Liquid-Liquid Phase Separation of a Surfactant-Solubilized Membrane Protein

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Recent studies of protein solutions have provided new and promising insights into the structural properties and the phase behavior of soft matter [1]. Thus far, the general attention has been mainly focused on soluble proteins, which perform basic enzymatic or transport tasks. Conversely, studies of the solution properties of membrane proteins, carrying on all primary transmembrane exchange functions such as specific ion channels, receptors for extracellular signals, energy harvesters, and linkers in cell adhesion [2], are totally lacking. Our knowledge of membrane proteins is rather poor even at the structural level, since obtaining good-quality crystals is extremely difficult [3]. The foremost limiting factor is that membrane proteins, which display large exposed hydrophobic regions often associated with lipids, are essentially insoluble in water. Stable solutions can be obtained only by exploiting the solubilizing properties of specific detergents used for extracting proteins from the supporting cell membrane. Therefore, surfactant self-assembly phenomena unavoidably coexist with, and strongly influence, membrane-protein solution behavior.

The Reaction Center (RC) is a 100 kDa bacterial pigment-protein complex, which spans the intracytoplasmic membrane and accomplishes the primary events of energy transduction by promoting photoinduced charge separation across the membrane [4]. RC, which was the first membrane protein to be crystallized, can be extracted from bacterial membranes by using lauryldimethylamino-N-oxide (LDAO), a surfactant acting as a very efficient solubilizer. In this Letter, we show that ionization of LDAO, tuned by the solution pH, induces strong cooperative electrostatic effects between surfactant and protein leading, in a narrow pH range, to phase segregation of the mixture into mesoscopic “droplets,” with a typical size of the order of a few μm and a relatively narrow size distribution. After a moderate initial growth, droplets persist in a quasistationary state for a very long time, with little further ripening or coalescence. It is therefore tempting to describe this liquid-liquid phase separation as a kind of “spontaneous emulsification.” At variance with what is found for most aggregation phenomena in complex fluids, phase segregation of RC/LDAO complexes is fully hindered by the addition of salt.

RC was isolated and purified from the purple bacterium Rhodobacter sphaeroides R-26 according to Gray et al. [5]. LDAO and buffer chemicals were obtained from Fluka, and used without further purification. We first discuss the qualitative phase behavior of RC/LDAO solutions observed in moderate acid conditions, at room temperature, and in the absence of added salt. From the original stock solution (RC concentration $c_{RC} = 110 \mu M$ at $pH = 8.0$), samples were prepared at $c_{RC} = 4.4 \mu M$ in the presence of 0.87 mM LDAO (value below the surfactant critical micellar concentration cmc $\approx 2 \mu M$), and buffered in the range $5 < pH < 8$ by adding HCl to 10 mM imidazole solutions. Samples prepared at $pH > 7$ look optically transparent and scatter light very weakly. By increasing acidity, a progressive growth of turbidity and a concomitant increase of small-angle light scattering are observed, until, for $pH < 6$, amorphous precipitation of the solute takes place in a few tens of minutes. Aggregation is, however, almost fully reversible: Rapid dissolution of most of the aggregates is indeed observed by titrating the solution back to $pH = 8$. For $pH \approx 5$, the precipitation kinetics becomes slower (taking place over some hours), but, when the solution is brought back to higher $pH$, partial aggregation can still be detected. In what follows, we shall mainly deal with the solution...
behavior for $6 < p\text{H} < 7.5$, which shows a very peculiar feature: In this region, samples persist in a strongly turbid state for days. The visual appearance of samples at $p\text{H} = 6.5$ is shown in the lower inset of Fig. 1 for different times after dilution of the stock solution. Strong turbidity is evident just after mixing, and is still present in the whole sample, with a barely perceptible increase, after 12 h. After 4 d, the position of the meniscus separating the turbid phase from the supernatant yields a sedimentation velocity $v_s = 1 \text{ mm/day}$. Severe problems with the preparation of homogeneous RC/LDAO solutions around $p\text{H} = 6.5$ have been previously reported [6], but no scrutiny of the effect has thus far been performed. RC absorbance, which is strong in the blue-violet and near-infrared regions, is about 0.03 cm$^{-1}$ at $\lambda = 633 \text{ nm}$, allowing for dynamic light scattering (DLS) measurements using a He-Ne laser. By progressively lowering the $p\text{H}$ of dilute RC/LDAO solutions from 8 to 6.5, the scattered intensity not only grows by orders of magnitude, but also becomes strongly forward peaked. DLS measurements concurrently show a dramatic slowing down of translational diffusion. While time-correlation functions $g_1(t)$ at $p\text{H} = 8$ (Fig. 1, main body) give a hydrodynamic radius $R_H = 5 \text{ nm}$ for the RC/LDAO complex, DLS measurements on dilute RC/LDAO solutions ($c_{\text{RC}} = 0.4 \mu\text{M}$) at $p\text{H} = 6.5$ suggest the presence of much larger objects.

Short-time cumulant fits to $g_1(t)$ yield indeed an average particle diameter $d = 550 \text{ nm}$ just after mixing, growing to $d = 1.5 \mu\text{m}$ after 1 h. Direct visualization, made by sealing samples into rectangular capillaries and using phase-contrast microscopy, show that the observed scattering is due to a relatively large number of spherical droplets with a typical diameter around 1–2 $\mu\text{m}$ (Fig. 1, upper inset). By counting the number of droplets within the depth of field of a 60 times, 0.7 numerical aperture objective, we estimate that samples at $p\text{H} = 6.5$ with $1 \mu\text{M}$ RC, $0.87 \text{ mM}$ LDAO, contain approximately a volume fraction $\Phi = 2 \times 10^{-3}$ of dispersed droplets. When filtered through a 0.2 $\mu\text{m}$ low protein-binding membrane, samples were observed to clarify considerably, meaning that most of the droplets are blocked by the filter. Indeed, DLS measurements of filtered samples essentially coincide with those obtained at $p\text{H} = 8$. By comparing absorption spectra of filtered samples at $p\text{H} = 6.5$ and $p\text{H} = 8$, it is possible to conclude that at least 95% of the total protein amount is confined within the droplets. Considering our estimate of $\Phi$, RC concentration within the droplets may therefore reach up to 2 mM.

We could not measure LDAO concentration within the separated phase but, since in this $p\text{H}$ range LDAO associates with RC in a 300/400 molar ratio [6], it should range between (15–20)% by weight.

The size range of the observed structures suggests the formation of droplets to be the initial stage of a macroscopic liquid-liquid phase separation, rather than, for instance, a supramolecular aggregation process leading to stable micellarlike objects. Growth of a new phase proceeds via Ostwald ripening and, possibly, droplet coalescence.

We have investigated by DLS the kinetics of the early-stage droplet growth for dilute RC/LDAO samples ($c_{\text{RC}} = 0.5 \mu\text{M}$), prepared at $T = 4 ^\circ\text{C}$ in order to limit droplet growth during the mixing process (see discussion of temperature effects below), and rapidly transferred to a scattering cell with $\ell = 1.5 \text{ mm}$ optical path, kept at $T = 22 ^\circ\text{C}$. Correlation functions were taken at a scattering angle $\theta = 90 ^\circ$, accumulating data only for 30–60 s, and simultaneously measuring the transmitted intensity $I_T$.

Figure 2 shows that both the droplet size and the scattering extinction coefficient $\alpha = -1/\ell \ln(I_T/I_{T0})$, where $I_{T0}$ is the intensity transmitted by the buffer, undergo a fast initial growth, followed by a relatively steady stage. The earliest growth is roughly consistent with a classical Lifshitz-Slyozov $t^{1/3}$ time dependence of the droplet-size (full line), but some kind of saturation effect seems to be present for $t \approx 1 \text{ h}$. On much longer time scales ($5–10 \text{ h}$), the apparent droplet size actually lessens, probably due to faster sedimentation of the larger droplets. The correlation functions, however, are perturbed by the presence of a small number of larger aggregates, making it difficult to extract reliable droplet-size values. Caution should also be taken with multiple scattering.

**FIG. 1.** Main body: DLS field correlation functions at $T = 22 ^\circ\text{C}$ for RC/LDAO solutions at $p\text{H} = 8$ (squares), and at $p\text{H} = 6.5$ measured 150 s (open dots) and 1 h (full dots) after mixing. Lower inset (from left to right): Pictures of RC/LDAO samples at $p\text{H} = 6.5$ taken 200 s, 12 h, and 4 days after mixing, compared to a sample at $p\text{H} = 8$ (last image), remaining fully transparent for indefinite time. Upper inset: Phase-contrast image of phase-separated droplets at $p\text{H} = 6.5$. Field of view is $80 \times 80 \mu\text{m}^2$. 
effects that, although mostly confined to smaller $\theta$, may yield errors on the estimate of $d$ at late stage, when $I_T/I_{T_0} \approx 0.6$.

In addition to the evaporation/condensation of independent droplets, coalescence may speed up the segregation of a new phase. In phase-separating mixtures, gravity plays an important role in thinning the interstitial film between two droplets, until they eventually merge due to short-range intermolecular forces. In the present case, however, the observed very low sedimentation rate implies tight density matching, and therefore quite slow gravity-driven coalescence. The density difference $\Delta \rho$ between the two phases, estimated from the measured sedimentation velocity and droplet-size interval, ranges between (0.01–0.05) g/cm$^3$. An independent evaluation, made by using the previous estimates for RC and LDAO concentration in a droplet, and the RC and LDAO material densities $\rho_{RC} = 1.35$ g/cm$^3$, $\rho_{LDAO} = 0.9$ g/cm$^3$, yields $\Delta \rho = 0.03$ g/cm$^3$. Taking advantage of the observation that droplet growth becomes very slow at late stage, we have performed a preliminary study of the segregation effect as a function of RC and LDAO concentration. The inset of Fig. 2 shows that $d$, measured 1 h after mixing, increases rather strongly with $c_{RC}$. Conversely, a less than 30% increase of the droplet size was observed by increasing $c_{LDAO}$ from 0.8 to 15 mM.

To check whether the observed behavior depends on temperature, we prepared RC/LDAO solutions at $T = 10^\circ C$, and then we progressively raised the temperature, before each measurement, until the turbidity had reached a quasistationary value. Figure 3 shows that $\alpha$, measured at pH values close to the onset of the “anomalous” region, strongly increases with $T$. For instance, RC solutions at pH = 7.25, looking fully transparent at room temperature, become considerably turbid for $T > 30^\circ C$. Liquid-liquid phase separation in RC/LDAO solutions takes place therefore by increasing temperature, at variance with demixing of most simple critical mixtures, but, similarly, for instance, to what happens (at much higher protein concentration) for sickle-cell hemoglobin (Hb) [7], where liquid-liquid separation precedes much more extensive protein association, in the form of Hb polymerization. In the present case, the inverted temperature dependence is probably due to temperature effects on the hydrogen-ion activity of the buffer, which modify the pH and therefore surfactant ionization [for imidazole-HCl buffers $d(pK_\alpha)/dT = -0.017^\circ C^{-1}$]. Inset 3(A) shows indeed that the $\alpha(T)$ for samples with different pH at $T = 25^\circ C$ collapses on a single curve when plotted versus the actual pH(T), directly measured with temperature-compensated electrodes. A final and very peculiar aspect of RC/LDAO liquid-liquid phase separation is that it is fully hindered by increasing the solution ionic strength $I$ adding KCl. Inset 3(B) shows indeed that the strong turbidity enhancement at pH = 6.5 is limited to moderately low electrolyte concentration, and totally vanishes by increasing $I$ up to 1 M.
The observed phase behavior for RC/LDAO solutions is strongly correlated with the ionization properties of the surfactant. LDAO is a pH-sensitive amphiphile, which is nonionic at neutral and basic pH, and becomes increasingly protonated (cationic) in acid conditions (reported \( pK_a = 5.0 \) [8]). No phase-segregation effect is observed for pure LDAO micellar solutions: LDAO protonation causes only moderate micellar growth, taking place in close proximity of the surfactant \( pK_a \), and in the presence of a sufficient amount of added salt [9]. However, addition of RC, which in this pH range is negatively charged, may be expected to lead to mutual neutralization of the protein and surfactant charges. A distinctive signature of charge complexation effects, commonly observed when polyacids are added to solutions of protonable surfactants or polybases [10, 11], is the shift to higher pH of the solution \( pK_a \), which for RC/LDAO solutions has indeed been reported to be \( pK_a = 6.2 \) [12]. Charge neutralization should weaken the electrostatic stabilization of the RC/LDAO complex, decreasing therefore its solubility. In addition, LDAO coordination around the protein hydrophilic caps should increase the complex effective hydrophobicity, due to exposure to water of the surfactant tails. A quite similar charge-neutralization mechanism has been invoked by Mel’nikova and Lindman to explain DNA coil-to-globule transition in the presence of LDAO [11], which is promoted by surfactant ionization. hindering of phase separation by the addition of salt (also observed for the DNA condensation effect reported in Ref. [11]), which should screen direct protein/surfactant interactions, is also consistent with LDAO complexation with charged amino acids within the RC hydrophilic caps.

An open question is why droplet growth becomes so slow at late stage. The different dependence of the droplet-size on RC and LDAO concentration suggests that protein and surfactant play a nonsymmetric role in the phase separation process. Therefore, rather than thinking of phase segregation as a condensation of structurally stable RC/LDAO complexes, it is probably more appropriate to regard the RC/LDAO/water system as a ternary mixture, where one of the components, the RC, is almost insoluble in water. Addition of weakly soluble species is often used to produce stable, monodisperse “mini-emulsions” [13]. As discussed in detail by Webster and Cates [14], Ostwald ripening may indeed be hindered if the droplets contain a “trapped” species, due to competition between Laplace pressure, favoring large-droplets growth, and osmotic pressure of the trapped component, preventing collapse of the small ones. Full equilibrium is predicted only if the trapped species is rigorously insoluble, but extremely slow late-stage kinetics is nonetheless expected in the presence of very weakly soluble components. The observed growth of the quasistationary droplet radius with RC concentration is consistent with the prediction for the equilibrium droplet size to be an increasing function of the trapped-species concentration. Full assessment of the soundness of this mechanism will require, however, quantitative comparison of the droplet growth kinetics with the predictions made in Ref. [14]. Hindering of Ostwald ripening still leaves coalescence as a phase-segregation mechanism. Besides density matching, the presence of a residual charge on the droplet surface may in principle prevent droplet merging [15]. Yet, we did not detect any appreciable droplet drift in samples inserted in microelectrophoretic cells, and subjected to electric fields with amplitude up to 40 V/cm. Therefore, we regard slow coalescence mainly as a kinetic effect.

As a final remark, we point out that we also observed noticeable effects of the phase-segregation process on RC photochemistry, concerning both the absorption spectrum and electron-transfer kinetics. Although a detailed discussion of these effects has to be deferred to a future publication, these findings demonstrate that RC photocycle depends on its aggregation state. The activity parameters of RC, when it is embedded in the intracytoplasmic membrane and surrounded by the concentrated macromolecular environment of the cell, may therefore appreciably differ from what is usually observed in dilute surfactant-stabilized RC solutions.

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