Solubilization of dodac small unilamellar vesicles by sucrose esters
A fluorescence study

N. Becerra, L.R. de la Nuez, A.L. Zanocco, E. Lemp, G. Günther *

Universidad de Chile, Facultad de Ciencias Químicas y Farmacéuticas, Departamento de Química Orgánica y Fisicoquímica, Casilla 233, Santiago 1, Chile

Abstract

A fluorescence method was employed to study the solubilizing interactions of several sucrose esters with Dioctadecyldimethylammonium chloride small unilamellar vesicles. In this paper we studied four different alkyl esters of sucrose, saturation and solubilization concentrations (C_{sat} and C_{sol}), the ester–DODAC molar ratio (R_e) and bilayer/aqueous partition coefficients (K) were measured by monitoring changes in laurdan generalized polarization values. A new critical surfactant concentration at lower values than saturation concentration was observed. All critical concentrations showed linear dependence with DODAC concentration. The decrease in the length of surfactant alkyl chain (upper cmc) led to an increase in its ability to saturate and solubilize vesicles and to a decrease in its bilayer affinity. Consequently the shorter alkyl chain (lauryl sucrose ester), the higher ability to saturate and solubilize the vesicles, whereas the longer chain (stearyl sucrose ester), exhibited the highest degree of partitioning into the vesicles.

Keywords: Liposomes solubilization; Sucrose esters; Laurdan; Lauryl sucrose; Palmityl sucrose; Myristyl sucrose; Stearyl sucrose; SUVs

1. Introduction

Great attention has been paid to understanding the principles governing the interaction of different surfactants with lipids, starting from simple model of membranes such as liposomes and vesicles [1–5] to more complex systems like stratum corneum [6]. Developed models relate the ability of surfactant to saturate and solubilize the bilayer, with its critical micellar concentration, cmc, and its distribution coefficients, K [7].

One of the most employed compounds in the lipidic membrane solubilization studies is the octyl glucoside [1,8–12]. A non-ionic surfactant with critical micellar concentration of 18–25.3 mM, a relatively high value [6,13]. Also, alkyl glucosides with different lengths of alkyl chain have been employed as surfactants [3,6]. Replacement of the octyl chain by longer alkyl groups improves the stability to hydrolysis and diminishes the toxicity of these compounds [14]. The surface-activity of alkyl glucosides is similar to that described for commercial mixtures of sugar esters, a related family of non-toxic, skin compatible, non-polluting and biodegradable surfactants. We are interested in evaluating the solubilization properties of pure sucrose esters by employing fluorescence methods.

The amphiphilic fluorophore 6-dodecanoyl-2-(dimethylamino)-naphthalene (Laurdan), incorporates into membranes with the fluorescent moiety localized in the region of the acyl bonds on the glycerol backbone for phospholipidic liposomes [15]. The spectroscopic behavior of this probe in the microaggregate has been related to the membrane polarity and/or the membrane fluidity (gel or fluid lamellar phases) [16], because of its sensitivity to the solvent dielectric relaxation effect. As a consequence any change in the microenvironment sensed by Laurdan is reflected in its emission spectra

* Corresponding author.

[17], allowing its use as a useful tool in observing physicochemical micro-properties in compartmentalized systems. To account for the emission spectra shifting of Laurdan, a currently used parameter is the generalized polarization, GP [16,18,19]. High values of GP are ascribed to a less fluid surroundings, where water penetration is blocked or limited [19].

In a previous work, we studied the sublityc alterations of DODAC large unilamellar vesicle bilayers upon addition of sucrose palmitate [20]. The incorporation of sucrose ester as co-surfactant promotes structural changes in the liposome bilayer. The voluminous sucrose head groups located on the liposome surface act as a physical barrier blocking the access of water molecules to the bilayer, with water structuration in the interface. These leads to changes in the polarity and fluidity of the probe surroundings with the consequent changes in Laurdan spectrum and its GP values.

In the present study, we report on the solubilization of DODAC small unilamellar vesicles by four sucrose esters with different alkyl chain lengths (lauryl $C_{11}H_{27}CO–$, miristyl, $C_{13}H_{27}CO–$, palmityl, $C_{15}H_{31}CO–$, and estearyl, $C_{17}H_{35}CO–$). We determine the effective molar ratio of surfactant to DODAC in the bilayers (R_e) and the partitioning coefficient (K) at different critical states of the solubilization process. We employ fluorescence methods instead of direct measurements of liposome size by static light-scattering [6,21]. We show the usefulness of the simpler and indirect fluorescence methods to study the interaction of liposomes with detergents. The methods used provide useful information about the effect the bulky hydrophilic head, the sucrose moiety, and the influence of the hydrophobic tail on the saturation and solubilization of vesicles.

2. Materials and methods

2.1. Chemicals

The sucrose monoesters, β-D-fructofuranosyl-6-*O*-lauryl-α-D-glucopyranoside (MLS), β-D-fructofuranosyl-6-*O*-miristyl-α-D-glucopyranoside (MMS), β-D-fructofuranosyl-6-*O*-palmityl-α-D-glucopyranoside (MPS), and β-D-fructofuranosyl-6-*O*-stearyl-α-D-glucopyranoside (MSS), were synthesized by a modification of the Osipow-Snell method [22,23] that yields a relatively complex mixture of mono- (mainly 6-*O* and presumably 1-*O*), di- and tri-esters. To isolate monoesters, chromatography on silica column was employed. Briefly, the reaction mixture solubilized in chloroform was eluted from a semipreparative silica gel column by using chloroform:methanol:water (20:5:0.7). Thin layer chromatography (using the same mobile phase and staining with a butanolic solution of urea-ortophosphoric acid) showed only one compound in the purified sample. The NMR spectra, obtained on a Bruker ADX 300 spectrometer, with DMSO-d_6 containing 5% of CH_3OD to avoid micellization, are in good agreement with previously reported spectra of monoesters [24]. Dioctadecyldimethylammonium chloride (DODAC) from Herga Ind. (Brazil) was purified as described elsewhere [25]. Laurdan from Molecular Probes was used as received. Solvents from Merck were HPLC quality. Water was treated with Milli-Q equipment from Waters.

2.2. Liposome preparation

Small unilamellar liposomes (SUVs) were obtained by ultrasonication of a 10 mM DODAC solution in Milli-Q water, with a Cole Parmer Ultrasonic Homogenizer. Final concentrations were fixed by water addition. DODAC SUVs with different sucrose ester concentrations were obtained by adding small aliquots of concentrated sucrose ester solutions in DMSO. Mixtures did not show spectral differences after been allowed to stand for 24 h to equilibrate, as it has been previously reported [3].

2.3. Fluorescence spectroscopy

Steady state fluorescence measurements of Laurdan were accomplished in a Fluorolog Tau-2 spectrofluorometer (SPEX, Jobin Ybon) at $25.0 \pm 0.5\,^{\circ}C$. The values of generalized polarization of Laurdan (LAU 3 μM, $\lambda_{ex} = 364$ nm, $\lambda_{em} = 440$ nm and 490 nm), not corrected against DMSO value, were determined by using the expression of Parassasi et al. [17,19] Eq. (1):

$$GP = \frac{I_{440} - I_{490}}{I_{440} + I_{490}} \quad (1)$$

2.4. Sucrose ester critical micellar concentration measurements

The values of the critical micellar concentration (cmc) were determined using the fluorescent properties of Laurdan. The plot of the changes in either emission intensity or GP values with the ester concentration gives the cmc parameter. All measurements were carried out at $25.0 \pm 0.5\,^{\circ}C$.

2.5. Determination of the effective molar ratio of surfactant to lipid in the bilayers (R_e) and the partitioning coefficient (K)

The equilibrium partition model [7,26,27], predicts a partitioning coefficient (K) for the distribution of a surfactant, between the lipid bilayer and the aqueous media. In a vesicular solution this parameter is defined as:

$$K = \frac{C_B}{(L + C_B)C_W}\,(mM^{-1}) \quad (2)$$

where C_B and C_W are the mM surfactant concentrations in the lipidic and aqueous pseudo phases, respectively and L is the lipid concentration (DODAC in our experiments). When

the surfactant concentration in the bilayer is on the order of the lipid concentration Eq. (2) can be rewritten as:

$$K = \frac{R_e}{(1 + R_e)C_W} \,(\mathrm{mM}^{-1}) \tag{3}$$

where R_e corresponds to the effective molar ratio of surfactant to lipid in the bilayers (C_B/L).

Solubilization of liposomes or vesicles can be characterized by two critical parameters, C_{sat} and $C_{sol}{\cdot}C_{sat}$ is the surfactant concentration necessary to saturate the liposome bilayer, and C_{sol} is the concentration needed to complete the bilayer solubilization into mixed micelles. In static light-scattering measurements for liposome solubilization, these parameters correspond to breakpoints observed when plotted against surfactant concentration. According to previous works [1,3,27,28], both critical concentrations exhibit a linear dependence on the lipid or phospholipid concentration, as stated in Eq. (4). This relation allows the determination of the effective surfactant to lipid molar ratios for surfactant-saturated vesicles (R_e^{sat}) and for lipid saturated mixed micelles (R_e^{sol}) as well as the concentration of monomeric surfactant (C_W) coexisting with the aggregates.

$$C_t^c = C_W^c + R_e^c L \,(\mathrm{mM}^{-1}) \tag{4}$$

In Eq. (4), C_t^c is the total surfactant concentration at each breakpoint, and the superindexes indicate the different critical points, saturation and solubilization.

3. Results and discussion

Fig. 1 shows the changes in the Laurdan generalized polarization (GP) value when the concentration of MMS in water increases. A linear behavior is observed before and after micellization, and the breakpoint in the curve at 4×10^{-5} M corresponds to the cmc value for MMS at 25 °C. Cmc for each sucrose ester were determined and are reported in Table 2.

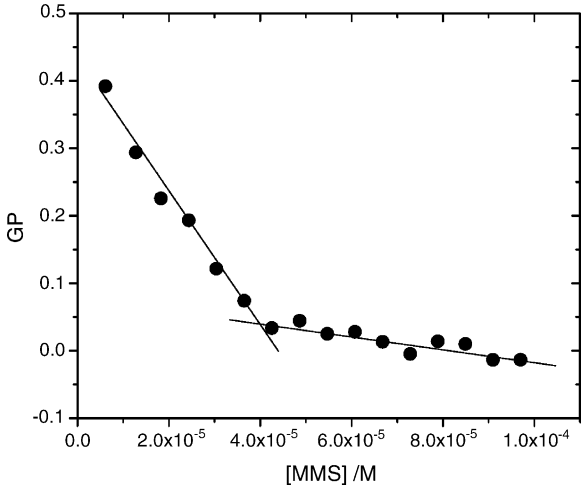

Fig. 1. GP dependence on MMS concentration, breakpoint indicates cmc.

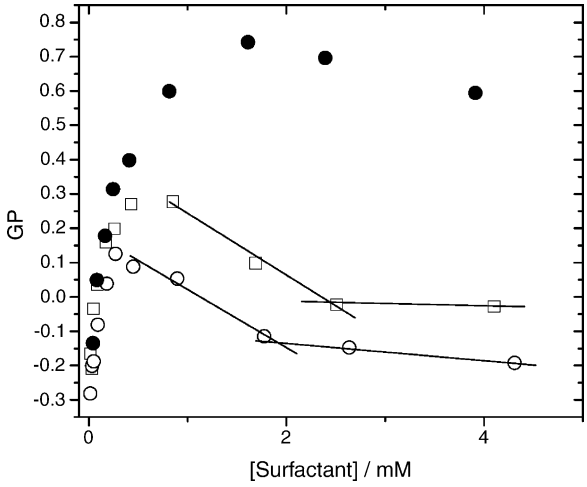

Fig. 2. Change of Laurdan generalized polarization of DODAC small liposomes (0.2 mM), induced by the presence of increasing amounts of MMS (◯), MPS (□) and MSS (•). Intersections correspond to (C_t^{sol}). Solubility of MSS does not allow to reach this critical concentration.

The measured values are in good agreement with the values previously reported by Vlahov et al. [24], determined using surface tension and spectrophotometric measurements, and in fairly good agreement with the values reported by Garofalakis et al. [29], data determined for products consisting in mixtures of more than one isomer of the same sucrose monoester, using surface tension measurements.

The solubilization of DODAC vesicles by the addition of surfactant was monitored by using GP. Fig. 2 shows the change in the GP value of DODAC SUVs due to the addition of different sucrose esters. MLS was not included because adequacy of scale. For all the surfactants employed (lauryl, miristyl, palmityl and stearyl sucrose esters) the changes in the GP values show a similar general behavior: (1) a fast increase in the GP value at low surfactant concentration, to reach a maximum. The initial increase of Laurdan GP is ascribed to the ester incorporation in the bilayer that first removes water and then blocks its access to the site of solubilization of Laurdan [20]. The maximum GP value is related with vesicle saturation, and the concentration of surfactant at this point corresponds to (C_t^{sat}); (2) after the maximum value, further addition of surfactant leads to a decrease in GP value until it reaches a plateau. At these sucrose ester concentrations mixed micelles and saturated vesicles coexist. Experiments carried out in parallel, show that Laurdan GP increases during the formation of sucrose ester-DODAC mixed micelles starting from pure ester micelles, in concordance with the reduction in GP values observed when the proportion of ester in mixed micelles increases. Mixed micelle formation has previously been suggested to explain the observed changes in the fluorescence spectra of Laurdan incorporated to DODAC large unilamellar liposomes when solubilization of microaggregate is performed with sucrose palmitate [20]. After vesicle saturation, the rate of Laurdan GP variation decreases and a change in slope at higher surfac-

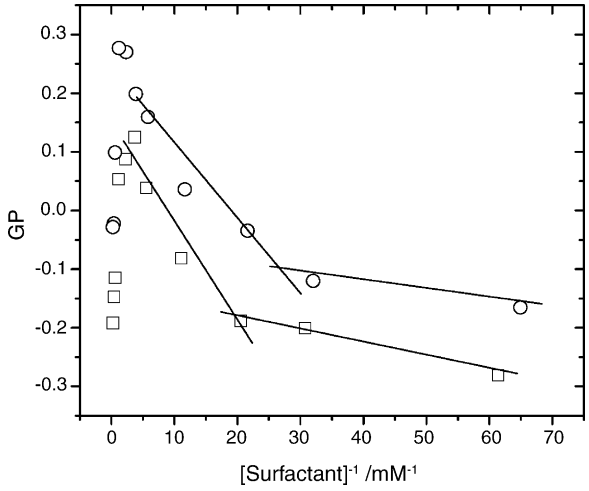

Fig. 3. Dependence of Laurdan GP on the reciprocal of sucrose ester concentration, for MMS (◯) and MPS (□). Intersections correspond to (C_t^{ss}).

tant concentrations is observed. At this point, solubilization of vesicles is complete, and surfactant concentration is denoted as (C_t^{sol}). Beyond this point no further changes in Laurdan GP were observed with sucrose ester addition.

Fig. 3 shows a plot of Laurdan GP against the reciprocal of surfactant concentration for MMS and MPS. The surfactants not plotted, MLS and MSS, behave in the same way. A maximum of GP, corresponding to the previously determined saturation concentration (C_t^{sat}) can be observed. Furthermore, the plot reveals a new and unexpected change of slope at lower surfactant concentrations (higher values at the *x*-axis), before saturation. The origin of this response, could be the formation of ordered surfactant structures over the liposome surface or a change in the packing of the sucrose groups, hindering the water access to the bilayer. At this stage, there are changes at the surface but the vesicle is not yet saturated. The concentration of surfactant at this point is called C_t^{ss} to indicate vesicle surface saturation.

Plots of Laurdan GP against surfactant concentration and the reciprocal of surfactant concentration at different DODAC concentrations (between 0.1 and 6 mM) allow the graphical determination of the critical surfactant concentrations C_t^{ss}, (C_t^{sat}), and (C_t^{sol}) for each DODAC concentration. Table 1 shows the critical concentration values for MMS.

Table 1
Critical concentration values ((R_e^{ss}), (R_e^{sat}) and (R_e^{sol})) for MMS at each DODAC concentration

[DODAC] (mM)	(R_e^{ss}) (mM)	(R_e^{sat}) (mM)	(R_e^{sol}) (mM)
0.1	0.046	0.247	0.860
0.2	0.065	0.272	1.800
0.5	0.222	0.561	3.339
0.8	–	–	5.766
1.0	0.200	1.293	6.744
1.5	–	1.596	–
2.0	0.500	2.283	–
3.0	–	–	–
4.0	0.830	–	–

Table 2
Critical micellar concentrations of sucrose monoesters at 25 °C

	cmc [24] (M)	cmc [29] (M)	cmc (M)
MLS	51.40×10^{-5}	21.00×10^{-5}	49.50×10^{-5}
MMS	8.80×10^{-5}	2.10×10^{-5}	4.00×10^{-5}
MPS	1.81×10^{-5}	0.41×10^{-5}	2.00×10^{-5}
MSS	–	–	1.00×10^{-5}

This methodology, where the fluorescent probe response is plotted against the concentration of surfactant, is analogous to the one employed when the measured property is scattered light [6].

In order to determine the surfactant to lipid molar ratio, R_e, and the free surfactant concentration, C_W, the experimentally determined critical surfactant concentrations were plotted against DODAC concentration (Eq. (4)). Although the data show a good linear fit, allowing to obtain good values for R_e from slope, the C_W values determined from the intercept, have a large error, in some cases being far from the corresponding cmc value. The reason for the poor agreement between C_W and cmc is probably due to the low cmc values (Table 2) for this family of non-ionic surfactants. The experimental constrain, surfactant concentration must be on the order of lipid concentration, forces surfactant concentrations to be higher than its own cmc. Thus this 'far' extrapolation, may be the origin of the uncertainty observed in the values for the intercept.

Table 3 shows the values for R_e at surface saturation, bilayer saturation, and vesicle solubilization for the different surfactants, obtained from the fit of data using Eq. (4). The R_e values clearly increase from surface saturation to complete solubilization. Also, for all the critical points the lauryl derivative shows the largest solubilization capacity and the stearyl derivative the lowest (solubility of MSS does not to reach its critical solubilization concentrations, consequently the last statement is based on trend). These results indicate that decreasing surfactant alkyl chain length corresponds to increasing ability to saturate or solubilize DODAC vesicles. The same trend has been found in the solubilization of phosphatidylcholine liposomes with alkylglucosides [3,6].

Evaluation of the bilayer/aqueous phase partitioning coefficients, *K*, with Eq. (3), requires knowing the free surfactant

Table 3
Surfactant to DODAC molar ratios (R_e), resulting in the interaction (surface saturation, bilayer saturation and solubilization) of sucrose esters with DODAC small unilamellar vesicles

	(R_e^{sol})	(R_e^{sat})	(R_e^{ss})
MLS	5.53 ± 0.03 (0.9853)	0.70 ± 0.02 (0.9988)	0.04 ± 0.02 (0.8259)
MMS	6.68 ± 0.07 (0.9963)	1.12 ± 0.10 (0.9913)	0.22 ± 0.04 (0.9577)
MPS	7.88 ± 0.13 (0.9565)	1.29 ± 0.19 (0.9774)	0.36 ± 0.03 (0.9875)
MSS	–	9.81 ± 0.35 (0.9987)	0.68 ± 0.08 (0.9798)

It also included the regression coefficients (r^2) of the straight lines obtained.

Table 4
Partition coefficients (K) in mM units, resulting in the interaction (surface saturation, bilayer saturation and solubilization) of sucrose esters with DODAC small unilamellar vesicles

	K^{sol}	K^{sat}	K^{ss}
MLS	1.71	0.84	0.078
MMS	21.80	13.20	4.80
MPS	44.60	28.60	13.40
MSS	–	90.70	40.60

concentration, C_W. The uncertainty in the experimental values for this parameter could be overcome by using cmc instead of C_W values. This approach is supported by previous data showing that free surfactant concentrations are always close to those of cmc [3,6]. The solubilization process of liposomes is mainly ruled by the formation of mixed micelles. In addition, the values for free surfactant concentrations are no more than 3%, which is below the cmc value for saturation and over the cmc value for solubilization.

The bilayer/aqueous phase partitioning coefficients in the surface saturation critical point, K_{ss}, have not been previously reported, which explains why there is no information about the relationship between values of (C_W^{ss}) and the cmc. However, for three of the four measured surfactants the intercept value of the fit with Eq. (4) yields a result close to the corresponding cmc (but always accompanied with a big value for the standard error). These results support the replacement of C_W in Eq. (3) with the corresponding cmc value, in order to obtain the partitioning coefficient at this critical point. The bilayer/aqueous phase partitioning coefficients calculated with these approaches are shown in Table 4. These data indicate an increasing affinity of the sucrose ester to the vesicle bilayer with the increase of the length of the alkyl chain, but besides this, the quantity of surfactant needed to saturate and solubilize the vesicle increases significantly. Also, the increase in alkyl chain length is accompanied by an increment in the concentration of surfactant required to solubilize from the saturation point. Comparison of the ratio between K^{sat} and K^{sol} for each surfactant shows a direct dependence with the surfactant alkyl chain length, indicating that both the partitioning of surfactants into vesicles and complete solubilization depend on this length. This behavior of sucrose esters is similar to the one observed for alkyl glucosides. The plot of Fig. 4 shows the dependence of K^{sat} on the cmc of the surfactant and includes data reported by Lopez et al. for the solubilization of phosphatidylcholine liposomes with alkylglicosides [3]. Despite the differences between DODAC SUVs and PC liposomes, clearly both sets of data follow the same trend supporting the applicability of the fluorescence method to determine the parameters describing the solubilization process. The results indicate that partitioning is mainly ruled by the surfactant cmc values, as has previously been reported for alkylglicosides, i.e. by the hydrophilic–lipophilic balance in the surfactant molecule.

On the other hand, an interesting point corresponds to the eventual applicability of sucrose esters in solubilization of

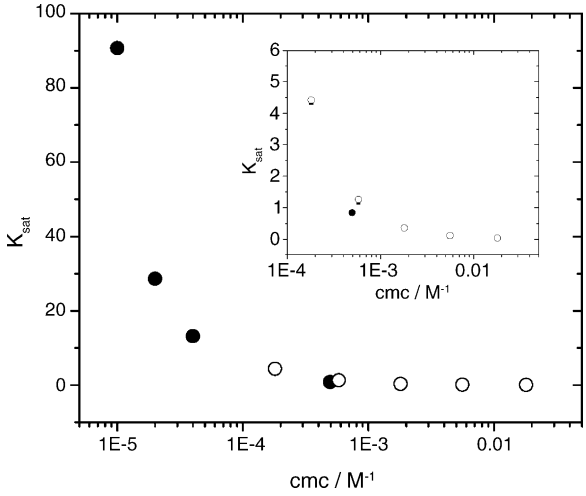

Fig. 4. Dependence of K^{sat} on the cmc of the surfactant. Data for solubilization of DODAC SUVs with sucrose esters (●) and for the solubilization of phosphatidylcholine liposomes with alkylglicosides (◯). Inset of a zoom for the high cmc section.

biological membranes. Comparison of the MLS concentration needed to saturate and solubilize DODAC SUVs with data for docecyl glucoside-phosphatidylcholine liposomes shows that when using MLS and DODAC SUVs, a concentration of one fourth than that needed of dodecyl glucoside produces bilayer saturation, and a slightly lower concentration is necessary to reach the solubilization critical point, regardless the limitations arising from the differences in the microaggregates (charged DODAC SUVs versus neutral phosphatidylcholine liposomes). These findings imply that the hydrophilic–lipophilic balance make MLS an adequate surfactant in membrane solubilization processes. Based upon our data sucrose esters with shorter alkyl chains (C_8–C_{10}) would be more convenient alternatives with respect to the use of octyl glucoside.

4. Conclusions

Measurements of Laurdan generalized polarization enable fluorescence techniques to be used as an attractive, simpler and useful option to determine the parameters associated with liposome solubilization by surfactants. Sensitivity of fluorescent methods, involving Laurdan as a probe, allows to detect possible morphological changes on vesicle surfaces prior to bilayer saturation. For the interaction of sucrose esters of different alkyl chain with DODAC SUVs, a new critical concentration (observed as a breakpoint in the plot of GP against the reciprocal of surfactant concentration) is detected before saturation of the vesicle. The values for lipid to surfactant ratio and partition coefficient are well measured; however, for surfactants with very low cmc, such as sucrose esters, this method is not adequate to determine C_W of saturation or solubilization because they are obtained with significant error.

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