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Dielectric measurements on cytochrome c containing AOT water droplets: drastic changes in the percolation threshold

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Abstract. In the present paper changes in the percolation threshold of AOT–isooctane–water droplets by solubilizing a protein such as cytochrome c are reported. It is demonstrated that the critical percolation factors (volume fraction, temperature, water content) change by cytochrome c addition. This is attributed to the increase in the attractive interactions between droplets by solubilizing cytochrome c in water droplets.

Surfactants, S, dissolved in organic solvents, form spheroidal aggregates called reverse micelles [1]. Reverse micelles can be formed both in the presence and in the absence of solubilized water. However, if the medium is completely free of water the aggregates formed are very small and polydisperse. The presence of water is necessary to form large aggregates called microemulsions or water droplets. Water is readily solubilized in the polar core, forming a so-called 'water pool', characterized by W, the ratio of water concentration over surfactant concentration ($W = [H_2O]/[S]$). More often the surfactant used to form reverse microemulsions is sodium sulfosuccinate usually called Aerosol OT or AOT. For AOT in isooctane, the maximum amount of bound water in the micelle corresponds to a water–surfactant molar ratio $W = [H_2O]/[\mathrm{AOT}]$ of about ten. Above $W = 15$, the water pool radius, r_w, is found to be linearly dependent on the water content. This can be explained by a geometrical model [2] assuming that the water droplets are spheres: the volume of the sphere is attributed to the volume of water molecules, V, and the surface of the sphere to the total surface area of the surfactant, S. This is supported by the fact that the area per surfactant molecule is constant and that all surfactant molecules participate in the interface. From this model, in the case of AOT–isooctane–water, the relationship found is: $r_w = 3V/S = 1.5\ W$. Thus, as the size of the droplet increases, the concentration of discrete micelles decreases while the water content, W, increases. The reverse microemulsion concentration, [RM], is the ratio of the AOT concentration to the aggregation number and, at a given water content W, is directly related to the polar volume fraction, φ_w, by the following:

$$[\mathrm{RM}] = 3 \times 10^3 \varphi_w / 4N\pi r_w^3$$

(N is the Avogadro number). The polar volume fraction is the ratio of the volume of water to the total volume.

The interactions between water droplets (AOT–water–isooctane), largely studied at relatively high AOT concentrations [3], favour the formation of aggregates of microemulsions: dimers, trimers, and so forth. If the microemulsion volume fraction is large enough, an aggregate of macroscopic dimensions appears. Several groups [4] have shown that a divergence of the static dielectric permittivity and a sharp increase in the conductivity are attributable to a percolation transition. The percolation threshold corresponds to the maximum permittivity and to the onset of the conductivity. The formation of clusters explains the permittivity behaviour by a capacity effect before contact between droplets occurs [3, 4]: van Dijk *et al* explained the appearance of this divergence as originating from a significant additive capacity effect due to the vicinity of the droplets. Using the clustering model developed previously by van Dijk *et al* (1986) [3] for AOT reverse micelles, they analysed the problem by considering the contribution of the 'dipole–dipole' interactions to the polarizability of one microemulsion droplet. In this way they found a good description of the measured temperature and concentration dependence in terms of the polarizability of a single droplet, an activation energy and a prefactor. Hence the percolation threshold is attributed to the formation of an infinite cluster of water droplets allowing the Na^+ ions to percolate through the system. Hence temperature, T_p, polar volume fraction, φ_p, water content, W_p and percolation thresholds can be determined. Such a percolation transition is observed by increasing factors such as water content, temperature or polar volume fraction and keeping the other factors constant. The volume fraction percolation threshold, φ_p, decreases by increasing temperatures: at $W = 27$ φ_p was found by Bhattacharya *et al* [4] to equal 13.8%, 18.3% and 27.5% at T_p equal to 52 °C, 47 °C and 35 °C, respectively. These two factors (T_p, φ_p) decrease by increasing the water content, W (figure 3 in Bhattacharya *et al* [4]).

Microemulsions are able to serve as hosts to macromolecules in particular enzymes [5]. The solubilization of enzymes in organic solvents could provide many opportunities for the development of new biocatalysis synthetic reactions in the organic phase. One of the limiting steps is the removal of the surfactant at the end of the chemical reaction. The appearance of a phase transition at relatively low temperatures, which follows the percolation process, could be one of the ways of extracting the product and regenerating the chemical reaction.

To demonstrate that the presence of some proteins induces a change in the percolation threshold in AOT–water–isooctane microaggregates, we choose to solubilize cytochrome c in these microemulsions. This protein is a water soluble hemoprotein with a small molecular weight (12 400). It is responsible for several electron transfer reactions across membranes. Previously we have developed a geometrical model tested by SAXS [6] and a kinetic model [7] to determine the average location of low molecular weight proteins or enzymes in reverse microemulsions. We demonstrated that cytochrome c is located at the interface and its interfacial contribution increases with the water content [6, 7].

In this paper we report on an analysis of low frequency permittivity measurements of an AOT reverse micelle in the presence of cytochrome c. It is shown that the solubilization of small amounts of cytochrome c (10^{-4}–10^{-3} M) in reverse micelles favours a percolation process at lower temperatures and polar volume fraction values than observed with a protein-free water droplet system.

AOT was obtained from Sigma, cytochrome c and isooctane from Fluka and they were used without further purification.

The experimental investigations were carried out either at various water contents,

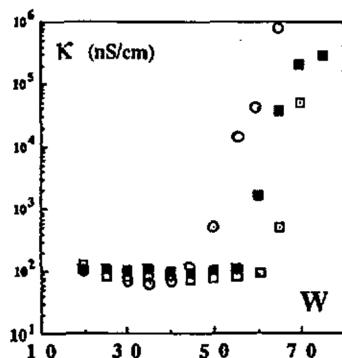

Figure 1. Variation with the water content of the conductivity of the AOT–isooctane–water solution in the absence and in the presence of varying and fixed cytochrome c concentrations. The cytochrome c concentrations are, at $W = 60$, respectively equal to: 3×10^{-4} M (□), 4.4×10^{-4} M (■) and 7×10^{-4} M (○).

W, or at a constant W value ($W = 40$) and a given cytochrome c number per micelle. The cytochrome c concentrations were equal to [RM], 2[RM] or 4[RM]. At $W = 40$, [AOT] = 0.1 M, [RM] is equal to 1.23×10^{-4} M. The samples used are optically isotropic in all the volume fractions and at the various temperatures.

The conductivity measurements have been made with a Tacussel CD 810 instrument. The dielectric measurements were carried out with a Hewlett Packard, HP 4191, impedance analyser in the 20–150 MHz frequency range, ω, with the use of a thermostated reflectometry cell. The measurements could not be made at lower frequencies because of the electrode polarization. Data acquisition was carried out by a Sirius S1 computer, and the data files were transferred and treated on a Sun station. The permittivity can be defined in its complex form by:

$$\varepsilon(\omega) = \varepsilon'(\omega) - \mathrm{i}(\varepsilon''(\omega) + \kappa/\omega\varepsilon_v).$$

$\varepsilon'(\omega)$, $\varepsilon''(\omega)$ and ε_v are the real, imaginary and vacuum forms of the permittivity, respectively. In order to determine the static value of epsilon, the treatment of the experimental results has been by means of Cole–Cole plots and the curves were fitted using a circle program (Rosenbrock). At, or just after, the onset of percolation, because of the conductivity increase, a deformation of the experimental Cole–Cole plots, called the Maxwell–Wagner effect, is observed.

The conductivity, $\sigma(\omega)$, is defined by its complex expression:

$$\sigma(\omega) = \sigma'(\omega) + \mathrm{i}\sigma''(\omega)$$

with $\sigma(\omega) = i\omega\varepsilon_v\varepsilon(\omega)$. The following can be derived:

$$\sigma'(\omega) = \omega\varepsilon_v(\varepsilon''(\omega) + \kappa/\varepsilon_v) \qquad \sigma''(\omega) = \omega\varepsilon_v\varepsilon'(\omega).$$

The real conductivity, $\sigma'(\omega)$, is plotted as a function of the imaginary term, $\sigma''(\omega)$. The same circle program has been used to fit and to obtain the low frequency limit of the conductivity subtracted from the imaginary part of the permittivity. In order to test the validity of our equipment we ran the same experiment as that published by Van Dijk *et al* in 1985 [4] and obtained the same data.

At room temperature, the conductivity of 0.1 M AOT in isooctane reverse micelles is very low. By increasing the water content, the changes in the conductivity are very weak and remain on the nanosiemens scale ($20 < \kappa < 100$ nS).

In the presence of cytochrome c, a very low conductivity is followed by a sudden rise in the conductivity as water is increased. Figure 1 and table 1 show that the sharp

Table 1. The water content threshold of 0.1 M AOT–isooctane–water at various cytochrome c concentrations determined from the increase in the conductivity at room temperature.

$[cyt]$ ($\times 10^4$ M)	0	0	4.9	7	3	4.4
W_p	40	40	40	40	60	54
φ_p (%)	>25	23	15	6.7	9.7	8.8
T (°C)	20	25	20	20	20	20

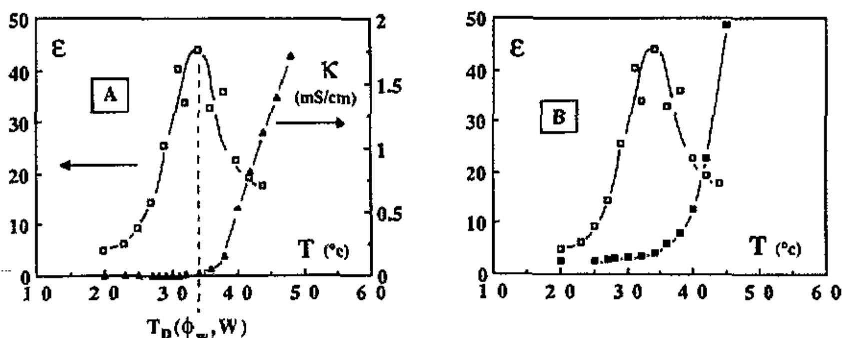

Figure 2. (A) Variation of the static permittivity (□) and conductivity (▲) with temperature; $W = 40$, $\varphi_w = 8.7\%$ and [cyt. c] = 4[RM]. (B) Variation with temperature of the static permittivity in the presence (□) and in the absence (■) of cytochrome; $W = 40$, $\varphi_w = 8.7\%$ and [cyt. c] = 4[RM].

increase in the conductivity occurs at lower water content by increasing cytochrome c concentration.

The static dielectric constant was measured at various temperatures for a given volume fraction ($\varphi_w = 8.7\%$) and a fixed water content ($W = 40$), in the absence and in the presence of cytochrome c ([cyt] = 4[RM]). The divergences in static dielectric permittivity with temperature are shown in figure 2.

In the presence of cytochrome c, the static dielectric constant reaches a maximum, which is associated with the conductivity onset (figure 2(A)). Such behaviour, characteristic of a percolation transition [4], indicates that similar percolation processes occur with filled and unfilled water droplets. In figure 2(B) the divergences in the permittivity in the presence and in the absence of cytochrome c are compared. It can be clearly seen that the increase in the permittivity occurs at lower temperatures with filled water droplets than with free protein water droplets. Figure 2 shows that, at a given polar volume fraction ($\varphi_w = 8.7\%$), the temperature threshold percolation is lower (T_p = 35 °C) in the presence of cytochrome than in its absence (T_p = 52 °C). This indicates that cytochrome c favours the percolation process, with a decrease in the percolation threshold by protein addition.

The measurement of the static dielectric constant at various polar volume fractions for a given temperature (T = 20 °C) and a fixed water content ($W = 40$) in the absence and in the presence of various cytochrome c concentrations is shown in figure 3. It is observed that the divergence in the static permittivity occurs at a lower volume fraction with filled water droplets than it does with unfilled ones. This takes place at a lower volume fraction as the protein concentration increases and confirms the data given above

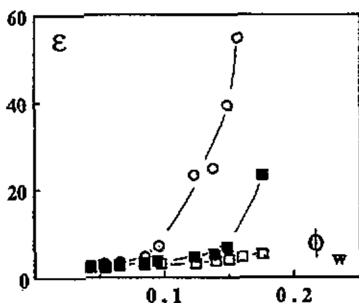

Figure 3. Variation of the static permittivity with polar volume fraction. $W = 40$ and $T = 20\,°C$, with cytochrome c concentrations: [cyt. c] = 0 (□); [cyt. c] = 2[RM] (■) and [cyt. c] = 4[RM] (○).

from which it has been deduced that the percolation threshold is lower in the presence of the protein. This decrease in the percolation onset is more important as the cytochrome c concentration increases.

By low-angle x-ray scattering measurements, the water pool radius determined at various volume fractions ($0 < \varphi_w \leqslant 0.20$) is unchanged and equal to 60 Å in the absence of cytochrome c and to 48 Å in its presence ([cyt] = 4[M]). This confirms the data previously published [2, 6] showing that the size of droplets decreases with increasing cytochrome c concentration. Such a decrease in the size of the droplet by cytochrome c addition was explained in terms of an increase in the total surface area due to the contribution of the cytochrome at the interface.

The diffusion coefficient of water droplets is determined by quasi-elastic light scattering, in the absence and in the presence of cytochrome c, at $W = 40$. In the absence of cytochrome c, water droplets interact via van der Waals attractive forces and short range repulsive forces. Van der Waals attraction is negligible at low polar volume fractions, and the overall interaction is of the hard sphere type [1]. In the presence of cytochrome, an additional attractive force is observed. At low polar volume fraction values, the diffusion coefficient D_c is related to the polar volume fraction, φ_w, [8] by: $D_c = D_0(1 + \alpha\varphi_w)$, where α is the virial coefficient and is directly related to the interaction potential, and D_0 is the diffusion coefficient at infinite dilution obtained by extrapolation. From the initial slope of the curve the virial coefficient is found to be equal to about -40 (for [cyt] = [RM]). The negative value indicates attractive interactions between droplets [8]. The origin of such attractive forces is still not well established: it has been attributed both to an enhancement of van der Waals interactions [9], and to solvent induced attraction [10]. In our case the latter can be excluded. It seems reasonable to conclude that the location of cytochrome at the interface induces an enhancement of van der Waals interactions. This can be related to the increase in the contribution of cytochrome at the interface [2, 6]. Another factor responsible for the increase in the attractive interaction is the strong dipole due to cytochrome c (about 300 D in aqueous solution) [11]; so part of the attractive potential could be a dipole–dipole interaction. These attractive interactions are large and promote phase separation well before the emulsification failure. D Chatenay *et al* [12] observed similar behaviour by solubilizing a non-water-soluble protein in microemulsion. However, in their system the attractive interactions were not strong enough to promote a phase transition. (From the hydrodynamic radii published [12], the virial coefficient calculated is close to -20.)

Above the percolation threshold, by increasing the water content or the protein concentration, a phase transition is reached. The two phases are optically transparent. The upper phase contains only isooctane, while the lower phase contains all the AOT,

Table 2. Temperature of the phase transition.

$[cyt]$ ($\times 10^4$ 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

water and cytochrome. The temperature of the phase transition, T_t, is dependent on cytochrome c concentration. Table 2 shows that at very high water contents, the phase transition is reached at room temperature.

Instead of cytochrome c, the solubilization of a water-soluble enzyme such as chymotrypsin located inside the water pool (no interaction with the interfacial wall [7]) does not induce any changes in the percolation thresholds and in the temperature of the phase transition ($\varphi_w = 10\%$, $W = 80$, $T = 37$ °C). In these two latter cases, the upper phase contains a small amount of the various compounds (AOT–water–isooctane and ±chymotrypsin), while the lower phase contains the major part.

In conclusion, because of its location at the interface, the addition of cytochrome c to water droplets induces an increase in the van der Waal attractive interactions between the microemulsion aggregates that promote percolation process. This usually takes place, using unfilled micelles, at larger polar volume fractions and higher temperatures. The percolation volume fraction under our experimental conditions ($T = 20$ °C, $W = 40$) is close to 10% and 20% in the presence (4[RM]) and in the absence of cytochrome c, respectively. The percolation temperatures at $W = 40$, $\varphi_w = 8.7\%$ are equal to 35 °C and 50 °C with filled and unfilled water droplets, respectively. Such changes in the percolation threshold are all the more important as the cytochrome c concentration is high and they are due to strong attractive interactions which promote a phase transition with two optically clear solutions. Such changes in the percolation process only seem to be observed with macromolecules located at the interface of the water droplet.

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