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Drastic change of reverse micellar structure by protein or enzyme addition

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Abstract. On addition of cytochrome *c* to a AOT reverse micellar solution, the percolation process usually observed at high temperatures and surfactant concentrations, occurs at room temperature. This is observed either at relatively high water content at a given cytochrome *c* concentration or at low content on increasing the cytochrome c concentration. On increasing the water content a phase transition is observed with two optically transparent phases. A similar phase transition is observed on solubilizing various enzymes. The temperature of the transition appears to be strongly dependent on the location of the macromolecule in the reserve micelle.

Key words: Reverse micelle – Percolation – Biotechnology

Introduction

AOT dissolved in organic solvents forms spheroidal aggregates called reverse micelles or droplets (Pileni 1989). Water is readily solubilized in the polar core, forming a so called "water pool", characterized by w, where $w=[H_2O]/[AOT]$. As the size increases, the concentration of discrete micelles decreases while the water content, w, increases. Reverse micelles have the ability to serve as hosts for macromolecules (Pileni 1989), particularly enzymes.

In this paper we show that the solubilization of a small amount of cytochrome *c* in reverse micelles induces strong attractive interactions between reverse micelles. This favours a percolation process at a lower temperature value and polar volume fraction than is obtained with unfilled micelles. The attractive interactions are strong enough to promote a two phase transition with an upper phase containing mainly isooctane while the lower phase contains all of the AOT, water and cytochrome.

This phenomen is extended to other enzymes such as chymotrypsin and ribonuclease.

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Experimental section

The preparation of protein containing microemulsions has been largely described in a previous paper (Brochette et al. 1988).

The two differents procedures used induce different changes:

i) An increase of the water content, w, keeping the surfactant concentration constant induces an increase in the reverse micelle size and a decrease in the reverse micelle concentration.

ii) An increase of the polar volume fraction, keeping w constant induces an increase of the same size in the reverse micelle molar concentration ([RM]). The latter was calculated from the value of the water pool radius determined by different techniques ($r_w=1.5$ w). In this case, the experiments were carried out at concentrations of 1, 2 or 4 cytochrome *c* per micelle. Thus, [cyt]=1, 2 or 4 [RM].

The phase transition described in the paper was determined visually

a. Materials

The macromolecules (cytochrome *c* from horse heart pancreatic ribonuclease, chymotrypsin), AOT (sodium di ethyl hexyl sulfosuccinate) and isooctane were purchased, respectively, from Fluka, Sigma and Merck and were used without further purification.

b. Methods

1. Absorption spectroscopy. The concentration of ferri cytochrome *c* was determined from its absorption spectrum measured using a Perkin Elmer lamda 5 spectrophotometer. The absorption spectra of cytochrome *c* under the conditions used in this paper are similar to those obtained in reverse micellar solutions and in aqueous solutions. The value of the extinction coefficient

is given a in the Biochemist's Handbook (1971). The absorption spectra were measured at 22 °C

2. Conductivity. The conductivity measurements were made using a Tacussel model CD 810. The cell constant, K, characterized by the ratio of the surface to the distance between electrodes was equal to 0.9 cm.

3. Dielectric measurements. These were carried out with a Hewlett Packard HP 4191 impedance analyser in the 20–150 Mhz frequency range, using a thermostatted reflectometry cell. Data acquisition was with a SIRIUS S1 computer and the data files were transferred and processed on a SUN station. To verify our experimental conditions we first performed the experiments published by Van Dijk (1985). The data obtained were similar to those previously published.

4. Small angle X ray scattering measurements. Two instruments were used. The first one was located in Saclay and the second at L.U.R.E., (Orsay University). With the latter, it was possible to reach about 6.10^{-3} Å^{-1} for the lowest values of the diffusion vector, q.
The apparatus and the methods used to determine the shape of the aggregate were largely as described previously (Pileni et al. 1985; Brochette et al. 1988).

The radii of the reverse micelles were calculated from the slope of a Guinier plot ($Ln\ I(q)$ versus q^2) and from the minimum of the Porod plot $I(q)\,q^4$ versus q. The similarity in the data obtained with both methods indicates that micelles are spherical.

5. Quasi elastic light scattering (Glatter and Hofer 1988). A sixty channel Malvern MD type correlator was used, driven by a CBM 3032 Commodore computer. In order to prevent the excitation of the protein around 410 nm, a 647.1 nm Helium-neon laser beam was used.

Experimental results

In the absence of protein, the limit of the water solubility in reverse micelles is generally obtained for $w=60$. By increasing w up to this value, a turbid solution is obtained, corresponding to a mixture of L_1 and L_2 phases (Pileni 1989). At low cytochrome c concentrations (below 10^{-4} M at $w=40$), the limit of the solubility of water in reverse micelles remains unchanged and equal to $w=60$.

By increasing the cytochrome c concentration the solubility limit increases to reach $w=80$. At such a water content, the system does not change to a lamellar phase, as is observed in the absence of protein or a low protein concentrations: a phase transition appears.

a. Conductivity variation with water content

At 20 °C in the absence of cytochrome c, the conductivity of AOT reverse micelles is very low. On increasing the water content, the change in the conductivity is very small and remains in the nS range. In the presence of cytochrome c a very low conductivity at low water content is observed. On increasing the water content, w, a drastic increase in the conductivity, about three orders of magnitude, is observed.

This correlates with the appearance of a phase transition, characterized by two optically transparent phases. This process depends on the cytochrome c and the water concentration: it occurs either at low water concentrations and high cytochrome c concentrations or at high water contents and low cytochrome c concentrations.

b. Dielectric permittivity

The static dielectric constant, ε_s or $\varepsilon\ (\omega=0)$, was measured at various volume fractions, ϕ_w at ($w=40$, $T=20$ °C). Fig. 1 shows the increase in the static dielectric constant with volume fraction, at various cytochrome c concentrations per micelle. It can be seen that there is a greater increase in the static dielectric constant, especially at low volume fractions, when the cytochrome c concentration is high. This increase in static dielectric constant is followed by an increase in conductivity. Similar behaviour (Van Dijk et al. 1984; Van Dijk 1985; Van Dijk et al. 1986; Van Dijk et al. 1986; Bhattacharya et al. 1985; Safran et al. 1985) to that observed in Fig. 1 was attributed to percolation onset. However, the critical volume fraction corresponding to percolation onset is found to be drastically changed by adding cytochrome c. The critical volume fraction is found to be equal to 0.18 (in the presence of cytochrome c, [AOT] = 0.1 M, [cyt] = 4. [RM], $w=40$) and up to 0.22 in unfilled micelles ([AOT] = 0.1 M, $w=40$). Similar results have been obtained by increasing the temperature. Under our experimental conditions ([AOT] = 0.1 M, $w=40$), the critical temperature value is lower (35 °C) in the presence ([cyt] = 4 [RM]) than in the absence of cytochrome (50 °C). This again indicates that cytochrome c favours the percolation process. The threshold decreases as the protein concentration increases.

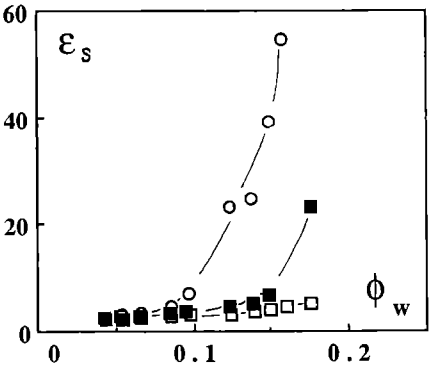

Fig. 1. Variation of the permittivity, $\varepsilon\ (\omega=0)$ or ε_s with the water content of AOT-isooctane-water solutions in the absence and in the presence of various cytochrome c concentrations. The concentrations of cytochrome c are: [RM] (□), 2 [RM] (■), 4 [RM] (○). (see experimental section)

c. Small angle X-ray scattering experiments

In the absence or in the presence of cytochrome *c* ([cyt]=4 [RM]), the water pool radius is independent of the volume fraction ($0<\phi_w \leq 0.20$) and is found to be close to 60Å and 48Å, respectively. The decrease in the reverse micelle size on cytochrome addition has been previously observed in dilute solution ([AOT]=0.1 M) and was interpreted in terms of the partition of cytochrome *c* at the interface inducing an increase in the total available surface (Pileni et al. 1985; Brochette et al. 1988).

d. Quasi elastic light scattering

The diffusion coefficient of the AOT reverse micelle was determined by quasi elastic light scattering, in the absence and in the presence of cytochrome *c*, at $w=40$, (Fig. 2). In the absence of cytochrome *c*, reverse micelles interact via van der Waals attractive forces and short range repulsive forces. Van der Waals attraction is frequently negligible and the overall interaction is of the hard sphere type (Pileni 1989). The hard sphere radius is found equal to 100Å, at $w=40$.

In the presence of cytochrome *c* an additional attractive force is observed. The diffusion coefficient decreases markedly with the polar volume fraction. The value of the diffusion coefficient extrapolated to zero volume fraction is greater than that obtained in the absence of protein (Fig. 2). For spherical monodispersed aggregates, the diffusion coefficient D_c is related to the water volume fraction ϕ_w, of the reverse micelle (Cazabat et al. 1982) by: $D_c=D_o(1+\alpha\,\phi_w)$, where α is the virial coefficient, which is directly related to the interaction potential, and D_o is the diffusion coefficient at infinite dilution obtained by extrapolation. From small angle X ray scattering, the water pool radius deduced from the various representations such as Porod and Guinier plots, and from the invariant could indicate a relative low polydispersity. Using empty micelles it has been evaluate as 15% in polydispersity. No changes were observed with filled micelles. From the initial slope of the curve in Fig. 2 the virial coefficient is found to be equal to about -40. The negative value indicates attractive interactions between reverse micelles. This value is large enough to lead to critical behavior and phase separation (Cazabat et al. 1982).

e. Phase transition

As noted above on increasing the water content in the presence of cytochrome, instead of reaching a turbid solution, a phase transition appears and is characterized by two optically transparent phases with an upper containing mainly isooctane and a lower phase containing all the compounds forming the reverse micelles.

A phase diagram gives the structural changes of 0.1 M AOT in isooctane solution at $w=80$ as a function of temperature and cytochrome *c* concentration. Figure 3 shows four different regions:

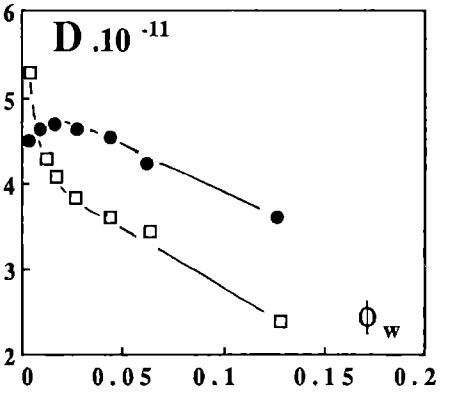

Fig. 2. Variation of the diffusion coefficient, D ($m^2\ s^{-1}$), with the polar volume fraction, ϕw, of AOT-isooctane-water solutions in the absence (●) and in the presence of cytochrome (□), [cyt]=[RM]

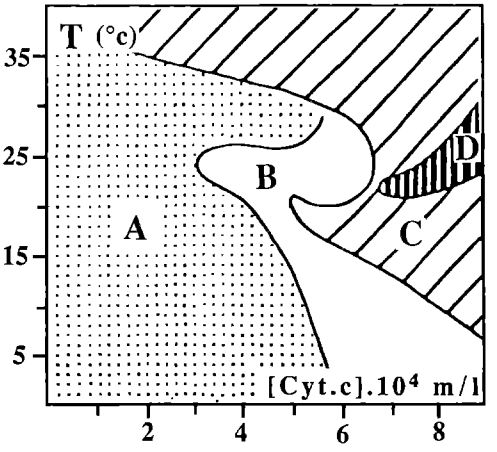

Fig. 3. Phase diagram for the AOT-isooctane-water system as a function of temperature and cytochrome *c* concentration. [AOT]=0.1 M; $w=80$

i) Region A is turbid (in the whole temperature range) as is usually observed in the 0.1 M AOT-isooctane-water system on solubilizing too many water molecules. It is usually attributed to a lamellar structure (Pileni 1989). No specific studies have been performed on this region.

ii) Region B is an optically transparent solution similar to that obtained at low water content, ($w<60$).

iii) In region C two optically transparent phases appear. Qualitatively it can be seen that most the cytochrome *c* is located in the lower phase whereas the upper phase contains little or no cytochrome *c*. At a given cytochrome *c* concentration, the two isotropic phases do not change on increasing the temperature.

iv) In region D three optically transparent phases are observed.

Such a phase transition is observed using two other low molecular weight and water soluble enzymes such as ribonuclease and chymotrypsin. However, the temperature of the phase transition changes with the enzyme. The temperature transition determined at $w=80$ and at a given concentration ([enzyme]=4.10^{-4} M) is equal to 20 °C for cytochrome *c*, to 31 °C for ribonuclease at pH 4 and 36 °C for ribonuclease at pH10, for chymotrypsin at neutral and alkaline pH and for unfilled micelles. These un-

Table 1. Variation of the upper and lower phase composition with total cytochrome *c* concentration solubilized in AOT-water-isooctane solution ([AOT] = 0.1 M; $w = 80$, $T = 20\,°C$)[a]

$[CYT] \times 10^{-4}$ (M)	5	6	7	9	10	11
Upper phase						
$[CYT] \times 10^{+4}$ (M)	2	1	0	0	0	0
$[AOT] \times 10^{+2}$ (M)	2	1	0	0	0	0
$[H_2O] \times 10^{+3}$ (M)	5000	2000	2	2	2	2
Lower phase						
$[CYT] \times 10^{+3}$ (M)	1	1.1	1.5	1.9	2.2	2.7
$[AOT] \times 10^{+2}$ (M)	10	17	18	19	22	25
$[H_2O]$ (M)	8	10	11	13	19	17

[a] The relative volume of the two phases changes on adding cytochrome *c*

Table 2. Temperature of the phase transition for the AOT-water-isooctane system in the presence of cytochrome *c*

$[cyt] \times 10^{+4}$ (M)	0	4	0	4	6	7	8
w	40	40	80	80	80	80	80
ϕ_w (%)	8.7	8.7	12.5	12.5	12.5	12.5	12.5
T (°C)	60	40	37	30	20	10	5

changed values in the temperature of the transition obtained in the latter cases are interpreted in terms of the location of the macromolecules in the reverse micelles: cytochrome *c* and ribonuclease at pH4 are located at the interface and ribonuclease at pH10 or chymotrypsin are located in the internal water pool (Pileni et al. 1985)

These phase transition are thermodynamically stable: by adding AOT to this new system the upper phase progressively decreases in volume while the lower increases to form a single phase solution. The process described above is reversible.

The composition of the two phases strongly depends on the experimental conditions. It was determined for the three macromolecules at a fixed w value ($w = 80$);

i) Using cytochrome *c* ($T = 20\,°C$), the composition of the two phases depends on the protein concentration initially solubilized in the AOT-isooctane-water solution (Table 1). It can be noticed that at a cytochrome concentration up to 6×10^{-4} M, the main part of the water and all of the AOT and cytochrome *c* are in the lower phase. The residual water concentration obtained in the upper phase is equal to that of water saturation in isooctane solution.

ii) Using ribonuclease and chymotrypsin, in contrast to what it is observed with cytochrome *c*, the concentrations of the various components do not change on increasing the initial amount of enzyme solubilized in the system. At 31 °C for ribonuclease at pH4 (located at the internal interface), and 36 °C with ribonuclease at pH 10 or chymotrypsin or empty micelles the AOT and water concentrations are equal to 2×10^{-2} M and 0.3 M in the upper phase and to 0.16 M and 13 M in the lower phase respectively. The enzyme distribution is close to 90% in the lower phase and 10% in the upper phase. These concentrations were determined either by NMR or by absorption. The experimental error is close to 10%.

Discussion

In contrast to what has been published before, the data presented above indicates that, in some cases, the solubilization of cytochrome *c* in reverse micelles induces strong perturbations of the micellar structure.

At up to $w = 20$, strong perturbations are observed: The conductivity of a reverse micellar solution in which cytochrome *c* has been solubilized increases either by increasing the cytochrome *c* concentration or by adding water molecules. The increase in the dielectric permittivity and in the conductivity observed in the presence and in the absence of protein at various volume fractions are, according to the data previously published (Van Dijk et al. 1984; Van Dijk 1985; Van Dijk et al. 1986; Van Dijk et al. 1986; Bhattacharya et al. 1985; Safran et al. 1985), associated with a percolation transition. The percolation transition occurs at a lower volume fraction in the presence of cytochrome *c* than in its absence. According to these data, and to those obtained from conductivity measurements, it seems reasonable to conclude that cytochrome *c* changes the percolation onset and favours the percolation transition which usually occurs at higher temperatures and AOT concentrations.

By SAXS, the good agreement between the water pool radii obtained from different Treatments (Guinier and Porod plots) indicates that the micelles remain reverse micelles at various volume fractions. The decrease in the reverse micelle size on cytochrome addition has been previously observed in dilute solution ([AOT] = 0.1 M) and was interpreted in terms of the partition of cytochrome *c* at the interface inducing an increase in the available total surface (Pileni et al. 1985; Brochette et al. 1988). Thus, in the presence of cytochrome *c*, [cyt] = 4 [RM], the reverse micelles are still formed although the conductivity is high. From permittivity data it seems reasonable to conclude that there is a percolation process and this is confirmed by the appearance of a phase transition either on increasing the water content or the cytochrome concentration.

The phenomena presented in this paper may be interpreted as follows: Cytochrome *c*, solubilized at the interface of the reverse micelles induces an increase in the attractive interactions between reverse micelles. These attractive interactions increase by increasing the cytochrome *c* concentration and by increasing the fluidity of the interface (the curvature radius of the interface is larger on increasing the size of the reverse micelle). This induces reverse micelle adhesion and the phase transition. The process observed at $w = 40$ in the presence of cytochrome appears to be a real percolation phenomenon: there is no destruction of the micellar interface when reverse micelles form clusters due to significant attractive interactions caused by the presence of cytochrome *c* at the interface of the reverse micelles.

Such a process can be extended to other macromolecules. However, it can be noted that the temperature transition is strongly dependent on the pH and on the macromolecule used. The interactions between reverse micelles are strongly dependent on the location of the solute: cytochrome *c* and ribonuclease at pH4, because of

their location at the interface, favour adhesion between reverse micelles and the phase transition temperature is relatively low. With enzyme and protein located in the water pool, such as ribonuclease at pH10 and chymotrypsin, no changes in the reverse micelle interactions take place, as the same phase transition temperature is observed with empty reverse micelles. The difference in the temperature transition, at a given macromolecule concentration (4×10^{-4} M), between cytochrome *c* (20 °C) and ribonucleae (31 °C) could be attributed to the difference in their location; cytochrome *c* acts as a surfactant whereas ribonuclease at pH4 is located at the internal interface and the attractive interactions induced by its solubilization are smaller than obtained with cytochrome *c*, (the virial coefficient is found to be equal to −18 (result obtained by F. Michel in our group) compared to −40 using cytochrome *c*).

Similar phenomenon were obtained previously by solubilizing either alcohols or monomers in reverse micelles. However, in these cases, the concentration added to induce such a process is three orders of magnitude greater than that required for cytochrome *c*, ribonuclease and chymotrypsin.

Conclusion

AOT reverse micelles are known to induce a percolation process at high surfactant concentrations and/or high water contents, and high temperature. In the present paper we have shown that the presence of a low concentration of protein in the reverse micelles favours the percolation process which takes place at very low volume fractions by increasing either the cytochrome *c* concentration or the water content. This process is thermodynamically stable. The solubilization of enzyme or protein located at the interface induces a phase transition at a lower water content and a lower polar volume fraction than for those located inside the water pool. In the latter case, percolation process occurs at the same polar volume fraction and same water content as for unfilled micelles.

This process could be very important for carrying out, in reverse micelles, a chemical reaction catalyzed by enzymes. Assuming that the reactants (A, B) and the product (C) are mainly solubilized in the bulk hydrocarbon phase and that the enzyme is located into the micellar core, the chemical reaction could occur in the water pool. At the end of the reaction, the product C is in the bulk phase. Addition of water to the micellar solution induces percolation and the appearance of a biphasic system. The upper phase would contain product C and all the other components (water, AOT, enzyme) would be in the lower phase. The upper phase could then be removed and be replaced by pure isooctane. Addition of AOT and the reactants (A and B) to this system would favour the reformation of reverse micelles containing the enzyme and thus give the enzyme the ability to play its catalytic role again.

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