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^{1}H NMR Diffusometry Study of Water in Casein Dispersions and Gels

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The self-diffusion coefficients of water in casein solutions and gels were measured using a pulsed-gradient spin−echo nuclear magnetic resonance technique (PGSE NMR). The dependence of the self-diffusion coefficient of water on the concentration and structure of casein is reported. The results were analyzed using a cell model. It was found that the water self-diffusion coefficient is insensitive to the structure of the casein in solution or in a gelled state. The influence of casein concentration on the water self-diffusion coefficient could be explained by obstruction from the casein molecule. Assuming a simple model with two water regions, each characterized by a specific water concentration and value of the water diffusion coefficient, the water mobility reduction induced by the casein can be rationalized.

KEYWORDS: Casein; self-diffusion; gel; NMR; obstruction; cell model for diffusion

INTRODUCTION

The state of water molecules in dairy protein systems is often discussed in terms of a multi-state model in which the water molecules are divided into different classes. However, in general, the different water classes are not well defined and most times they are defined on the basis of the method used to study the system. In the context of dairy protein systems, the water states most often discussed in the literature (*1*) are the following: (1) structural water, i.e., water molecules directly involved in the stabilization of the protein structure; (2) monolayer water, which refers to water molecules tightly bound to the protein surface; and (3) hydrodynamic hydration water which is transported with the protein during diffusion.

In most literature the structural and monolayer water are referred to as "bound" water, which implies a situation of essentially irreversible binding to the protein, at least on the time scale studied in the experiment. This concept has been used for a long time in connection with ^{1}H NMR relaxation measurements. Indeed, NMR relaxation parameters can potentially convey information about water mobility, but controversy arises because of the model-dependent interpretation of the relaxation data. The first model was the "two-fraction fast exchange model" (*2*) which assumes that water could be decomposed into bound water and free water, with fast exchange between the two water sites. The problem with this model is that the amount of bound water and its relaxation time are unknown. For bound water there exists efficient relaxation mechanisms such as chemical exchange and cross relaxation between the water protons and the protons on the protein. As a consequence, the amount of bound water obtained from NMR relaxation experiments is sometimes overestimated (*3*).

To remedy this situation, ^{17}O NMR relaxation could be used. Compared to proton or deuterium magnetic relaxation, ^{17}O relaxation has important advantages (*4*), because the ^{17}O relaxation is not influenced by either chemical exchange or cross relaxation, which simplifies the interpretation of the relaxation data. ^{17}O relaxation measurements have been performed to study water−protein interactions as well as protein−protein interactions (*5*); (*6*−*8*) as a function of different thermal and pH conditions. The average hydration determined for casein micelle at 21 °C and at pH 6.95 was 0.0065 g of water/g of protein. This amount of hydration water (which is lower than that of the BET monolayer, 0.06 g/g (*9*)) corresponds to water molecules internally "bound" or associated with micellar proteins. The observation of such a small amount of internal water molecules is also supported by recent ^{17}O NMR experiments (*10*, *11*)

An alternative way to study the water states in macromolecular solutions and gels is self-diffusion measurements of the water mobility. This can be done by means of the pulsed gradient spin−echo NMR technique (PGSE NMR). In comparison with the NMR relaxation technique, the interpretation of PGSE data is more straightforward. Moreover, the technique can be applied on most samples and provides structural information about the system investigated (*12*). The technique has been used to study water mobility in different protein and polysaccharide systems such as wheat starch gels (*13*−*15*),

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Table 1. Composition of the Protein Powders

	micellar casein powder	Na casein powder
total solid ($g \cdot kg^{-1}$)	901.8	955.5
total nitrogen matter ($g \cdot kg^{-1}$)	807.7	910
noncasein nitrogen ($g \cdot kg^{-1}$)	41.8	>10
nonprotein nitrogen ($g \cdot kg^{-1}$)	4.9	>5
lactose ($g \cdot kg^{-1}$)	16.6	0.7
ash ($g \cdot kg^{-1}$)	77.5	44

gellan gum gel (*16*), bovine serum albumin solutions and gels (*17*, *18*), and cheese (*19*).

Here we report results from water and protein NMR diffusometry measurements at various casein concentrations. The effects of the state of the casein and the effect of the gel structure on the water self-diffusion coefficients are also investigated.

MATERIALS AND METHODS

Materials. Na-caseinate powder (Armor Protein, St Brice en Coglès, France) and native phosphocaseinate powder (INRA, Rennes, France) were used throughout without any purification. The protein powder composition is summarized in **Table 1**. Sodium azide (Merck, Darmstadt, Germany), lactic acid, etc., were all used without any purification.

Preparation of Solutions. Re-hydration of the powders was performed at room temperature for micellar casein dispersion and at 45 °C for sodium caseinate with a NaCl/water solution (0.1 M). Sodium azide was added (0.02% w/w) to each solution to prevent any bacterial development. The solutions were studied without pH adjustment. For example, the pH of the Na-caseinate solution was 6.51 for a concentration of 0.026 g/g and 6.41 at 0.138 g/g. The pH of the micellar casein dispersion was higher, ranging from 7.14 at 0.038 g/g to 6.90 at 0.19 g/g.

Gels Preparation. Rennet and acid gels were prepared from the micellar casein dispersion. For the rennet gel, the pH was adjusted to 6.60 ± 0.05 with lactic acid (7.5% (w/w), Fluka Chemie, Buchs, Switzerland) added drop by drop under continuous stirring. The dispersions were equilibrated overnight, and the pH was controlled. Then the dispersions were preheated at 40 °C, and rennet (Ch Hansen, Arpajon, France) was added to a final concentration of 1 μL/g. The chymosin concentration of the rennet was 55 mg/L. After addition of the rennet, the dispersions were vigorously shaken and small amounts were transferred to 5-mm NMR tubes (≈1 mL). All the samples were kept in a water bath at 40 °C for 1 h, then cooled at 25 °C. For a few samples, the whey phase was extracted from the gel. The extraction was performed as follows: after 1 h the gel was unstuck from the glass tube and kept in a water bath at 40 °C during the night. The gel shrunk and a small amount of the water phase could be extracted. The pH was controlled and no changes were observed.

The acid gels were obtained by addition of glucono-δ-lactone (GDL) (Sigma Chemical, St. Louis, MO). The amount of GDL added was 2.4% (g/g). After the mixture was shaken vigorously, 1 mL was taken out for NMR measurements. To allow equilibration, the samples were maintained at room temperature overnight and the pH was adjusted to 3.88.

NMR Measurements. All NMR measurements were performed on a 200 MHz Bruker spectrometer equipped with a field gradient probe. For water self-diffusion measurements, ^{1}H NMR spectra were recorded with 20-ppm windows and 2-K data points in the time-domain. A total of 4 scans were collected with a recycling delay of 1 s. In each run, 8 dummy scans were first applied to the sample before the actual experiment was carried out. For protein self-diffusion measurements, the spectral width was 20 ppm and 16 scans were collected with a recycling delay of 2 s. NMR tubes (5-mm) were used and all the measurement were performed at 25 ± 0.1 °C. The diffusion experiments were performed using the stimulated spin−echo sequence (STE), described by Tanner (*20*). Diffusion coefficients were obtained using

$$I(\delta,\Delta,g) = I_0 \exp\left[-\gamma^2 g^2 \delta^2 \left(\Delta - \frac{\delta}{3}\right) D\right] \quad (1)$$

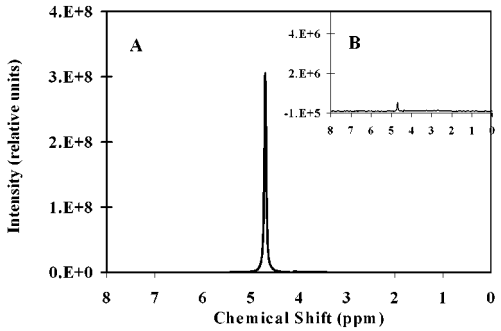

Figure 1. ^{1}H NMR spectra from water in a casein dispersion (0.12 g/g) acquired with the PFG STE sequence: (A) with a field gradient strength $g = 0.36$ T m^{-1}; (B) with $g = 2.9$ T m^{-1} (for additional experimental parameters see Materials and Methods) at 25 °C.

where $I(\delta,\Delta,g)$and I_0 are the stimulated echo intensities in the presence of gradient pulses of strength g and in the absence of gradient pulses, respectively. The length of the gradient pulse is δ, Δ is the distance between the leading edges of the gradient pulses, and γ is the gyromagnetic ratio (for protons, $\gamma = 26.7520 \times 10^7$ rad T^{-1} s^{-1}). The values of Δ and δ used in the water self-diffusion measurements were 20 ms and 0.5 ms, respectively, while δ was 5 ms in the protein self-diffusion measurements. The delay τ_1 between the first 90° pulse and the gradient pulse was fixed at 100 μs, and the delay between the gradient pulse and the second 90° pulse was fixed at 900 μs. In the experiments, g was incremented from 0.18 to 2.9 T m^{-1} and from 0.6 to 9.63 T m^{-1} for water and protein measurements, respectively. The pure water self-diffusion was measured to $2.29 \times 10^{-9} \pm 0.04$ m^2 s^{-1}.

The experimental NMR data were analyzed by nonlinear least-squares regression of eq 1 using the Levenberg−Marquardt algorithm. For the water self-diffusion experiments, the fitting equation used is

$$I = I_0 \exp(-kD) \quad (2)$$

where k is defined as $k = \gamma^2 g^2 \delta^2 (\Delta - \delta/3)$ and I_0 is the normalized intensity (normalized to 1 for $k = 0$). For the case of protein self-diffusion experiments, the fact that the protein is polydisperse in size has to be taken into account (*21*). Therefore, the signal attenuation is written as follows:

$$I = \int P(D) \exp(-kD) dD \quad (3)$$

where $P(D)$ is the probability to find a component with a self-diffusion coefficient of D. $P(D)$ is assumed to be described by a log-normal distribution function

$$P(D) = \frac{1}{D\sigma\sqrt{2\pi}} \exp\left[-\frac{(\ln(D) - \ln(D_0))^2}{2\sigma^2}\right] \quad (4)$$

where σ is the width of the distribution. The Levenberg−Marquardt method was used in the fitting procedure. The errors were estimated by a Monte Carlo analysis according to Alper and Gelb (*22*) and errors quoted correspond to a 90% level of confidence.

Dry Matter Determination. The dry matter of all the samples was estimated by measuring the weight variation after drying in a oven at 103 °C for 16 h.

RESULTS AND DISCUSSION

Micellar Casein Dispersion. Two ^{1}H NMR spectra of water in a micellar casein dispersion with a concentration of 0.12 g/g (g_{casein}/g_{water}) are shown in **Figure 1**. These spectra were obtained from the stimulated spin−echo sequence with two different field gradient pulse strengths: $g = 0.36$ T m^{-1} (A) and $g = 2.9$ T m^{-1} (B). The narrow peaks correspond to the water protons. At this casein concentration, the protein proton peaks were too small to contribute significantly to the water proton peak. Conse-

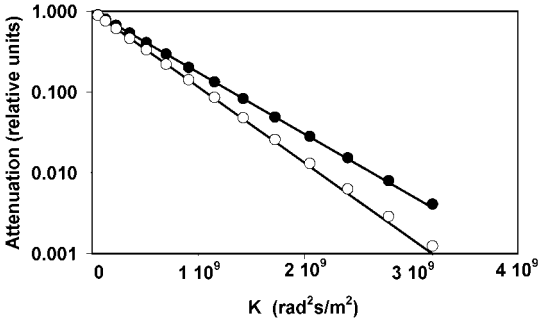

Figure 2. Echo attenuation for water in 0.035 g/g (○) and 0.12 g/g (●) casein dispersions as a function of $(2\pi q)^2(\Delta - \delta/3)$. The lines are the results of the fit of eq 2 to the data.

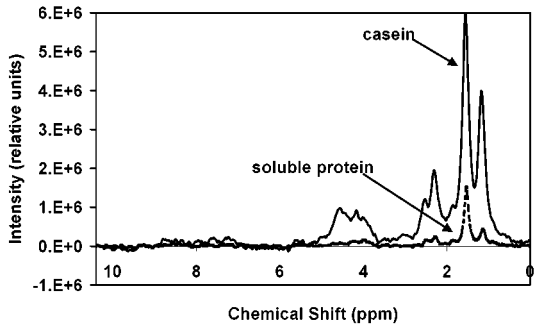

Figure 3. ^{1}H NMR spectra from protein in a casein dispersion (0.19%) and from the soluble protein fraction acquired with the PFG STE sequence at 25 °C. The field gradient strength g was fixed at 0.6 T m^{-1} (for additional experimental parameters see Materials and Methods).

quently, the intensity of the water peak for different gradient strengths can be used to determine the water self-diffusion coefficient. An example of a semilogarithmic plot of the echo intensities as a function of k is given in **Figure 2**. A straight line was observed for all the protein concentrations, and also for different Δ values in the range from 20 to 200 ms. This demonstrates that the majority of the water molecules are not confined in compartments or affected by the presence of barriers and can diffuse freely over a length scale, given by the relation $\langle r_z^2 \rangle = 2D\Delta$. The length scale corresponds to 29 μm for a micellar casein concentration at 0.03 g/g and 24 μm for 0.19 g/g.

The ^{1}H NMR spectrum of a micellar casein dispersion obtained from the stimulated echo sequence with a gradient strength $g = 0.6$ T m^{-1} is shown in **Figure 3**. Because of the high dynamic range of the water signal, the application of a strong gradient pulse effectively suppressed the water peak and the ^{1}H protein spectra could be obtained without any distortion of the spectra. The interpretation of the proton spectrum of casein micelles has been discussed previously (*23*−*25*). The NMR spectra is the superposition of a spectrum with a relatively small line width and a strongly broadened spectrum. In our case, only the narrow line width contribution is observed because the NMR signal was acquired from a stimulated echo experiment, and the protein protons with the shortest relaxation times, T_2, were almost completely relaxed. According to Rollema et al. (*23*), 50% of the spectrum with narrow line width can be attributed to κ-casein which is located mainly at the surface of the micelle and corresponds to the so-called "hairy" part of the micellar casein. However, in the spectrum in **Figure 3**, contributions from the soluble proteins and amino acids cannot be excluded because of the presence of a small amount of nonmicellar protein in the powder. To prove the presence of

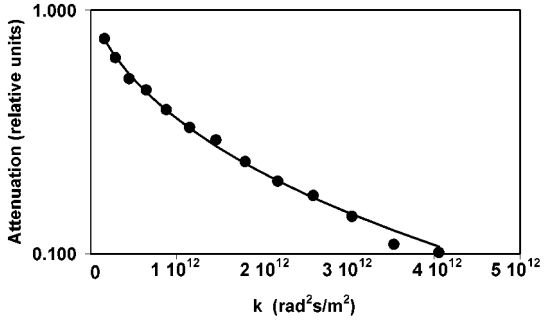

Figure 4. Echo attenuation for protein in a 0.08 g/g (●) casein dispersion as a function of $(2\pi q)^2(\Delta - \delta/3)$. The lines are the results of the fit of eq 3 to the data.

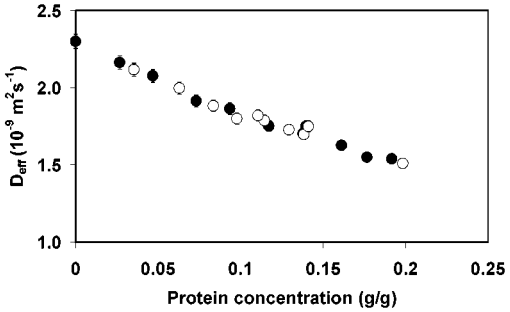

Figure 5. Observed water self-diffusion coefficients as a function of the protein concentration (g/g) for Na-caseinate solutions (○) and micellar casein dispersions (●).

this contribution, the casein dispersion was renneted and the water phase was separated after the shrinking of the gel. During this process, the composition of the water is constant and only the soluble components are released. Subsequently, a ^{1}H NMR spectrum was acquired with the same experimental parameters previously used for the acquisition of a spectrum from the casein dispersion. Both spectra are given in **Figure 3**. Although contributions from soluble proteins and amino acids to the NMR spectra are small, they cannot be neglected.

To determine the protein self-diffusion coefficient, the integrated area of the peak between 0.46 and 2.4 ppm was plotted versus k, and the result is presented in **Figure 4**. A large deviation from linearity is observed, which is not due to a restricted diffusion behavior, but is an effect of the micelle size distribution and the presence of a small amount of noncasein protein, with the size distribution of the micelle casein constituting the main effect. This conclusion is supported by the results of Morr et al. (*26*). These authors determined the self-diffusion coefficient of micellar casein by inelastic light scattering for two relatively monodisperse casein micelle size fractions. At 20 °C, the self-diffusion for the smaller size fraction ($R_h = 76.7$ nm) with a concentration of 0.037 g/100 mL was 2.8×10^{-12} m^2 s^{-1} and 0.9×10^{-12} m^2 s^{-1} for the bigger size fraction ($R_h = 216.5$ nm) with a concentration of 0.06 g/100 mL. From our NMR results, the protein mean self-diffusion coefficients were calculated according to eqs 3 and 4 to 3.4×10^{-12} m^2 s^{-1} with $\sigma = 1.4$ for a concentration of 0.08 g/g. These values are in agreement with previously published results (*26*, *27*).

Effect of the Protein Structure on the Water Self-Diffusion in Solution. The water self-diffusion coefficients for micellar casein and Na-caseinate dispersions as a function of protein concentration are shown in **Figure 5**. As expected, the water self-diffusion coefficient decreased when the protein concentration increased. However, we did not observe any difference between the water self-diffusion coefficient in a Na-caseinate

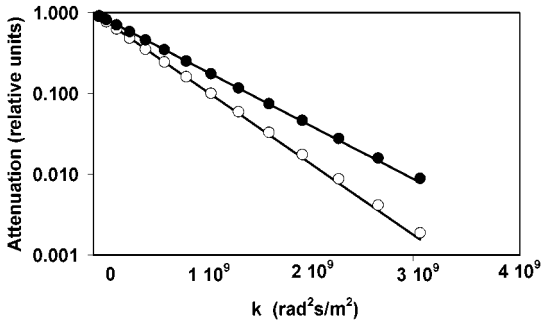

Figure 6. Echo attenuation for water in 0.038 g/g (○) and 0.19 g/g (●) casein rennet gels as a function of $(2\pi q)^2(\Delta - \delta/3)$. The lines are the results of the fit of eq 2 to the data.

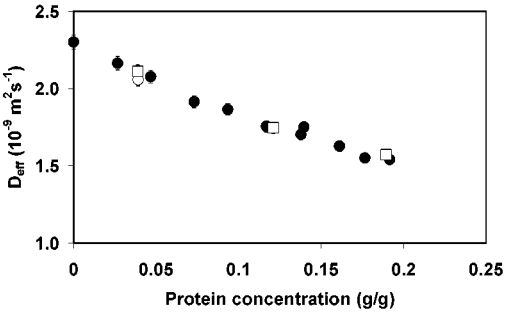

Figure 7. Observed water self-diffusion coefficients as a function of the protein concentration (g/g) for micellar casein dispersions (●), for acid gels (○), and for rennet gels (□).

solution and that in a micellar casein dispersion, despite the size difference of the colloidal particles in the two systems.

Casein micelles are colloidal complexes of proteins, salts, and water. Many models have been proposed to describe the casein micellar structure (*28*) but it appears generally accepted that the casein micelle is a roughly spherical, fairly swollen particle with a "hairy" outer layer (*29*). The differences between the various models proposed concern mainly whether sub-micelles exist inside the micelle or not. A casein micelle is a large aggregate with a diameter of 150 nm, highly hydrated with about 4−6 g water/g protein (*30*, *31*). In contrast to micellar casein, Na-caseinate is a soluble protein, roughly spherical, with a diameter of ≈10 nm (*30*−*32*). However, the voluminosity of Na-caseinate is similar to the micellar casein voluminosity (*30*, *31*).

Considering the delay between the two applied pulse gradients in the NMR experiment was 20 ms, the average distance probed by the water molecule was at least 13 μm. Consequently, the distance covered by the water molecules is very large compared to the average micellar diameter (0.15 μm) and to the average size of the sub-micellar casein proteins (0.01 μm). In other words, during the time scale of the experiment, a water molecule diffuses around or through many micellar and sub-micellar casein particles. However, no effects from the quartenary or higher structures are observed on the water diffusion data. This is rather surprising because there is a large difference in size and structure between a casein micelle and a Na-caseinate protein. In conclusion, it seems clear that the water self-diffusion is mainly influenced by protein concentration, and not on the aggregation state of the casein.

Water Self-Diffusion in Gel. The logarithm of the echo attenuation as a function of k is given in **Figure 6** for a rennet gel at two casein concentrations. A straight line is observed for both acid and rennet gels. Consequently, the formation of the gel induced no restriction in the diffusion of the water molecule. Moreover, no differences were observed between the water self-diffusion in the dispersion or in the gels throughout the protein concentration range investigated (**Figure 7**).

The gel results further confirmed that the water mobility during the time scale of the NMR experiment is insensitive to the structure of the casein aggregates and to the heterogeneous structure of the gel. Indeed, according to Van Vliet and Walstra (*33*) the casein gels are heterogeneous on several length-scales. First, on the length-scale of the casein particle, second, at the level of the casein strands and nodes formed by the aggregated casein particles, and, finally, at the level of the small and large aggregates formed by these strands and nodes. We note that the water permeability coefficient is comparable for acid and rennet gels (*34*, *35*) and the permeability of acid gels is unaffected by heating of the milk, despite the heating effect on the gel microstructure (*36*). Thus, our results clearly indicate that the water mobility is not affected by the macrostructure of the gel or by the aggregates, but by the organization of the casein molecules themselves. This is in agreement with the description of casein gels as particle gels: networks built up of casein micelles or marginally modified micelles (*37*).

Description of the Water Mobility. To explain the water diffusion in gels or polymer solutions, at least two effects should be considered: (i) the obstruction effect induced by the impenetrable slow-moving polymer molecules, and (ii) the hydration effect, i.e., the lowering of the water diffusion on account of water−protein interactions.

Models based on different physical concepts such as obstruction effects, free volume concepts, and hydrodynamic interactions have been proposed to describe the reduction of the water mobility (*38*−*40*). Several of them require numerous physical parameters relating to the system under study or are based on scaling concepts. Other theories are based on solution of Fick's first law for different geometries. According to our results, it seems clear that casein could be described as a micro-gel, and only the structural organization of this micro-gel need to be considered in order to explain the reduction of the water mobility. Unfortunately, this organization is still unknown and the structural parameters needed to apply the free volume or hydrodynamic concepts are unavailable.

To facilitate the theoretical treatment of our experimental results, the obtained diffusion data are divided into two classes, dependent on the casein concentration. A simple model of the system, at low protein concentrations, is to consider a casein aggregate (micelle) as a spherical particle with a high internal concentration of water. The internal structure and the water concentration in an aggregate can be assumed constant as long as there is free water between the particles (**Figure 8**). This means that the concentration and transport properties of the internal water in a casein micelle can be assumed concentration-independent at protein concentrations less than the close packing limit which is around 10% w/v (*32*). This gives the upper limit of the first concentration range. In the second concentration range, which starts at the close packing limit, no free water exists between the casein aggregates. This means that the aggregates are compressed when the water concentration is reduced. An obvious result of this compression is a reduction of the internal water concentration and also a reduction of the water diffusivity in the casein aggregates.

A simple model of the macroscopic water self-diffusion, at low protein concentrations, is to assume that the two regions, aggregates and surrounding solution, are characterized by different but constant water concentrations and self-diffusion coefficients (cf. **Figure 8**). For this situation, the effective

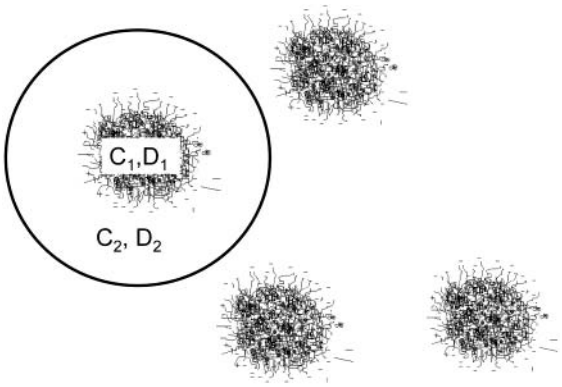

Figure 8. Illustration of the two water regions according to the cell model. The cell is divided into regions 1 and 2. Region 1 corresponds to water molecules inside the micelle and region 2 corresponds to water molecules outside the micelle (pure water). Taken together, the regions represent the macroscopic behavior of the water diffusion.

diffusion has been discussed by Jönsson et al. (*41*) within the framework of a cell model. The approach has been successfully applied to surfactant−water systems (*42*). Within this model, obstruction effects and water−protein interactions manifest themselves in the values of the diffusion coefficients in the different regions. When the self-diffusion coefficient of the particle is small compared to the water diffusion coefficient, the observed self-diffusion coefficient, D_{eff} is given by the following:

$$D_{\text{eff}} = D_2 \frac{1}{1-\left(1-\frac{C_1}{C_2}\right)\phi} \frac{1-\beta\phi}{1+\frac{\beta\phi}{2}} \tag{5}$$

with

$$\beta = \frac{D_2C_2 - D_1C_1}{D_2C_2 + 0.5D_1C_1} \tag{6}$$

where C_1 and D_1 are the water concentration and self-diffusion coefficient inside the spherical particle and C_2 and D_2 characterize the same properties in the region surrounding the particle. We stress that the water diffusion in the casein aggregates (cf. **Figure 8**) does not necessarily have to be "homogeneous" in the sense that it is described by the same local diffusion coefficient in all parts. A situation with several different sites in which the water diffusion has different values is perfectly feasible, as long as the exchange of water between the regions is rapid and the rms distance diffused by the water is larger than the region, such that any inhomogeneities are averaged out.

The parameters in eqs 5 and 6 and the volume fraction ϕ occupied by the aggregates can also be written as follows:

$$C_1 = \frac{m^{\text{cas}}H^{\text{cas}}}{m^{\text{cas}}v^{\text{cas}} + m^{\text{cas}}H^{\text{cas}}v^{\text{water}}} \tag{7}$$

where m^{cas} and v^{cas} are the protein mass (g) and the specific volume of casein, 0.75 cm^3 g^{-1}, respectively (*43*). v^{water} is the specific volume of water (1 cm^3 g^{-1}) and H^{cas} is the water amount in the casein micro-gel in grams of water per gram of casein.

$$\phi = \frac{m^{\text{cas}}v^{\text{cas}} + m^{\text{cas}}H^{\text{cas}}v^{\text{water}}}{m^{\text{cas}}v^{\text{cas}} + m^{\text{water}}v^{\text{water}}} \tag{8}$$

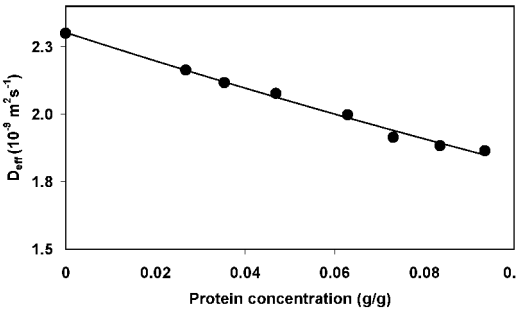

Figure 9. Variation of the self-diffusion coefficient D_{eff} as a function of the casein concentration (g/g). The line corresponds to the best fit from eq 9a with $K = 2.07$.

If we assume that there is pure water surrounding a micelle, $D_2 = 2.3 \times 10^{-9}$ m^2 s^{-1} and $C_2 = 1$ g/cm^3. If these values, plus the expressions in eqs 7 and 8, are used in eq 5, the concentration dependence of the effective water self-diffusion coefficient becomes

$$D_{\text{eff}}^{\text{water}} = 2.3\times10^{-9}\left(1+0.75\frac{m^{\text{cas}}}{m^{\text{water}}}\right)\frac{\left(1+0.75\frac{m^{\text{cas}}}{m^{\text{water}}}-K\frac{m^{\text{cas}}}{m^{\text{water}}}\right)}{\left(1+0.75\frac{m^{\text{cas}}}{m^{\text{water}}}+K\times0.5\frac{m^{\text{cas}}}{m^{\text{water}}}\right)} \tag{9a}$$

where

$$K = (v^{\text{cas}}/v^{\text{water}} + H)\beta \tag{9b}$$

K is the only unknown parameter in eq 9a, and its value can be obtained by fitting eq 9a to the experimental data. In this process we must keep in mind that the assumption of constant internal aggregate properties can be used only as long as there is "free" water surrounding the casein particles. Above this concentration a more detailed model of the internal properties of a casein aggregate needs to be defined.

The result of the fitting process is presented in **Figure 9**. The K value that gives the best fit is $K = 2.07$. However, from this value it is not possible to determine both the concentration and diffusion coefficient of the water in a casein micelle; one of the parameters needs to be specified. In the further evaluation of our data, H^{cas} is fixed at 5 g of water per g of casein. At first sight this value seems to be rather high, but as already pointed out in the Introduction, a casein micelle is a porous structure. The voluminosity of a casein micelle, as determined from SAXS, is between 4 and 6 g/g (*30*, *31*) and recently, from osmotic pressure measurements (*32*). If H^{cas} is fixed at 5 g of water per g of casein, the water diffusion coefficient inside a casein micelle becomes 1.45×10^{-9} m^2 s^{-1}.

To be able to construct a model that can be used to determine the effective water self-diffusion coefficient also at higher casein concentrations, a detailed description of the internal aggregate structure at different water concentrations is required. Because this information is not available at present, two rather simple models of the water transport inside a casein aggregate will be discussed.

The two models are as follows. (I) A model where all protein molecules in a casein micelle are assumed to be spherical particles with water molecules "weakly bound" in a surface layer. In the model we also assume that there is an exchange of water molecules between the surface layer and a surrounding

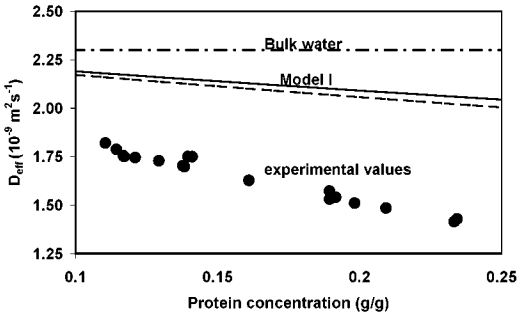

Figure 10. Variation of the self-diffusion coefficient D_{eff} as a function of the casein concentration above the close packing limit. The two lines correspond to the D_{eff} values calculated from eq 10 with two different $D_1/D_2 = 0$ and $D_1/D_2 = 0.67$ values assuming the model I.

water network. The water molecules can, in the model, move rather unrestricted in the water network that surrounds the protein molecules, but are immobile in the surface layer. The volume of the protein molecules, as well as the amount of water in the surface layers, is assumed to be independent of the protein concentration. (II) A model where the casein aggregate is characterized by water-rich regions in a water-poor matrix. Each water-rich region is assumed to be surrounded by a water-poor region. This means that a water molecule needs to diffuse through a water-poor region when transported from one water-rich region to another. The concentration and the self-diffusion coefficient for the water in the water-poor matrix is assumed to be constant and independent of the total protein concentration.

The models can be seen as two borderline cases, and a combination of the two models would perhaps constitute a better description. In what follows we will apply the two models to our experimental data.

The equations presented above for the effective diffusion coefficient in a spherical cell system with two regions characterized by different transport properties, eqs 5 and 6, can also be used to model the intrinsic water diffusion in the two new models. In model I we assume that the water molecules are either associated with the protein molecules, or behave as "free" molecules in the connected water network surrounding the protein molecules, $C_2 = 1$ g/cm^3 and $D_2 = 2.3 \times 10^{-9}$ m^2/s. The amount of water associated with the protein molecules is mainly dependent on the number of water molecules in the first monolayer around the protein molecules. An analysis of BET isotherms gives $C_1 \approx 0.06$ (g/g) (*9*). The lateral diffusion coefficient for the water molecules in the first water layer depends mainly on the polar heterogeneity of the protein surface and is not known for the studied system. However, D_1 is in the range $0 < D_1 < 0.67\ D_w$, where 0.67 is the obstruction factor introduced by the restricted diffusion on a surface of a spherical protein (*41*).

If these values are used in eq 5, the equation for the effective self-diffusion coefficient of water in a casein micelle as a function of the casein concentration becomes

$$D_{eff} \approx 2.3 \times 10^{-9}\left(\frac{1}{1 + (0.59 - 0.14 \times D_1/D_2) \times m_c/m_w}\right) \ (m^2/s) \quad (10)$$

where the numerical factors come from inserting relevant parameter values and changing concentration variables in eqs 5 and 6.

The concentration dependence implied by eq 10 is plotted in **Figure 10** together with the limiting values for $D_1/D_2 = 0$ and $D_1/D_2 = 0.67$. As can be seen from the figure, model I severely overestimates the water mobility in a casein micelle. The assumptions in model I of an interconnected water network around all protein molecules in a casein micelle is probably too severe a simplification. Therefore, we proceed to test model II.

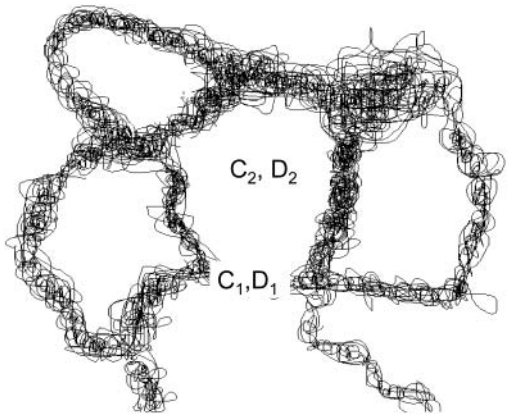

Figure 11. Illustration of the two water regions according to the cell model for protein concentration above the close packing limit. The cell is divided into regions 1 and 2. Region 1 corresponds to pure water and region 2 corresponds to water molecules close to the protein molecule or the micelle aggregates in case of a gel.

In this model the center of a cell corresponds to a region of pure water, which is surrounded by a region with a lower water content (**Figure 11**). Effectively, we model a situation where a given water molecule diffuses through successive zones characterized by different water concentrations and diffusion coefficients. It is important to remark that this representation could also be applied to describe the water mobility in the gel on a macroscopic scale and also to describe the water mobility in the micelle on a sub-microscopic scale. For the situation depicted in **Figure 11**, eq 5 is still applicable, but now C_1 and D_1 characterize the region with pure water while C_2 and D_2 describe the region with a lower water concentration.

As a starting point we assume that the water concentration in the protein matrix surrounding a pure water region is independent of the total protein concentration, at least at the protein concentrations studied here. Thus, we assume that the pure water regions are compressed when the protein concentration is increased at protein concentrations above the aggregate overlap concentration. If we further assume that all pure water regions are squeezed out from a casein micelle at the highest protein concentration used in our investigation, 0.23 g casein/g water, then $C_2 = 0.2$ g/cm^3 and $D_2 = 1.4 \times 10^{-9}$ m^2/s. If these values are inserted into eqs 5 and 6, the concentration dependence of the effective water self-diffusion constant in this model becomes

$$D_{eff} \approx 2.3 \times 10^{-9}\left(\frac{1}{1 + 2.3 \times m_c/m_w}\right) \ (m^2/s) \quad (11)$$

as in eq 10, the numerical factors come from inserting relevant parameter values and changing concentration variables in eqs 5 and 6.

The concentration dependence from eq 11 is plotted in **Figure 12** together with the experimentally obtained water self-diffusion values in the concentration range $0.1 < m_c/m_w < 0.23$.

In conclusion, by assuming a simple model with two water regions characterized by specific water concentrations and diffusion coefficients, the water mobility reduction induced by

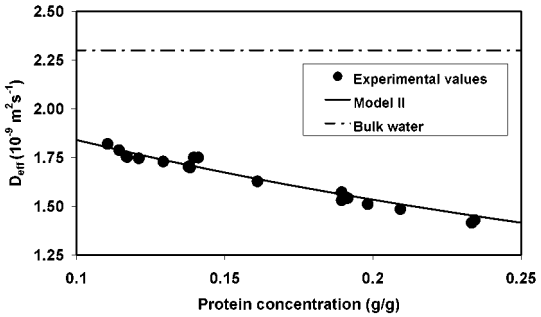

Figure 12. Variation of the self-diffusion coefficient D_{eff} as a function of the casein concentration above the close packing limit. The line corresponds to the D_{eff} values calculated from eq 11 assuming the model II.

the casein can be rationalized. From external data pertaining to the water concentration inside the micelle structures or submicellar structures we demonstrate that (i) on a macroscopic level, the water diffusion can be described by two self-diffusion fluxes, one around the micelle and one through the micelle; (ii) inside the micelle the water mobility is probably reduced by regions with low water contents, no specific water−protein "binding" needs to be invoked to describe the lowering of the water mobility. Our results are in agreement with recent NMR relaxation measurements (*11*) which showed that the amount of water molecule hindered by specific water−protein "binding" is quite small (a few water molecules/protein), and consequently, their contributions to the macroscopic flow seem to be negligible.

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