with a resonance frequency of 320 kHz, a driving amplitude of 1.25 V at a scan rate of 1.0 Hz, and a tip consisting of P-doped silicon (radius less than 10 nm). For sample preparation, a drop of casein solution ( $c_{\text{casein}} = 1 \text{ g L}^{-1}$ ) with pH = 12 was placed onto a carefully cleaned silicon wafer (Wacker AG, Burghausen/Germany) and allowed to dry in  $\text{N}_2$  flow. The sample was then placed on the AFM stage and imaged in air.

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