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pH-dependent structures and properties of casein micelles

Yan Liu, Rong Guo *

School of Chemistry and Chemical Engineering, Yangzhou University, Yangzhou, 225002, PR China

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ABSTRACT

The association behavior of casein over a broad pH range has first been investigated by fluorescent technique together with DLS and turbidity measurements. Casein molecules can self-assemble into casein micelles in the pH ranges 2.0 to 3.0, and 5.5 to 12.0. The hydrophobic interaction, hydrogen bond and electrostatic action are the main interactions in the formation of casein micelles. The results show that the structure of casein micelles is more compact at low pH and looser at high pH. The casein micelle has the most compact structure at pH 5.5, when it has almost no electrostatic repulsion between casein molecules.

1. Introduction

Biomaterials based on biologically derived polymers, including peptides, protein, lipids, and oligonucleotides [1–4], have been shown to exhibit properties beneficial to food, cosmetic and medical science and will have significant implications in advances of future generations of materials.

In milk the most abundant protein, casein, is easily available in a high purity at a low cost with several possibilities of technological applications. These applications arise from the multifunctional properties of casein that depend on the supramolecular structure. The casein constituents, α_{s1}-, α_{s2}-, β-, and κ-casein, exist in proportions of approximately 4:1:4:1 by weight. Approximately, caseins can be thought of as amphiphilic block copolymers consisting of blocks with high levels of hydrophobic or hydrophilic amino acid residues [5,6]. Caseins exhibit a strong tendency to self-assemble into casein micelles because of its amphiphilic property in aqueous solution.

The mechanism of casein assembly has been the subject of much speculation, and the casein micelle structure is not yet established up to now. Various models of casein micelle structure have been proposed over the last 50 years [7–10]. Among them, two conflicting models for the internal structure of casein micelles have arisen. The submicelle model emphasized the role of hydrophobic interactions in giving rise to submicelles. However, the other model relies solely on the interactions between the caseins and calcium phosphate to hold the micelle together. In the later model, the calcium phosphate is in the form of nanoclusters and the interaction sites on the caseins are the phosphoseryl clusters of the calcium-sensitive caseins (e.g. αs1- and β-casein).

It is well-known that microenvironment such as pH, salt and surfactant can significantly alter the behavior and overall performance of biomolecules [11–16]. In earlier researches, numerous techniques have been employed to study the effect of pH on the properties of casein micelles, including viscosity measurement [17,18], particle size analyzer [19,20], dynamic light scattering [21,22], zeta potential measurement [18] and turbidity measurement [23]. These studies, however, have sometimes produced conflicting results, and the effect of pH on the properties of casein micelle remains unclear. Two explanations can be offered for the inconsistencies among those studies. First, in some of the studied milk system, the presence of other constituents in milk often makes the studied system become complicated, and the effect of pH on the complex of whey protein and casein is actually studied. Second, the concentration of casein, temperature and the ionic strength all affect the properties of casein micelles, which also makes the effect of pH on the casein micelle more complex.

As we all know, fluorescent molecules are useful probes for sensing the microdomain property of polymeric systems [24–27]. Recently, we have studied the effect of surfactant on the structure and property of casein micelles mainly by fluorescence spectra from molecular level [14,15]. In this article, we study the self-assembly process of casein and the effect of pH on the properties of casein micelle by means of

* Corresponding author. Tel.: +86 514 7971858; fax: +86 514 7311374.
E-mail address: guorong@yzu.edu.cn (R. Guo).

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fluorescence measurement (including intrinsic fluorescence spectra and fluorescence spectra of extrinsic probes such as pyrene and 1-anilino-8-naphthalenesulfonate (ANS)) together with DLS and turbidity measurements. Therefore, we can obtain detailed and deep-going information of the casein micelle structure and the structure–property relationship of casein micelles at different pHs on molecule level, which will broaden the application range of casein in food, cosmetic and medicine domains.

2. Materials and methods

2.1. Materials

Casein was purchased from Sigma (90%). Pyrene and 1-anilino naphthalene-8-sulfonate (ANS) (99%) were obtained from Aldrich. Casein powders were dispersed in Milli-Q water under stirring at 50 °C, and the dispersions were stored at 4 °C overnight to allow complete hydration. Then, the pH was adjusted to desired values by adding appropriate 1 M NaOH and 1 M HCl. The casein solutions were filtered through a Millipore filter with a 2.0 μm pore size and thermostated at 25 °C for at least 0.5 h before use. All other reagents used were of analytical grade.

2.2. Turbidity measurements

Turbidity measurements were carried out with a Shimadzu 1601 PC UV/vis spectrometer. The turbidity of the casein solutions was monitored by the transmittance at 450 nm. A cuvette with 1 cm pathway was used. All the measurements were conducted at (25±0.1) °C.

2.3. Dynamic light scattering (DLS)

Dynamic light-scattering measurements were made at 25.0±0.1 °C and at a scattering angle of 90° to the incident beam, using an ALV 5022 laser light-scattering instrument equipped with a 22 mW He–Ne laser at 632 nm (JDS model 1145P) in combination with an ALV-5000 digital correlator with a sampling time range of 1.0 μs to 1000 ms. Experiment duration was in the range of 5–10 min, and each experiment was repeated two or more times.

2.4. Steady-state fluorescence

Steady-state fluorescence experiments were performed with a RF-5301 luminescence spectrometer (Japan Shimadzu Company) equipped with a thermostated water-circulating bath. During the experiments, for casein solution, the excitation and emission slits were fixed at 3.0 and 1.5 nm, respectively, the excitation wavelength was set at 295 nm, and the emission spectra were collected from 300 to 500 nm. In all the measurements, the scan rate was selected at 240 nm/min.

Pyrene was used as the probe to determine the microenvironmental polarity of casein micelles by observing its fluorescence fine structure. The emission spectra were measured in the range of wavelength 350–600 nm with the excitation wavelength being 338 nm. The pyrene concentration was 1.0×10^{-6} M.

The ANS emission spectra were measured in the range of wavelength 400–560 nm with the excitation wavelength being 380 nm. The ANS concentration was 1.0×10^{-5} M.

3. Results and discussion

3.1. The formation of casein micelles probed by pyrene fluorescence

Pyrene has a very low solubility in water (7.0×10^{-7} M), and as a result, it is selectively solubilized in the hydrophobic region or microphases existing in aqueous medium. The vibration fine structure of its mononer fluorescence spectra in solution makes pyrene an excellent probe of local environment polarity changes. The intensity ratio of the first peak to the third (I_1/I_3) of the fluorescence spectrum of pyrene shows the microenvironmental polarity where the probe exists [28]. The abrupt change of I_1/I_3 as a function of surfactant concentration has been commonly used to determine the critical micelle concentration (cmc) of surfactant solutions. Furthermore, an excited monomer can encounter a ground-state pyrene to form an excimer, which produces a broad band at about 450 nm. The ratio of the maximum emission intensity of the excimer (I_e) to the monomer (I_1) for pyrene, I_e/I_1, can be used to judge the efficiency of excimer formation, which can provide further information about the hydrophobic domain in the micelle.

Fig. 1A shows the fluorescence spectra of pyrene in casein solution with different casein concentrations at pH 7.0. Fig. 1B shows I_1/I_3 and I_e/I_1 of pyrene against casein concentration at pH 7.0. As shown in Fig. 1A, the value of I_1/I_3 decreases gradually with increasing concentration of casein over the wide range of concentration from 0.1–1.0 mg/ml. A plateau in the I_1/I_3 vs casein concentration plots appears beyond 1.0 mg/ml. The concentration 1.0 mg/ml can be considered as the critical micellar concentration (cmc). A significant aspect is the gradual decrease in I_1/I_3 between concentrations 0.1 and 1.0 mg/ml, which is unlike the behavior of typical surfactants such as SDS and CTAB. This gradual decline implies that premicellar aggregates are formed and the aggregation number of the premicelle increases with the casein concentration.

Fig. 1B also shows that I_e/I_1 has a maximum at a particular concentration just below 1.0 mg/ml, and diminishes with increasing concentration of casein. It has been reported that a 2×10^{-6} mol/l ethanol solution of pyrene does not exhibit the excimer emission. However, the results in this study show that, compared with ethanol solution, a lower pyrene concentration (1×10^{-6} mol/l) suffices in these systems for efficient excimer formation. Pyrene molecules can be assumed to be dissolved in hydrophobic microdomains, where the local concentration is much higher than the bulk concentration. The appearance of the maximum excimer emission of pyrene in dilute casein solutions below the cmc shows that premicellar aggregates provide a space where the local concentration of pyrene is much higher than the bulk concentration. The pyrene excimer is considered to have formed when pyrene molecules are close to each other in casein aggregates. When the aggregation number of the premicelles is small enough, the pyrene molecules are in intimate contact in a restricted hydrophobic environment. Above cmc, the micelles grow to an infinitely large size to solubilize the pyrene molecules separate. Consequently, I_e/I_1 decreases at this concentration. The pyrene excimer results here also confirm the formation of submicelles of casein.

The pyrene fluorescence spectra were further used to study the self-assemble behavior of casein in the pH ranges of 2.0–3.0, and 5.5–12.0. Fig. 2A and B shows the ratios I_1/I_3 and I_e/I_1 of pyrene against casein concentration at two typical pHs, i.e. pH 2.0 and 12.0. As shown in Fig. 2, a plateau in the I_1/I_3 and the maximum I_e/I_1 vs casein concentration both appear too, which indicates that the casein micelle is formed with the increase of casein concentration at the two pHs. Fig. 2 also shows that the ratios I_1/I_3 and I_e/I_1 of pyrene differ to some extent at different pHs, which will be discussed deeply in the following section. Similar pyrene fluorescence results also suggest the formation of casein micelles at other pHs (data not shown).

The above results clearly indicate that casein micelles are formed at high pH, even at pH 12.0. Contrary to our results, Vala et al. found that casein micelles were disrupted at pH higher than 9.0 [23]. They suggested that the strong electrostatic repulsive might lead to the dissociation of casein micelles. In our case, the hydrophobic interaction between the hydrophobic portions of casein molecules still plays an important role in the self-assembly of casein molecules, and hence the formation of casein micelles at higher pH. This is in agreement with the results of Zhong et al. [29]. In their research, they indicated