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SHORT COMMUNICATION

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Influence of the level of cholesteryl sulfate in the solubilization of stratum corneum lipid liposomes by sodium dodecyl sulfate

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Abstract The role played by cholesteryl sulfate (Chol-sulf) in the solubilization of liposomes modeling the stratum corneum (SC) lipids by sodium dodecyl sulfate (SDS) was studied. We determined the surfactant-to-lipid molar ratios and the bilayer/aqueous phase surfactant partition coefficients of this interaction by varying the proportion of Chol-sulf, the relative proportions of the others lipids remaining constant. These parameters were determined by monitoring the changes in the static light scattering of the system during solubilization. The fact that the free surfactant concentration was always similar to its critical micelle concentration indicates that the liposome solubilization was mainly ruled by the formation of mixed micelles. The SDS ability to saturate and solubilize SC liposomes decreased as the proportion of Chol-sulf in the bilayers increased until a minimum was reached for a Chol-sulf proportion of about 15%. Inversely, the SDS partitioning into liposomes (or affinity with these bilayers) increased as the proportion of Chol-sulf increased until a maximum was reached at similar Chol-sulf proportions (10–15%). Hence, in these Chol-sulf proportions (similar to that existing in the intercellular lipids, which was 10%) the ability of SDS molecules to interact with liposomes exhibits a minimum despite their enhanced partitioning into liposomes. These effects may be related to the reported dependencies of the level of Chol-sulf on the abnormalities in the skin barrier function and on the SC intercellular cohesion.

Key words Stratum corneum lipid liposomes · Sodium dodecyl sulfate · Stratum corneum liposome solubilization · Influence of cholesteryl sulfate in stratum corneum liposome solubilization · Dynamic light scattering changes

Introduction

The stratum corneum (SC) forms a continuous sheath of alternating squamae (protein-enriched corneocytes) embedded in an intercellular matrix enriched in nonpolar lipids displayed as lamellar sheets. The proportion of cholesterol and cholesteryl sulfate (Chol-sulf) in these lipids is claimed to play an important role in the stability properties of the SC (cohesion and desquamation) and in the regulation of the skin barrier function [1–5]. Thus, patients with recessive X-linked ichthyosis show elevated proportions of Chol-sulf due to steroid sulfatase deficiency [6], whereas tissues with extremely tenacious intercellular cohesion also present higher Chol-sulf proportions than that existing in skin lipids [7]. SC lipid liposomes have been used to study the role played by each lipid in the SC lipid phase behavior [8–11]; however, the

molecular mechanism by which Chol-sulf affects SC shedding is not clear.

Sodium dodecyl sulfate (SDS) has frequently been used as a model substance to induce structural changes in the epidermal surface and in the SC transcutaneous permeability barrier [12–15]. The interaction of SDS with lipid bilayers leads to the breakdown of lamellar structures and to the formation of lipid–surfactant mixed micelles [16–18]. A significant contribution in this area has been made by Lichtenberg et al. [19], who postulated that the surfactant/lipid molar ratio (*Re*) producing liposome solubilization depends on the surfactant critical micelle concentration (cmc) and on the bilayer/aqueous medium partition coefficients (*K*).

We studied the formation of liposomes using a mixture of four lipids modeling the SC composition and the interactions of alkyl sulfates and mixtures of SDS/alkyl betaines with these liposomes [20–23]. We also investigated the role played by the ceramides in the interaction of SDS with SC lipid liposomes [24]. Here, we seek to extend these studies by characterizing the influence of the Chol-sulf on the resistance of SC lipid liposomes to be solubilized by SDS. To this end, we determined the *Re* and *K* parameters of this interaction at lytic level by varying the proportion of Chol-sulf in the bilayers. This information may shed light on the possible correlation between the level of Chol-sulf in the bilayers and the abnormalities in the skin barrier function and in the SC cohesion.

Materials and methods

SDS was obtained from Merck and was further purified by column chromatography [25]. Piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) was obtained from Merck (Darmstadt, Germany). PIPES buffer was prepared as 20 mM PIPES containing 110 mM Na_2SO_4 and adjusted to pH 7.20 with NaOH. Polycarbonate membranes and membrane holders were purchased from Nucleopore (Pleasanton, Calif.). Reagent grade organic solvents, ceramides type III (Cer), cholesterol and palmitic acid were supplied by Sigma Chemical Co. (St Louis, Mo.). Chol-sulf was prepared by reaction of cholesterol with excess chlorosulfonic acid in pyridine and was purified chromatographically. The molecular weight of Cer used in the lipid mixtures was determined by low-resolution fast-atom-bombardment mass spectrometry using a Fisons VG Auto Spec Q (Manchester, UK) with a caesium gun operating at 20 kV. A molecular weight of 671 g was obtained for the major component of the ceramides used (Sigma). This value was used to calculate the molarity of the lipid mixture investigated. The lipids of the highest purity grade available were stored in chloroform/methanol 2:1 under nitrogen at −20 °C until use.

Liposome preparation and characterization

Liposomes formed by mixtures of SC lipids by varying the percentage of Chol-sulf from 1 to 25%, the relative proportions of the other lipids remaining constant, were prepared following the method described by Wertz et al. [8]. The lipid compositions investigated are given in Table 1. After preparation the liposomes were annealed at 60 °C for 30 min and incubated at 25 °C under a N_2 atmosphere. The final volumes of the liposomes were adjusted with PIPES buffer to provide a final lipid concentration ranging from 0.5 to 5.0 mM. Experiment 3 corresponded to the composition of the intercellular lipids, in accordance with the data reported by Wertz et al. [8]. The lipid composition of the liposomes after preparation was determined by thin-layer chromatography coupled to an automated flame ionization detection system (Iatroscan MK-5, Iatron Lab., Tokyo, Japan) [20, 26].

Table 1 Liposome lipid composition corresponding to the six experiments, in which the percentage of cholesteryl sulfate (*Chol-sulf*) varied from 1 to 25% and the relative proportions of the other lipids remained constant

Exp no.	Liposome lipid composition (%)			
	Ceramides type III	Cholestrol	Palmitic acid	Chol-sulf
1	44.0	27.5	27.5	1.0
2	42.2	26.4	26.4	5.0
3	40.0	25.0	25.0	10.0
4	37.8	23.6	23.6	15.0
5	35.6	22.2	22.2	20.0
6	33.4	20.8	20.8	25.0

In order to find out whether all the lipid mixture components formed liposomes, vesicular dispersions were analyzed for these lipids [26]. The dispersions were then spun at 140 000 *g* at 25 °C for 4 h to remove the vesicles [27]. The supernatants were tested again for these components. No lipids were detected in any of the supernatants. The phase transition temperatures of the lipid mixtures forming liposomes were determined by proton magnetic resonance, showing values ranging from 55 to 59 °C [20]. The size distribution and polydispersity index (PI) of the liposomes after preparation were determined by dynamic light scattering measurements using a photon correlator spectrometer (Malvern Autosizer 4700c PS/MV). The samples were adjusted to the appropriate concentration range with PIPES buffer. Measurements were taken at 25 °C at a scattering angle of 90°.

Parameters involved in the interaction of SDS with SC lipid liposomes

In the analysis of the equilibrium partition model proposed by Schurtenberger et al. [28] for bile salt/lecithin systems, Lichtenberg et al. [19] and Almog et al. [27] have shown that for mixing lipids, at a lipid concentration L (millimoles) and surfactant, at a concentration S_T (millimoles), in dilute aqueous media, the distribution of surfactant between lipid bilayers and aqueous media obeys a partition coefficient K, given (in reciprocal millimoles) by

$$K = S_B/[(L + S_B)S_W] \quad , \tag{1}$$

where S_B is the concentration of surfactant in the bilayers (millimoles) and S_W is the surfactant concentration in the aqueous medium (millimoles). For $L \gg S_B$, the definition of K, as given by Schurtenberger et al., applies:

$$K = S_B/(LS_W) = Re/S_W \quad , \tag{2}$$

where Re is the effective molar ratio of surfactant to lipid in the bilayers ($Re = S_B/L$). Under any other conditions, Eq. (2) has to be employed to define K; this yields

$$K = Re/S_W[1 + Re] \quad . \tag{3}$$

This approach is consistent with the experimental data offered by Lichtenberg et al. [19] and Almog et al. [27] for different

surfactant lipid mixtures over wide ranges of *Re* values. The validity of this model for the system investigated is studied in the Results and discussion section. The solubilization of liposomes was characterized by two parameters, termed Re_{SAT} and Re_{SOL} (according to the nomenclature adopted by Lichtenberg et al. [19]), corresponding to the *Re* ratios at which static light scattering (SLS) starts to decrease with respect to the original value and shows no further decrease. These parameters corresponded to the surfactant/lipid molar ratios at which the surfactant saturated liposomes and led to a complete liposome solubilization. Equal volumes of SDS solutions were added to the liposome suspensions and the resulting mixtures were left to equilibrate for 24 h at 25 °C. The final surfactant concentration was calculated from each mixture. This time was chosen as the optimum period needed to achieve a complete surfactant/liposome equilibrium in the lipid concentration range used [29, 30]. The temperature of 25 °C was selected by the following reasons; the reasonable stability of the SC liposomes in these conditions; similar experimental conditions to those used to study the interaction of this surfactant with phosphotidylcholine (PC) liposomes; these experimental conditions are generally used in "in vivo" tests to study the interaction of alkyl sulfates with skin [12, 13, 15]. SLS measurements were made at 25 °C using a spectrofluorophotometer (Shimadzu RF-540, Kyoto, Japan) with both monochromators adjusted to 500 nm [31]. The assays were carried out in triplicate and the results given are the average of those obtained.

The determination of the *Re* and S_W parameters was carried out on the basis of the linear dependence existing between the SDS concentrations required to saturate and solubilize liposomes and the lipid concentration (L), which can be described by the equations

$$S_{SAT} = S_{W,SAT} + Re_{SAT}[L] \quad , \tag{4}$$

$$S_{SOL} = S_{W,SOL} + Re_{SOL}[L] \quad , \tag{5}$$

where S_{SAT} and S_{SOL} are the total surfactant concentrations. The surfactant/lipid molar ratios Re_{SAT} and Re_{SOL} and the aqueous concentration of surfactant $S_{W,SAT}$ and $S_{W,SOL}$ are in each curve, respectively, the slope and the ordinate at the origin (zero lipid concentration). The K_{SAT} and K_{SOL} parameters (bilayer/aqueous phase surfactant partition coefficient for saturation and complete liposome solubilization) were determined from Eq. (3).

Results and discussion

We previously reported the CMCS of SDS in the working medium, which was 0.5 mM [31]. The characterization of the geometric properties of liposomes used in the present study was reported previously [20]. This study demonstrated that these liposomes were formed by unilamellar vesicles in all cases. Furthermore, the vesicle size distribution of the liposomes after preparation varied very little, showing in all cases a similar value of about 100 nm (PI lower than 0.1), thereby indicating that the size distribution was very homogeneous. The size of the vesicles after addition of equal volumes of PIPES buffer and equilibration for 24 h always showed values similar to those obtained after preparation with a slight PI increase (between 0.11 and 0.13). Hence, the SC lipid liposomes investigated were reasonably stable in the absence of SDS under the experimental conditions used.

A systematic study based on SLS variations of liposomes by varying the level of Chol-sulf and due to the action of SDS was performed to shed light on the possible dependencies between the level of Chol-sulf in skin lipids and the skin function barrier abnormalities. To this end, the percentage of Chol-sulf was varied from 1 to 25% (lower and higher than the proportion reported by Wertz et al. [8] for this lipid in the SC, which was 10%), the relative proportion of the other lipids remained constant (Table 1).

To determine the partition coefficients of SDS between bilayers and the aqueous phase, we first studied the validity of the equilibrium partition model proposed by Lichtenberg et al. [19] and Almog et al. [27] based on Eq. (1) for the systems investigated. This equation may be expressed by $L/S_B = (1/K)(1/S_W) - 1$. Hence, this validity requires a linear dependence between L/S_B and $1/S_W$; this line should have a slope of $1/K$, intersect with the L/S_B axis at -1 and intersect with the $1/S_W$ axis at K.

To test the validity of this model for the systems investigated, the liposomes were mixed with varying sublytic SDS concentrations (S_T). The resultant surfactant-containing vesicles were then spun at 140 000 *g* at 25 °C for 4 h to remove the vesicles [27]. No lipids were detected in the supernatants [26]. The SDS concentration in the supernatants (S_W) was determined by high-performance liquid chromatography [32] and its concentration in the lipid bilayers was calculated ($S_B = S_T - S_W$). The results of the experiments in which S_B and S_W were measured (at the same range of lipid and surfactant concentrations used to determine K) were plotted in terms of the dependence of L/S_B on $1/S_W$. Straight lines were obtained for each lipid mixture tested (r^2 = 0.093, 0.990, 0.988, 0.993, 0.994 and 0.992 for experiments 1, 2, 3, 4, 5 and 6, respectively, Table 1). These straight lines were dependent on L and always intersected with the L/S_B axis at -0.95 ± 0.11. Both the linearity of these dependencies and the proximity of the intercept to -1 support the validity of this model to determine K for these surfactant/liposome systems.

To determine the *Re* and S_W parameters the SLS variations in the SC liposomes due to the action of SDS were studied by varying the lipid composition (Table 1), the lipid concentration ranging from 0.5 to 5.0 mM. The SLS curves obtained for experiment 3 are given in Fig. 1. The addition of surfactant led to an initial increase and a subsequent fall in the SLS intensity of the system until achieving a low constant value. The curves obtained from the different experiments (Table 1) showed similar trends to those exhibited for experiment 3 (results not shown). This SLS behavior is similar to that reported for the interaction of the same surfactant with PC liposomes [33], although showing in all cases a more pronounced initial SLS increase.

The surfactant concentrations producing 100% (S_{SAT}) and 0% (S_{SOL}) of SLS were obtained for each lipid concentration by graphical methods. The arrows A and B (curve for 5.0 mM lipid concentration, Fig. 1)

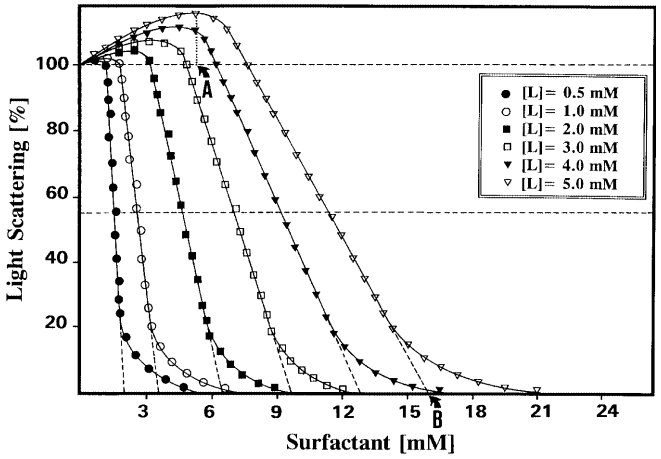

Fig. 1 Percentage changes in static light scattering (SLS) of stratum corneum (SC) liposomes, (lipid composition for experiment 3, Table 1) the lipid concentration ranging from 0.5 to 5.0 mM, induced by the presence of increasing amounts of sodium dodecyl sulfate (SDS). *Symbols*: Lipid concentrations 0.5 mM (●), 1.0 mM (○), 2.0 mM (■), 3.0 mM (□), 4.0 mM (▼), 5.0 mM (▽)

correspond to these parameters. When plotting the S_{SAT} and S_{SOL} values thus obtained for each experiment versus the lipid concentration the curves shown in Fig. 2A and B were obtained, respectively, in which an acceptable linear relationship was established in all cases. The error bars given in these figures are standard deviations and represent the error of three replicates. The straight lines obtained corresponded to Eqs. (4) and (5), from which the *Re* and S_W values were determined. The results obtained for each experiment including the regression coefficients of the straight lines (r^2) are given in Table 2.

The free surfactant concentrations ($S_{W,SAT}$, $S_{W,SOL}$) were always comparable to the SDS cmc, also showing similar values to those reported for the interaction of this surfactant with PC liposomes [33]. These findings extend to the SC liposomes investigated the generally admitted assumption for PC liposomes that the free surfactant concentration must reach its cmc for solubilization to occur and indicate that liposome solubilization was mainly ruled by the formation of mixed micelles [19, 33]. Furthermore, the rise in the Chol-sulf percentage in liposomes resulted in a slight increase in both $S_{W,SAT}$ and $S_{W,SOL}$.

The variations of *Re* and *K* versus the proportion of Chol-sulf in the bilayers are plotted in Fig. 3A and B, respectively. The increase in the Chol-sulf proportion resulted in a clear increase in the *Re* parameters until a maximum was reached for a Chol-sulf proportion of about 15% (Fig. 3A), this increase being more pronounced for Re_{SOL}. The rise in the proportion of this lipid also resulted in a rise in the partitioning of SDS into these bilayers (Fig. 3B) until a maximum was achieved when the Chol-sulf proportion was 10–15%.

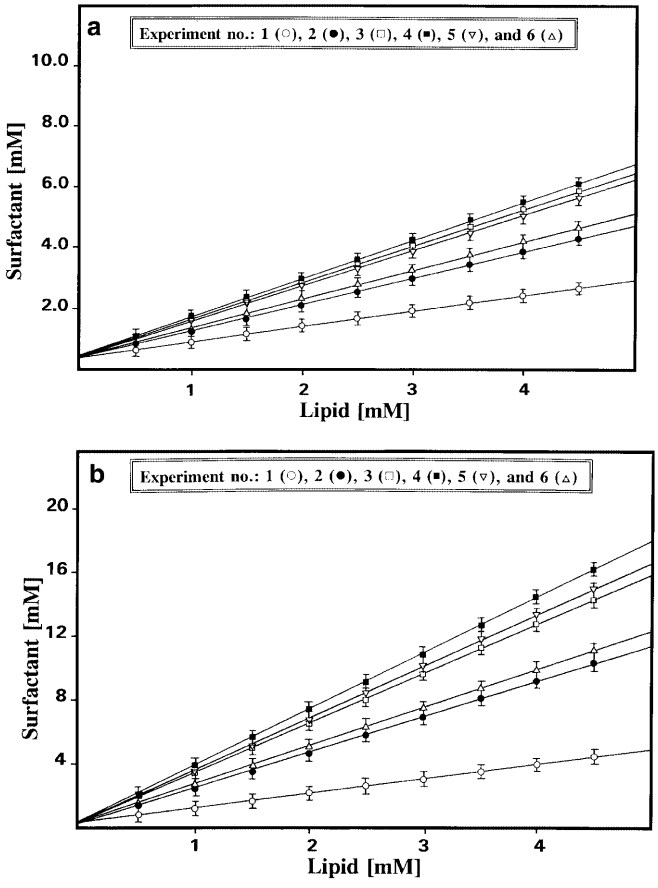

Fig. 2 **A** Surfactant concentrations resulting in saturation (100% SLS) of SC liposomes versus lipid concentration and due to the action of SDS for the experiments of Table 1. *Symbols*: experiments 1 (○), 2 (●), 3 (□), 4 (■), 5 (▽) and 6 (△). **B** Surfactant concentrations resulting in complete solubilization (0% SLS) of SC liposomes versus lipid concentration and due to the action of SDS for the experiments of Table 1. *Symbols*: experiments 1 (○), 2 (●), 3 (□), 4 (■), 5 (▽) and 6 (△)

Given that the surfactant capacity to saturate or solubilize liposomes is inversely related to the *Re* values, these capacities decreased as the proportion of Chol-sulf increased, showing a minimum at a Chol-sulf proportion of about 15% (optimum resistance of liposomes to be saturated and solubilized by SDS). Inversely, the surfactant affinity with SC liposomes (SDS partitioning into liposomes) exhibited a maximum at similar Chol-sulf proportions. Thus, at lower and higher Chol-sulf proportions than 15% the ability of the SDS molecules to saturate or solubilize the SC liposomes increased despite their reduced partitioning into liposomes. This effect, which was specially pronounced at low Chol-sulf proportions, emphasizes the fragility of the Chol-sulf impoverished bilayers against SDS. The increased SDS partitioning and *Re* values in liposomes containing Chol-sulf similar to that in the SC lipids [8] underlines the stability of these bilayers against SDS in spite of the

Table 2 Surfactant to lipid molar ratios (*Re*), partition coefficients (*K*) and surfactant concentrations in the aqueous medium (S_W) resulting in the interaction of sodium dodecyl sulfate with stratum corneum liposomes by varying the proportion of Chol-sulf from 1 to 25 %, the proportions of the other lipids remaining constant. Lipid compositions in Table 1

Exp. no.	$S_{W,SAT}$ (mM)	$S_{W,SOL}$ (mM)	Re_{SAT} mol/mol	Re_{SOL} mol/mol	K_{SAT} (mM^{-1})	K_{SOL} (mM^{-1})	r^2 (SAT)	r^2 (SOL)
1	0.40	0.48	0.50	0.90	0.83	0.98	0.995	0.993
2	0.42	0.49	0.85	2.20	1.10	1.40	0.992	0.999
3	0.44	0.50	1.20	3.08	1.24	1.50	0.992	0.994
4	0.46	0.51	1.25	3.50	1.20	1.52	0.996	0.996
5	0.47	0.51	1.15	3.25	1.13	1.50	0.994	0.997
6	0.48	0.51	0.92	2.35	0.99	1.37	0.996	0.998

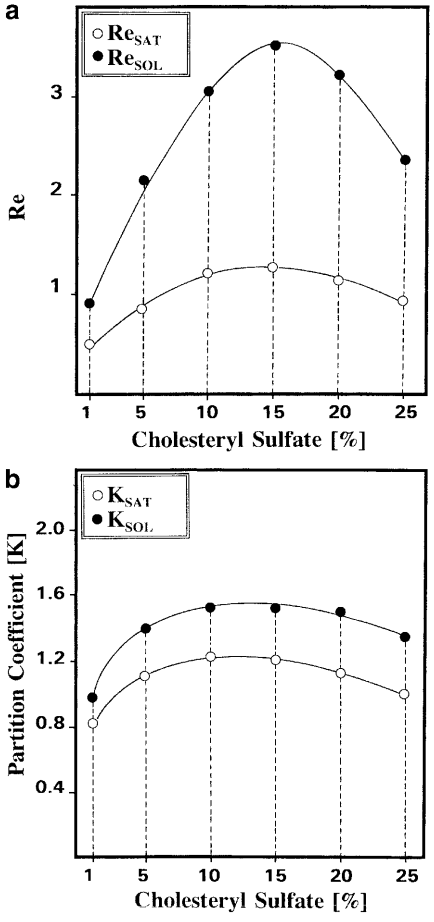

Fig. 3 A Effective surfactant-to-lipid molar ratios (Re_{SAT} and Re_{SOL}) in SC liposomes for SDS versus the percentage of cholesteryl sulfate (*Chol-sulf*) in the liposomes. *Symbols*: Re_{SAT} (○) and Re_{SOL} (●). **B** Partition coefficients (K_{SAT} and K_{SOL}) in SC liposomes for SDS versus the percentage of Chol-sulf in the liposomes. *Symbols*: K_{SAT} (○) and K_{SOL} (●)

enhanced number of surfactant molecules incorporated into the liposomes. These findings are in line with those recently reported by Hatfield and Fung [11], who demonstrated that the inclusion of this proportion of Chol-sulf in SC lipid liposomes reduced the intervesicle lipid interaction and helped to prevent the collapse of fused vesicles into other structures. The present findings may be related to the reported dependencies between the alterations in the level of Chol-sulf in the intercellular lipids and the abnormalities in the SC barrier function [1, 2, 6]. In fact, insufficient or excessive Chol-sulf content would alter the liquid-crystalline "melting point" of these lipids, thereby producing nonphysiological phase transitions that would affect the skin barrier function.

Comparison of the present data with that reported for the sublytic interaction of this surfactant with SC liposomes by varying the proportion of Cer [24] shows noticeable parallelisms and differences. Thus, although in both cases the rise in the proportion of these two lipids increased *Re* and *K* only in the case of Chol-sulf, the lowest activity and the highest affinity took place at the lipid composition around that of the intercellular lipids [8]. In the case of Cer the increasing presence of this less polar lipid increased the *Re* values even at proportions higher than that existing in the SC. Hence, changes in the lipid mixture affecting the lipid–surfactant electrostatic interactions (as occurred when the proportion of the more polar lipid, Chol-sulf exceeded that of the SC lipids) seem to affect more drastically the vesicle stability against SDS than when the exceeding lipid exhibits less polar characteristics as occurred with Cer. Hence, the electrostatic interactions seem to play an important role in this interaction and, consequently, in the stability of these vesicles against SDS.

In conclusion, this model has proved to be useful in establishing a correlation between the proportion of Chol-sulf in bilayers and the stability of these structures against SDS. The level of Chol-sulf in liposomes plays an important role both in the resistance of these vesicles to be saturated and solubilized by SDS and in the surfactant affinity with these structures. These findings may be related to the reported dependencies between the alterations in the level of Chol-sulf in the intercellular lipids and the abnormalities in the SC barrier function.

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