Micelle Formation and Solubilization of Fluorescent Probes in Poly(oxyethylene-*b*-oxypropylene-*b*-oxyethylene) Solutions

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ABSTRACT: Micellization of poly(oxyethylene-*b*-oxypropylene-*b*-oxyethylene) triblock copolymers (Pluronic polymers F68, P85, and F108) in aqueous solutions was studied, and critical micellization concentrations (cmc) were determined using surface tension measurements and fluorescent probes (pyrene, 1,6-diphenyl-1,3,5-hexatriene). The dependence of cmc on temperature was observed, and critical micellization temperatures characterizing temperature-dependent transitions of Pluronic unimers to multimolecular micelles were measured. The molecular characteristics of P85 and F108 micelles including their dimensions, molecular masses and surfactant aggregation numbers were determined using light-scattering and ultracentrifugation techniques. Depending on the type of Pluronic, the micelles had an average hydrodynamic diameter ranging from about 15 to about 35 nm, a molecular mass of about 200 kDa and aggregation numbers ranging from one to several dozens. The partitioning of fluorescent probes between aqueous and micellar phases was analyzed within the frame of a pseudophase model, and the partitioning coefficients were determined using the fluorescence data. The results are compared with previous reports and are discussed in relationship to the application of block copolymer micelles as microcontainers for drug delivery.

Introduction

Poly(oxyethylene-*b*-oxypropylene-*b*-oxyethylene) triblock copolymers (Pluronic polymers[1]) represent nontoxic polymeric surfactants that have been used in a number of biomedical applications. In particular, these polymers serve as emulsifiers for perfluoroganic carriers of oxygen in "artificial blood" formulations.[2–4] Immunoadjuvant[5–7] and antitumour[7,8] activities of Pluronic polymers have been also demonstrated. Pluronic-based gels are being tested as sustained release systems for *in vivo* administration of biologically-active compounds.[9]

An important application of Pluronic micelles as microcontainers for drug delivery has been developed recently.[10] The drug molecules are noncovalently incorporated into the hydrophobic core of the micelle formed by poly(oxypropylene) (PPO) chain blocks. The external shell of such micelles is formed by nontoxic and nonimmunogenic poly(oxyethylene) (PEO) blocks which prevent the interactions of the drug with blood components and therefore increase their stability in the blood system. The resulting micelles are small in size (15–35 nm) and undergo receptor-mediated endocytosis into the cell.[11] They also enhance the transport of a model, low molecular weight compound across the cell membrane.[12] The *in vivo* targeted delivery of a micelle-incorporated neuroleptic across the undamaged blood–brain barrier has been demonstrated recently.[10,13] Application of Pluronic micelles for targeting of other molecules cannot be predicted, and further studies in this area are necessary. In order to understand the behavior of micellar drugs during interaction with cells and upon administration in organisms, the following questions must be addressed.[13] How stable are the micelles during dissolution in biological fluids? How firmly are the drug molecules associated with the micelle (i.e. do the micelles remain intact or is drug released while in the bloodstream or during interaction with the target cell)? What is the size range of the drug-containing micelles, and are there size-related issues regarding the penetration of the micelles in the capillary walls? Do the micellar carriers interact with various components of biological fluids (e.g. with serum proteins, etc)? These questions are to a significant extent the subject of the physico-chemical analysis of critical micellization parameters, micelle dimensions, drug partitioning, etc. At the same time, they give a basis for consideration of the effects of various molecular parameters of the micellar microcontainers on their pharmacological behavior.

The behavior of Pluronic polymers in aqueous solutions has been intensively studied during the past several years.[14–34] Those works addressed a number of important problems such as kinetics and thermodynamics of micellization and solubilization, structural aspects, effects of temperature, and system composition and created a comprehensive picture of Pluronic systems.[23,28,29] However, some specific results of these works are not in good agreement with each other. Particularly, there are some contradictory data on the critical micellization concentration (cmc) which has been mentioned in the early publication by Schmolka.[1] Two decades later this controversy was again discussed in the recent papers by Alexandris *et al.*,[33,34] and to our opinion, it remains unresolved. Less information is available about critical micellization temperatures (cmt), and again, some controversy in cmt values has been indicated.[33] Solubilization of hydrophobic molecules in

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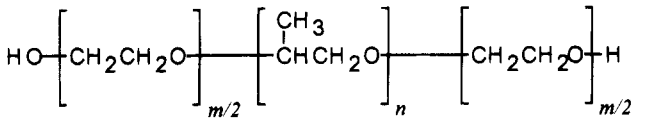
Table 1. Characteristics of Various Pluronic Polymers



code	block length n	block length m	molecular mass (Da)	HLB[a]
F68	30	159	8800	24
P85	38	51	4500	12–18
F108	56	295	16200	>24

[a] HLB calculated from glc relative retention ratios.[36]

the block copolymer micelles has been well documented and theoretically analyzed.[23] Meanwhile, not much experimental data are available about the partitioning of these molecules in Pluronic systems. Finally, interaction of Pluronic micelles with blood components (e.g. serum proteins) practically is not explored, and advancement in this field is needed.

We have undertaken this work in the course of our drug delivery studies to characterize the micelles of Pluronic polymers F68, P85, and F108. The cmc and cmt are determined using surface tesion measurements and fluorescent probes (pyrene and 1,6-diphenyl-1,3,5-hexatriene, DPH). These techniques resulted in cmc data which are in very good agreement with each other as well as with other published values.[15,35] The micelle dimensions and other molecular parameters are determined for P85 and F108 using light-scattering and ultracentrifugation techniques. Our results for P85 are consistent with the previous observations.[26,31] The effect of serum proteins on Pluronic micelles is analyzed. Solubilization and partitioning of fluorescent probes (pyrene, DPH) that mimic drug molecules is studied using fluorescence techniques. The results are discussed in relation to the application of block copolymer micelles as microcontainers for drug delivery.

Experimental Section

Materials. Pluronic polymers were supplied by Serva and used without further purification. Their characteristics are presented in Table 1. Pyrene and DPH were purchased from Sigma.

Sample Preparation. The 10% Pluronic stock solutions were prepared by dissolving Pluronic polymers in bidistilled water with a partial conductivity of 0.056 ms/cm purified by a "Milli-Q" device (Millipore). To obtain sample solutions containing fluorescent probes a known amount of pyrene or DPH in acetone was added to volumetric flasks and the acetone was evaporated. Each flask was then supplemented with a known amount of the Pluronic stock solution and bidistilled water. The stoppered flasks were incubated overnight at corresponding temperatures prior to further experiments.

Surface Tension. The surface tension in Pluronic solutions (0.0001–10 wt %) was determined by the ring-tearing technique using torsion weights with a thermostatic cell unit (the error in temperature determination was ±0.1 °C).

Fluorescence Spectroscopy. The fluorescence spectra of the micellar solutions were obtained at various temperatures using a Hitachi F 4000 spectrofluorometer with a thermostatic cell unit equipped with a stirring device. Prior to fluorescence measurements Pluronic solutions with fluoroescent probes added were left in the dark at corresponding temperatures from 16 to 24 h.

Light Scattering. One hour prior to light-scattering measurements, the Pluronic sample solutions (from 0.5 to 10 wt %) were purified from powder by filtration through a GS Millipore 0.2 μm filter. The z-average coefficients of translation diffusion (D_z) and the hydrodynamic radii (R_h) of Pluronic micelles were measured at various temperatures by the quasielastic light scattering method using an Autosizer 2c (Malvern) small-angle laser photometer with a 10 mW He–Ne laser (633 nm). The autocorrleation function of fluctuations of scattering intensity was determined using a Correlator K 7032-05 (Malvern). The D_z values were calculated by the cumulants method. The R_h values (radii of equivalent hydrodynamic spheres) were calculated on the basis of translation diffusion coefficients using the Stokes–Einstein equation:

$$R_h = kT/6\pi\eta D_z \quad (1)$$

where k is the Boltzmann constant, T is the temperature, and η is the solvent viscosity.

Ultracentrifugation. The sedimentation coefficients of Pluronic micelles were determined at 37 °C using a Beckman E ultracentrifuge with a Schlieren-optic registration system. The rotation rate of an An-D rotor with a 12 nm monosector cell was equal to 56 000 rpm. The sedimentation coefficients were calculated from the experimental data using the "Ninal" program. Some sedimentation experiments (see below) were performed in the scanning mode using a Beckman E ultracentrifuge equipped with a photoelectric scanning device with a monochromator and a multiplexor. The scanning was carried out at absorption wavelengths of the solubilized fluorescent probes—pyrene (338 nm) and DPH (360 nm) or proteins (280 nm).

Partial Specific Volume. The partial specific volume of Pluronic P85 and F108 micelles in aqueous solution was determined at 37 °C using a pycnometer.

Results and Discussion

Critical Micellization Concentration. The micellization of block copolymers in aqueous solutions depends on their concentration. Therefore, the application of micelles as drug carriers raises the fundamental question about their stability during dissolution of body fluids.[10,13] At a given temperature, the multimolecular micelles are formed at Pluronic concentrations equal to or exceeding cmc, which may serve as a thermodynamic parameter characterizing the micelle stability during dissolution. Some contradictory data on Pluronic cmc can be found in the literature;[1,33,34] the reported cmc values differing by a factor of 1 or 2 for the same Pluronic polymers. Such a tremendous difference raises serious questions about the state of Pluronic micellar carriers during administration in the organism. Therefore we employed several independent methods for cmc determination, based on the surface tension measurements and solubilization of the fluorescent probes (pyrene and DPH).

(a) Surface Tension Measurements. This method implies that an increase in a surfactant concentration leads to a decrease in the surface tension; the micelle formation at cmc causing a break in this dependence.[37] In this case, the cmc value is determined by the crossing point of the straight lines that continue the surface tension vs log concentration curves before and after the break. However, several authors have contested the applicability of such consideration in the case of Pluronic polymers.[24,32,34] According to these works, not one but at least two breaks are observed at the surface tension curves for Pluronic polymers. Therefore, controversy over which break represents the formation of the multimolecular micelles has arisen, see also ref 16.

Surface tension data for F68, P85, and F108 at various temperatures are presented in Figure 1. While the dependencies for P85 in the studied range of concentration resembled the classic one-break curve, the behavior of F68 and F108 was different. In the latter cases steplike curves were observed which revealed several characteristic breaks. Increasing the temperature resulted in a gradual transformation of the steplike curves into the one-break type. This transfor-

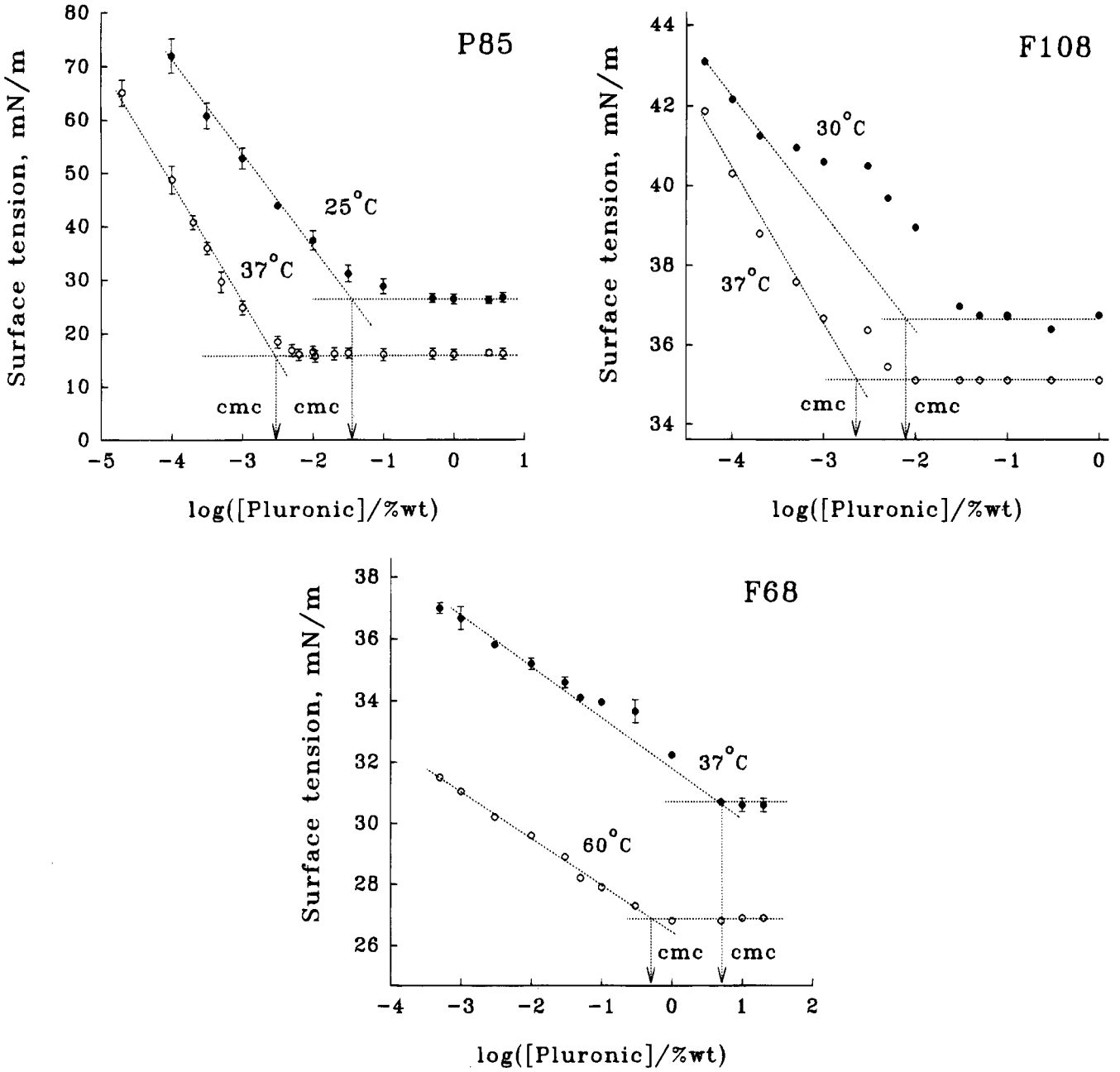


Figure 1. The isotherm of surface tension in the aqueous solutions of Pluronic polymers F68, P85, and F108 at various temperatures. Values are means ± SD.

mation was observed at 50–60 °C for F68 and at 30–40 °C for F108. Generally, the steplike curves observed in this work were similar to those reported by some other authors.[24,32,34] Like in our case, these steps revealed the tendency for smoothing out with elevation of the temperature. Recently, studies by Alexandris *et al.*[34] reported surface tension measurements for several Pluronic polymers, including P85 and F108. For F108, a similar steplike curve was observed at 25 °C which was smoothed out at 35 °C. However, the concentration range in which this step was observed in Alexandris and colleagues studies (from 0.01% to 1%) was not consistent with our data (from 0.0002% to 0.01%). Furthermore, the authors observed a steplike curve for P85 which was not detected in our present studies. These authors accounted for the higher concentration break (the end of the step) for the formation of multimolecular micelles (cmc) on the basis of the following reasons: (*i*) these values are in agreement with the cmc values determined earlier by the same group using the DPH fluorescence technique; (*ii*) the plateau is observed in the surface tension isotherms after this break; (*iii*) the position of this break is temperature-dependent. As for the DPH solubilization data, this technique can produce some artifacts which we discuss below. As for the two other reasons, they do not seem very convincing to us, since the observations (*ii*) and (*iii*) may have some other explanation, which we also discuss.

Several possible reasons have been analyzed in the literature for the appearance of the steplike curves: (*i*) concentration-dependent formation of the unimolecular micelles (collapsed unimers) in the bulk;[16] (*ii*) polydispersity of Pluronic polymers;[32] (*iii*) formation of the copolymer aggregates in the bulk;[34] (*iv*) concentration-dependent change in the configuration of the block copolymer at the air/water interface.[34] We will consider these hypotheses consequently. (*i*) Prasad *et al.*[16] explained the low-concentration breaks in the surface tension isotherms of Pluronic polymers by the formation of unimolecular micelles in the bulk due to the collapse of the PPO chain blocks. Recently, Alexandris *et al.*[34] concluded that such a transition at low concentrations

of the block copolymers is very unlikely since it has no apparent thermodynamic reasons. Generally speaking, there may be a reason for the concentration dependence of this process since it may account for intermolecular interactions (collisions) of the block copolymer chains. However, some recent studies caused concerns about the unimer collapse at temperatures lower than 30 °C where the steps in the surface tension curves are observed.[27] Moreover, the increase in the temperature should promote the collapse of the unimers,[26,27] which is not consistent with smoothing of the steps at high temperatures. (*ii*) The step-by-step micellization of the components with different surfactant activity[32] should result in a consequent (or even monotonous) decrease of the slope of the surface tension curves which does not explain their steplike shape (decrease of the slope, followed by a steeper slope). On top of that, the recent theoretical work by Linse[29] reveals that the impurity of PEO and PPO homopolymers as well as the PEO–PPO diblock copolymer only marginally affects cmc (less than 20%) which cannot explain the 10- to 1000-fold difference in the position of the breaks in the surface tension isotherms. (*iii*) The formation of dense PEO aggregates which are in the equilibrium with the unimers have been predicted recently.[41] Alexandris *et al.*[34] proposed that these aggregates may also form in the solutions of Pluronic polymers, which may explain the change in their activity at low concentrations. Similar aggregates have been registered previously by the light-scattering technique which has been accounted for by composition heterogeneity of the commercial samples of the block copolymers.[20] We also observed these aggregates in the size vs temperature plots below and in the vicinity of the cmt (data not presented here), their dimensions lying in the range from several hundred nanometers to several micrometers and their portion being less than 1% of the overall amount of the block copolymer in the solution. While the aggregates can be separated by ultracentrifugation or ultrafiltration, after 30–60 min they form again, which is indicative of an equilibrium process. Interestingly enough, these aggregates were registered at the same temperature range in which the steplike isotherms of surface tension were observed (blow 25–30 °C for P85, 35–40 °C for F108, 50–60 °C for F108). At the same time, the aggregate formation gives no clear explanation for the shape of the surface tension curves. (*v*) The most consistent consideration has been advanced by Alexandris *et al.*[34] It implies that at low concentrations (below the low-concentration break) the decrease of the surface tension is conditioned by the adsorption of the monomers of the block copolymer at the air/water interface. At a bulk concentration of approximately 10^{-3}% the interface becomes fully covered by the block copolymer. This is followed by some structural transition of the block copolymer at the interface resulting in its compaction, which accounts for the change in the slope of the surface tension curve. After the high-concentration break the multimolecular micelles are formed in the bulk and the surface tension attains a steady value.

The hypothesis that variations in the bulk concentration of the block copolymer may result in changes of the configuration of its adsorbed chains has been advanced previously by Lee *et al.*[42] and it seems quite reasonable. The formation of unimolecular two-dimensional micelles ("jellyfishes") from expanded polymer chains ("starfishes") has been reported recently for the adsorption layers of block polyelectrolytes.[43] It may well happen that such a transition also takes place in Pluronic systems at the low-concentration break. It is worth mentioning that the shape of the surface tension vs log concentration plots is very similar to that of the "π-a" isotherms obtained for the adsorption layers of block polyelectrolytes.[43] It is possible that the similarity between the surface tension and surface pressure curves has a fundamental basis. The monotonous increase of the two-dimensional pressure in "π-a" isotherms is accounted for by the expanded films of the starfishes at the interface, this region being similar to the region observed before the low-concentration break in the surface tension isotherms. The plateau in the "π-a" curves corresponds to the "starfish–jellyfish" transition (coexistence of starfishes and jellyfishes) and is related to the shallow slope (or plateau) area in the surface tension curve. The steep increase in π after the plateau is due to formation of compressed films and is similar to the sharp decrease in the surface tension before the high-concentration break.

However, we disagree with the conclusion of Alexandris *et al.*[34] that the high-concentration break is the cmc, because this conclusion is not based on an experimental observation. Studies by Zhu *et al.*[43] have described very significant kinetic effects and hysteresis phenomena for the two-dimensional micellization. The shape of the "π-a" isotherms (particularly, the size and position of the step) significantly changes with variation of the rate of the monolayer compression, this effects lasting for at least 10 h. Lee *et al*[42] and Alexandris *et al.*[34] have also mentioned some kinetic effects on the surface tension isotherms which they suggested was a fast equilibration processes at the surface of the Wilhelmy plate. However, no study of the effects of the storage of the block copolymer solution on the surface tension measurements was conducted. Furthermore, on the basis of the data of Zhu *et al.*,[43] one can expect that the surface tension isotherms may be influenced by the kinetic effects and, in particularly, by the history of the block copolymer solutions prior to the measurements. Such nonequilibrium phenomena may well explain the differences in the cmc data reported by various authors, as well as the differences between the surface tension isotherms for P85 and F108 reported in the present study and work by Alexandris *et al.*[34] As far as the temperature dependence of the surface tension measurements is concerned, it may result from the contribution of several phenomena among which are (*i*) the temperature dependence of the "starfish–jellyfish" transition at the interface, (*ii*) the temperature dependence of the kinetics of relaxation processes at the interface, and (*iii*) the temperature dependence of the block copolymer micellization in the bulk.

We presumed that relaxation processes do not affect significantly the surface tension before and after the step. In this case the correct way to analyze the surface tension data is to ignore the steps and determine the cmc by the crossing point of the straight lines that continue the surface tension vs log concentration curves before and after the step. As a first approximation, this point corresponds to the conditions of equality of the chemical potentials of the block copolymer molecules in the multimolecular micelles and its unimers in solution. Application of this approach for the analysis of the data presented by Alexandris *et al.*[34] would result in the following cmc for P85: 0.02–0.03% at 25 °C and 0.003–0.004% at 35 °C. These values are in very good agreement with our data: 0.03–0.04% at 25 °C and 0.005–0.007% at 37 °C. In the case of F108 it results in cmc values equal to 0.06% at 25 °C and 0.01% at 35 °C compared to our data: 0.03–0.05% at 25 °C and 0.003–0.007% at 37 °C. The cmc values obtained by

Table 2. cmc Values for Various Pluronic Polymers (in wt %)[a]

pluronic	T (°C)	surface tension	fluorescent probes: pyrene	fluorescent probes: DPH[b]	lit. data[c]
F68	25	not obs			
	30	≥10			
	37	4–5		10	
	45	1.0			3
	50	0.9		1–2	0.9
	60	0.5			
P85	25	0.03–0.04	0.04		4
	30	0.01			0.9
	37	0.005–0.007	0.006	0.004	
	45	0.006			0.014
	50	0.004			
F108	20			0.1	
	25	0.03–0.05	0.03		4.5
	30	0.009–0.011			0.8
	37	0.003–0.007	0.005	0.008–0.010	
	45	0.005–0.006			0.008
	50	0.003–0.005			

[a] Each experimental point in the surface tension isotherms or fluorescence intensity plots was repeated 2–5 times, and an experimental error for the cmc of 20 to 30% was estimated. In some cases we present the range of the values obtained in several independent experiments. [b] Systems were incubated 16 h after addition of the probe. [c] The data reported by Alexandris et al.[33] using the DPH solubilization technique are presented.

us using this approach are listed in the Table 2. These values are in good agreement with the data obtained using the pyrene and DPH probes.

(b) Pyrene Solubilization Technique. Pyrene solubilization has been used previously for the determination of the cmc in block copolymer solutions.[38,39] The fluorescence of this probe is sensitive to changes in the microenvironment which permits monitoring its incorporation in micelles at concentrations exceeding the cmc. The typical emission spectra of pyrene and the plot of its fluorescence intensity versus Pluronic concentration are represented in Figures 2 and 3, respectively. The intensity values remained virtually constant below the cmc. Above that concentration, the fluorescence increased substantially, reflecting incorporation of pyrene in the hydrophobic core of the micelle. As is seen from Table 2, the cmc values obtained for P85 at 25 °C and 37 °C using this technique are in a very good agreement with the surface tension data.

(c) DPH Solubilization Technique. The behavior of DPH in Pluronic solutions was similar to that of the pyrene probe (Figures 4 and 5). At Pluronic concentrations lower than the cmc, the DPH fluorescence was not registered; meanwhile, the micelle formation was accompanied by a sharp increase in the probe fluorescence. However, in this case the significant time dependencies of the fluorescence were observed. For example, the apparent cmc value obtained for solutions incubated at a constant temperature for 1 h after addition of the probe was more than 10 times higher than that obtained for solutions which were incubated for 16 h (Figure 5). At the same time, the latter values were consistent with the data obtained using the surface tension and pyrene techniques (Table 2). The relaxation process in DPH micellar solutions lasted up to 5–7 h depending on the composition of the system. It is well-known that DPH may form supermolecular complexes in aqueous solutions, which differ in fluorescent properties. It is probable that the relaxation processes which we have observed are due to DPH complexation/decomplexation during incorporation in the Pluronic micelles. Therefore one should carefully analyze the micellization data obtained using this technique, since it can produce some artifacts. We have not encountered this problem in the

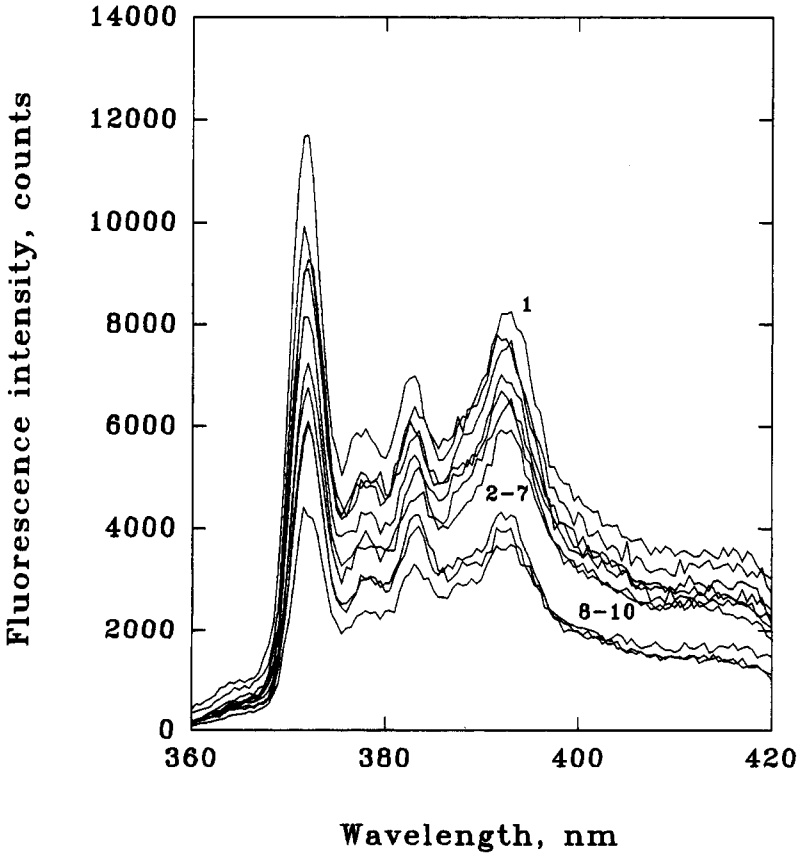


Figure 2. Fluorescence emission spectra (λ_{ex} = 333 nm) of pyrene (5×10^{-7} M) registered at 25 °C in the presence of various concentrations of Pluronic P85 (% wt): (1) 1.22; (2) 0.82; (3) 0.81; (4) 0.55; (5) 0.40; (6) 0.37; (8) 0.24; (9) 0.20; (10) 0.17.

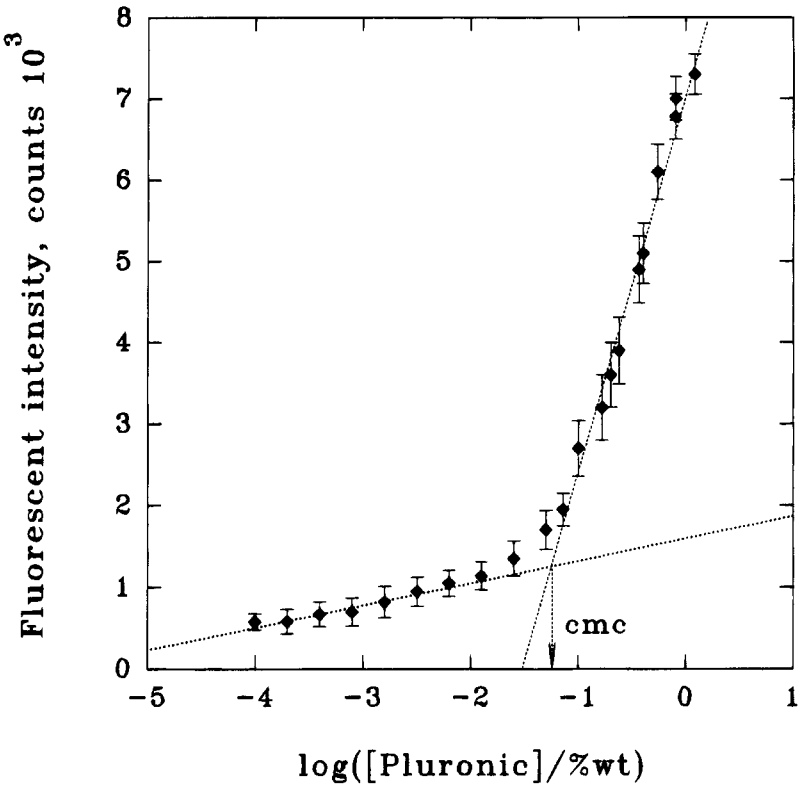


Figure 3. Plot of the fluorescence emission intensity of pyrene (5×10^{-7} M) versus the Pluronic P85 concentration at 25 °C (λ_{ex} = 333 nm, λ_{em} = 395 nm). Values are means ± SD.

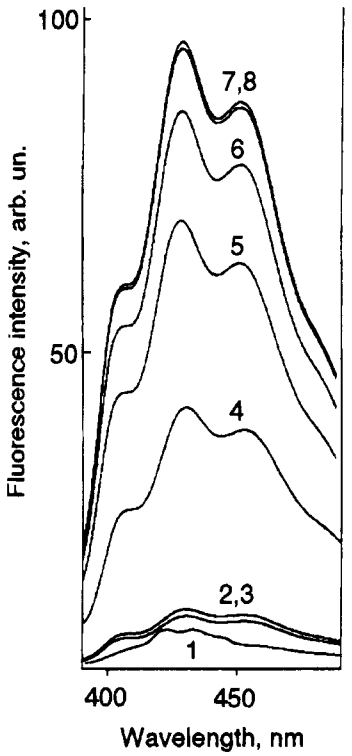


Figure 4. Fluorescence emission spectra (λ_{ex} = 380 nm) of DPH (10^{-7} M) registered at 37 °C in water (1) and in the presence of various concentrations of Pluronic P85 (% wt): (2) 0.0001; (3) 0.001; (4) 0.01; (5) 1; (6) 2; (7) 5; (8) 10.

case of pyrene solubilization, and therefore the pyrene technique seems to be more reliable.[40]

Our cmc data (Table 2) are in reasonable agreement with the values reported by Schmolka and Raymond[15] for F68, P85, and F108. They are also consistent with the data obtained by Zhou and Chu[21] and Nakashima *et al.*[35] for F68. The work by Alexandris *et al.*[33] in which the Pluronic micellization was studied using the DPH probe reports the cmc values that are generally higher than the data obtained by some other authors[15,35,44] and in this work (Table 2). For example, for F68 they report the cmc value of 7% at 40 °C compared to 2.5% at 39 °C determined by Zhu and Chu[21] using the light-scattering technique. While our cmc values for F68 differ from those reported by Alexandris *et al.*[33] by approximately 2–3 times, the discrepancy of the cmc for P85 and F108 is more substantial (Table 2). Comparison of these data reveals that the difference is maximal at lower temperatures. For example, at 30 °C we obtained a cmc of 0.01% for P85 and 0.03–0.05% for F108 compared to 0.9% for P85 and 0.8% for F108 reported by Alexandris *et al.*[33] At higher temperatures, the difference is less significant, e.g. at 45 °C we obtained 1.0% for F68, 0.006% for P85, and 0.005–0.006% for F108 compared to 3% for F68, 0.014% for P85, and 0.008% for F108 reported by Alexandris *et al.*[33] It should be noted that Alexandris *et al.*[33] incubated the DPH solutions for 3 h prior to the fluorescence measurements. Therefore, it cannot be excluded that some cmc values in their work were affected by the relaxation process.

Critical Micellization Temperature. The micelle formation in Pluronic solutions is very sensitive to temperature.[33] At a given concentration, the multimolecular micelles are formed at temperatures exceeding the cmt. At lower temperatures, Pluronic polymers exist in a unimer form.[21,26] Using the surface tension measurements, we determined cmc values for F68, P85, and F108 at various temperatures. The cmc decreased with temperature elevation (Figure 6a). These curves establish a relationship between cmc and cmt and produce phase diagrams for Pluronic micellization that is schematically illustrated in Figure 6b. Similar dependencies have been observed recently in Linse and Malmsten,[25] Mortensen *et al.*,[26] and Alexandris *et al.*[33] Such behavior is explained by the decreasing hydration

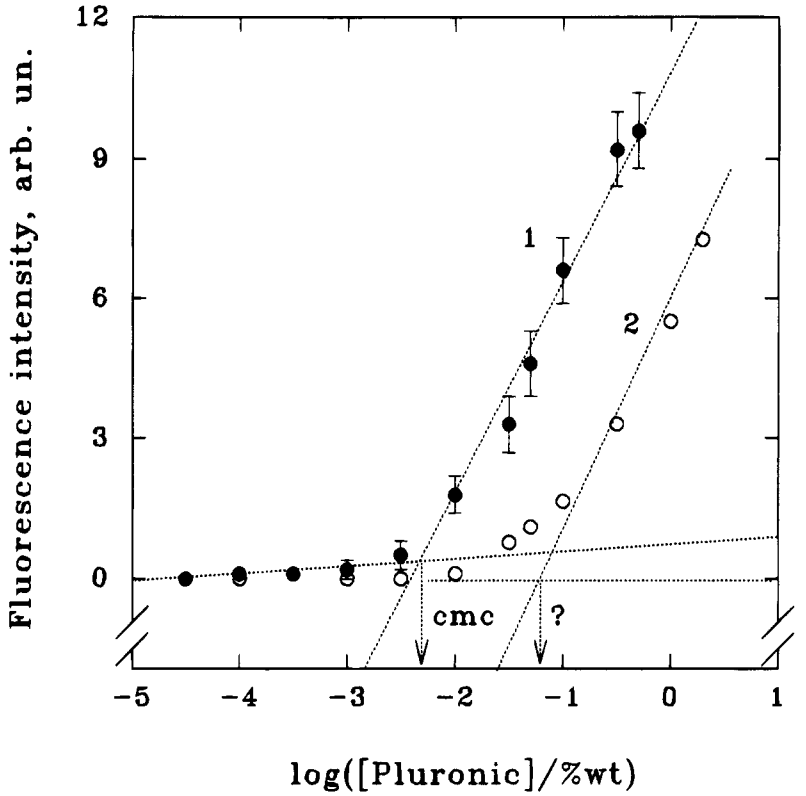


Figure 5. Plots of the fluorescence emission intensity of DPH (0.5 μM) versus the Pluronic P85 concentration at 37 °C determined (1) 16 h and (2) 1 h after solubilization of the probe (λ_{ex} = 380 nm, λ_{em} = 430 nm). Values are means ± SD.

and, consequently, by the increasing hydrophobicity of Pluronic molecules and temperature elevation.[21] Model calculations on the basis of the Flory–Huggins theory were performed[25,29] that accounted for the hydration of Pluronic blocks and agree reasonably well with the experimental dependence of cmc on temperature. The molecular mechanisms of the temperature effects are also discussed in detail by Hatton *et al.*[28,33] These theories give the relationships between the critical micellization parameters and lengths of PEO and PPO blocks. The attempt to establish these correlations experimentally has been made recently by Alexandris *et al.*[33]

Molecular Characteristics of Pluronic Micelles. The size of Pluronic micelles is one major parameter affecting their interactions with cells and distribution in the organism during application as drug carriers.[11,13] The dimensions, molecular mass and other characteristics of P85 and F108 micelles at temperatures exceeding the cmt were determined. (These characteristics of F68 micelles were recently studied by Zhou and Chu[21]). The values of z-average translation diffusion (D_z) and the sedimentation (S) coefficient of micelles were measured at various block copolymer concentrations using the light-scattering and ultracentrifugation techniques. D_z° and S° corresponding to the infinite dilution were determined from the concentration dependencies obtained and the molecular mass (M) of micelles were calculated using the Svedberg equation:

$$M = S^\circ RT/D_z^\circ(1 - \nu\varrho) \qquad (2)$$

where ν is the partial specific volume of micelles and ϱ is the solvent density.

Using the values of molecular masses, the average aggregation numbers (N) of micelles were calculated. On the basis of these values, the dimensions of hydrophobic core and hydrophilic corona of micelles were estimated as described by Zhou and Chu.[21] These calculations were performed under the assumption that (*i*) the micelles represent spherical aggregates in which the hydrophobic core is formed by condensed (liquid-like) PPO blocks and the hydrophilic corona by hydrated PEO chains; (*ii*) the radius of hydrophobic core (r_{core}) is expressed by the equation

$$r_{core} = [3nNM_{PO}/(4\pi N_A \varrho_{PO})]^{1/3} \qquad (3)$$

where M_{PO} (=58) is the molecular mass of the oxypropylene units and ϱ_{PO} (=1.01 g/cm^2)[21] is the density of liquid PPO; (*iii*) the thickness of the PEO corona is estimated as a numerical difference between the micelle hydrodynamic radius and r_{core}; (*iv*) the mean volume per oxyethylene (v_{EO}) in the micelle shell is expressed by the equation

$$v_{EO} = 4\pi(R_h^3 - r_{core}^3)/3nN \qquad (4)$$

The characteristics of P85 and F108 micelles at 37 °C are listed in Table 3. This table also presents for comparison the data previously obtained for F68 at 54 °C.[21] The Brown and Mortensen groups[26,31] have recently studied P85 micelles using small-angle neutron scattering, Rayleigh–Brillouin, and dynamic light scattering and reported the molecular parameters (R_h is about 8 nm,[31] r_{core} varies from 3 nm at 5 °C to 5 nm at 50 °C[26], N changes from 37 at 20 °C to 78 at 40 °C[26]). These values are in good agreement with our results (R_h = 7.3 ± 0.3 nm, r_{core} = 3.7 ± 0.3 nm, and N = 57 ± 16 at 37 °C). Figure 7 depicts a model of a Pluronic micelle at physiological temperatures. According to this model, the micelle hydrodynamic diameter lies in the range from about 15 nm (P85, F68) to about 35 nm (F108). The diameter of the hydrophobic core, consisting of liquid-like PPO, lies in the range from about 5 nm (F68, F108) to about 7.5 nm (P85). The thickness

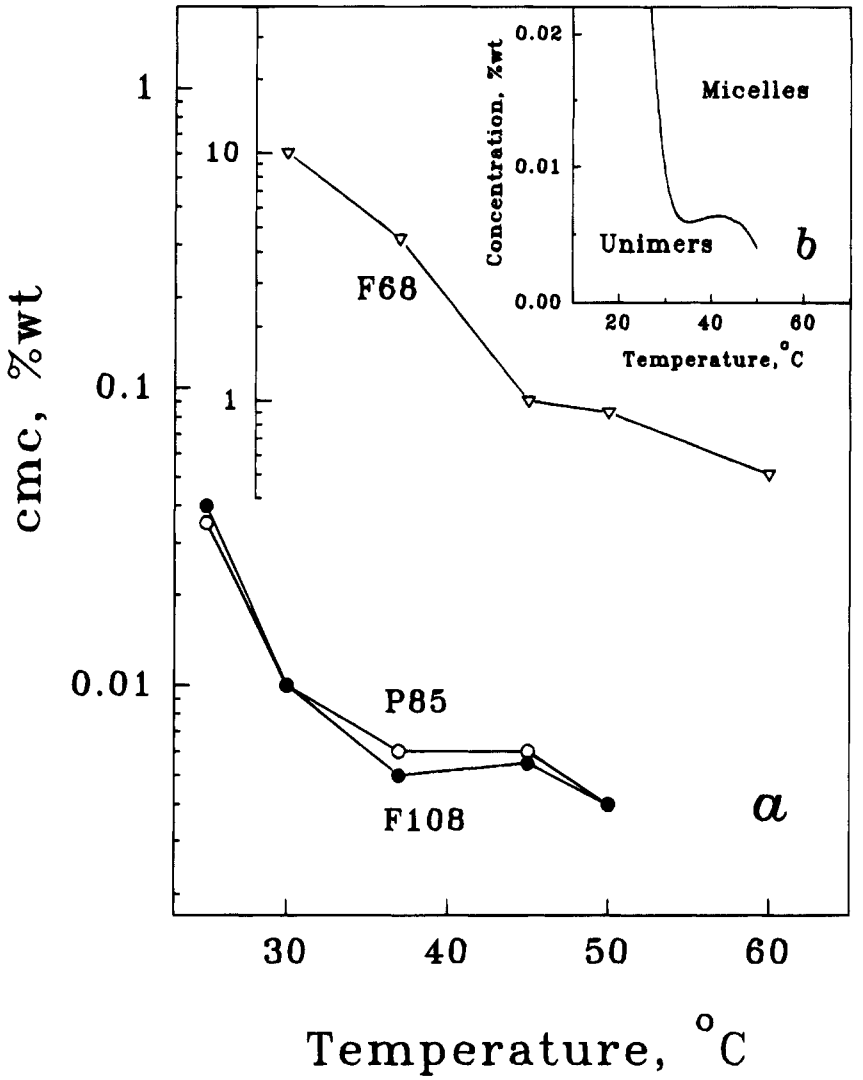


Figure 6. (a) Temperature dependencies of cmc for Pluronic polymers F68, P85, and F108 and (b) the phase diagram for Pluronic F108 derived from the temperature dependence of cmc. The cmc values were determined using surface tension measurements.

Table 3. Molecular Characteristics of the Pluronic Micelles

pluronic	P85[a]	F108[a]	F68[b]
partial specific volume, ν (cm^3/g)	0.84 ± 0.01	0.60 ± 0.01	n.d.
translation diffusion coefficient, $D_z°$ (cm^2/s)	3.70(±0.4) × 10^{-7}	2.0(±0.2) × 10^{-7}	6.0 × 10^{-7}
sedimentation coefficient, S ° (s)	5.9(±0.7) × 10^{-13}	6.7(±0.8) × 10^{-13}	n.d.
molecular mass, M (kDa)	256 ± 74	215 ± 43	190
aggregation no., N	57 ± 16	13 ± 3	22
hydrodynamic radius, R_h (nm)	7.3 ± 0.3	17.5 ± 2.5	7.8
hydrophobic core radius, r_{core} (nm)	3.7 ± 0.3	2.5 ± 0.2	2.5
hydrophilic corona thickness (nm)	3.6 ± 0.6	15.0 ± 2.7	5.3
mean volume per oxyethylene unit, v_{EO} (nm^3)	0.49 ± 0.16	5.8 ± 1.8	0.55

[a] Characteristics of the micelles at 37 °C (measured or calculated by us). Values are means ± SD. Absolute errors for the calculated parameters are obtained from the SD values of the directly measured parameters using eqs 2–4. [b] Characteristics of the micelles at 54 °C determined by Zhou and Chu.[21]

of the hydrophilic corona varies from about 3.5 nm (P85) to about 15 nm (F108). The estimated mean volumes per oxyethylene in the micelle shell (Table 3) are from 1 (P85, F68) to 2 (F108) orders of magnitude higher than those of the oxyethylene monomer proper (about 0.06 nm^3); i.e. the PEO shell is highly swollen and traps large amounts of water.[21] The theoretical model proposed recently by Linse[29] established relationships between the lengths and PEO and PPO blocks and the molecular parameters of the Pluronic micelles formed at various concentrations and temperatures. Generally, this theory is consistent with experimental observations; however, some minor discrepancies can be mentioned. For example, according to Linse[29] at 37 °C the aggregation number of P85 equals 10.5, which is lower than the experimental value. At the same time the predictions of this theory are significantly closer to reality than those of the previous model advanced by Nagarajan and Ganesh[18] who obtained very large aggregation numbers of 500–1000.

Micelle Interaction with Serum Proteins. Being introduced in the blood serum, Pluronic polymers may interact with the plasma components, particularly with serum proteins.[13] Some previous results indicate that hydrophobic Pluronic polymers (such as L101 or L121 (HLB = 1 to 7)) form water-insoluble complexes with proteins.[44] Therefore, we studied the effect of serum proteins on micellization in Pluronic solutions. The data

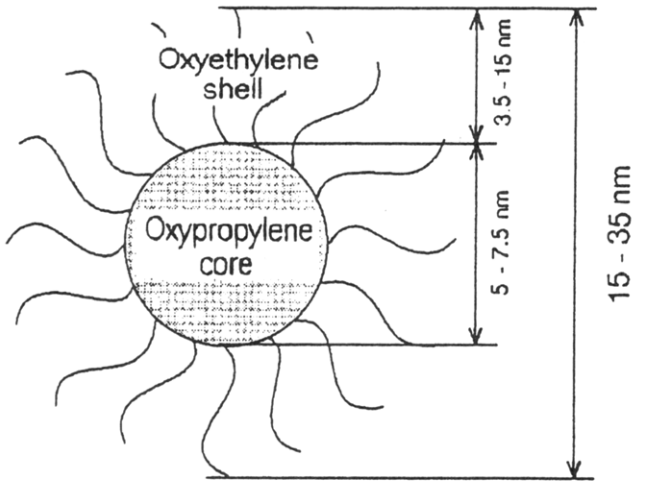


Figure 7. Pluronic micelle at the physiological temperature (37 °C). Micelles represent spherical aggregates of from about one to several dozen Pluronic molecles; the micelle hyrophobic core consists of liquid-like PPO blocks; the hydrophilic shell is formed by swollen PEO chains trapping large amounts of water.

Table 4. Sedimentation Coefficients of Pluronic P85 Micelles and Serum Proteins at 37 °C

	sedimentation coeff (10^{-13} s)		
system studied	protein (scanning mode)	protein (Schlieren mode)	micelles (Schlieren mode)
2% Pluronic P85			3.1
3% BSA	n.d.	7.0	
2% Pluronic P85 + 3% BSA	n.d.	6.5	3.0
1 mg/mL of IgG	9.0	n.d.	
2% Pluronic P85 + 1 mg/mL of IgG	8.5	n.d.	3.0

obtained revealed that the critical micellization parameters of P85, F68, and F108 remained virtually unchanged in the presence of 3% bovine serum albumin (BSA) and 1 mg/mL of IgG that represent major serum proteins. The micelle dimensions (in 1−10% Pluronic solutions, 37 °C) were not affected in the presence of 3% BSA. (The light-scattering measurements of micelle sizes in the presence of IgG were not possible because of formation of large protein aggregates in aqueous solutions of immunoglobulins). The sedimentation experiments were performed in order to determine whether serum proteins can bind to micelles. The sedimentation of micelles and proteins was monitored using the Schlieren technique or in the scanning mode by measuring the optical density. As is shown in Table 4 using the example of P85 (which is the most hydrophobic one among the block copolymers studied in this paper), the sedimentation coefficients of BSA and IgG did not change significantly in the presence of micelles. (Some decrease in these coefficients can be probably explained by an the increase in the viscosity in Pluronic solutions). The micelle sedimentation coefficients also did not change in the presence of the proteins. Therefore, under the conditions studied, the proteins do not bind to the Pluronic micelles. This result is consistent with the previous statements that water-soluble Pluronic polymers do not interact with serum proteins (in particular with serum albumins).[44,45]

Partitioning of Low Molecular Weight Fluorophores. The concept of Pluronic-based drug delivery systems[10−13] implies that drugs are noncovalently incorporated (solubilized) in the micelle carriers. Therefore application of Pluronic micelles as drug carriers requires the study of solubilization and partitioning of drug molecules. In this work, the fluorescent probes (pyrene, DPH) were used as model drugs and their partitioning in micellar solutions of Pluronic polymers P85 and F108 that differ in hydrophobicity was studied. The direct proof of the probe incorporation in the micelles was obtained during ultracentrifugation experiments performed in the scanning and Schlieren modes. The micelles proper practically do not absorb light in the UV range, and their sedimentation cannot be monitored in the scanning mode. However, the addition of probes, such as pyrene and DPH, permits "visualization" of the micelles. The border corresponding to the sedimenting micelles appeared at the sedimentation curves when scanning was performed at the absorption wavelength of the probe. The sedimentation coefficients determined in this case were virtually equal to those measured using the Schlieren technique in the absence of the probe (data not shown). These experiments revealed that both pyrene and DPH were incorporated in the micelles.

The comprehensive theoretical model for solubilization in PPO−PEO micelles has been developed by Nagarajan and Ganesh.[23] In order to determine the parameters characterizing the probe partitioning in P85 and F108 micelles from the fluorescence data, a simple pseudophase model which can be derived as an approximation from Nagarjan and Ganesh theory[23] was used. A similar approach has recently been used by Winnik's group for description of the partitioning of the pyrene probe in polystyrene−PEO solutions.[38] According to this model, the probe partitioning in the aqueous phase and micellar microphase is characterized by the partitioning coefficient (P):

$$P = [\text{Probe}]_m/[\text{Probe}]_w \tag{5}$$

where $[\text{Probe}]_m$ and $[\text{Probe}]_w$ are the local probe concentrations in the micellar and aqueous phases, respectively. Using the material balance equation, the bulk concentrations of micellar (C_m) and aqueous (C_w) forms of the probe can be expressed as

$$C_m = \theta P C_o/[1 + (P - 1)\theta] \tag{6}$$

and

$$C_w = (1 - \theta)C_o/[1 + (P - 1)\theta] \tag{7}$$

where C_o is the total probe concentration and θ is the volume portion of the micellar phase ($\theta = 0.01\nu$([Pluronic] − cmc)). Therefore, the portion of the micellar probe (α) is given by the equation

$$\alpha = C_m/C_o = \theta P/[1 + (P - 1)\theta] \tag{8}$$

According to eq 8, the increase in the Pluronic concentration results in an increase in the portion of the micellar probe in the system. This leads to an elevation of the fluorescence emission intensities (I) of the probes, the intensity dependence of the Pluronic concentration being described by the curves with "saturation" (Figure 8). Under saturating conditions, virtually all probe molecules were entrapped in the micelles; the emission reached the maximal value (I_{max}) and did not change with a further increase in the block copolymer concentration. The I_{max} value linearly depended on the probe concentration (Figure 9). At the same time, within the studied ranges of probe concentrations, similar linear dependencies were also observed for the fluorescence

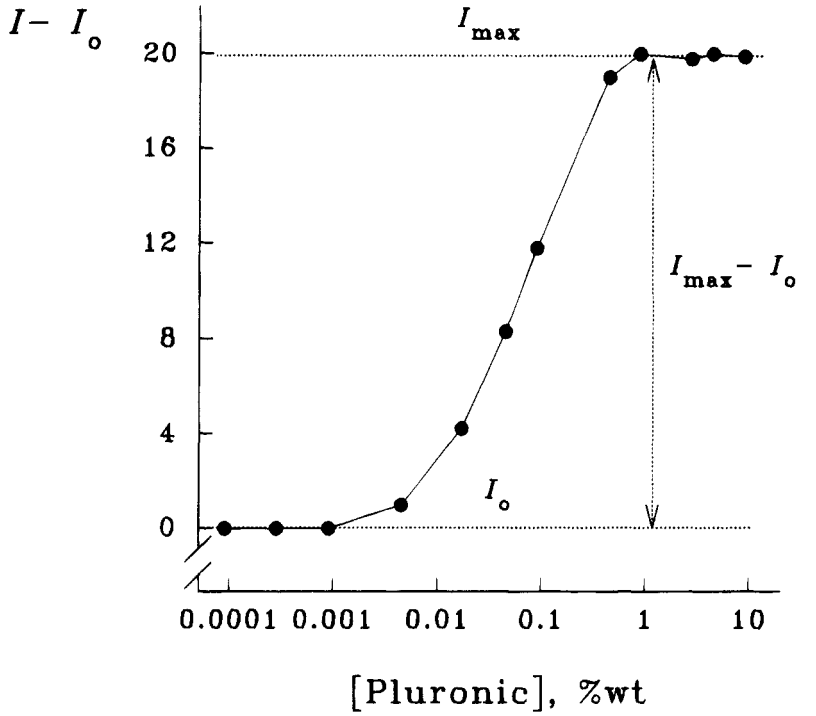


Figure 8. Dependence of the intensity (I) of pyrene fluorescence (λ_{ex} = 333 nm, λ_{em} = 395 nm) on Pluronic P85 concentration at 37 °C. I_o is the fluorescence in the absence of Pluronic; I_{max} is the fluorescence in the presence of Pluronic "saturating" concentrations.

intensities of pyrene and DPH in Pluronic-free aqueous solutions (I_o). Therefore, I_o and I_{max} can be expressed as follows: $I_o = f_w C_o$ and $I_{max} = f_m C_o$, where f_w and f_m are the molar coefficients of emission corresponding to the probe in the bulk aqueous solution and micellar microphase, respectively. The emission in Pluronic solutions as a first approximation consists of the emissions of the aqueous and micellar portions of the probe and is given by $I = [(1 - \alpha)f_w + \alpha f_m]C_o$. This leads to the following expression for α:

$$\alpha = (I - I_o)/(I_{max} - I_o) \qquad (9)$$

Combination of eqs 8 and 9 yields a simple relationship

$$(I_{max} - I_o)/(I - I_o) - 1 = 1/(P\theta) - 1/P \qquad (10)$$

that was used for determination of P values from the fluorescence emission data. The experimental plots corresponding to this relationship are represented in Figure 10. The P values determined using the above described approach are represented in Table 5. In the cases of both Pluronic polymers, the partitioning coefficients of DPH were essentially higher than those of less hydrophobic pyrene. At the same time in the cases of pyrene and DPH, the partitioning coefficients increased with the increase in the Pluronic hydrophobicity. This suggests that the hydrophobic interactions with Pluronic micelles play a major role in the solubilization of the probes. The comparison of P values obtained for pyrene at 20 and 37 °C reveals a significant increase in the probe interaction with the micelles with temperature elevation. This result is consistent with the previous considerations[21,26,27] that the temperature elevation decreases the hydration of Pluronic chains and, therefore, elevates the hydrophobicity of the micellar core. The same phenomenon apparently underlies the decrease in cmc with temperature elevation.[28,29] The portion of the micellar probe at a given Pluronic concentration can be determined from the relationship (8) using the experimental P values obtained. The

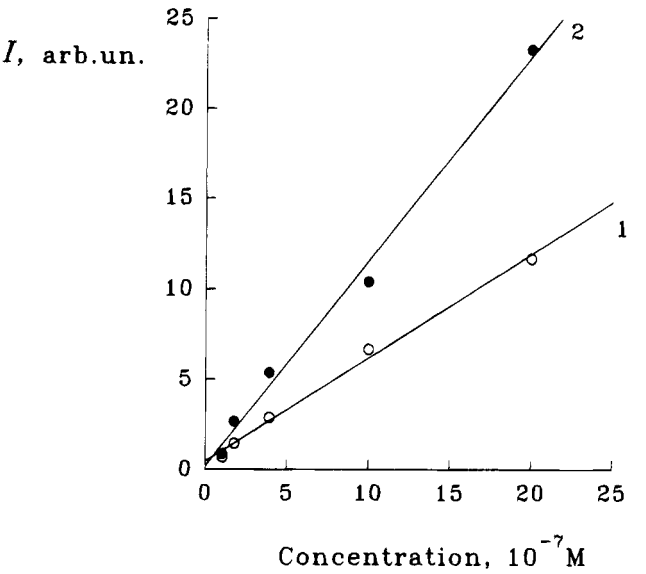


Figure 9. Dependence of the fluorescence emission intensity on (1) pyrene and (2) DPH concentration in 2% Pluronic P85 solution at 37 °C. (1) λ_{ex} = 333 nm, λ_{em} = 395 nm; (2) λ_{ex} = 380 nm, λ_{em} = 430 nm.

dependencies of α on the Pluronic concentration are represented in Figure 11. For example, at 37 °C in 2% P85 solution, the micelles incorporate 98.2% of pyrene and 99.2% of DPH added to the system. Under the same conditions, the F108 micelles solubilize 95.2% of pyrene and 99.0% of DPH. In other words, virtually all probe molecules introduced in these systems were located inside the micellar species. Using the N values determined, the micelle concentrations can be estimated as about 10^{-4} M. Therefore, at the studied probe concentrations (10^{-7} to 10^{-16} M), the average probe to micelle molar ratios ranged from 1:1000 to 1:100 and the portion of probe-containing micelles did not exceed 0.1−1.0%. Since no changes in the sedimentation coefficients and dimensions of these micelles were

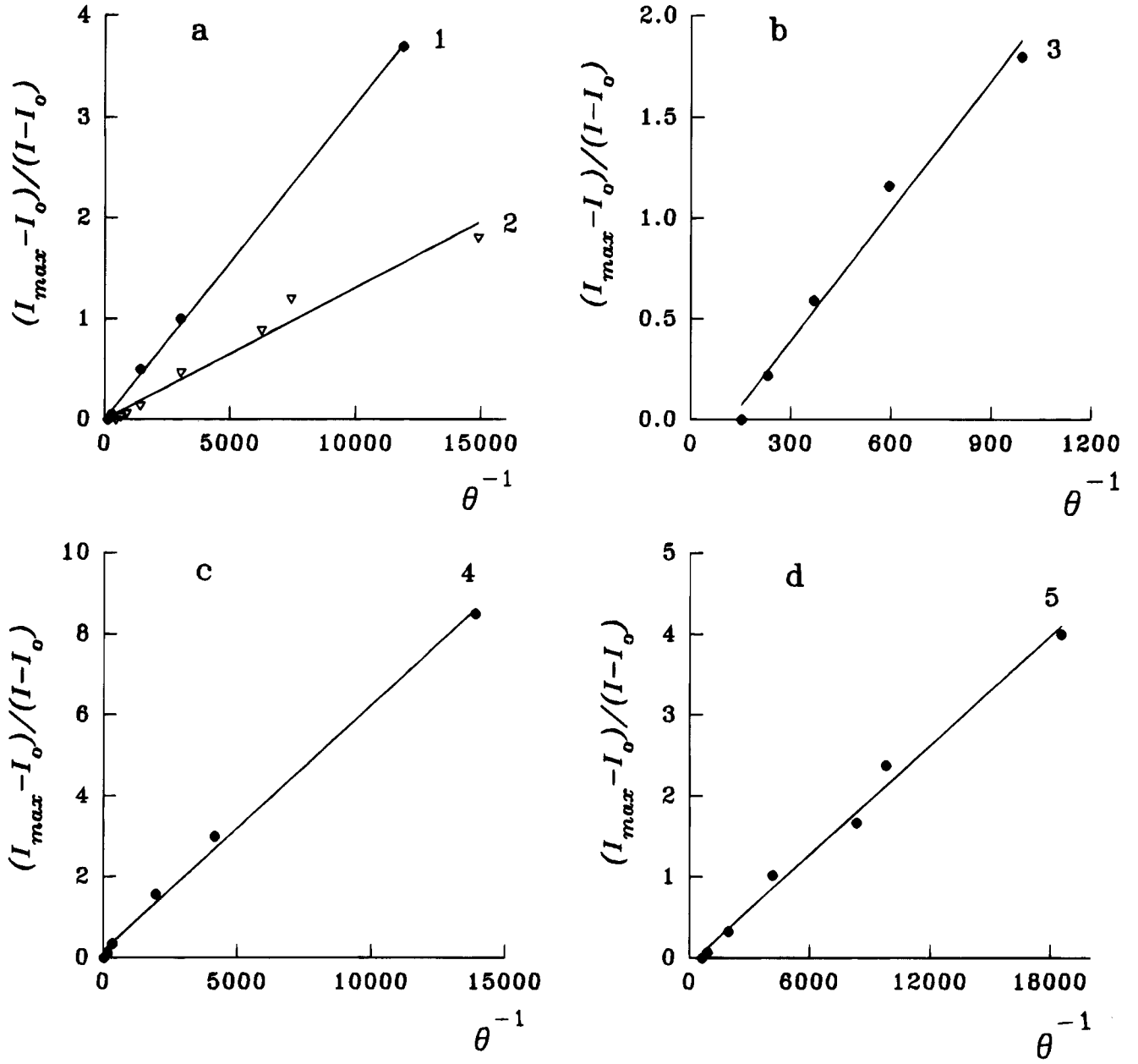


Figure 10. $(I_{max} - I_o)/(I - I_o)$ versus $1/\theta$ plots obtained for DPH (1, 4) and pyrene (2, 3, 5) solubilized in Pluronic F108 (a) and P85 (b–d) micelles at 37 °C (1, 2, 4, 5) and 20 °C (3). DPH: λ_{ex} = 380 nm, λ_{em} = 430 nm; pyrene: λ_{ex} = 333 nm, λ_{em} = 395 nm.

Table 5. Partitioning Coefficients of Pyrene and DPH in Pluronic Micelles

fluorescent probe	Pluronic	partitioning coeff, P^a 20 °C	37 °C
pyrene	P85	460 ± 30	3260 ± 260
	F108	n.d.	1640 ± 90
DPH	P85	n.d.	7630 ± 550
	F108	n.d.	4450 ± 190

[a] The P values are determined from the slope of the experimental curves presented in Figure 10 using the Sigma Plot 5.0 fitting program. The values are mean ± SD.

registered under these conditions, incorporation of such small amounts of probes does not affect the micelle characteristics.

Conclusion

Micellization of Pluronic polymers F68, P85, and F108 in water solutions was studied and cmc values were measured. The dependence of cmc on temperature was observed, and the cmt values characterizing temperature-dependent transitions of Pluronic unimers to multimolecular micelles were determined. The cmc and cmt parameters determine the micelle stability during dissolution and temperature changes and they must be taken into account while using micelles as microcontainers for targeted drug delivery. According to our data at physiological temperature of 37 °C the cmc for P85 and F108 lie in the range 0.003–0.007% compared to the cmc of 4–5% for F68. The molecular characteristics of P85 and F108 micelles, i.e. size, molecular masses, and surfactant aggregation numbers, were determined using light scattering and ultracentrifugation. The data for P85 are consistent with those reported previously. Depending on the Pluronic type, the micelles had an average hydrodynamic diameter ranging from about 15 to about 35 nm, a molecular mass of about 200 kDa, and aggregation numbers ranging from one to several dozens. During application as drug carriers, such particles could easily penetrate into capillaries in body tissues and fit inside the endocytic vesicles of target cells. The partitioning of probe molecules between the

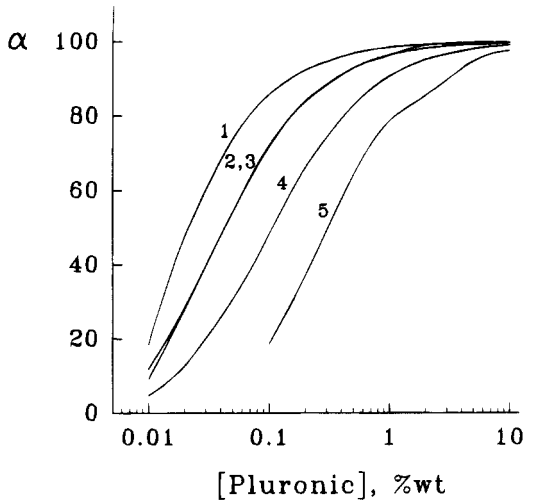


Figure 11. Dependencies of the portion of the micellar probe (a) on the Pluronic concentration obtained using the experimental values of the partitioning coefficients presented in Table 5: (1) DPH in Pluronic P85 at 37 °C; (2) DPH in Pluronic P108 at 37 °C; (3) pyrene in Pluronic P85 at 37 °C; (4) pyrene in Pluronic P108 at 37 °C; (5) pyrene in Pluronic P85 at 20 °C.

aqueous and micellar phases was analyzed within the frame of a pseudophase model, and the partitioning coefficients were determined from the fluorescence data using pyrene and DPH. Behavior of Pluronic polymers and model compounds in aqueous solutions does not necessarily predict their utilization as drug carriers and much of the work in this direction should be carried out.

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