Water-in–Carbon Dioxide Microemulsions: An Environment for Hydrophiles Including Proteins

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Carbon dioxide in the liquid and supercritical fluid states is useful as a replacement for toxic organic solvents. However, nonvolatile hydrophilic substances such as proteins, ions, and most catalysts are insoluble. This limitation was overcome by the formation of aqueous microemulsion droplets in a carbon dioxide–continuous phase with a nontoxic ammonium carboxylate perfluoropolyether surfactant. Several spectroscopic techniques consistently indicated that the properties of the droplets approach those of bulk water. The protein bovine serum albumin (BSA) with a molecular weight of 67,000 is soluble in this microemulsion and experiences an environment similar to that of native BSA in buffer.

The ability to design surfactants for the interface between water and carbon dioxide (CO_2) offers new opportunities in protein and polymer chemistry, separation science, reaction engineering, environmental science for waste minimization and treatment, and materials science. Supercritical CO_2 (critical temperature and pressure are 31°C and 73.8 bar, respectively) is a useful replacement for organic solvents to minimize waste and reduce volatile organic carbon (VOC) emissions. Not only is CO_2 more environmentally acceptable and naturally abundant than organic solvents, but it is also nonflammable, essentially nontoxic, and the least expensive solvent after water. Practical applications for CO_2 include extraction of caffeine from coffee, homogeneous (*1*) and heterogeneous (*2*, *3*) polymerization, production of submicrometer particles and fibers (*4*), spray painting and coating, and utilization as a feedstock to synthesize various organic substances (*5*). Our objective here was to make CO_2 accessible to proteins and other nonvolatile hydrophilic substances such as ions by producing water-in-CO_2 microemulsions that consist of thermodynamically stable and optically transparent aqueous domains. These polar domains can solubilize the protein BSA.

The properties of CO_2 are much different from those of water or nonpolar organic solvents. Unlike water, CO_2 has no dipole moment. Even when highly compressed, CO_2 has far weaker van der Waals forces than those of hydrocarbon solvents, making it more like a fluorocarbon or fluoroether. Consequently, both lipophilic and hydrophilic substances are often insoluble. It should be possible to form a dispersion of either a hydrophilic or lipophilic phase in a CO_2-continuous phase. The dispersions may be stabilized with surfactants containing "CO_2-philic" tails, such as fluorinated compounds. Recently, an organic latex, polymethylmethacrylate, was synthesized in CO_2 with a fluorocarbon stabilizer, demonstrating the ability to disperse an organic phase (*2*). However, the ability to disperse water into CO_2 as a microemulsion has been elusive, despite attempts with more than 150 surfactants during the last decade (*6*, *7*).

The design of surfactants for conventional water-in-oil microemulsions is reasonably well understood, even for supercritical fluid alkane solvents (*8*–*10*). However, little is known about designing surfactants with the proper balance of molecular interactions for the CO_2-water interface. A key advance was the extraction of a moderately polar interfacially active dye, thymol blue, into reverse micelles in CO_2 with a newly synthesized fluorinated sulfosuccinate surfactant (*11*). The amount of water, if any, present in the micelles was unknown, because the dye is soluble in water-free reverse micelles (*12*). In contrast to thymol blue, proteins are not soluble unless the reverse micelle cores are swollen with water to form microemulsion droplets. Recently, Harrison *et al.* (*13*) showed enhanced dissolution of water into CO_2 with a fluorocarbon-hydrocarbon hybrid surfactant. Although this suggests the possibility of the aggregation of water into a microemulsion, the nature of the water was not studied, because the surfactant was rapidly hydrolyzed.

Very few lipophilic or hydrophilic surfactants are soluble in CO_2. We chose surfactants that are active at the fluorocarbon-water interface (used in blood substitutes) given that the weak van der Waals forces of CO_2 match those of fluorocarbons. We have used an ammonium carboxylate perfluoropolyether (PFPE) surfactant, $CF_3O(CF_2CF(CF_3)O)_3CF_2$-$COO^-NH_4^+$ (*14*) (commercially available in the COOH form), with an average molecular weight of 740. PFPE carboxylate surfactants with molecular weights from 2500 to 7500 are soluble in CO_2 at pressures below 300 bar (*15*), but their ability to form microemulsions has not been reported. The biological inertness of PFPE surfactants complements the nontoxic nature of CO_2.

To prepare a microemulsion, we added water to a 1.4 weight % solution of PFPE in CO_2, yielding a water concentration of 0.48 weight %. Phase boundaries were determined for a given solution of constant composition with a stainless-steel view cell (28 ml) containing a sapphire window (*10*). At high pressures, the single-phase microemulsions were optically transparent. As the pressure was lowered at constant temperature by moving a piston (*10*), the loss in density and hence in solvent strength of CO_2 caused the solution to become turbid. Table 1 lists the cloud-point pressure and corresponding pure CO_2 density as a function of temperature. We calculated the number of moles of water to those of surfactant, W_0, by subtracting the independently known amount of water soluble in pure CO_2 without surfactant from the total amount of water. The total moles of water per mole of surfactant was 14 for all temperatures. A W_0 of 10 suggests that some type of aggregated water domain is present, because a single surfactant molecule would not be hydrated by so many water molecules.

To show that such a domain truly exists and to characterize its nature, we describe below the results of four different spectroscopic techniques. Fourier-transform infrared (FTIR) spectroscopy has been used to characterize water environments in microemulsions, specifically interfacial "bound"

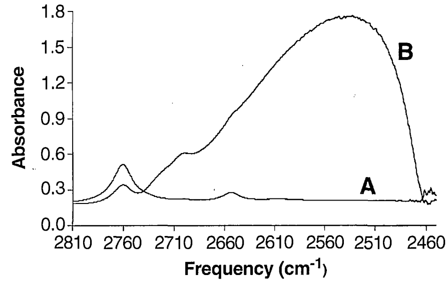

Fig. 1. The FTIR spectra of D_2O in CO_2 without (trace A) and with (trace B) the addition of PFPE surfactant at 31°C and 162 bar, W_0 = 20 (uncorrected). The IR bands of CO_2 have been removed by computer subtraction.

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Table 1. Cloud-point pressure (P) for a given temperature (T) for a microemulsion containing 0.15 g of PFPE, 0.052 g of water, and 10.4 g of CO_2.

T (°C)	P (bar)	CO_2 density (g/ml)	W_0
35	116	0.758	10.2
40	131	0.745	9.1
45	142	0.725	8.5
50	153	0.708	7.9
60	174	0.674	5.6

water close to the surfactant head groups and "bulk-like" water in the droplet core (*16*). We used D_2O because the O–H stretching bands are obscured by CO_2 and PFPE absorptions (*17*). Figure 1 shows the asymmetric and symmetric bands for free D_2O dispersed in a saturated solution of CO_2 (trace A, 2761 and 2653 cm^{-1}). A dramatic effect was seen upon addition of PFPE to the solution (trace B) with the appearance of a large broad band centered at 2535 cm^{-1}. This low-frequency broad band can be assigned to highly hydrogen bonded water in the microemulsion core, because the O–H (or O–D) stretching frequency is known to decrease in proportion to the hydrogen bond energy. The O–D stretching frequency approaches that of pure bulk D_2O at ~2500 cm^{-1}. The second water environment in the microemulsion structure, interfacial water, may be tentatively assigned to the shoulder at 2697 cm^{-1} (trace B). This intermediate frequency, between those of free and bulk-like D_2O, might be expected from the more strained interfacial environment for hydrogen bond formation.

Spectral shifts were measured with the spectroscopic probe methyl orange [$(CH_3)_2NC_6H_4N{=}NC_6H_4SO_3Na$]. As the local environment of the probe becomes more polar, the absorbance maximum λ_{max} shifts to longer wavelengths. Methyl orange in methanol was introduced into a stainless-steel cell with two sapphire windows, and the solvent was fully evaporated. The PFPE surfactant (0.026 M) and various amounts of water were loaded into the cell. The spectra obtained at 276 bar are shown in Fig. 2 for various values of W_0. Benchmarks were placed for λ_{max} of (surfactant-free) solvents, including methanol (421 nm), water (464 nm), and aqueous carbonic acid (502 nm). As the value of W_0 increased, there was a pronounced shift in λ_{max}, which indicates that methyl orange resides in a highly polar aqueous environment. This result confirms the existence of the bulk-like water seen, as shown above, with FTIR spectra. The shoulder in the spectra at 540 nm indicates the presence of an acidic environment due to carbonic acid.

Given a stable aqueous environment within CO_2, we examined next solubilization of a model protein, acrylodan-labeled BSA (BSA-Ac) (*18, 19*). This particular system was chosen because BSA is moderately large (67,000 daltons) and the strong Ac fluorescence provides a measure of both BSA conformation and concentration in solution (*20*). We checked for any residual emission of pure supercritical CO_2 and found essentially none. Weak fluorescence at ~390 nm was observed in a binary mixture of CO_2 and water (Fig. 3A). With the addition of PFPE to form a microemulsion (W_0 = 9), the BSA-Ac dissolved and the emission was substantial (Fig. 3B, solid line). For comparison, we show spectra of native BSA-Ac (*19, 21*) and of BSA-Ac in bis(2-ethylhexyl) sodium sulfosuccinate (AOT) water-in-oil microemulsions formed in liquid *n*-heptane at W_0 = 20 (*21*). For convenience, each spectrum was normalized to the maximum of the BSA-Ac + PFPE + water + CO_2 fluorescence.

The blue-shifted emission band in Fig. 3A relative to that of native BSA-Ac (Fig. 3B) is consistent with a small amount of BSA-Ac suspended into wet CO_2 and with an Ac residue experiencing a nonpolar environment. Exposure of the Ac residue to CO_2 indicates a conformational change in the suspended BSA. Upon forming a water-in-CO_2 microemulsion with PFPE, the BSA-Ac fluorescence is very strong and is similar to that of native BSA-Ac in buffer at pH 7.0. After recovery, the BSA-Ac is still recognized by the BSA antibody. Together, these results show that BSA-Ac is solubilized within the aqueous microemulsion droplets in an environment similar to that of bulk-buffered water and remains biologically active. These results are especially intriguing because BSA-Ac in AOT microemulsions at high water loadings (Fig. 3B) is conformationally altered with respect to native BSA-Ac. Finally, we estimate that about 9% of the total BSA-Ac is encapsulated within the PFPE microemulsion droplets (*22*) and that the remainder is likely at the bottom of the vessel.

X-band electron paramagnetic resonance (EPR) of 4-hydroxy-2,2,6,6-tetramethyl piperidino-1-oxy (4-hydroxy-TEMPO) was used to probe the local environment of the PFPE assemblies (*23*). At low CO_2 density (<13 M), nitrogen hyperfine splitting constants (called A_N) for 4-hydroxy-TEMPO in CO_2 solutions containing water and PFPE are much higher than those in CO_2 alone, which indicates that a local environment around the probe is much more polar than pure CO_2 (Fig. 4). Also, the EPR spectral shapes reflect highly anisotropic motion, as evidenced by the diminished high-field

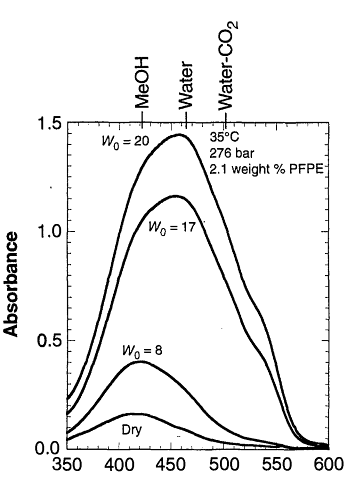

Fig. 2. Absorbance of methyl orange in the PFPE + water + CO_2 system as a function of added water. Conditions are as listed; MeOH, methanol.

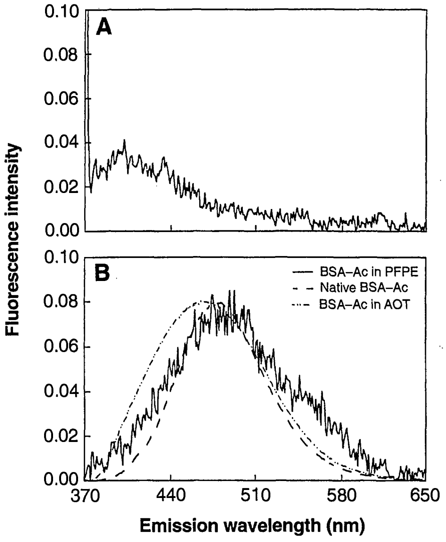

Fig. 3. (**A**) Emission of BSA-Ac in CO_2 + water and (**B**) encapsulated in a PFPE-stabilized water-in-CO_2 microemulsion. In (B) are also spectra for native BSA-Ac (dashed line) and BSA-Ac encapsulated in an AOT water-in-oil microemulsion at W_0 = 20 (dotted and dashed line).

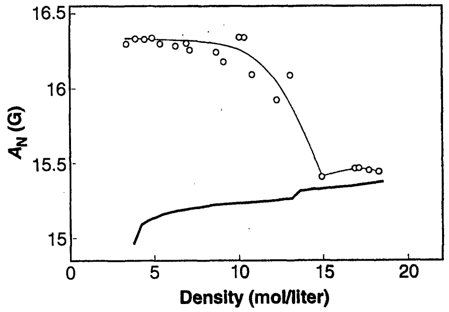

Fig. 4. Nitrogen hyperfine splitting constants (A_N) for 4-hydroxy-TEMPO in CO_2 at 35°C. The thick line indicates water-saturated CO_2 with no added surfactant. The points indicate a PFPE-stabilized water-in-CO_2 microemulsion (W_0 = 14). For comparison, the value of A_N in water is 17.05 G.

peak. Both the splitting constants and spectral shapes show that PFPE is present as an organized assembly. In contrast, the motion of the probe in water-saturated CO_2 (without surfactant) is isotropic.

At CO_2 densities >13 M, the apparent A_N in the surfactant solution drops to near that in neat CO_2. Thus, the observed A_N results predominately from the presence of 4-hydroxy-TEMPO molecules found outside the microemulsion droplets. The shapes of the spectra support this explanation. The lack of a polar environment for 4-hydroxy-TEMPO is a result of the rapid reduction of the nitroxide by reaction with NH_4^+ within the polar microemulsion core. The reduction in dense CO_2 surfactant solutions follows pseudo first-order kinetics with an apparent rate constant, k, of 6.8×10^{-4} s^{-1}. For comparison, k is equal to 2.0×10^{-5} s^{-1} for solutions of 4-hydroxy-TEMPO in trifluoroacetic acid and ammonium acetate (pH 3.0). The much larger rate constant may be attributed, in part, to a high degree of nitroxide orientation in the restricted environment of the microemulsion core. The strong sensitivity of the reaction rate to CO_2 density is due to the requirement that a water pool be present before significant ionization and subsequent reduction reaction can occur.

Our FTIR, ultraviolet-visible absorbance, fluorescence, and EPR experiments demonstrate the existence of an aqueous domain in CO_2 with a polarity approaching that of bulk water. Thus, not only do PFPE-based microemulsions offer a thermodynamically stable aqueous domain in a CO_2 phase, but they also appear to offer simultaneously a microenvironment that may not alter significantly the conformation of sequestered proteins. By comparing these results with those of previous studies, the successful formation of a microemulsion with PFPE may be attributed to the weak van der Waals forces of the surfactant tail, the strong tendency of the ionic head group to leave CO_2, the CF_3 branching and the small size of the head group, which favor bending of the interface about water, and the low solubility of the fluoroether tails in water. Given these guidelines, it should be possible to synthesize additional surfactants for environmentally benign applications in dense CO_2.

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17. The microemulsion solutions were prepared in a miniature stainless steel spectroscopic cell (2.8 mm, path length) with CaF_2 windows (10 mm thick) [S. M. Howdle and M. Poliakoff, in *Supercritical Fluids: Fundamentals for Applications*, NATO ASI Series, Applied Sciences 273, E. Kiran and J. M. H. Levelt-Sengers, Eds. (Kluwer Academic, Dordrecht, 1994), pp. 527–540]. The PFPE + D_2O + CO_2 mixtures were prepared at 31°C in conditions similar to those of the above phase behavior measurements. The IR spectra were recorded on a Nicolet 730 interferometer with a mercury cadmium telluride detector (2 cm^{-1} resolution).
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20. All experiments were conducted with stirred high-pressure optical cells and equipment described previously [T. A. Betts and F. V. Bright, *Appl. Spectros.* **44**, 1196 (1990)]. Excitation was at 360 nm, and excitation and emission band passes were at 8 nm. The PFPE concentration was always 1.4 weight % as in the above phase equilibria measurements. BSA-Ac was added to as a solid to produce a concentration of 100 nM (6.7 μg/ml) based on the volume of the cell. Water was added with a micropipette before charging with CO_2.
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22. This estimate is based on a calibration based on BSA-Ac in liquid water. It should be considered a lower limit as the Ac quantum yield within microemulsions at all values of W_0 is somewhat less than that in liquid water (*21*).
23. The molar ratio of water to 4-hydroxy-TEMPO was ~1700:1. Spectra were recorded in a high-pressure recirculating cell [T. W. Randolph and C. Carlier, *J. Phys. Chem.* **96**, 5146 (1992)] at a microwave power of 10 mW, modulation frequency of 100 kHz, and a 1.0-G modulation amplitude.
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Crystal and Molecular Structures of Hexamethyltungsten and Hexamethylrhenium

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The structure of hexamethyltungsten, $W(CH_3)_6$, was determined by x-ray single-crystal diffraction at −163°C. The molecule has a strongly distorted trigonal prismatic structure with C_{3v} symmetry. This irregular structure is not a result of intermolecular forces, but rather represents its true molecular structure. A similar structure, which deviates less from the ideal trigonal prismatic structure, was determined for hexamethylrhenium, $Re(CH_3)_6$. Although these structures violate the simplistic models used to predict the geometry of molecules, they are at least in part explainable by the molecular orbital model.

Homoleptic molecules, which have only one kind of ligand surrounding a central atom, are important for checking bond theory against experimental data because structure, stability, and reactivity are not influenced by different ligands. The methyl group is the simplest organic ligand in organometallic chemistry, and permethyl metal derivatives are often surprisingly stable because β eliminations are impossible. However, only three neutral hexamethyl compounds are known to exist: $W(CH_3)_6$, $Re(CH_3)_6$, and $Te(CH_3)_6$. $Te(CH_3)_6$ has only recently been prepared (*1*) and has an octahedral structure (*2*), as expected. $W(CH_3)_6$ and $Re(CH_3)_6$ are unstable compounds that were first synthesized (but not structurally characterized) by Wilkinson and colleagues (*3*). The finding that isoelectronic $Zr(CH_3)_6^{2-}$ in $[Li(CH_3)_2N{-}CH_2{-}CH_2{-}N(CH_3)_2{}^+]_2Zr(CH_3)_6^{2-}$ is trigonal prismatic (*4*) led to increased interest in the structures of hexamethyl compounds, especially because it had been theoretically predicted that certain hexacoordinative d^0 complexes may not be octahedral (*5–9*). This prediction was supported by the results of a recent gas phase structure determination by electron diffraction, which showed $W(CH_3)_6$ to be trigonal prismatic (*10*).

Crystallographically, $W(CH_3)_6$ and $Re(CH_3)_6$ posed a challenge. The compounds are thermally unstable, even explosive, if pure; $Re(CH_3)_6$ is even more sensitive than $W(CH_3)_6$. Moreover, crystals from solutions

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