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The pH Induced Sol−Gel Transition in Skim Milk Revisited. A Detailed Study Using Time-Resolved Light and X-ray Scattering Experiments

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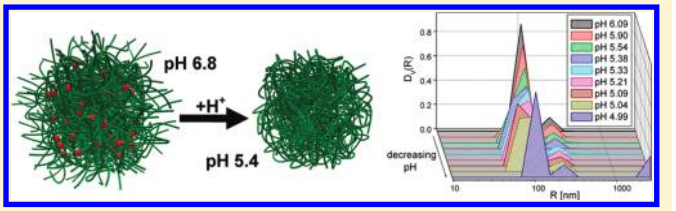
Supporting Information

ABSTRACT: We present a detailed study of the evolution of the size, structure and stability of casein micelles upon acidification of skim milk typically applied in yogurt-making processes using a combination of time-resolved light and small-angle X-ray scattering experiments. While most of the available light scattering studies on casein acidification have been restricted to transparent and therefore highly diluted samples, we now profit from a newly developed multiangle 3D light scattering instrument, which allows for time-resolved measurements in highly turbid samples. Our experiments clearly demonstrate the presence of two parallel pH-dependent processes, micellar reassembly and aggregation. Using a systematic investigation of the effect of casein concentration, acidification rate, and ionic strength, we are able to decouple these two processes and obtain detailed information about the pH-induced restructuration of the casein micelle structure that occurs prior to destabilization. Moreover, our experiments also unambiguously demonstrate that these micellar reassembly processes are highly concentration dependent, and that typical light scattering studies conducted under highly diluted conditions are resulting in findings that may not be relevant for the situation encountered in industrial processes at higher concentrations. Experiments conducted with covalently cross-linked micelles, where the pH-induced reassembly has been suppressed, further confirm our findings.



1. INTRODUCTION

Skim milk is a colloidal suspension of casein micelles, formed via self-assembly of four casein proteins and calcium phosphate, which are dispersed in an aqueous solution of salts, lactose and whey proteins.[1,2] The casein micelles are spherical with a relatively broad size distribution between about 30 to 300 nm in radius.[1] The high colloidal stability of the casein micelles—i.e., the stability against intermicellar aggregation—derives from a surface brush of κ-caseins extending their hydrophilic parts into the solution.[3,4] This steric layer can be modeled as a polyelectrolyte brush because each molecule carries about 14 carboxylic acid groups which are dissociated at physiological pH. Because of the high ionic strength in skim milk (80 mM),[5] the charges are highly screened and contribute only little to the colloidal stability of the micelles via direct electrostatic repulsion. However, the charges are promoting the solubility of the polymer and thus the steric layer can be viewed as a salted brush and the stabilization as electrosteric.[6]

The structure of casein micelles has been intensively investigated in the past, and numerous models have been postulated.[7−12] In a recent development, the internal assembly and structure of the casein micelle has been described by the dual-binding model.[8] Here two distinct forms of binding are involved in the micellar assembly. These are on the one hand attractions between the hydrophobic regions of the caseins and on the other hand cross-links between phosphoserine clusters of α_{S1}-, α_{S2}-, and β-caseins and colloidal calcium phosphate clusters. This leads to a growing protein network that is then terminated by the binding of κ-casein molecules, which do not possess phosphoserine clusters. The overall structure of the casein micelle can then be seen as a quite loose spherical protein network that resembles polymeric microgels rather than homogeneous colloids, stabilized by a polyelectrolyte brush formed by the surface layer of κ-caseins.

Gel formation is an important process used in a variety of industrial applications. The different processes used in the production of milk gels all rely on the elimination of the electrosteric stabilization of the casein micelles. In the production of cheese, the hairy layer is cut off by the proteolytic action of chymosin during renneting. In the yogurt making process the pH of the milk is lowered by the action of lactobacilli, which convert lactose into lactic acid. Because of the shift in pH the hairy layer loses its charge and eventually collapses onto the surface of the micelle.

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Analogies have been drawn to aggregation and gelation in colloidal suspensions in order to better understand these processes. On the basis of this analogy, the casein micelles can thus be regarded as adhesive spheres with a very strong repulsion at very short distances and a subsequent short-range attraction.[13−15] The attractive well changes in depth as a result of external influences during the destabilization. The produced gels are often considered as particle gels where the integrity of the micelles is still preserved in the final gel.[16] However, especially in the case of acidification there are significant deviations from these simple models. The shift in pH does not only influence the stabilizing polymer brush but has also other effects. First of all, the colloidal calcium phosphate starts to dissociate because of increased aqueous solubility.[17,18] Since this material is largely responsible for maintaining the integrity of the micelle, its dissociation in turn causes dissociation of individual casein molecules. The loss of negative charges of the proteins eventually reverses the process and leads to intramicellar precipitation followed by aggregation and gelation. The ongoing intramicellar rearrangements thus cannot be described by simple adhesive sphere and particle gel models, which assume constant internal structure of the particles involved.[9] Moreover, this might also have effects on the macroscopic properties of the final product. It was shown by Horne that the evolution of the shear modulus in rennet gels agrees well with the predictions of the mean field model while the acid gels do not.[19] This was attributed to the internal changes in the micelle, the loss of calcium and phosphate and the changes in localized net charge, during the shift in pH.

Here we are presenting detailed investigations on the sequence of internal structural rearrangements and aggregation phenomena that occur during skim milk acidification. While in the case of renneting only a slight decrease in size due to the cutoff of the κ-casein layer can be observed,[20−23] the behavior during acidification is much more complex. We use a combination of light and X-ray scattering methods that allow for a time-resolved and in situ investigation of the temporal evolution of the micellar size and internal structure during the early stages of the acidification and gelation process. Light scattering experiments have previously not been possible for measurements at concentrations typically used in milk processing due to the high turbidity. Here we use a novel instrument that also allows for time-resolved static and dynamic light scattering in turbid samples.[24] Our newly developed multiangle 3D light scattering instrument is based on a cross-correlation scheme that efficiently suppresses multiple scattering and thus permits us now to investigate the micellar size distribution and the micellar structure as a function of pH in samples that have previously not been accessible for such studies.

These experiments provide us with convincing evidence that the structural rearrangements upon acidification are indeed different if the milk is diluted in its own serum. Therefore, it is important to look at the process at its natural concentration, which for milk is in the regime of excessive multiple scattering. We found a decreasing particle size as the pH is lowered. The drop in size is accompanied by a simultaneous loss of mass. This is interpreted as the effect of dissolution of the colloidal calcium phosphate and subsequent loss of caseins. The effect is very pronounced if the milk is diluted and much less distinct in undiluted milk, where the micelles are shrinking in size mainly due to a reduced net charge of the proteins. We show that there is a redistribution of the scattering mass within the micelle during acidification. At neutral pH the mass is rather concentrated in the center of the micelles. At lower pH they become smaller and more homogeneous. Changes in the size distribution as a function of the pH and the influence of salts are studied as well. Our experiments also show that the acidification rate has an influence on the degree of dissolution of the casein micelles. Faster acidification reduces the rate of dissolution of the micelles. The aggregation in turn sets in at the same pH at all investigated acidification rates. This demonstrates the presence of two parallel processes, one being the internal reassembly of the micelles and the other one being the particle−particle aggregation due to a collapse of the stabilizing electrosteric layer.

2. EXPERIMENTAL SECTION

A. Skim Milk Preparation. A 9.46 wt % sample of Nilac skimmed low-heat milk powder (NIZO, The Netherlands) was dispersed in Milli-Q water at room temperature and stirred at 45 °C for 1 h. This leads to a protein solution with a concentration of 3.47 wt % and a casein concentration of 2.78 wt %, referred to as "undiluted milk". After adding 0.02 wt % of sodium azide the solution was kept for 12 h at 4 °C. For lower casein concentrations the skim milk was diluted in its own serum. The nomenclature of the diluted samples is $(x + y)$, where x is the relative volume of stock solution and y the relative volume of serum. The serum was obtained by ultrafiltration through a regenerated cellulose membrane (YM10, MWCO 10 000 Da) from Millipore Corp. (Billerica, MA). Prior to acidification the milk was allowed to equilibrate at least for 1 h at 25 °C. The pH shift was induced by the addition of glucono-δ-lacton (GDL, ≥99%, Sigma).

Cross-linked casein micelles were obtained by adding a 2 wt % transglutamine (Activa WM, 100U/g, Ajinomoto Foods Europe SAS) solution to a whey protein free skim milk (Micellar Caseins produced by the Hungarian Dairy Research Institute, MPI-85MC, Lot 07031). The activity of the enzyme in the milk was set to 50 U/g casein. The sample was incubated for 1 h at 40 °C. To inactivate the enzyme the milk was heated for 30 min at 90 °C. After cooling under tap water the sample was stored at 4 °C.

B. Methods. All experiments were performed at a temperature of 25 °C. The light scattering experiments were done in a home-built multiangle instrument which fully implements the 3D-cross-correlation scheme.[25−28] It allows for time-resolved measurements at four angles simultaneously. The 3D-cross-correlation technique suppresses multiply scattered light, and thus allows for detecting scattered intensities and correlation functions which originate from singly scattered light only. A Coherent Compass 415M-200 diode-pumped solid-state laser (532 nm) was used as light source. The primary laser is split in two parallel beams, which are both focused into the same scattering volume within the sample cell (cylindrical cuvettes, inner diameter 2.4 mm, immersed in a decalin index matching vat). At each angle two optical fibers were placed exactly at the same q-vector relative to the incoming beams. The collected light was detected by eight photomultiplier tubes (four angles with two detectors each). To correct for fluctuations in the primary laser intensity a part of the laser was split off and detected separately by a photodiode. The signals of each pair of detectors at the same angle were cross-correlated to obtain four intensity correlation functions. Scattered intensities corrected for multiple scattering were then obtained following established procedures using the reduction in signal intercept as a measure of the degree of multiple scattering.[27] To set the static scattering curve on an absolute scale, the detectors were calibrated by a toluene reference measurement. The transmission, measured with a power meter (PM100 from Thorlabs), was used to correct for the loss of singly scattered intensity in turbid samples. Because of the relatively small sample cell, the transmission was measured separately in a dedicated setup using flat cells with a thickness of 1 mm in parallel to

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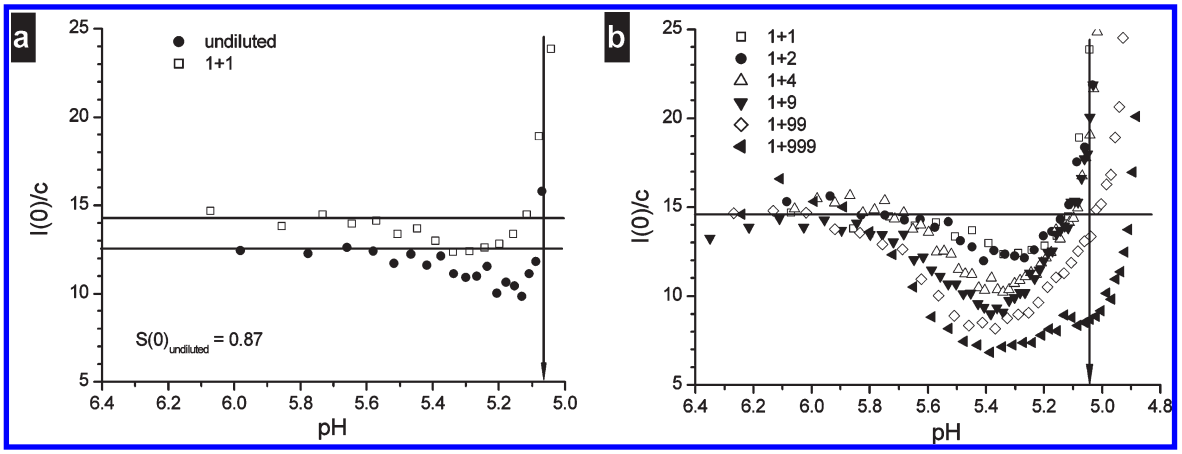

Figure 1. Evolution of the concentration normalized forward scattering intensity $I(0)/c$ as a function of pH during acidification for different casein concentrations. The experiments were started at neutral pH. With progressing time the pH shifts toward lower values. The vertical arrows in both graphs indicate the pH at which $I(0)$ starts to significantly increase in undiluted milk due to aggregation. (a) Results for the two highest concentrations. Because of repulsive interactions $I(0)/c$ of the undiluted sample is lower compared to the diluted samples. Here dilution $(1 + 1)$ is shown. (b) Results for different degrees of dilution. At higher dilution values $I(0)/c$ does not change significantly at the onset of acidification, i.e., at neutral pH. However, the full curves $I(0)/c$ vs pH now exhibit a strong dependence on casein concentration.

the scattering experiment in order to avoid systematic errors due to lensing effects caused by the high curvature of the small cylindrical scattering cells. Thus, we are able to measure static scattering intensities on an absolute scale even in highly turbid samples. A detailed description of the instrument used is given elsewhere.[24] The forward scattering intensity $I(0)$ was determined by a Guinier extrapolation[29] of the q-dependent intensities toward a scattering angle of zero. A part of the sample was used to record the pH as a function of time during the acidification process with a pH-meter (pH 2100 series, Oakton Instruments, IL, USA).

The small-angle X-ray scattering experiments were performed at the Swiss Light Source (SLS of the Paul Scherrer Institute) at the cSAXS instrument. The samples were filled in quartz capillaries with a diameter of 1 mm and a wall thickness of 0.01 mm. The incident beam had a wavelength of 0.101 nm (12.24 keV) and a size of 202 × 18 μm. The scattering pattern was recorded at a sample−detector distance of 7.05 m by a PSI-developed Pilatus 2 M detector (1475 × 1679 pixels of 172 × 172 μm) which operated in single-photon counting mode and thus allowed operation without read-out noise. At least 50 2D images were taken, azimuthally integrated and corrected according to established procedures provided by the PSI. The q-scale was calibrated by a measurement of silver behenate. No absolute calibration was done for the X-ray data.

3. RESULTS AND DISCUSSION

A. Influence of Dilution. In the yogurt making process, the pH of the milk decreases from about 6.8 until the electrosteric layer of the casein micelles collapses and they start to aggregate and form a gel. Rheological measurements are normally done at the natural casein concentration, but provide macroscopic information only. Investigations of the acid-induced changes by light scattering on the other hand give access to the properties of the single particles, but usually require a significant dilution of the sample in order to avoid multiple scattering. Thus, we now aim at extending the applicability of light scattering to high concentrations of industrial relevance. As one of our primary tasks is also to validate the previously made investigations at lower casein concentrations, we first investigate the influence of the casein concentration on the processes occurring upon acidification. GDL was used in order to slowly reduce the pH of the skim milk samples in a controlled fashion that allows for parallel in situ detection of the changes in the micellar properties in response to the decreasing pH. The use of GDL instead of lactobacilli, which are usually used for the yogurt production, makes the experiments much more reproducible. The cyclic ester GDL slowly hydrolyses to the corresponding carboxylic acid when solved in water and thus causes a pH shift. The GDL concentration was varied to reach pH 5 after approximately 3 h at all casein concentrations investigated ($(1 + 0)$: 1.5%, $(1 + 1)$: 1.2%, $(1 + 2)$: 1.0%, $(1 + 4)$: 0.8%, $(1 + 9)$ to $(1 + 999)$: 0.6% GDL, where in $(x + y)$ x indicates the amount of the stock solution and y the amount of the added serum in milliliters).

Figure 1, where the concentration normalized forward scattering intensity, $I(0)/c$, is plotted as a function of pH, clearly demonstrates the considerable effect of dilution on the micellar properties during the early stages of the acidification process. We monitor the forward scattering intensity $I(0)$ as a measure of the aggregate mass M of the scattering particles, the overall mass concentration c, and the interactions between the particles. Here interaction effects on $I(0)$ are linked to the structure factor $S(0)$ through $I(0) \propto NM^2S(0)$ or $I(0) \propto cMS(0)$, where N is the number concentration of particles and c the mass concentration given by $c = NM$. We observe that in skim milk at its natural concentration the forward scattering decreases upon acidification by approximately 20% until aggregation sets in (Figure 1a). If the milk is diluted in its own serum, which is usually done to avoid multiple scattering, the resulting $I(0)$ versus pH curves look significantly different. As for the concentration-normalized scattering intensity $I(0)/c$ differences are related either to changes in the average molar mass of the micelles or to interaction effects, we have to distinguish between three different effects as possible sources for the evolution of $I(0)$: (i) intermicellar interaction effects ($S(0)$); (ii) intramicellar rearrangements; (iii) onset of aggregation.

The effects from intermicellar interactions are best estimated at neutral pH, where previous studies have demonstrated that casein micelles behave very similarly to colloidal hard spheres up to quite high concentrations.[30,31] At a weak $(1 + 1)$ dilution, $I(0)/c$ is shifted to higher values while the shape of the curve remains unchanged (Figure 1a). The slightly lower $I(0)/c$ of the

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undiluted milk compared to the diluted ones most probably arises from the influence of the structure factor $S(0)$, whose effect completely vanishes at higher dilutions. At neutral pH, where the casein micelles are known to behave like hard spheres, we can quantitatively estimate the effect of $S(0)$ using the corresponding polydisperse hard sphere model employing liquid state theory and the commonly used Percus−Yevick closure relation.[32,33] Details about the calculation can be found in the Supporting Information (chapter A). The results of the calculations are values of $S(0) = 0.52$ for undiluted milk, and $S(0) = 0.72$ for a dilution factor of 2, in agreement with our measurements. At higher dilutions there is no significant further change in $I(0)/c$ at neutral pH. This clearly demonstrates that the strong effects of dilution that are visible in Figure 1 cannot be the result of interaction effects, but reflect concentration-dependent restructuration and aggregation events.

Some concentration-dependent changes in the evolution of $I(0)$ are easy to directly associate with the aggregation process initiated by the loss of the stabilizing power of the salted brush at low pH and can thus be interpreted by making an analogy to colloidal model particles. The steep increase at low pH, which appears due to the onset of aggregation, shifts to lower pH if the milk is diluted 100 times or more. This shift most probably arises from a lower collision probability of casein micelles at very high dilution. In general, the aggregation rate for completely destabilized particles in the so-called fast aggregation regime decreases linearly with concentration. This results in a characteristic aggregation time that is 1000 times slower for the most diluted sample when compared to the milk at its natural concentration. At the later stages of the acidification process the dilution obviously does not affect the behavior of the casein micelles other than through a simple colloid-like reduction of the collision probability.

However, the situation is completely different at the early and intermediate stages of the acidification, where the pH dependence of $I(0)/c$ is distinctly different from the behavior encountered for destabilized synthetic colloids. While for destabilized colloids $I(0)/c$ increases monotonically with time or degree of destabilization, for the casein micelles, we first observe a well-pronounced decrease followed by a distinct minimum that indicates intramicellar rearrangements prior to aggregation This minimum of $I(0)/c$ is getting deeper and shifts to larger pH values (undiluted at a pH of about 5.2, $(1 + 999)$ at about pH 5.4) if one is diluting the milk sample. We tentatively interpret the minimum as a result of the partial loss of the internal integrity of the micelles due to the dissolution of colloidal calcium phosphate, followed by a reassembly due to reduced charge of the individual casein proteins that form the constituents of the micelles at even lower pH. The pronounced concentration dependence might be explained by the finite solubility of calcium phosphate and the existence of a corresponding equilibrium distribution that results in a concentration dependence of the rates of change of the micellar structure and composition with pH. It would of course be the ultimate goal to quantitatively understand the casein self-assembly and to model the binding effect within the casein micelles due to the phosphate clusters. We assume that at neutral pH the Ca^{2+}-concentration in the milk serum is in equilibrium with the Ca−phosphate clusters. A dilution with serum at this pH does therefore not lead to any dissolution of Ca−phosphate. At decreasing pH, however, it is known that the solubility of Ca−phosphate increases. While we can use this information qualitatively to interpret the pH-dependence and the influence of dilution on the rate of dissolution, we lack the necessary quantitative information to attempt any model building. For this we would need to monitor the Ca^{2+}-concentration in the serum during the process, which was not done in the current study. It will thus be vital to combine time-resolved scattering experiments with an in situ determination of pH and Ca^{2+}-concentration during similar acidification experiments at different casein concentrations in the future. These results clearly demonstrate that it is dangerous to investigate highly diluted solutions if one is interested in the behavior of milk at its natural concentration. Nevertheless, it is still possible to gain important insight into the general behavior of casein micelles by investigating these highly interesting concentration dependent phenomena. In the following sections we therefore show how the additional information that can be obtained from time-resolved multiangle light scattering measurements can be used to arrive at a detailed understanding of the different restructuring and aggregation processes that occur upon acidification of skim milk.

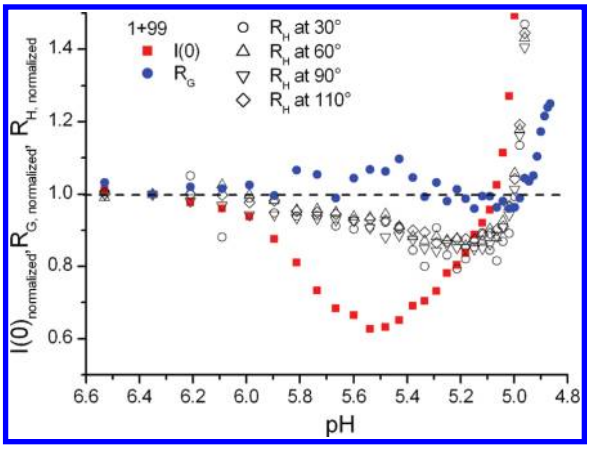

Figure 2. Forward scattering intensity $I(0)$, radius of gyration R_G and hydrodynamic radii R_H as a function of pH for the acidification of skim milk which was diluted 100 times in its own serum. All parameters were normalized to a value of 1 at the beginning of the experiment.

B. Changes of Micellar Size and Internal Structure as a Function of pH. While the reduced forward intensity $I(0)/c$ only yields information about the pH dependence of the average overall mass of the particles present and thus does not allow us to obtain a detailed and unambiguous model of the reassembly processes that occur during acidification, our multiangle instrument also provides information on the hydrodynamic radii R_H at different angles and the radius of gyration R_G as measures of the overall size and structure of the casein micelle as a function of pH. In Figure 2 the whole data set is shown for a series performed at a relatively high dilution $(1 + 99)$. As we are interested in the evolution of the different parameters as a function of pH rather than the absolute values of R_H and R_G, all quantities are normalized by their value at a pH of about 6.3. One can clearly see that not only $I(0)$ but also R_H, which is a measure for the physical size of the particles, drops with decreasing pH. However, while in these experiments $I(0)$ decreases by 40%, R_H decreases by 15% only. In contrast to the average overall mass and the micellar radius given by R_H we see from Figure 2 that R_G, which provides information on the internal distribution of the scattering mass, remains approximately constant until aggregation sets in. Given the fact that for homogeneous spheres we expect a constant ratio $R_G/R_H = 0.775$, this is at first sight a rather unexpected finding. Figure 2 shows that the ratio R_G/R_H changes with decreasing pH

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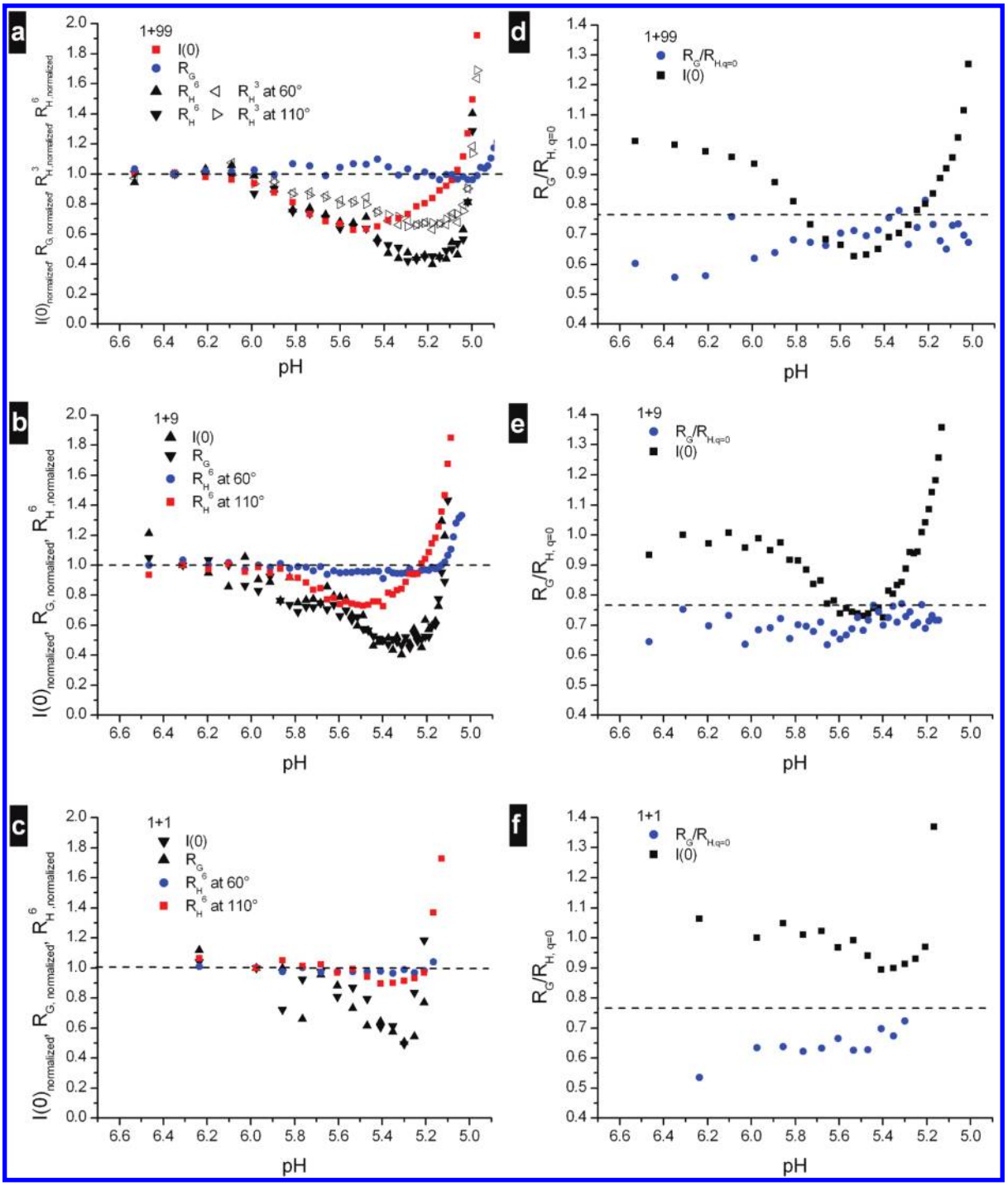

Figure 3. (a) Comparison of the forward scattering intensity $I(0)$ with the hydrodynamic radii R_H to the power of 3 and R_H to the power of 6. The initial decrease of $I(0)$ closely follows the curve R_H^6, thus indicating a decrease in micellar mass at constant number of micelles (see text for details). (a, b, and c). Forward scattering intensity $I(0)$, radius of gyration R_G and hydrodynamic radii R_H to the power of 6 for the acidification of skim milk, at varying concentrations of casein. All parameters were normalized to a value of 1 at the beginning of the experiment. (d, e, and f) Parameter R_G/R_H as a function of the pH in the acidification of skim milk at varying casein concentrations. For comparison the forward scattering intensity is shown as well. The dashed line indicates the value which is expected for a homogeneous sphere, lower values are a sign of inhomogeneous particles with a scattering mass which is rather concentrated in the center.

and thus indicates a redistribution of mass within the particles or a change in shape as the acidification proceeds.

Our experiments thus show that the acidification of skim milk not only results in an initial decrease of the average micellar mass and radius, but also in a redistribution of the mass within the micelles. In a next step we thus have to analyze these changes in a quantitative way and try to build a structural model that is capable of providing a self-consistent explanation for the concentration and pH dependence of all the data. First we need to understand the influence of the pH on the micellar size and mass during the

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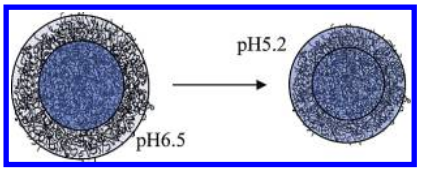
initial stage of the acidification process. We start from a simple model where we assume the micelles at all stages to be spherical with a constant mass density profile. While we already know from the pH dependence of the characteristic ratio R_G/R_H that this is not correct, it nevertheless provides us with a good starting point in our attempt to quantitatively connect the initial pH dependence of $I(0)$ and R_H. Given the self-assembled nature of the casein micelles, there are then two possible scenarios to consider in the framework of this primitive model. In the first scenario, we consider dissolution of parts of the casein micelle material into individual monomers (casein proteins), which would then no longer contribute significantly to the scattering signal. This would lead to a constant number concentration of casein micelles, however with a smaller micellar size and mass. Given the fact that the scattering intensity from a single particle is proportional to V^2 or M^2, where V and M are the volume and the mass of the particles, respectively, $I(0)$ should then be proportional to ${R_H}^6$ during this initial restructuring period. In a second possible scenario we assume that the micelles split up into smaller units, with the same total concentration of protein mass present in micelles, but with a smaller mass of the individual micelles. Because of the fact that the scattering intensity from an ensemble of particles with weight concentration c and mass M is proportional to cM, $I(0)$ should then be proportional to ${R_H}^3$.

In order to differentiate between these two cases we can compare the evolution of $I(0)$ with ${R_H}^3$ and ${R_H}^6$, respectively. In Figure 3a, the corresponding data for a $(1 + 99)$ dilution are plotted for two selected angles (60 and 110 deg). One clearly sees that the overlap of the initial decay of $I(0)$ is much better with ${R_H}^6$ than with ${R_H}^3$. This indicates that the first proposed mechanism that leaves the number concentration of the micelles unmodified, i.e., the dissolution of small units of material from the individual micelles, caused by the loss of colloidal calcium phosphate at lower pH, may be responsible for the reduction of the micellar size and mass.

After reaching a pH of about 5.5, $I(0)$ starts to increase while the size is still decreasing. This can be explained by a reassembly of the previously dissolved casein molecules, who now gradually lose their charge and become more hydrophobic. The increasing mass of the scattering particles is reflected by the increasing forward scattering intensity. At the same time, the size is still decreasing. This is also due to the decreasing charge of the proteins. With decreasing pH the net charge is getting smaller and allows the network to shrink. A very similar effect is frequently found for cross-linked microgels. The shrinking is easily possible because the casein micelle is a highly swollen object, containing about 75% of solvent at neutral pH. The increasing mass of the micelles leads to an increase of $I(0)$ even if the size is getting smaller due to compaction. Finally, if the micelles recover their original mass one would expect $I(0)$ to reach the starting value regardless of their size. It is important to point out that these changes observed during the pH shift cannot be caused by changing the interaction potential as for a decrease in repulsion both $I(0)$ as well as the apparent hydrodynamic radius R_H should increase. Furthermore, these effects should be much more prominent for undiluted or weakly diluted samples and absent for highly diluted samples.

The assumption that there is a change in the internal mass distribution is confirmed by the evolution of R_G. The ratio R_G/R_H, where R_H was extrapolated to a scattering angle of zero, is a measure for the inhomogeneity of the particles (provided the particles are spherical). In Figure 3d, it is shown that at the beginning of the experiment the value for R_G/R_H is rather low (approximately 0.6) which means that the scattering mass is rather concentrated in the center of the micelle and the outer parts of the network are rather loose. With decreasing pH, the ratio increases and finally reaches almost the value one expects for a homogeneous sphere (0.775). This means that during these initial stages of skim milk acidification the casein micelles are getting smaller and more homogeneous with a higher average density. This is shown schematically in Scheme 1.

Scheme 1. Decreasing Size and Redistribution of Mass within the Casein Micelle upon Acidification



We have not only investigated the evolution of the average size and the radius of gyration, but also determined the size distribution as a function of pH. The details of this analysis can be found in the Supporting Information, chapter B.

We now move on to higher casein concentrations, where we see that the hydrodynamic radius is decreasing as well. Actually, the decrease in particle size seems to be basically independent of the concentration. Skim milk that was diluted 10 times (Figure 3b) still shows a distinct minimum in $I(0)$ at pH 5.5. However, the decrease is less pronounced than for the higher dilutions. R_H decreases about 15% similar to the samples which were diluted 100 and 1000 times. At even higher casein concentrations $(1 + 1$, Figure 3c) the minimum in $I(0)$ nearly vanishes. However, like in the diluted samples, the decrease in R_H is about 15%. The very shallow minimum in $I(0)$ points at only a small loss of mass of the scattering particles. The observed reduction of size thus originates mostly from shrinkage of the micelles. This happens because of the reduced net charge of the protein network at decreasing pH and the corresponding decrease in solvent quality. Contrary to the diluted samples, where we observe a considerable loss of material, the integrity of the micelles seems to be well preserved at high concentrations.

In the case of the undiluted milk one also faces the problem of the significant contribution of the structure factor, which changes as the particle size and the effective volume fraction are altered. Therefore, only the diluted samples up to a dilution of $(1 + 1)$ are quantitatively discussed here.

The information that can be obtained from light scattering is limited to overall quantities such as M, R_G and R_H due to the accessible q-range. The situation is quite different for SAXS, where larger q-values, corresponding to smaller distances in real space, are probed. Small-angle X-ray scattering can thus be used to determine the internal structure of the casein micelles. In the Supporting Information, chapter C, a description of the SAXS measurements, the evaluation method and a discussion of the results can be found. The analysis of the SAXS data of casein micelles provides us with an estimate of the radial scattering length density distribution $\Delta\rho(r)$. The thus obtained $\Delta\rho(r)$ indicates a core−shell structure with a rather dense core and a rather loose shell in neutral milk (pH 6.17). When the pH is shifted to lower values the core becomes less dense and eventually, when a pH of about 5 is reached, one gets an almost

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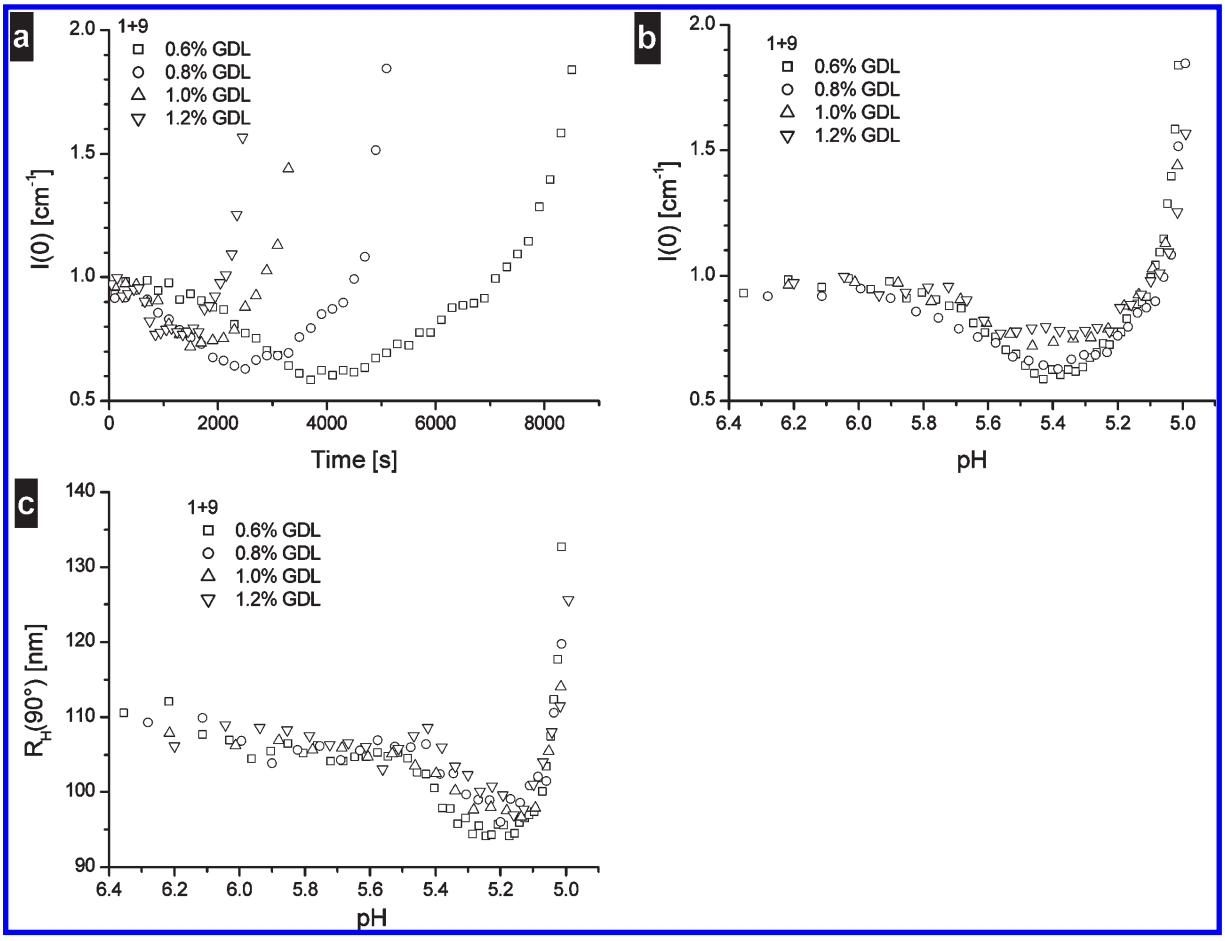

Figure 4. (a) Forward scattering intensity $I(0)$ versus time as a function of the acidification rate (concentration of GDL). (b) $I(0)$ versus pH as a function of the acidification rate. (c) Hydrodynamic radii versus pH as a function of the acidification rate.

constant contrast profile. In summary the results from X-ray scattering experiments are in perfect agreement with the light scattering. The fact that different types of radiation, different q-regimes, and a completely different type of data analysis were used for both methods gives strong support for the validity of the results.

C. Kinetics of Micellar Rearrangements versus Aggregation. In Figure 4 experiments carried out at different rates of acidification (induced by different GDL concentrations) are shown. One can clearly see that the minimum in $I(0)$ gets less pronounced if the process is faster (Figure 4a). If the same data is plotted versus pH instead of time (Figure 4b), it becomes obvious that the onset of aggregation occurs at the same pH at all rates. Only the depth of the minimum is changing, indicating that the system is not completely in equilibrium in the case of relatively fast acidification. A similar dependence on GDL concentration can be observed for R_H (Figure 4c). These experiments clearly indicate that intramicellar rearrangements and intermicellar aggregation are largely independent. The intramicellar rearrangement processes require the dissolution of calcium phosphate clusters that act as internal cross-links, the diffusion of individual casein proteins out of the internal network structure and the internal rearrangement of proteins and are thus relatively slow. The colloidal stability with respect to intermicellar aggregation and sol−gel transition however depends on the state of the κ-casein brush only and thus adjusts almost instantaneously to the actual pH value. While intramicellar rearrangements may also alter the strength of the attraction between micelles and thus the resulting strength of the final protein gel, the onset of aggregation is determined by the loss of colloidal stability due to the κ-casein brush collapse only. These findings will be further supported by experiments investigating the influence of the ionic strength and using internally cross-linked casein micelles shown below.

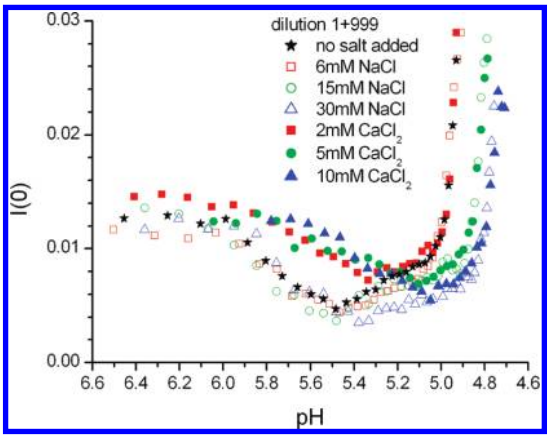

Figure 5. Forward scattering intensity for the acidification of skim milk at high dilution with varying concentrations of salt added (NaCl, open symbols; $CaCl_2$, filled symbols). The symbols in the same color represent experiments carried out at the same ionic strength.

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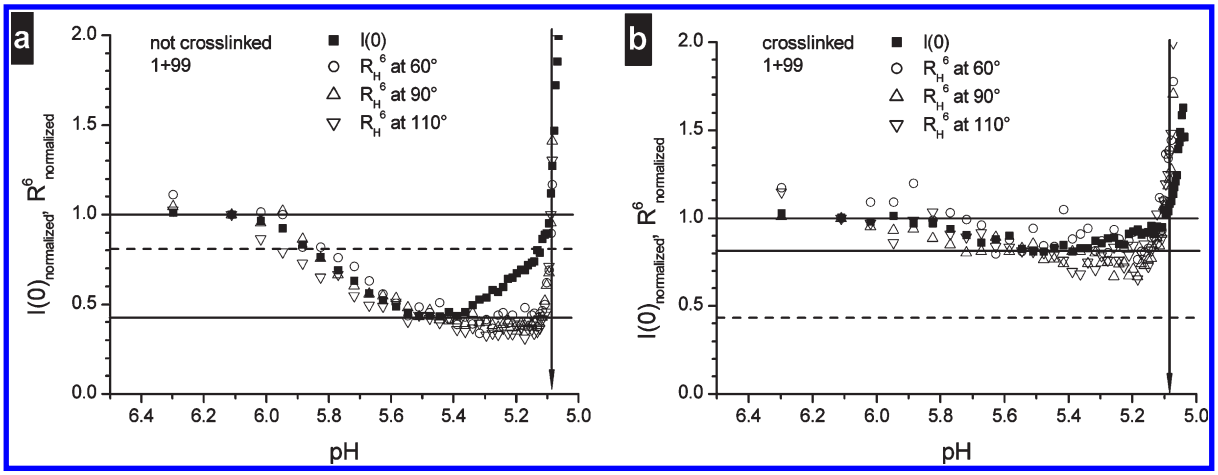

Figure 6. Forward scattering intensity $I(0)$ and hydrodynamic radii R_H during the acidification of casein micelles, which were diluted 100 times in their own serum. All parameters were normalized to a value of 1 at pH 6.1. (a) Reference sample, no enzyme was added but the same temperature sequence was applied. (b) Casein micelles, where covalent cross-links between the proteins within the micelles were introduced enzymatically.

D. The Effect of Salt. We have further investigated these two processes by studying the effect of added salt. Here we have monitored the dependence of the forward scattering intensity $I(0)$ as a function of pH for two different types of salt, NaCl and $CaCl_2$, at three different values of the ionic strength. As shown in Figure 5, we observe two completely different regimes at high and low pH, respectively. At pH values between 7 and 5, it is mainly the nature of the salt that plays a dominant role. Here the presence of Ca^{2+} ions seems to reduce the pH-induced intramicellar rearrangements, presumably via a corresponding shift in the Ca^{2+} equilibrium that reduces the dissolution of the internal calcium phosphate clusters and thus helps to preserve the micellar integrity. The situation changes quite dramatically at lower pH. Here we see a clear effect of the overall ionic strength on the onset of micellar aggregation, while the nature of the cation does not seem to matter as the curves for the two types of salt of equal ionic strength now overlap. While the fact that a higher salt concentration seems to stabilize the micelles against aggregation appears surprising at first sight, it simply reflects the titration curves of classical carboxylated colloids.[34] Here one observes that for a given pH the surface charge density of the particles is larger at higher ionic strength. While for normal charge-stabilized colloidal particles the stability against aggregation or flocculation is nevertheless dominated by the ionic strength and we thus observe a lower stability for higher ionic strength at a given pH value, the situation apparently is different for electrosterically stabilized particles such as the casein micelles. Here it is the solubility of the κ-casein brush that determines the stability, which is directly linked to the number of charges on the chains and thus to the titration curve, while the intermicellar screening by the salt has no measurable influence. These findings have also been corroborated by a subsequent investigation of the stability ratio of the casein micelles as a function of pH and ionic strength.[35]

E. Internally Cross-Linked Casein Micelles. While our experiments have clearly indicated that intramicellar rearrangements and intermicellar aggregation appear to be rather independent processes, the presence of micellar rearrangements nevertheless makes it difficult to directly study the aggregation process in analogy to well-known model colloid systems.[34,36] To allow for the application of established procedures to analyze aggregation kinetics, it would thus be extremely helpful to avoid micellar rearrangements. This can be done by introducing covalent cross-links between the casein molecules within the micelles. The enzyme Transglutaminase was used for this purpose.[37,38] After cross-linking, the enzyme has to be deactivated thermally to stop the reaction, avoid intermicellar cross-linking, and guarantee a reproducible sample preparation. To avoid complicating side effects from the whey proteins, which would denature during the heat treatment, only whey protein free milk was used for these experiments. Although caseins are known to be intrinsically unstructured proteins and are thus much less heat sensitive than globular proteins a certain effect of the heat treatment can not be excluded a priori. In order to investigate the occurrence of heat-induced effects on the casein micelles, a reference sample was made by adding water instead of the enzyme solution to a part of the sample, which then passed through the same temperature sequence as the cross-linked sample. Figure 6a shows that the heat-treatment has no measurable influence on the non cross-linked sample; we indeed recover the same sequence of events as previously observed for milk which has not been heat-treated: an initial loss of mass combined with a decrease of the micellar size, a subsequent readdition of proteins accompanied by a compaction of the micelles visible through the continuing decrease of R_H, and finally the destabilization and aggregation at pH $\approx$ 5.1. For the cross-linked micelles however we observe a totally different behavior (Figure 6b). The micellar mass and the hydrodynamic radius still decreases slightly, but the effect is drastically reduced. The colloidal destabilization, however, occurs at exactly the same pH value as observed with the untreated micelles, once again confirming our observations that micellar rearrangement and micellar aggregation are largely independent processes.

A small but measurable increase of the scattering intensity $I(0)$ by 7% indicates that a small amount of intermicellar cross-links may also have been created. The observed changes during the pH shift can, however, by now means be explained by this small increase in polydispersity and are solely due to the internal cross-linking of the casein micelles. The cross-linked micelles will thus allow us in the future to quantitatively investigate the interactions between casein micelles as a function of pH and explore colloid analogies in an attempt to better understand the pH-induced sol−gel transition in the yogurt-making process.

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4. CONCLUSION

Our new light scattering instrument has enabled us to do time-resolved static and dynamic light scattering in acidified skim milk despite the fact that these samples are highly turbid. While most of the light scattering studies which were done on skim milk before were restricted to highly diluted samples, we now present an extensive study on skim milk acidification without this restriction. Indeed, we were able to show that the micellar rearrangements of casein micelles during the acidification are dependent on the casein concentration. Experiments done at high dilution do not allow drawing direct conclusions for undiluted milk.

When one is attempting to draw conclusions on the interaction potential of casein micelles as a function of the pH from such studies one clearly needs to consider two processes. The first is the internal reassembly of the casein micelles as a result of the pH shift. The self-assembled nature of the casein micelles causes an immediate change of particle size, voluminosity and aggregation number as the environment of the particles is changed. The second effect is the actual particle−particle aggregation due to the collapse of the stabilizing electrosteric layer at a pH of about 5.

Our experiments unambiguously demonstrate that both effects are virtually not coupled and can be studied and understood independently. For the first time, we are now in a position to monitor the influence of the rate of acidification, the ionic strength and the nature of the ions on micellar reassembly and intermicellar aggregation. In particular the case of internally cross-linked casein micelles, where intramicellar rearrangements are strongly suppressed, here provides us with an ideal model system to investigate pH induced casein micelle aggregation. These experiments show that the aggregation of casein micelles can be seen in quantitative analogy to colloidal model systems. Internal rearrangements do not influence the aggregation process which is solely determined by the interface properties of the particles.

■ ASSOCIATED CONTENT

Supporting Information. Text containing details about small-angle X-ray scattering from casein micelles, the pH dependent size distribution of casein micelles, and the polydisperse structure factor of casein micelles, including figures showing the size distribution of micelles and SAXS curves, scattering intensities, and radii of gyration, and radial density distributions. This material is available free of charge via the Internet at http://pubs.acs.org.

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