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ORIGINAL CONTRIBUTION

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Solubilization of phospholipid bilayers by C_{14}-alkyl betaine/anionic mixed surfactant systems

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Abstract The mechanisms governing the solubilizing interactions between zwitterionic/anionic mixed surfactant systems at different molar fractions of the zwitterionic surfactant ($X_{zwitter}$) and unilamellar liposomes were investigated. Solubilization was detected as a decrease in static light-scattering of liposome suspensions. Three parameters were regarded as corresponding to the effective surfactant/lipid molar ratios (Re) at which the surfactant system a) saturated the liposomes Re_{sat}; b) resulted in 50% solubilization of liposomes $Re_{50\%}$, and c) led to a complete solubilization of these structures Re_{sol}. These parameters corresponded to the Re at which light scattering stars to decrease, reaches 50% of the original value and shows no further decrease. From these parameters the surfactant partition coefficients for these three steps (K_{sat}, $K_{50\%}$ and K_{sol}) were also determined. The mixed systems were formed by N-tetradecyl-N,N-dimethylbetaine (C_{14}-Bet) and sodium dodecyl sulphate (SDS) in PIPES buffer at pH 7.20. Liposomes were formed by egg phosphatidylcholine and phosphatidic acid (9:1 molar ratio). When the range of $X_{zwitter}$ was about 0.4–0.6 Re and K parameters showed a maximum, whereas the critical micelle concentration (CMC) of these systems exhibited a minimum. Given that the ability of the surfactant systems to solubilize liposomes is inversely related to Re_{sol}, this capacity appeared to be directly correlated with the CMC of the systems. The progressive rise of K during the process indicates that an increasing surfactant partition equilibrium governs the interaction process from the saturation until the solubilization of vesicles, the free surfactant concentration remaining almost constant with similar values to the CMC for each mixed system studied.

Key words Liposome solubilization – interaction liposome/(tetradecyl betaine/sodium dodecyl sulphate) mixed systems – light scattering changes – effective surfactant/phospholipid molar ratios – partition coefficients

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Introduction

Zwitterionic surfactants have a strong interaction or complex formation with ionic surfactants in aqueous solutions [1, 2]. Betaines, which are capable of accepting a proton, interact much more strongly with anionic than with cationic surfactants. These interactions lead to mixed surfactant solution systems that show greater surface activity than that attainable with any of the individual surfactants of the mixture at the same concentration and consequently exhibit synergism. Thus, zwitterionic surfactants have been used as boosters of several anionic surfactants in industrial applications and their mixed properties have also been reported [3, 4]. The effect of the micelle solution phase of these surfactant mixtures in avoiding or at least reducing the level of anionic/protein interaction has been suggested by several workers as a way of slowing down the irritation potential of the anionic surfactants [5–7].

The interaction of surfactants with phospholipid bilayers in excess water leads to the breakdown of lamellar structures and to the formation of lipid-surfactant mixed micelles [8, 9]. This process is commonly denoted as "solubilization". Many studies have been devoted to the understanding of the principles governing this complex process [10–16]. A significant contribution has been made by Lichtenberg [17], who postulated that the critical effective surfactant/lipid ratio producing solubilization depends on the surfactant critical micelle concentration (CMC) and on the bilayer/aqueous medium distribution coefficients (K) rather than on the nature of the surfactants. Urbaneja et al. [18] demonstrated that when performed systematically, light-scattering measurements constitute a very convenient technique for the quantitative study of the bilayer solubilization by surfactants.

In recent papers, we reported studies on the solubilizing interactions of the N-tetradecyl-N,N-dimethylbetaine and sodium dodecyl sulphate systems with unilamellar liposomes when interacted individually with these liposomic structures [19, 20]. In the present work, we seek to extend these investigations by characterizing the solubilization of liposomes by mixtures of these surfactants in order to correlate the synergism existing between these two types of surfactants and their solubilizing capacity when applying to a phosphatidylcholine unilamellar vesicles. The solubilization process was assessed as a decrease in the light-scattered by the liposome/surfactant systems. To evaluate the light-scattering variations, three parameters were determined, namely, Re_{sat}, $Re_{50\%}$ and Re_{sol} according to the three-stage model adopted by Lichtenberg [9, 17]. The knowledge of the different phases involved in these solubilizing interactions could be useful in improving our understanding of the synergism in these binary systems, and in establishing a criterion for the evaluation of their activity on biological membranes.

Experimental

Materials

Phosphatidylcholine (PC) was purified from egg lecithin Merck (Darmstadt, FRG) according to the method of Singleton et al. [21] and was shown to be pure by thin-layer chromatography (TLC). Phosphatidic acid (PA) from egg yolk lecithin was purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Both lipids were stored in chloroform under nitrogen at $-20\,^{\circ}C$ until use.

N-tetradecyl-N,N-dimethylbetaine (C_{14}-Bet) was specially prepared by Albright and Wilson, Ltd. (Warley, West Midlands, UK); the active matter was 30% in aqueous solution and the free amino contents was 0.50%. Sodium dodecyl sulphate (SDS) was obtained from Merck and further purified by a column chromatographic method [22].

Piperazine-1, 4-bis-(2-ethanesulphonic acid) (PIPES buffer) obtained from Merck was prepared as 20 mM PIPES adjusted to pH 7.20 with NaOH, and contained 110 mM Na_2SO_4. Water was purified by the Milli-Ro system (Millipore, Milford, M.A.). Polycarbonate membranes and membrane holders were purchased from Nucleopore (Pleasanton, California, USA).

Methods

Liposome preparation

Unilamellar liposome vesicles of a defined size (about 100 nm) were prepared by the extrusion of large unilamellar vesicles previously obtained by the reverse-phase evaporation method [23, 24] based on an earlier method described by Szoka and Papahadjopoulos [25]. A lipid film was formed by removing the organic solvent by rotatory evaporation from a chloroform solution of lipids (lipid composition PC/PA 9:1 molar ratio). The lipids were then redissolved in diethyl ether, and the PIPES buffer was added to the solution of lipids. Gentle sonication led to the formation of a water-in-oil (W/O)-type emulsion. After evaporating the ethyl ether under reduced pressure, a viscous gel was formed. Elimination of the final traces of the organic solvent at high vacuum transformed the gel into a liposome suspension in which no traces of ether were detectable by NMR. Ether was washed in advance with the PIPES buffer and stored in bottles over

water containing bisulfite in order to obtain a total elimination of the ether in the liposome suspensions [26].

Unilamellar vesicles (of a uniform size distribution) were obtained by extrusion of vesicle suspensions through 800, 400, 200 and 100 nm polycarbonate membranes to achieve a uniform size distribution [27]. The range of phospholipid concentration in the liposome suspension studied was 0.5–5.0 mM.

Determination of lipid bilayer concentration

The lipid bilayer concentration of the liposome suspensions after preparation was determined using thin-layer chromatography (TLC) coupled to an automated ionization detection (FID) system (Iatroscan MK-5, Iatron Lab. Inc. Tokyo, Japan) [28].

Determination of vesicle size and stability of liposome suspensions

The mean vesicle size and the polydispersity of the liposome suspensions were determined with a Photon correlator spectrometer (Malvern Autosizer 4700c PS/MV; Malvern, England). The mean vesicle size was established by particle number measurement. The samples were adjusted to the appropriate concentration range with PIPES buffer and the measurements were taken at 25 °C at a reading angle of 90°.

Surface tension measurements and CMC determinations

Surface tensions of buffered solutions of single surfactants and mixed surfactant systems were measured by the ring method [29] using a Krüss tensiometer (processor tensiometer K-12) which determines directly the real surface tension values at equilibrium. The critical micelle concentration values (CMC) for single surfactants and mixed systems in PIPES buffer were determined by plotting the surface tension values versus the log surfactant concentration.

Solubilizing parameters

The perturbation produced by the surfactants in phospholipid bilayers leads to the solubilization of the lipid components via mixed micelle formation [17]. This solubilization results in changes in light scattering of these systems, which depend on the nature of both surfactant and lipid components. This can be monitored by measuring the variations in light scattering during the solubilizing processes [18, 30].

When defining the parameters related to the solubilization of liposomes it is essential to consider that the mixing of lipids and surfactants is not ideal due to the specific interactions between both components, which has been demonstrated for a variety of amphiphiles [31, 32]. To evaluate the alterations caused by the C_{14}-Bet/SDS mixed surfactant systems on phosphilipid bilayers, the effective surfactant/lipid molar ratio Re in an aggregate (liposome or micelle) is defined as follows [17]:

$$Re = \frac{S_T - S_W}{PL - PL_{monomer}}, \tag{1}$$

where PL is the lipid concentration (mM), S_T is the total surfactant concentration (mM) and S_W is the surfactant concentration in the aqueous medium (mM). The monomeric PL concentration ($PL_{monomer}$) is negligible due to the low solubility of PL in water. Likewise, it is generally admitted that an equilibrium partition of surfactants between bilayer and the aqueous medium governs the incorporation of surfactants into liposomes, thereby producing saturation and solubilization of these structures.

In the analysis of the equilibrium partition model proposed by Schurtenberger [33] for bile salt/lecithin systems, Lichtenberg [17] and Almog et al. [13] have shown that for a mixing of lipids, in dilute aqueous media, the distribution of surfactant between lipid bilayers and aqueous media obeys a partition coefficient K, given in $(mM)^{-1}$ by

$$K = \frac{S_B}{[PL + S_B]S_W}, \tag{2}$$

where S_B is the concentration of surfactant in the bilayers (mM). For PL $\gg S_B$, the definition of K, as given by Schurtenberger, applies:

$$K = \frac{S_B}{(PL \cdot S_W)} = \frac{Re}{S_W}, \tag{3}$$

Re is the above-mentioned ratio of surfactant to phospholipid in the vesicle bilayer: ($Re = S_B/PL$). Under any other conditions, Eq. (2) has to be employed to define K; this yields:

$$K = \frac{Re}{S_W[1 + Re]}, \tag{4}$$

This approach is consistent with the experimental data offered by Lichtenberg [17] and Almog [13] for different surfactant phospholipid mixtures over wide ranges of Re values. Given that the range of phospholipid concentrations used in our investigation is similar to that used by Almog to test this equilibrium partition model, the K parameter has been determined using this equation.

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The overall solubilization process of phospholipid bilayers by surfactants can be characterized by three parameters termed Re_{sat}, $Re_{50\%}$ and Re_{sol}, according to the nomenclature adopted by Lichtenberg [9, 17], corresponding to the surfactant/lipid molar ratios at which light scattering starts to decrease, reaches 50% of the original value, and shows no further decrease. These parameters correspond to the *Re* at which the surfactant: a) saturates the liposomes, b) results in 50% of solubilization of vesicles, and c) leads to total solubilization of the liposomes.

The determination of these parameters can be carried out on the basis of the linear dependence existing between the surfactant concentrations which are required to achieve these parameters and the phospholipid concentration in liposomes. The equations describing the surfactant concentration needed to saturated the bilayer (Eq. 5), to solubilize the 50% of liposomes (Eq. 6) or to achieve the complete solubilization of liposome structures via mixed micelles formation (Eq. 7) are given as:

$$S_{T,sat} = S_{W,sat} + Re_{sat} \cdot PL \quad (5)$$

$$S_{T,50\%} = S_{W,50\%} + Re_{50\%} \cdot PL \quad (6)$$

$$S_{T,sol} = S_{W,sol} + Re_{sol} \cdot PL\,, \quad (7)$$

where the Re_{sat}, $Re_{50\%}$ and Re_{sol} and the aqueous surfactant concentrations $S_{W,sat}$, $S_{W,50\%}$ and $S_{W,sol}$ are, in each curve, respectively the slope and the ordinate at zero phospholipid concentration. The surfactant partition coefficients (K_{sat}, $K_{50\%}$ and K_{sol}) have been determined from Re and S_W parameters by applying Eq. (4). These parameters show the distribution of surfactant molecules between the different lipid aggregates (bilayers or mixed micelles) and the aqueous medium in the three transition stage studied.

Liposome suspensions were adjusted to the proper lipid concentration (from 1.0 to 10.0 mM). To these, equal volumes of the appropriate surfactant mixed solutions were added and the resulting mixtures were left to equilibrate for 24 h. Light-scattering measurements were made at 25 °C with a Shimadzu RF-540 spectrofluoro-photometer equipped with a thermoregulated cell compartment, with both monochromators adjusted to 500 nm. The assays were carried out in triplicate and the results given are the average of those obtained.

Results and discussion

Mean vesicle size and stability of liposome suspensions

The mean vesicle size of liposome suspensions after preparation (phospholipid concentration ranging from 0.5 to 5.0 mM) varied little (around 100 nm). The polydispersity index (PI), defined as a measure of the width of the particle size distribution curves obtained from the "cumulant analysis," was in all cases lower than 0.1. Given that PI values higher than 0.25 correspond to a non-homogeneous distribution of particles, the liposome suspensions used showed a homogeneous size distribution in all cases. The size of vesicles after the addition of equal volumes of PIPES buffer and equilibration for 24 h at 25 °C showed in all cases values similar to those obtained after preparation, with a slight increase in the PI (from 0.12 to 0.14). Hence, the liposome preparations appeared to be reasonably stable in the absence of surfactants under the experimental conditions used in solubilization studies.

Critical micelle concentration (CMC)

Figure 1 shows the variation of the surface tension as a function of the total surfactant concentration for mixed surfactant systems at different molar fractions of the zwitterionic surfactant. The surface tension values decrease as the total surfactant concentration increases although these

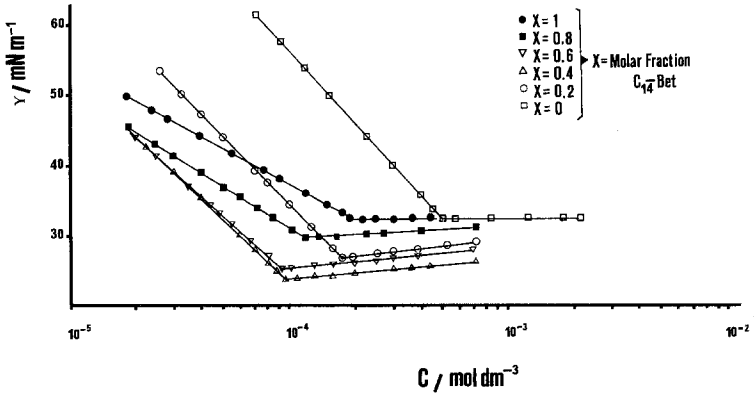


Fig. 1 Surface tensions versus total surfactant concentration for different molar fractions of zwitterionic surfactant for *N*-tetradecyl-*N*,*N*-dimethyl-betaine/sodium dodecyl sulphate mixed systems

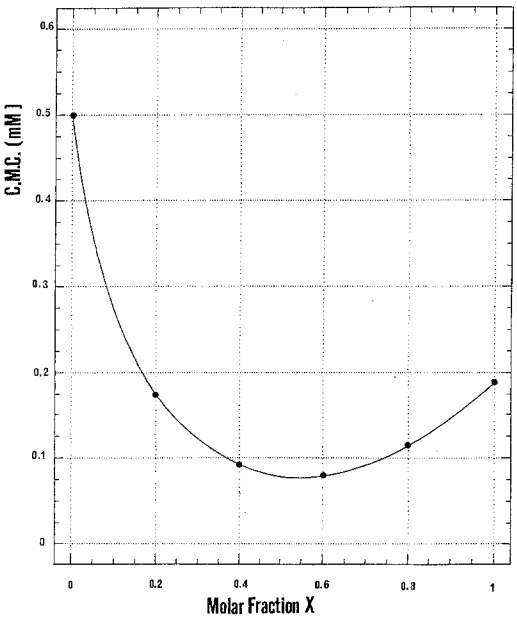


Fig. 2 Relationship between the critical micelle concentration values (CMC) and the different molar fractions of the zwitterionic surfactant for *N*-tetradecyl-*N*,*N*-dimethylbetaine/sodium dodecyl sulphate mixed systems

systems show a minimum in the vicinity of the CMC. Such a minimum has also been reported [1, 34] in studies for zwitterionic-anionic mixed systems.

When plotting the CMC values against the $X_{zwitter}$ (Fig. 2), the CMC values of mixed systems decrease with increasing $X_{zwitter}$ and show a minimum approx at $X_{zwitter} = 0.50–0.55$. It has been reported [1, 2] that the CMC of a mixture of two oppositely charged surfactants becomes notably smaller than that of respective surfactants due to the association of the surfactants induced by the electrostatic attraction. The present result can also be explained by assuming that the association of the surfactant occurs easily by electrostatic attraction between the cationic portion of the betaine and the dodecyl sulphate ion in the mixed system.

Solubilization studies

In order to determine the solubilizing capacity of the C_{14}-Bet/SDS mixed systems on unilamellar liposomes, a series of experiments was carried out studying the disrupting effect caused by these mixed systems at various $X_{zwitter}$.

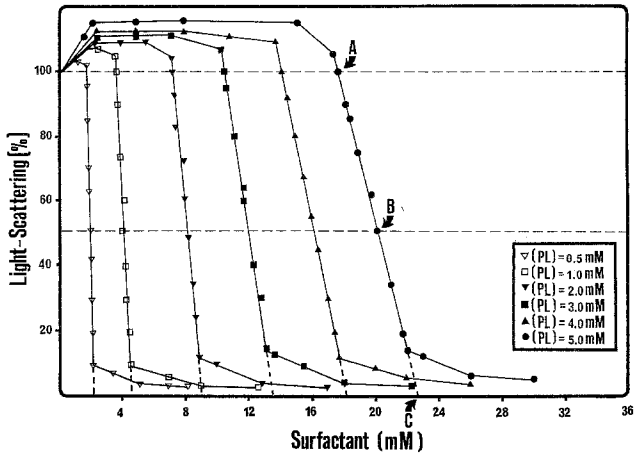
The solubilization process was studied by monitoring the variation in the light-scattered by the surfactant/liposome systems as a function of surfactant concentration. In accordance with the procedure described by Urbaneja et al. [18], changes in the light-scattering were determined 24 h after addition of the surfactant to liposomes at 25 °C.

Figure 3 shows the percentage change of light-scattering corresponding to the solubilization curves of unilamellar liposome suspensions (lipid concentration from 0.5 mM to 5.0 mM) arising from the addition of different concentrations of mixed surfactant system with $X_{zwitter} = 0.4$. From these curves, the surfactant concentration producing the saturation ($S_{T,sat}$), 50% solubilization ($S_{T,50\%}$) and complete solubilization of the liposomes ($S_{T,sol}$), for each lipid concentration can be obtained by graphical methods. The arrows A, B and C (curve for lipid concentration 5.0 mM.) correspond to these parameters. When plotting the extrapolation of the solubilization curves (lipid concentrations 0.5 and 5.0 mM.) versus the surfactant concentration at various $X_{zwitter}$, the three-dimensional picture (Fig. 4) is obtained. It is interesting to note that the surfactant concentration needed to achieve the saturation or solubilization of liposomes shows a maximum value for the $X_{zwitter}$ values ranging 0.4–0.5.

By plotting the surfactant concentrations previously obtained at various $X_{zwitter}$ versus phospholipid concentration straight lines are obtained, in which an acceptable linear relationship is established in each case. These straight lines correspond to the aforementioned Eqs. (5), (6) and (7) from which the *Re*, and *K* parameters were determined.

The *Re* and *K* parameters including the regression coefficients of the straight lines and the CMC values for

Fig. 3 Percentage change in light-scattering of unilamellar liposomes, the bilayer lipid concentration ranging from 0.5 to 5.0 mM., versus surfactant concentration (*N*-tetradecyl-*N*,*N*-dimethylbetaine/sodium dodecyl sulphate mixed system) for the molar fraction of the zwitterionic surfactant $X_{zwitter} = 0.4$.



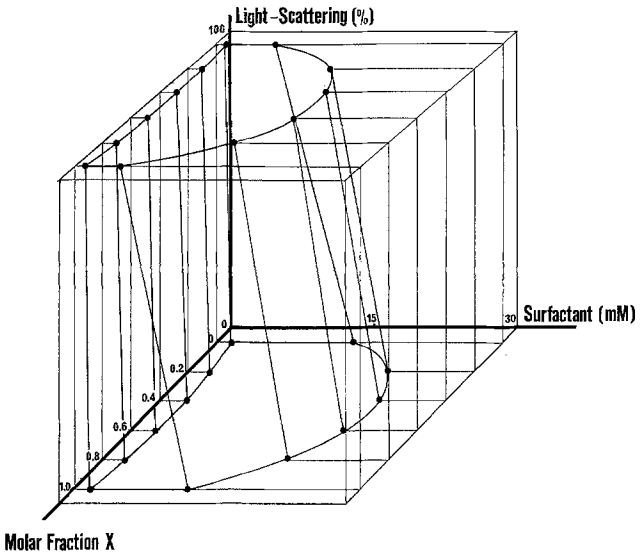


Fig. 4 Percentage change in light-scattering of unilamellar liposomes, (bilayer lipid concentration 0.5 and 5.0 mM), versus surfactant concentration (*N*-tetradecyl-*N*,*N*-dimethylbetaine/sodium dodecyl sulphate mixed system) for different molar fractions of the zwitterionic surfactant

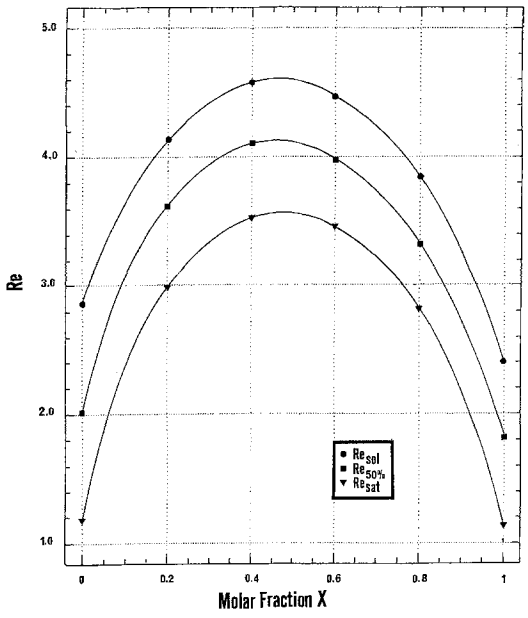


Fig. 5 Effective surfactant/lipid molar ratios Re_{sat} $Re_{50\%}$ and Re_{sol} corresponding to the interaction of liposomes with the mixed surfactant systems *N*-tetradecyl-*N*,*N* dimethylbetaine/sodium dodecyl sulphate versus the molar fraction of the zwitterionic surfactant

the single surfactants and the mixed surfactant systems are shown in Table 1. From these data it is interesting to note that the values of the *Re* and *K* parameters increased as the molar fraction of the zwitterionic surfactant roses, showing the highest values in the range of $X_{zwitter}$ between 0.4 and 0.6.

When plotting the variation of the Re_{sat}, $Re_{50\%}$, and Re_{sol} parameters versus the $X_{zwitter}$ (Fig. 5) maximum values were attained approximately for the $X_{zwitter} = 0.45$–0.50. Given that the *Re* value decreases as the surfactant activity increases (both in the saturation and the solubilization of lipid bilayers) this $X_{zwitter}$ range corresponded to the minimum activity of the surfactant systems with respect to the lipid bilayers. Thus, the binary surfactant systems showed lower solubilizing activity than that of the single surfactants. These results are in agreement with those reported by Tanaka et al. [35] in studies on the interaction of carboxybetaine/SDS mixtures with liposome membranes.

The surfactant concentration in the aqueous medium was always comparable to the CMC of the mixed surfactant system. These results support the generally admitted assumption that the concentration of free surfactant must reach the CMC for solubilization to occur [17].

As for the partition coefficients, it is noteworthy that the *K* values increase progressively during the transitional interaction steps studied (from bilayer saturation K_{sat} to complete solubilization of bilayers K_{sol}), regardless of the molar fraction of zwitterionic surfactant. From these findings we may assume that an increasing partition equilibrium governs the incorporation of surfactant molecules into the lipid bilayers and the subsequent association of

Table 1 Solubilizing parameters (*Re* and *K*) of unilamellar liposomes treated with the mixed surfactant systems *N*-tetradecyl-*N*,*N*-dimethylbetaine/sodium dodecyl sulphate for different molar fractions of the zwitterionic surfactant. The CMC values of the single surfactants and the mixed surfactant systems and the regression coefficients of the straight lines obtained are also included.

C_{14}-Bet $X_{Zwitter}$	CMC (mM)	$S_{W,sat}$	$S_{W,50\%}$	$S_{W,sol}$	Re_{sat}	$Re_{50\%}$	Re_{sol}	K_{sat}	$K_{50\%}$	K_{sol}	r^2
0	0.50	0.49	0.51	0.52	1.18	1.99	2.84	1.10	1.30	1.42	0.992
0.2	0.17	0.17	0.17	0.17	2.96	3.64	4.16	4.39	4.61	4.74	0.994
0.4	0.09	0.09	0.09	0.09	3.54	4.12	4.56	8.66	9.84	9.11	0.993
0.6	0.08	0.08	0.08	0.08	3.46	3.92	4.48	9.69	9.95	10.21	0.998
0.8	0.11	0.11	0.11	0.11	2.83	3.31	3.85	6.71	6.98	7.21	0.997
1.0	0.19	0.19	0.20	0.22	1.10	1.80	2.40	2.75	3.21	3.20	0.992

the surfactant molecules with the lipid building liposomes to form mixed micelles. Thus, the affinity of surfactant molecules by lipids appears to be greater in the bilayer solubilization (micellization process) than during the previous step of bilayer saturation.

Comparison of Figs. 2 and 5 shows that *Re* and CMC values display contrary tendencies showing minimum CMC and maximum *Re* values for the same range of molar fractions ($X_{zwitter} = 0.45–0.55$). From these results, the ability of each mixed surfactant system to saturate or solubilize liposomes is directly related to its CMC. These findings support the hypothesis that the solubilizing capacity of the single surfactants or mixed surfactant systems depends on the concentration of active surfactant molecules in the aqueous medium capable of interacting with the phospholipid structures; (i.e., surfactant monomers or "ion pairs"). The synergism which decreases the CMC of the mixed system also decreases the concentration of single surfactant monomers or ion pairs in the aqueous medium. As a consequence, this synergism is related to the decrease in the capacity of these systems to solubilize liposomes. These results may explain the decrease in the irritation potential of these systems with respect to the irritation potential for the single anionic surfactant [5–7].

In light of our results, the $X_{zwitter}$ appears to be a very mandatory parameter regulating the physico-chemical properties of these binary systems. The specific properties of C_{14}-Bet/SDS mixtures has also been reported in different studies on viscosity, NMR line widths and solubilization of water-insoluble dyes, in which the mixed systems exhibit maximum values and minimum CMCs for the same $X_{zwitter}$ [1].

Conclusions

From our results, we conclude that the solubilization of unilamellar PC liposomes by C_{14}-Bet/SDS at different molar fractions of zwitterionic surfactant is related to the concentration of surfactant monomers or "ion pairs" in the aqueous medium which is directly linked with the CMCs of the surfactant mixtures. Thus, the lower the CMC of the systems the higher the *Re* parameters and consequently, the lower their ability to saturate or solubilize liposomes. These results may account for the decrease in the irritation potential of these mixed systems with respect to that of the single anionic surfactant.

Given that the CMC of these mixed systems depends on the $X_{zwitter}$, the molar fraction appears to be a mandatory parameter regulating the physico-chemical properties of these binary systems. The range of $X_{zwitter}$ between 0.45–0.55 correspond to the minimum of both the CMC and the solubilizing activity of these mixed systems.

The rise of the surfactant partition coefficients during the solubilization process means that an increasing partition equilibrium governs the saturation of lipid bilayers by surfactant molecules and the subsequent interaction of the mixed surfactant systems with the lipids building liposomes, which leads to the lipid-mixed micelle formation. The aqueous surfactant concentration during the overall process remains almost constant with a concentration similar to that of the CMC for the mixed system studied.

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