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ORIGINAL CONTRIBUTION

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Evaluation of partition coefficients of low molecular weight solutes between water and micelles of block copolymer of ethylene oxide based on dialysis kinetics and fluorescence spectroscopy

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Abstract Application of micelle-forming surfactants in various fields of technology and use of micelles as carriers for drug targeting requires simple evaluation of partitioning coefficients of different solutes between water solution and micellar microphase. A number of methods of partitioning evaluation have been already proposed, but most of them are applicable only for fluorescent dyes or require complicated and expensive equipment, such as NMR spectrometers. In the present work two methods of the molar fraction of solubilized material determination are compared. One of the techniques used is based on the changes in fluorescence spectra during solubilization. The other approach is based on the dialysis slowing down, which occurs if the solute is associated with large particles (e.g. micelles). Graphic method of partition coefficient (P) evaluation is proposed and P values of a widely used anti-tumor drug doxorubicin and highly hydrophobic fluorophore perylene are determined. The comparison shows that the results of both methods give very close coincidence. The solubilization data were used for the calculation of the important parameter – the ratio of micelle volume to aggregation number, which makes sense of packing density of the detergent molecules in the micelle. The comparison of this parameter calculated from the solubilization data with that obtained from light scattering experiments is discussed. It is assumed that the dialysis technique could be used for partitioning evaluation of all substances, which may be detected in solution.

Key words Micelles – partition coefficient – pluronic P85 – doxorubicin – perylene – dialysis

Introduction

Micellar systems are now widely used as microcontainers for drug delivery [1–6]. So, a number of approaches to the evaluation of partition coefficients of drugs and other solutes between water solution and micellar microphase have already been suggested [7–11]. The majority of the methods have been developed for fluorescent dyes and are based on the changes in electronic or fluorescent spectra occurred after transfer of the solute from aqueous media to micellar microphase [7–9]. In some cases fluorescence is quenched by oxygen present in the water media [9], in the other works quenching was achieved by salts presented in water [7–8].

NMR spectroscopy was also used for evaluation of partitioning between water medium and surfactant micelles [10]. This approach is feasible not only for the

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fluorescent dyes but also for the majority of substances, nevertheless, it is rather complicated and expensive. Obviously that is why only few examples of magnetic resonance spectroscopy application for partitioning evaluation are available in the literature.

Another approach is based on the measurement of critical micellization concentration (CMC) in the presence of solubilized hydrophobic solutes [11]. This method is rather simple but can be applied for very hydrophobic substances, which strongly interact with the micelle structure and change the CMC values.

We suggest a simple method of determination of partition coefficient feasible for all substances that can be detected in solution.

Dialysis has been proposed at least 15 years ago for the evaluation of drug release from colloidal and polymeric carriers [12]. This method appears to have evolved from experiments designed to study release from systems, which are applied topically. The main principle of partitioning evaluation between bulk phase and colloidal particles from dialysis kinetics data has already been suggested [13]. This approach is based on the measurement of the initial rate of diffusion of the solute from the micellar solution across the semi-permeable membrane. Colloid particles are large enough not to penetrate across the membrane; thus entrapment of the solute into the particles results in the decrease of the diffusion rate. The measurement of the diffusion slowing down allows to calculate the partition coefficients. Recently, it has been applied for the evaluation of partitioning of the fragrances between micelles of pluronic P85 and bulk solution [14]. In the present work we have compared two methods of the partition coefficients evaluation and applied the solubilization data for the determination of the surfactant micelle packing density.

Among micellar systems special attention is paid to the micelle-forming block copolymers of ethylene oxide and propylene oxide (pluronics) [14–19]. These polymers are widely explored as microcontainers for drug targeting [1–6]. So, we investigated the ability of these micelles to solubilize anti-tumor antibiotic, doxorubicin, and highly hydrophobic fluorophore perylene.

Materials

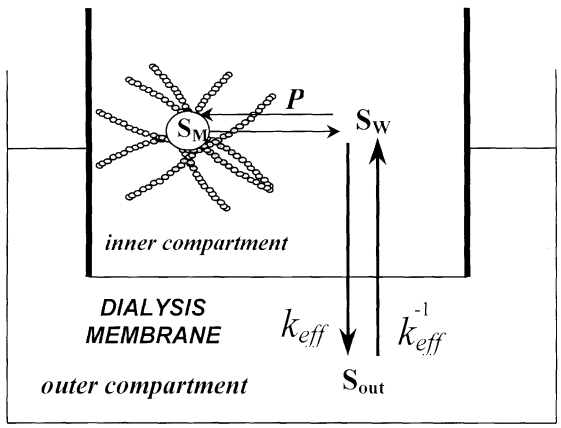
Block copolymer of ethylene oxide and propylene oxide (pluronic P85) was purchased from "Serva". Doxorubicin hydrochloride (95% purity according to liquid chromatography) was purchased from Russian Antibiotic Institute. Perylene was purchased from "Sigma". Samples of cellulose dialysis membranes were kindly presented by Dr. A.I. Dyachkov from "Polymersynthesis Ltd." (Russia).

Methods

Dialysis kinetics measurement

200 μl of buffer solution (10 mM NaH_2PO_4), 150 mM NaCl, and 7.4 (PBS)), containing 40 μM of the solute (doxorubicin or perylene) and pluronic P85 (0.02–5% (w/v)) were placed into the tube covered with dialysis membrane (Fig. 1). The tubes (inner diameter 4 mm) were fixed in the disposable fluorescent cuvettes ("Sarstedt"), containing 1 ml of PBS solution of pluronic (0.02–5% (w/v)) and lactose (40 μM). The composition of the outer compartment was determined by the necessity of the osmosis compensation. It should be mentioned that partition coefficient was calculated from the initial rate of the diffusion which does not depend on the outer compartment composition, whereas osmosis strongly interferes with the diffusion kinetics. The cuvettes were shaken vigorously at 37 °C and the solute concentration was detected in the outer solution at fixed time intervals. In our experiments we used fluorescent solutes, so fluorescent spectroscopy was used for its concentration estimation. Doxorubicin and perylene concentrations were detected at excitation wavelengths 490 and 350 nm and emission wavelengths 590 and 443 nm, correspondingly. The final fluorescence intensity (I_∞) was measured after mixing of the inner and outer solutions. First-order kinetic constants of dialysis of the solute from pluronic solutions at various pluronic concentrations were calculated. In the control experiments

Fig. 1 Schematic representation of the method of evaluation of the molar portion of the solubilized material based on dialysis kinetics. Inner solution contains micelle forming detergent and the solute. Outer solution contains the same concentration of the detergent and some inert solute (e.g. lactose in our experiments) which compensate osmosis. Molar portion of the solubilized material α and partition coefficient P can be calculated from the initial dialysis rate constants at various micelle concentrations



rate constant of the solute dialysis from aqueous solution was determined.

Special attention should be paid to the standardization of the experimental procedure. All samples containing various pluronic concentrations should be shaken with equal intensity. Membranes should be characterized with narrow pore size distribution to achieve reproducibility of experimental data. A number of commercially available membranes do not give satisfactory results.

Fluorescence spectroscopy measurements

Fluorescence spectra of doxorubicin and perylene were measured using Hitachi S-4000 spectrofluorometer with thermostattable cuvette holder. Pluronic solutions of various concentrations in 0.01 M phosphate buffer with 150 mM NaCl pH 7.4, containing 10 μM of doxorubicin or 1 μM of perylene were incubated at 37 °C for at least half an hour and the fluorescence spectra were measured. Excitation wavelength was 490 nm for doxorubicin and 350 nm for perylene.

Partition coefficient calculation

Partition coefficient P of the solute between water phase and micellar microphase is defined as the ratio of the concentrations of the solute in the corresponding phases:

$$P = \frac{[S]_m}{[S]_w}, \tag{1}$$

where $[S]_m$ and $[S]_w$ are the molar solute concentrations in the micellar and water phases, calculated per volume of the corresponding phase. Denote specific volume of the micellar phase as $\Theta = V_m/(V_m + V_w)$, where V_m and V_w are the volumes of micellar and water phases, respectively. Thus, we can present partition coefficient via molar concentrations of the solute in micellar (C_m) and water (C_w) phases, calculated per total system volume.

$$P = \frac{C_m}{C_w}\left(\frac{1-\Theta}{\Theta}\right). \tag{2}$$

Thus, the molar portion of the solute associated with micelles α can be presented by P and Θ

$$\alpha = \frac{C_m}{C_m + C_w} = \frac{P\Theta}{P\Theta + 1 - \Theta}. \tag{3}$$

The molar portion of the solute associated with micelles is an experimental value and can be calculated from dialysis kinetics or fluorescent spectra. Specific volume of the micellar phase (Θ) may be expressed using the pluronic percent concentration (C_{pl}), its critical micellization concentration (CMC), micelle aggregation number (q), average volume of one micelle (v_o), molecular weight of pluronic (M) and Avogadro number (N_A)

$$\Theta = \frac{10 N_A v_o (C_{pl} - \mathrm{CMC})}{qM}. \tag{4}$$

Equations (3) and (4) can be transformed to give a simple expression which determines a linear dependence

$$\frac{1}{\alpha} = \frac{qM}{10P(C_{pl} - \mathrm{CMC})N_A v_o} + 1 - \frac{1}{P}. \tag{5}$$

Thus, a partition coefficient P can be evaluated from the intercept of the linear graph of α^{-1} versus $(C_{pl} - \mathrm{CMC})^{-1}$.

Determination of α from the dialysis kinetics is based on the fact that dialysis rate is proportional to the solute concentration in the water phase (S_w) of the micellar solution

$$\frac{d[S_{out}]}{dt} = k[S_w]. \tag{6}$$

$[S_w]$ being a function of α, Eq. (6) could be transformed into

$$\frac{d[S_{out}]}{dt} = k([S_o] - [S_{out}])(1 - \alpha), \tag{7}$$

where $[S_{out}]$ is the solute concentration in the outer dialysis vessel $[S_o]$ is the final concentration of the solute, achieved after equilibration of the dialysis and k is the first-order kinetic constant of the process. Integration of this equation leads to the expression (8)

$$\ln\frac{[S_o] - [S_{out}]}{[S_o]} = -k(1-\alpha)t. \tag{8}$$

So, the effective dialysis rate constant

$$k_{eff} = k(1-\alpha) = k\frac{1-\Theta}{1-\Theta+P\Theta}. \tag{9}$$

The last expression can be used for the determination of the molar portion of the solute associated with micelles, α, from the kinetic data.

Another way of α determination is based on the observation that solubilization of fluorescent dyes in micelles results in fluorescent spectra changes [17]. The dependence of fluorescence intensity on the micelle concentration is represented as hyperbolic curve: if all dye is solubilized in the micelles its fluorescence spectrum does not change upon the increase of the micelles concentration. So, one could assume that the intensity corresponding to the

bounding level (I_∞) corresponds to the fluorescence of the solute associated with micelles. Thus, the molar portion of the solute associated with micelles can be calculated as

$$\alpha = \frac{I - I_0}{I_\infty - I_0}, \tag{10}$$

where I is the fluorescence intensity of the sample, containing fluorescent dye and pluronic, and I_o is the fluorescence intensity of aqueous solution of the pure dye.

α values determined by both the described techniques were used for graphic evaluation of partition coefficients. Linear graph of $1/\alpha$ as a function of $(C_{pl} - CMC)^{-1}$ cuts off an intercept on the ordinate axis equal to $1 - 1/P$.

Evaluation of the solute partition coefficients in biphasic *n*-octanol–water system

Log P value of perylene was taken from review of Leo et al. [20]. The same value for doxorubicin was measured according to the standard procedure [20]. Briefly, *n*-octanol ("Sigma") was saturated with phosphate buffer saline (PBS) by thorough mixing of equal volumes of octanol and buffer and doxorubicin (0.025 mM) was dissolved in this system. The biphasic system was shaken for 24 h at room temperature, the phases were separated and doxorubicin concentrations in both phases were detected by fluorescent method. Calibration curves were measured in octanol saturated with PBS and in PBS, saturated with octanol.

Results

Evaluation of partition coefficients by fluorescence spectroscopy

Fluorescence spectra of doxorubicin and perylene alter strongly upon the increase of pluronic P85 concentration (Fig. 2). In the case of perylene solubilization of the substance in pluronic micelles results in the increase of the total quantum yield and fluorescence intensity at 443 nm (Fig. 2a). So, changes in the fluorescence intensity at this wavelength depend on the polarity of the dye microenvironment. Intensity is growing up when pluronic concentration increases levelling at 1–2% of pluronic (Fig. 3a). Molar fraction of solubilized perylene was calculated according to Eq. (10). In contrast to hydrophobic perylene, fluorescence intensity of more hydrophilic doxorubicin is less sensitive to pluronic concentration (Fig. 2b). So, in this case the ratio of intensities at two wavelengths (590 and 550 nm) is a more sensitive parameter characterizing the microenvironment of the doxorubicin (Fig. 3b). This ratio was used for the calculation of the molar fractions of solubilized doxorubicin according to Eq. (11), which is analogous to Eq. (10)

$$\alpha = \frac{(I^{590}/I^{550}) - (I^{590}/I^{550})_0}{(I^{590}/I^{550})_\infty - (I^{590}/I^{550})_0}. \tag{11}$$

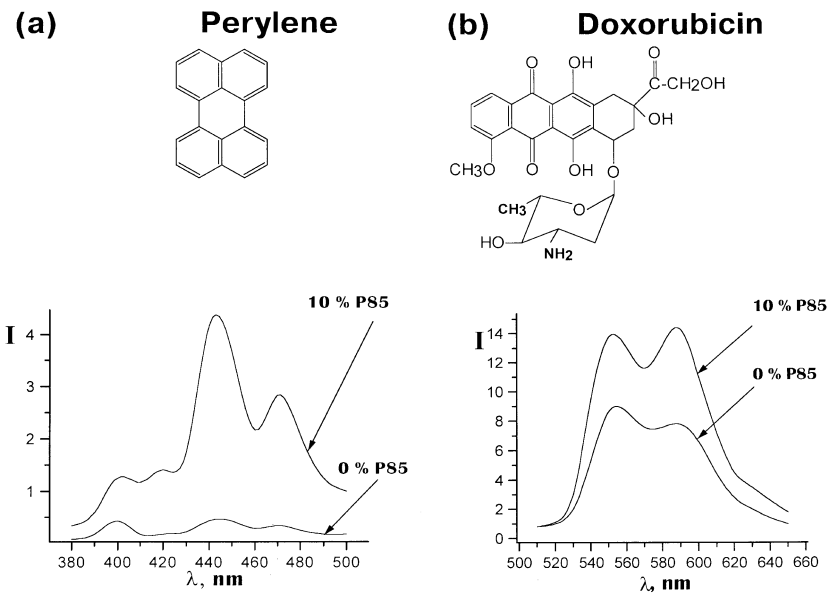


Fig. 2 Fluorescence spectra of perylene (a) and doxorubicin (b) in phosphate buffered saline in the presence and in absence of pluronic micelles. Excitation wavelength was 350 nm for perylene and 490 nm for doxorubicin

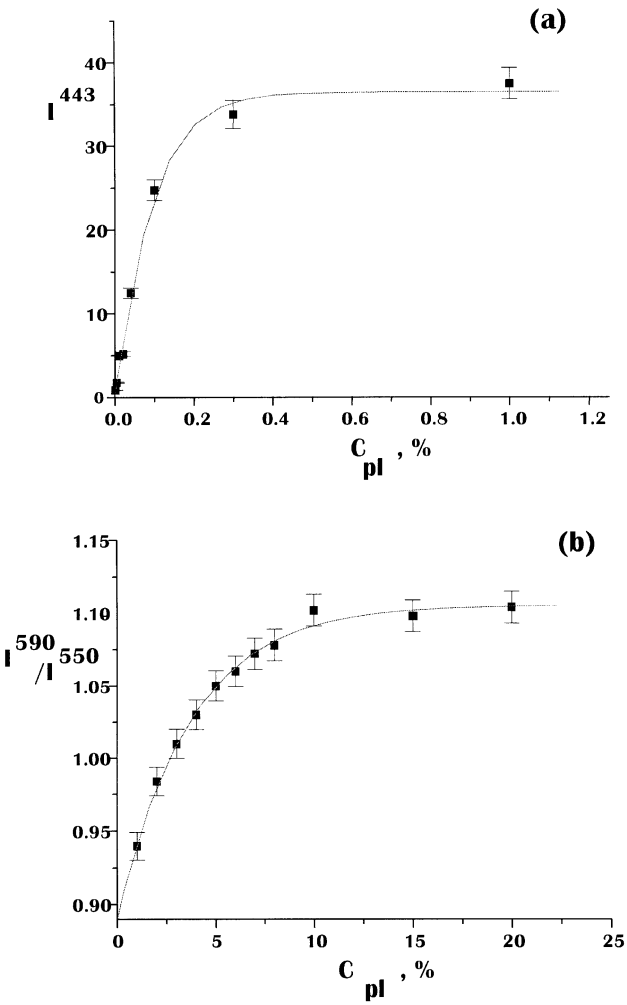


Fig. 3 Dependence of fluorescence intensity at 443 nm of perylene (a) and the ratio of intensities at two wavelengths (590 and 550 nm) of doxorubicin of pluronic P85 concentration (phosphate buffered saline, pH 7.4, 37 °C)

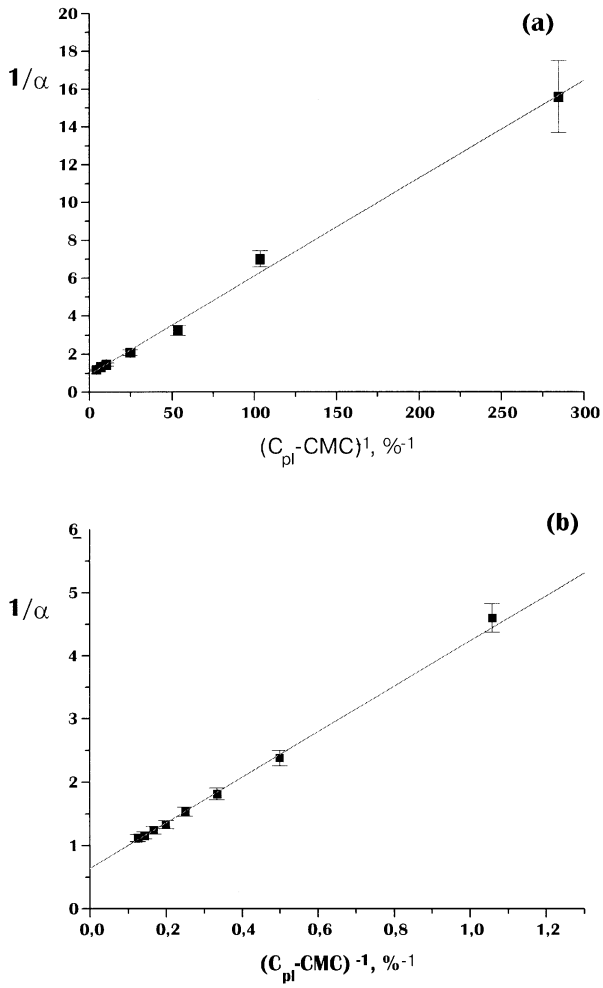


Fig. 4 Linearization of the dependence of the molar portion of solubilized perylene (Fig. 4a) and doxorubicin (Fig. 4b) (α) versus pluronic concentration. α was determined from the fluorescent data. Experimental conditions are given in the legend to Fig. 2

Linearization of the dependence of α versus pluronic concentration is shown in Fig. 4. Calculation of the partition coefficient from this data gives values of 2.5 ± 0.2 for doxorubicin. It means that doxorubicin interaction with micelles is very weak. On the contrary, perylene partition coefficient cannot be calculated from the intercept because of its very high value. Nevertheless, partition coefficient of highly hydrophobic perylene can be evaluated from the slope of the graph on Fig. 4a. As it follows from Eq. (5), the slope of this line depends on the partition coefficient and the parameters of micelle structure: aggregation number, micelle volume and pluronic molecular weight. This combination of micelle parameters can be evaluated from graph 4b, where partition coefficient can be determined from the intercept on the ordinate axis. The calculation of the perylene partition coefficient gives the value of 193 ± 25, which is obviously much higher than the value obtained for doxorubicin.

Measurement of the partition coefficients by dialysis kinetics technique

The method of α determination from the decrease of the solute dialysis rate is based on the measurement of the first-order constants of the solute diffusion across the dialysis membrane (Fig. 5). Control experiments revealed that adsorption of the solute on the membrane gives negligible contribution to the overall dialysis kinetics. Certainly, the absolute diffusion rate strongly depends on the form of the dialysis apparatus, pore size, thickness of the dialysis membrane and intensity of shaking of the solution during the

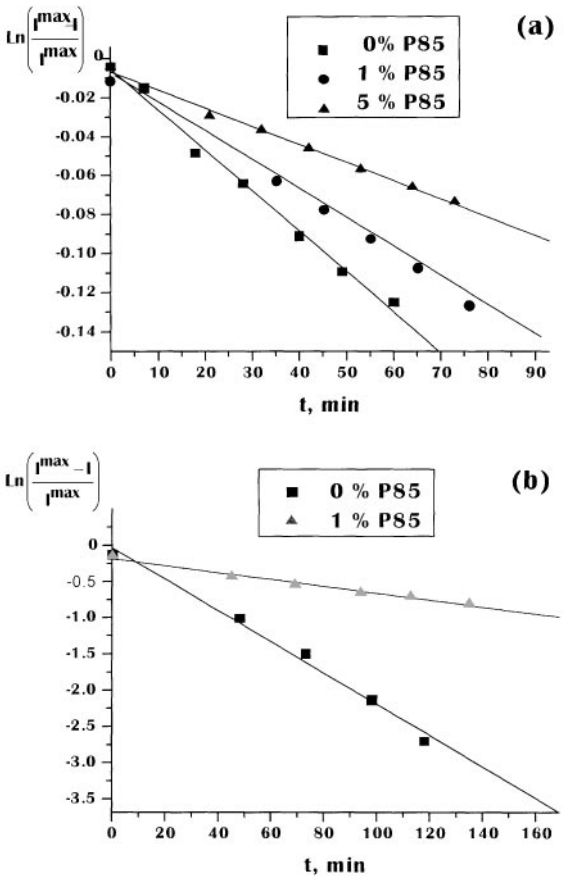


Fig. 5 First-order kinetics of doxorubicin (a) and perylene (b) dialysis from the pluronic solutions (phosphate buffered saline, pH 7.4, 37 °C)

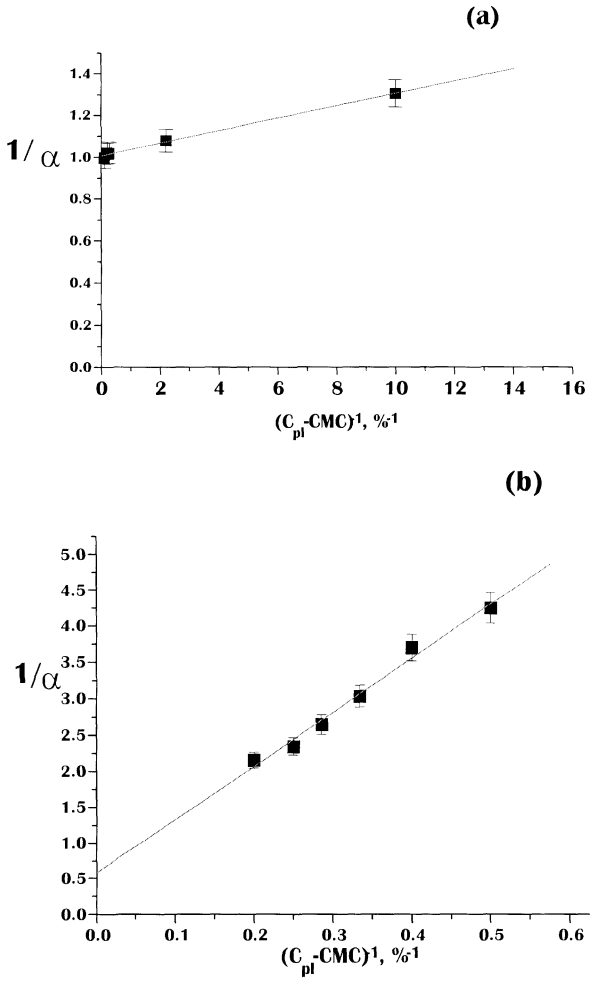


Fig. 6 Linearization of the dependence of the molar portion of solubilized perylene (Fig. 6a) and doxorubicin (Fig. 6b) (α) versus pluronic concentration. α was determined from the dialysis kinetics data (phosphate buffered saline, pH 7.4, 37 °C)

dialysis as well. Nevertheless, all these parameters being constant, dialysis rate decreases if the pluronic concentration increases (Fig. 5). Evaluation of α from Eq. (9) gives $\alpha = 1 - k_{\text{eff}}/k^0_{\text{eff}}$, where k_{eff} is the effective first-order constant of the solute dialysis from the micellar solution and k^0_{eff} is the first-order constant of the solute dialysis from regular water solution. Both the constants should be determined in the same experiment, so as to avoid deviations of the experimental conditions. Each constant was evaluated as average mean of six samples taken in one experiment.

Treatment of the dependence of α versus pluronic concentration (Fig. 6) gives the partition coefficient value of 2.4 ± 1.2 for doxorubicin which is quite similar to that obtained by fluorescent method. The value of perylene partition coefficient determined by dialysis technique ($P = 215 \pm 56$) is also very close to the value measured by fluorescent spectroscopy ($P = 193 \pm 25$). So, one can assume the dialysis kinetics technique does work and can be widely used for the partition coefficients determination of substances which have no specific fluorescence spectrum.

Discussion

Partition coefficients determined in our work give a quantitative measure of interaction of the solute with polymeric micelles. The partition coefficients of both the investigated solutes, determined by the two independent methods, are shown in Table 1. It should be mentioned that the results of both the methods are in good agreement. The interaction of low molecular weight solutes with pluronic micelles depends on their total hydrophobicity. The evaluation of perylene and doxorubicin hydrophobicities according to Leo et al. [20] shows that doxorubicin is much more hydrophylic substance than perylene (Table 1). Therefore, the most substantial driving force of solubilization in pluronic micelles is hydrophobic interaction.

Table 1 Perylene and doxorubicin partition coefficients between water and pluronic P85 micelles estimated by the two different methods and their partition coefficients in *n*-octanol–water biphasic system

Substance	Log *P* micelles P85–water estimated by the dialysis method	Log *P* micelles P85–water estimated by the fluorescent spectra method	Log *P* *n*-octanol–water, according to Leo et al. [20]
Perylene	2.33 ± 0.10	2.28 ± 0.05	6.4 ± 0.3
Doxorubicin	0.38 ± 0.17	0.44 ± 0.02	-0.95 ± 0.03

Table 2 Pluronic P85 micelle parameters determined by different methods

Method of α evaluation	v_o/q [nm^3]
Dialysis kinetics	42 ± 15
Fluorescence spectra changes	75 ± 7
Low-angle light scattering and neutron scattering technique	38[a)]

[a)] as determined in [20].

Equation (5), which determines the dependence of the solubilized material molar portion on the pluronic concentration, gives an opportunity to evaluate the structural parameters of micelle – ratio of micelle volume and its aggregation number. This ratio is a measure of a portion of micellar volume per one detergent molecule. In other words, it is a measure of the packing density of the detergent molecules in the micelle. Obviously this parameter does not depend on the nature of the solubilized substance and is determined by the structure of the surfactant and its micelles. Nevertheless, this parameter depends on the method of α evaluation. Comparison of the v_o/q parameters of pluronic *P*85, determined by different methods, given in Table 2, leads to the conclusion that the dialysis method gives v_o/q values very close to that obtained by traditional small-angle neutron scattering technique. On the contrary, v_o/q ratio, determined by dialysis kinetics proves to be 1,8 times less, than the parameter, evaluated from the fluorescence spectra changes (Table 2).

The reasons of such a phenomenon can be elucidated considering that the dialysis rate decreases when the solute adsorbs on the micelle surface whereas fluorescence spectra may undergo changes when the environment polarity decreases. Such changes in the polarity of microenvironment can take place near the micelle and not only inside its hydrophobic core. Therefore, the dialysis method of α determination gives more reliable information about packing density of the detergent molecules in the micelle, whereas fluorescence technique obviously gives higher α compared to real values. Nevertheless, this diversity between the two methods is not fundamental and the both techniques give rather close values of the partition coefficients.

We suppose that the dialysis kinetics method of the determination of the molar portion of the micelle-associated solute is feasible not only for the investigation of the partitioning in the polymeric surfactants solutions, but also could be used for measuring interaction of any detergent micelles or other molecular aggregates with low molecular weight molecules. The method can also be applied for the evaluation of the partitioning of substances which have no characteristic fluorescent spectra but could be determined by chromatographic or radioactive methods. So, we hope that the dialysis technique will be a useful tool in different physico-chemical and biochemical laboratories.

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