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Detergents induce raft-like domains budding and fission from giant unilamellar heterogeneous vesicles A direct microscopy observation

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Abstract

The effect of detergents on giant unilamellar vesicles (GUVs) composed of phosphatidylcholine, sphingomyelin and cholesterol and containing liquid-ordered phase (l_o) domains was investigated. Such domains have been used as models for the lipid rafts present in biological membranes. The studied detergents included lyso-phosphatidylcholine, the product of phospholipase A_2 activity, as well as Triton X-100 and Brij 98, i.e. detergents used to isolate lipid rafts as DRMs. Local external injection of each of the three detergents at subsolubilizing amounts promoted exclusion of l_o domains from the GUV as small vesicles. The budding and fission processes associated with this vesiculation were interpreted as due to two distinct effects of the detergent. In this framework, the budding is caused by the initial incorporation of the detergent in the outer membrane leaflet which increases the spontaneous curvature of the bilayer. The fission is related to the inverted-cone molecular shape of the detergent which stabilizes positively curved structures, e.g. pores involved in vesicle separation. On the other hand, we observed in GUVs neither domain formation nor domain coalescence to be induced by the addition of detergents. This supports the idea that isolation of DRM from biological membranes by detergent-induced extraction is not an artifact. It is also suggested that the physico-chemical mechanisms involved in l_o domain budding and fission might play a role in rafts-dependant endocytosis in cells.

Keywords: GUV; Rafts; DRM; l_o phase; Detergents; Budding; Fission; PLA_2

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1. Introduction

The interest towards biological membrane microdomains of 'raft'-type is due to their functional role in fundamental cell biology processes such as signal transduction (Simons and Toomre, 2000), protein and lipid sorting (Simons and van Meer, 1988), cholesterol transport (Simons and Ikonen, 2000) and endocytosis (Sharma et al., 2002). In parallel, their implication in various biomedical subjects is getting impressive. This includes cancer physiopathology (Waugh et al., 2001), virus infection, Alzheimer and prion diseases (Fantini et al., 2002). An intriguing feature is that the biological membrane "rafts", while relatively well identified functionally and biochemically, still remain quite enigmatic as structural entities. Rafts are biochemically identified as DRMs, i.e. membrane fragments that remain unsolubilized after low temperature detergent extraction of membranes (Brown and London, 1998). The high level of sphingolipids and cholesterol as well as the detergent resistance of such isolates has suggested that these might correspond to phase-separated domains containing a liquid-ordered lipid phase l_o, similar to the one that can be observed in artificial membranes with such lipid composition. However, rafts seem so difficult to observe directly as structural elements in living cell membranes that eventually their existence has been challenged (Munro, 2003). In particular, the question has been raised whether DRMs are native membrane structures or are formed during the detergent isolation process. Of course, alternative explanations for the difficulties in direct structural identification of functional rafts in biological membrane might be their small size and dynamical organization. The size of rafts in biomembranes may be at the limit of direct optical microscopy methods (although these may be patched into larger structures). Rafts dynamical organization might be coupled to their functions and involves change in composition, size fluctuations, coalescence and fragmentation as well as diffusion within the membrane or into and off the membrane as functional vesicles.

The possibility that rafts lipids may occur as a liquid-ordered l_o phase has prompted many efforts to mimic raft-associated phenomena in artificial membranes. Recently, several authors have used giant unilamellar vesicles (GUVs) prepared by electroformation (Angelova and Dimitrov, 1986) and containing cholesterol and sphingolipids to visualize raft-like liquid-ordered microdomains by optical microscopy and to study biologically relevant phenomena (Dietrich et al., 2001; Staneva et al., 2004; Bacia et al., 2004a). The cholesterol and sphingomyelin-enriched l_o domains that can be visualized by optical microscopy on GUVs are probably larger than biological rafts. However, several studies suggest that these domains might be partially representative of the behaviour of rafts in situ. Indeed, several biological processes involving lipid rafts have been mimicked with liquid-ordered domains-containing GUVs, including glycosphingolipid (Dietrich et al., 2001) and intrinsic proteins (Bacia et al., 2004b), lateral heterogeneity, cholera toxin binding (Bacia et al., 2004a) and cholesterol exchange (Puff et al., 2005).

Rafts have been suggested to play an important role in cellular events that involve membrane budding and fission, i.e. endocytosis and exocytosis (Sharma et al., 2002). In a previous article, we have attempted to mimic rafts associated budding phenomena in GUVs. We have shown that the bee venom PLA_2 was able to trigger, develop and finalize the budding and fission process of raft-like liquid-ordered domains in GUV (Staneva et al., 2004). It was found that both the binding of PLA_2 to the bilayer and its enzymatic products are involved in this process. In particular, we proposed that the fission event was mainly due to the effect of produced LysoPC that promotes hydrophilic pore formation at the interface of the l_o and l_d lipid phases due to its inverted-cone molecular shape. Here, in order to further document the physico-chemical mechanisms of budding and fission phenomenon of raft-like l_o domains induced by biological agents, we have studied in more detail the effect of LysoPC and other cone-shaped amphiphilic molecules, i.e. detergents, on such multidomain GUVs. This study is also relevant to the problem of the characterization of rafts in biological membranes since detergents are used to isolate rafts as DRMs. One may wonder what is the mechanism involved in DRM extraction, especially since the possibility that DRMs might be artifacts produced by detergents has not been ruled out.

In this article, we present direct optical microscopy evidence that detergents can induce raft-like vesicles budding and fission from giant unilamellar heterogeneous vesicles.

2. Materials and methods

2.1. Reagents

Lipids were obtained and used without further purification as follows: egg yolk L-α-phosphatidylcholine (PC), egg yolk sphingomyelin (SM) and cholesterol (Chol) were from Sigma; the fluorescent lipid analogue C_{12}–NBD–PC (PC*) and egg yolk lysophosphatidylcholine (LysoPC) were from Avanti Polar Lipids. The detergents Triton X-100 and Brij 98 were from Sigma.

2.2. Vesicle preparation

GUVs were made by the electroformation method (Angelova and Dimitrov, 1986) in a temperature-controlled chamber following the particular protocol for unilamellar giant heterogeneous vesicles formation previously described in Staneva et al. (2004). GUVs are formed from PC/PC*/SM/Chol 55/5/20/20 mol/mol at 34 °C, if not otherwise stated. The raft-like (l_o domain) is visualized in fluorescence as previously described in Staneva et al. (2004). Briefly, the chain-labeled lipid analogue PC* is excluded from the ordered (l_o) phase and partitions predominantly in the disordered (l_d) phase. That makes the l_o domain to appear as a dark round-shaped spot within the bright vesicle membrane.

2.3. Video microscopy

A Zeiss Axiovert 200M microscope (fluorescent unit fluo arc N HBO 103, Zeiss), equipped with a Lambda 10-2 unit (Sutter Instrument Co.), plus a CCD B/W chilled camera (Cool SNAP HQ), was used for GUV imaging. The set-up was piloted by Methamorph 6.0 software (Ropper Sci.). The morphology transformations and dynamics of the heterogeneous GUV membrane were followed in phase contrast and in fluorescence by Zeiss filter set 16 (E_x/E_m = 485/>520 nm).

2.4. Microinjection of detergents

The microinjection was carried out with an Eppendorf FemtoJet. The microcapillary for performing local microinjection to a GUV had a 1.2–1.4 μm inner diameter. A detailed study of the influence of injection parameters was initially performed with buffer and the allowed range of injection pressures, times and distance was chosen in order not to perturb the shape of the vesicle during the injection. Injection was performed from a distance of about 30–50 μm from the GUV. Injected volumes of detergent stock solutions were of the order of a few picoliters (1 pl = 1×10^{-12} l) and required the use of very concentrated detergent stock solutions. It should be noted that the exact injected volume is not known with precision but is reproducible as a function of injection pressure and time and can be varied proportionally by varying the injection time. The observations presented below are based on at least 5–10 experiments of the same kind.

Stock solution of 256 mM Triton X-100 (=15%, v/v), 97 mM Brij 98 and 92 mM LysoPC was prepared in 0.5 mM Hepes buffer, pH 7.4, identical to that in which the vesicles were formed.

3. Results

Our strategy for the study of detergent-associated effects on rafts was to monitor the effects of detergent addition on GUVs containing liquid-ordered domains. The heterogeneous vesicles were made of equimolar proportions of PC and SM with cholesterol contents ranging from 10 to 20%. As mentioned in Staneva et al. (2004), it is possible to resolve l_o-phase domains up to 39 °C, but the characteristic size decreases with increasing temperature. Fig. 1 gives an example of a GUV displaying such domains at the physiological temperature of 37 °C as visualized by fluorescence microscopy using the fluorescent probe PC*. As already described, this probe is excluded from the more ordered lipid phase (l_o phase) which appears as dark round-shaped spots within the bright l_d liquid-disordered phase. While the domains are clearly visible at this temperature, these were found to be too small for detailed observation of the detergent-associated effects. The advantage of lower temperatures is the larger l_o domain sizes which makes them more suitable for optical microscopy observation. Qualitatively, the detergents effects discussed below are similar for temperatures from 30 to 37 °C. No spontaneous transformation was observed at any temperature on vesicles neither flacid or tense.

We chose to study the effect of three detergents, namely: (i) LysoPC, i.e. a natural detergent which

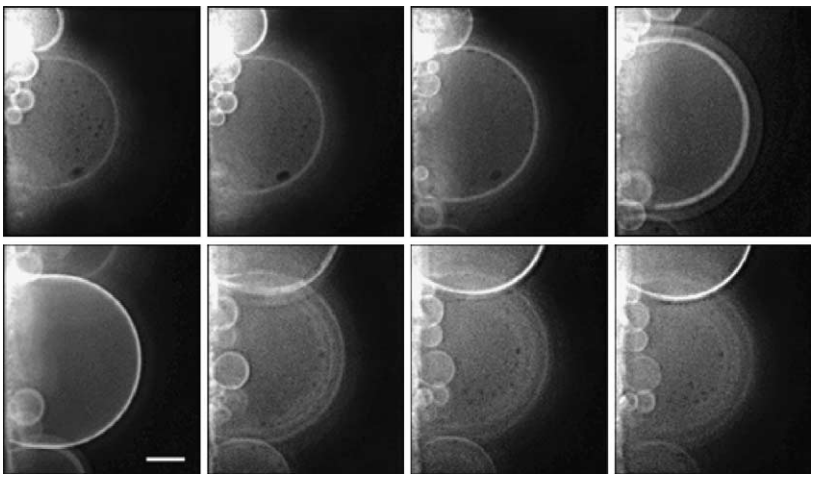

Fig. 1. Visualization of domains in l_o phase by *z*-scan fluorescence, GUV composed from PC/PC*/SM/Chol = 55/5/20/20 at 37 °C. Bar, 20 μm.

is also the product of the enzymatic activity of the PLA_2; (ii) Triton X-100, the most typical and strongest detergent for low temperature raft extraction which yields DRMs highly enriched in SM and Chol; (iii) Brij 98, a milder and moderately selective detergent, which yields less selectively enriched DRMs at 37 °C which appear to be similar in composition (Drevot et al., 2002; Schuck et al., 2003) to the so-called "non-detergent" lipid rafts (Pike, 2004).

The effect of each detergent was studied by successive local injections of picoliter quantities of detergent stock solution in the vicinity of individual vesicles. For each detergent, the range of added detergent quantities was chosen by trial and error in order to start from the minimal quantity eliciting a visible morphological effect (each detergent stock solution concentration was ultimately chosen in order to allow for similar injected volumes). In all cases, such quantities elicited no or very few apparent solubilization of the lipid bilayer except when purposely increased. An estimation of the effective detergent/lipid ratios will be given in Section 4.

3.1. LysoPC

Fig. 2a and b shows a heterogeneous vesicle (PC/SM/Chol 45/45/10 mol/mol) at 30 °C, respectively, in phase contrast and fluorescence microscopy. The white arrows in Fig. 2b point at two dark oval, l_o domains of different sizes: a small one—about 10 μm in diameter, and a larger one—about 30 μm in diameter. The effect of a local delivery to the vesicle of exogenous LysoPC is to trigger the budding of the small l_o domain and leads to its complete fission as a l_o-phase vesicle, as shown in Fig. 2c–f by phase contrast microscopy (note that budding can only be visualized by phase contrast and if the domains are located at the vesicle equator). A further indication that budding involves the l_o domain is the simultaneous disappearance of the small dark spot in the parent vesicle (Fig. 2g). As also visible in Fig. 2g, the larger l_o domain remains in the parent vesicle. Fission of this latter domain was neither achieved by a second LysoPC injection (Fig. 2h–l) although a limited and reversible budding was apparent. It was only the third LysoPC injection which succeeded in yielding a complete budding and fission of the domain (Fig. 2m–r). Increased membrane thermal fluctuations of the l_d, as well as of the l_o phases, were occasionally observed after budding (Fig. 2n–q). This is presumably due to bilayer bending rigidity decrease after detergent incorporation and/or to internal pressure decrease of the most tense vesicles that results from water efflux due to transient membrane opening. The data described above illustrate a general trend in our experiments, namely that small domains required less detergent to undergo complete budding than large domains.

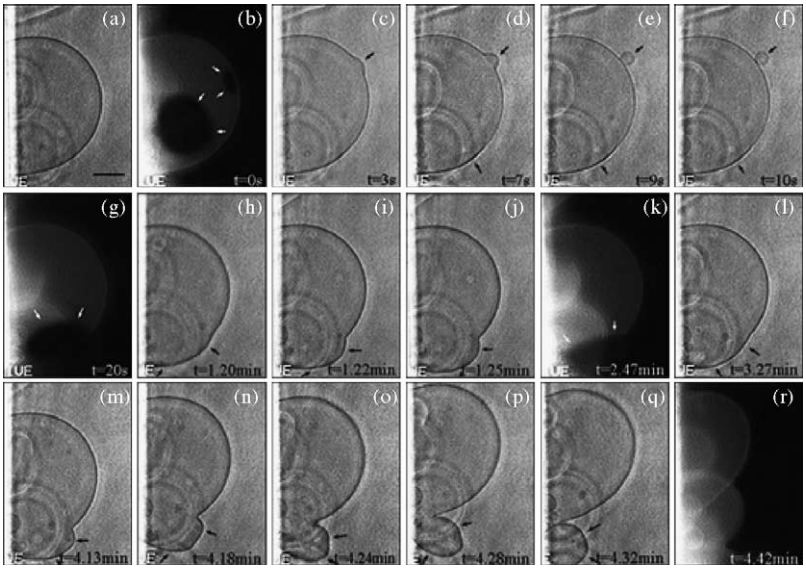

Fig. 2. Raft vesicle budding and fission promoted by LysoPC from heterogeneous GUV (PC/SM/Chol = 45/45/10 at 30 °C). The first injection (injection parameters: $\Delta p = 200$ hPa; $\Delta t = 2$ s; micropipette–vesicle distance, $l = 50$ μm) of LysoPC was sufficient to trigger the process of raft vesicle budding and fission for the small domain (a–g): (a) initial raft–GUV in phase contrast; (b) the white arrows point at two dark l_o domains: a small one (about 10 μm in diameter) and a big one (30 μm); (c–f) microinjection, small l_o domain budding and complete fission; (g) no small dark domain left in the mother vesicle (the fission was completed in 10 s). The large domain fission was achieved neither after the first (a–g) nor after the second (h–l) LysoPC injection. It was the third one which succeeded to get the fission completed (m–r). Bar, 20 μm.

3.2. Triton X-100

A similar experiment performed with Triton X-100 on GUVs (PC/PC*/SM/Chol 55/5/20/20 mol/mol) is presented in Fig. 3. Here, an injection performed at 4 °C is shown since this temperature is used for preparation of DRMs with this detergent. As already described (Dietrich et al., 2001; Staneva et al., 2004), at such temperature, only one (Fig. 3a) or several huge dark domains are present in each vesicle. As previously shown for LysoPC at 30 °C, addition of Triton X-100 by local microinjection (Fig. 3b) gives rise to budding of the l_o domain (Fig. 3c–g) followed by complete fission (Fig. 3 h and i), the l_o domain being excluded as an exocytic-like vesicle. No dark domain is observed in the mother GUV membrane after the fission, and no fluorescence is detected at the location of the budded vesicle (Fig. 3j). The process of raft vesicle budding and fission took about 10 s and was occasionally accompanied by increased vesicle membrane thermal fluctuations. Qualitatively similar results were obtained at higher temperature.

3.3. Brij 98

Figs. 4 and 5 present the case of Brij 98, a detergent used for "warm" DRM extraction. Experiments were performed at 34 °C. Fig. 4 presents a unique sequence of simultaneous budding and fission of two l_o-phase vesicles. In the first second after providing the detergent in the vesicle vicinity, budding initiates for two domains of similar size (Fig. 4c), develops (Fig. 4d–f) and terminates with the fission of l_o domain vesicles from the parent vesicle (Fig. 4g and h). In the fluorescence image (Fig. 4i), no dark domains were left in the parent vesicle. In Fig. 5, we have purposely increased the detergent quantity by increasing the injection time in order to provide a direct observation of the different stability with respect to the detergent of the two lamellar phases, l_d and l_o. While the budding event occurs as usual, one additional phenomenon is the solubilization of a part of the mother vesicle (Fig. 5d–f). The solubilization is localized in the l_d-phase region (see black arrows). In the same time, the budding l_o-phase domain is only slightly destabilized in shape—the membrane thermal

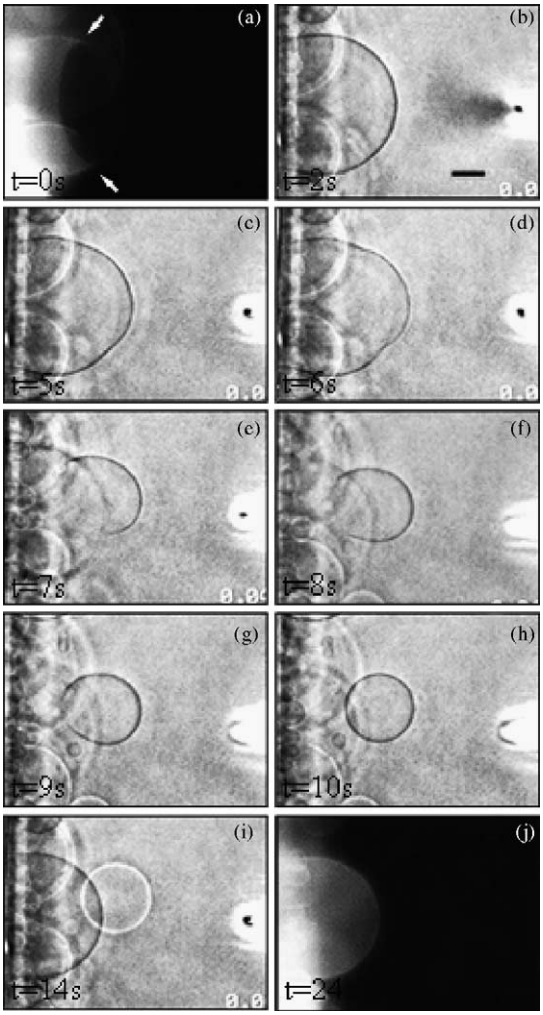

Fig. 3. Interaction of heterogeneous GUV (PC/PC*/SM/Chol = 55/5/20/20) with 15% Triton X-100 at 4 °C. Visualization of domain budding and fission. (a and b) Initial raft–GUV in fluorescence (one huge dark domain is observed) and in phase contrast. (b) Local microinjection (injection parameters: $\Delta p = 200$ hPa; $\Delta t = 1$ s; micropipette–vesicle distance, $l = 50$ μm); (c–g) l_o domain budding; (h–j) complete fission; (j) no dark domain is observed in the mother GUV membrane after fission, and no fluorescence is detected at the location of the ejected vesicle (white arrow). The process of raft vesicle budding and fission took about 10 s. Bar, 20 μm.

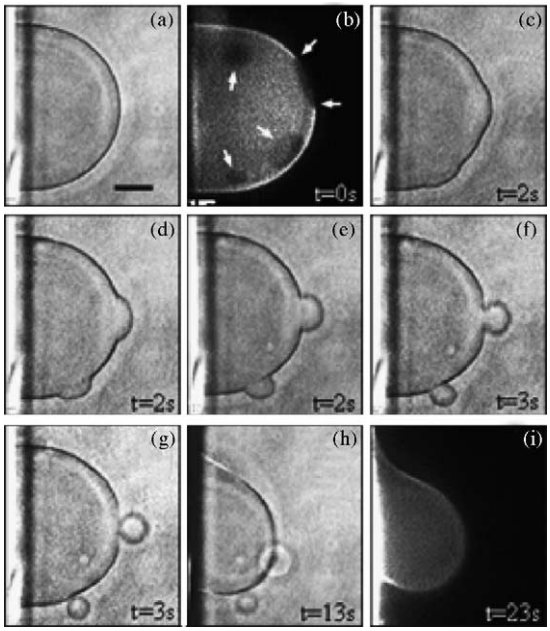

Fig. 4. Interaction of heterogeneous GUV (PC/PC*/SM/Chol = 55/5/20/20) with 15% Brij 98 at 34 °C. Visualization of two budding events: (a) initial raft–GUV in phase contrast; (b) initial raft–GUV in fluorescence (four dark domains are pointed by the arrows); (c–g) budding initiates, develops and terminates with fission (note that c and d are taken within 1 s) (injection parameters: $\Delta p = 200$ hPa; $\Delta t = 1$ s; micropipette–vesicle distance, $l = 50$ μm); (h) shrinking of the mother vesicle; (i) no dark domains left in the mother vesicle in fluorescence. The process of raft vesicle budding and fission took about 5 s. Bar, 20 μm.

fluctuations at its site increase for a while (Fig. 5c–e). This transient solubilization (lasting about 20 s) is probably correlated with the time of detergent diffusion to the bulk of the working chamber and the related decrease of detergent local concentration at the vesicle membrane. The mother vesicle regains its spherical shape but at smaller diameter due to the loss of lipids for the formation of the ejected l_o vesicle, as well as for the mixed lipid/detergent micelles formation during l_d-phase partial solubilization. It is worth noting that the solubilization occurs more slowly and is not correlated in time with the budding since the solubilization occurs while budding is already in progress. Fig. 5g–i also illustrates a rare event in which the fission of the vesicle is not completed since a thin filament remains connecting the budded vesicle to the parent vesicle. The budded vesicle is forced to remain in the equatorial plane as can be visualized in phase contrast. In fluorescence, no dark spot is left in the parent vesicle at the filament location and no fluorescence is observed at the position of the budded vesicle. This is a further confirmation that the budded vesicle originates from the dark l_o domain.

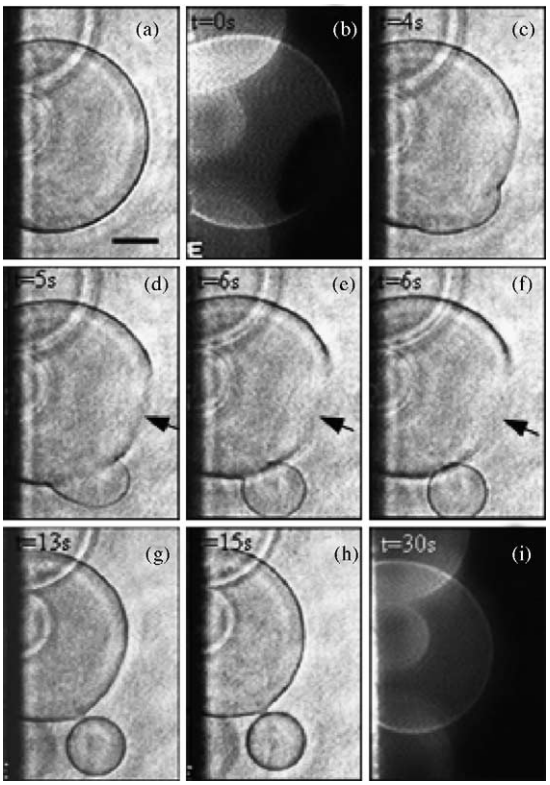

Fig. 5. Interaction of heterogeneous GUV (PC/PC*/SM/Chol = 55/5/20/20) with 15% Brij 98 at 34 °C. Visualization of one budding event and transient partial solubilization of the l_d phase. The process of raft vesicle budding and fission took about 20 s (injection parameters: $\Delta p = 200$ hPa; $\Delta t = 2$ s; micropipette–vesicle distance, $l = 50$ μm). The fission of the vesicle is not completed since a thin filament remains connecting the budded vesicle to the parent vesicle. Bar, 20 μm.

4. Discussion

The present study was initiated as a continuation of our previous work which showed that budding and fission of l_o domain vesicles could be promoted from heterogeneous GUVs by the action of PLA_2 (Staneva et al., 2004). This situation is in contrast to what is observed when similar experiments are performed on homogenous GUVs (Wick et al., 1996) suggesting that the presence of domains is favorable to budding, as predicted theoretically (Lipowsky, 1992, 2002). It was found that budding could be induced by the sole presence of the enzyme in the absence of enzymatic activity, an effect which was likely due to its exterior binding to the membrane. On the other hand, fission of the budded vesicle could only be observed with the active enzyme and therefore requires the presence of one or both its reaction products. This prompted us to examine the effect of LysoPC alone, as well as other detergents, on l_o domain-containing GUVs. Our results show that LysoPC alone, as well as Triton X-100 and Brij 98, is able as such, to induce the same effect as the active PLA_2, namely both budding and fission of l_o domain vesicles from GUVs. This indicates that external addition of the detergent and its binding to the membrane induces to some extent an effect similar to binding of PLA_2 which promotes l_o domain budding. As proposed for PLA_2, this may be related to asymmetric binding of the detergent and differential expansion of the outer leaflet. This first effect is distinct from the second effect induced by the detergent and illustrated both here and in our previous work, namely the fission of the bud.

In order to get an insight into the mechanisms underlying both effects, the mode of interaction of detergent to the lipid matrix must at first be evaluated. Detergents are known to partition into bilayer without promoting solubilization provided that a certain effective detergent to lipid mole ratio in the membrane (R_{sat}) is not reached (Lichtenberg et al., 2000). R_{sat} depends to some extent on the detergent but more strongly on temperature and on the physical state of the lipids. Among the vast literature data on the subject, the most relevant are those of Heerklotz (2002) who studied Triton X-100 solubilization of LUVs made of equimolar mixture of POPC/SM/Chol for which both l_o and l_d phases are co-existing. These authors proposed a phase diagram from which values of R_{sat} of 0.005 and 0.03 can be estimated at 4 and 34 °C, respectively. A rough upper limit of an average effective detergent to lipid ratio in the treated vesicle in our experiments can be calculated as 0.01 (from the average injected detergent quantity, the vesicle diameter and the average volume of the cone in which the detergent is injected and neglecting detergent diffusion outside the cone and aqueous partition).[1] This indicates that most of our conditions

[1] The following values were used in this calculation: cone base diameter and height, 160 and 60 μm; detergent concentration, 256 mM (corresponding to the case of Triton X-100); average injected volume, 5 pl; GUV diameter, 80 μm; surface area per lipid, 0.7 nm^2, yielding 5.7×10^{10} lipids per GUV.

likely correspond to bilayer insertion of the detergent without solubilization or with little solubilization. Furthermore, as already mentioned and further discussed below, the budding effect of the detergent is likely to be related to its initial insertion into the outer bilayer leaflet while solubilization into mixed micelles requires its transbilayer diffusion (le Maire et al., 1987; Kragh-Hansen et al., 1998) and therefore occurs on a slower time scale. Such conclusions are borne out by our experimental results which shown that detergent-induced l_o domain budding occurs without visible bilayer solubilization except in Fig. 6 in which a higher detergent amount was purposely injected and in which partial solubilization (limited to the l_d phase) occurs while budding is already completed. Interestingly, these data support another prediction of Heerklotz (2002), namely that the l_d phase gets solubilized more easily than the l_o phase.

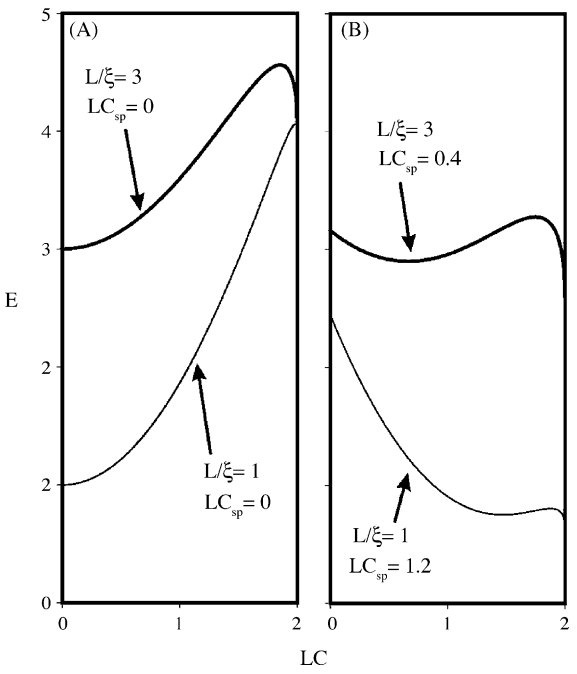

Fig. 6. Reduced energy of budding for a small domain (thin curves) and a large domain with three times larger diameter (thick curves) in the absence (A) or in the presence (B) of a spontaneous curvature induced by a constant amount of detergent. *LC* is the parameter which represents the budding since *C* is the curvature of the bud and *L* is the diameter of the flat domain ($C = 2/L$ for a complete bud). The spontaneous curvature is represented by the LC_{sp} parameter which is proportional to *L*.

Asymmetric binding of the detergent to the bilayer requires its transbilayer diffusion to be relatively slow. Very few data exist on detergent flip–flop in membranes. LysoPC flip–flop is of the order of hours in homogenous bilayers (Bhamidipati and Hamilton, 1995). While it is probably highly increased in our case by packing defects due to the co-existence of two phases, it probably remains of the order of minutes (John et al., 2002). For non-ionic detergents, transbilayer diffusion rates appear to be strongly dependent on the polar headgroup. Half-times ranging from 350 ms to several minutes have been estimated for octaethylene glycol monododecyl ether and dodecylmaltoside, respectively (le Maire et al., 1987; Kragh-Hansen et al., 1998). Such timescales suggest that in our experiments, there is a period of the order of seconds were there remains an excess detergent in the outer monolayer relative to the inner monolayer after the initial asymmetric exterior binding. Under such circumstances, it appears possible to explain the l_o domain budding induced by detergents by the same arguments used previously for PLA_2, namely an increase of the inherent tendency of the l_o domain to bud due to the spontaneous curvature afforded by the differential lateral constraints associated with the asymmetric detergent binding.

Indeed, budding appears to be an intrinsic property of multicomponent membranes (Lipowsky, 1992, 2002). In a two-phase membrane, the difference in composition of the two phases is usually associated with a free energy term, which is proportional to the length of the interphase boundary (the density of this free energy term being the line tension at the interphase boundary). This provides a driving force for budding of one domain from the lipid bilayer matrix, since this process leads to a decrease of the length of the interphase boundary. Whether budding actually occurs is related to the interplay of the opposing effects of the free energy of bending and that of the interphase boundary (Lipowsky, 2002). Any mechanical lateral tension applied on the membrane adds to the process. Our experiments show that the presence of a liquid-ordered domain per se is not sufficient for budding to appear in GUVs. In contrast, detergent addition triggers budding at the l_o domain sites. The destabilizing effect of the detergent can be attributed to modification of the membrane local spontaneous curvature. The asymmetric constraint generated on the coupled lipid bilayer by the excess detergent in the external leaflet induces a

positive spontaneous curvature (i.e. towards the exterior). Put in simpler terms, this means that the initial insertion of the detergent in the outer leaflet promotes a lateral tension of this leaflet which cannot be relieved by lateral expansion since the two leaflets must remain together. This promotes a tendency to increase the outside/inside surface ratio of the two leaflets by budding. Budding occurs preferentially at the l_o domains since it also has the effect of decreasing the boundary between the two phases, a process which is also energetically favorable (e.g. due to differences in bilayer thickness (Kuzmin et al., 2005)). The fact that asymmetrically located amphiphiles can modify the shape of membranes was demonstrated in 1976 and forms the basis of the "bilayer couple hypothesis" (Sheetz and Singer, 1974; Iglic and Hagerstrand, 1999).

An intriguing feature of our experiments is the observation that under identical conditions, small l_o domains bud more easily than larger l_o domains, i.e. require less detergent to bud. Indeed, normally, when all other parameters are equal, larger domains have a higher tendency to bud since their boundary energy is higher (Lipowsky, 2002). However, this is not obligatory true anymore if the spontaneous curvature increases with decreasing domain size which is the case here. In fact, a particular aspect of the detergent-induced effect is that the spontaneous curvature induced by an identical amount of detergent is higher for smaller domains. Simple geometrical arguments indicate that if a bud is formed from a 10–30 μm diameter domain in a ∼100 μm GUV, the variation in monolayer surface difference in the parent vesicle is negligible in comparison with the monolayer surface difference in the budding domain. This means that detergent binding to the outer monolayer only has an effect on the spontaneous curvature of the budding domain. Under such circumstances, it is possible to express the spontaneous curvature of the bud at first order as (Farge, 1995; Farge et al., 1999):

$$C_{sp} = \frac{\Delta s}{2hS} = \frac{2\,\Delta s}{\pi h L^2} \quad (1)$$

where Δs is the *unconstrained* surface difference between the two leaflets associated with the binding of a particular detergent amount in the GUV outer leaflet (i.e. the surface difference that detergent binding to the GUV would induce on the domain if its edges were unconstrained, roughly proportional to the number of detergent bound molecules and the molecular area of the detergent), h the membrane thickness and S and L are the surface and diameter of the domain. This spontaneous curvature is inversely related to the square of the domain size. Qualitatively, this illustrates the fact that for a smaller domain, it would take a more pronounced budding to achieve the surface difference that relieves the outer monolayer compression induced by the detergent.

According to Lipowsky (1992), the total energy of a budding domain as a function of its curvature C is given by:

$$E = (LC - LC_{sp})^2 + \left(\frac{L}{\xi}\right)\left[1 - \left(\frac{LC}{2}\right)^2\right]^{1/2}$$

where ξ is a size-independent parameter that describes the balance between bending modulus and line tension. The term that governs the spontaneous curvature effect is LC_{sp} which, according to equation (1), is inversely proportional to L. Fig. 6 illustrates a situation in which a small domain would bud under the influence of detergent while a three times larger domain would not bud. Values of L/ξ and LC_{sp} are similar to those used by Lipowsky (1992). Fig. 6a is the situation in the absence of detergent. Although none of the domains actually buds (as found in our experiments in the absence of detergent), it would appear that the larger domain would have a higher propensity to bud. However, as shown in Fig. 6b, in the presence of an identical amount of detergent (i.e. of a domain size-dependent spontaneous curvature), the smaller domain is predicted to bud completely while the larger domain only experiences a limited budding. This is due to the fact that LC_{sp} is three times larger for the smaller domain than for the larger domain. This is exactly the situation which is observed in Fig. 2 with LysoPC. The fact that the incompletely budded larger domain retracts is probably due to the slow flip–flop of LysoPC which progressively abolishes the spontaneous curvature of the bud.

Our previous work with PLA_2 (Staneva et al., 2004) indicates that asymmetric binding of an external agent which increases the membrane spontaneous curvature of the bilayer is not sufficient for completing the vesiculation of the l_o domain. It is therefore likely that, in the present study, detergents besides the effect on bilayer spontaneous curvature that promotes budding also have

a distinct effect which induces fission of the budded domain. The fact that fission is observed with all the detergents studied here suggests that the ability to induce fission is a general property of detergent molecules. Detergents are amphiphilic molecules that form micelles due an inverted-cone molecular shape (Israelachvili, 1985). These are therefore expected to stabilize membrane region with positive spontaneous curvature of one monolayer and to preferentially insert into such regions (note that the spontaneous curvature of the monolayers, related to the shape of their molecular species, is in part distinct from the spontaneous curvature of the bilayer, invoked above and linked to the relative number of molecules in each monolayer). These are also expected to stabilize positively curved membrane edges in contact with water. At the current stage of knowledge, only tentative proposals can be made on how such properties promote fission. Fission initially requires the formation of a fission neck (Kozlovsky and Kozlov, 2003). Since this neck is characterized both by negative curvature (along the neck profile) and positive curvature (around the neck), it is difficult to predict the effect of inverted-cone like molecules and opposite reports in this regards have been obtained in other types of fission events (Barr and Shorter, 2000; Kozlov, 2001). Fission can in principle be completed by two different mechanisms: (1) self-fusion of the internal monolayer of the neck to form a hemifission intermediate which is then ruptured; (2) simple rupture of the fission neck followed by resealing of the two produced vesicles. A detailed analysis of the mechanism of the first type of fission has been recently carried out and it was predicted that increase of the spontaneous curvature of the membrane monolayers would favor fission by destabilizing the neck more than the hemifission intermediate (Kozlovsky and Kozlov, 2003). This would be consistent with our results since stated above, such a spontaneous curvature effect is expected from detergents. Alternatively, direct rupture of the fission neck is also a possible mechanism for detergent induced fission of l_o domain buds. Indeed, rupture of the fusion neck is expected to arise from formation and growing of defects or pores in the bilayer. Such defects or pores are known to occur at the boundary of two co-existing membrane phases (Antonov et al., 1980). The effect of the detergents would then be to stabilize the pores, thereby contributing to the decrease of the line tension alongside the budding process. Such a stabilizing effect of detergents has already been observed for osmotically induced pores (Karatekin et al., 2003). More hypothetically, such detergent-stabilized pores may begin to form during the budding process so that the progressive decrease of domain boundary would lead to progressive coalescence of the pores (pore coalescence might actually contribute energetically to the budding process). This would unite budding and fission into a single continuous process.

To the extent that l_o domains can be considered as models for rafts in biomembranes, our work has several biological consequences. First, we observed neither domain formation nor domain coalescence to be induced by the addition of detergents to GUVs. Our experiments with higher amount of detergent suggest that the l_d phase is more susceptible to solubilization as is observed for the preparation of DRMs. Therefore, the experiments presented here above support the idea that: (i) no detergent associated artifacts occur during isolation of DRM from cells and (ii) temperature effects may occur (it cannot be excluded that the low temperature used in Triton X-100 extraction may have an effect on raft size, although rafts have also been isolated at room temperature using Brij 98). It is in fact possible that DRM extraction from cells occurs as it is observed here, i.e. by budding of rafts prior to solubilization of the non-raft membranes. Second, an important amount of data has recently pointed at the existence of clathrin-independant endocytic processes involving lipid rafts (for review, see Sharma et al., 2002). Budding of several enveloped viruses also appears to occur at raft sites (Fantini et al., 2002). Although the detailed mechanism probably depends on the involved specific protein–protein and protein–lipid interactions, our work suggests that it is worth examining whether simple physico-chemical mechanisms may not play a role in such processes. As shown here and in our previous work (Staneva et al., 2004), any phenomenon which tends to modify the surface ratio of the two bilayer leaflets and therefore the spontaneous curvature, such as changes in surface protein binding or enzymatic modification of lipids, is expected to promote budding of rafts domains. Additionally, any process that produces lipid species with inverted-cone shape would tend to favor structures with positive curvatures possibly involved in fission. In this regard, it has also been shown that endocytic sorting of lipids appears to be associated with their cone or inverted-cone shape

(Mukherjee et al., 1999). The fact that the natural "detergent", LysoPC, has the capacity to promote l_o domains fission suggests that it might play a role in cell rafts trafficking. Indeed, phospholipase A_2 (PLA_2) enzymes have been recently found to be mediators of membrane shape and function in membrane trafficking (Brown et al., 2003).

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