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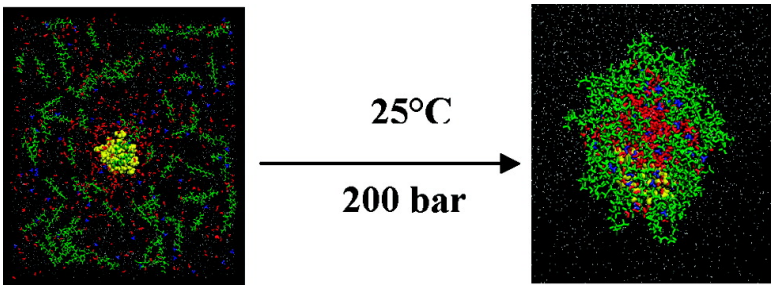


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# Self-Assembled Reverse Micelles in Supercritical $CO_2$ Entrap Protein in Native State

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***Abstract:*** Molecular dynamics simulations of random quaternary mixtures of protein-water-$CO_2$-fluorosurfactants show the self-assembly of reverse micelles in supercritical carbon dioxide where the protein becomes entrapped inside the aqueous pool. Analyses show that the protein native state remains intact in the water pool. This is because of the bulk nature of the enclosed water that provides a suitable environment for the extracted protein. Results from ab initio calculations imply that the existing fluorosurfactants can be made more effective in stabilizing water-in-$CO_2$ microemulsions by a partial hydrogenation in their tails. A Lewis acid−Lewis base interaction among $CO_2$ and the surfactant tails enhances the stability of the aqueous droplets substantially. The study can help accelerate the search for surfactant process for environmentally benign applications in dense $CO_2$.

## Introduction

Carbon dioxide in the supercritical state has been identified as a promising green solvent because it is nontoxic, nonflammable, and potentially recyclable. But, many important classes of substances, including water, exhibit low solubility in supercritical carbon dioxide ($scCO_2$). Recently discovered fluorosurfactants have overcome this inherent inability of $CO_2$ by creating reverse micelles, the little pockets of water that are soluble in $CO_2$.[1−3] In this study, we simulate a random mixture of protein-water-$scCO_2$-fluorosurfactants and found that the protein becomes entrapped spontaneously inside the water pool of a self-assembled reverse micelle (RM) in $scCO_2$. In a parallel attempt, we simulated a random mixture of water and $scCO_2$ and observed a distinct phase separation. Once we added fluorosurfactants into this mixture, the water became solubilized in the $CO_2$ phase in the form of RM through the surfactants. Addition of the model protein, a Trp-cage in this mixture, again showed a spontaneous diffusion of the protein into the aqueous core of the RM. In both the cases, the protein native state remained unaltered. The root-mean-square deviation (rmsd) from the NMR structure of this protein was only 1.4 Å. Our results match well with the experimental work of Johnston et al.,[1] where the protein bovine serum albumin was reported to be soluble in water-$CO_2$ (W/C) microemulsions without a serious loss of its activity. Our study not only complements the experimental findings but also provides a wealth of detailed information at the atomic level that is certain to accelerate the search for a surfactant process for environmentally benign applications in dense $CO_2$.

The molecular aggregation itself is a complex phenomenon that is impossible to study in detail experimentally. Computer simulation, on the other hand, can demonstrate the entire process of self-aggregation even in atomic detail. However, simulation of self-assembly at atomic detail is a large challenge due to its requirement of very accurate potential models and enormous computer time. Until recently, simplified potential models were therefore used to mimic aggregation of surfactant-like molecules into micelles,[4] bilayers,[5,6] and even vesicles.[7] These models could induce a faster aggregation, but they often lack accuracy. A few atomistically detailed MD simulations were attempted by placing the surfactants in their target places.[8,9] These simulations again produced results that depend on the initial conditions. The self-assembly in $scCO_2$ has received recent attention due to the growing application of $CO_2$ as a solvent. Thus, Salaniwal et al.[8] studied the self-assembly and aggregation dynamics of a ternary mixture composed of a dichain surfactant, water, and $CO_2$. The surfactants were found to form small reverse micelle-like aggregates at a span of about 1 ns starting from a dispersed state. Whether this short period of time enabled the system to reach equilibrium was unclear from this study. Senapati and Berkowitz[9] investigated the structural properties

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of preassembled reverse micelles in $scCO_2$. Their work involved the generation and modification of certain potential parameters that describe the surfactant-$CO_2$ interactions accurately. On the basis of the potential model of Senapati and Berkowitz, very recently, Lu and Berkowitz[10] showed a successful aggregation of a RM from MD simulations. Although no detailed analysis of the kinetics of aggregation is given, the authors concluded that the fluorinated surfactants are effective in creating W/C microemulsions.

Successful protein extractions have been reported in various systems employing RM as encapsulating hosts for proteins.[11,12] Most of these studies on reverse micellar extraction have employed various organic solvents that are toxic, carcinogenic, and implicated in ozone depletion. An occasional protein deactivation has also been reported to occur during the process.[13] The application of environmentally benign $scCO_2$ in protein extraction has therefore received tremendous attention in the past few years.[1,14] Now the question remains: Can the extracted protein keep its native state intact? Our results show that it indeed does. To avoid any dependence on initial conditions, we started our simulations from a completely random configuration, and for a higher accuracy, we carried out all-atom dynamics simulations. In the course of the simulations, we observed the self-assembly of RM that spontaneously entrapped the protein in its native state.

## Model and Simulation Details

We performed a series of unrestrained, all-atom MD simulations of systems composed of protein, water, $scCO_2$, and surfactants. We chose the Trp-cage as the model protein, as it is the most stable miniprotein reported to date[15] (PDB ID: 1L2Y). As the surfactants, we first chose commercially available $CF_3$-(O-$CF_2$-CF($CF_3$))$_3$-O-$CF_2$-$COO^-NH4^+$ (PFPE), which has been reported to form aqueous RM in $scCO_2$.[1] The well-tested SPC/E water model and an economical single-site $CO_2$ model were among the other models used in the simulations. The single-site $CO_2$ model was tested to make sure that it reproduced the equation of state in the supercritical region.[16] Our first simulation was started from a random mixture of 1 Trp-cage, 554 waters, 66 surfactants ($W_o$ = 8.4), and 6359 $CO_2$ molecules (system 1). The second simulation was started from a random binary mixture of 554 waters and 6359 $CO_2$ molecules (system 2). The surfactants and the protein were added to this system subsequently, to mimic the experimental procedure of Johnston et al.[1] rather closely. All the simulations were carried out at 25 °C and 200 bar pressure. These values for temperature, pressure, and $W_o$ are the same as were used in the experimental studies conducted by Johnston et al.[1] The number of water and surfactants was also estimated from the reported experimental data[1] (see Supporting Information). To enable volume variation, the simulations were performed in the NPT ensemble using a Nose-Hoover thermostat and barostat. Both system 1 and system 2 were simulated for at least 50 ns. All minimization and MD steps were performed using the Amber 9.0 package[17] with Amber force fields.[18] The missing interaction parameters in the surfactant and $CO_2$ were introduced using antechamber tools in Amber. These parameters can be found in ref 9.

Ab initio calculations were carried out using the Gaussian 03 package.[19] All the geometry optimizations and energy calculations were performed at the second-order Moller−Plesset (MP2) level using the aug-cc-pVDZ basis set. Dissociation energies were calculated using the supermolecule method as the difference between the energy of the complex and the sum of the isolated energies of the monomers.[20] Geometry and energy corrections for the basis set superposition error (BSSE) were calculated at the MP2 level of theory, using the counterpoise correction method of Boys and Bernardi.[21] The atom-centered point charges were determined via fits to the electrostatic potentials obtained from the calculated wave functions using the CHelpG subroutine of Gaussian 03.

## Results and Discussion

Figures 1 and 2 illustrate the process of spontaneous encapsulation of the Trp-cage into the aqueous domain of self-assembled RM in $scCO_2$. The snapshots in Figure 1 correspond to the evolution of system 1 from a scattered starting configuration. The aggregation process seems to take place in a few stages—a fast initial clustering of protein-water-PFPE in the $CO_2$ continuous phase in about 200 ps, followed by a much slower merging of the small aggregates to form three or four small reverse micelles after 3 ns. This was followed by a much slower process during which three or four small micelles merged into two after 10 ns. The final single RM first appeared after 22 ns. The protein, Trp-cage, was found to be entrapped in the aqueous pool and remained so during the remainder of the simulation. We noted that at a few instances during the initial phase of aggregation, such as in Figure 1b, a small fraction of the protein surface did not become well-hydrated. However, the rearrangement of waters took place in a time scale of nanoseconds, which is much shorter than the folding/unfolding rates of the Trp-cage.[22] This transient exposure, therefore, did not affect the state of the protein. The snapshots in Figure 2 correspond to the evolution of system 2 that simulated a random mixture of water and $CO_2$ at the beginning. A distinct phase separation with the water phase dispersed in blocks in the $CO_2$ continuous phase was evident throughout the 2 ns simulation. We then added 66 PFPE randomly into this mixture and followed the process of aggregation. Micellar self-assembly was again observed with the formation of a spherical RM in about 17 ns. When the resulting solution was in contact with the Trp-cage aqueous solution, the protein was found to diffuse spontaneously into the water core of the RM and remained stable for the rest of the simulation. The resulting structure looked similar to the one presented in Figure 1c.

Thus, simulations of system 1 and system 2 implied that the starting configuration had little correlation with the long-time dynamics of the micelle systems. Our results also indicated that the aggregation process followed a two-step mechanism: fast hydration of polar components (surfactant headgroups, $NH_4^+$ ions, and protein), followed by slow aggregation of hydrated molecules. Since the protein becomes hydrated in the fast first

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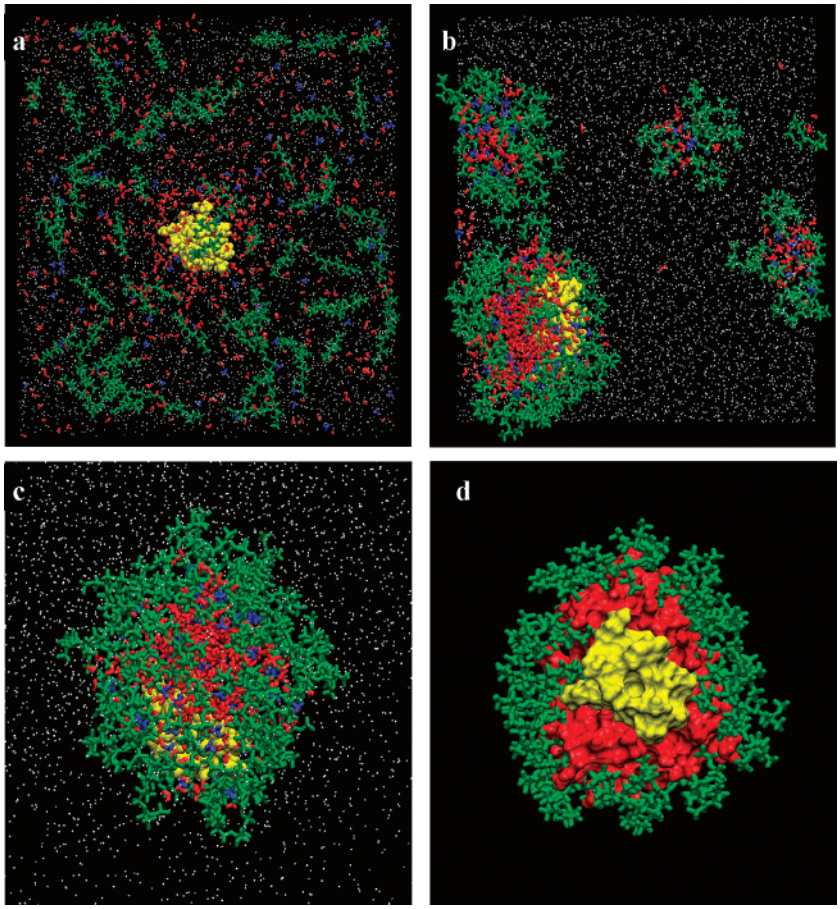


***Figure 1.*** Time evolution of system 1. Snapshots are taken at (a) 0 ps, (b) 3 ns, (c) 30 ns, and (d) 50 ns. The snapshot in panel d is a cross-section to show the protein environment clearly. Color scheme: yellow, protein; red, water; green, surfactants; and white, $CO_2$. In panel d, $CO_2$ is omitted for clarity.

step and does not have a role in the slow second step, we do not speculate that the protein would accelerate the micelle formation. As a matter of fact, we see the micelle formation occurring in system 1 and system 2 at similar times (around 20 ns). The aqueous core provides an environment for the protein that is similar to bulk water. The calculated water density in system 2, before it was in contact with the protein solution (state corresponding to Figure 2b), was about 1 g/cm$^3$ near the center of the micelle. The water-to-water tetrahedral hydrogen-bonding network was also found to be retained in this region. Figure 3 shows the density profile of water in this system, as a function of the distance from the headgroup surface. The headgroup surface, formed by the carboxylate carbon atoms of PFPE, is located 17 Å from the center of the micelle. The profile was generated by calculating the local densities of water as a function of the distance. An interfacial layered structure, consistent with previous studies,[23,24] is also seen.

Finally, we checked that the Trp-cage retains its native state during both simulations. The $C_\alpha$ rmsd from the NMR structure of this protein was only 1.4 Å. All the secondary structure elements—α-helix composed of residues 2−8, $3_{10}$ helix composed of residues 11−14, and the polyproline II helix—remained intact. The hydrophobic core with the indole ring of Trp6 at the center and flanked by the side chains of Tyr3, pro12, and pro18 also remained unaffected. This is shown in Figure 4. Our comparison of the Trp-cage structure with two other short simulations carried out on the Trp-cage aqueous solution at (1) 25 °C, 1 bar pressure and (2) 25 °C, 200 bar pressure indicates that the protein structure remains unaltered even at 200 bar pressure.

To search for a new surfactant and to test the dependence of micelle stability on surfactant type, additional simulations were performed using hydrogenated and partially fluorinated analogues of PFPE. Note that the stability of a microemulsion depends on the inter-droplet interactions. A better solvation of surfactant tails in $CO_2$ would result in weaker inter-droplet interactions and hence a more stable microemulsion.[25] From the quantum mechanical calculations on model systems $CF_3$-O-$CF_3$ + $CO_2$, $CF_2H$-O-$CF_2H$ + $CO_2$, and $CH_3$-O-$CH_3$ + $CO_2$, we found that partially fluorinated surfactant tails yielded a maximum $CO_2$-philicity. This is because $CO_2$ can act as a weak Lewis acid as well as a Lewis base while interacting with the >CFH moiety.[26] Both the fluorine atoms (through C−F···$C_{CO_2}$ interactions) and the electron deficient C−H bonds (through C−H···$O_{CO_2}$ interactions) contribute to the overall $CO_2$-philicity. The MP2/aug-cc-pVDZ optimized geometries for the three model complexes are presented in Figure 5. The dissociation energies for each of the three complexes are included in Table 1. An enhanced $CO_2$-philicity in a partially fluorinated chain is

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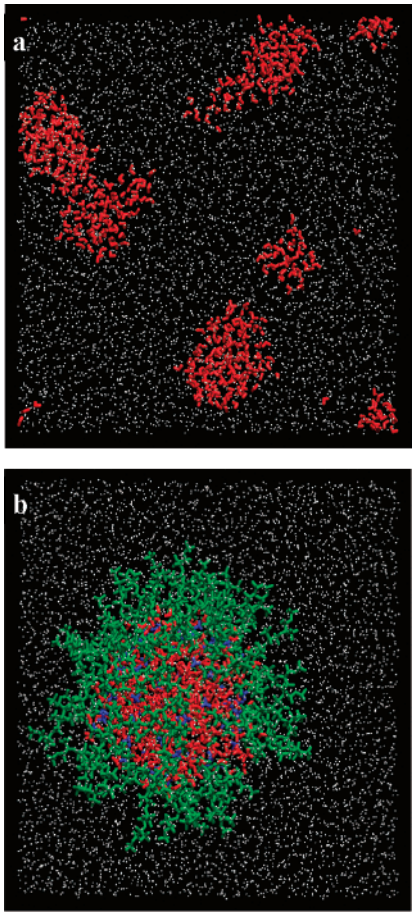


***Figure 2.*** Time evolution of system 2. Snapshots are taken at (a) 1 ns and (b) 20 ns. Color scheme is the same as in Figure 1.

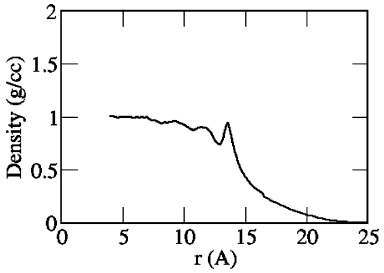


***Figure 3.*** Density distribution of water as a function of distance. The center of the micelle is at (0,0), and the headgroup surface is located at 17 Å. The existence of a bulk phase is evident.

evident from these data. This is rightly reflected in the 13% increased solvation of the $CF_2H$-(O-CFH-$CF(CF_2H))_3$-O-CFH-$COO^-NH4^+$ (PTFPE) tails as compared to the PFPE surfactant in our subsequent reverse micellar simulations. The solubility of the hydrogenated analogue (PE surfactants), as obtained from simulation, was about 33% lower than PFPE.[27] Note that PTFPE and PE simulations were also carried out with 554 waters, 66 surfactants, and 6359 $CO_2$ molecules. We have taken the average number of $CO_2$ molecules in the first shell of the surfactant tail as a measure of solvation.[9] Moreover, although we observe self-assembly into RM-like aggregates with PE, PFPE, and PTFPE surfactants, a huge 32% exposed aqueous core area in $CO_2$ was found in the simulation with the PE surfactant.[28] This inability to effectively separate the water and $CO_2$ phase from each other apparently makes the hydrogenated surfactants a poor performer of W/C microemulsions.[29] The corresponding exposure was only

(27) The stronger tail−tail interaction in PE surfactant simulation leaves less room for $CO_2$ to penetrate between the tails, which may lead to complete insolubility or poor solubility of these surfactants. However, a set of distinct although weak peaks in the radial distribution functions between carbon dioxide and PE surfactant tails implies that the polypropylene oxide surfactant tails can exhibit a certain degree of solubility in $CO_2$. A similar observation was reported by Senapati and Berkowitz (Figure 15 in first part of ref 9).

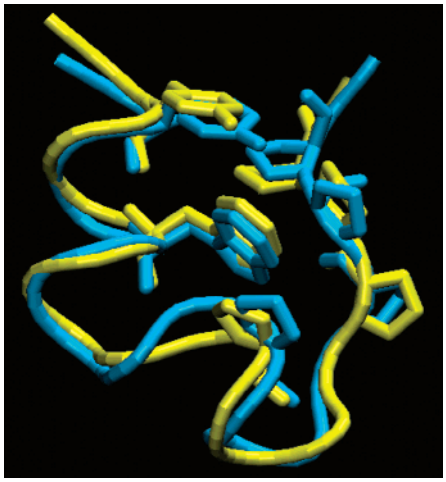

***Figure 4.*** Backbone structures of the Trp-cage. Color scheme: yellow, simulation and cyan, NMR. Only side chains that form the Trp-cage are shown.

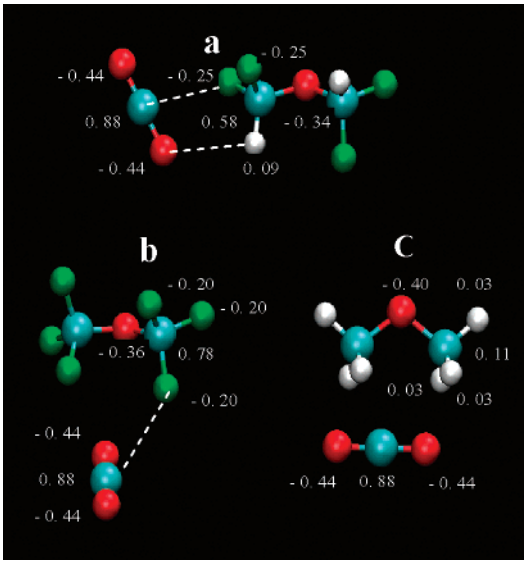


***Figure 5.*** Optimized geometries of the complexes of $CO_2$ with (a) $CF_2H$-O-$CF_2H$, (b) $CF_3$-O-$CF_3$, and (c) $CH_3$-O-$CH_3$. Color scheme: cyan, carbon; red, oxygen; and white, hydrogen. The dashed lines represent the possible modes of interactions among the monomers, and the numbers represent the partial atomic charges obtained from ab initio calculations.

***Table 1.*** BSSE-Corrected Dissociation Energies (kcal/mol) of the Complexes at the MP2/aug-cc-pVDZ Optimized Geometries

| solute | no. of $CO_2$ molecules | dissociation energy |
|---|---|---|
| $CF_2H$-O-$CF_2H$ | 1 | 3.019 |
| | 2 | 4.443 |
| $CF_3$-O-$CF_3$ | 1 | 1.393 |
| | 2 | 2.370 |
| $CH_3$-O-$CH_3$ | 1 | 0.433 |
| | 2 | 0.829 |

(28) Exposed aqueous core area was estimated by rolling a probe across the surface of the interior water core in the presence and absence of the surfactant molecules. See ref 9 for details.

12% in PFPE and 7% in the case of the partially fluorinated PTFPE surfactant simulation. Note that a direct water/$CO_2$ contact increases the surface tension and, therefore, the free energy of the micelle, thus destabilizing the microemulsion.[10] All these facts imply that the partially fluorinated surfactants could be most effective in stabilizing W/C microemulsions. It is worth mentioning here that the newly designed partially fluorinated surfactant, PTFPE, could successfully reproduce the results corresponding to Figures 1−4, implying that the self-assembled RM in $scCO_2$ indeed entrap protein in native state.

## Conclusion

To summarize, we observed the spontaneous encapsulation of a miniprotein, Trp-cage, inside the water pool of a self-assembled RM in $scCO_2$ from our 50 ns MD simulations. The aqueous core provides an environment for the protein that is similar to bulk water, and thus, the Trp-cage structure remains unaffected. MD simulations in conjunction with ab initio calculations were proven to be useful tools in predicting new surfactants that stabilize W/C microemulsions. This strategy has applications in protein extraction, selective encapsulation of drugs for targeted delivery, and as media for reactions between polar and nonpolar molecules.

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**Supporting Information Available:** Complete refs 17 and 19 and formulas that estimate the number of water and surfactants for our systems based on known experimental parameters. This material is available free of charge via the Internet at http://pubs.acs.org.

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