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# Ordered-disordered domain coexistence in ternary lipid monolayers activates sphingomyelinase by clearing ceramide from the active phase

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ABSTRACT

We explored the action of sphingomyelinase (SMase) on ternary monolayers containing phosphatidylcholine, sphingomyelin (SM) and dihydrocholesterol, which varied along a single tie line of phase coexistence. SMase activity exhibited a higher rate and extent of hydrolysis when the film is within the liquid-expanded (LE)/liquid-ordered (LO) coexistence range, compared to monolayers in the full LO phase. Since Alexa-SMase preferably adsorbs to the LE phase and there was no direct correlation found between enzymatic activity and domain borders, we postulate that the LE phase is the active phase for ceramide (Cer) generation. The enzymatically generated Cer was organized in different ways depending on the initial LE/LO ratio. The action of SMase in Chol-poor monolayers led to the formation of Cer-enriched domains, while in Chol-rich monolayers it resulted in the incorporation of Cer in the LO phase and the formation of new Chol- and Cer-enriched domains. The following novel mechanism is proposed to provide an explanation for the favored action of SMase on interfaces that exhibit an LE–LO phase coexistence: the LO phase sequesters the product Cer causing its depletion from the more enzyme-susceptible LE phase, thus decreasing inhibition by the reaction product. Furthermore, LO domains function as a substrate reservoir by allowing a rapid exchange of the substrate from this phase to the SM-depleted LE phase.



## 1. Introduction

Over recent years several studies have related the activation of sphingomyelinase (SMase), and the action of its product ceramide (Cer), to cell signaling events mediated by the presence of cholesterol (Chol)- and sphingomyelin (SM)-enriched liquid-ordered (LO) domains [1,2]. Based on indirect evidences, it was proposed that the production of Cer by SMase may take place within hypothetical LO domains in living cells [3]. However, biophysical studies have shown that the activity of SMase is low when the substrate SM is organized in the LO phase compared to liquid-disordered (Ld) membranes [4,5], despite the mechanism accounting for this effect not being well understood. Some studies have explored the hypothesis that SMase was activated by the presence of domain borders, but no correlation has been found between the extent of the domain borders and enzyme activation [5,6]. Furthermore, fluorescent-labeled SMase is not enriched in liquid-condensed (LC) domain borders, but instead uniformly distributed in the liquid-expanded (LE) phase [6]. In order to investigate this topic, we examined the action of SMase on ternary mixtures containing phosphatidylcholine (PC), SM, and the non-oxidizable analog of Chol dihydrocholesterol (Dchol) in model lipid monolayers. The lipid proportions of the ternary mixtures chosen varied along a single tie line of phase coexistence, which contained a 1:1:1 composition, thus spanning from a homogeneous LE phase to a completely LO phase. This feature implies that, by varying simultaneously the proportions of all three components, the ratio of LE/LO phases (and also the amount of domain borders) is changed while the composition of each phase remains constant.

Our experimental approach was based on the phase diagram and tie lines reported for PC/SM/Chol bilayers [7], and we extrapolated this concept to the monolayer system and substituted some lipids for non-oxidizable analogs. Even with these considerable changes, in previous studies we demonstrated, from detailed analysis of the mean molecular area of the lipid components in each phase and phase proportions, that our system still behaved like a single tie line [8]. Furthermore, using compression isotherms and Brewster angle microscopy, the dipole potential and monolayer thickness and composition of each coexisting phase were calculated in the ternary lipid system as a function of surface pressure. We also provided a theoretical framework for the pressure-induced liquid–liquid miscibility transition point observed in these Chol-containing monolayers.

Although similar systems have been previously studied [5,9–12], with some of the findings described here being previously observed

*Abbreviations:* SM, sphingomyelin, pSM, N-palmitoylsphingomyelin; Cer, ceramide; pCer, N-palmitoylceramide; Chol, cholesterol; Dchol, dihydrocholesterol; PC, phosphatidylcholine; dlPC, dilauroylphosphatidylcholine; LE, liquid-expanded; LO, liquid-ordered; LC, liquid-condensed; LD, liquid-disordered; BAM, Brewster angle microscopy; SMase, *Bacillus cereus* sphingomyelinase

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in bilayer systems, in the present study we considered several complementary points of view (SMase activity, adsorption preferences and surface dynamic reorganization) in order to respond to the following open questions. Why do LO domains, in coexistence with disordered phases, activate SMase action while pure LO phase cannot? Is the presence of domain borders a structural activator for SMase? (In this case, why do only LO domain borders work while condensed or gel domain borders cannot?) Is the enzyme acting on these borders, and therefore are they essential for Cer production? Similar to other studies on bilayers [5], the data reported in the present work show that SMase activity against ternary monolayers exhibited a higher rate and extent of hydrolysis when the state of the film was within the LE/LO coexistence range and showed an abrupt fall when all the monolayer is in the LO phase state. We suggest that the LE phase, rather than domains borders, is probably the active location of Cer generation in films containing LO domains. Based on studies of PC/Cer/Chol bilayers reported in the literature [13], on the previous mechanism of action described for SMase in LE monolayers [6] and on the results described herein, we propose the following mechanism that explains SMase regulation by the presence of LO domains; a membrane that exhibits coexistence of the LE–LO phases offers a favorable topographic surface for SMase action, by allowing rapid dynamic clearance of products from the active phase and enhanced accessibility of the enzyme to the SM molecules. The results and discussion in the present work provide a comprehensive interpretation of the processes involved in the SMase action on LO domain-containing membranes.

## 2. Materials and methods

### 2.1. Chemicals

The lipids palmitoylsphingomyelin (pSM), dilauroylphosphatidylcholine (dlPC) and Dchol were purchased from Avanti Polar Lipids (Alabaster, AL). These were found to be more than 99% pure by thin-layer chromatography and were therefore used without further purification. The Chol analog Dchol was utilized instead of Chol because it minimizes air oxidation during experiments and shows an interfacial behavior similar to that of Chol [14–16]. Choline methyl-$^{14}$C bovine SM (52 mCi mmol$^{-1}$) was obtained from Perkin Elmer Life and Analytical Science (Boston, USA). *Bacillus cereus* sphingomyelinase (SMase) (EC 3.1.4.12) was purchased from Sigma-Aldrich (St. Louis, MO), and the amine-reactive probe Alexa Fluor 488 carboxylic acid succinimidyl ester was obtained from Invitrogen (Eugene, OR). Solvents and chemicals were of the highest commercial purity available, and the water was purified using a Milli-Q and Milli-Rho combined system (Millipore, Billerica, MA). The absence of surface-active impurities was routinely checked as described elsewhere [17]. Lipid stock solutions were kept under $N_2$ at −70 °C until use.

### 2.2. Ternary monolayers

The lipid proportions of the ternary mixtures studied in this work varied along a tie line that included the 1:1:1 composition point, as was previously demonstrated in Ref. [8]. These spanned from the full LE ternary mixture M1 (dlPC/pSM/Dchol 74:22.5:3.3), through the ternary mixtures that showed LE/LO phase coexistence (M2, dlPC/pSM/Dchol 60:26:14; M3 dlPC/pSM/Dchol 34:33:33 and M4 dlPC/pSM/Dchol 15:37:48), and finally to the ternary monolayer that revealed only the LO phase at the surface pressure studied (20 mN m$^{-1}$) M5 (dlPC/pSM/Dchol 4:40.3:55.7). Thus, the ternary mixtures exhibited LE (M1), LE/LO (M2–M4) or LO (M5) phases, with both phases keeping the same lipid proportion and only changing the relative amount of each phase along the compositional axis (the greater the Dchol content, the more the surface area was covered by the LO phase) [8].

### 2.3. Determination of SMase activity in Langmuir monolayers

Monolayers of dlPC/pSM (where 10% of the pSM content was replaced by choline-labeled $^{14}$C-SM) and Dchol were spread from lipid solutions in chloroform/methanol (2:1) over a subphase of 10 mM Tris/HCl, 125 mM NaCl, 3 mM $MgCl_2$, pH 8, until reaching a pressure of less than ~0.5 mN m$^{-1}$. After solvent evaporation (5 min), the monolayer was slowly compressed (at ~3 Å$^2$ molecules$^{-1}$ min$^{-1}$) to the desired surface pressure (typically 20 mN m$^{-1}$) and the enzyme was injected under stirring into the subphase of the reaction compartment (18 ml; 17 cm$^2$) of an all-Teflon trough, whose surface was connected by a narrow and shallow slit to a compartment that served as a substrate monolayer reservoir. After injection of SMase (final subphase concentration 1.3 nM), SM was converted at a constant surface pressure of 20 mN m$^{-1}$ to $^{14}$C-phosphocholine, which dissolved into the subphase with Cer remains at the interface. As the reaction proceeded, the total monolayer area was continuously adjusted in order to keep the surface pressure constant by means of automatic movement of a barrier in the reservoir compartment [18]. For the determination of the reaction kinetics, aliquots of 0.5 ml of subphase were collected at different times, and the radioactivity was quantified (Liquid Scintillation counter Rackbeta 1214, Wallac).

### 2.4. Alexa dye conjugate of SMase

Conjugation of SMase with the dye was performed in 100 mM $NaHCO_3$ (pH 8.3). SMase was incubated with the amine-reactive dye Alexa Fluor 488 carboxylic acid succinimidyl ester, which was previously dissolved in DMSO and immediately added to the protein solution. The reaction mixture was incubated at room temperature under stirring for 1 h in the dark. The conjugated protein was separated from the unreacted dye by filtering through an Amicom Ultra 0.5 10 K filter (Millipore Corp., USA). Conjugates were labeled with an average ratio of 2:1 dye/protein molecule (calculated from absorbance measurements), and the recovery of the labeled protein was ~80%. The Alexa-conjugated enzyme retained 72% of the original activity, which was stored in the dark at 4 °C until use.

### 2.5. Visualization of Alexa-SMase adsorbed to monolayers

Lipid monolayers were formed by deposition of a chloroformic solution of lipids at the air/buffer interface of a circular compartment (3 ml; 3.14 cm$^2$), until reaching the desired surface pressure (20 mN m$^{-1}$). The subphase used was 10 mM Tris/HCl, 130 mM NaCl, pH 8. The absence of the cofactor $Mg^{+2}$ allowed visualization of adsorbed SMase, thus avoiding SM degradation or surface structural changes. After solvent evaporation (5 min), Alexa-SMase was injected into the subphase to achieve a final concentration of 13 nM. After 15 min of incubation, Langmuir–Blodgett films were assembled using alkylated glass as solid supports. Coverslips (12 mm-diameter) were previously alkylated with octadecyltrichlorosilane to self-assemble a covalently linked monolayer of octadecylsilane [6] in order to obtain a hydrophobic surface. To perform the transference, the coverslips were held horizontally above the monolayer and slowly lowered until touching the film. After a few seconds, the coverslips were pushed through the monolayer while the film pressure was kept constant by the surface barostat, before being gently washed and observed through the microscope (always maintained under aqueous solutions to avoid collapse of the monolayers). Epifluorescence visualization of transferred monolayers was carried out on a Zeiss Axioplan microscopy (Carl Zeiss, Oberkochen, Germany) using a mercury lamp (HBO 50) and a 63× water-LD objective. Images with exposure times of about 300 ms were taken with a CCD Olympus MX10 camera (Olympus Corp., USA). No membrane structural differences were observed in the presence or absence of $Mg^{2+}$ in the subphase, and the topography pattern maintained the surface features of the fresh monolayer (not shown). At least two independent samples

were imaged for each monolayer composition. All the experiments were carried out in an air-conditioned room (23 ± 2 °C).

### 2.6. BAM observation of ternary monolayers treated with SMase

Monolayers were prepared as described above using a KSV Minitrough apparatus (KSV, Helsinski, Finland). The Langmuir equipment was mounted on a Nanofilm EP3 Imaging Ellipsometer (Accurion, Goettingen, Germany) used in the Brewster Angle Microscopy (BAM) mode. Zero reflection was set with a polarized 532λ laser incident on the bare aqueous surface at the experimentally calibrated Brewster angle (≈53.1°). After monolayer formation and slow compression (~3 $Å^2$ $molecules^{-1}$ $min^{-1}$) to the desired surface pressure (20 mN $m^{-1}$), unlabeled SMase was injected in the subphase to obtain a final concentration of 0.15 nM. The reflected light was collected with a 20× objective at different times until no further changes were observed in the monolayer structure (typically 45–50 min). The reflectivity of each lipid phase ($R$) is given by the ratio of the intensity of the incident and reflected light at the Brewster angle to the air/water interface, and was calculated from the gray level of selected areas within each phase of the BAM images (typically $R = (\text{gray level} - 13.3) \times 10^{-7}$ with calibration factors being checked for each individual experiment according to the manufacturer). The reflectivity of each phase is related to the thickness ($d$) of the film as $R \propto a \times d^2$, where the coefficient $a$ depends on the refraction index of the subphase and the film [8,19]. The quantification of the gray levels of each phase was performed using the Image J 1.43u software (NIH, USA), and the extent of area covered by each phase and domain borders was calculated by interactive image processing routines written in IDL (Interactive Data Language, ITT, Boulder, CO) [20,21].

## 3. Results

### 3.1. SMase activity and the extent of hydrolysis is favored by the presence of LO domains

The aim of this work was to evaluate the influence of LO domains (and domain borders) on SMase action to further the understanding of the mechanisms involved. To this end, we required a ternary monolayer that contained the substrate SM, a sterol (condition for LO phase formation) and a glycerophospholipid to provide an expanded (disordered) liquid phase. Thus, SMase activity was assayed against dlPC/pSM/Dchol monolayers with lipid proportions belonging to the tie line that contained the 1:1:1 composition, as was previously determined in Ref. [8]. These ternary monolayers spanned the phase coexistence region along all the tie line, from a homogeneous LE phase (mixed film M1) to a completely LO phase (M5) via a LE/LO coexistence region (M2–M4, see Section 2.2). Fig. 1a shows the time course of hydrolysis of SM measured by the release of $^{14}C$-labeled phosphocholine in the subphase. It can be observed that after a short lag time (about 1 min), all the substrate monolayers, independent of their phase coexistence, showed a steady state velocity followed by a slowdown in the reaction rate. The maximum reaction rates, which were calculated from the slope of the linear portion of the curve after the lag time, are shown in Fig. 1b. These rates can be considered to be roughly analogous to the initial rates of a kinetic process that does not show lag time [22].

From previous studies, we could estimate that pSM was enriched in the LO phase with a content of ~40 mol%, compared to ~24 mol% of the LE phase [8]. As substrate concentration increases in the ternary monolayers, higher maximum reaction rates are to be expected. This can be observed in Fig. 1b, up to an SM content of 37 mol%. Further increases in the proportions of SM caused the entrance into the fully LO phase region of the phase diagram, where no LE phase could be observed by microscopy (see below). This point coincided with a marked drop in the maximum reaction rate, and it could not be recovered by further increasing the substrate proportions in the

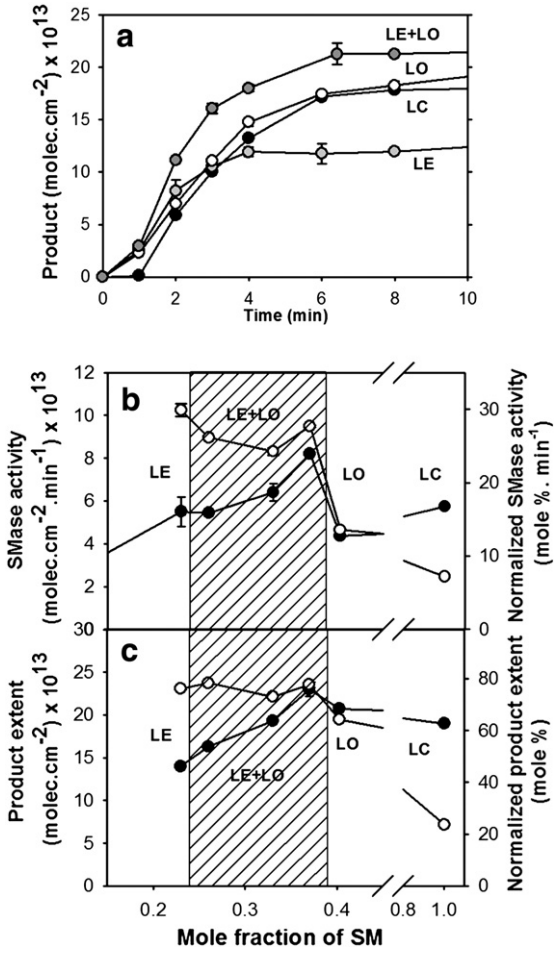


**Fig. 1.** SMase activity against ternary monolayers along the tie line. a) Time curves of Cer generation by SMase in monolayers, where 10% of the pSM content was replaced by choline-labeled $^{14}C$-SM (at 20 mN $m^{-1}$). Monolayer compositions were pure SM (black circles), M1 (LE, light gray circles), M4 (LE + LO, dark gray circles) and M5 (LO, open circles). For details in the composition of the mixtures M1–M5 see Section 2.2. b–c) Closed circles denote maximum hydrolysis rate (b) or final degradation extent (c) of SM in ternary monolayers and in pure SM monolayer plotted as a function of the initial substrate proportions at the interface. Open circles show hydrolysis rates (b) or degradation extent (c) normalized to the content of SM in the monolayer. The dashed area represents the LE/LO coexistence region. The values are the average of three independent experiments ± SEM.

film. As a comparison, we also assayed SMase activity against pure SM monolayers al 20 mN $m^{-1}$. Under this condition the film is in a liquid-condensed (LC) state [23]. In agreement with previous reports on bilayers [4,24], Fig. 1 shows that SMase was poorly active when SM was organized in such a rigid structure. A similar scenario was observed when the extent of SM degradation was analyzed (Fig. 1c). The total amount of Cer produced increased almost linearly with the original substrate concentration and throughout the LE/LO coexistence region (shadow area), but decreased in the fully LO phase at a higher SM content. To give a better comparison, in Fig. 1b and c the enzymatic activities and extents for the different ternary mixtures were normalized to the initial SM content of the substrate monolayer. These results indicate a similar good activity and accessibility to the substrate in monolayers that showed pure LE or LE/LO phase coexistence. In contrast, when the substrate monolayer was fully in the LO phase or in the LC phase (pure SM at 20 mN $m^{-1}$), the enzyme exhibited diminished reaction rates and extents of degradation.

### 3.2. Fluorescent-labeled SMase adsorbs preferentially to the LE phase

In order to investigate the differences of enzymatic activity observed when SMase was acting in the LE and LO phase, we further

explored the partition properties of SMase in the different phases. To this end, we labeled the enzyme with Alexa-fluor conjugate and incubated it under a ternary monolayer with a composition within the LE/LO coexistence range (M2). After equilibration (15 min), and to eliminate the excess fluorescence in the subphase, the lipid/enzyme monolayer was transferred to alkylated glass which acts as a solid support (see Section 2.5), washed and observed by epifluorescence microscopy (Fig. 2). As a control, enzyme-free, transferred M2 monolayers, labeled with a fluorescent probe that preferentially partitioned to the LE phase, were analyzed as shown in Fig. 2a. The LO domains appeared as dark circles surrounded by a continuous bright (LE) phase. The size and distribution of the LO domains in Fig. 2a (and also in Fig. 2b) differed from the unlabeled, untransferred monolayers observed by Brewster angle microscopy (see Section 3.3 and in previous studies [8]), with difference likely being induced by flux of the lipid film on the plane (and also the subphase) resulting from the transferring process [25], which may promote LO domain collision and fusion. The labeled-SMase monolayers (Fig. 2b) showed that the continuous phase (LE) was preferred by the enzyme, compared to the LO domains. An analysis of the fluorescence intensity of the LE and LO phases indicated a decrease in the adsorption of SMase to the LO phase of $11.7 \pm 0.2\%$ compared to the value of the LE phase. In Fig. 2b bright spots can be observed corresponding to SMase aggregates that were located preferentially along the LE/LO interface. It is worth noting that the experimental setup did not include the activator $Mg^{+2}$ in order to allow the evaluation of enzyme adsorption to the initial interface without reaction (and also to avoid surface changes induced by Cer generation, see Section 3.3). In Fig. 2b, to provide a better visualization, a 10 fold higher concentration of protein was used in the subphase (final 1.3 nM) than the concentration normally used for measuring SMase activity, as shown in Fig. 1. However, the visualization of Alexa-SMase in this latter subphase condition was beyond the technical capability of our equipment. This is easily explained if we take into account that the surface SMase concentration in equilibrium with the subphase barely reached ~100 molecules $\mu m^{-2}$ (calculated from a partition constant of $7 \times 10^4$ [22] and a subphase concentration of 0.13 nM). When the concentration of labeled-SMase was decreased by half the value used in Fig. 2b (but still five-fold that employed for SMase activity), the occurrence of bright spots of SMase was scarce, and did not appear along the LE/LO boundary (Fig. 2c).

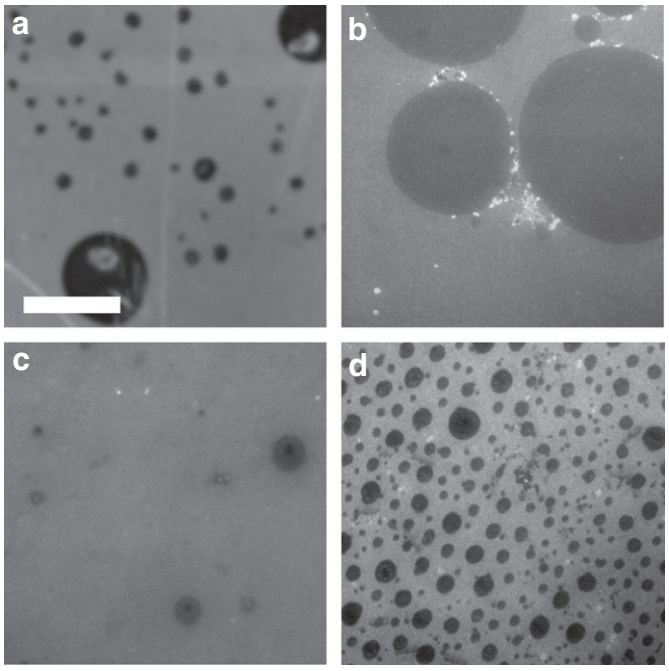


**Fig. 2.** Fluorescence-enzyme adsorption to two-phase monolayers. a) Enzyme-free ternary monolayer M2 containing 1 mol% of PE-NBD fluorescent probe; b–c) Alexa-SMase adsorbed to ternary monolayer M2 at a subphase enzyme concentration of 13nM (b) and 7.5nM (c) ; d) Alexa-SMase adsorbed to binary monolayer composed of pSM/pCer (80:20), which shows LE–LC coexistence [23] at a subphase enzyme concentration of 13 nM. Bar size is 30 μm.

Additionally, we assayed the adsorption of Alexa-SMase to monolayers composed of SM/Cer (80:20) at 10 mN $m^{-1}$, which showed LC domains containing Cer and SM (at a ratio ~2:1) surrounded by an SM-enriched LE phase, as previously reported [6,23,26] (Fig. 2d). According to these studies [6], Alexa-SMase also adsorbs preferentially to the LE phase when coexisting with a LC phase, and in agreement we found a reduced adsorption preference of SMase for the LC domains of about $21 \pm 3\%$ compared to the LE phase. Therefore, if only the phase state is considered, the results for the adsorption of SMase indicated a preference in the order LE > LO > LC.

### 3.3. The changes of surface structure produced by SMase depend on the LE/LO proportion

The changes of surface structure after treatment of ternary monolayers with SMase were explored using Brewster angle microscopy (BAM). When a polarized radiation hits the air/water surface at the Brewster angle (which contains a nanometer thick film), the ratio of the intensity of the incident to reflected light determines the reflectivity of the sample. This analysis evidences some physical properties of the film since the reflectivity (and also the gray level of each pixel of a BAM image) is related to both the refraction index and the thickness of the film. It also has the advantage of reporting surface features of the film without the addition of any marker, thus allowing identification of different lipid phases independent of probe preference. Moreover, it prevents alteration of the sample, since it has been reported in some systems (in particular when the area of the preferred phase for the probe partitioning is small) that the addition of only 1% of a fluorescent probe to lipid monolayers that exhibit phase coexistence may alter the distribution of the components between both phases [27].

We first explored the surface changes induced by SMase on a monolayer of pure pSM at surface pressures at which the film was present in the LE phase (10 mN $m^{-1}$). As previously reported in fluorescent-labeled monolayers [20,26], Cer generation under these conditions led to nucleation and growth of Cer-enriched condensed domains over time (see Fig. 3a), with reflectivity values being four-fold higher than those in the surrounding LE phase ($t$ = final Fig. 4a and c). These domains (referred to as Cer domains) appeared in the BAM images as bright areas with characteristic non-circular shapes (Fig. 3a) that covered about 28% of the area at $t$ = final (when no further changes in surface structure were observed, see also Fig. 5) and were surrounded by a dark (and thin) LE phase. The enzymatic action on full LE ternary monolayers (M1, Fig. 3b) at 20 mN $m^{-1}$ or ternary monolayers with high proportions of LE phase and small LO domains (that can be seen as circular gray domains in ternary monolayer M2, Fig. 3c) resulted in both Cer and circular domains of the LO phase (that could be differentiated by their shape and reflectivity, see Fig. 4a, b and c). It was also frequent to find Cer domains located along the linear interface between the LE and LO phases (Fig. 3c, long times).

In the 1:1:1 ternary mixture (M3), when the LO phase occupied ~70% of the initial monolayer area (Fig. 5a), the action of SMase did not lead to the formation of Cer-domains, but very bright spots were observed after long times that were usually located along the LE/LO linear interface (see Fig. 3d). These bright spots saturated the range of acquisition of our CCD camera. Therefore, although it was not possible to assign to them a defined reflectivity, approximate calculations suggested that they were not likely to correspond to a

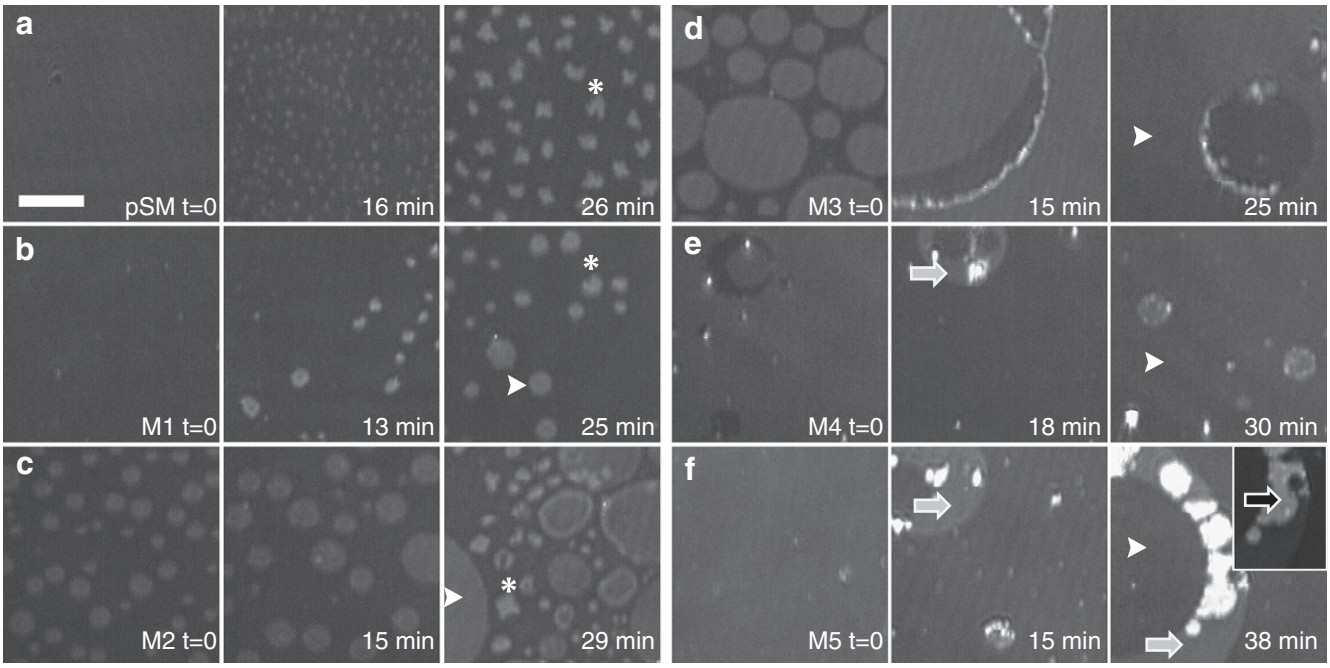


**Fig. 3.** Changes of surface structure produced by the action of SMase on ternary monolayers. BAM images of pure pSM at 10 mN $m^{-1}$ (a) and ternary monolayers M1 (b), M2 (c), M3 (d), M4 (e) and M5 (f) at 20 mN $m^{-1}$ are shown, before ($t=0$) and after SMase treatment (time from SMase injection as indicated). For a better visualization, the lower 0–100 gray level range (from the 0–255 original scale) was selected in order to keep the gray level–film thickness relationship, with the exception of the inset in (f) which the original 0–255 scale is shown. Arrowheads highlight the LO phase, while asterisks mark the Cer domains. Gray and black arrows in (e) and (f) indicate domains with an intermediate and high reflectivity, respectively. Images are representative of two independent experiments. Bar size is 30 μm.

monolayer structure (which would give an optical thickness >6 nm, if a refraction index of 1.5 is assumed). In fact, the bright spots observed represented a very low proportion of the area of the interface (~0.4%), while the LO area increased as the reaction progressed, at the expense of the LE area (decreasing from 32% to 20%, see Fig. 5). This effect was also observed for the M4 monolayer, which showed a reduction of the LE area from 12% to 2% after SMase treatment. This finding suggests that the LO phase was able to incorporate the new Cer molecules produced by SMase in the LE phase. When the amount of LO phase predominated (ternary mixtures M4 and M5), the action of SMase induced the formation of new structures.

A new phase that exhibited rounded borders and intermediate reflectivity values (between that of the Cer domains and the LO phase) occurred at long times (see Fig. 3f at long times and Fig. 4d), and will be referred to as intermediate reflectivity domains. Additionally, a second new phase was observed (see Fig. 3e and f at long times) that showed irregular borders and a higher reflectivity than any other monolayer structure (4–5 fold higher than the continuous LO phase, see Fig. 4e), and will be referred to as high reflectivity domains. The presence of bright spots was observed at low proportions from $t=0$ and increased at $t=$final. Further analysis of Fig. 4 showed that when intermediate and high reflectivity domains were present in the monolayers (M4 and M5), the reflectivity of the continuous LO phase diminished compared to that observed for the monolayers at $t=0$, and the LO in mixtures M2 and M3. This suggests that these thick structures sequestered the longer and/or more condensed components of the mixed monolayers, thus depleting the remaining LO phase.

It should be noted that the structural changes observed by BAM occurred at long times (25 to 40 min) and closer to the equilibrium compared with the reaction times reported in Fig. 1. Other authors [11,28] in addition of ourselves [29] have previously reported that the rapid conversion of SM to Cer by SMase drives the membrane structure into a metastable state, which thereby relaxes to a new organization with a kinetic that may exceed the reaction times and proceed even further when the Cer production has stopped [29].

We next investigated if the topographic features observed in SMase-treated ternary monolayers were also present in monolayers where a proportion of pSM was originally replaced by pCer. Fig. 6a shows the BAM image of an M2 monolayer that contained 20 mol% of pCer, at the expense of pSM, thus only 6% of the total lipid composition being pSM. Similar to that observed in Fig. 3c at long times, three phases can be distinguished: a continuous LE phase, LO domains and Cer domains (see also Fig. 6d). However, the premixed quaternary monolayer showed a more regular arrangement of the Cer domains (which did not locate along the LE/LO linear interface) and smaller LO domains. A quaternary M3 monolayer where 20% of pSM was replaced by pCer showed (similar to the enzyme-generated monolayer) a larger area covered by the LO phase than the untreated (Cer free) monolayer (compare Fig. 6b with Fig. 3d). In contrast with the SMase-treated monolayer, this one showed large LO domains surrounded by a thin line of LE phase that acted as the continuous phase. Bright spots can be observed but these were isolated and covered a very low proportion of total area. The analysis of M3 + pCer monolayers (as shown in Fig. 6b) revealed that both LE and LO phases increased their reflectivities when pCer was present (Fig. 6e), indicating that both phases were altered by the incorporation of pCer. Finally, we examined the ternary monolayer M5, where 32% of the total lipid concentration was replaced by pCer at the expense of pSM. This showed a continuous LO phase, with the presence of isolated and regularly distributed bright spots, and eventually led to the occurrence of intermediate and high reflectivity small domains (Fig. 6c and f).

## 4. Discussion

### *4.1. Cer production occurs mainly in the liquid-expanded phase, rather than at domain borders*

The aim of this work was to achieve a deeper understanding of the action of SMase on the lipid interfaces that exhibit phase coexistence. Whether SMase acts preferentially in the more expanded lipid phase

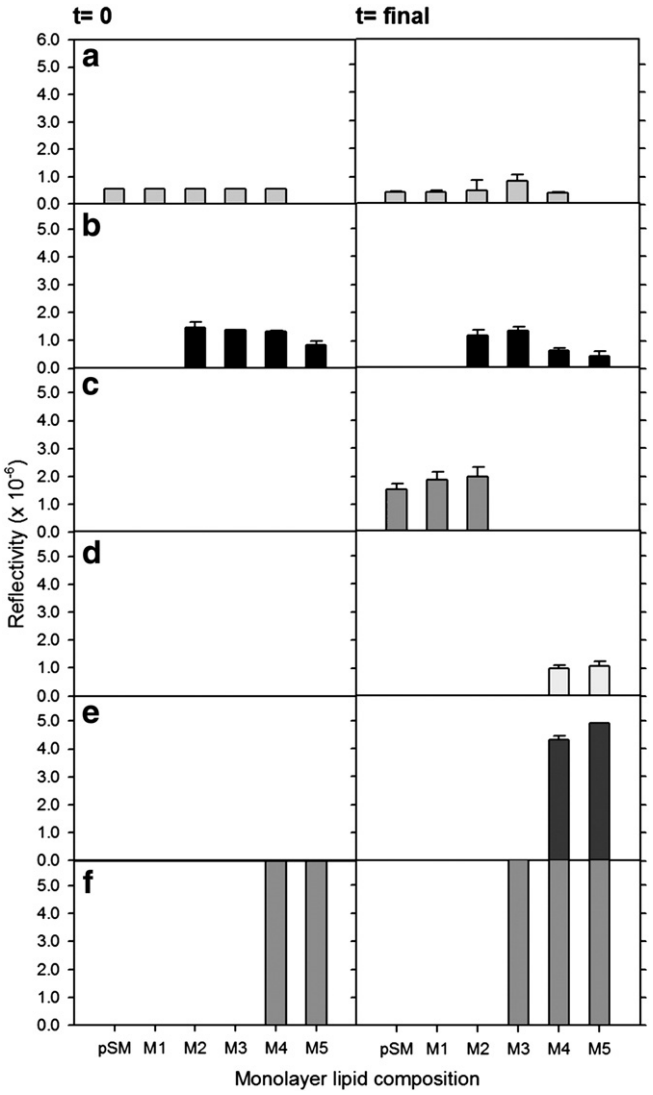


**Fig. 4.** Quantification of reflectivity in BAM images of ternary monolayers under SMase treatment. The reflectivity of each phase identified from BAM images shown in Fig. 3 are from before ($t=0$) and after ($t=$final) SMase treatment, with its occurrence as follows: a) LE phase, b) LO phase, c) Cer domains, d) intermediate reflectivity domains, e) high reflectivity domains and f) bright spots. The reflectivity of the phases in panels b–f is normalized to the reflectivity of the LE phase at $t=0$. The bars indicate the average of 10–12 images from two independent experiments ± SEM.

(called liquid-disordered (LD) in bilayers and LE in monolayers), in the LO phase, or along domain borders, is a matter of discussion. Some authors have proposed that SMase acts preferentially at the domain borders, based mainly on topographic observations [12,30]. In these studies, Cer-enriched domains were observed located at preexistent LO domain borders after SMase treatment of ternary monolayers. Although these changes were undoubtedly a consequence of SMase action, they do not provide conclusive proof about the location of the actual SM degradation site. This is due to the fact that the new Cer molecules may have been generated at any phase of the membrane, and by diffusion through the liquid phase become segregated into Cer-enriched domains that nucleated along a LD/LO linear interface. In addition, since nucleation of a new phase is a kinetically dependent event [31], it may be favored by domain borders acting as seeds for the new phase domains.

Prieto et al. observed an enhanced SMase activity in vesicles with a composition within the LD/LO phase coexistence range [5], coinciding with our results using monolayer systems (Fig. 1). Based on the finding that homogeneous LD or LO phases do not activate the enzyme [4,5], this work suggested that the presence of linear interfaces might constitute a structural activation factor for SMase. However, the same authors [5] and others [24] noted that the presence of linear interfaces generated by gel domains did not result in activation. An equivalent observation has also been reported, where no activation of SMase was found in the presence of LC domains in monolayers. Furthermore, a constant reaction rate was determined during the time course of SM degradation, concomitant with an increase in the amount of domain borders, with thereby no correlation between the extent of linear interface and SMase activity having been found in monolayers [6,20].

In the present work, the adsorption preference of Alexa-labeled SMase for ternary monolayers that show LE/LO phase coexistence was investigated in conditions where SMase catalysis was inhibited (Fig. 2). We found that Alexa-SMase had a moderate preference for the LE phase, when compared to the LO phase or with Cer/SM condensed domains (comparative fluorescence ratio 1/0.89/0.82 respectively). This result indicates a preference of SMase adsorption in the order LE > LO > LC, coinciding with SMase activity preference against bilayers [4]. A detailed inspection of Alexa-SMase-treated monolayers transferred to a solid support showed that the LO domains borders did not reveal an enhanced fluorescence that would allow a preferential adsorption to the linear interface to be observed (Fig. 2b), at least within our resolution limit, even when the subphase SMase concentration was ten times greater that used for the analysis of SMase activity. In fact, only a few SMase aggregates could be seen adsorbed

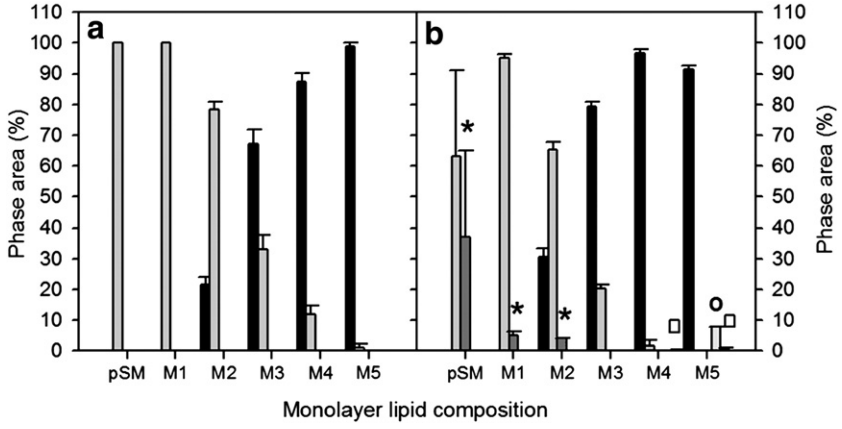


**Fig. 5.** Quantification of the area occupied by each phase in BAM images of ternary monolayers under SMase treatment. The phase areas are shown as before (a) and after (b) SMase treatment. The bars corresponds to the area occupied by the LE phase (light gray bars), LO phase (black bars), Cer domains (gray bars highlighted by *), intermediate reflectivity domains (light gray bars highlighted by°) and high reflectivity domains (gray bars highlighted by □). Bright spots occupy less than 1% of the area for mixtures M3, M4 and M5 (not shown). The bars indicate the average of 10–12 images from two independent experiments ± SEM.

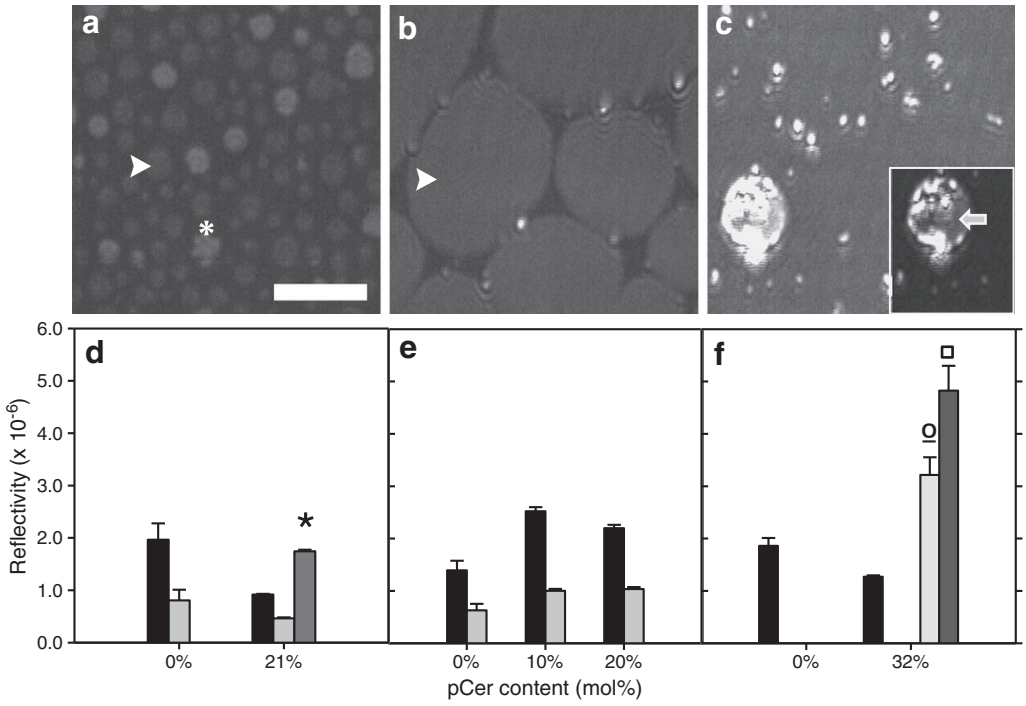


**Fig. 6.** BAM images of enzyme-free quaternary monolayers. Representative images of ternary mixtures where a proportion of pSM was replaced by pCer at 20 mN m$^{-1}$, in order to obtain the following mixtures: a) M2 + pCer 21 mol%, b) M3 + pCer 20 mol% and c) M5 + pCer 32 mol%. For a better visualization, the lower 0–100 gray level range (from the 0–255 original scale) was selected in order to keep the gray level–optical film thickness relationship, except for the inset in (c). Images are representative of two independent experiments. Arrowheads highlight the LO phase while asterisks mark Cer domains. Gray arrow in (c) indicates a domain with an intermediate reflectivity. Bar size is 30 μm. d–f) Analysis of the reflectivity values of the phases observed in ternary monolayers M2 (d), M3 (e) and M5 (f), where pSM was replaced by pCer at the total proportion indicated. The bars corresponds to the reflectivity of the LO phase (black bars), LE phase (light gray bars), Cer domains (gray bars highlighted by *), intermediate domains (light gray bars highlighted by °) and high domains (dark gray bars highlighted by □). Bright spots occurring in mixtures M3 and M5 show reflectivity $>2\times10^{-5}$ (not shown).

at the linear interface (see Fig. 2b), which were reduced significantly when the SMase concentration was lowered (but still kept five times above the concentration used for measuring SMase activity). Similar results were previously found in Alexa-SMase treated SM/Cer monolayers [6].

Although we did not demonstrate a preferential adsorption of SMase at the linear interface, the question remains of whether such a small amount of SMase molecules located at the domain borders may have had an enhanced activity to account for the high activity observed in the LE/LO coexistence region. To examine this issue, we analyzed the dependency of SMase activity on the domain border extent (calculated as linear interface per image area) for ternary monolayers that showed LE/LO coexistence. Now, if the SMase molecules that located along this border were exceptionally active, we should observe an increase of the overall activity of the sample with an increase in the linear interface. However, as can be concluded from Fig. 7, no clear correlation is apparent between SMase activity and an increased extent of the LE/LO linear interface (instead, an inverse correlation, if any, is suggested from a composition of 25 mol% SM upwards, where LO domains increased). Therefore, these results rule out the hypothesis that SMase acts preferentially at the domain borders. The discussion above also provides an answer to the previously unsolved question regarding the location of Cer production in a complex interface such as the one studied in this work, and we may conclude that Cer production occurs mainly in the LE phase, rather than at domain boundaries. Likewise, we propose that LO domain borders have an active role in substrate and product redistribution between the active LE phase and the substrate reservoir (LO) phase (see Section 4.3). This hypothesis is essential for our interpretation of the processes involved in the SMase regulation, as will be pointed out below in Section 4.3.

### 4.2. SMase-driven topographic changes in heterogeneous lipid interfaces

The changes induced by SMase action on ternary monolayers were also explored. As commented in Section 3.3, the rapid action of SMase

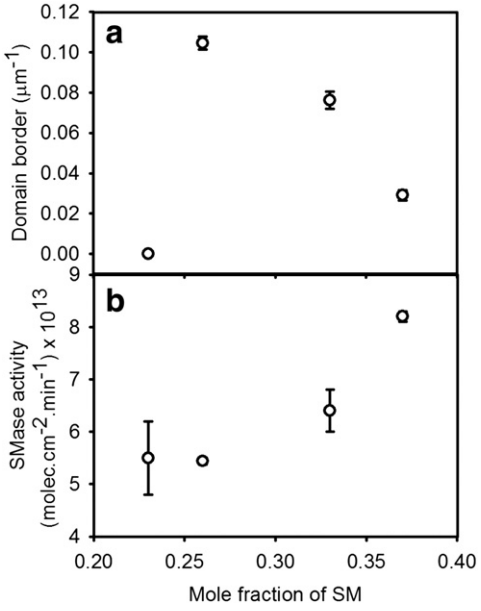


**Fig. 7.** Lack of correlation between SMase activity and the extent of the domain boundary. a) Quantification of domain borders from BAM images of ternary monolayers at 20 mN m$^{-1}$ (no enzyme added) as linear interfaces (μm)/image area (μm$^2$); b) SMase activity on ternary monolayers (from Fig. 1). The data correspond to an average of 10–14 images from two independent experiments (a) or the average of triplicates (b) ± SEM.

on a lipid interface led the system to an out-of-equilibrium condition [29], resulting in reordering of the surface structure. This phenomenon has been explored previously by other authors on vesicles that show LD/LO phase coexistence [5] but the system used did not permit direct visualization of the membrane or measurement of the extent of the domain boundaries. In addition, several papers have reported the changes induced by SMase on supported bilayers by AFM [9,10,12] but direct enzyme activity measurements were not available. Lateral reorganization in supported bilayers may be influenced by reduced lateral diffusion in contrast with free standing bilayers or air/water monolayers. The present work advances the study of this issue, because the use of the BAM technique on air/water lipid monolayers allows monitoring of the structural features of the surface in a dynamic way as the reordering of the membrane proceeds. In this way, unreported structures of Cer enriched interfaces could be observed.

Our results revealed different landscapes depending on the initial ratio of the LE/LO phase areas (Fig. 3). When the LE phase occupied a large proportion of surface area and behaved as the continuous phase (at low Dchol content), the action of SMase resulted in the formation of Cer-enriched domains, which have been previously characterized in initially full LE monolayers [20,21,26] and LD bilayers [28,32–34]. These enzymatically-generated Cer domains were also related to Cer-SM condensed domains obtained non-enzymatically [23,26], in the presence or absence of LO domains [32,35–38]. When LO domains were initially present (Dchol content <25 mol%), the enzymatically generated Cer molecules induced the nucleation of Cer-enriched domains located at the LE/LO linear interface (Fig. 3c). This result is equivalent to the observation of a thick phase at the LO domain borders in AFM studies of supported bilayers treated with SMase [10,12,30]. As described above, this may have been due to a favorable nucleation of the new Cer-enriched phase at the LO domain borders. Supporting this theory, Cer domains obtained non-enzymatically (and generated in an equilibrium condition) distributed more homogeneously and were not related to LO domain borders (see Fig. 6 and Refs. [12,30,32]). Comparable results were obtained in SMase treated supported bilayers studied by fluorescence microscopy [9], where stable and non-stable probe-depleted domains were observed throughout a complex time-dependent structural rearrangement of the membrane.

At high Dchol contents (>33 mol%, corresponding to mixtures M3–M5), the action of SMase did not result in the formation of Cer domains, but in a reduction of the extent of the LE area at the expense of an increase of the LO area (Figs. 3d,e and 5). This suggests that the newly generated Cer was being incorporated mainly into the LO phase. This observation is in agreement with elegant fluorescence studies of PC/Chol/Cer vesicles, which indicate that Chol-rich membranes solubilize Cer domains [13], suggesting a higher solubility of Cer in Chol-rich membranes compared to poor ones. Similar conclusion can be drawn from fluorescence microscopy and calorimetric studies of SM/Chol/Cer vesicles [39], where at Chol contents >25 mol% all three components merged into a single LO-like phase. These studies highlight that the Chol/Cer mole ratio may be a highly determinant factor for membrane structuring, rather than the absolute concentration of the two lipids. Furthermore, the presence of Cer has been reported to stabilize LO domains in vesicles, thus shifting the LO/LD miscibility threshold to higher temperatures [40,41]. In agreement, our compression isotherm experiments (not shown) on quaternary monolayers exhibited an enhanced stability of LO domains by Cer (the presence of 12 mol% of Cer raised the miscibility threshold pressure by 5 mN $m^{-1}$). This finding supports the hypothesis of a Cer enrichment in the LO domains, and might have functional relevance in the stabilization of LO domains in the cell environment [42]. At Dchol contents ≥48%, the SM→Cer conversion resulted in a monolayer enriched in Dchol and Cer, but poor in SM and PC. This reveals a so far unreported topographic complexity, mainly because of the technical difficulties in obtaining stable membranes with high contents of Chol and in finding a fluorescent marker that partitioned into the Chol enriched phases [39]. For BAM, which overcame the latter limitation, we demonstrated the presence of a liquid LO-like phase, with intermediate reflectivity and rounded domain boundaries, which was reproduced by substitution of pSM with pCer (to reach 30 mol% of Cer) in the M5 mixture (see Fig. 6c and f). In addition, a low proportion of solid-like domains of very high reflectivity were found, which were also observed in premixed quaternary monolayers. In fact, the high reflectivity values observed in such domains (eight times the reflectivity of the surrounding LO phase, see Fig. 4e) might have been caused by both an increase in the thickness and/or by a variation in the reflection index of the membrane. Recent detailed studies investigating the aqueous dispersion of Cer/Chol mixtures have reported a crystalline phase with a stoichiometry that lay between 45:55 and 35:65, with a repeat distance of 3.50 nm that may have been arranged in low-hydrated single layers not usually observed for other bilayer-forming lipids [43]. On the other hand, when intermediate or high reflectivity phase domains were present, the continuous LO phase showed reflectivity values that were lower than before the SMase action (Fig. 4b and Fig. 6f). This may indicate that the former phases reorganized from the longer/more condensed components of the original LO phase.

Finally, we should mention the bright spots observed at high Dchol contents, when the LO phase became the continuous phase (Figs. 3d–f and 4). These spots appeared discretely before SMase treatment, and occurred more frequently when the initial monolayer was compressed from 0 to the target surface pressure at a higher speed (not shown). After SMase treatment, the occurrence of bright spots was observed more often in Dchol-rich monolayers, but decreased when the SMase concentration in the subphase was reduced five-fold (not shown). Therefore, taking these results together, we found a correlation of the occurrence of bright spots with out-of-equilibrium processes, which may be related to instability and the formation of metastable structures that were previously reported in Chol and Cer enriched vesicles [13,43].

The results above and the high reflectivity found for these spots (which actually saturated our detection systems and made it impossible to determine their reflectivity values) support the conclusion that these spots correspond to nuclei of non-planar lipid structures protruding from the monolayer. A similar finding was reported for PC/SM/Chol supported bilayers treated with SMase, where a 3D feature (which could be labeled with several membrane-markers) was proposed to be an active SM degradation center [9,44]. It is worth bearing in mind that such 3D structures were only observed at long times, after a first stage of Cer generation, and could not have been involved in the regulation of the initial activity observed at short times (Fig. 1 and Ref. [5]). Furthermore, if the bright spots observed in our experiments were actually 3D membrane arrangements (of the HII type), we can speculate that the occurrence of such structures in a bilayer might favor the connectivity between both hemilayers. In agreement, a detailed analysis of the SMase activity experiments in PC/SM/Chol bilayers along a tie line [5] indicates that the SM degradation observed in the LO/LD coexistence region reached such an extent that can only be explained by it involving both hemilayers. A crude extrapolation of this phenomenon to a cellular environment may link SMase activity with the loss of compositional asymmetry of the plasma membrane during apoptotic cell death [45].

### *4.3. The presence of LO domains modulates the action of SMase by a mechanism that involves substrate supply and the maintenance of a low product concentration in the active phase*

In the present work, we investigated the mechanism by which the presence of LO domains in ternary mixtures regulates SMase activity. We proposed a mode of action of SMase (illustrated in a simplified manner in Fig. 8) by which the enzyme acts preferentially in the LE phase, as demonstrated by several studies and discussed in Section 4.1. In a previous work [6], we analyzed the action of SMase on chemically and structurally simpler interfaces of pure SM monolayers. In order to

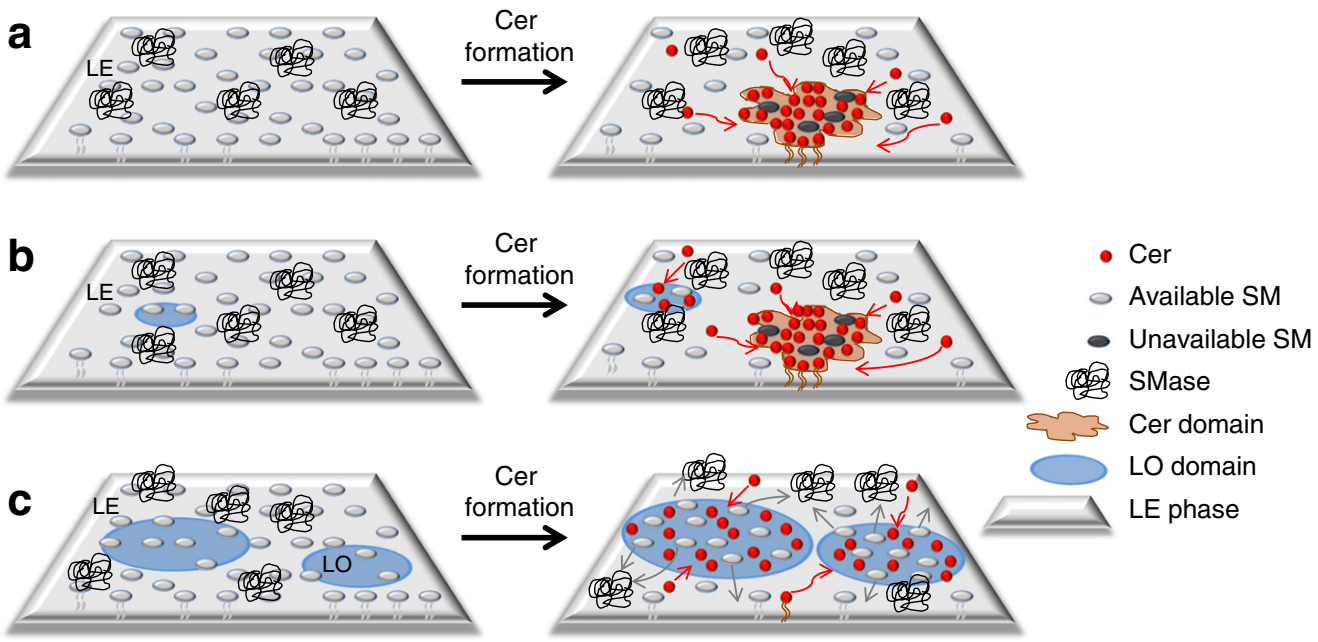


**Fig. 8.** Simplified illustration for the mechanisms of action of SMase acting in the liquid-expanded phase of homogeneous or heterogeneous membranes (see text in Section 4.3): a) When SMase acts on a homogeneous LE phase, the Cer molecules formed show a low solubility in the LE phase and segregate into Cer domains that sequester SM, which becomes unavailable to the enzyme. b) When SMase acts in the LE phase of an interface containing a low proportion of Chol (and the area covered by LO domains is small) the overall Cer solubility in the membrane is still low and Cer domains are formed, leading to the coexistence of three phases. c) When SMase is acting on the LE phase of Chol-rich interfaces that show a large area of the LO phase, the Cer molecules produced in the LE phase rapidly diffuse to become integrated in the LO phase. SM depletion in the LE phase causes molecules of SM to diffuse to the LE phase from the LO domains, thus becoming available for SMase degradation.

facilitate the interpretation, we hypothesized that the new Cer molecules generated in homogeneous LE monolayers show low solubility in this phase (~2 mol%), and when the reaction proceeds beyond such a threshold, the nucleation of Cer domains is thermodynamically driven (Fig. 8a). This nucleation is a kinetically controlled process which requires some time to reach stable nuclei, thus contributing to explain the lag time of SMase activity. Therefore, fast reaction rates result in stronger nucleation-driven forces, together with shorter lag times to achieve SMase full activity [6,21]. A scarce solubility of Cer in disordered phases was also reported for bilayers, even in the presence of Chol at low proportions [13].

Taking into account all the above factors together with our current results, we propose the following mechanism for SMase acting on (initially) heterogeneous membranes: when the Chol content of the membrane is low (and the LO phase is scarce), the Cer molecules produced mainly in the LE phase that surpass the solubility limit in the monolayer nucleate into a new phase, thereby producing Cer-enriched LC domains that grow as a consequence of the incorporation of new Cer molecules [5,6,12,30,34] (Fig. 8b). In the cases of Chol-rich membranes, where a large area of LO phase is already present, Cer can be directly incorporated into the LO phase where it shows high miscibility [13,43], thus freeing the LE phase from the (oversaturated) Cer, and consequently no Cer-domains are produced (Fig. 8c). This allows maintaining an environment for SMase acting in the LE phase that is poor in Cer, and thereby the inhibition by product is minimal.

SMase inhibition by Cer has been previously reported [18,46]. As a consequence of SM depletion in the LE phase, SM molecules from the LO phase will rapidly diffuse to the LE phase. In this manner, a large area of LO phase will serve as a substrate reservoir and most SM molecules in the monolayer can be hydrolyzed, even when SMase acts only in a relatively small region of the membrane. In Chol-poor membranes, the Cer-domains formed by SMase action after reaching the Cer solubility threshold in the LE phase sequester SM up to a Cer/SM molar ratio of 1:2 [23,29,47]. These condensed domains show shape annealing that indicates slow SM diffusion within the Cer-domains (with equilibration times being in the range of tens of minutes) [29]. The slow diffusion of SM within LC domains implies a rather slow rearrangement of SM molecules after depletion of SM in the LE phase. Therefore, compared to the LO phase, the condensed Cer-enriched domains are not able to act as efficient substrate reservoirs and a significant proportion of the total substrate in the membrane will remain unavailable for hydrolysis by SMase. In addition, in Chol-poor membranes (either bilayers or monolayers at high surface pressures) that are enriched in the substrate SM, the formation of LC or gel SM-enriched phases is induced [7,23]. These SM-enriched condensed phases are not a good substrate for SMase [4,5,18] (see also Fig. 1), which is expected to show slower diffusion properties. As a consequence, the SM molecules included in this phase will be poorly accessible for hydrolysis. Therefore, the presence of Chol in the membrane prevents the formation of a Cer-SM condensed phase [7], allowing SM to become accessible to the enzyme by means of rapid diffusion in the liquid phase.

As discussed above, the action of SMase on Chol-rich membranes will lead to the formation of Cer-enriched, SM-depleted LO domains. Several works support a displacement of Chol from the LO phase in the presence of Cer [40,41,48], with it being reported that the incorporation of Cer can induce a displacement of roughly 50% of Chol from the LO domains when the Chol content is 15 mol% [40] and a full displacement (dissolution of the LO phase) when the Chol content is only 10 mol% [41]. At a high Chol content (as is the case of plasma membrane, where Chol can reach about 50 mol% [42]) and a low LD fraction, the displacement of Chol from the LO phase is expected to occur to a lower extent. This is because the Chol solubility threshold in the (scarce) Ld phase would soon be reached. Interestingly, a close correlation between Chol and Cer solubility thresholds in bilayers has been reported by Ali and coworkers [49]. These authors demonstrated that lipid bilayers present a combined solubility limit of Chol + Cer such that the sum of both lipids cannot be greater than 67 mol%. Above this threshold, Cer and/or Chol crystals are formed in equilibrium with bilayers. Furthermore, an early report highlighted a close interrelation between Cer production and Chol distribution at the cell level, with SMase treatment of cells leading to Chol displacement to the cell interior [50].

As a consequence of the above observations, the Chol content of a membrane can determine not only the extent of the LO phase but also the final equilibrium composition of the phases in coexistence after Cer production. This may imply that subtle changes in Chol (or Cer) content will lead to different Cer-enriched LO domains with different physical and functional properties, both in model membranes and at the cell level. In this way, it was recently reported that the affinity of sterol to bilayers is affected in a dose-dependent manner by Cer content [37]. In turn, our model indicates that SMase activity can be regulated by subtle changes of both the lipid ratios and the relative proportions of the phase domains. Furthermore, the variations of lipid composition generated by the enzyme at the local level are amplified by changes in the surface topography. The mechanism proposed here provides simple novel concepts toward a better understanding of the complex action of SMase and the consequences of the generation of Cer in membranes.

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