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Solubilization of human erythrocyte membranes by non-ionic surfactants of the polyoxyethylene alkyl ethers series

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Abstract

In the present study, we investigated the interaction of the non-ionic surfactants polyoxyethylene alkyl ethers (C_nE_m) with erythrocyte membranes. For this purpose we have performed hemolytic assays under isosmotic conditions with five surfactants in the 8 polyoxyethylene ether series. By applying to the hemolytic curves a quantitative treatment developed for the study of surface-active compounds on biomembranes, we could calculate the surfactant/lipid molar ratios for the onset of hemolysis (R_e^{sat}) and for complete hemolysis (R_e^{sol}). This approach also allowed the calculation of the binding constants for each surfactant to the erythrocyte membrane. Results in the C_nE_m series were compared to those obtained for Triton X-100, a well-known non-ionic surfactant with values of *cmc* and HLB in the range of the alkyl ethers studied. Inside the series the lytic effect increased with the more hydrophobic homologues ($C_{10}E_8 < C_{12}E_8 < C_{14}E_8 < C_{16}E_8 < C_{18}E_8$), with *Re* values between 3:1 and 0.03:1. The effect of $C_{10}E_8$ and $C_{12}E_8$ was found to be in the range of that caused by Triton X-100, proving that C_nE_m surfactants are strongly hemolytic.

Keywords: Erythrocyte; Membrane; Surfactants; Hemolysis; Solubilization

1. Introduction

Most non-ionic surfactants are ethylene oxide derivatives and they have an enormous economic importance worldwide since their detergency, wetting and foaming properties allow their use in industrial and household products [1]. Other applications of non-ionic surfactants include the extraction of membrane proteins [2–6] or preparation of drug-delivery formulations [7,8].

Non-ionic surfactants are also used in membrane studies, since they can induce solubilization of the bilayer [5,9–11] leading to mixed-micelle formation. In 1985, Lichtenberg proposed a treatment to evaluate the solubilizing effect of surface-active

Abbreviations: *cmc*, critical micelle concentration; C_nE_m, polyoxyethylene alkyl ether; HLB, hydrophilic–lipophilic balance; Ht, hematocrit; PBS, phosphate buffered saline; M_w, molecular weight; Triton X-100, *t*-octylphenoxypoly ethoxyethylene.

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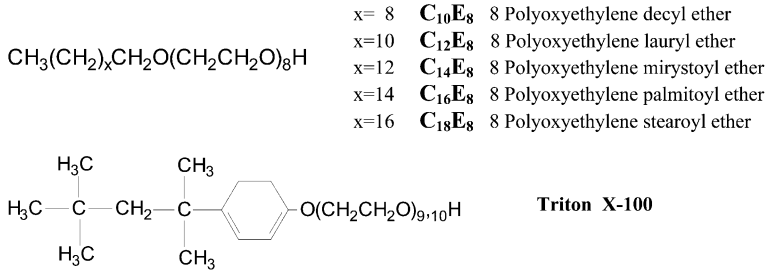

Fig. 1. Chemical structure of the non-ionic surfactants studied.

compounds on biomembranes [12] allowing the determination of the effective surfactant/lipid molar ratio in the membrane for the lytic effect. Although many studies in the literature have reported the lytic effect of detergents such as Triton X-100 [13–15], Tween [16–18], Texofor [19] and Renex [11], only the group of Goñi [10,20–22] applied this treatment to the study of solubilization of lipid and biological membranes by non-ionic surfactants.

The present investigation is a quantitative study of the interaction of non-ionic 8 polyoxyethylene alkyl ether surfactants (C_nE_8) with erythrocyte membranes using Lichtenberg's treatment [12]. The solubilizing properties of five homologous compounds in this series (Fig. 1) are presented and compared to those of Triton X-100, the best studied non-ionic surfactant so far, whose MW, *cmc* and HLB are in the range of those for the surfactants studied.

2. Materials and methods

The surfactants ($C_{10}E_8$, $C_{12}E_8$, $C_{14}E_8$, $C_{16}E_8$, $C_{18}E_8$ and Triton X-100) were obtained from the Sigma Chemical Co.

2.1. Erythrocytes

Freshly obtained human blood was collected into a stock solution containing 26.3 g/l trisodic citrate, 3.27 g/l citric acid, 31.9 g/l dextrose, 2.2 g/l sodium phosphate and 0.275 g/l adenine, and washed three times in 5 mM PBS buffer, pH 7.4.

2.2. Hemolytic assay

The surfactants were added to erythrocyte suspensions (0.075–0.45% hematocrit, diluted in PBS), and the samples were kept for 15 min at 37 °C. After centrifugation at $260\times g$ for 3 min, the released hemoglobin concentration in the supernatant was measured at 412 nm. The hemolytic effect, expressed as percentage hemolysis (%), was determined as described by Malheiros et al. [23]. Triplicate test tubes and at least two different blood samples were used for each curve.

2.3. R_e (*surfactant/lipid ratio*) *calculation*

The analysis of membrane solubilization proposed by Lichtenberg [12] was applied, considering C^{sat} and C^{sol} as the surfactant concentration required to induce saturation (the onset of hemolysis) and total membrane solubilization (total lysis), respectively [23,24]. These concentrations were determined from the hemolytic curves, and plots of C^{sat} and C^{sol} as a function of membrane lipid concentration allowed the determination of R_e, the effective surfactant/lipid molar ratio for initial (saturation, R_e^{sat}) and total hemolysis (solubilization, R_e^{sol}), according to Eq. (1) [10,25]:

$$D_t = R_e[L + 1/K_b(R_e + 1)] \quad (1)$$

where D_t is the total surfactant concentration (C^{sat}, C^{sol}) and L is the lipid concentration in the system. The slope of the resulting straight lines allows R_e calculation while the y intercept corresponds to D_w, the concentration of free detergent in water, [12,26]. Finally, K_b (M^{-1}), the molar binding constant of the surfactants to the erythro-

cyte membrane, could easily be derived from Eq. (2) [12,26]:

$$R_e = K_b \cdot D_w / (1 - K_b \cdot D_w) \quad (2)$$

As in previous publications [10,23,24] K_b was calculated just from the value of R_e^{sat}, since at 100% solubilization (R_e^{sol}) no more membrane, but mixed-micelles are present [51], which may give rise to different K_b values.

3. Results and discussion

Non-ionic polyoxyethylene alkyl ethers are ethylene oxide adducts of linear alcohols (Fig. 1) that have been used with different approaches such as membrane protein extraction [27–31], phase-separation of chemical compounds [1,32], or as a co-adjuvant in drug-delivery systems [7,8,33].

While lipid membrane solubilization triggered by Triton X-100 is a fairly well described phenomenon [10,20,34,35] and its hemolytic action has been studied [13,14,36–38], the interaction of polyoxyethylene alkyl ethers with lipid membranes [39,40] has not been investigated to any great extent. Even papers devoted to the study of the effect of C_nE_m on erythrocyte membranes [41–47] were mainly related to the pre-lytic stage or did not present a quantitative approach to the hemolytic phenomena.

Hemolysis is the disruption of the red blood cell and can be caused by the interaction of chemical compounds with the membrane. The effect of amphiphilic compounds on the stability of erythrocyte membranes tends to be biphasic; at small drug/lipid molar ratios they protect erythrocytes against hypotonic lysis, whereas at higher molar ratios in the membrane they induce hemolysis [24,48–50].

Fig. 2 presents the hemolytic curves obtained with increasing concentrations of $C_{10}E_8$ and $C_{18}E_8$ in erythrocyte suspensions (Ht = 0.075, 0.15, 0.30 and 0.45%) under isotonic conditions and at pH 7.4. Arrows indicate the surfactant concentration for the onset (C^{sat}) and 100% (C^{sol}) solubilization on the curve of $Ht = 0.075\%$.

Table 1 lists C^{sat} and C^{sol} values obtained from hemolytic curves as those illustrated in Fig. 2 for all the detergents studied. The values of C^{sat} and C^{sol} for Triton X-100 lay between those for $C_{10}E_8$ and $C_{12}E_8$ solubilization. Inside the polyoxyethylene alkyl ethers, C^{sat} and C^{sol} decreased with increases in the hydrophobic portion of the surfactant, which reflects the importance of the hydrophobic interaction in the hemolytic process. Increased erythrocyte membrane binding with detergents of longer hydrophobic tails has been described before—at hyposmotic condition—for C_nE_m, anionic, cationic and zwitterionic series [43]. It is interesting to note that in contrast to the criticism of other authors relative to hemolytic and anti-hemolytic studies [16,17,19,45], our results show that inside the C_nE_m series, HLB (Table 2) is a good parameter to evaluate the ability of a detergent to bind to the membrane and to be a good lytic agent; the lower the HLB, the smaller the C_nE_m concentration for membrane solubilization (C^{sat} and C^{sol}).

Solubilization involves hydrophobic interactions between the surfactant chains and the lipid or proteins of the membrane. According to Helenius and Simons [9], membrane solubilization by surfactants is dependent on aggregation. In general, it is accepted that the amount of surfactant required to solubilize the membrane increases with the *cmc* of the surfactant [5,13]. In fact, the relationship between the aggregative and solubilization properties of amphiphiles has been very well characterized [12], and reviewed by us in a recent paper [50]. We have also demonstrated that Lichtenberg's treatment could be useful to describe the hemolytic effect of non-classical surfactants such as the phenothiazine trifluoperazine [23,24].

Table 2 presents some physicochemical properties of the surfactants studied. The *cmc* and *HLB* values of Triton X-100 show its amphiphilic character comparable to those of $C_{10}E_8$ and $C_{12}E_8$. It is interesting to note that the HLB of the C_nE_8 compounds studied vary between 11.9 and 14.5—approximately equal to the value of Triton X-100, a good lytic detergent [50]—while the less lytic non-ionic surfactants Renex [11] and Tween [18] show higher HLB values, from 14 to 19.

C^{sat} and C^{sol} were plotted as a function of lipid concentration in Fig. 3, to give the straight lines predicted by Eq. (1). The corresponding surfactant/lipid molar ratios in the membrane, R_e, were

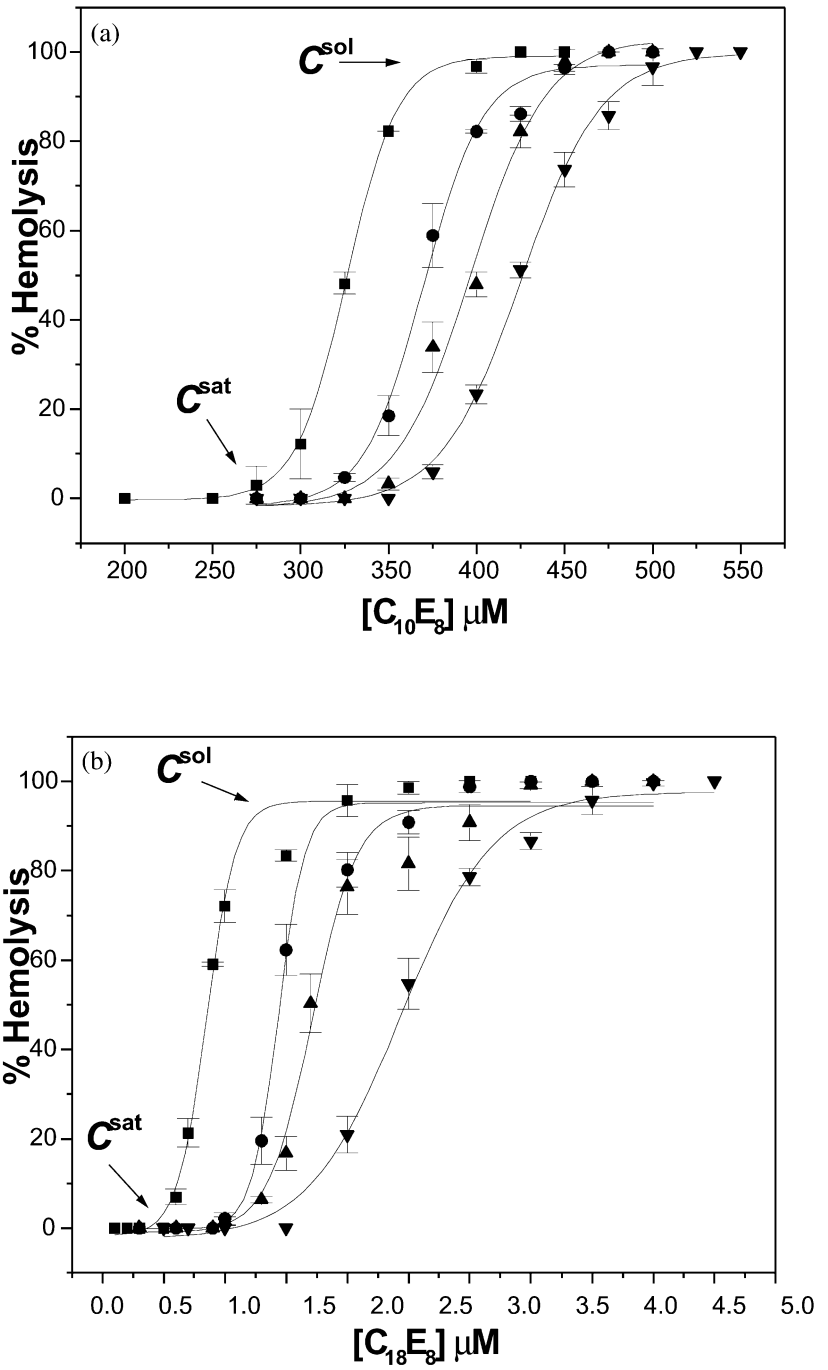

Fig. 2. Hemolytic effect of $C_{10}E_8$ (a) and $C_{18}E_8$ (b) upon human erythrocytes in 5 mM PBS buffer, pH 7.4, 37 °C. Ht = 0.075 (■), 0.15 (●), 0.30 (▲) and 0.45% (▼). The arrows show C^{sat} and C^{sol} (see text) determination in the 0.075% curve.

Table 1
Hemolytic effect of polyoxyethylene alkyl ethers, C_nE_8 surfactants and Triton X-100 upon human erythrocyte membranes

Surfactants	C^{sat} (μM)				C^{sol} (μM)			
Ht (%)	0.075	0.15	0.30	0.45	0.075	0.15	0.30	0.45
L (μM)	6.5	13	26	39	6.5	13	26	39
$C_{10}E_8$	295	335	352	375	354	406	441	475
$C_{12}E_8$	30.2	32.1	35.1	38.8	37.0	37.3	40.3	43.5
$C_{14}E_8$	5.9	6.1	7.4	10.2	6.6	6.8	8.5	12.5
$C_{16}E_8$	1. 9	2.2	2.9	6.8	2.8	2.8	5.2	8.2
$C_{18}E_8$	0.6	1.2	1.3	1.6	1.1	1.7	2.1	3.2
Triton X-100	117	131	169	184	192	222	213	255

L=lipid concentration in erythrocyte membranes corresponding to each hematocrit, calculated according to Malheiros et al. [24].

readily calculated from the saturation and solubilization lines in Fig. 3 and are listed in Table 3, as well as K_b. The values of D_w were calculated from the intercept of the straight lines in Fig. 3.

In the hemolytic tests some surfactants seems to be more affected by the micellization process, making it difficult to precisely determine the values of (and differences between) C^{sat} and C^{sol}. That would be the case of the more lytic $C_{16}E_8$ and $C_{18}E_8$, with the smallest *cmc* values; the energy involved in the transfer of monomers of surfactant and membrane lipids to the mixed-micelles are easily overcome, and C^{sat} and C^{sol} values verge upon each other, explaining the deviation from linearity in the straight lines predicted by Eq. (1) (Fig. 3b).

In a recent review, Lichtenberg et al. [51] mention that the D_w values reported in the literature are always smaller than the measured values of *cmc* ($D_w^{sat} < D_w^{sol} < cmc$), since the lipids offer an additional driving force for micelle formation, decreasing the *cmc* (in the presence of the membrane). In that way, the values of D_w presented in Table 3 are in very good agreement with literature data on Table 2.

The hemolytic process induced by surface-active compounds can be described as a bilayer-to-micelle transition. At low surfactant/lipid ratios, the lamellar structure of the erythrocyte membrane is maintained; at quite high ratios, mixed-micelles (composed by the surfactant, phospholipids and membrane-bound proteins) are formed and at intermediate ratios of surfactant/lipid, mixtures of these two types of aggregates are detected. R_e^{sat} and R_e^{sol} represent the limits, in terms of the detergent/lipid ratios, for the co-existence of mixed-membranes and mixed-micelles [51].

The R_e values obtained seem quite reasonable, considering the physicochemical properties of Triton X-100; the values of 1.58 and 2.15 for the onset of solubilization and for complete solubilization, respectively, are comparable to those obtained by other authors in the solubilization of liposomes. In 1974, Dennis [52] described that

Table 2
Physicochemical properties of the non-ionic surfactants: C_nE_8; and Triton X-100

Name	Abbrev.	MW	HLB[a]	*cmc* (M)[b]
8 Polyoxyethylene decyl ether	$C_{10}E_8$	510.7	14.47	9.7×10^{-4}
8 Polyoxyethylene lauryl ether	$C_{12}E_8$	538.8	13.71	8.8×10^{-5}
8 Polyoxyethylene miristoyl ether	$C_{14}E_8$	566.8	13.04	8.0×10^{-6}
8 Polyoxyethylene palmitoyl ether	$C_{16}E_8$	594.9	12.42	7.3×10^{-7}
8 Polyoxyethylene stearoyl ether	$C_{18}E_8$	622.9	11.86	5.9×10^{-8}
Octylphenoxypolyethoxyethylene	Triton X-100	625.0	13.50	2.5×10^{-4}

[a] Calculated according to Griffin [62].
[b] Data from Berthod et al. [1] for the C_nE_8 series, and Neugebauer [2] for Triton X-100.

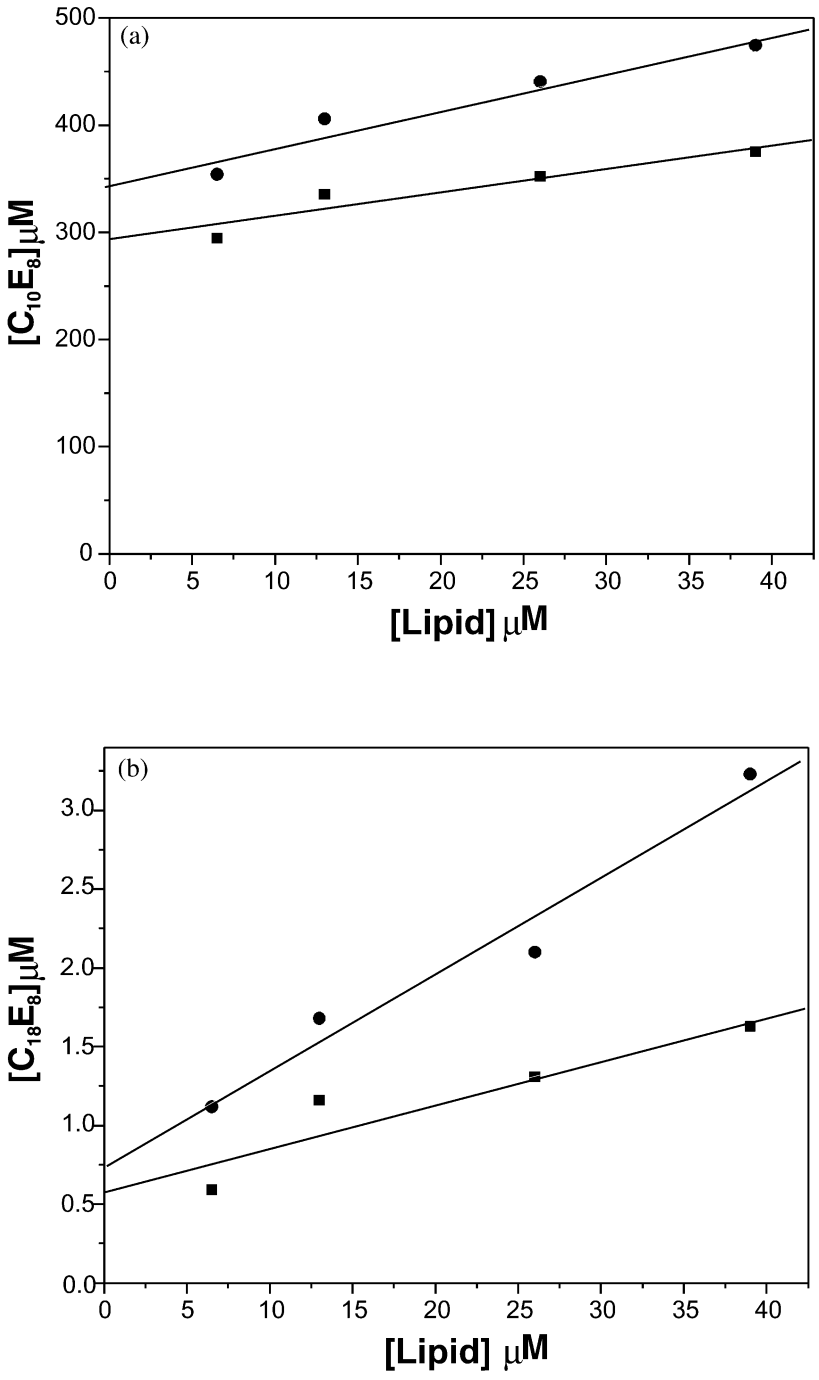

Fig. 3. Effective surfactant/lipid molar ratios for erythrocyte membrane saturation and solubilization: (a) $C_{10}E_8$; (b) $C_{18}E_8$; C^{sat} (■) and C^{sol} (●) were plotted as a function of erythrocyte lipid concentration. R_e values were taken from the slope of the straight lines.

Table 3
Effective surfactant/lipid molar ratios (R_e), *cmc* (D_w) and binding constant (K_b) in the lysis of erythrocytes by C_nE_8 surfactants and Triton X-100. Experimental condition as in Fig. 3

	$C_{10}E_8$	$C_{12}E_8$	$C_{14}E_8$	$C_{16}E_8$	$C_{18}E_8$	Triton X-100
R_e^{sat}	2.23	0.21	0.13	0.05	0.03	1.58
R_e^{sol}	3.45	0.26	0.18	0.13	0.06	2.15
D_w^{sat} (μM)	291.7	28.7	4.5	1.51	0.61	104.1
D_w^{sol} (μM)	346.4	35.1	4.7	1.56	0.75	188.0
K_b ($\times 10^3$ M^{-1})[a]	2.4	6.0	25.5	31.5	47.7	5.9

[a] Taken from the saturation curves in Fig. 3.

egg phosphatidylcholine bilayers were able to incorporate Triton X-100 up to a detergent/lipid molar ratio of 1:1; the author also reported that at ratios above 2:1, all the lipids were solubilized in mixed micelles. In a further work, Partearroyo et al. [10] described the solubilization of the same phosphatidylcholine vesicles in terms of the effective detergent/lipid molar ratios in the membrane for saturation and solubilization, giving R_e^{sat} and R_e^{sol} values of 0.7:1 and 3:1, respectively [10]. In erythrocytes, Loizaga et al. [37] calculated a Triton X-100/protein ratio of 4.7:1, corresponding to a 4:1 Triton X-100/lipid molar ratio at 50% membrane solubilization.

R_e values for the C_nE_m detergents prove that the extent of solubilization of biological membranes is related to the concentration of the detergent inside the membrane [9], since the higher the binding constant (Table 3), the stronger the lytic effect ($C_{18}E_8 > C_{16}E_8 > C_{14}E_8 > C_{12}E_8 > C_{10}E_8$) in the series.

K_b values reveal high affinities for all the C_nE_m surfactants studied to the erythrocyte membranes, explaining their stronger hemolytic effect, comparable to that of Triton X-100 [38].

The literature reports the anti-lytic effect of the $C_{10}E_8$ to $C_{16}E_8$ surfactants [42,43] and Triton X-100 upon human erythrocyte membranes; at sub-lytic concentrations, C_nE_m induced protection against hyposmotic hemolysis at lower concentrations than SDS, CTAB and zwitterionic detergents of equivalent hydrophobic tails. The pre-lytic stage of surfactant–membrane interaction is directly associated with the amphiphile's insertion into the bilayer [42,43]. In fact, the concentration for maximum protective effect reported for $C_{10}E_8$ to $C_{16}E_8$ surfactants in hyposmotic erythrocyte membranes at Ht = 1.5% [43] are directly related to the values of C^{sat} and C^{sol} shown here ($R^2 = 0.99994$ and 0.99998, respectively; $n = 4$). We conclude that as for the pre-lytic stage, lysis is directly associated with the amphiphile's insertion into the bilayer.

Altered ion permeability [41,45,46,48], changes in the asymmetric distribution of components in the outer and inner monolayers of biological membranes [53,54], leading to changes in flip-flop rates [46,47,55–57] and to morphological alterations [24,41,58–61], are events associated with the protective or pre-lytic stage of hemolysis.

The lamella–micelle phase transition induced by surfactants is not so well described and understood. What are the main driving forces in the solubilization process and which steps are involved in the movement of surfactant molecules in-between membrane, water and micelle phases is not yet clear [50]. With the results presented here we have contributed for the understanding of the membrane solubilization process using the model provided by Lichtenberg [12] for the quantitative analysis of hemolysis, showing that hydrophobic interaction and micellization are the main driving forces in the process. Further work devoted to the determination of the structural and dynamic properties of the mixed membranes and mixed micelles (in preparation) should help us to determine the specific events involved in the hemolytic phenomenon, considered in terms of bilayer solubilization.

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