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Novel Zwitterionic Reverse Micelles for Encapsulation of Proteins in Low-Viscosity Media

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Abstract: Large proteins remain inaccessible to structural NMR studies because of their unfavorable relaxation properties. Their solubilization in the aqueous core of reverse micelles, in a low-viscosity medium, represents a promising approach, provided that their native tertiary structure is maintained. However, the use of classical ionic surfactants may lead to protein unfolding, due to strong electrostatic interactions between the polar head groups and the protein charges. To design reverse micelles in which these interactions are weakened, a new zwitterionic surfactant molecule was synthesized and studied by high-resolution NMR spectroscopy, for which cytochrome C and ^{15}N-labeled ubiquitin were used as guest candidates. At different ionization states, both proteins are encapsulated in the absence of salts or other additives, in a folded conformation similar to the native one.

Keywords: micelles · NMR spectroscopy · proteins · surfactant · zwitterions

Introduction

The study of large biomolecular systems by high-resolution NMR spectroscopy is impeded by their slow rotational motion, which leads to a very efficient transverse relaxation and, thus, to line broadening and shortening of signal lifetime. Relaxation-interference methods based on chemical-shift anisotropy-dipole–dipole cross-correlation in transverse relaxation optimized spectroscopy (TROSY)-type experiments,[1] combined with the extensive deuteration of ^{13}C- and ^{15}N-labeled macromolecules, have been used to extend the size limit of the molecules studied by solution-state NMR spectroscopy.

In 1998, A. J. Wand and co-workers proposed a quite innovative approach for the structural study of large proteins (>30 kDa).[2] This involves the substitution of water as bulk solvent by a low-viscosity medium, which would accelerate the solute's Brownian motion and, therefore, lengthen its T_2 relaxation time. To maintain the natural environment of the protein for the study of its native three-dimensional structure, it was proposed to encapsulate it with some hydration layers into reverse micelles.

Although this ambitious approach was validated for ubiquitin (8 kDa) in reverse micelles of sodium bis(2-ethylhexyl)sulfosuccinate (AOT), it has not yet been applied to the structural studies of large proteins. Indeed, the problems encountered are numerous, for example: 1) some surfactants lack the swelling capacity necessary to encapsulate large molecular objects, 2) certain micellar systems suffer from time-instability, 3) the proteins may be encapsulated, but in a non-native state. Such a conformational transition of proteins encapsulated in AOT micelles could be explained by the presence of strong electrostatic interactions between the surfactant polar head groups and the charged residues of the guest molecule. Therefore, the attenuation of these interactions through the design of new surfactants may facilitate protein structural studies in reverse micelles. Here, a comparison of AOT, CTAB (cetyltrimethylammonium bro-

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mide), and a newly synthesized zwitterionic surfactant, 2-ammonioethyl 2,3-bis(3-ethylheptanoyloxy)propyl phosphate, is presented. Unlabeled cytochrome C was chosen as a guest candidate, and ^{1}H NMR spectroscopy was used to confirm not only that the protein was encapsulated, but also that it had maintained its folded structure.

Results and Discussion

The choice of commercially available cytochrome C for our experiments was dictated by reasons of simplicity, as the solution becomes red upon solubilization of the protein. Moreover, cytochrome C, being a basic protein (pI = 10.2) of 104 amino acids, is strongly positively charged at neutral pH. Due to the presence of several hydrophilic residues at its external surface, cytochrome C remains highly soluble in water, even at pH conditions close to its isoelectric point.

For unlabeled proteins, the NOESY experiment can give a rapid estimation of their conformational state. The presence of numerous cross-peaks between amide protons combined to an expanded amide region indicate a folded protein. This is the case of the oxidized paramagnetic ferrocytochrome C, at basic or neutral pH, as shown in Figure 1a and b. The increase in spectral quality achieved by lowering the pH from 9.3 to 6.4 reflects the decrease in proton-exchange rates between water and protein amide groups.[3]

For the design of our new surfactant, some characteristics were desired. Firstly, it should be able to form reverse micelles in apolar solvents and to encapsulate proteins of different isoelectric points at the pH values used for NMR studies in aqueous solution. Secondly, the use of additives, such as linear-chain alcohols, should be avoided if possible. Indeed, with respect to the final objective of the study of large proteins, the addition of such cosolvents in significant proportions could become a drawback as they increase the viscosity of the medium. Finally, the micellar system should not require a buffer or high salt content.

Clearly, the design of a new surfactant capable of encapsulating various protein types in organic solvents is somewhat empirical. In dealing with the shape of the aggregates, some basic rules exist, such as consideration of the packing parameter, the hydrophilic–lipophilic balance, or use of the flexible surface model.[4] However, no accurate prediction of the objects formed can be made easily, which is even more the case when a protein is present, even at low concentration.

For the choice of the polar head, some considerations from previous works were applied. The article of Shioi et al. on protein extraction[5] suggests that ionic polar heads favor the formation of reverse micelles in organic solvents, the transfer of the protein into the aqueous core being governed by electrostatic interactions. However, if the protein is strongly charged, these interactions are also likely to occur inside the formed micelles between these charges and the anionic or cationic polar heads.

For example, in the case of AOT micelles in alkanes, it has been proposed that the protein is located in the interfacial region of the reverse micelles, due to strong electrostatic attractions between cytochrome C and the negatively charged surfactant layer of AOT.[6,7] This local environment leads to a conformational transition of cytochrome C from its folded structure to an amphiphilic entity.[8] Hence, the NOESY spectra of cytochrome C in AOT micelles dissolved in pentane, shown in Figure 1c and d, exhibit a very peculiar behavior. Whereas the spectrum in AOT/pentane at pH 9.3 reflects a folded protein (without indication of its three-dimensional structure), the spectrum at pH 6.4 is more indicative of a molten globule. A reduced amide-proton region, similar to that of Figure 1d, was also observed by the group of Wand.[9] By comparing the experiments made in aqueous solution (Figure 1a and b), the rates of proton exchange with the solvent cannot be responsible for this spectral degradation. The variation in protein charge between the two pH values (net charge +5.3 and +9.8, for pH 9.3 and 6.4, respectively), leading to different electrostatic strengths, remains the only explanation for the appearance of the NOESY maps displayed in Figure 1c and d.

To confirm that the sulfosuccinate function of AOT is actually responsible for this effect, an AOT analogue with the same head group, but a different hydrophobic tail, providing higher solubility in apolar solvents,[10] was synthesized. The NMR spectra of cytochrome C in micelles of this compound at two pH values seem very similar to those displayed in Figure 1c and d (see Supporting Information), indicating that the nature of the head group is probably the cause of the observed effects. This also suggests that the influence of the surfactant tail on the protein conformational state is rather minor.

The encapsulation of ^{15}N-labeled cytochrome C in cationic CTAB reverse micelles was reported recently.[11] In such an environment, the native conformation of the protein is preserved, because the electrostatic interactions between the protein and the CTAB head groups are now repulsive.[12] However, in the search for a unique surfactant system capable of encapsulating proteins of different isoelectric points, this cannot represent our final solution. We suspect that cationic micelles would unfold acidic proteins if the pH of the aqueous core is largely inferior to the protein isoelectric point. However, Wand et al. have successfully encapsulated a mutant of flavodoxin (pI ~ 4.2) in a folded state in CTAB micelles. Nevertheless, the formation of CTAB micelles in short-chain alkanes is quite difficult and requires the addition of cosolvents, such as *n*-hexanol at a proportion of 8–10% of the total volume, thereby penalizing the viscosity of the solution.[13] In the case of flavodoxin in CTAB/*n*-hexanol micelles, the presence of the alcohol may dilute the charge of the micelle, and may explain the folded form of the protein. In the absence of cosolvent, we were unable to solubilize cytochrome C in CTAB/pentane with a water-to-surfactant molar ratio W of 20, even after considerable sonication. After addition of *n*-hexanol at 10% v/v, the solution turns slightly red, however, a proportion of the protein remains

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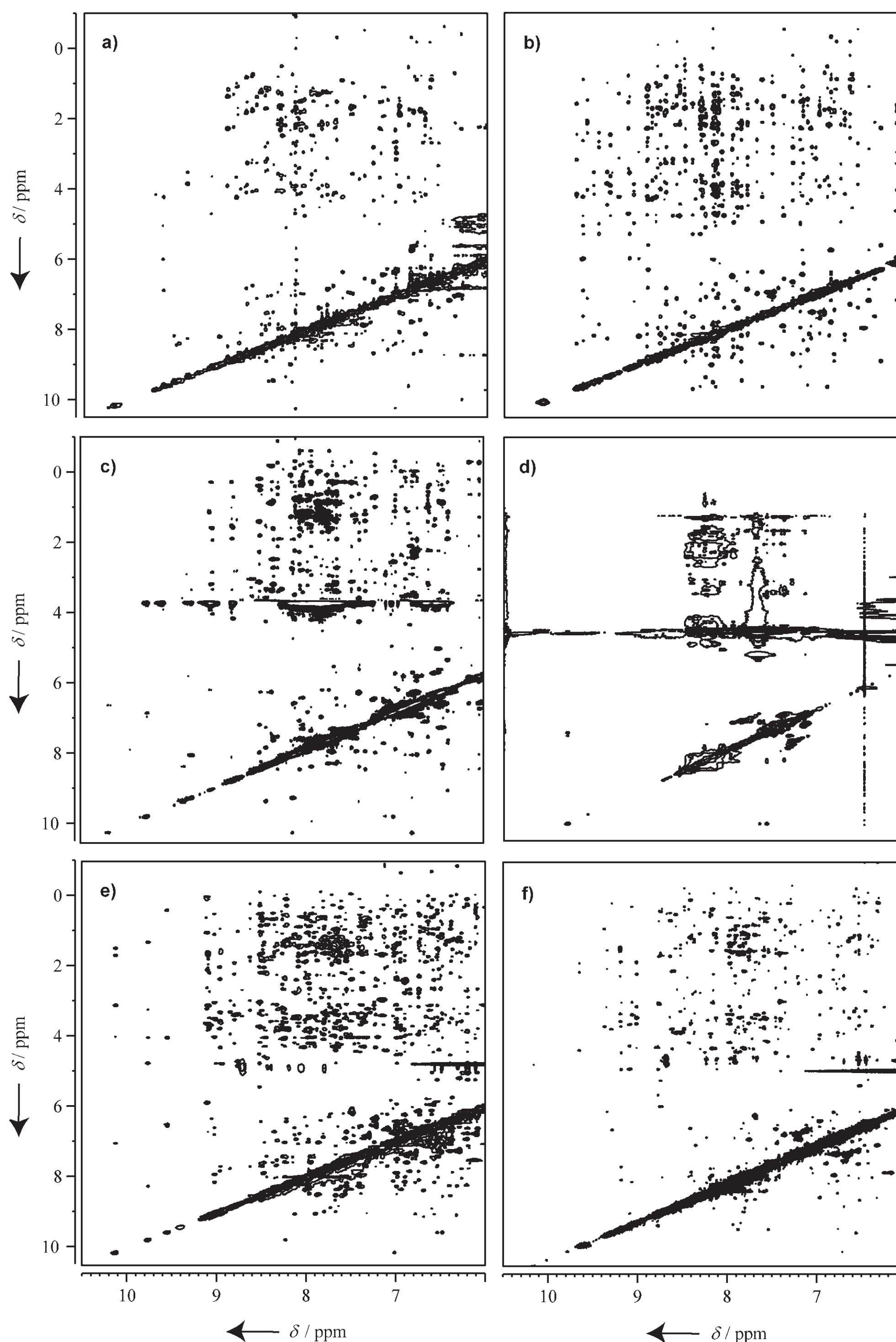

Figure 1. Partial contour plots of two-dimensional NOESY spectra of cytochrome C at 298 K (mixing time 150 ms). Each sample contained around 1 mg of protein, except for (f), in which it was estimated as 0.3 mg. (a) and (b): Spectra recorded in H_2O/D_2O of 95:5; (a) pH 9.3, (b) pH 6.4. (c) and (d): Spectra recorded in the AOT/[D_{12}]pentane system; (c) pH 9.3, $W=10$, (d) pH 6.4, $W=11.7$. (e) and (f): Spectra recorded in the surfactant **1**/[D_{12}]pentane system; (e) pH 9.3, $W=8$, (f) pH 6.4, $W=12.0$.

precipitated (see Supporting Information for the recorded NOESY spectrum). This surfactant is, therefore, not suitable for our purpose.

Mixtures of anionic, cationic, and even nonionic surfactants have been proposed recently by the group of Wand to encapsulate proteins in low-viscosity solvents, such as liquid ethane.[13,14] These surfactant mixtures appear to increase the stability of the micelles formed, as they facilitate the encapsulation of proteins in low-density solvents. The pressures required to stabilize the microemulsions can be de-

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creased if an appropriate cosolvent is used. Thus, a real breakthrough is achieved by the lengthening of the transverse relaxation time. However, in view of the work of several teams who use catanionic reverse micelles for the synthesis of hierarchical nanostructures, the risk of a charge redistribution/segregation along the inner surface of the micelle cannot be neglected. The cationic-to-anionic surfactant ratio influences the structure of the aggregates, and this value is taken into account in the modification of the size and shape of the nanocrystals growing in these aggregates (see, for example, reference [15]). Moreover, adaptation of the micellar charges according to the surface-charge distribution of the protein is possible.

Another solution that combines the presence of charged groups in the polar head and the decrease in micellar net global charge is to conceive a zwitterionic surfactant. It has been reported that lecithin, a zwitterionic natural phospholipid (a mixture of phosphatidylcholines with acyl chains of different lengths and degrees of saturation), self-assembles in alkanes into long, wormlike objects in the presence of small amounts of water.[16] Such behavior observed at high surfactant concentrations could arise from the large size of the polar head group (containing a $N(CH_3)_3^+$ group) relative to that of the hydrophobic tail. For this reason, the new surfactant molecule, 2-ammonioethyl 2,3-bis(3-ethylheptanoyloxy)propyl phosphate (compound **1**), possesses a smaller head group (NH_3^+), namely that of phosphatidylethanolamine, the phospholipid located primarily at the external surface of biological membranes. The zwitterionic head prevents the release of counter ions into the water pool and the balance between positive and negative fixed ions gives a neutral character to the internal wall, making the protein–micelle interactions weaker than those with other ionic surfactants. The alternation of fixed opposite charges on the internal surface of the micelle reduces perturbation on the protein due to electrostatic interactions. Clearly, the risk of redistribution of micellar charge according to the protein surface potential, as can be the case for a mixture of surfactants (see below), is avoided. This surfactant may act itself as a buffer for pH levels between its two pK values: ~5 for the phosphate group and ~8 for the ammonium function.[17]

Based on geometric considerations and basic predictions, we have kept an AOT-like hydrophobic tail to create the curvature required for an inverse micelle. The new zwitterionic surfactant, 2-ammonioethyl 2,3-bis(3-ethylheptanoyloxy)propyl phosphate (compound **1**), was prepared by using a synthetic pathway that allows for a future perdeuteration (Figure 2).

During preparation of the samples of cytochrome C encapsulated in micelles of either AOT or **1**, a remarkable difference was observed. Whereas the dissolution of the protein after addition of AOT micelles is immediate, in the case of **1**, it takes much longer (several hours, even with ultrasound). For the same reason as stated previously, this may be due to the zwitterionic nature of the surfactant polar head, which could hide the net electrostatic interactions required to form the reverse micelles.

Figure 1e and f displays the NOESY contour maps of cytochrome C initially prepared at pH 9.3 and 6.4, respectively, in reverse micelles of surfactant **1**. The spectra are similar (the lower signal-to-noise ratio in spectrum f is due to a lower protein concentration). As for the aqueous solution studies, and contrary to the spectrum recorded in AOT at neutral pH, the amide protons cover a wide spectral region, which characterizes a folded protein.

Experiments to encapsulate larger proteins are underway in our laboratory. The use of nonionic surfactants from the poly(oxyethylene) alkyl ether family (C_iE_j) to encapsulate cytochrome C at various pH values was also successful in our hands, however, the resulting spectra gave broad peaks

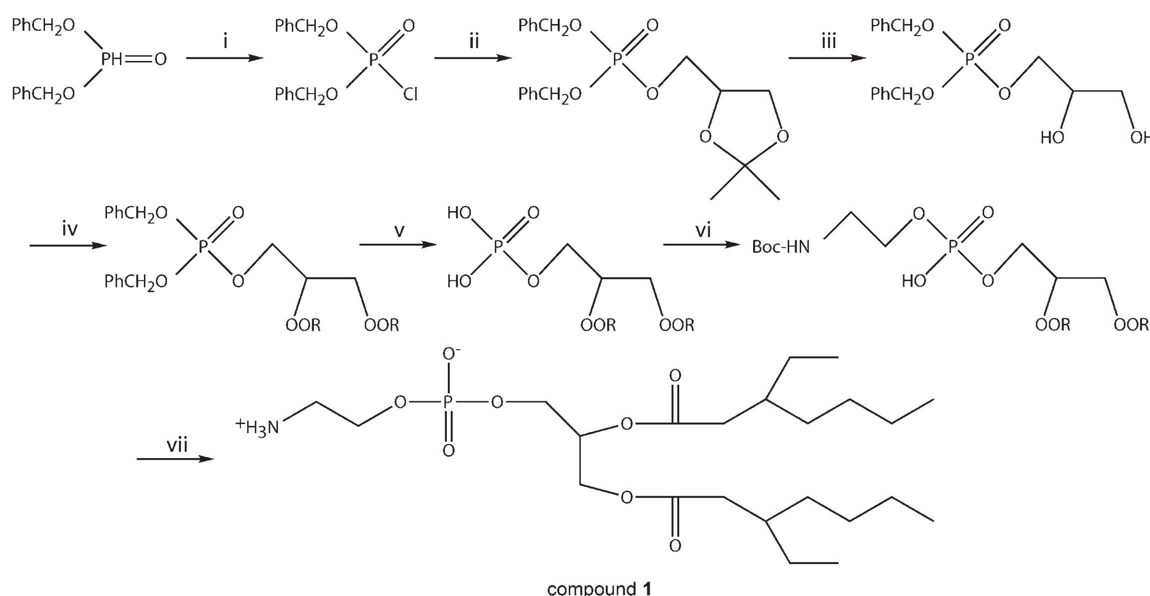

Figure 2. Pathway for synthesis of surfactant **1**. Details of steps i–vii are given in the Experimental Section.

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and the protein was not fully solubilized. Moreover, the region of the phase diagram indicating reverse micelles in short-chain alkanes is very sharp and depends strongly upon temperature.[18] For these last experiments, the addition of AOT to $C_{12}E_4$ could have improved the encapsulation of cytochrome C, as electrostatic interactions between the surfactant head groups and the protein are paradoxically important for this.[5]

Another critical point in the usefulness of our new surfactant in the approach of Wand is its solubility in low-viscosity solvents and the time-stability of the micelles containing a protein. Indeed, in the subcritical region, for a chemical family, such as *n*-alkanes, viscosity of a medium is strongly related to its solvating power through the cohesive energy densities, as introduced by Hildebrand.[19] Thus, the solubility of this new surfactant was evaluated in an ethane/propane/pentane mixture (ca. 45:22:33 v/v). ^{15}N-labeled ubiquitin, a protein of 76 amino acids (pI = 6.6) used as a model compound by Wand, was chosen. The ^{1}H,^{15}N-HSQC spectrum displayed in Figure 3 resembles that of ubiquitin in water, thereby indicating a similar three-dimensional structure. The spectrum obtained with eight scans per transient, by using a 500 MHz spectrometer equipped with a classical inverse probe head, exhibits an excellent signal-to-noise ratio. This is encouraging for future studies of larger proteins by using triple-resonance techniques. The stability of the micellar system was confirmed, as the same experiment repeated one month later showed no alteration in the HSQC spectrum.

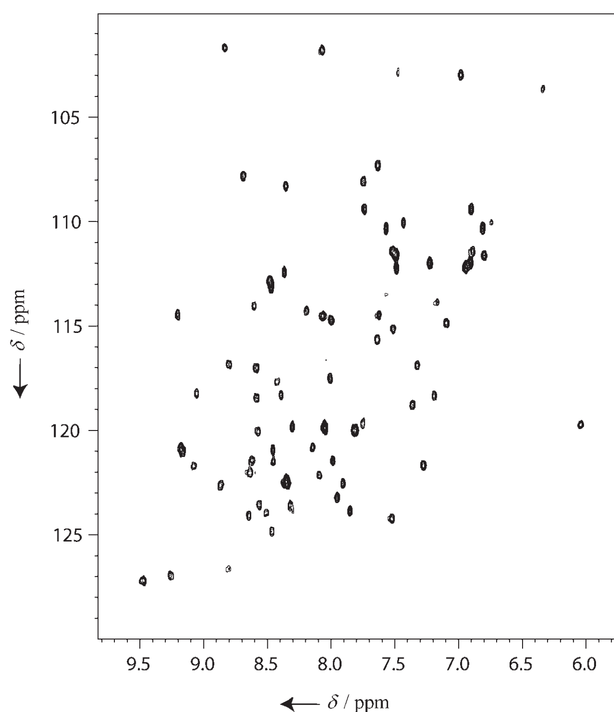

Figure 3. ^{1}H,^{15}N-HSQC spectrum of ubiquitin encapsulated in micelles of surfactant **1** dissolved in an ethane/propane/[D$_{12}$]pentane mixture of 45:22:33 v/v. Eight scans were recorded over 160 t_1 increments.

Conclusion

This new zwitterionic surfactant appears to facilitate the encapsulation of proteins of low or high isoelectric point in the absence of any buffer or salt. The formation of reverse micelles occurs without the need for any cosurfactant. For both ubiquitin and cytochrome C, the line widths of the ^{1}H or ^{15}N NMR spectra show that small micellar objects are formed in short-chain alkanes, with *W* values close to 10. These reverse micelles are stable over a very long period: after several months no precipitation or phase separation was observed. The encapsulation of larger proteins and their NMR analysis in low-viscosity solvents is now underway in our laboratory. It is also expected that modification of the aliphatic tail to increase solubility of this surfactant in apolar solvents would improve its capacity to encapsulate proteins in greater quantities, or in solvents of lower viscosity.

Experimental Section

Syntheses

Reagents, solvents, and cytochrome C were purchased from Fluka. ^{15}N-labeled ubiquitin was purchased from VLI Research and deuterated solvents from Eurisotop.

Synthesis of the AOT analogue: The AOT analogue was synthesized according to S. Nave et al.,[20] overall yield of 66.5 %. ESI-MS: $m/z = 467.2$ [M+Na] corresponding to $m/z = 444.2$ [M]. The purity of the product (white solid) was verified by NMR spectroscopy.

Synthesis of compound 1

Step i: dibenzyl phosphoryl chloride: Diphenyl phosphonate (7.5 g, 28.5 mmol) and chlorosuccinimide (3.75 g, 28.5 mmol) were dissolved in 50 mL of anhydrous toluene and stirred for 2 h at ambient temperature. The mixture was filtrated in an anhydrous atmosphere and the solvent was evaporated under reduced pressure. Due to its instability, the crude product was used in the subsequent reaction without any purification.

Step ii: dibenzyl (2,2-dimethyl-1,3-dioxolan-4-yl)methyl phosphate: Solketal (2.5 g, 18.5 mmol) was dissolved in 45 mL of anhydrous pyridine and the mixture was cooled in an ice bath. Dibenzyl phosphoryl chloride was introduced under vigorous stirring and the mixture was left for 2 h at 0 °C. Unreacted dibenzyl phosphoryl chloride was hydrolyzed by the addition of aqueous sodium carbonate. After extraction with dichloromethane and purification by flash chromatography using hexane/ethyl acetate (6:4 v/v), 2.1 g of the product was obtained (29 %, based on solketal).

Step iii: dibenzyl 2,3-dihydroxypropyl phosphate: Dibenzyl (2,2-dimethyl-1,3-dioxolan-4-yl)methyl phosphate (2.1 g, 5.35 mmol), previously obtained, was mixed with 1.5 g of Dowex 5W-X8(H^+) resin and 40 mL of methanol. After 4 h, 1.5 g of resin was added and the reaction was left for 48 h under stirring. The mixture was filtrated and purified by flash chromatography using ethanol/ethyl acetate (1:9 v/v) to yield 1.03 g of product (55 %).

Step iv: 3-(bis(benzyloxy)phosphoryloxy)propane-1,2-diyl bis(3-ethylheptanoate): Dicyclohexylcarbodiimide (3.352 g) dissolved in 10 mL of dichloromethane was added at 0 °C under stirring to a solution of dibenzyl 2,3-dihydroxypropyl phosphate (1 g, 3.125 mmol), 3-ethyl-heptanoic acid (1.169 g, 7.40 mmol, obtained in 63 % yield by chain elongation from 2-ethylhexyl bromide), and dimethylaminopyridine (763 mg, 6.25 mmol) in 20 mL dichloromethane. The reaction proceeded for 10 min at 0 °C and overnight at ambient temperature. After filtration of the precipitated urea and evaporation of the solvent, the crude mixture was purified by

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flash chromatography using pentane/diethyl ether (2:1 v/v) to yield 1.26 g of product (63 %).

Step v: 2,3-bis(3-ethylheptanoyloxy)propyl phosphate: An amount of 5 % palladium on charcoal (125 mg) was added to a solution of 3-(bis(benzyloxy)phosphoryloxy)propane-1,2-diyl bis(3-ethylheptanoate) (1.26 g) in methanol, and the mixture was stirred overnight under a hydrogen atmosphere. After Celite filtration and solvent evaporation, the crude mixture was purified by flash chromatography using dichloromethane/methanol (9:1 v/v) to yield 0.522 g of product (58 %).

*Step vi: 2-*tert*-butoxycarbonylamminoethyl-2,3-bis(3-ethylheptanoyloxy)-propyl phosphate*: Triisopropylbenzene sulfonyl chloride (200 mg) dissolved in 4 mL of anhydrous pyridine was added to a solution of 2,3-bis(3-ethylheptanoyloxy)propyl phosphate (100 mg, 0.22 mmol) and *N*-Boc-ethanolamine (69 µl, 0.44 mmol, BOC = *tert*-butyloxycarbonyl) in 6 mL of anhydrous pyridine. After 4 h of stirring, the reaction was quenched by the addition of water. Following solvent evaporation under reduced pressure, diethyl ether was added and the precipitated triisopropylbenzene sulfonic acid was filtered out. Purification by flash chromatography using dichloromethane/methanol (95:5 v/v) gave 134 mg of product (99 %).

Step vii: 2-ammonioethyl-2,3-bis(3-ethylheptanoyloxy)propyl phosphate: Deprotection of the amino function was carried out by using a solution of 1 mL of trifluoroacetic acid in 10 mL of dichloromethane. After 30 min, the solvent was evaporated under reduced pressure and the product was purified by flash chromatography using dichloromethane/methanol (95:5 v/v) to yield 107 mg of final product (95 %).

ESI-MS: $m/z = 518.3$ $[M+\mathrm{Na}]$ corresponding to $m/z = 495.3$ $[M]$. The purity of the product (white solid) was verified by NMR spectroscopy.

Sample preparation: The samples of cytochrome in reverse micelles were prepared by the solid–liquid transfer method.[21] The protein was dissolved in water, the pH was adjusted by the addition of HCl, and the protein was lyophilized. It was then added to a solution containing the surfactant, deionized water, and $[D_{12}]$pentane (98 % enriched). At this step, the amount of water added was that required for *W* to reach a value of 10. The final *W* values, estimated from the ^{1}H NMR spectra, are indicated in the legend of Figure 1c–f. The AOT concentrations were 70 mM in 500 µL. For spectra c and d, 1 mg of protein was used. The concentrations of **1** were 113 mM in the case of spectrum e (final *W* value of 8), and 37 mM for spectrum f, due to the limited quantities of the new surfactant. In the latter case, the final *W* value was 12, as measured from the ^{1}H NMR spectrum. The amounts of cytochrome C were 1 and 0.3 mg for spectra e and f, leading to concentrations of 0.15 and 0.05 mM, respectively.

For the ubiquitin sample, the micellar solution was prepared by the injection method.[21] The lyophilized protein (1.02 mg) was hydrated by 5.2 µL of deionized H_2O, added by using a Hamilton syringe placed directly into the NMR tube. A solution containing 17.9 mg of the zwitterionic surfactant dissolved in 500 µL of pentane was added to the hydrated protein. Manual shaking allowed homogenization of the solution, and a fast ^{1}H,^{15}N-HSQC analysis was performed to ensure that the protein was encapsulated in a folded state. An evaporation method[11] was then used to obtain the final solvent mixture. All of these steps were performed in an NMR sapphire tube, purchased from the laboratory of A. Merbach.[22] A dedicated pressure line with a manual pump and pressure gauges was constructed and used to fill the NMR tube with propane, then ethane. The solvent volume was determined by measurement of the height of the liquid in the NMR tube. The propane/ethane ratio was estimated by relative integration on the ^{1}H NMR spectrum, and the *W* value was measured on the final sample at pH 6.2. No precipitation appeared at the bottom of the tube, however, a small viscous droplet could be observed some minutes after shaking. The maximum concentration of ^{15}N-enriched ubiquitin observed in Figure 3 is, thus, 0.25 mM, if one considers that all of the protein is solubilized.

NMR spectroscopy: The conformational state of cytochrome C in various environments was assessed by conducting NOESY experiments. In the absence of ^{15}N-enriched protein, the results of these ^{1}H NMR experiments gave an insight into both the encapsulation and the folding state of the protein. However, due to the presence in the NMR spectra of the intense surfactant peaks (concentration ~ 100 mM), a dedicated pulse sequence was required for the spectra shown in Figure 1c–f. It was essentially the classical NOESY pulse scheme followed by a frequency-selective spin echo (Gradient–Soft 180°–Gradient) prior to acquisition. The soft pulse selectively inverted the 6–11 ppm region and enabled observation of through-space polarization transfer involving the amide/aromatic protons. As the ^{1}H spectrum covers a range from −25 to +25 ppm, clearly some amide protons are excluded from the plot. These NMR experiments were performed by using a Bruker DRX800 spectrometer (18.7 T) equipped with a 5-mm *z*-gradient HCN cryoprobe.

To characterize the conformational state of ^{15}N-labeled ubiquitin in micelles of **1**, sensitivity-enhanced ^{1}H,^{15}N-HSQC spectroscopy was performed at 293 K by using a Bruker DRX500 spectrometer (11.7 T) equipped with a 5-mm *xyz*-gradient broadband inverse probehead.

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