Polymer 50 (2009) 6024–6031

Contents lists available at ScienceDirect

Polymer

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

# Micelle formation in beta-casein solutions

Adel Aschi [a,*], Patrick Calmettes [b], Mohamed Daoud [c], Roger Douillard [d], Abdelhafidh Gharbi [a]

[a] Physic Department, Faculté des Sciences de Tunis, Campus Universitaire, 2092, Tunis, Tunisia
[b] Laboratoire Léon Brillouin, CEA Saclay 91191 Gif-sur-Yvette cedex, France
[c] Service de Physique de l'Etat Condensé, CEA Saclay 91191 Gif-sur-Yvette cedex, France
[d] Equipe de Biochimie des Macromolécules Végétales, Centre de Recherche Agronomique, 2 Esplanade R. Garros, BP 224, 51686 Reims cedex 2, France

ARTICLE INFO

*Article history:*
Received 17 May 2009
Received in revised form
8 October 2009
Accepted 9 October 2009
Available online 25 October 2009

*Keywords:*
Denaturant concentration
Micelle
Small angle neutron scattering

ABSTRACT

We have studied by Small Angle Neutron Scattering the structure of beta-casein in aqueous solutions in a wide range of temperatures and denaturant concentrations. For higher temperatures and low denaturant concentrations, we find micelles. These may be described by using a two shell model with constant concentration in every shell. When either temperature is lowered or denaturant concentration is raised, the aggregation number of the micelles decreases, until it reaches unity at a critical temperature or a critical concentration. Beyond this threshold, single isolated casein molecules are present. For both cases, the results are interpreted in terms of synthetic polymers, by a copolymer model. We introduce an excluded volume parameter describing the hydrophobic sequences. We assume that it depends on both temperature and denaturant concentration, that varies and eventually changes sign as either of these parameters is varied. This leads to some power law variations that are in reasonable agreement with the experiments.



## 1. Introduction

Beta-casein is made of 209 amino acids. Because of the hydrophilic and hydrophobic nature of its constituents, it has interesting surface and bulk properties. The protein is strongly attracted to the interface between the solution and air. Far from the surface, in the bulk, it forms micelles. The presence of micelles in the bulk of the solution has been studied by neutron scattering [1–5]. The authors showed that in the absence of salt and near room temperature, about 30 casein molecules cluster to form a micelle and that temperature has a strong effect on the structure of these micelles. It was found that as the temperature decreased, there was a decrease in the aggregation number of the micelles until a critical temperature was reached, approximately 4 °C, below which the aggregation number was unity, i.e. the micelles disappeared. The fact that the aggregation number decreased with temperature is a strong indication that clustering is related to hydrophobic interactions. Therefore, one expects that the presence of a denaturing salt should also lead to a similar effect. In what follows, we study both experimentally and theoretically the structure of $\beta$-casein dissolved in a buffer solution, in the absence and in the presence of a strong chemical denaturant, guanidine hydrochloride (GdmCl). We prolonged our work to study the temperature effects. The GdmCl strongly decreases interactions between the monomers (amino acids) of the polypeptide chain. The transition between the micellar and excluded volume states is determined as a function of the GdmCl concentration. We determine the values of the apparent radius of gyration for micelles and monomers of $\beta$-casein. We also determine the aggregation number, number of proteins in the micelle, $f$ and the size of the core, $R_{core}$ and corona $R_{corona}$ using a core–corona model [6–10].

In order to understand these results, we adopted a unifiyng approach very similar to what was done recently with synthetic macromolecules, and more precisely with copolymers. In what follows, we consider the protein as a diblock copolymer, made of successive sequences of hydrophobic and hydrophilic monomers and can form core/shell/corona micelles in water. We made the strong assumption that what is important in the study of such object is not the precise chemical nature of the primary amino acid sequence, but the fact that each amino acid, considered as a "monomer", is either hydrophilic or hydrophobic. We decided first to have a "black and white" approach, by neglecting any possible continuous variation in a hydrophilic/hydrophobic scale, and to assume that only two possible states are possible. The hydrophobic or hydrophilic nature of each amino acid is known [11–13], as is their exact sequence along the beta-casein molecule. What is considered as a monomer however in what follows is not one single amino acid, but a short sequence, made of seven of them

* Corresponding author. Fax: +216 71 885 073.
*E-mail address:* aschi13@yahoo.fr (A. Aschi).

0032-3861/$ – see front matter 
doi:10.1016/j.polymer.2009.10.051

(effective monomer). This allows to neglect the local rigidity of the molecule, and to assume that the orientation of two successive effective monomers is random and uncorrelated. The effective monomer was assumed to be either hydrophilic or hydrophobic by a simple majority rule: if most of the amino acids in it are hydrophilic, it is assumed to be hydrophilic, and conversely in the opposite case. Other choices are possible, but this one optimized the random orientation assumption and the necessity of having a sufficiently large number of monomers. We also decided not to use directly the number of monomers as a variable, but to eliminate it whenever possible, and this was always possible in the present study. We finally note that the question of the definition of the effective monomer also exists for synthetic macromolecules [14], and does not lead to any difficulty. It is then straightforward to account for the presence of micelles in the solution. As for synthetic copolymers, it is possible to assume that hydrophobic sequences may be described by a negative excluded volume parameter in a Flory approach [14]. The fact that the aggregation number of the micelles is found to change with temperature $T$ and denaturing agent concentration $C_D$ leads us to assume that the Flory parameter also depends on these variables [12]. More precisely, as the size of the micelles decreases, we are led to assume that this parameter decreases in absolute value, and eventually changes sign as either temperature is decreased or $C_D$ is increased.

In what follows, we will first briefly remind the properties of beta-casein. In section '' effect of guanidine hydrochloride on β-casein solutions'' the consequences of the variation of denaturing agent concentration are discussed while in section ''effect of temperature on β-casein solutions'' deals with the experiments at varying temperature. In the final section, we present a rough interpretation of these results in terms of changing Flory excluded volume parameter.

## 2. Experimental section

β-casein, with high purity, was obtained from the skimmed milk of a single homozygous cow for the four major caseins ($\alpha_{s1}$B, $\alpha_{s2}$B, βB, κB), provided by the INRA Reims Laboratory, purified according to the method of Mercier et al. [15] and freeze-dried. Acid precipitated casein was fractionated by ion exchange chromatography on DEAE column (5PW, Waters) using NaCl gradient in a 20 mM imidazole buffer pH 7 including 3.3 M urea and 1 mM dithiothreitol (DTT). The fraction corresponding to $\beta$-casein was rechromatographed in the same conditions and its purity checked by polyacrylamide gel electrophoresis. The extinction coefficient used to determine the volume concentration of this protein is $E^{1\%}_{1cm} = 4.6$ at 278 nm.

β-casein was exhaustively dialysed against $D_20$ buffered, with 0.1 M phosphate, containing 0.1 M NaCl to reduce electrostatic interactions. The pH of the solution was adjusted to 7. When dissolved in water or in a buffer solution, casein forms micelles with spherical shape, as already observed by Leclerc et al. [2] and discussed below.

Guanidine hydrochloride (Pierce, Sequanal Grade) was purified by crystallization. The molar concentration [GdmCl] of guanidine hydrochloride was obtained from the measurement of the index of refraction of the sample using the relation [16]:

$$[\mathrm{GdmCl}] = 57.141\Delta n + 3.68(\Delta n)^2 - 91(\Delta n)^3 \quad (1)$$

where $\Delta n$ is the difference between the index of refraction of the buffer with and without GdmCl.

In our case, we prepared a stock solution of GdmCl concentration, from which one can dilute into a working concentration, equal to 6.1 M, where the indices of refraction of deuterated GdmCl and buffer are equal to 1.436 and 1.328 respectively.

Samples for SANS experiments were prepared as detailed elsewhere [17]. The SANS measurements were carried out at the Laboratoire Léon Brillouin (CEA Saclay France) with the PACE spectrometer. This apparatus was equipped with 30 concentric annular detectors and a central circular detector measuring the scattered and transmitted beam intensities, respectively. Incident neutrons had a wavelength $\lambda = 11.0$ ∼ and different sample-to-detector distances gave access to wavenumber transfers ranging from about 0.006 to 0.07 $\sim^{-1}$. The wavenumber transfer, defined as $q = (4\pi/\lambda \sin\theta/2)$, where $\theta$ is the scattering angle. The samples were contained in fused silica cells of 5 mm inner path length. The scattering data were corrected for the solvent contribution and for excess incoherent scattering. Detector non-uniformity was corrected by normalization to the scattering from a 1 mm thick light water sample. The duration of data collection was chosen according to the protein concentration, in order to get the same statistical accuracy for all measurements. For instance, 6 h was needed when the protein concentration was 7.5 mg $cm^{-3}$. The final spectrum of each sample corresponds to the average of all the spectra obtained during consecutive 30 min runs. This enabled us to check that the forward scattered intensity $I(0)$ stayed constant. The protein denaturing agent, GdmCl, was deuterated as described elsewhere [3,17]. In aqueous solution deuterated GdmCl has almost the same neutron scattering-length density as heavy water (D2O) so that the excess scattering mainly arises from the protein. Denaturant concentrations ranged from 0 to 4 M.

The solutions that will be considered below are diluted, and the average distance between the various micelles is much larger than the size of each micelles. For such dilute systems, where the distance between scattering micelles is large enough so that no interference occurs between scatterings from different micelles, the scattered intensity is simply given by the product of the number $N$ of micelles, and the scattered intensity by a single micelle. In the general case of a dilute solution of identical objects and in the case of a spherically symmetric object, one may write the scattered intensity in the following form:

$$I(q) = (N/V)\langle k^2 A^2(q)\rangle \quad (2)$$

Where, $A(q)$ is the amplitude form factor of each object (a micelle in our case), $k^2$ is the contrast factor, $N$ the number of objects, as mentioned above, $V$ is the scattering volume.

For very low values of $q$ and for very low guanidine hydrochloride concentrations, the Guinier approximation is very often used to determine the apparent radius of gyration, $R_g(c)$, of the scattering elements [17]. It is a very useful simplification of the scattered intensity for small values of $q$. It is, in general, acceptable as long as $qR_g(c) \leq 1$ and is written [18]:

$$I(q,c) = I(0,c)\exp\left(-\frac{q^2 Rg(c)^2}{3}\right) \quad (3)$$

The plot of ln[I($q$,c)] versus $q^2$ allows the determination of the forward scattered intensity I(0,c) and of the apparent radius of gyration $R_g(c)$.

The flexible chain is a rather simplistic polymer model without rigidity. The expression of the form factor of an ideal chain was established by Debye [19]:

$$P_D(x) = \frac{2}{x^2}\left(e^{-x} - 1 + x\right) \quad (4)$$

where $x = (qR_g)^2$. For large values of $x$, this expression has the following asymptotic form:

$\lim_{q\to\infty} P_D(q) = \frac{2}{qR_g^2}$. In the presence of excluded volume interactions, the Debye approximation (Eq. (4)) remains valid as long as $x \leq 9.31$. This function can be approximated as [20]:

$$\left[\frac{I(q,c)}{I(0,c)}\right]^{-1} \cong 1 + 0.359\left[qR_g(c)\right]^{2.206} \tag{5}$$

where the numbers 0.359 and 2.206 give to this approximation an accuracy of better than ±0.4%, for $1 \leq x \leq 13$.

In this way high $q$ data are incorporated in the fitting range. These data are much less affected by interparticle interference than low $q$ ones [20]. The scattering spectra of highly denatured $\beta$-casein were fitted to this expression. A plot of $I(q, c)^{-1}$ versus $q^{2.206}$ gives a straight line and allows the apparent radius of gyration $Rg(c)$ and the apparent scattered intensity $I(0, c)$ to be obtained from its slope and intercept. The Debye law is basically valid for a Gaussian chain [20] but it can also be used for an excluded volume chain [14,21].

Many authors [2,4,5,22–24] found that micelles of β-casein can be considered as spherical objects made up of a core of radius $R_{core}$, surrounded by a corona, with external radius $R_{corona}$, with different but constant scattering-length densities. The latter assumption is, in principle, in contradiction with what was found for long polymer chains either in star shaped configuration or for linear chains grafted on a solid surface. In such cases, we know that there is a concentration profile and that the concentration is not constant but varies as a power law as a function of the distance to the center. The latter results however are valid for long chains. In the present case, the proteins are rather short polymers. Moreover, inspection of the radius of the core and corona (see Fig. 6a and b) shows that the extension of the corona is typically 70 ∼, with an internal radius of 60 ∼ for the core, and an overall radius of 130 ∼. For such rather small object, it is possible to accept that the concentration profile [25] may be neglected, and that a core–shell model may be used, Fig. 1. Thus, in what follows, we assume that the micelle has a density profile $\phi(r)$, given by:

$$\begin{aligned} \phi(r) &= \phi_1 = 3\phi_0/4\pi R_1^3 && \text{if } 0 \leq r < R_{core}, \\ \phi(r) &= \phi_2 = 3(1-\phi_0)/4\pi\left(R_2^3 - R_1^3\right) && \text{if } R_1 \leq r < R_{corona}, \\ \phi(r) &= 0 && \text{if } r > R_{corona}. \end{aligned} \tag{6}$$

In these expressions $\phi_0$ is the proportion of amino acids in the core of the micelle, compared to the total number. Using the latter relations for $\phi(r)$, we find the following form factor for a micelle:

$$A(q) = \frac{4\pi}{q}\left[\phi_1 \int_0^{R_{core}} r\sin(qr)\mathrm{d}r + \phi_2 \int_{R_{core}}^{R_{corona}} r\sin(qr)\,\mathrm{d}r\right] \tag{7}$$

The form factor of micelle is thus:

$$P(q) = \left[\frac{3\phi_0}{q^3R_{core}^3}F(qR_{core}) + \frac{3(1-\phi_0)}{q^3(R_{corona}^3 - R_{core}^3)}[F(qR_{corona}) - F(qR_{core})]\right]^2 \tag{8}$$

where,

$$F(x) = \int x\sin x\mathrm{d}x = \sin x - x\cos x \tag{9}$$

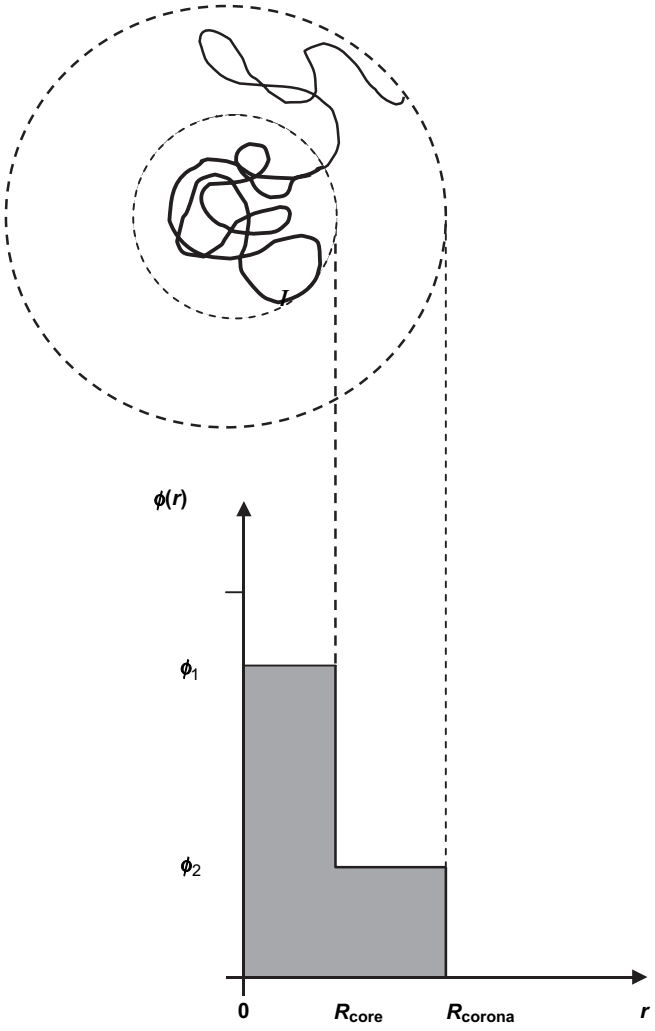


**Fig. 1.** sketch of $\beta$-casein spherical micelle made of a hydrophobic core (region I) and a hydrophilic corona (region II) in a selective solvent which is good for (II). We assume constant concentrations in each region, with $\phi_1 > \phi_2$.

## 3. Results and discussion

### 3.1. Effect of guanidine hydrochloride on β-casein solutions

In the experiments discussed in this section, the concentration of β-casein was 7.5 mg cm$^{-3}$ and temperature was set at 20 °C. Typical neutron scattering spectra are shown in Fig. 2. Two types of spectrum shapes are observed. The first ones, corresponding to low salt concentrations, have a high forward intensity, $I(0,c)$. This may be attributed to the existence of micelles of protein molecules, with larger molecular weights. The second ones, for larger GdmCl concentrations, have smaller forward intensity, and may be interpreted as being the scattering by smaller micelles, and eventually by isolated casein molecules, with much smaller molecular weight than those involved in the micelles observed in the previous case.

For $\beta$-casein in lower GdmCl concentrations ([GdmCl] ≤ 1 M), we used the Guinier approximation, Eq. (3). This approximation describes the scattered intensity in the appropriate $q$-range very well. For $\beta$-casein in higher GdmCl concentrations ([GdmCl] > 1molL$^{-1}$), we used the Debye corrected approximation, Eq. (5). Note that the Guinier approximation is valid whatever the object that is considered, linear chain or micelle. Eq. (5) on the other hand assumes that the object is a linear chain. This is in agreement with the (independent) estimate for the mass of the object that is made from the intensity at zero angle. As discussed below, the number $f$ of molecules in a micelle may be determined

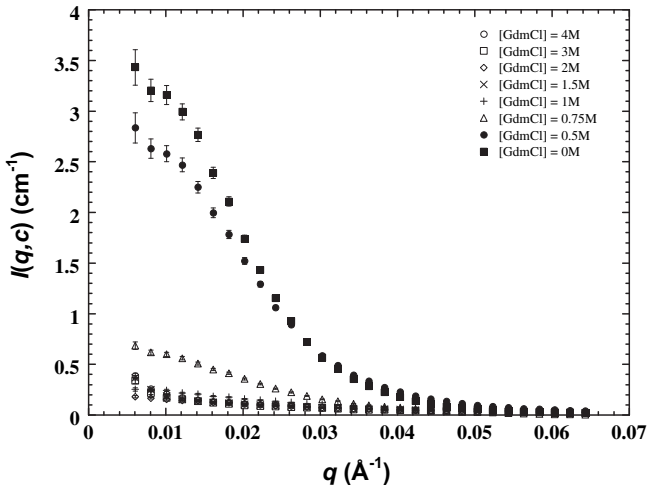


**Fig. 2.** Experimental scattering spectra, $I(q,c)$, of $\beta$-casein for various concentrations of GdmCl at 20 °C. The protein concentration is $c = 7$, 5 mg cm$^{-3}$.

from the forward intensity. For large concentrations in denaturing agent, both our results and previous studies [3,20] showed that isolated proteins only are present. We finally note that in these systems, the smallest species that may be present are the casein molecules, as they cannot be broken into smaller parts.

From these curves, it is possible to get both the radius of gyration and the aggregation numbers of the micelles. This is done in Fig. 3, which shows the transition curve (or conformation change) assessed by the variation of apparent radius of gyration $Rg(c)$ and the number of $\beta$-casein monomers involved in an aggregate, $f$, as a function of the GdmCl concentration. The number $f$ is given by the ratio $f = I_f(0,c)/I_1(0,c)$, where $I_f(0,c)$ and $I_1(0, c)$ are the forward intensities by a micelle and by a single molecule, respectively. The value used for $I_1(0, c)$ is that obtained at 4 M GdmCl. The transition midpoint is at $C_M = 1.2$ M and the curve may be described as a simple two-state transition, i.e. the micellar state and monomer state. Indeed, the radius of gyration remains constant for GdmCl concentrations larger than approximately $C_M$. Moreover, for these concentrations, the aggregation number is unity. This demonstrates that only isolated β-casein molecules are present in solution.

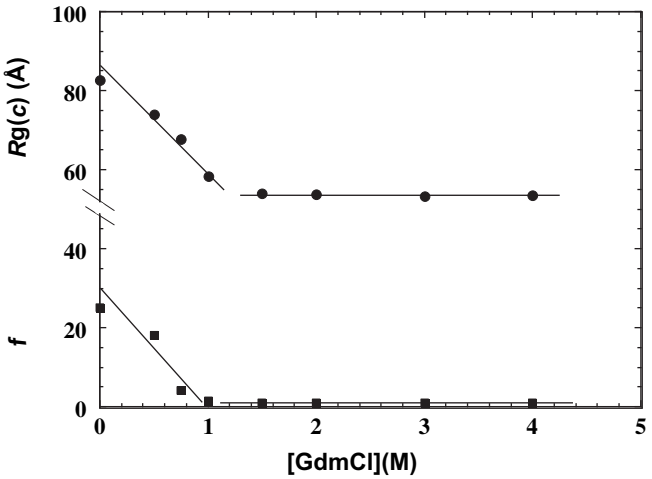


**Fig. 3.** Radius of gyration, $R_g(c)$, and aggregation number, $f$, versus the GdmCl concentration. The straight lines are merely guides for the eye.

Thus, we conclude from Fig. 3 that at a temperature of 20 °C, micelles are present in the solution for denaturing agent concentrations smaller than a critical value, on the order of 1.2 M. The transformations in micelle morphology can be explained by changes in solvent quality, i.e. the GdmCl–protein hydrophobicity. A similar study reported a decrease in the radius of gyration when the urea concentration increases or in the case of a decrease of temperature [1]. For larger concentrations, single isolated, protein molecules only are present. Their structure will not be considered here. We will merely note that we find a radius of gyration for such isolated macromolecules approximately equal to $Rg(0) = 62.2 \pm 2.0$ ∼, and that the structure looks like that of a synthetic chain with excluded volume interactions. Previous report has given value $69 \pm 2$ ∼ at 4 M of GdmCl and at 4.4 °C [3]. Also, small angle X-ray scattering data yield a predicted radius of gyration of $54 \pm 3$ ∼ [25]. This value is slightly lower than ours, which obtained for deuterated solution, but is still within the error bars of our measurements.

### 3.2. Effect of temperature on β-casein solutions

Fig. 4, shows the scattered intensity obtained at various temperatures for 7.5 mg cm$^{-3}$ concentration of β-casein in 1 M of denaturing agent concentration, near the transition midpoint. The scattered intensity values along the temperature cycles have been taken at thermodynamic equilibrium by letting the system to equilibrate after each temperature step. The data have been taken for steps of 10 °C. As for Fig. 2, two sets of curves may be observed for low and high temperatures. This indicates the presence of large micelles in the former case, i.e. when the temperature increases the $\beta$-casein monomers interact together, change of quality of solvent, and encourages the transition, $f \times$ monomers → micelle, as described by de kruif et.al [26].

Fig. 5 shows the resulting radius of gyration $R_g(c)$, and the aggregation number $f$, as a function of temperature. At low temperature (10–20 °C) the flat scattering curves of isolated "unimers" are observed. The radius of gyration $R_g(c)$ and the aggregation number $f$ are almost constant. For temperatures above 20 °C (Fig. 4), the scattered intensity increases at low $q$ values and the formation of a side maximum typical for spherical micelles is observed. The radius of gyration and the aggregation number increase and micelles are present. The changes in the micellar sizes

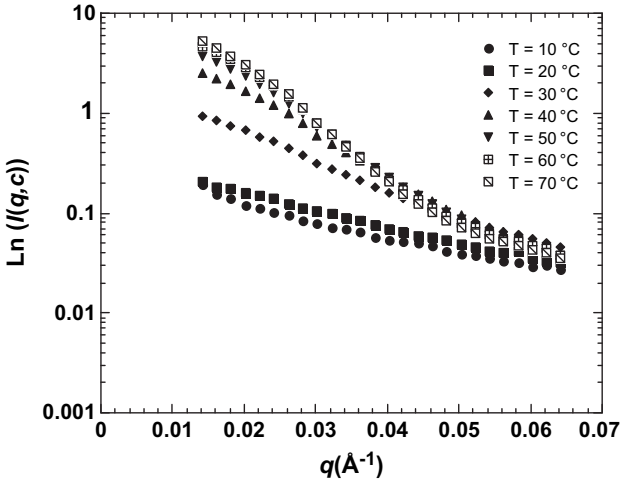


**Fig. 4.** Scattering Functions, $\ln(I(q, c))$ versus, $q$ for an aqueous solution of $\beta$-casein, obtained in the 10–70 °C temperature range. The concentration of $\beta$-casein is 7.5 mg cm$^{-3}$. The $\beta$-casein was dissolved in phosphate buffer with 0.1 M Na Cl and [GdmCl] = 1 M.

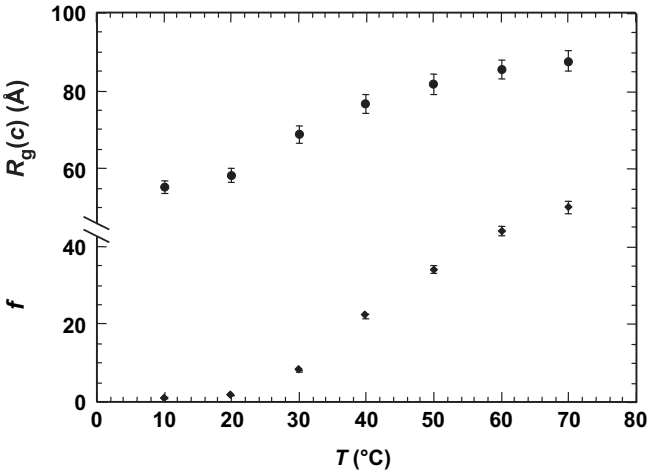


**Fig. 5.** The radius of gyration $R_g$(c) and the aggregation number $f$, evaluated according to eqs. (3) and (5), plotted against temperature. The concentration of $\beta$-casein is 7.5 mg cm$^{-3}$ and [GdmCl] = 1 M.

with temperature show that thermodynamic equilibrium may be reached. This allows optimising the aggregation number as temperature changes, as we will discuss in the last section. This may be done for various temperatures at a given denaturing agent concentration $C_D$ or for various $C_D$ at a given $T$. It leads to the variations of the parameters as a function of $T$ and $C_D$ respectively.

The SANS data presented in Fig. 4 can be also analyzed with Porod plot ($q^4I(q, c)$ vs $q$). Typical curve is shown in Fig. 6, for $T = 50$ °C and [GdmCl] = 1 M. The scatter of the experimental points and their uncertainty (error bars) increase with the increase of $q$. This is due to the reduction of SANS with the increase of scattering angle. The solid line was obtained by a fit (Eq. (8)) of the experimental data and so is a reasonable approximation here.

The variations of the core and corona sizes respectively with temperature and denaturing agent concentration, determined by the eq. (8), are shown on Fig. 7a and 7b. These figures show clearly that the radiuses of core and corona increase when the temperature increases and on the contrary for the chemical denaturant case. It is true since we know that denaturant play an inverse role than the

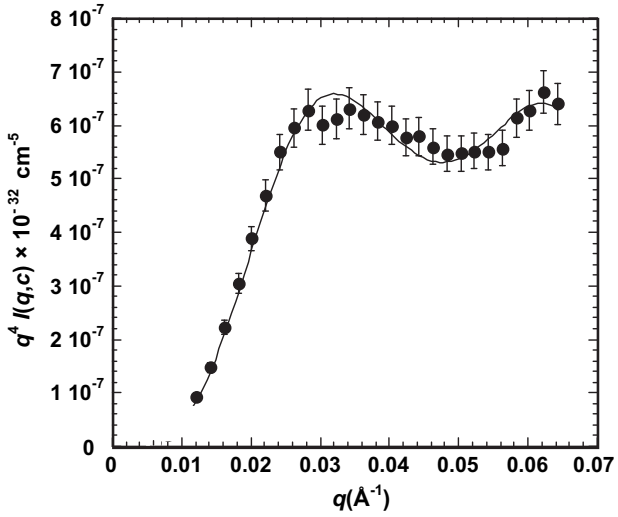


**Fig. 6.** Porod plot, $q^4I(q, c)$ vs. $q$, of SANS curve from β-casein in 1 M GdmCl at 50 °C. The solid line is the result of the Eq. (8). The radius of core, $R_{core}$ and corona, $R_{corona}$ and the forward intensity $I$ (0,$c$) are equal to 56.58 ∼, 127.05 ∼ and 5.30 cm$^{-1}$ respectively.

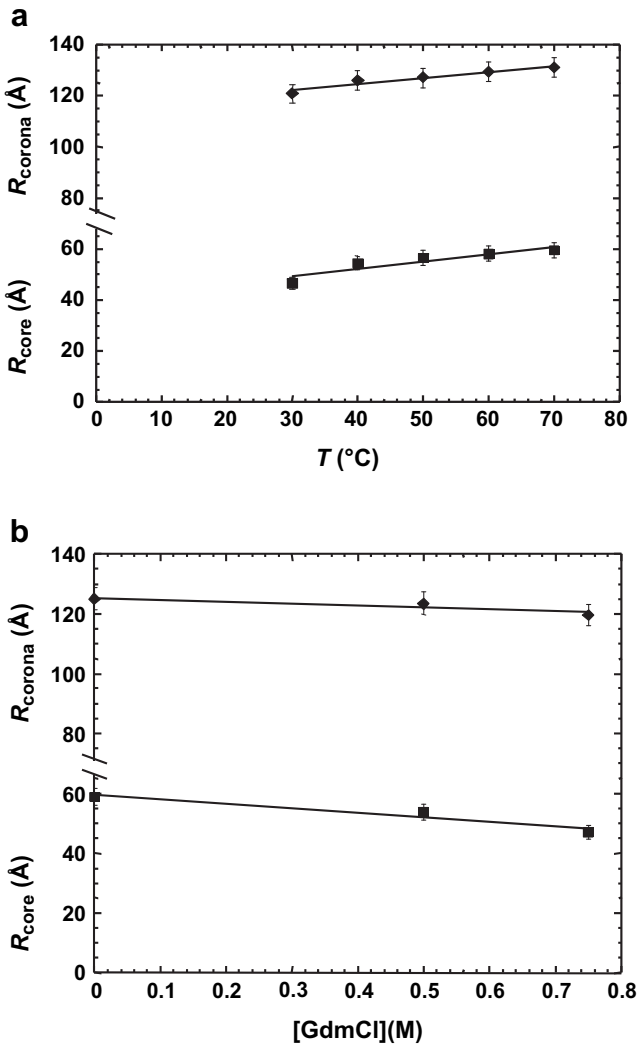


**Fig. 7.** (a) Variations of the respective core and corona radius of $\beta$-casein micelle, $R_{core}$ (●) and $R_{corona}$ (◆) versus temperature. The solid line is a guide for the eyes.(b). Variation of radius of the core, $R_{core}$ (●), and the corona, $R_{corona}$ (◆), versus GdmCl concentration, $T = 20$ °C. The solid line is only a guide for the eyes.

temperature for this type of protein [26]. Combining these results, it is possible to get the variation of the sizes with the aggregation number $f$, Fig. 8. These are discussed below.

### 3.3. Discussion

Both the influence of temperature and denaturing agent concentration might be explained by adopting an approach that was used for synthetic polymers. We would then be led to assume that as one changes either of these parameters, the quality of the solvent changes: for moderately large temperatures or low concentrations in denaturing agent, water is a poor solvent for the "hydrophobic" sequences. In the opposite limits, water becomes a good solvent for all sequences in the protein. Therefore, in the latter conditions, casein has the same behaviour as a self avoiding walk. This was already discussed by Leclerc et al. [2,27]. In the following, we would like to discuss the consequences of this change in the nature of the solvent on the conformation of the micelles. As a first, very rough, assumption, we will assume that the chain is infinite, and we introduce the usual Flory excluded volume interaction parameter $v$ for the hydrophobic part. (The hydrophilic one

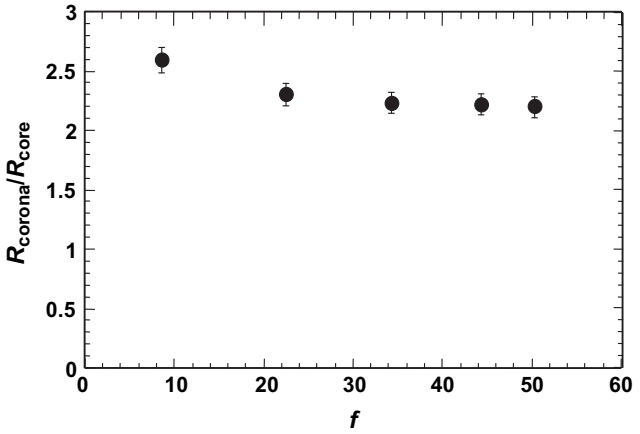


**Fig. 8.** Variation of the ratio of the core, $R_{core}$, and corona, $R_{corona}$, sizes versus the aggregation number $f$.

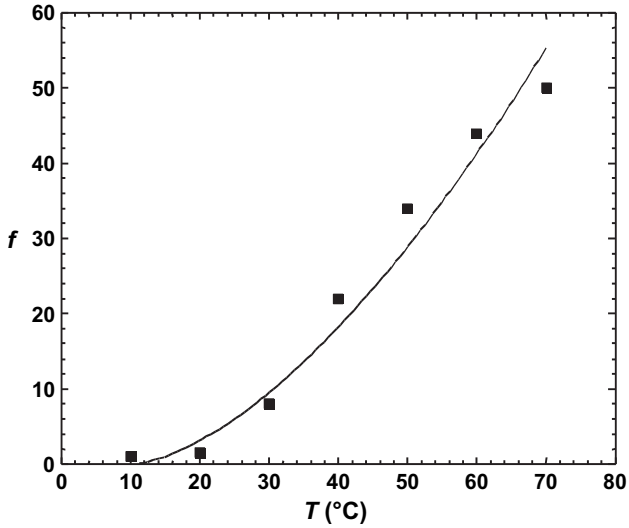


**Fig. 9.** Test of relation (21) with the experimental results.

is assumed to be always in a very good solvent). We assume that it depends on both temperature $T$ and denaturing agent concentration $C_D$. From the results obtained above, we may assume that there is a line $v(T, C_D) = 0$ in a temperature–concentration plane. Note that for an infinite chain, this implies a line of theta points $\Theta(C_D)$, where the two point interaction vanishes. In our case, this implies that on the large concentration side, the solvent is good for all sequences, excluded volume effects are present, and the protein is completely denatured and behaves as a self avoiding walk. On the low $C_D$ side on the contrary, hydrophobic effects are present and lead eventually to micelle formation. We discuss the effect for varying temperature, at a given value of $C_D$. A similar discussion may be given for given $T$ and variable $C_D$. We also assume that $v$ has a linear change with temperature in the vicinity of the theta temperature:

$$v \sim (T - \Theta)/\Theta \equiv \tau \tag{10}$$

It has been known for a long time that what is important for discussing the conformation of a linear chain of N elements not too far away from the theta temperature is neither temperature itself nor N, but a combination of both [28,29], namely $\tau N^{1/2}$ . In a poor solvent, where this variable is larger than unity, this led to the introduction of temperature blobs, made of g units, such that

$$\tau\gamma^{1/2} \sim 1 \tag{11}$$

For large values of $\tau$, and of $N$, it was assumed that the chain is a collapsed sequence of blobs. But each blob has Gaussian behaviour. Introducing the radius $B$ of a blob and $R_L$ for the chain, we have:

$$B \sim \gamma^{1/2} \sim \tau^{-1} \tag{12}$$

and

$$R_L \sim (N/g)^{1/3} B \sim (N/\tau)^{1/3} \tag{13}$$

The above variations may be used to understand the variation of the size of the casein micelles. In order to do this, we remind very briefly the micelle formation in a polymeric system [6–8]. It is assumed here that we are dealing with a dilute solution of triblock copolymers (core, shell and corona), with a block in a very good solvent, a block in $\theta$-solvent, while the other one is in a very poor solvent. We consider the symmetric case where the blocks are made of $N$ effective monomers. In this approach, one considers the free energy of the resulting micelles. This has in principle three terms, corresponding respectively to the core, the corona and the interface ($\theta$-condition). It was shown that only the first two terms are relevant. Assuming that the free energy, $F$ of the corona is similar to the one of a star branched polymer, it was found:

$$F = \gamma R_{core}^2 + f^{3/2} \tag{14}$$

where $\gamma$, $R_{core}$ and $f$ are respectively the surface tension between the solvent and the solvophobic block, the radius of the core, and the number of triblocks in the micelle. Assuming that there is no solvent in the core, one may relate $f$ and $R_{core}$: the volume of the core is the total number of solvophobic effective monomers.

$$R_{core}^3 \sim Nf \tag{15}$$

where, $N$ is the number of units in each of the sequences of the triblock (effective monomers), which we assume to be symmetric. Minimizing the free energy with respect to $f$, and using (15), we find, for a very poor solvent

$$f \sim N^{4/5}\gamma_0^{6/5} \tag{16}$$

Finally, in order for the micelles to exist, one has to have a concentration in casein that is above the critical micelle concentration $\Phi_{CMC}$. The latter is determined by comparing the chemical potentials of a triblock copolymer when inserted into a micelle and in the free – isolated – state. The latter is merely its entropy:

$$F_1 = \ln \Phi/\Phi_1 \tag{17}$$

where $\Phi_1$ is a concentration to be discussed below.

The former is, using relations (14) and (16):

$$F_2 = N^{2/5}\gamma^{3/5} \tag{18}$$

Comparing these two quantities leads to the critical micelle concentration

$$\Phi_{CMC} \sim \Phi_1 \exp N^{2/5}\gamma^{3/5} \tag{19}$$

$\Phi_1$ is a reference concentration. As this discussion is valid in the poor solvent regime, one may assume that it is identified to the branch of the coexistence curve between water and the

**Table 1**
Variations of the internal, external radius and the ratio of the core, $R_{core}$, and corona, $R_{corona}$ versus temperature ($R_{core}$) ≅ 2.6.($R_{corona}$)$^{5/8}$, [GdmCl] = 1 M.

| $T$ (°C) | $R_{core}$ (Å) | $R_{corona}$(Å) | $(R_{corona})^{5/8}$ | $R_{core}/(R_{corona})^{5/8}$ |
|---|---|---|---|---|
| 30 | 46.576 | 120.93 | 20.026 | 2.3258 |
| 40 | 54.397 | 125.91 | 20.537 | 2.6487 |
| 50 | 56.583 | 127.05 | 20.653 | 2.7397 |
| 60 | 58.060 | 129.50 | 20.901 | 2.7778 |
| 70 | 59.471 | 131.13 | 21.065 | 2.8232 |

corresponding hydrophobic sequences. Note that as the theta temperature is approached, this becomes of the same order as the overlap concentration of the "hydrophobic" sequences. This may be generalized easily to a solvent that is moderately poor by using the blobs that were introduced above. One has then to realize that some solvent is present inside these, and that the blobs are densely packed inside the core. There is no change for the corona, but this implies that the core is filled of blobs instead of monomers:

$$R_{core}^3 \sim (N/g) f B^3 \tag{20}$$

Similarly the interfacial tension becomes $\gamma_0/B^2$.

Minimizing the resulting free energy and using relation (12) leads to

$$f \sim N^{4/5} \gamma_0^{6/5} \tau^{8/5} \tag{21}$$

Similarly, the critical concentration also depends on $\tau$

$$\Phi_{CMC} \sim \Phi_1 \exp\{N^{2/5} \gamma^{3/5} \tau^{4/5}\} \tag{22}$$

Therefore, as $\tau$ decreases, the number of chains in the micelle also decreases, until it reaches a critical micelle temperature $\tau$.

$$\tau \sim N^{-1/2} \gamma_0^{-3/4} \tag{23}$$

Relation (21) was tested with our experimental values. The theta temperature was set at 10 °C. The results are shown on Fig. 9, and exhibit a rather good agreement between the theoretical and experimental results. Based on high-sensitivity differential scanning calorimetry measurements, Mikheeva et al. [30] give a detailed analysis of the $\beta$-casein micellation and their result is remarkably similar to the data points of Fig. 9, except for a shift which may be due to a difference in composition and especially in concentration. The Fig. 9 shows that the micelles may grow indefinitely, for temperatures higher than 70 °C, this is a consequence of the applied model, which assumes a linear variation of the excluded volume parameter with temperature. Obviously this is only valid in the vicinity of the theta point only, and the variation flattens for large temperatures.

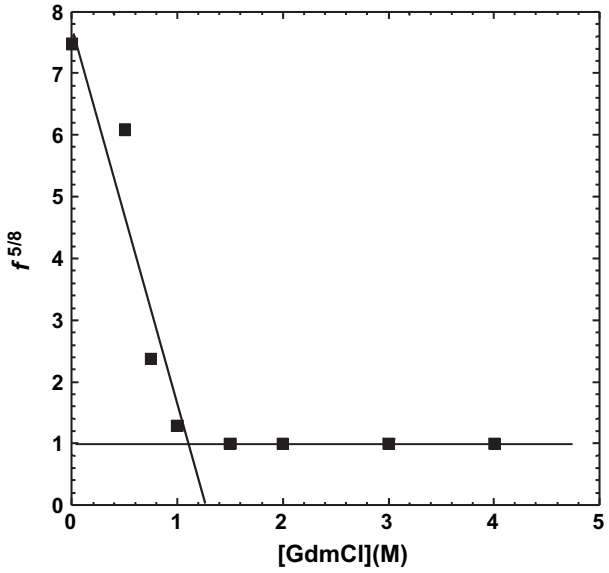


**Fig. 10.** Test of relation (29) with the experimental results. The former implies a linear variation of $f^{5/8}$ with the concentration in denaturing agent.

Finally, the radius of the core may be deduced from relations (20) and (21). We find:

$$R_{core} = R_0 \tau^{1/5} \tag{24}$$

Combining relations (21) and (24), and eliminating $\tau$, we find

$$R_{core} = R_1 f^{1/8} \tag{25}$$

For long star shaped polymers, it was found theoretically, and experimentally, that the outer radius of the corona varies [31] as $f^{1/5}$. This implies that as temperature varies, the variations of the internal and external radius are not similar, and that as far as temperature variations are concerned, we predict, by eliminating $f$,

$$R_{core} \sim {R_{corona}}^{5/8} \tag{26}$$

The latter prediction was compared with our experimental results. This is shown in Table 1, and we find experimentally that

$$R_{core} \sim 2.6 {R^{5/8}}_{corona} \tag{27}$$

Let us note that a similar discussion may be done with the denaturing agent concentration $C_D$. It was shown that for large $C_D$, the protein behaves as a polymer in a good solvent. This implies that the excluded volume parameter vanishes, and eventually changes sign for the "hydrophobic" groups at some value $C_D^C$. Assuming that $v$ varies linearly with $C_D$, i.e.

$$v(T, C_D) \sim \Gamma \equiv (C_D - C_D^C)/C_D^C \tag{28}$$

The same theoretical arguments as above apply, and we find a relation identical to (21), with $\Gamma$ replacing $\tau$. Then at a given temperature, we find, as a function of $\Gamma$:

$$f \sim N^{4/5} \gamma^{6/5} \Gamma^{8/5} \tag{29}$$

Therefore, as the denaturing agent concentration increases towards $C_D^C$, $\Gamma$ decreases, until a concentration difference $\Gamma^*$, where the micelles disappear. Equating $f$ to unity in the last relation leads to:

$$\Gamma^* \sim N^{-1/2} \gamma_0^{-3/4} \tag{30}$$

Relation (29) was compared with the experimental results by plotting $f^{5/8}$ as a function of the concentration in denaturing agent. This is shown on Fig. 10, and is in reasonable agreement with the linear variation implied by relation (29).

Note that because of the similar variations found in relations (21) and (29), relation (25) is valid when either temperature or denaturing agent concentration is varied in the regions where micelles are present.

## 4. Conclusion

The structure of beta-casein molecules was observed by SANS both at a given concentration in denaturing agent and varying

temperature and at a given temperature as a function of the concentration in denaturing agent. Micellar aggregates are found for larger temperatures in the first case, and for low concentrations $C_D$ in denaturing agent in the second one. The aggregation number decreases, as either temperature decreases or $C_D$ increases. This implies that the critical casein micellar concentration depends on both parameters. In a ($T$, $C_D$) diagram, there is a line separating a region, for low $T$ and large $C_D$ where only isolated beta-casein molecule are present, from another one where micelles coexist with beta-casein molecules. These results were interpreted by using a simple copolymer model where we assumed that the excluded volume parameter for the initially hydrophobic sequences depends on both $T$ and $C_D$. This is a first attempt to describe the behaviour of partially denatured proteins by a simple model that applies for synthetic polymers. The only relevant parameter that we kept is an effective excluded volume parameter $v$ related to the "hydrophobic" sequence. This is needed in order to explain the existence of micelles. Because their size varies with temperature and concentration in denaturing agent, we also assumed that $v$ varies and eventually changes sign: the quality of the solvent changes for the hydrophobic sequence as either of these parameter varies. This leads to some predictions that are in reasonably good agreement with the SANS observations.

We note that some limitations should apply to the above considerations. The ideas that were used are valid for very large chains. The sequences however are finite, and rather short. This may imply two consequences: the first one is the possible presence of a bending energy, related to the fact that we are dealing with a triblock copolymer rather than a diblock. Therefore some sequences start from and come back to interface between the core and the corona. A second one is related to the fact that the sequences are not very large, so that a limitation may appear, related to the size of the largest "hydrophobic" sequence.

Finally, although the approach that was used is very crude, it seems to lead to satisfactory results. We end this discussion by noting the possible existence of a surface of theta points as both temperature and denaturing agent concentration are varied.

## Acknowledgements

Our thanks to MENRT (France) and MESRST (Tunisia) for supporting the stay of A. Aschi in France in the frame of the research and teaching network 07/317/TN.

## References

[1] O'Connell JE, Grinberg VYA, de Kruif CG. J Colloid Interface Sci 2003;33:258.
[2] Leclerc E, Calmettes P. Phys Rev Lett 1997;78:150.
[3] Calmettes P, Durand D, Receveur V, Desmadril M, Minard P, Douillard R. Physica B 1995;213/214:754.
[4] Leclerc E, Calmettes P. Physica B 1997;234:207.
[5] Leclerc E, Calmettes P. Physica B 1998;241:1141.
[6] Halperin A. Macromolecules 1987;20:2943.
[7] Halperin A. Macromolecules 1989;22:3806.
[8] Halperin A. Macromolecules 1991;24:1418.
[9] Borisov OV, Halperin A. Macromolecules 1996;29(7):2612.
[10] Leckband DE, Borisov OV, Halperin A. Macromolecules 1998;31(7):368.
[11] Cornette JL, Cease KB, Margalit H, Spouge JL, Berzofsky JA, DeList C. J Mol Biol 1987;195:659.
[12] Dickinson E. Int Dairy J 1999;9:305.
[13] Horne DS, Curr O. Colloid Interface Sci 2002;7:456.
[14] Flory PJ. Principles of polymer chemistry. Ithaca: Cornell University Press; 1953.
[15] Mercier MC, Maubois JL, Posnanski S, Ribadeau-Dumas B. Bull Soc Chim Biol 1968;50:521.
[16] Nozaki Y. Methods Enzymol 1970;26:43.
[17] Calmettes P, Durand D, Smith JC, Desmadril M, Minard P, Douillard R. J Phys IV (Paris) 1993;3(C-8):253.
[18] Guinier A, Fournet G. Small angle scattering of X-rays. New York: Wiley Interscience; 1955.
[19] Debye J. J Appl Phys 1944;15:338.
[20] Calmettes P, Durand D, Desmadril M, Minard P, Receveur V, Smith JC. Biophys Chem 1994;53:105.
[21] des Cloizeaux J, Jannink G. Polymers in solution: their modeling and their structure. Oxford: Clarendon Press; 1990.
[22] Brendan T, Kennedy O, Mounsey JS. J Agric Food Chem 2006;54(15):5637.
[23] Panouille M, Durand D, Nicolai T. Biomacromolecules 2005;6(6):3107.
[24] Huppertz T, Smiddy MA, de Kruif CG. Biomacromolecules 2007;8(4):1300.
[25] Andrews AL. Biopolymers 1979;18:1105.
[26] de kruif CG. Langmuir 2002;18:4885.
[27] Leclerc E. Thesis. University of Paris VI; 1996.
[28] Daoud M, Jannink G. J de Phys 1976;37:973.
[29] Cotton JP, Nierlich M, Boue F, Farnoux B, Daoud M, Jannink G, et al. J Chem Phys 1976;65:1101.
[30] Mikheeva LM, Grinberg NV, Grinberg VYA, Khokhlov AR, de Kruif CG. Langmuir 2003;19:2913.
[31] Daoud M, Cotton JP. J de Physique 1982;43:531.