Supramolecular Association in the System Water−Lysozyme−Lithium Perfluorononanoate

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Mixtures containing water, lysozyme (LYS), and a fluorinated surfactant (LiPFN) have been investigated in a wide range of protein-to-surfactant ratios. Depending on composition, sample consistency and coexistence of the different phases, different experimental methods were used. Volumetric, viscometric, surface tension, potentiometric (by a home-built ion-selective electrode), turbidity, optical polarizing microscopy, and ^{19}F NMR experiments were used. The results obtained from the above methods have been interpreted in terms of a combination of electrostatic and hydrophobic contributions to the stability of the different phases formed in the water−LYS−LiPFN system. Solutions, gel phases, and precipitates have been observed in the range investigated in more detail. Multiphase regions have also been observed. Such rich polymorphic behavior implies the existence of interactions between the protein and surfactant. The gel phase is presumably formed by interconnections between micelles and protein-surfactant complexes, held together by protein-bound micelles and forming, presumably, interconnected necklace structures. The overlapping of different protein-surfactant aggregates to form gels requires a significant amount of time. Its formation obeys a volume fraction statistics; the width of the gel phase, in fact, is controlled by the amount of protein−surfactant complex.

Introduction

The physical chemistry of complex colloid mixtures composed by surfactants (or lipids) and macromolecules has recently got renewed interest and is being systematically investigated.[1,2] Studies reported so far deal with homo-polymers and ionic surfactants,[3−10] hydrophobically modified polymers (sometimes referred to as poly-soaps)[11] and surface-active molecules,[12,13] block copolymers and detergents,[14,15] polyelectrolytes and oppositely charged amphiphiles,[16−18] etc.

As can be seen from the above list, Coulombic effects are dominant in some cases. In others, hydrophobic contributions control the rich polymorphic behavior of polymer-surfactant systems, PSS, in water and are responsible for the supramolecular organization of the components into complex structures. Depending on the macromolecule and the surfactant, different and sometimes conflicting interaction modes between the components occur. Such differences are reflected in the phase behavior and self-organization of the components.

Even more complex are the interactions between proteins (or DNA) and surfactants or lipids.[19−21] Proteins bear a pH-dependent net charge, allowing them to interact electrostatically with oppositely charged surface active species.[22] Attention is focused on the formation of protein−surfactant complexes [23] for surfactant-assisted protein recovery.[24−25] Proteins obtained in this way are generally inactive, because of the combined effect of Coulombic, hydrogen bond, and hydrophobic interactions on their conformation and folding.[26,27]

Systematic studies on the interactions between biopolymers and surface active agents have been reported. Precipitates, gels and solutions were obtained and characterized by different physicochemical methods.[28−30] The rich polymorphic behavior observed in such mixtures depends on protein-to-surfactant ratio, ionic strength, pH, temperature, etc. It is not easy to ascertain univocally which contribution is dominant and controls the polymorphism of water−protein−surfactant systems.

To clarify still open questions, we present and discuss the resuls obtained by a systematic investigation on a system composed by water (or brine), a globular protein (lysozyme, LYS), and a totally fluorinated surfactant. Interactions of this protein with surface-active agents are known[23,31] and may be compared with data reported in the present contribution.

The hydrophobic and oleophobic character of the fluorinated surfactant influences the interactions with the protein. Such interactions shall be presumably controlled by electrostatic contributions, whereas hydrophobic interactions between the apolar regions of the protein and the surfactant can be ruled out. Hydrophobic contributions, conversely, are relevant in fluorocarbon−fluorocarbon interactions. This behavior will be reflected in the phase behavior, in the self-organization of the components and in the stability of the different phases.

Fluorinated molecules (fluoro-alcohols, for instance) are protein stabilizers.[32] Not much is known on the effect of fluorosurfactants on protein stability and conformation. In the present work, a systematic physicochemical investigation on the system water−LYS-lithium perfluorononanoate, LiPFN, at 25 °C, is reported. The role of fluorinated surfactants in the supramolecular organization of proteins and protein-surfactant complexes is considered. LiPFN has been formerly studied and micelle formation, aggregation numbers, counterion binding, phase diagram, and interactions with hydrophilic homopolymers [8,33,34] are well-known.

The interactions between the components have a pronounced effect on the physicochemical properties of the above system, as well as on the structure of adducts and complexes that could occur. That's why different experimental methods were used to investigate and clarify the observed behavior.

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Experimental Section

Materials. Lysozyme, LYS, Sigma, was purified by dialysis. About 4.0 g of the protein were dissolved in 100 mL of 0.15 *m* NaCl and the solution was dialyzed overnight. Lysozyme was recovered, lyophilised, dried, and kept under vacuum over P_2O_5 until use. The solutions were prepared by weight and corrected for buoyancy.

The membranes for dialysis were immersed in water for 4−5 h and washed with a 0.3 wt Na_2S solution at 80 °C and hot water. They were held in a 0.2 vol H_2SO_4 solution at 60 °C for a few minutes and washed with bidistilled water, at r.t.

LiPFN was prepared from the acid, Aldrich, 99.5% purity. It was dissolved in ethanol and reacted with aqueous LiOH. The end-point of the titration was checked by phenolphthalein as an indicator. The solvent was removed under reduced pressure, and the resulting paste was lyophilised. The product was crystallized in ethanol−acetone, dried at 80 °C, and stored in the presence of P_2O_5.

Ethanol, acetone, PVC (molecular mass 2.8 10^5 Dalton), THF and $CHCl_3$, RPE, were from Carlo Erba. Tetraoctylammonium chloride (TOACl), LiCl, HCl, Tris, H_3PO_4, and LiOH, of analytical grade, were from Fluka. The salts were dried and kept in a desiccator until use.

High quality water ($\chi \approx 10^{-7}$ S cm^{-1} at 25 °C) was obtained by distilling deionized water in a Pyrex glass apparatus over alkaline $KMnO_4$. Deuterium oxide, 99.95% isotopic enrichment, from Merck, was used as received. When required, water was replaced by D_2O on a mole fraction basis.

The lithium-based buffer was prepared by reacting phosphoric acid solutions with lithium hydroxide, to get pH values close to 7.0. Other buffers were prepared by mixing LiCl, Tris, and HCl, to get pH close to 8.0 or 4.5.[35] Lithium-based buffers avoid the precipitation of perfluorononanoate ions in the presence of sodium salts. The Krafft temperature, T_K, of NaPFN, in fact, is close to 25 °C [36,37] and increases upon addition of salt in excess. The T_K of lithium salt, conversely, is much lower.

Individual samples were prepared by weight in glass vials and equilibrated at room temperature. In some instances, water was replaced by LiCl, LiCl and lithium-based phosphate buffer (LiH_2PO_4 and Li_2HPO_4), at pH 7.0, or LiCl−HCl−Tris, at pH 4.5 or 8.0, respectively. Care was taken to ascertain whether samples had reached thermodynamic equilibrium. Visual or nephelometric investigation and optical microscopy (in white and polarized light) checked the appearance of samples located in different regions of the phase diagram on a weekly basis, over six months. The multiphase samples were centrifuged at 6000 rpm, and the precipitates were filtered, dried, and investigated by optical microscopy and X-ray diffraction.[38]

Methods

Turbidity. The nucleation of precipitates was determined by a PC Compact photometer (Aqualytic). Apparent turbidity versus log [LiPFN] plots determine the amount of surfactant required forming and dissolving the complex.

Volumetric Properties. Densities, ρ, were determined at 25.000 ± 0.002 °C by an Anton Paar vibration densimeter, mod. DMA 60, equipped with a thermostatic unit. The temperature, *T*, was measured by a digital thermometer, A. S. L. Densities were obtained by

$$\Delta\rho = \rho - \rho^\circ = (1/A)(\tau^2 - \tau^{\circ 2}) \qquad (1)$$

where τ° and τ are the vibration frequencies of the solvent and the solution; ρ and ρ° are the solution and solvent density, respectively. *A* is a constant, obtained by fitting eq 1 for liquids of known density, i.e., water,[39] acetone, ethanol, p-dioxane, ethylene glycol [40] and trifluoro-ethanol.[41] The accuracy on density values is about 5×10^{-6} g cm^{-3}.

Apparent, $\Phi_{V,2}$, and partial, V_2, molal volumes, in cm^3 mol^{-1}, were obtained by

$$\Phi_{V,2} = (MW)/\rho - [10^3(\rho\text{-}\rho^\circ)/\rho\rho^\circ m] \qquad (2)$$

$$V_2 = \partial(m\Phi_{V,2})/\partial m \qquad (3)$$

where *m* is the surfactant molality and MW is its molar mass. The accuracy on apparent and partial molal quantities is between 0.2 and 1.8 cm^3 mol^{-1}, depending on surfactant content. Volumetric data are reported in Table 1; plots of $\Phi_{V,2}$ values versus $m^{1/2}$ are drawn in Figure 1.

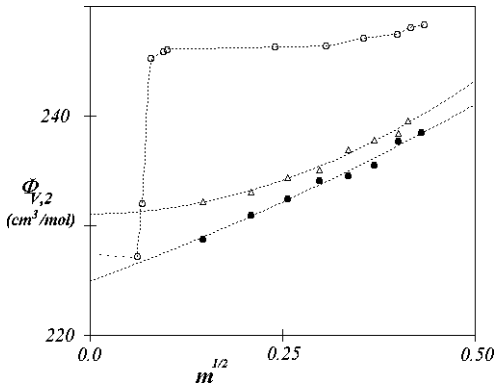

Figure 1. Apparent molal volumes of LiPFN, $\Phi_{V,2}$, in cm^3 mol^{-1}, versus $m^{1/2}$, in water (○), 1.00 wt. LYS and 0.15 *m* LiCl (△), 1.00 wt. LYS in 0.15 *m* LiCl and pH 4.5 (●), at 25.00 °C. Curves are for visual purposes only.

TABLE 1: Molality, *m*, mol kg^{-1}, and the Apparent Molal Volume, $\Phi_{V,2}$, cm^3 mol^{-1}, of LiPFN in Water, Left, and in 1.00 wt Aqueous Lysozime, Right Columns, at 25.00 °C[a]

water		1.00 LYS wt.	
$10^3 m$	$\Phi_{V,2}$	$10^3 m$	$\Phi_{V,2}$
3.708	227.2	21.47	222.5
4.529	232.0	43.47	232.5
6.115	245.3	65.76	234.7
9.139	245.9	88.51	237.7
10.06_2	246.1	111.9	240.6
57.49_2	246.3	135.4	233.7
93.82_3	246.4	159.9	234.4
125.87	247.1	184.9	242.0
159.21	247.5		
173.46	248.1		
188.37	248.3		

[a] The pH was the spontaneous value of the protein in solution.

Viscosity. The viscosity was determined by Ubbelohde viscometers, Schott, equipped with a semiautomatic reading unit (Schott, mod. 400). Flow times, *t*, in the range of 200−300 s minimize spurious kinetic effects. Measurements were determined with an accuracy of ±0.03 s. The viscometer was located in a thermostatic bath at 25.00 ± 0.01 °C. Care was devoted to avoid bubble formation. Each datum is the average of, at least, five independent measurements. The relative viscosity, η_{rel}, was calculated by

$$\eta_{rel} = (t\rho)/(t^\circ\rho^\circ) \qquad (4)$$

where *t* and t° are the solution and solvent flow times, respectively.

TABLE 2: Weight Percent, wt, and the Relative Viscosity, η_{rel}, of LiPFN Solutions in Different Systems, at 25.00 °C[a]

1.00 wt %LYS		1.00 wt %LYS with 0.15 *m* LiCl		1.00 wt %LYS with 0.15 *m* LiCl, pH 4.5	
LiPFN wt.	η_{rel}	LiPFN wt.	η_{rel}	LiPFN wt.	η_{rel}
0.000	1.000	0.000	1.000	0.000	1.000
0.999	1.039	1.001	1.125	1.000	1.123
2.002	1.086	2.000	1.198	2.001	1.231
2.998	1.138	3.002	1.200	3.001	1.235
3.994	1.189	3.999	1.232	4.000	1.269
4.996	1.260	5.001	1.276	4.995	1.315
5.983	1.314	5.998	1.326	6.003	1.370
6.992	1.386	7.004	1.384	7.002	1.423
7.994	1.473	8.000	1.443	7.798	1.466

[a] Data refer to 1.00 wt aqueous LYS, left columns; 1.00 lysozime wt in 0.15 *m* aqueous LiCl solution, central columns, and 1.00 wt LYS in aqueous 0.15 *m* LiCl solution at pH 4.5, right columns. The accuracy on individual relative viscosity values is ± 0.002.

Viscosity data are reported in Table 2. Values relative to micellar solutions and time-dependent findings (relative to the gel) are reported in Figures 2 and 3, respectively.

Surface Tension. The apparatus for measuring surface tension, σ (mN m^{-1}), is a MGW Lauda unit equipped with a Pt−Rh du Noüy ring. Its properties and setup procedures are described elsewhere.[42] The measuring vessel is thermostated to within 0.1 °C by an external jacket. The du Noüy ring was flame cleaned before use, washed in 0.1 M HCl, and kept in doubly distilled water. The surface tension of H_2O is 71.8 mN m^{-1}, at 25 °C, in agreement with the literature.[43] The solutions were equilibrated some minutes, to ensure the attainment of equilibrium between bulk and surface phases. No drift in surface tension values is found. Five individual measurements per point were performed. The data accuracy is to 0.2 mN m^{-1}.

Surface tension data were analyzed by the Gibbs adsorption isotherm, according to

$$\partial\sigma = -\Gamma_2\, \partial\mu_2\, \partial \approx \Gamma_2\, RT\, \partial ln m_2 \qquad (5)$$

where Γ_2 is the surface excess concentration of LiPFN, μ_2 is its chemical potential, and R is the gas constant. In eq 5, the activity, a_2, is replaced by the corresponding molality, m_2.

NMR Spectroscopy. Proton NMR spectra were recorded at 300 K on a BRUKER AMX 600 unit. Setup procedures are reported elsewhere.[44] The samples were located into 5-mm NMR tubes and flame sealed, taking care to avoid thermal degradation of the protein.

A Varian unit, INOVA 500, measured ^{19}F NMR spectra, according to a previously described procedure.[45] The CF_3 peak was chosen as a reference signal. The chemical shifts, extrapolated to infinite dilution, are reported in Table 3. A progressive number indicates the position of different nuclei with respect to the carboxylate group. Those in position 2 and 9 are sensitive to polar and hydrophobic interactions, respectively. ^{19}F NMR spectra are reported in Figure 4.

Optical Polarizing Microscopy. A CI Ceti optical microscope equipped with removable polarizers, Bertrand lenses, a thermostatic chamber (Linkam, mod. TP 93, with a HFS 91 heating stage), and a camera (Pentax, ME super) [46] was used. Optical microscopy, in white and polarized light, was determined on samples homogenized by stirring. A selected photograph is reported in Figure 5. The photograph in the above figure gives merely qualitative information on the gel structure. It can be compared with those observed by TEM in complexes formed by peptides and surfactants[47] and with those reported in dye-surfactant complexes (obtained by optical polarizing microscopy).[48]

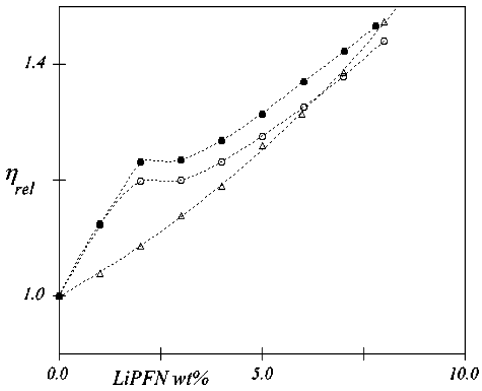

Figure 2. Dependence of the relative viscosity, η_{rel}, on LiPFN weight percent in water (△), 1.00 wt. LYS in 0.15 *m* LiCl (○), 1.00 wt. LYS in 0.15 *m* LiCl and pH 4.5 (●), at 25.00 °C.

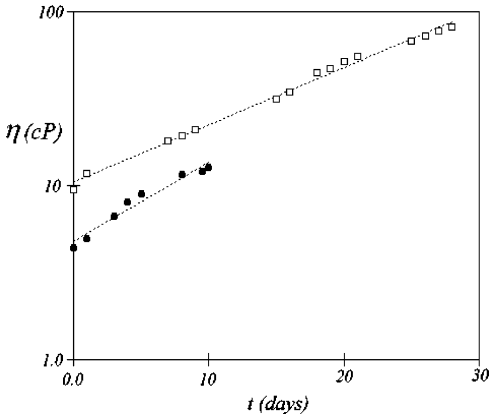

Figure 3. Dependence of viscosity, η (cP), on aging (days) in gels containing 6.50 wt. LYS and 7.02 wt. LiPFN, (□), or 7.01 wt. LYS and 7.53 wt. LiPFN, (●), at 25.00 °C.

TABLE 3: Fluorine Chemical Shifts, δ, (in ppm) for Different Groups in the Perfluoro-methylene Chain, Extrapolated to Infinite Dilution

carbon atom	$\delta^{19}F$ chemical shift[§]
2	−39.71[a]
3	−45.42[a]
4	−44.00
5	−44.01
6	−44.14
7	−44.94
8	−48.25[a]
9	−03.01[a]

[a] More used signals.

Potentiometry. Ion-selective electrodes were prepared by mixing aqueous LiPFN with equivalent amounts of tetraoctylammonium chloride. The cloudy TOAPFN dispersion was extracted with acetone and precipitated by water. The water insoluble part was dried and mixed under stirring with a PVC-THF solution. The resulting paste was allowed staying 3 days in an air oven, at 45 °C. Films obtained in this way (thickness, 1 mm) were cut and located in the potentiometric tube.

The scheme of the electrode is

Ag|AgCl||reference solution|PVC membrane|sample||AgCl|Ag

$$\begin{pmatrix} 10^{-3}\text{ m LiPFN} \\ \text{in 0.15 m LiCl} \end{pmatrix} \begin{pmatrix} \text{TOAPFN} \\ \text{in PVC-THF} \end{pmatrix}$$

The potentiometer is a Philips unit.

Inner solutions contain 10^{-3} *m* LiPFN in 0.15 *m* LiCl. The slope of emf values versus log [LiPFN] below the CMC is 57.4

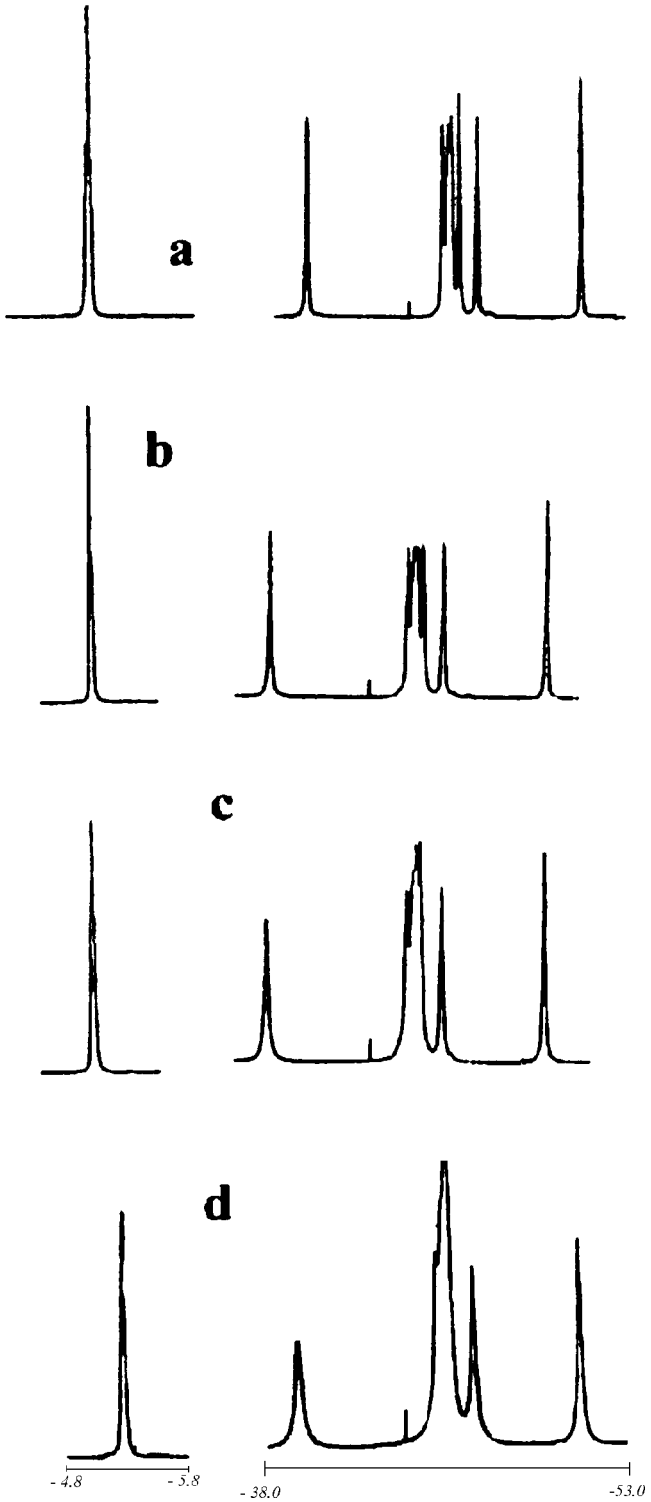

Figure 4. ^{19}F NMR spectra relative to systems containing 7.97 LYS and 2.02 LiPFN, (a), 7.13 LYS and 2.64 LiPFN, (b), 7.25 LYS and 3.49 LiPFN, (c), 7.12 LYS and 4.39 LiPFN wt., (d), at 300 K. Signals of the CF_3 group are reported on the left side of the figure. The signal assignment is given in Table 3.

$\pm$ 0.2 mV per concentration unit, at 25 °C. Details on electrode preparation and conditioning are reported elsewhere.[49] The time response of the electrode depends on LiPFN concentration, added electrolyte, stirring and conditioning. In dilute solutions, the time required to get stable emf values is 2 min, inset in Figure 6. In concentrated solutions, it can be longer, depending on the presence of precipitate(s) and/or sample fluidity. Above the CMC ($\approx 9 \times 10^{-3}$ m), changes in slope are observed. In the presence of interactions with the protein and precipitation of the PS complex, the behavior depicted in Figure 6 is observed.

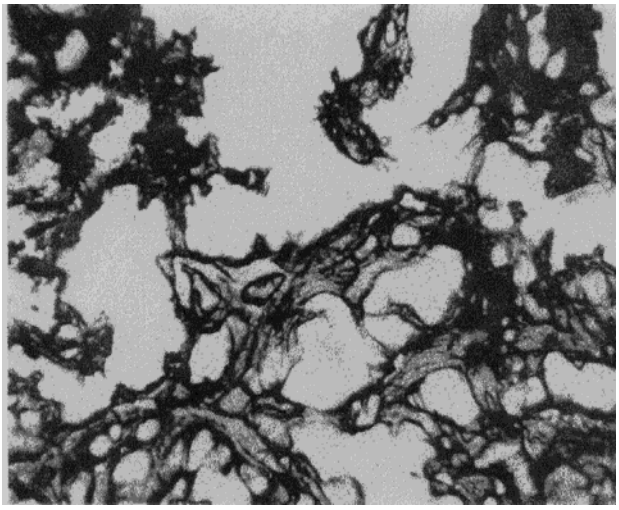

Figure 5. Optical polarized light micrograph relative to a gel containing 10.0 LYS and 4.97 LiPFN wt., at 25 °C. Magnification is 100×.

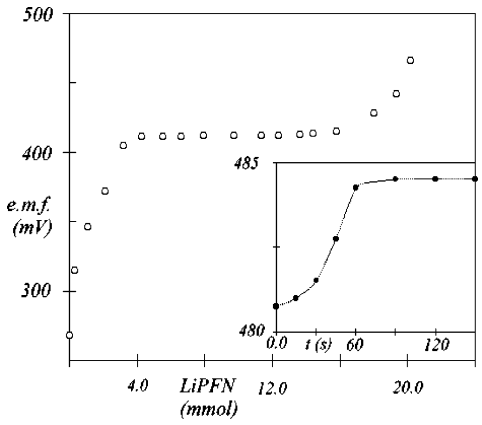

Figure 6. Potentiometric data, mV, relative to a solution containing 0.89 LYS wt. as a function of LiPFN molality. The interaction region is characterized by constant emf values. In the inset are reported data relative to a 4.54×10^{-3} LiPFN m solution as a function of time (in sec) for a conditioned electrode, at 25.0 °C, under stirring.

Results and Discussion

Phase Diagram and Macroscopic Phase Properties. The partial phase diagram of the system water−LYS−LiPFN is reported in Figure 7. Less refined diagrams relative to pseudo-ternary systems containing LiCl, LiCl + lithium phosphate, or LiCl + HCl + Tris have been drawn too. Pseudo-ternary phase diagrams in the presence of salt, or buffer, are slightly different from the $H_2O-LY-LiPFN$ one, but the phase sequence is similar and the width of the different phases is comparable. The more relevant features due to LiCl or pH are slight, but significant, changes in the width of the solid and gel phases.

In the concentration range, we have investigated, the following regions are observed:

(1) A tiny protein-rich solution phase, $L°$, at low LiPFN content. Its width is difficult to get univocally, and the phase border was drawn by extrapolation of emf and turbidity data, obtained at low protein content. In such a phase, LYS exists in molecular and dimeric form, depending on concentration;[50,51]

(2) A two-phase region, with a white precipitate. In the presence of salt, competition of PFN^- with the Cl^- occurs, and the phase width is modified;

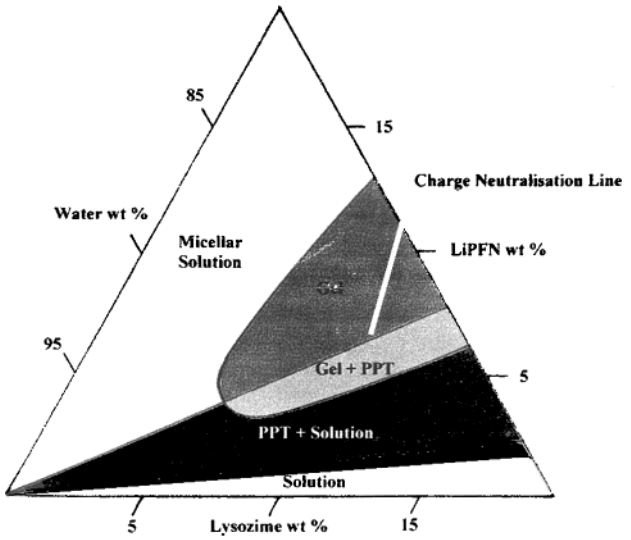

Figure 7. Partial phase diagram of the system water, lysozime, lithium perfluorononanoate, at 25 °C. The black color refers to the precipitate region, the light gray to precipitate dissolved in the gel, the gray to the gel, and white to solutions. The small area on the lysozime-rich side indicates a solution region: the related phase width is uncertain. The line cutting the gel phase in two indicates the charge ratio 1/1.

TABLE 4: Critical Association Concentration, T_1, the Critical Redissolution Concentration, T^*, and the Critical Micellar Concentration, T_2, in Water−LYS Systems, at 25.0 °C[a]

LYS wt.	T_1	T^*	T_2
0.00			8.0×10^{-3}
0.01	7.0×10^{-5}	1.8×10^{-3}	1.0×10^{-2}
0.01[b]	3.7×10^{-4}	7.4×10^{-4}	8.9×10^{-3}
0.05[b]	2.3×10^{-4}	1.9×10^{-3}	9.0×10^{-3}

[a] Quantities are in mol kg^{-1}. [b] Data in 0.15 molal aqueous LiCl.

(3) The precipitate imparts a bluish color to the gel. A similar behavior, ascribed to solid particles, has been observed in the water−SDS−LYS system;[28]

(4) A transparent gel is observed at high concentrations, above the charge neutralization line, Figure 7. It does not show optical birefringency or disperse particles. Addition of LiCl reduces the width of the gel phase;

(5) At still higher surfactant content, a micellar solution occurs. Surfactant in excess dissolves the $[LYS(PFN)_X]$ precipitate. The micellar region, L_1, is observed for LiPFN/LYS mass ratios higher than 3/2.

We do not know to what extent protein−protein association is relevant in the interactions with surfactants and gel formation. It is worth mentioning that the minimum amount of LYS required to form the gel is close to the protein−protein association concentration.[51]

Dilute Regime. The critical association concentration [52] (CAC or T_1) is the threshold at which interactions between protein and surfactant begin to occur. Some T_1 data are reported in Table 4. The redissolution concentration, T^*, was determined by turbidity. The critical thresholds were determined with an appreciable accuracy at low protein content, less than 1 wt. As can be seen from the data in Table 4, no univocal links between T_1, T_2 (the CMC in the presence of protein), or T^* values and protein concentration or ionic strength can be found. In a first approximation, T_1 values increase with protein content and added salt; T_2 is sensitive to both variables. T^* values, conversely, are the result of a complex balance between pH, protein concentration, and ionic strength effects.

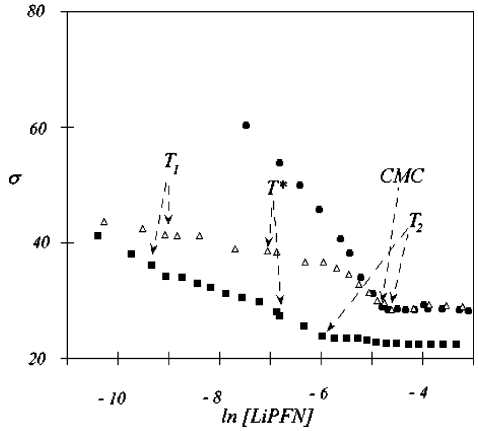

Figure 8. Dependence of surface tension, σ, (mN m^{-1}) on surfactant content, ln [LiPFN], in water (●), 0.1 LYS wt. (△) and 0.1 LYS wt. in 0.15 *m* LiCl, (■), at 25.0 °C. Symbols indicate the critical association, T_1, the critical redissolution, T^*, and the critical micellar concentration, T_2, or CMC, respectively.

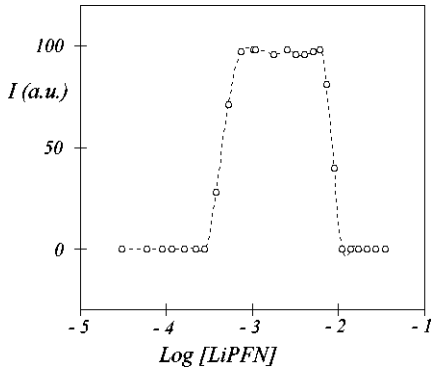

Figure 9. Relative turbidity, *I* (a.u.), as a function of LiPFN molality, in logarithmic form, in 0.15 m LiCl. The concentration of LYS in the solvent is 0.65 wt.

Above T_1 values, the slope of the surface tension progressively reduces and, at the same time, the solution turbidity increases because of an incipient precipitation, Figure 8. On thermodynamic grounds, surface adsorption and precipitation of $[LYS(PFN)_X]$ complexes are in competition, as previously observed in polymer−surfactant systems.[53−55]

In dilute regimes, Coulombic effects control the interactions between LYS and PFN ions, whereas hydrophobic ones are negligible. Hydrophobic interactions between surfactant and lyozime do not occur because of the unfavorable energy of mixing terms between hydrocarbon and fluorocarbon moieties. It is worth mentioning that mixtures of hydrocarbons and fluorocarbons are strongly non ideal and show the occurrence of consolute phenomena or (micro) phase separation.[56−58]

Precipitation of $[LYS(PFN)_X]$ complexes occurs at surfactant/protein mass ratios close to 0.5. This behavior is controlled by the charge neutralization of the protein amino groups from the surfactant. Redissolution requires three times more surfactant. It is tentatively ascribed to interactions between fluoro-alkyl groups of PFN^- bound to the protein (and facing toward the bulk) with surfactant in excess. This hypothesis implies the occurrence of nonspecific interactions[59] between the precipitate and micelles.

Effect of Added Electrolytes. Electrolytes influence the interactions between LYS and the surfactant. As indicated in Table 4 and Figure 10, added LiCl shifts the surfactant adsorption to lower concentrations and reduces the width of the interaction region. This effect is due to competition between

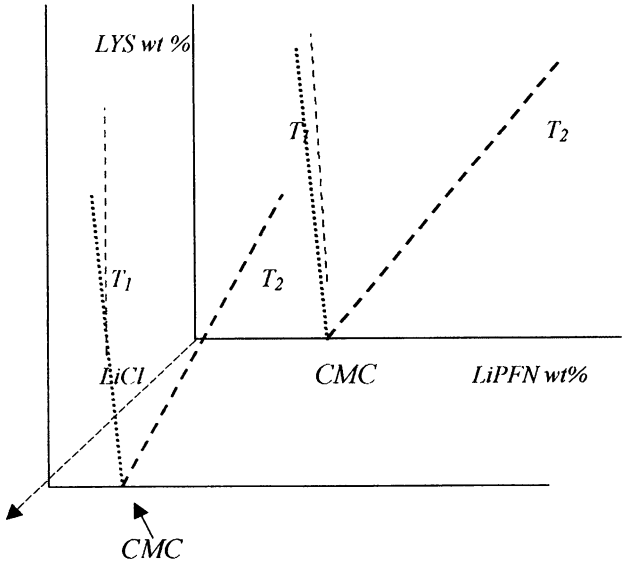

Figure 10. Effect of added LiCl on micelle formation and interaction between LYS and LiPFN. T_1 is the CAC line, T_2 the CMC line in the presence of protein. The related slopes are sensitive to the medium ionic strength. The dotted lines indicate the precipitation threshold, T^*.

PFN^- and Cl^- ions toward the protein charged groups. At the same time, added LiCl shifts micelle formation to lower concentration. All of these effects exert a role on ion competition, counterion binding, and micelle formation.

Addition of electrolytes modifies the following equilibria:

$$NM \rightleftarrows M_N \qquad \text{(a)}$$

$$XM + LYS \leftrightarrows LYS(M)_X \qquad \text{(b)}$$

where $N \neq X$, shifts the former toward lower Gibbs energy values and allows N values to increase.[60,61] At the same time, electrolytes reduce the width of the interaction region.[54,62] A scheme of the overall process is in Figure 10.

Volumetric and Viscosity Findings. The occurrence of electrostatic interactions between protein and surfactant can be put in evidence by volumetric findings, Figure 1. In the presence of LYS (alone, in acid form and/or with added LiCl), $\Phi_{V,2}$ values are noticeably lower than in water. This behavior implies the occurrence of electrostrictive effects. The data in Figure 1 reflects the intricacies associated with the overlapping of contributions responsible for micelle formation and protein−surfactant interaction. Such effects become more evident at pH $\approx$ 4.5, when almost all NH_2 groups on the protein are ionized. The volume change of micelle formation is steep and significant in fluorinated surfactants.[34,63,64] In the presence of LYS, ΔV_{mic} is progressively reduced and decreases in proportion to protein content, pH, and ionic strength. The effect of protonation on ΔV_{mic} can be ruled out, because fluorocarboxylates are strong acids.

Viscosity data, Figure 2, indicate that electrostatic contributions are relevant. The behavior of η_{rel} as a function of surfactant content indicates the occurrence of steep changes at low LiPFN content. The effect, not observed in the H_2O−LiPFN system,[9,34] becomes more relevant in the presence of added electrolyte and/or when the protein is in acid form. Similar peculiarities were found in some water−gelatine−SDS mixtures.[65]

The increase in η_{rel} values may be ascribed to a micelle-assisted cross-linking. Alternatively, the increase in viscosity is explained in terms of electro-viscous effects, due to charging processes of protein−surfactant complexes by PFN^- ions.

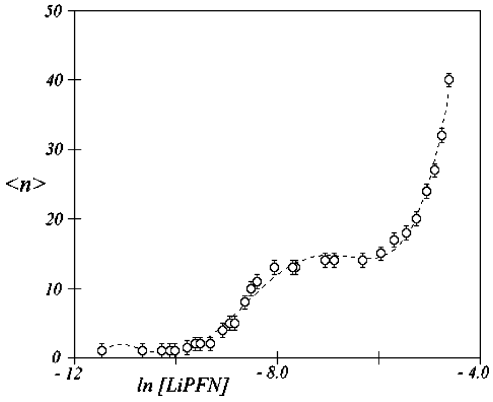

Figure 11. Binding isotherm calculated from emf values. In the figure are reported $\langle n \rangle$ values as a function of LiPFN molality. Data refer to a 0.28 LYS wt. mixture, at 25 °C. The curve is a guide to the eye.

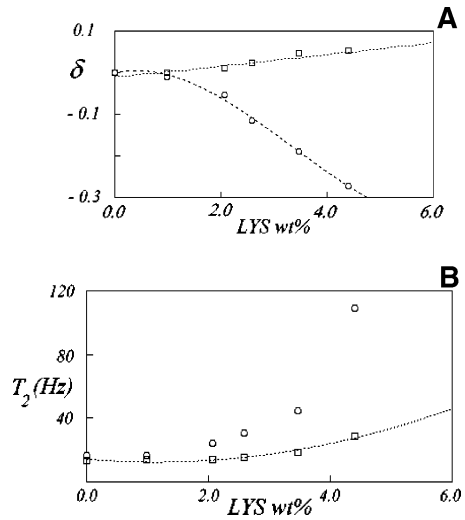

Figure 12. a, Relative fluorine chemical shifts, δ (in ppm), of a 4.5 LiPFN wt. solution as a function of the wt. of added lysozime, for C_9 (□), and C_2 fluorine nuclei (○). In b is reported the line width at half-height, T_2, of the same nuclei as a function of LYS wt.

Electro-viscous effects are significantly screened upon addition of electrolytes in excess. If the latter hypothesis holds, addition of surfactant in slight excess will favor cross-linking.

Potentiometric Studies. The interactions between LYS and LiPFN were inferred from the changes in slope of emf values.[66,67] Some data are reported in Figure 6. The width of the interaction region is grossly proportional to protein content and can be easily distinguished. According to emf findings, the interaction between LYS and LiPFN is a true phase separation. In fact, the surfactant ion activity remains nearly constant up to complete redissolution of the precipitate. The slopes of emf curves are different above and below the precipitation threshold, as expected.

The binding isotherm can be obtained from data in Figure 6. Proper fitting of emf findings gives $\langle n \rangle$ values,[17,68] as indicated in Figure 11. Errors in $\langle n \rangle$ values are to ±5%, but the general features are reasonable. According to the fit in Figure 11, 12−14 ions interact with LYS, in fairly good agreement with the protein net charge.[28] Above the saturation threshold, $\langle n \rangle$ values diverge, approaching those peculiar to free micelles.

NMR Findings. ^{19}F NMR gives information on the dynamic properties of micelles and gels. According to Figure 12, parts a and b**,** both chemical shift and transverse relaxation times (estimated from the line broadening of ^{19}F peaks) change upon addition of LYS. In Figure 12a is observed a systematic decrease

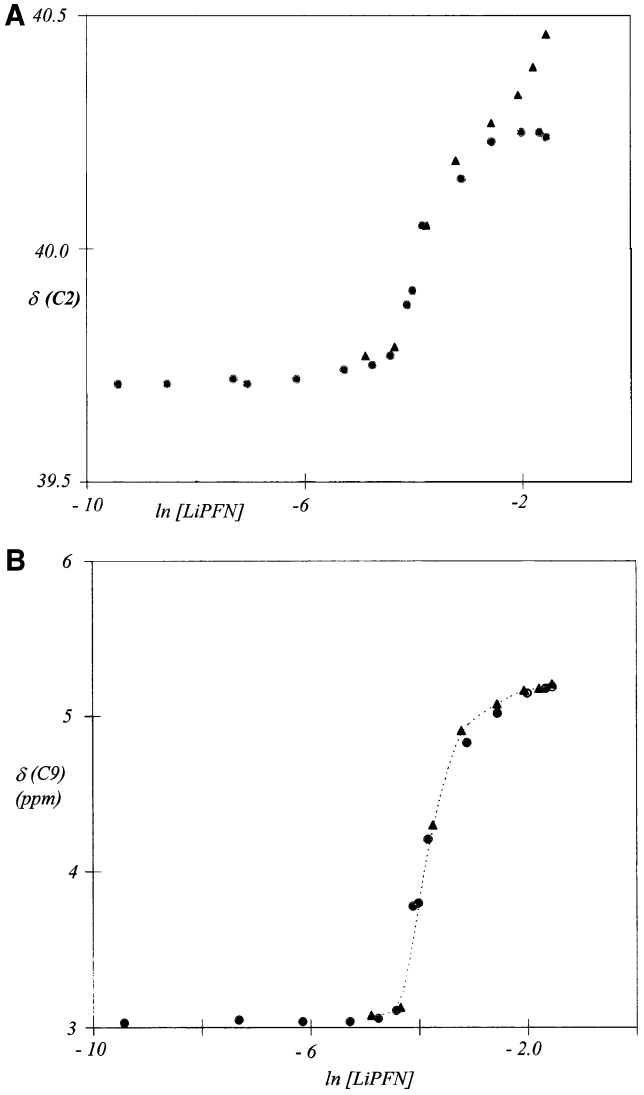

Figure 13. ^{19}F chemical shift of fluorine nuclei in water (■), and 1.02 LYS wt. (▲), at 300 K, as a function of surfactant molality, in logarithmic scale. Data in a refer to C_2 nuclei, those in b to C_9 ones.

of chemical shift, δ, of fluorine nuclei bound to C_2 carbon atoms upon addition of LYS, whereas those relative to C_9 nuclei do not change above the gelation threshold. This is a clear indication of the occurrence of Coulombic interactions between the LiPFN and the protein. Strictly related features are also found in the line broadening of ^{19}F nuclei, Figure 12b.

Micelles interact with selected loci on the protein, giving rise to an extended flexible network, formed by interconnection of different protein−surfactant complexes. It is questionable, at this stage, whether they assemble to form necklaces[69] or other structures.[47] The possibility for PFN^- ions to denature the protein can be ruled out, since the gel rapidly and reversibly forms a clear solution upon dilution. Even more convincing is evidence from ^{19}F chemical shift, reported in Figure 13a,b. It indicates that no direct interactions between fluorinated moieties and the a-polar protein residues occur.

Structure of the Gel. The gel becomes stiffer with aging, as inferred from the dependence of viscosity on time, Figure 3. The increase of viscosity can be fitted in a first-order kinetic equation. The dependence of gelling kinetics on composition is not large. Gelling kinetics is controlled by the volume fraction of the disperse phase and by the [LYS]/[LiPFN] mass ratios. This behavior indicates that the process depends on the number of links and/or cluster size. Such hypothesis is qualitatively supported by optical microscopy, showing that the size of gel fibrils increases with time. The interpretation of a string of pearl morphology bridged by fluorinated micelles in presumably not unequivocal. Recent work on protein−surfactant systems, in fact, showed that a ribbonlike supramolecular morphology is typical of 1−1 complexes.[47]

Gelling requires significant amounts of protein and surfactant, Figure 7, and is controlled by stoichimetric ratios between the components. Its formation presumably implies the formation of a network, where micelles join together different protein−surfactant complexes. The present behavior indicates that proteins and surfactants (or lipids) form interconnected networks. It finds analogy with systems containing slightly hydrophobically modified polymers and surfactants.[70] Surfactant in excess destroys the gel structure, in analogy with the system ethylhydroxyethylcellulose−sodium dodecyl sulfate−water.[71] In the present case, the protein−surfactant complex behaves as a hydrophobically modified entity.

Conclusions

The ternary system containing water, lithium perfluorononanoate, and lysozyme has been investigated and the region of existence of the different phases was determined. Solution, precipitate, and gel phases have been observed, together with poly-phasic regions. The width of the different phases is modulated by pH and by medium ionic strength. According to experimental evidence, the interactions between the surfactant and LYS are mostly electrostatic. This hypothesis is supported by volumetric and viscometric findings, as well as from the effect of addedd electrolytes on the width of the gel phase.

Volumetric properties indicate that hydrophobic hydration becomes progressively less relevant in the presence of LYS, added electrolyte, and low pH. The volumetric and hydrodynamic features indicate the presence of both hydrophobic and Coulombic contributions.

NMR findings indicate that significant interactions occur between the surfactant polar heads and the protein. They also put in evidence the role of micellar aggregates in the redissolution of the precipitate. In the same time, they rule out the occurrence of interactions between fluorocarbon surfactants and the apolar moieties of the protein. This effect may imply that LYS is nondenaturated. This hypothesis, however, needs to be demonstrated by proper experiments.

The gel phase occupies a significant portion of the phase diagram and disperses the $LYS(PFN)_X$ precipitates. The gel strength increases with time. Gelling is presumably due to micelle-mediated interconnections between polymer-surfactant complexes, in analogy with gels formed by hydrophobically modified polysaccharides and surfactants.[70,71] A reasonable explanation can be made by assuming the formation of interconnected networks, where micelles act as junctions between different protein−surfactant complexes.

Biological implications of gelling and micelle assisted redissolution of the protein surfactant complex are relevant in the recovery of proteins and in the formation of "ad hoc" protein-based gels.

Evidence from thermodynamic and transport methods, as well a from NMR, indicates that the equilibria between LYS and the surfactant are controlled by a delicate balance of different contributions. Thermodynamic properties, unfortunately, do not give information on the selective role of certain interaction modes, and more information on the gel structure and on the activity of the protein in gels are required. On this purpose,

NMR, structural,[38] biochemical, dielectric, and rheological investigations are in progress.

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Supporting Information Available: Further data on fluorine chemical shifts, Table V, fluorine line widths, Table VI, and data relative to the dependence of viscosity on ageing, Table VII, are reported as Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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