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Partitioning of Triton X-100, deoxycholate and $C_{10}EO_8$ into bilayers composed of native and hydrogenated egg yolk sphingomyelin

Fredrik Ollila*, J. Peter Slotte

Department of Biochemistry and Pharmacy, Åbo Akademi University, P.O. Box 66, FIN 20521 Turku, Finland

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Abstract

We have used isothermal titration calorimetry (ITC) to study the thermodynamics of Triton X-100 ($T_{X\text{-}100}$), deoxycholate and decyl octaethylene glycol ($C_{10}EO_8$) penetration into bilayers composed of native (ESM) and hydrogenated egg yolk sphingomyelin (DHSM). Light scattering measurements were used to study the point of saturation ($R_{e,sat}$) and the onset of solubilization of membranes by the detergents. We found that DHSM bilayers at 25 °C were much more resistant to detergent partitioning (lower K) and gave higher reaction enthalpies (ΔH) for all three detergents compared to the ESM bilayer system. Because DHSM lacks double bonds (Δ^{4trans} and some *cis* bonds as well), attractive acyl chain interactions are favored in membranes of this lipid class. The high stability and cohesion of DHSM in membranes could be a crucial functional property of this lipid as it is enriched in eye lens membranes.

Keywords: Isothermal titration calorimetry; Phospholipid; Bilayer; Detergent; Partitioning

1. Introduction

Sphingolipids constitute, together with glycerophospholipids and cholesterol, a major lipid class in cell membranes. Natural sphingolipids have a tendency to segregate laterally in the lipid bilayer and they have been found to play a central role in the formation of lateral heterogeneity and so called domains together with cholesterol [1–3]. The formation of lateral heterogeneity is thought to be a result of several different interactions, with intermolecular hydrogen bonding and hydrophobic forces predominating. The chemical structures of these lipids reflect their behavior in membranes. For instance, natural sphingomyelins (SM) differ from most biological glycerophospholipids in containing long, mostly saturated acyl chains, which enable them to pack tighter than natural glycerophospholipids in membranes [4,5]. This packing property gives natural sphingolipids much higher melting temperatures (T_m) than membrane glycerophospholipids, which are rich in unsaturated acyl chains [4]. Glycerophospholipids are the major lipid class in the membranes of the eye lens epithelium, while the lens fibers have been shown to mainly contain cholesterol and SMs [6–10]. A large part of the SM found in the eye lens fibers was furthermore shown by Byrdwell and Borchman [9] to belong to the dihydrosphingomyelin (DHSM) species. They showed in a mass-spectrometric study that 76.9% of the sphingolipids in the human eye lens membranes account for 4,5-dihydrosphingomyelin compared to other biological membranes where DHSM, on average, accounts for only 5–10% of the total SM mass.

The available data on the biophysical properties of DHSM is scarce as compared to that of SM [9]. DHSM lacks the *trans* double bond between carbons 4 and 5, which is present in SM (Scheme 1). We showed recently that the bilayer melting temperature of D-*erythro*-*N*-palmitoyl-SM was 41.2 °C whereas it was 47.7 °C for D-*erythro*-*N*-palmitoyl-DHSM [11]. We also presented results showing that cholesterol interacts more favorably with DHSM compared to acyl-matched SM [11]. This finding could in part explain why the human lens membranes are highly enriched in cholesterol and have more cholesterol relative to phospholipid than any other tissue [12]. The mechanism of the enrichment of DHSM in the human eye lens membranes is still unknown. The high affinity of cholesterol for SM and DHSM also leads to the formation of condensed domains in model bilayer membranes [11]. Not surprisingly, the domains formed with cholesterol and DHSM were more

Abbreviations: DHSM, hydrogenated egg yolk sphingomyelin; ESM, egg yolk sphingomyelin; $T_{X\text{-}100}$, Triton X-100

* Corresponding author. Fax: +358-2-215-4010.
E-mail address: follila@abo.fi (F. Ollila).

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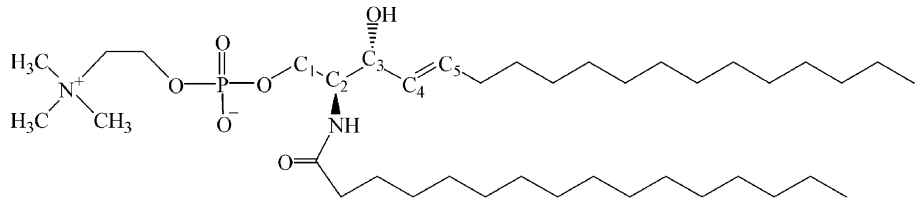
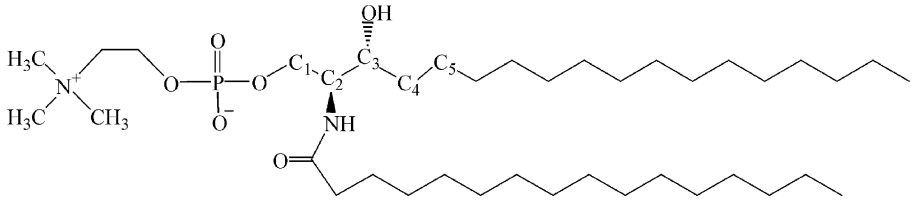


Sphingomyelin



Dihydrosphingomyelin

Scheme 1.

stable than the domains formed between cholesterol and SM, and could withstand higher temperatures before "melting".

The amphipathic properties of detergents enable them to partition into or bind to lipid membranes [13]. The partitioning process, like almost all physico-chemical processes, is associated with heat effects that can be measured and studied by means of titration calorimetry. We have used titration calorimetry to study the partitioning thermodynamics of Triton X-100 ($T_{X\text{-}100}$), deoxycholate and $C_{10}EO_8$ penetration into bilayers composed of native and hydrogenated egg yolk sphingomyelin. By studying the detergent penetration into these two membrane types, we wanted to learn more about the properties of the interfacial region of the SM/DHSM membranes, and about the possible role of DHSM/s in forming detergent-resistant "domains" or "rafts" in biological membranes.

2. Experimental procedures

2.1. Material

The sodium salt of deoxycholate (3α, 12α-dihydroxy-5β-cholanoic acid) and $T_{X\text{-}100}$ was purchased from Sigma Chemicals (USA) and $C_{10}EO_8$ was obtained from Fluka (Buchs, Switzerland). Egg yolk sphingomyelin (ESM) was purchased from Avanti Polar Lipids (Birmingham, AL). Egg yolk dihydrosphingomyelin (DHSM) was prepared from ESM by hydrogenation using palladium oxide (Aldrich Chemicals Co., Milwaukee, WI), as catalyst [14]. The purity of the DHSM solution was furthermore analyzed by means of reversed phase chromatography with a LiChrospher 100 RP-18 column (5-μm particle size, 250 × 4 mm column dimensions) using methanol at a flow of 1 ml/min to assure full hydrogenation of ESM to DHSM. All chemicals were used without further purification. The water used for the isothermal titration calorimetry (ITC) and fluorometric experiments was purified by reverse osmosis and a Millipore UF Plus water purification system, to yield a product with a resistivity of 18.2 MΩ cm. If not otherwise specifically denoted, the buffer composition used was 10 mM Tris, 140 mM NaCl, pH 7.4.

2.2. ITC

Titration calorimetry was performed using a high-sensitivity isothermal titration calorimeter 4200 from Calorimetric Science Corporation (Provo, Utah). The ITC was calibrated electrically. The data were acquired by computer software provided by the manufacturer. All experiments were performed under constant stirring (300 rpm) driven by a stepping motor coupled to the ITC. The sample cell volume was 1335 μl in all experiments. Detergent solutions were used at different concentrations below their critical micelle concentration (cmc). The experiments were carried out as described by Wenk et al. [15]. In control experiments, 10-μl aliquots of phospholipid vesicle solution (20 mM) were injected into the sample cell containing buffer without detergent. The measured dilution heat was small (below − 12 μJ/inj.) compared to the heat of reaction acquired from the partitioning experiments. The dilution heat was corrected for during the data analysis.

2.3. Determination of the cmc for $C_{10}EO_8$

The cmc for $C_{10}EO_8$ was determined with ITC using a demicellization protocol. The injection of micellar detergent dispersion into buffer allows measurement of the demicellization heat derived form the dissociation of the detergent molecules from the micelles. The demicellization heat was then used to define the cmc of $C_{10}EO_8$, which was calculated to be 0.85 ± 0.05 mM. The cmc for $T_{X\text{-}100}$ was

taken as 0.23 mM [16] and for deoxycholate a cmc of 1.8 ± 0.2 mM was used [17].

2.4. Differential scanning calorimetry (DSC)

An aliquot of the vesicle solution that was used for the ITC experiments was diluted to a final lipid concentration of about 1 mM (0.7 mg/ml). The lipid solutions and buffer were loaded into the sample and reference cell, respectively, of a Nano II high-sensitivity scanning calorimeter (Calorimetric Science). Heating and cooling scans from 20 to 60 °C at a scan rate of 0.3 °C/min were obtained. Three consecutive heating scans were made on both samples, giving identical thermograms.

2.5. Preparation of large unilamellar vesicles (LUVs)

LUVs were prepared from either ESM or DHSM using a Lipextruder (Lipex Biomembranes, Vancouver, BC) and the extrusion technique described by Hope et al. [18]. In brief, lipids were suspended and briefly sonicated in buffer to yield multilamellar vesicles (MLVs). The MLVs were then extruded at 65 °C through 100-nm polycarbonate filters (Costar Corp., Cambridge, MA) for 10 cycles to yield a homogeneous solution with unilamellar vesicles [19]. The phospholipid vesicles were size analyzed by means of dynamic light scattering with a Malvern 4700 (Malvern Instruments Ltd, Malvern, Worcestershire, UK) using an argon laser (514.5 nm) at a 90° angle to define the vesicle size. Our vesicle extrusion system produced an average vesicle size of 110 ± 30 nm. The lipid concentrations were determined with phosphorus analysis [20] and the LUV dispersion was diluted with buffer to 20 or 60 mM. The LUV and detergent solutions were prepared with the same buffer to minimize dilution heat.

2.6. Detection of detergent solubilization of vesicles with light scattering

Light scattering experiments were performed to assure that the detergent solutions used in the ITC experiments did not solubilize the phospholipid vesicles. The experiments were performed using a PTI Quantamaster 1 spectrofluorimeter operating in the T-format with both excitation and emission wavelengths set at 300 nm. The experiments were performed by injecting aliquots of an ESM or DHSM vesicle solution into a cuvette containing 1335-μl $T_{X\text{-}100}$, deoxycholate or $C_{10}EO_8$ at 25 °C. The light scattering intensity was monitored after every injection. Control experiments were carried out by injecting the vesicle solution into the same volume of buffer.

We also used light scattering to determine the detergent/lipid ratio at which the membranes of ESM and DHSM vesicles were saturated with detergent ($R_{e,sat}$), and when solubilization was initialised. These experiments were carried out by injecting aliquots of 10 mM $T_{X\text{-}100}$, deoxycholate or $C_{10}EO_8$ into 0.5 mM phospholipid vesicle solutions at 25 °C. The light scattering was measured with both excitation and emission monochromators set to 300 nm. The degree of solubilization was assessed from the changes in the light scattering.

3. Results and discussion

3.1. Bilayer stability in the presence of detergent molecules

To ensure that the phospholipid bilayers were stable in the presence of detergent molecules, we determined potential vesicle solubilization by measuring changes in light scattering from the vesicles in a fluorescence spectrophotometer (Fig. 1). Experiments were performed by titrating 10-μl aliquots of ESM or DHSM (20 mM) vesicles into the cuvette containing $T_{X\text{-}100}$ (0.15 mM), deoxycholate (0.3 mM) or $C_{10}EO_8$ (0.5 mM) at 25 °C. Titration of vesicles into detergent-free buffer was used as a reference. The cuvette volume in the scattering experiments was equal to the volume in the ITC sample cell (1335 μl). We found that

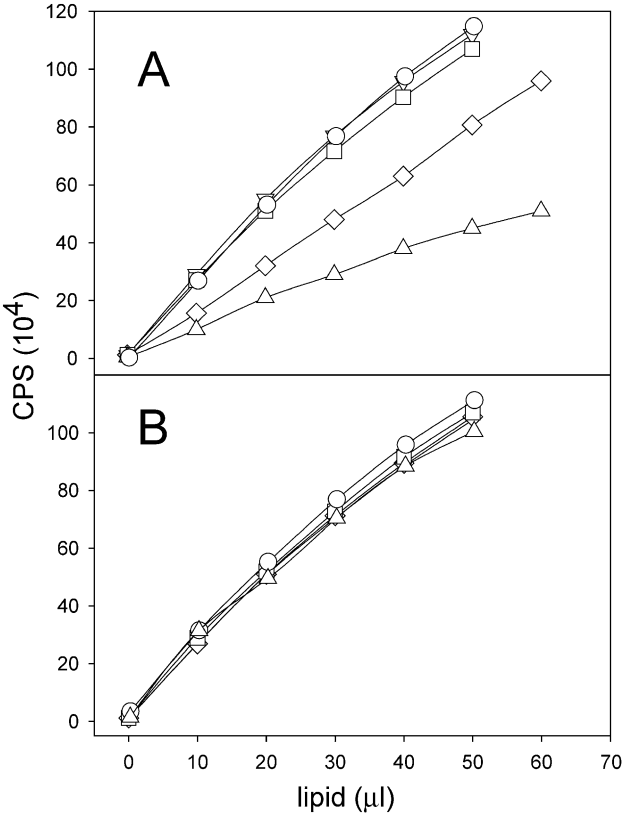


Fig. 1. Detection of vesicle solubilisation by means of light scattering. Titrations of (▽) buffer, (○) $T_{X\text{-}100}$ (0.15 mM), (◇) deoxycholate (0.3 mM), (△) deoxycholate (0.6 mM) and (□) $C_{10}EO_8$ (0.5 mM) with vesicles composed of ESM (panel A) or DHSM (panel B) as detected by light scattering. The experiments were performed by titrating 10-μl aliquots of vesicle solution (20 mM) into the detergent solution (V = 1.335 ml). The solutions were constantly stirred and the measurements performed at 25 °C.

T_{X-100} (0.15 mM) and $C_{10}EO_8$ (0.5 mM) at the indicated concentrations did not solubilize ESM or DHSM vesicles, whereas deoxycholate at 0.3 mM caused the ESM vesicles to scatter light with a slightly lower intensity. At 0.6 mM deoxycholate, the light scattering intensity from ESM vesicles was markedly lower (Fig. 1). To further clarify the effect of deoxycholate on ESM and DHSM vesicles, we determined possible solubilization with titration calorimetry. A vesicle–micelle transformation was clearly observed when ESM vesicles (60 mM) were injected into deoxycholate (present at 0.6 mM; Fig. 2). The DHSM bilayers remained intact in presence of deoxycholate and presented a pure partition equilibrium in contrast to ESM vesicles, which were solubilized. The vesicle solubilization-membrane reformation process has been intensively studied [21–26]. The first injections of vesicles into the detergent solution result in a complete solubilization of the vesicles by the detergent molecules giving rise to lipid–detergent micelles, as confirmed by the light scattering experiment. Vesicle solubilization is an endothermic process as can be seen in Fig. 2. After reaching a critical detergent/membrane ratio (0.33 mole ratio) the exothermic membrane formation process compensates the endothermic detergent partitioning signal to a large enough extent to turn the injection peaks into the exothermic territory. The titration experiment corresponds to pure partition equilibrium when all the lipid–detergent micelles have transformed into bilayers. When using a deoxycholate concentration of 0.3 mM, despite the finding that the light scattering signal was slightly diminished for ESM membranes, we did not observe solubilization with ITC (Fig. 3). Partition studies were consequently

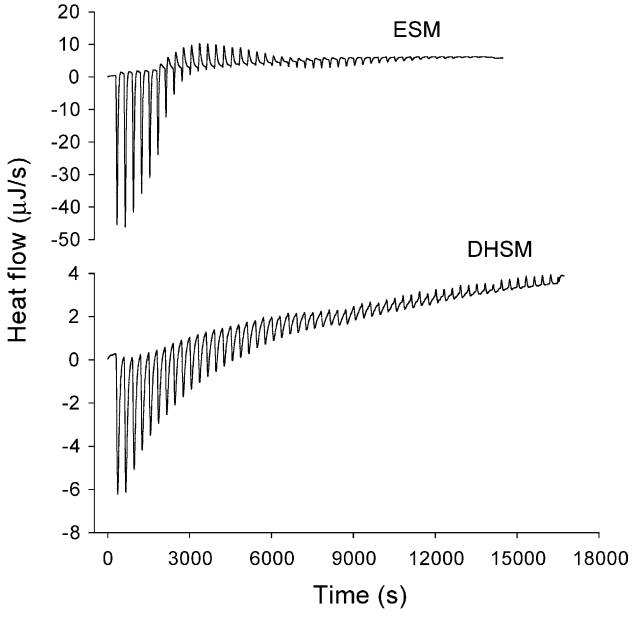


Fig. 2. Detection of vesicle solubilisation by means of titration calorimetry. Titration of deoxycholate (0.6 mM) with large unilamellar vesicles composed of ESM (60 mM) or DHSM (60 mM) in buffer. The experiments were performed by titrating 5-μl aliquots of vesicles into the detergent solution (V = 1.335 ml) in the sample cell at 25 °C.

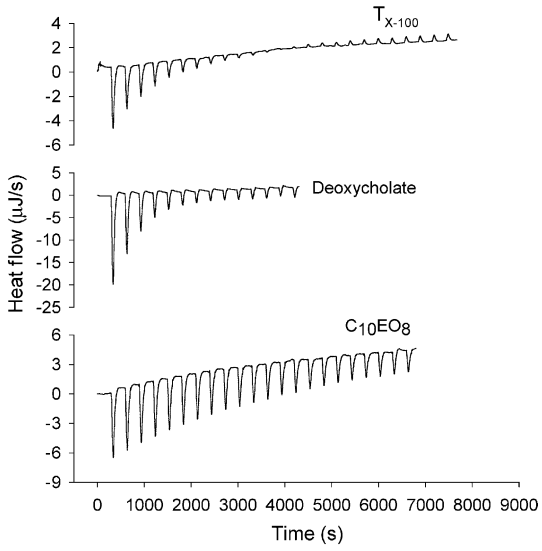


Fig. 3. Partitioning of detergents into ESM vesicle membranes. Titration of T_{X-100} (0.15 mM), deoxycholate (0.3 mM) and $C_{10}EO_8$ (0.5 mM) with large unilamellar vesicles composed of ESM (20 mM) in buffer was performed at 25 °C. The experiment was performed by titrating 10-μl aliquots of vesicles into the detergent solution (V = 1.335 ml) in the sample cell.

performed with detergent concentrations that were under their respective cmc values and that did not cause apparent vesicle solubilization. We used 0.3 mM for deoxycholate (despite the light scattering findings at this concentration for ESM vesicles) since the signal/noise ratio for the partition experiments became rather small and did not allow a further decrease in detergent concentration.

3.2. Onset of detergent solubilization of ESM and DHSM vesicles

Light scattering was also used to determine the detergent/sphingolipid ratio at which the membranes of the ESM and DHSM vesicles were saturated with detergent ($R_{e,sat}$), and when solubilization was initialised. The experiments were performed by measuring the light scattering intensity of the phospholipid vesicle solution as aliquots of T_{X-100}, deoxycholate or $C_{10}EO_8$ was being added to the cuvette (Fig. 4). The initial light scattering intensity (before any detergent was added) was similar in both ESM and DHSM vesicle solutions. The DHSM membranes proved to be more stable during the initial injections of detergent. The intensity was stable until a certain detergent concentration was reached at which the intensity started to decrease. T_{X-100} and deoxycholate saturated the membranes at quite low detergent/lipid ratios, while $C_{10}EO_8$ reached $R_{e,sat}$ at a markedly higher value (Fig. 4). There was a clear difference in the onset of solubilization of the ESM and DHSM membranes. The DHSM membranes were in all cases more stable and showed higher $R_{e,sat}$ values ($T_{X-100} = 0.525 \pm 0.025$, deoxycholate = 0.85 ± 0.05 and $C_{10}EO_8 = 1.7 \pm 0.01$) compared to ESM ($T_{X-100} = 0.39 \pm 0.01$, deoxycholate = 0.425 ± 0.025 and $C_{10}EO_8 = 1.175 \pm 0.025$).

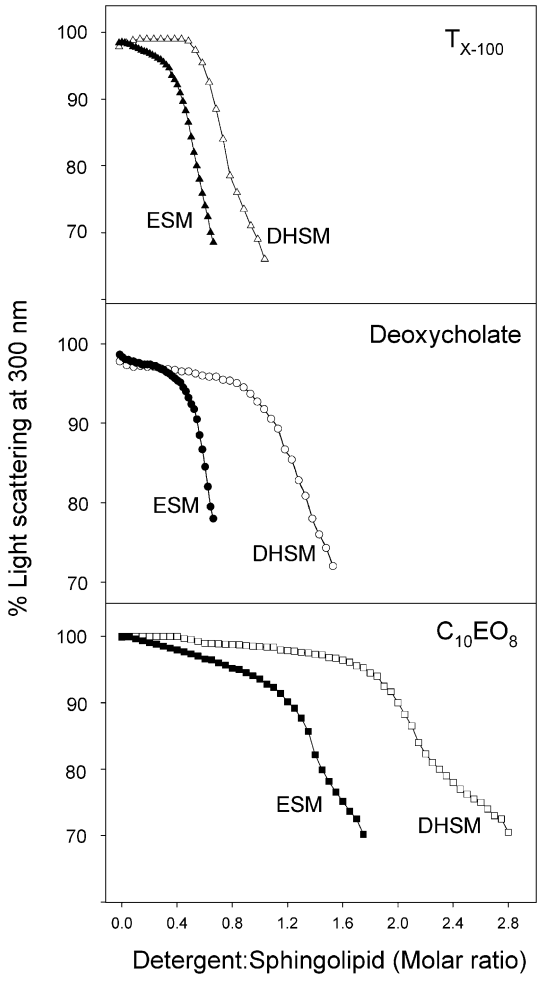


Fig. 4. Onset of vesicle solubilisation detected by light scattering. Titration of T_{X-100}, deoxycholate and $C_{10}EO_8$ with vesicles composed of ESM or DHSM. The experiments were performed by titrating 5-μl aliquots of detergent solution (10 mM) into the vesicle solution (0.5 mM) in buffer (V=2 ml). The solutions were constantly stirred and the measurements performed at 25 °C.

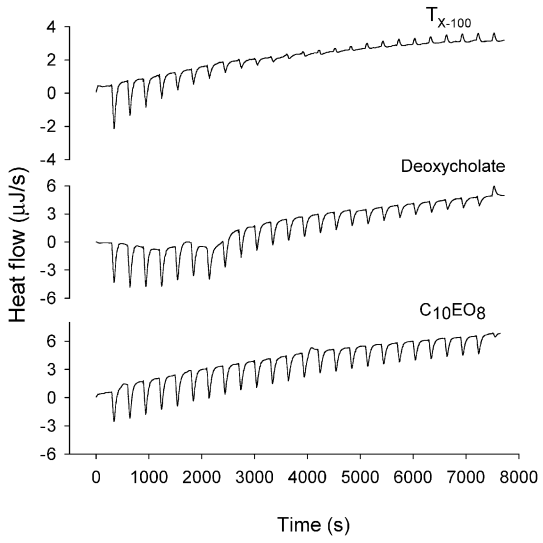


Fig. 5. Partitioning of detergents into DHSM vesicle membranes. Titration of T_{X-100} (0.15 mM), deoxycholate (0.3 mM) and $C_{10}EO_8$ (0.5 mM) with large unilamellar vesicles composed of DHSM (20 mM) in buffer was performed at 25 °C. The experiment was performed by titrating 10-μl aliquots of vesicles into the detergent solution (V=1.335 ml) in the sample cell.

3.3. Partitioning of T_{X-100}, deoxycholate and $C_{10}EO_8$ into bilayers composed of ESM or DHSM

The partitioning of T_{X-100}, deoxycholate and $C_{10}EO_8$ into large unilamellar vesicles composed of ESM or DHSM was conducted to see how the Δ^{4trans} double bond in the long-chain base and the *cis* unsaturations (about 7% of the SM species contained one single *cis* mono-unsaturation), which are present in ESM but not in DHSM, would affect partitioning of these detergents into the bilayers. All three detergents showed a substantially higher degree of partitioning (higher K values) into ESM compared to DHSM membranes (Figs. 3 and 5, Table 1). This suggests that the detergent molecules were transferred into the bulk hydrocarbon core of the ESM membranes or bound to the ESM membrane interface to a higher degree compared to the DHSM membranes. It was also found that the ΔH was markedly lower for the partitioning of the detergents into ESM compared to DHSM membranes.

The partitioning of detergent molecules into phospholipid bilayers has recently been presented and thoroughly discussed by several research groups, including ourselves [13,16,27–31]. The partitioning process can be described to result from a hydrophobic effect in which the reduction of the intermolecular hydrogen bonding capacity of water, caused by the hydrophobic moieties of the detergent molecules, forces the detergent molecules to be distributed between the hydrophobic environment in the membranes and the bulk water. It is therefore the removal of the

Table 1
Partitioning of Triton X-100, deoxycholate and $C_{10}EO_8$ into ESM and DHSM membranes

	K (M^{-1})	ΔH (kJ mol^{-1})	ΔG (kJ mol^{-1})	ΔS (J K^{-1} mol^{-1})
ESM				
Triton X-100	2000 ± 100	6.5 ± 0.5	− 18.8	85
Deoxycholate	2200 ± 100	13.2 ± 2.8	− 19.1	109
$C_{10}EO_8$	160 ± 10	24 ± 2	− 12.6	123
DHSM				
Triton X-100	550 ± 50	13 ± 0.5	− 15.6	96
Deoxycholate	200 ± 2	29 ± 2	− 13.1	141
$C_{10}EO_8$	60 ± 2	42 ± 2	− 10.1	175

Aliquots (10 μl) of ESM or DHSM vesicles (20 mM) in buffer were injected into the sample cell of the containing a Triton X-100 (0.15 mM), deoxycholate (0.3 mM) or $C_{10}EO_8$ (0.5 mM) solution at 25 °C. The partition coefficients are calculated under the assumption that all phospholipid is available for partitioning. Values are given as the average value ± range from three different experiments.

hydrophobic moieties from the polar phase to the bulk hydrocarbon in the lipid membrane that increases the overall entropy of the system and makes partitioning possible [15,28]. If the detergent is applied at low enough concentration, $c_D < \text{cmc}$, membrane partitioning is the only process to be considered [15], as is the case of this study.

The observed differences in ESM and DHSM membranes clearly reflect the effect of saturation (in DHSM) on packing density and cohesion among the acyl chains. The higher ΔH values seen for DHSM membranes with all three detergents reflect the higher energy barrier to be overcome during partitioning of detergents into the highly saturated DHSM membranes. The large difference in partitioning seen for $T_{X\text{-}100}$ and $C_{10}EO_8$ are most likely a result of the hydrophobic function of the molecules (the aromatic ring in $T_{X\text{-}100}$ compared to the aliphatic hydrocarbon arrangement in the hydrophobic part of $C_{10}EO_8$). The extent of partitioning is not only a function of the accepting membrane, but naturally also a function of the "activity" of the detergent. Lichtenberg [22] has demonstrated a correlation between the membrane/water partitioning coefficients of detergents and their respective cmc. Although $T_{X\text{-}100}$ and $C_{10}EO_8$ have similarities in their structures, they have quite different cmc/s ($T_{X\text{-}100} = 0.23$ mM and $C_{10}EO_8 = 0.85$ mM), which also partly explains why $T_{X\text{-}100}$ showed a higher degree of partitioning compared to $C_{10}EO_8$.

The partitioning of $T_{X\text{-}100}$ and $C_{10}EO_8$ into phospholipid bilayers has been investigated in several studies. Pantaler et al. [32] recently presented results showing that $T_{X\text{-}100}$ and $C_{10}EO_8$ partitioned into erythrocyte membranes with K values of 2386 ± 416 and 287 ± 16 M^{-1}, respectively. Heerklotz and Seelig [16] showed that $T_{X\text{-}100}$ and $C_{10}EO_7$ partitioned into POPC membranes with K values of 3000 and 770 M^{-1}. These results are in reasonable agreement with our values taken into account that it is known that $C_{10}EO_7$ has higher affinity for phospholipid bilayers compared to $C_{10}EO_8$ [16].

3.4. Phase transition thermodynamics of ESM and DHSM in the presence and absence of detergent

To investigate the thermodynamics of the gel-to-liquid transition of ESM and DHSM bilayers, DSC was used. The thermograms were acquired with a heating scan rate of 0.3 °C/min. The thermograms in Fig. 6 show the heating scans obtained for dispersions of ESM and DHSM in the absence and presence of detergent. The main transition occurred at a transition temperature (T_m) of 46.0 °C for pure DHSM and the enthalpy (ΔH) of the main transition was 34.3 $kJ \cdot mol^{-1}$. The heating scan for ESM showed the main transition at a T_m of 39.6 °C and the change in ΔH was 23.5 $kJ \cdot mol^{-1}$. These results clearly show that membranes composed of DHSM were much more cohesive (more attractive interactions among the acyl chain segments) as compared with ESM membranes, a finding which is very consistent with the detergent partitioning data and the onset of solubilization data presented previously. The DSC thermograms of ESM and DHSM in the presence of $T_{X\text{-}100}$, deoxycholate and $C_{10}EO_8$ induced structural reorganization of the membranes. Although the detergent molecules markedly affected the phase transition dynamics, the membranes still remained in the gel phase at 25 °C. We took three experimental approaches when performing the DSC experiments. (i) The detergent solution was added to the lipid solution after the extrusion of the lipids and incubated for 1 h on a water bath set at 25 °C or (ii) incubated for 1 h on a water bath at 60 °C. (iii) The detergent was added before the lipids were sonicated and extruded to yield unilamellar vesicles with an equal distribution of detergent on both hemileaflets. The detergent and lipid concentrations that were used in the DSC runs were comparable to the conditions in the ITC experiments after six or seven consecutive injections (10 μl) of vesicles into the sample cell. The phase transition patterns from the DSC runs of the extruded lipid/detergent suspensions were almost identical compared to the patterns where detergent was added after extrusion (data not shown). There was, however, one exception. We found a change in the phase transition pattern when the DHSM was extruded in the presence of $C_{10}EO_8$, in comparison to the experiments where vesicles were generated in the absence of this detergent (Fig. 6). This could be an indication that flip-flop of $C_{10}EO_8$ does not occurs at 25 or 60 °C in DHSM membranes within this experimental time frame, but it might as well reflect a difference in the detergent-induced lateral segregation of the lipids when the detergent is present on both leaflets compared to only one leaflet.

$T_{X\text{-}100}$ did not alter the membrane transitions of ESM and DHSM as markedly as deoxycholate and $C_{10}EO_8$. The T_m of ESM and DHSM was only a few degrees lower and the transition remained relatively sharp in the presence of $T_{X\text{-}100}$. Marked broadening and double peak formation was on the other hand observed in the thermograms for ESM/deoxycholate, ESM/$C_{10}EO_8$ and DHSM/$C_{10}EO_8$, which suggests local instabilities and lateral heterogeneity in the bilayer membranes. The transitions of DHSM/deoxycholate and DHSM/$C_{10}EO_8$ resembled very much the transition pattern of DPPC/$C_{12}EO_8$ (2:1 mole ratio) that was acquired by Otten et al. [33].

3.5. Detergent flip-flop

When phospholipid vesicles are added to a solution containing detergent, the detergent molecules partition into the outer monolayer of the membrane, but not into the inner monolayer unless the detergent molecule undergoes flip-flop between the two hemileaflets. The transbilayer movement of $T_{X\text{-}100}$, deoxycholate and $C_{10}EO_8$ has been shown to be fast enough to assume an equal distribution between membrane hemileaflets in fluid membranes [16,34]. Hildebrand et al. [35] suggested in a recent publication that the negatively charged bile salts sodium cholate and sodium

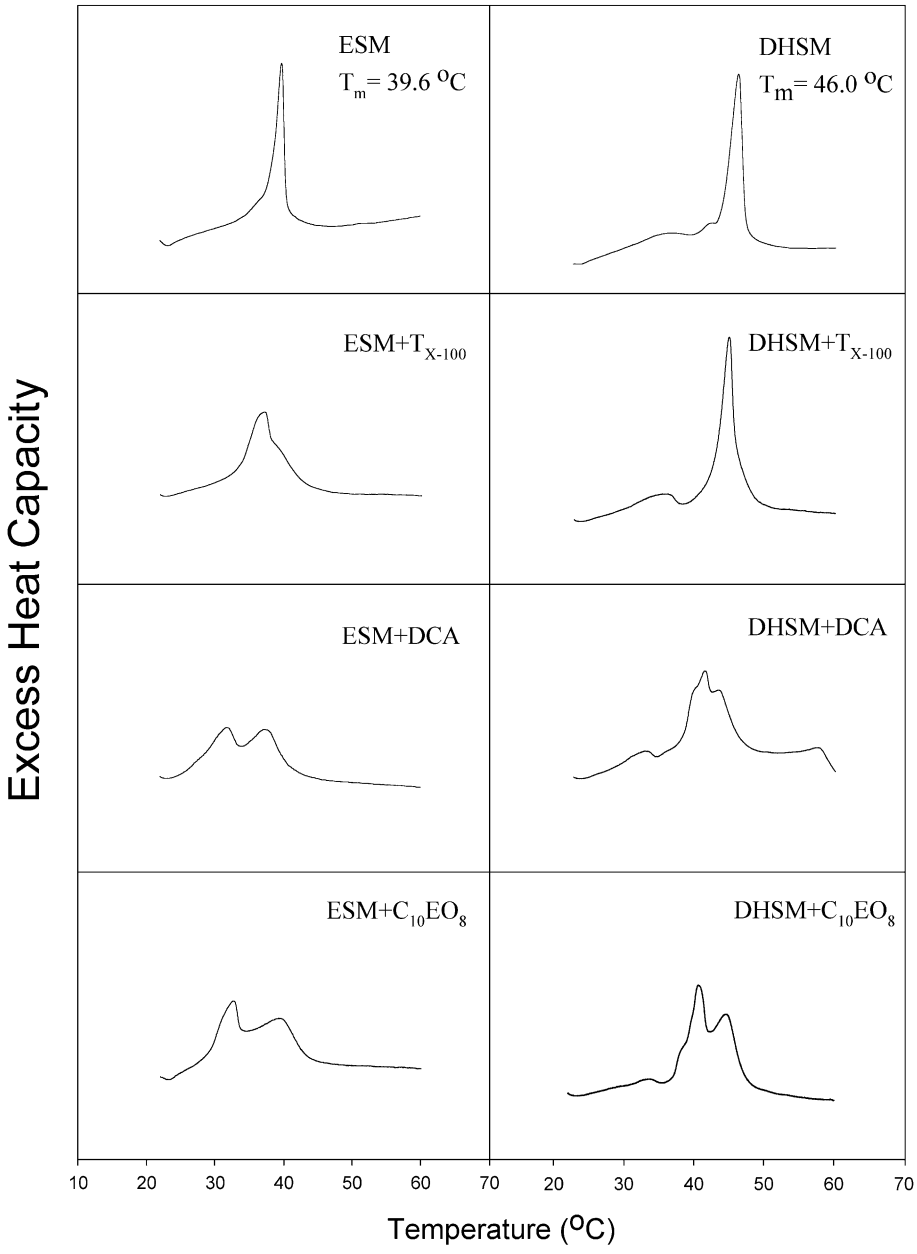


Fig. 6. Differential scanning calorimetry of ESM and DHSM. Heating curves of ESM and DHSM membranes in the absence and presence of T_{X-100} (0.15 mM), deoxycholate (DCA; 0.3 mM) and $C_{10}EO_8$ (0.5 mM). The membrane transition temperature for ESM (1 mM) or DHSM (1 mM) vesicles was studied using a scanning speed of 0.3 °C/min.

deoxycholate undergo flip-flop in DPPC membranes, with half times of 4–5 min, at 60 °C. They also suggested that the rate of flip-flop for the detergents were ~30 times slower at 25 °C, which is not unexpected and that a high degree of detergent binding promotes flip-flop. These results would indicate that sodium deoxycholate could flip-flop within the time frame of an ITC experiment, taken into account the high degree of binding in the beginning of the ITC experiment. Other results on the flip-flop of detergents in membranes in the gel phase have, to our knowledge, not been presented. Heerklotz [36] has noted that the flip-flop is not easily measured, although many attempts have been made to define the translocation phenomena using several different methodological approaches.

The fact that almost all the DSC runs with detergent added before or after extrusion were almost identical, together with the membrane instabilities observed in the transition patterns, suggests that the detergent molecules did undergo flip-flop under these conditions in these two types of membranes. Especially the high detergent-to-lipid ratio in the beginning of the ITC experiment altered and destabilized the membrane structure to an extent that most likely induced flip-flop of the detergent molecules in the bilayer. Although the ESM and DHSM membranes are in the gel

state in the presence of detergent, all the thermodynamic parameters presented in Table 1 were computed with the assumption that these three detergents distribute freely across the acceptor membranes due to the detergent-induced perturbation of the membrane.

In conclusion, ours results clearly show that DHSMs are, relatively speaking, more resistant to detergent partitioning and detergent-induced solubilization as compared with SMs having the Δ^{4trans} double bond and occasional *cis* mono-unsaturations. The high stability and cohesion of DHSM in membranes could be a crucial functional property of this lipid as it is enriched in lens membranes where it, together with cholesterol, provides a fully integrated protective barrier for both chemical and physical stress.

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