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Enhanced Solubilization of Bovine Serum Albumin in Reverse Micelles by Compressed CO_2

Xiaoying Feng, Jianling Zhang, Jing Chen, Buxing Han,* and Dong Shen[a]

Abstract: The effect of compressed CO_2 on the solubilization of bovine serum albumin (BSA) in water/sodium bis-(2-ethylhexyl) sulfosuccinate (AOT)/isooctane reverse micelles was studied by observing phase behavior and recording UV-visible spectra under different conditions. The pH values within the water cores of reverse micelles at different CO_2 pressures were also determined. The solubilization capacity of the reverse micelles for the protein increased considerably as CO_2 pressure increased within the low-pressure range, but decreased at higher CO_2 pressures, so that the micelles eventually lost their ability to solubilize the protein. The effect of CO_2 on the stability of the reverse micelles played an important role in the relationship between pressure and protein solubility. A "multicomplex" model was proposed to explain these effects. The different solublization capacities within different pressure ranges demonstrates the unique advantage of using compressed CO_2 in the extraction of proteins with reverse micelles.

Keywords: BSA · carbon dioxide · micelles · solubilization · surfactants

Introduction

Reverse micelles are thermodynamically stable surfactant aggregates with polar cores, which are formed spontaneously as certain types of surfactants dissolve in nonpolar solvents.[1] They have attracted considerable attention due to their ability to host hydrophilic components in organic solvents. These systems are suitable media for various processes, such as chemical reactions,[2] preparation of fine particles,[3] and enzyme-activity studies.[4] Extraction and fractionation of proteins from aqueous solutions by using reverse-micellar solutions has been studied extensively.[5] It has been shown that the extraction efficiency depends mainly on the hydrophobic and electrostatic interactions between the charged headgroups of the surfactants and the protein molecules. These interactions are determined by the properties of the proteins, the nature of the surfactants, the properties of the organic solvents,[6] and are significantly affected by the ionic strength and pH of the aqueous phase.[7] Thermodynamically, three main factors affect the solubilization of proteins; the free-energy change due to the electrostatic interactions between the charged headgroups of the surfactant and the proteins, the entropy change due to protein entrapping and the reconstitution of the reverse micelles, and the interfacial energy change.[8]

As to the solubilization mechanism, a general view, accepted by most authors in this field, is that the solubilization of protein leads to protein-containing reverse micelles, and this process varies according to the hydrophility of the proteins.[9] However, as for the detailed structural organization, two contrary models have been proposed. One is the water-shell model reported by Luisi's group.[10] Another model, proposed by Martinek and co-workers, is defined as the induced-fit or fixed-size model and functions according to the size of the water cores.[11] In addition, Dordick and co-workers have proposed an electrostatic complex model to explain the solubilization of chymotrypsin (CMT) in sodium bis-(2-ethylhexyl) sulfosuccinate (AOT) reverse micelles.[12] Nevertheless, the mechanism for the solubilization of proteins requires further study.

Some compressed gases, such as CO_2, are quite soluble in a number of organic solvents. This causes the solvent to expand and the gas acts as an antisolvent. The gas-antisolvent technique has been widely used in fractionation and separation,[13] particle formation and precipitation,[14] and recovery of proteins from reverse micelles.[15] In a gas-antisolvent process, the properties of the liquid solvent can be tuned by changing the pressure, and the separation of gas-antisolvent and liquid solvent can be achieved easily by depressing the system.[16]

[a] X. Feng, Dr. J. Zhang, J. Chen, Prof. Dr. B. Han, Dr. D. Shen
The Center for Molecular Sciences, Institute of Chemistry
Chinese Academy of Sciences, Beijing 100080 (China)
Fax: (+86) 10-6255-9373
E-mail: Hanbx@iccas.ac.cn

We have demonstrated previously that compressed CO_2 can induce the formation of reverse micelles from polymer surfactant,[17] and can also enhance the stability of reverse micelles formed from both nonionic[18] and ionic[19] surfactants. The main reason for this is that CO_2 can penetrate into the tail region of the surfactant molecules and increase the rigidity of the interfacial films of the reverse micelles.

Disadvantages in using reverse micelles for extracting proteins are the overall low efficiency and the difficulty in reusing the reverse-micellar solutions.[20] This restricts the further application of this technique. Here, we studied the effect of CO_2 on the solubilization of bovine serum albumin (BSA) in water/AOT/isooctane reverse-micellar solutions. Interestingly, compressed CO_2 can enhance significantly the solubilization of BSA at suitable pressures, and the protein can be recovered completely at higher pressures, while the AOT remains in solution. Therefore, by controlling CO_2 pressure, both extraction and recovery efficiencies for the protein can be increased. Furthermore, the ability of reverse micelles to extract the protein depends strongly on the stability of the micelles. We propose a qualitative "multicomplex" model to explain this observation.

Results

Phase behavior of reverse-micellar solution in the presence of CO_2: The reverse-micellar solutions can be expanded by the dissolution of CO_2. The increase in volume can be expressed by the volume-expansion coefficient $\Delta V=(V-V_0)/V_0$, in which V_0 and V represent the volumes of the CO_2-free and CO_2-saturated reverse-micellar solutions, respectively. We determined the ΔV of the water/AOT/isooctane reverse-micellar solutions at 297 K and at different W_0 and AOT concentrations. The dependence of ΔV on CO_2 pressure is demonstrated in Figure 1. As expected, ΔV increases as pressure increases, because the solubility of CO_2 in the solution is greater at higher pressures. A slight decrease in ΔV is observed as the concentration of AOT increases, which may result from the poor miscibility of CO_2 and AOT. In addition, the variation in W_0 has a negligible effect on the ΔV.

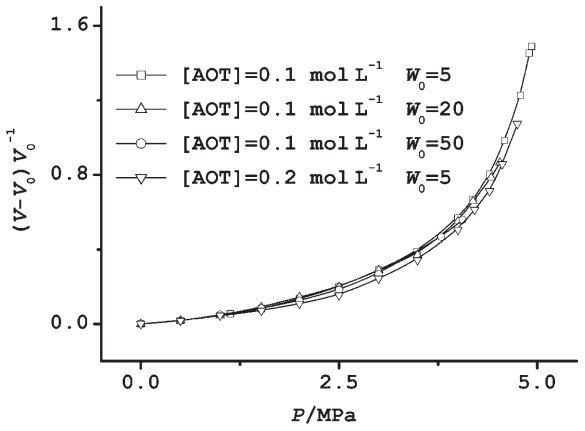

Figure 1. Dependence of the volume-expansion coefficient ΔV of water/AOT/isooctane reverse-micellar solutions on CO_2 pressure at 297 K.

AOT can be precipitated if the CO_2 pressure is sufficiently high, and the pressure at which the surfactant begins to precipitate is called cloud-point pressure (Pc). The Pc values for water/AOT/isooctane reverse-micellar solutions under different conditions are listed in Table 1. The Pc decreases as W_0 and the concentration of AOT increase. All of the BSA solubilization experiments and UV-visible studies were carried out at pressures lower than Pc.

Table 1. The Pc values of the water/AOT/isooctane reverse-micellar solutions at 297 K and at different W_0 levels and AOT concentrations.

[AOT] [mol L^{-1}]	0.1	0.1	0.1	0.2
W_0	5	20	50	5
Pc [MPa]	4.93	4.53	4.07	4.75

Effect of compressed CO_2 on the solubilization of BSA: W_0 and surfactant concentration are important parameters in the characterization of reverse-micellar solutions, and may affect the extraction of proteins considerably.[21] Here, the maximum concentration of BSA, $[BSA]_{max}$ (under saturation conditions), in the reverse-micellar solutions with various W_0 values and AOT concentrations were determined at different CO_2 pressures. The results are shown in Figure 2. Interestingly, at each of the W_0 values and AOT concentrations, $[BSA]_{max}$ increases as CO_2 pressure increases within the low-pressure range. Therefore, compressed CO_2 can enhance considerably the solubilization of BSA in the water/AOT/isooctane reverse micelles within a suitable pressure range. It is also apparent that $[BSA]_{max}$ in all the reverse-micellar solutions decreases after reaching a maximum. Here, we define the CO_2 pressure at which the reverse micelles host the largest quantity of BSA as $P^{max}_{CO_2}$. It is evident that $P^{max}_{CO_2}$ shifts to lower pressure as the W_0 value increases. The detailed mechanism will be discussed in the following sections.

At a fixed AOT concentration (0.1 mol L^{-1}), the $[BSA]_{max}$ increases as W_0 increases, in the absence of CO_2, as shown in Figure 2. This is because larger water cores can host more protein. Figure 2a and d shows that the effect of pressure on $[BSA]_{max}$ at [AOT] of both 0.1 and 0.2 mol L^{-1} is similar. However, the reverse-micellar solution with the higher AOT concentration can host a larger quantity of BSA at the same W_0, because the solution contains more water and AOT.

Verification of solubilization of BSA by UV-visible studies: Due to the strong absorption of tyrosine residues at around 280 nm, the absorbance spectrum of BSA can be employed to detect BSA concentration. This provides a simple means to quantify BSA without the need for derivatization. Here, we also studied the effect of CO_2 on the solubilization of BSA by using UV-visible methods. Figure 3 shows the UV-

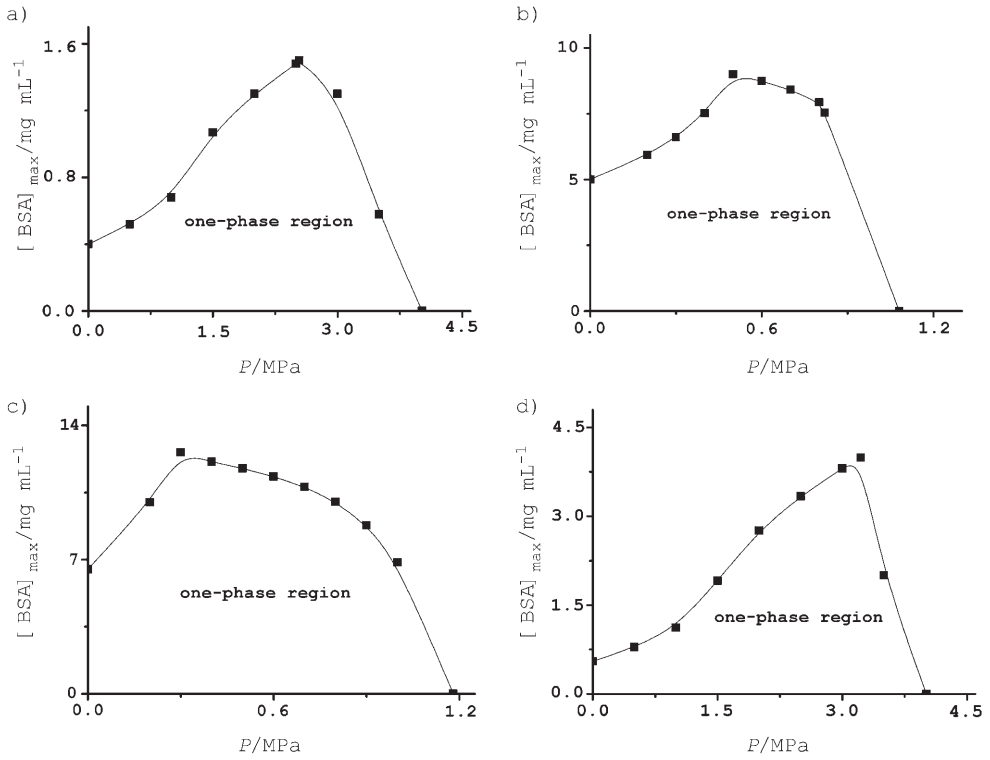

Figure 2. Dependence of maximum BSA concentration, $[BSA]_{max}$, in the AOT reverse-micellar solutions on CO_2 pressure at 297 K. a) $[AOT]=0.1\ mol\,L^{-1}$, $W_0=5$; b) $[AOT]=0.1\ mol\,L^{-1}$, $W_0=20$; c) $[AOT]=0.1\ mol\,L^{-1}$, $W_0=50$; d) $[AOT]=0.2\ mol\,L^{-1}$, $W_0=5$.

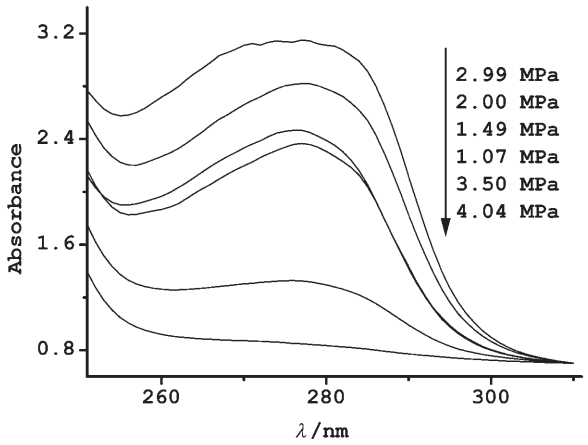

Figure 3. UV-visible absorption spectra of BSA in the AOT reverse-micellar solutions under different CO_2 pressures at 297 K ($[AOT]=0.2\ mol\,L^{-1}$, $W_0=5$).

visible absorption spectra of BSA in the reverse micelles ($[AOT]=0.2\ mol\,L^{-1}$, $W_0=5$) at some typical CO_2 pressures. In all experiments, the reverse-micellar solutions were saturated with BSA, and, therefore, the absorbance reflects the maximum concentration of the protein in the solution. The intensity of absorbance of the protein at around 280 nm[15a,22] increases gradually as the pressure increases from 1.07 to 2.99 MPa, indicating that more BSA is solubilized in the reverse micelles at higher pressures within this pressure range. If the pressure exceeds 2.99 MPa, the absorbance decreases sharply, indicating the precipitation of BSA from the reverse micelles. The turning point of the pressure in Figure 3 (2.99 MPa) is consistent with the $P^{max}_{CO_2}$ (3.10 MPa) shown in Figure 2d.

pH of the water cores in the reverse micelles: Because CO_2 can dissolve in the water cores of the reverse micelles to form carbonic acid, the pH of the water droplets in the reverse micelles should change after the addition of CO_2, which might affect the solubilization of BSA. We measured the pH value of the water cores inside the reverse micelles at different CO_2 pressures in the absence of BSA by using UV-visible methods, and an acid-sensitive indicator, bromophenol blue (BPB), as the probe.[23] The UV-visible spectra of BPB in citric acid buffers with different pH values were first determined to obtain the working curve. The UV-visible spectra of BPB in water cores of the reverse micelles were then measured at 297 K and at different CO_2 pressures under equilibrium conditions. The pH values of the water cores were obtained from the working curve, and the results are plotted in Figure 4. The pH of the water cores decreases as the pressure of CO_2 increases. At a given CO_2 pressure, the pH inside the reverse micelles decreases gradually as W_0 increases. As can also be seen from Figure 4, the pH value is lower at higher AOT concentrations.

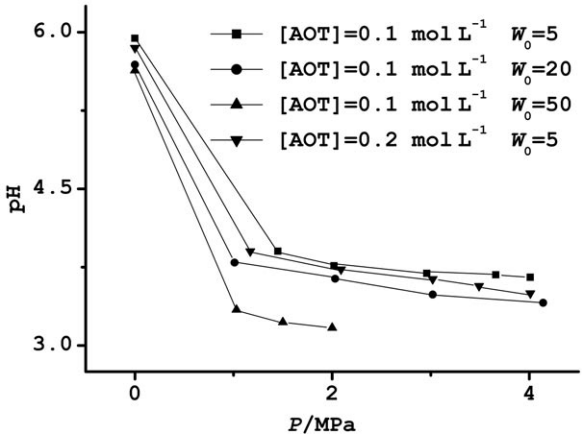

Figure 4. Dependence of the pH values in the water cores of the water/AOT/isooctane reverse micelles on CO_2 pressure at 297 K.

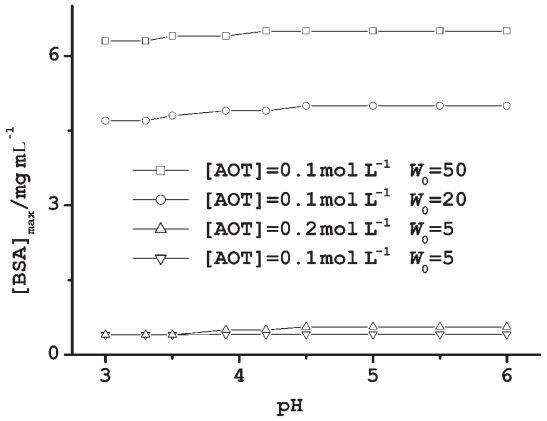

Figure 5. The effect of pH on the maximum concentration of BSA, $[BSA]_{max}$, in water/AOT/isooctane reverse-micellar solutions at 297 K.

Discussion

Many researches have demonstrated that ionic strength[24] and pH[25] in the water cores of reverse micelles are important parameters for the extraction of proteins. Dissolution of CO_2 in the water cores can produce ions (H^+, HCO_3^-) that alter the ionic strength and pH in the water cores. Moreover, our recent research has shown that compressed CO_2 at suitable pressures can stabilize AOT reverse micelles,[19] which may also affect solubilization of the protein. Therefore, CO_2 may affect the solubilization of BSA in two ways; by altering the ionic strength and pH in the water cores of the reverse micelles, and by affecting the stability of the reverse micelles. It is difficult to assess without conducting further experiments which of these two factors is dominant. Hence, we studied the effect of pH on the solubilization capacity in the absence of CO_2 by using aqueous BSA solutions of different pH, adjusted by addition of HCl solution. As shown in Figures 2 and 4, an increase in CO_2 can reduce the pH of the water cores, although the pH is higher than 3.2 for the ranges of CO_2 pressure and W_0 studied in this work. Therefore, the selected pH values of the water cores are higher than 3.0 for the reverse-micellar solution. The $[BSA]_{max}$ at different pH values is demonstrated in Figure 5, which reveals that the $[BSA]_{max}$ is almost independent of pH within the pH range studied. The ionic strength of the CO_2 solution is the same as that of the HCl solution at the same pH value, because the ionization of HCO_3^- is negligible. Therefore, it can be deduced that changes in pH and ionic strength originating from the dissolution of CO_2 is not the reason for the complex relationship between $[BSA]_{max}$ and CO_2 pressure shown in Figure 2. Thus, the effect of CO_2 on $[BSA]_{max}$ must result mainly from the variation in stability of the reverse micelles caused by the dissolved CO_2, that is, the stability of the reverse micelles has a pronounced effect on the protein-solubilization capacity of the reverse micelles.

The stability of the reverse micelles is affected by the dissolution of CO_2 in two opposite ways. Firstly, CO_2 at suitable pressures can stabilize the reverse micelles of AOT in alkanes with different chain lengths.[19] This is because CO_2 is a small, nonpolar, linear molecule that can penetrate into the surfactant-tail region to increase the rigidity of the surfactant film, thus making the reverse micelles more stable. Secondly, the dissolved CO_2 reduces the strength of the solvent, which leads to inadequate interaction between the solvent and the surfactant tails.[26] In other words, CO_2 acts as an antisolvent, which is evidenced by the precipitation of AOT as CO_2 pressure reaches cloud-point pressure, as shown by the data in Table 1. Within the low-pressure region, the first factor is dominant, and the reverse micelles become more stable as pressure increases, which enhances the solubilization of BSA. By contrast, the second factor becomes dominant within the high-pressure range, and the $[BSA]_{max}$ in reduced. Therefore, there is a maximum in each $[BSA]_{max}$ vs pressure curve in Figure 2. In other words, the maxima in the $[BSA]_{max}$ vs pressure curves provide convincing evidence that the stability of the reverse micelles considerably affects the $[BSA]_{max}$.

Figure 2 also demonstrates that the pressure at which the reverse micelles host the largest quantity of BSA ($P_{CO_2}^{max}$) is reduced as W_0 increases. A reasonable explanation is that the size of the reverse micelles is greater at larger W_0, and the curvature of the interface of the reverse micelles is small. Therefore, the gap between the surfactant tails in the reverse micelles is relatively small at large W_0. The curvature of the interface becomes larger as the W_0 decreases, and the gap between the tails is large, especially the portion close to the solvent. Therefore, the rigidity of the interfacial film can be increased more effectively at smaller W_0 by the penetration of CO_2 into the tail region, due to the larger gap between the tails. Hence, the reverse micelles with smaller W_0 values can be stabilized more efficiently, and higher pressure is required for the antisolvent effect.

The mechanism by which the stability of reverse micelles affects solubilization capacity is another interesting topic that has been widely studied. Nevertheless, we have not identified a model that can link the solubilization capacity

directly with the stability of reverse micelles. Here, we propose a multicomplex model to explain qualitatively this relationship. We assume that there are different protein–reverse micelle complexes in the solution, each of which complex contains a reverse micelle and a fixed number of protein molecules. In other words, the numbers of surfactant and water molecules in all complexes are the same, but the number of protein molecules differs. The formation of the complexes is reversible, as is expressed in Scheme 1, in

$$\begin{aligned}
&P + R \rightleftharpoons \text{Complex (1)}\\
&P + \text{Complex (1)} \rightleftharpoons \text{Complex (2)}\\
&P + \text{Complex (2)} \rightleftharpoons \text{Complex (3)}\\
&\vdots \quad \vdots \qquad\qquad \vdots\\
&P + \text{Complex } (n\text{-}1) \rightleftharpoons \text{Complex } (n)
\end{aligned}$$

Scheme 1. Formation of the protein (P)–reverse micelle (R) complexes.

which P and R stand for the protein and reverse micelle, respectively. The sensitivity of the solubilization capacity to the stability of the reverse micelles is directly related to the interactions between the reverse micelles and the protein molecules. In other words, the extent of the effect of stability on solubilization capacity depends on the nature of the components and the operation conditions. For example, the stability of the reverse micelles should not influence noticeably the solubilization capacity if the interactions between the species in the reverse micelles and the proteins are weak and the reverse micelles are sufficiently stable. On the other hand, micelle stability should affect solubilization capacity if these interactions are strong and the reverse micelles are not sufficiently stable. In other words, the stability of the reverse micelles dominates the solubilization capacity. In the system studied here, the interactions between the protein and reverse micelles are sufficient for the stability to affect the solubilization capacity significantly. Therefore, the solubilization capacity can be controlled by CO_2 pressure, because the stability of the reverse micelles depends on CO_2 pressure.

For the extraction of proteins by using reverse-micellar solution, one of the crucial questions is how to increase the solubilization capacity of the proteins in the reverse micelles. In addition, the recovery of the products should be a simple procedure. As discussed above, the solubilization of BSA in the AOT reverse micelles can be enhanced considerably by using compressed CO_2 at suitable pressures. The protein can be precipitated at the higher pressures, while the AOT remains in the solution, which is supported by the fact that the protein can be precipitated completely at pressures lower than cloud-point pressure (Table 1 and Figure 2). Consequently, high extraction and recovery efficiency of the protein can be achieved by controlling CO_2 pressure.

Conclusion

The effect of compressed CO_2 on the solubilization of BSA in water/AOT/isooctane reverse micelles at different W_0 values and AOT concentrations was studied. The maximum solubilization of BSA increases markedly as CO_2 pressure increases within the low-pressure range. However, as the pressure reaches the high-pressure range, solubililzation decreases, and eventually the protein can be precipitated totally, while the AOT remains in the solution. Therefore, high extraction and recovery efficiencies of the protein can be achieved simply by controlling CO_2 pressure.

The stability of reverse micelles can influence the maximum solubilization of the protein considerably, and a multicomplex model is proposed to explain this phenomenon. Based on this model, the sensitivity of the solubilization capacity to the stability of the reverse micelles is directly related to the interactions between reverse micelles and the protein. In the system studied here, these interactions are strong enough for the solubilization capacity to be affected notably by the stability of the reverse micelles. Therefore, the solubilization capacity can be controlled by CO_2 pressure because the stability of the reverse micelles depends on CO_2 pressure.

Experimental Section

Materials: AOT (99% purity) was purchased from Sigma. CO_2 (>99.995% purity) was provided by the Beijing Analytical Instrument Factory. The BSA (Part V, $M_w = 66400$, pI = 4.9) was obtained from the Sino-American Biotechnology Corporation and was used without further purification. Bromophenol blue (BPB, 90% purity) and isooctane (analytical grade) were purchased from the Beijing Organic Chemical Plant. Double-distilled water was used throughout the experiments.

Volume expansion of reverse-micellar solution in the presence of CO_2: Dissolution of CO_2 in the reverse-micellar solution causes the volume of the solution to increase. The apparatus for determining the volume-expansion curves was the same as that used previously.[27] It consisted mainly of a view cell (34.0 mL), a high-pressure pump (DB-80), a constant-temperature water bath, and a pressure gauge. The accuracy of the pressure gauge, which was composed of a transducer (FOXBORO/ICT) and an indicator, was ± 0.01 MPa within the pressure range of 0–7 MPa. The temperature of the water bath was controlled by using a HAAKE D8 controller with an accuracy of ± 0.1 K.

The reverse-micellar solutions were prepared by dissolving the desired amount of AOT and water in isooctane. All the experiments were carried out at 297 K and a magnetic stirrer was used. In a typical experiment, the reverse-micellar solution (5 mL) was loaded into the view cell. After the thermal equilibrium was reached, CO_2 was charged slowly into the view cell. The volume of the liquid phase did not change with time until the phase equilibrium was reached. The pressure and the volume under equilibrium conditions were recorded. More CO_2 was added and the volume of the liquid phase at other pressures was determined. The volume-expansion coefficients were calculated on the basis of the volumes of liquid phase before and after dissolution of CO_2.

Preparation of reverse-micellar solutions containing BSA: A certain amount of AOT/isooctane solution was prepared initially in a glass tube, and the desired amount of aqueous BSA solution was added. The tube was then immersed in the water bath (297 K) and the mixture was stirred until the solution became transparent.

Solubilization of BSA in reverse-micellar solutions in the presence of compressed CO_2: The apparatus and procedures were the same as those used for determining the volume expansion described above. In a typical experiment, the reverse-micellar solution (5 mL) containing excessive BSA was loaded into the high-pressure view cell, which was immersed in the water bath (297 K). After the thermal equilibrium was reached, CO_2 was pumped slowly into the cell until the solution became absolutely transparent and clear, indicating that BSA was completely solubilized. As the CO_2 pressure reached a sufficiently high value, the solution became turbid again, which was indicative of the precipitation of BSA.

UV-visible studies of solubilization and precipitation of BSA: The apparatus and procedures were similar to those described previously,[28] which were adopted to study the intermolecular interactions in supercritical fluids. The apparatus consisted mainly of a gas cylinder, a high-pressure pump, a pressure gauge, a temperature controller, a high-pressure UV cell, valves, and fittings. The UV/Vis spectrometer was produced by the Beijing Instrument Company (TU-1201). In a typical experiment, the double-distilled water, AOT/isooctane solution, and excessive amount of BSA were added into the UV cell. The CO_2 was then pumped into the sample cell until the sample cell was full and the pressure was recorded. The UV/Vis spectrum of the solution was recorded every 10 min until it became constant, indicating equilibrium conditions.

pH of the water cores in the reverse micelles in the presence of compressed CO_2: The pH value of the water cores in the reverse micelles was determined at different CO_2 pressures in the absence of BSA, and UV/Vis spectra were recorded. The experimental procedures were similar to those described above. The main difference was that aqueous BPB solution was added to the AOT/isooctane solutions and the pH meter (PHS-313, Shanghai REX Instrumental) was calibrated with the citric acid buffers.

Acknowledgements

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