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Solubilization of human erythrocyte membranes by ASB detergents

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Understanding the membrane solubilization process and finding effective solubilizing agents are crucial challenges in biochemical research. Here we report results on the interaction of the novel linear alkylamido propyl dimethyl amino propanosulfonate detergents, ASB-14 and ASB-16, with human erythrocyte membranes. An estimation of the critical micelle concentration of these zwitterionic detergents (ASB-14 = 100 µM and ASB-16 = 10 µM) was obtained using electron paramagnetic resonance. The amount of proteins and cholesterol solubilized from erythrocytes by these detergents was then determined. The hemolytic activities of the ASB detergents were assayed and the detergent/lipid molar ratios for the onset of hemolysis (R_e^{sat}) and total lysis (R_e^{sol}) were calculated, allowing the determination of the membrane binding constants (K_b). ASB-14 presented lower membrane affinity (K_b = 7050 M^{-1}) than ASB-16 (K_b = 15610 M^{-1}). The amount of proteins and cholesterol solubilized by both ASB detergents was higher while R_e^{sat} values (0.22 and 0.08 detergent/lipid for ASB-14 and ASB-16, respectively) were smaller than those observed with the classic detergents CHAPS and Triton X-100. These results reveal that, besides their well-known use as membrane protein solubilizers to enhance the resolution of two dimensional electrophoresis/mass spectrometry, ASB-14 and ASB-16 are strong hemolytic agents. We propose that the physicochemical properties of ASB detergents determine their membrane disruption efficiency and can help to explain the improvement in the solubilization of membrane proteins, as reported in the literature.

Key words: Hemolysis; Protein solubilization; Erythrocyte; Zwitterionic detergents; Critical micelle concentration; Cholesterol solubilization

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Introduction

The solubilization of biological membranes by detergents has been investigated for many years (1-6). Detergents are amphiphilic compounds that solubilize membrane proteins and lipids, leading to disruption of cell membranes at high enough concentrations. According to their physicochemical properties - such as charge, critical micelle concentration (CMC), aggregation number and hydrophilic-lipophilic balance (HLB) - different interactions occur and membrane proteins are denatured or not (4,7). As for the lipids, quantitative studies of membrane solubilization show a strong correlation between these physicochemical properties of detergents (2,8-10) and their lytic effect.

In the last decades, new detergents have appeared, being designed to improve the solubilization of membrane proteins. The zwitterionic amidosulfobetaine detergents ASB-14 and ASB-16 (linear alkylamido propyl dimethyl amino propanosulfonates) with 14 and 16 atoms of carbon in the acyl chain, respectively, were synthesized by Chevallet et al. (11). They have contributed to the advance of two-dimensional gel electrophoresis analysis, improving the solubilization of many hydrophobic proteins such

as erythrocyte band III (12,13) and others (11,14-16).

Despite the contribution of ASB detergents to the proteomic sciences, their role in the membrane solubilization process still remains unclear. In the present article, we studied the interaction of ASB-14 and ASB-16 with human erythrocyte membranes, using the quantitative approach described by Lichtenberg (2) to describe cell disruption and membrane solubilization.

Material and Methods

The alkylamido propyl dimethyl amino propanosulfonate detergents ASB-14 and ASB-16 were obtained from Calbiochem (USA). CHAPS and 5-doxyl stearic acid (5-SASL) were purchased from Sigma Chemical Co. (USA). All other reagents were of analytical grade.

Determination of critical micelle concentration

5-SASL films were prepared by evaporating stock chloroform solution of the spin label, under a stream of N_2. PBS (147 mM NaCl, 5 mM phosphate buffer, pH 7.4) was added and the samples were vortexed for about 5 min. Aliquots of ASB-14 or ASB-16, prepared in PBS, were added to the tubes to produce 1-5000 µM of each detergent and 50 µM of 5-SASL. Electron paramagnetic resonance (EPR) spectra were recorded in 0.2-mL flat quartz cells, at room temperature, in a Bruker EMS spectrometer, operating at 9 GHz and 3.4 kG.

Isotonic hemolytic assay

Freshly obtained human blood from healthy donors 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 the cell were washed three times in 5 mM PBS buffer, pH 7.4, by centrifugation.

ASB-14 (10-100 µM) or ASB-16 (1-20 µM), prepared in isotonic PBS, pH 7.4, were added to the erythrocyte suspensions (0.15-0.60% hematocrit, diluted in PBS), and the samples were kept for 15 min at 37°C. After centrifugation at 260 *g* for 3 min, membrane disruption, reported as hemolysis percentage, was measured by the hemoglobin released in the supernatant, as described by Malheiros et al. (17). Each triplicate measurement was carried out on at least two different blood samples.

Solubilization of protein and cholesterol

Blood was collected and cells washed as described above. Packed cells suspended in PBS were filtered through α-cellulose/microcrystalline-cellulose. Ghost membranes were prepared by hypotonic hemolysis, resuspending erythrocytes in lysis buffer (5 mM sodium phosphate, 0.5 mM EDTA, 5 mM diisopropyl fluorophosphate, pH 8.0) at 4°C, followed by centrifugation at 26,000 *g* for 20 min and repeated washings with the same buffer, according to Dodge et al. (18). Protein concentration was determined with bicinchoninic acid (Pierce Biotechnology, USA), using bovine serum albumin as standard.

Purified ghost membranes (1 mg/mL, final protein concentration) were mixed with ASB, CHAPS or Triton X-100, prepared in PBS, for 30 min at 37°C. The samples were then centrifuged at 15,000 *g*, for 30 min at room temperature and the supernatant solution was used for the measurement of solubilized protein and cholesterol. Cholesterol was measured using a colorimetric assay kit (R-Biopharm Italia srl, Italy; #10139050035) for lutidine-dye (3,5-diacetyl-1,4-dihydrolutidine), which has a stoichiometric relationship with the amount of cholesterol in the sample and is produced after enzymatic reactions catalyzed by cholesterol oxidase and catalase. Untreated samples of ghosts in PBS were used as control. Triplicate measurements on at least two different blood samples were carried out. The 100% values in Figure 2A,B correspond to the total protein and cholesterol concentrations, respectively, of the untreated ghosts in PBS.

Results and Discussion

Electron paramagnetic resonance experiments and CMC determination

Figure 1 presents the EPR spectra of 5-SASL in PBS buffer (0 ASB-14) and in the presence of increasing concentrations of ASB-14. At low ASB-14 concentrations (up to 75 µM), the isotropic 5-SASL signal refers to the probe in the aqueous phase. Once the CMC of ASB-14 is reached, the spin label increasingly enters the newly formed micelles, giving rise to: 1) spin-spin interactions (mainly visible as changes in the baseline of the spectra at 100 to 150 µM ASB-14), and 2) broad lines and highly anisotropic signals of 5-SASL inside the micelles, easily observed at ASB-14 concentrations greater than 150 µM. In addition, the spin probe signal (area under the peaks) is increased, due to the increased solubility of 5-SASL in the micelles. We have determined the CMC of ASB-14 to be 100 µM, the lowest concentration of detergent in which a spectrum with motional restricted spin label was observed. Similar results were found for ASB-16, but micelle formation was registered at concentrations about 10 times lower (CMC = 10 µM) than that of ASB-14. This is the first report in the literature of the CMC of ASB-14 and ASB-16. The values reported here, in PBS buffer, are quite reasonable compared with the CMC determined for other sulfobetaine

zwitterionic homologues, Zwit 3-14 and Zwit 3-16, with equivalent acyl chains: CMC = 121 and 11.3 µM, respectively, in water (19) and 140 and 14 µM, respectively, in 0.1-0.2 M NaCl (20).

HLB is a parameter frequently used to describe the physicochemical properties of non-ionic detergents. Here we have calculated HLB values for ASB-14 and ASB-16, from the ratio of mass between the hydrophilic/hydrophobic groups on the molecules, according to Griffin (21). Intermediate values (11.6 and 10.9, respectively) reveal the well-balanced amphiphile character of these detergents, the smaller HLB of ASB-16 being due to the contribution of its longer hydrophobic tail.

Solubilization of membrane components

Figure 2A shows that the total amount of proteins solubilized from erythrocyte ghosts by ASB-14 and ASB-16 is higher than that obtained with CHAPS or Triton X-100, even at the highest concentrations of detergents tested. This result agrees well with the two dimensional electrophoresis data in the literature that show an improvement in the resolution of many integral membrane proteins (11-16) with ASB-14 and ASB-16 compared with classic detergents such as Triton and CHAPS (22,23). We have tested both detergents in the sample buffer used for two-dimensional gel electrophoresis of red blood cells, and the results indicate that different proteins were solubilized by using these detergents, and therefore ASB detergents are useful to achieve a more detailed assignment of the proteins (24).

Figure 2B reports the amount of cholesterol released in the supernatant after treatment of red blood cell membranes with the detergents. It is interesting to note that, under the experimental conditions (starting material containing 1 mg/mL membrane proteins or ca. 1.3 mM total lipids), the maximum amounts of cholesterol solubilized after treatment with ASB-14 or ASB-16 were reached when the detergent:lipid molar ratio was approximately 4:1, while for Triton X-100 and CHAPS the ratios were higher (20:1 and 40:1, respectively), indicating that ASB detergents are stronger cholesterol solubilizers.

The higher amounts of cholesterol in the supernatant indicate that ASB detergents did not induce the formation of detergent-resistant membrane (DRM), known to be enriched in cholesterol and sphingolipids, as described for Triton X-100 (25-27), possibly explaining the improved solubilization of proteins eventually involved in DRM (Figure 2A). In addition, preliminary results from our laboratory indicate that a low buoyant density fraction in sucrose gradient was not formed when ASB detergents were used to solubilize erythrocyte membranes at low temperature

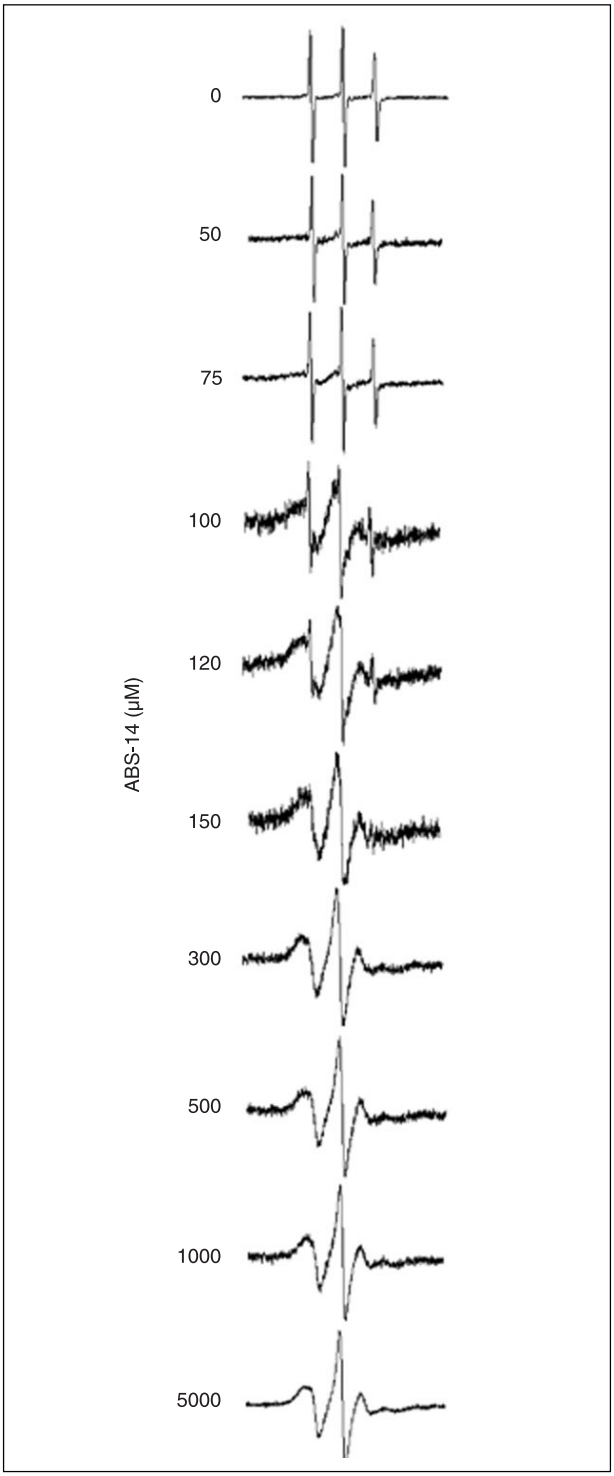

Figure 1. EPR spectra of 5-SASL in 5 mM PBS buffer, pH 7.4 (0 ASB-14), and in the presence of increasing ASB-14 concentrations. Line broadening and anisotropic spectra (beyond 150 µM - see text) indicate the critical micelle concentration. Abscissa = 100 gauss windows around the magnetic field center (3.4 kG).

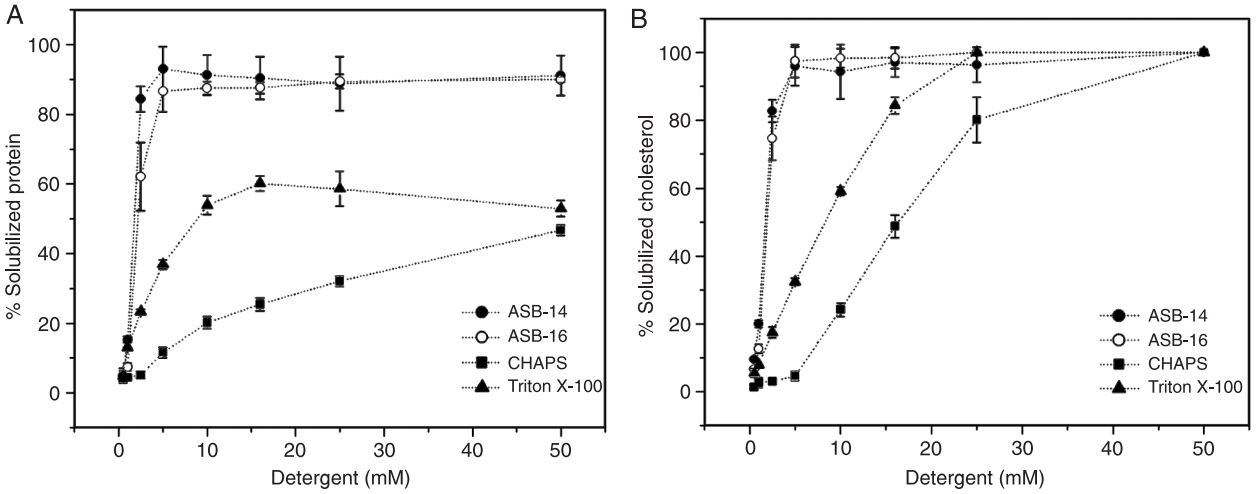

Figure 2. Protein (A) and cholesterol (B) content solubilized from erythrocyte ghosts by detergents ASB-14, ASB-16, CHAPS, and Triton X-100. Samples of ghosts containing 1 mg/mL of protein were incubated with the detergents in 5 mM PBS buffer for 30 min at 37°C and centrifuged at 15,000 *g* for 30 min, at room temperature. Untreated samples of ghosts in PBS were used as control. The 100% values in panels A and B correspond to the total protein and cholesterol concentrations, respectively, in the untreated ghosts' samples, in PBS.

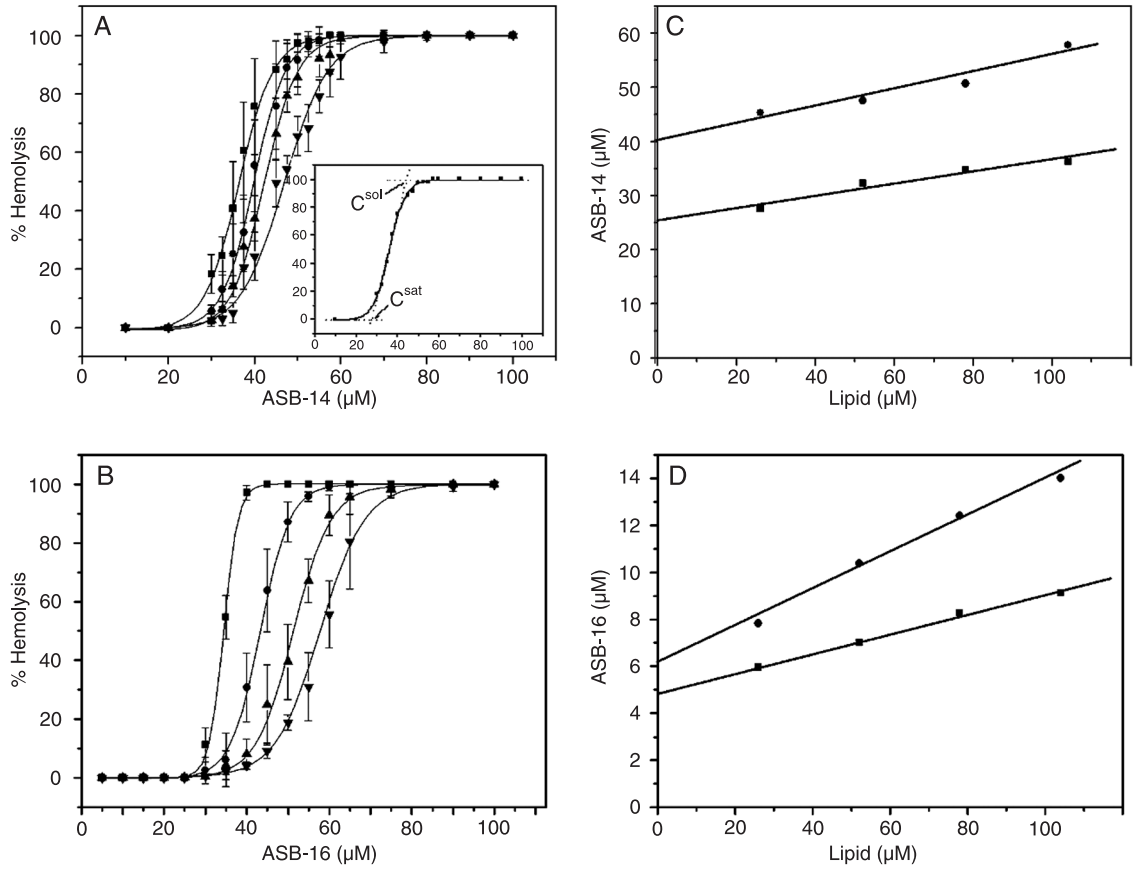

Figure 3. Hemolytic curves of ASB-14 (A) and ASB-16 (B) on human erythrocytes in 5 mM PBS buffer, pH 7.4, 37°C (15 min) at hematocrits 0.15% (squares), 0.30% (circles), 0.45% (triangles), and 0.60% (inverted triangles). Effective detergent/lipid molar ratios for erythrocyte membranes saturation and solubilization by ASB-14 (C) and ASB-16 (D). The inset in *A* shows the C^{sat} and C^{sol} determination from the hemolytic curve at 0.15% hematocrit. C^{sat} = concentration required to induce membrane saturation (onset of hemolysis); C^{sol} = detergent concentration required for total membrane solubilization (100% lysis).

(4°C), indicating their greater power to disrupt the membrane compared with Triton X-100 (Domingues CC, Ciana A, de Paula E, Minetti G, unpublished results).

To further understand the membrane solubilization process, we have applied the quantitative approach proposed by Lichtenberg (2) to analyze ASB-induced erythrocyte hemolysis, as follows.

Hemolytic experiments

The hemolytic curves obtained with increasing concentrations of ASB-14 and ASB-16 in erythrocyte suspensions are shown in Figure 3A,B. Membrane disruption, reported as % hemolysis, was determined from the amount of hemoglobin released in the supernatant solution, as previously described (17).

The quantitative analysis of the membrane solubilization process proposed by Lichtenberg (2) was applied to the hemolytic curves, considering C^{sat} and C^{sol} (inset in Figure 3A) to be the detergent concentration required to induce membrane saturation (the onset of hemolysis) and total membrane solubilization (100% lysis), respectively (2,6,10,17,28).

Plots of C^{sat} and C^{sol} as a function of membrane lipid concentration (Figure 3C,D) allowed the determination of R_e, the effective detergent/lipid molar ratio for initial (R_e^{sat}) and total hemolysis (R_e^{sol}). The straight line obtained in each case is predicted by Equation 1 (8,9):

$$D_t = R_e[L+1/K_b(R_e+1)] \quad \text{(Equation 1)}$$

where, D_t is the total detergent concentration (C^{sat}, C^{sol}) and L is the lipid concentration in the membrane (28). R_e values were calculated from the slope of the resulting straight lines while the y-intercept corresponds to D_w, the concentration of free detergent in water (2,29). K_b (M^{-1}), the molar binding constant of the detergent to the erythrocyte membrane, was derived from R_e^{sat} and D_w^{sat} values, according to Equation 2 (2,29):

$$R_e = K_b \cdot D_w/(1-K_b \cdot D_w) \quad \text{(Equation 2)}$$

Table 1 lists the C^{sat} and C^{sol} values obtained from the hemolytic curves of ASB-14, ASB-16 and CHAPS, a classic detergent of identical polar head group but with a steroidic hydrophobic portion (30). The small C^{sat} and C^{sol} values found for ASB-14 and ASB-16 reflect their high capacity to solubilize erythrocyte membranes, being more potent hemolytic agents than CHAPS. The differences in C^{sat} and C^{sol} values observed between ASB-14 and ASB-16 are consistent with the results reported by Preté et al. (10) within a homologue series of detergents, in which C^{sat} and C^{sol} decreased with C_xE_8 polyoxyethylene alkyl ethers of longer hydrocarbon chains. The smaller C^{sat} and C^{sol} values found for ASB-16 show that hydrophobic interactions between the detergent acyl chains and the erythrocyte membrane components are strongly involved in the solubilization process.

The hemolytic process induced by detergents could be described as a bilayer-to-micelle transition in which, according to Lichtenberg et al. (31), R_e^{sat} and R_e^{sol} determine the limits for the coexistence of mixed-membranes and

Table 1. Hemolytic effect on human erythrocytes induced by ASB-14, ASB-16 and CHAPS.

Ht (L)	C^{sat}			C^{sol}		
	ASB-14 (μM)	ASB-16 (μM)	CHAPS (mM)	ASB-14 (μM)	ASB-16 (μM)	CHAPS (mM)
0.15% (13 μM)	27.6	6.0	4.3	45.3	7.8	7.4
0.30% (26 μM)	32.3	7.0	5.6	47.6	10.4	8.4
0.45% (39 μM)	34.7	8.3	5.8	50.7	12.4	8.7
0.60% (52 μM)	36.3	9.1	6.0	57.9	14.0	9.2

Experimental condition as in Figure 3. L = lipid concentration in erythrocyte membranes corresponding to each hematocrit (Ht), calculated according to Malheiros et al. (28). C^{sat} = concentration required to induce membrane saturation (onset of hemolysis); C^{sol} = detergent concentration required for total membrane solubilization (100% lysis).

Table 2. Effective detergent/lipid molar ratios and related parameters in the hemolysis of erythrocytes by ASB-14, ASB-16, CHAPS, and Triton X-100.

	ASB-14	ASB-16	CHAPS	Triton X-100[b]
R_e^{sat}	0.22	0.08	40.62	1.58
R_e^{sol}	0.31	0.16	43.77	2.15
D_w^{sat}	25.6 μM	4.9 μM	4.1 mM	0.10 mM
D_w^{sol}	40.2 μM	6.0 μM	7.0 mM	0.18 mM
K_b (M^{-1})[a]	7050	15610	236	5900

Experimental conditions as in Figure 3. R_e^{sat} = detergent/lipid molar ratio for the onset of hemolysis; R_e^{sol} = detergent/lipid molar ratio for the total lysis; D_w^{sat} = concentration of free detergent in water at the onset of hemolysis; D_w^{sol} = concentration of free detergent in water at total lysis; K_b = molar binding constant of the detergent to the erythrocyte membrane. [a]Taken from the saturation curves in Figure 3C,D. [b]Data from Prete et al. (6).

mixed-micelles. Table 2 reports the quantitative parameters calculated from the hemolytic curves in Figure 3C,D. The R_e values smaller than unit determined for ASB-14 and ASB-16 (Table 2) confirm that these detergents present stronger lytic effects than CHAPS (R_e = 40.62; Table 2) or the non-ionic Triton X-100 (R_e = 1.58; Ref. 6). In fact, the R_e values herein described for the ASB detergents are similar to those reported by Preté et al. (10) for alkyl ether (C_xE_8) homologues of equivalent acyl chains, but higher HLB.

The binding constants of ASB-14 (7050 M^{-1}) and ASB-16 (15610 M^{-1}) in Table 2 show the different affinities of these detergents to the erythrocyte membrane, explaining their distinct lytic effects (ASB-16 > ASB-14). These results are in good agreement with those previously reported for the C_xE_8 homologues (10), when a straight relationship between the K_b value and lytic effect was observed.

Moreover, the CMC values found for ASB-14 and ASB-16 disclosed the correlation between the solubilization and the aggregative properties of these detergents (1,4,5,32). Lichtenberg et al. (31) demonstrated that D_w values, the concentration of free detergent in water, are related but always smaller than the CMC values, since the membrane lipids offer an additional driving force for detergent aggregation, decreasing the concentration for micelle formation. By the same reasoning, to have an estimation of the CMC, the values of D_w^{sat} are preferable to those of D_w^{sol}, which may be influenced by the mixed micelles formed at higher detergent concentrations (33). Thus, the D_w values found for ASB-14 and ASB-16 (25.6 and 4.9 µM, respectively; Table 2), are consistent with the CMC values determined from the EPR data. The determined D_w value for CHAPS (4.14 mM) was also smaller than its CMC (6-10 mM; Ref. 34).

According to Heerklotz and Seelig (33), the membrane disruption potency of detergents can be assessed by the relationship between their CMC and binding constants, in which strong detergents (K_b x CMC <1) are able to solubilize lipid membranes at detergent-to-lipid molar ratios smaller than 1. Thus, by applying the product K_b x CMC to classify detergents according to their membrane disruption potency (33), ASB-14 and ASB-16 can be designated as strong detergents, and their R_e values (Table 2) smaller than 1 corroborate this classification.

This research shows that ASB-14 and ASB-16 present a high affinity to the erythrocyte membrane, being a useful tool for the solubilization of its components (both proteins and lipids), compared to classic detergents such as CHAPS or Triton X-100. Our results suggest that the relationship between membrane disruption efficiency (Table 2) and the physicochemical properties of ASB detergents (CMC, HLB) can explain the improvement in the solubilization of membrane proteins, as described in proteomic research papers (11-16,22,23).

To further understand their role in the specific solubilization of membrane proteins and lipids, other experiments are being conducted by our group to evaluate the effect of ASB-14 and ASB-16 on the formation/disruption of DRM from erythrocyte membranes, using specific lipid raft markers.

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