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Pressure-induced Shape Change of Phospholipid Vesicles: Implication of Compression and Phase Transition

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Abstract. A microscopic study has allowed the analysis of modifications of various shapes acquired by phospholipid vesicles during a hydrostatic pressure treatment of up to 300 MPa. Giant vesicles of dimyristoylphosphatidylcholine / phosphatidylserine (DMPC/PS) prepared at 40°C mainly presented a shape change resembling budding during pressure release. This comportment was reinforced by the incorporation of 1,2-dioleyl-sn-glycero-3-phosphatidylethanolamine (DOPE) or by higher temperature (60°C) processing. The thermotropic main phase transition (Lα to Pβ′) of the different vesicles prepared was determined under pressure through a spectrofluorimetric study of 6-dodecanoyl-2-dimethylamino-naphtalene (Laurdan) incorporated into the vesicles' bilayer. This analysis was performed by microfluorescence observation of single vesicles. The phase transition was found to begin at about 80 MPa and 120 MPa for DMPC/PS vesicles at, respectively, 40°C and 60°C. At 60°C the liquid-to-gel transition phase was not complete within 250 MPa. Addition of DMPE at 40°C does not significantly shift the onset boundary of the phase transition but extends the transition region. At 40°C, the gel phase was obtained at, respectively, 110 MPa and 160 MPa for DMPC/PS and DMPC/PS/DOPE vesicles. In comparing volume data obtained from image analysis and Laurdan signal, we assume the shape change is a consequence of the difference between lateral compressibility of the membrane and bulk water. The phase transition contributes to the membrane compression but seems not necessary to induce shape change of vesicles. The high compressibility of the Lα phase at 60°C allows induction on DMPC/PS vesicles of a morphological transition without phase change.

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Introduction

During the last ten years, numerous studies have shown that hydrostatic pressure treatment can drastically affect microbial physiology and viability. However, the mechanisms leading to the microorganisms' inactivation need to be elucidated. Intracellular protein structures like ribosomes or cell membranes have been pointed out as determinant in pressure sensitivity (Hayert, Perrier-Cornet & Gerrais, 1997; Niven, Miles & Mackey, 1999). Other work has shown that cell membranes are permealized under pressure, leading to abnormal mass transfer (Perrier-Cornet, Hayert & Gervais, 1999). In order to understand the biophysical modification of cell membrane under pressure, the comportment of artificial vesicles was analyzed. Giant vesicle bilayers containing phosphatidylcholine (PC) and phosphatidylethanolamine (PE) phospholipids were used to imitate cell comportment.

Many studies of the mechanical properties of model membranes have revealed the influence of physical parameters such as temperature or pressure on phospholipid structural organization and particularly in closed bilayers (Ichimori et al., 1998). It has already been demonstrated that hydrostatic pressure induces different phase changes of phospholipid bilayer at ambient temperature and numerous temperature, pressure phase diagrams have been established, reflecting the phase existence for defined pressure and temperature ranges (Wong et al., 1988).

A closed bilayer shell naturally adopts a spherical shape, which represents an equilibrium between the surface and volume forces. The shape modifications

of vesicles are generally explained in terms of the minimum-bending-energy concept of closed shells, the change in area-to-volume ratio and the area difference between the opposing monolayers (Käs & Sackmann, 1991; Sackmann, 1994). Beney and colleagues (Beney et al., 1997) have shown the area-to-volume ratio involvement in shape transition obtained with pressure treatment of egg-yolk phosphatidylcholine vesicles. The bulk compressibility difference between the vesicle and its water content has been demonstrated to induce a spherical to pear shape transition.

The aim of this study was to understand the processes involved in modifications of vesicle shape observed under high-pressure treatment and especially to relate the vesicle shape bending to the phospholipids' phase transition during a pressurization and release cycle. In a first stage, a fluorescent probe (Laurdan) sensitive to the dynamic relaxation of its environment was used to estimate the main phase transition pressure and phase coexistence on giant unilamellar vesicles. Then, the effects of high-pressure treatment on shape and volume variations were observed directly with a microscopic visualization system. The relation between vesicle behavior and parameters like phospholipid composition, treatment temperature or pressure level were tested.

Materials and Methods

Phospholipids

The 6-dodecanoyl-2-dimethylamino-naphtalene (Laurdan) was purchased from Molecular Probes (Eugene, OR). L-a-Dimyristoylphosphatidylcholine (DMPC), phosphatidylserine (PS) and 1,2-dioleyl-sn-glycero-3-phosphatidylethanolamine (DOPE) were purchased from Sigma-Aldrich (France).

Preparation of Phospholipid Vesicles

Giant unilamellar vesicles (GUVs) were obtained using a modification of the technique described by Reeves and Dowben (1969). Preparations were carried out at a constant temperature of 40°C or 60°C, corresponding to experiment conditions. Oligolamellar / unilamellar vesicles were prepared by suspending each type of phospholipid in chloroform/methanol (4:1). This solution was expelled with a stream of nitrogen. After 5 h of solvent evaporation, the thin film of phospholipids formed at the bottom of the Erlenmeyer flask was rehydrated with distilled water carried by nitrogen flux for 3 h. Vesicles were collected in distilled water and stored overnight at controlled temperature.

Two kinds of phospholipid composition were used: binary (DMPC/PS) and ternary mixture of lipids such as DMPC/DOPE/PS (in mass proportion 7:2,5:0,5). 10% or 5% (w/w) of phosphatidylserine was added in the preparation of mixtures to stabilize liposomes (Akashi et al., 1996).

The fluorescent probe Laurdan was incorporated into phospholipid membrane to optimize the observation of shape transformation and to study spectral properties of bilayers during compression/decompression cycles. Laurdan was initially dissolved in order to obtain a concentration of 9 g/l in chloroform, and then this solution was premixed with lipids in chloroform/methanol solution at the beginning of vesicle preparation (final Laurdan/lipid ratio was 1:50 (mol/mol)). The probe repartition on the membrane surface is uniform. This technique allows the formation of large (> 5 μm), spherical, oligo- or unilamellar and fluorescent vesicles.

Observation of Liposomes under High Hydrostatic Pressure

A special device developed in our laboratory was used to observe vesicles and biological cells through a light microscope during high-pressure treatment. Its working principle has been previously described by Perrier-Cornet (Perrier-Cornet et al., 1995). In order to carry out experiments in isothermal conditions (40°C and 60°C), the visualization chamber was beforehand heated by hot air and maintained at a constant temperature (40°C or 60°C). A thermocouple device (Top Industrie, France) allowed the measurement and control of the temperature in the sample.

A few microliters of vesicle solution were put in the visualization chamber and the top was gently tightened. This high-pressure chamber was placed on the stage of an epifluorescent light microscope (DMLB Leica, Germany), allowing observation of vesicle behavior during pressure treatments. The fluorescence observation requires the use of a filter set (E1) corresponding to excitation and emission wavelengths of incorporated probes. An electronic shutter (Lot Oriel, E.U) was used to minimize fading of the probe. A CCD camera (Kodak, Megaplus ES 1.0, E.U) connected to the microscope was used to record vesicle behavior during compression/decompression cycles, allowing the acquisition of images (30 images/s) on-line through an image-acquisition system (Genesis, Matrox, Canada).

Fluorescence Study

This method allows the detection of phase changes of each type of vesicle prepared at 40°C and 60°C and the definition of the physical state of the membrane bilayer at different pressure levels (Bagatolli & Gratton, 1999). For this study, the sensitivity of the emission spectrum of 6-dodecanoyl-2-dimethylamino-naphtalene (Laurdan) to the environment polarity was utilized. Using an excitation spectrum centered at 340 nm, the probe emission-spectrum shifts from blue (452 nm) in the lipid gel phase to a longer wavelength (Parasassi et al., 1990) when the phospholipid membrane adopts a liquid crystalline structure.

As described by Parasassi (1990), the excitation generalized polarization parameter (GP_{ex}) was calculated to determine phase transition pressure of each type of vesicle studied. Steady-state emission spectra were measured by a spectrofluorimeter (Instaspec IV, Oriel) connected to the microscope. The frequency of opening the light shutter (LOT-Oriel) was modulated to minimize fluorescence extinction and to ensure complete data acquisition during a compression/decompression cycle. Microscopic fluorescence spectrum analysis allows one to focus on individual vesicles and to follow each liposome's structure modification.

Liposome Area and Volume Measurements

Images were processed with an image-analysis software (Matrox Inspector 3.0, Matrox, Canada). As the form factor stayed close to 1, liposomes were considered as spherical and were individually analyzed to find their projected area. Therefore, vesicle volume could be calculated from these data. For each type of liposome, the measurement of mean volume was performed on at least six liposomes.

Results and Discussion

Two different experiments were carried out on phospholipid vesicles. A spectrofluorimetric study under the microscope was performed to determine the phase diagram of the artificial vesicle under pressure. A microscopic measurement of shape and the corresponding volume of different liposomes was then carried out during pressure cycles.

Effect of Hydrostatic Pressure on DMPC/PS and DMPC/PS/DOPE GUVs as Given by Laurdan Fluorescence

Laurdan is an interesting tool for the characterization of lipid pressure transition because its fluorescence is maintained in spite of drastic pressure conditions. Chong and Wong (1993) have shown that this probe remains incorporated in the membrane bilayer up to 2.5 GPa.

Phospholipid vesicles were submitted to pressure levels up to 300 MPa under isothermal conditions (40°C or 60°C). During compression, an emission spectrum shift was clearly observed under the microscope: the sample, which presented a green fluorescence, became blue when pressure was increased. This phenomenon was reversible with pressure release.

Laurdan fluorescence spectra obtained for each phospholipid mixture confirm the blue shift during pressurization. The emission spectrum initially centered at 502 nm (characterizing the Lα phase) shows a shift of about 50 nm towards lower wavelength (characterizing the gel phase). Calculation of the general polarization of excitation parameter (GP_{ex}) allows the demonstration of phase transition pressures of each GUV type studied (Bagatolli & Gratton, 1999).

Variations of GP_{ex} of DMPC/PS vesicles (Fig. 1*A*) show a phase transition pressure near 80 MPa. Similar pressure transition values on pure DMPC vesicles are mentioned by Ichimori and coworkers (1998) who had also set up a pressure-temperature phase transition diagram of DMPC bilayer membrane. As expected, the hydrostatic pressure of the main transition increases with higher temperature, i.e., the transition pressure of DMPC/PS vesicles prepared at 60°C is increased more than 100 MPa in comparison with DMPC/PS vesicles prepared at 40°C. This phase transition observed in the spectrofluorimetric study seems to be related to a liquid-crystalline (Lα)/ ripple phase gel (Pβ′) change.

Figure 1*B* shows that the addition of DOPE in proportion of 25% allows an increase of phase transition pressure (about 10–20 MPa) compared to DMPC/PS liposomes. The phase transition region between the two states is also extended (50 MPa for DMPC/PS and 80 MPa for DMPC/PS/DOPE).

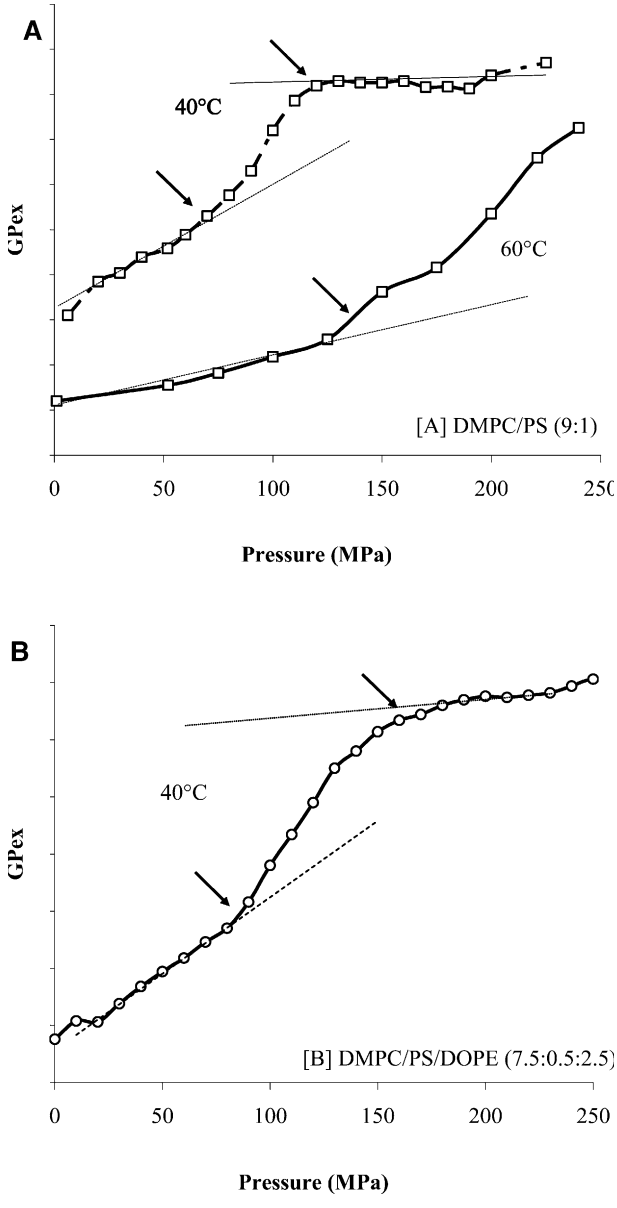

Fig. 1. Variations of general polarization excitation ratio (GP_{ex}) obtained from Laurdan fluorescence of vesicles according to their phospholipid composition and temperature. (*A*) DMPC/PS 40°C (□); and 60°C. (*B*) DMPC/PS/DOPE, 40°C. Boundaries of phase transition are indicated by an arrow, dashed and dotted lines represent regression of, respectively, liquid (Lα) and gel (Pβ′) phase.

Landwehr and Winter (1994) have also reported such an increase of transition interval, but in studying the temperature effect on phospholipid binary mixture DMPC/DPPC and DMPC/DSPC at excess of water.

Morphological Behavior of Vesicles During a Pressure Cycle

All vesicles studied were submitted to a compression/ decompression cycle reaching 300 MPa. Microscopic

Table 1. Descriptive summary of vesicle behavior during compression according to phospholipid composition and temperature

Temperature	Phospholipid composition	Observed vesicle behavior
40 °C	DMPC/PS	Bidding shape transition during compression (5%)
		Budding and pear shape transition during decompression (60%)
		No modification during pressure treatment (35%)
	DMPC/PS/DOPE	Budding shape transition (100%)
60 °C	DMPC/PS	Budding shape transition (100%)

observations of phospholipid show specific shape changes depending on lipid composition and temperature conditions (Table 1). In most cases, a dramatic shape change from spherical to pear-shaped and even budding vesicle was observed during pressure release. These vesicles keep a spherical shape throughout pressure loading, while during the release phase, at about 150 MPa, as given by shape factor analysis, vesicles lose their shape and exhibit a budding transition of type 1, as defined by Käs and Sackmann (1991) (Fig. 2). Such pressure shape hysteresis has been previously observed by Beney and colleagues (Beney et al., 1997) on egg yolk vesicles. These authors have interpreted this change by pressure-induced intravesicular water exit and subsequent vesicle surface-to-volume ratio increase during pressure release.

The DMPC/PS/DOPE vesicles always exhibit such a budding comportment. In contrast, DMPC/PS liposomes prepared at 40°C could exhibit minor alternative behaviors:

- Vesicles show similar shape change but it happens during pressure loading (5% of occurrence).
- No morphological modifications during the whole cycle (35% of occurrence).
- In most cases (60%), the DMPC/PS GUVs show similar shape change as observed with ternary mixture except that morphological transformations are less pronounced.

At a temperature of 40°C, it seems necessary to achieve a pressure level exceeding the complete phase transition to observe shape modifications during decompression. Compression/decompression cycles reaching 250 MPa were carried out on DMPC/PS GUVs at 60°C. Observations of the morphological behavior of these vesicles under pressure reveal the preservation of the spherical shape during compression and a pronounced shape transition when pressure decreases. Numerous compression/decompression cycles reaching successively 50 MPa, 100 MPa, 150 MPa and 200 MPa were tested on a same vesicle. A cycle treatment reaching a pressure level of 50 MPa or 100 MPa did not lead to a shape modification during depressurization, whereas compression/decompression cycles up to 150 MPa or 200 MPa (less than phase transition pressure) induce a pear-shaped transition back to atmospheric pressure. So, contrary to DMPC/PS GUVs formed at 40°C, vesicles present a morphological deformation even without complete phase change.

Compression Measurements of Prepared Vesicles

Beney and coworkers (1997) have postulated that difference in compression between phospholipid bilayer and intravesicular water could be responsible in a water exit and a subsequent change in surface to volume ratio. In order to verify this hypothesis on our vesicles, a measurement of cell volume variations during compression was also carried out.

As the vesicles remained spherical during pressure increase, volume changes of vesicles, obtained from image analysis (Table 2), resulted essentially from lateral compression of the phospholipid bilayer. The compression ratios obtained by this optical technique were, of course, different from apparent compression data obtained by the dilatometry technique, which represent a measurement of global volume variations including the hydration shell. The pressure-induced water exit was estimated by the maximum difference between vesicle compression and theoretical water-specific volume.

These results show a remarkable compression for all the vesicles up to 100 MPa. This volume decrease is far higher than expected from water bulk compressibility and should induce an outflow of water because of the spherical shape.

In order to relate this difference in compressibility with phospholipid composition and temperature, the phase diagram of the phospholipid mixture has to be considered. The liquid crystalline phase ($L\alpha$), which represents the initial atmospheric state of all vesicles, was known to be much more compressible than $P\beta'$ gel. The phase transition from $L\alpha$ to $P\beta'$ was also accompanied by an abrupt volume change in a small pressure level interval. These considerations could explain the higher volume change of DMPC/PS at 40°C for a pressure variation from 0.1 to 100 MPa because this pressure range included $L\alpha$ compression and the phase transition. From 100 MPa to 200MPa, the mean volume does not vary significantly because of the slight compressibility of the gel phase. As ex-

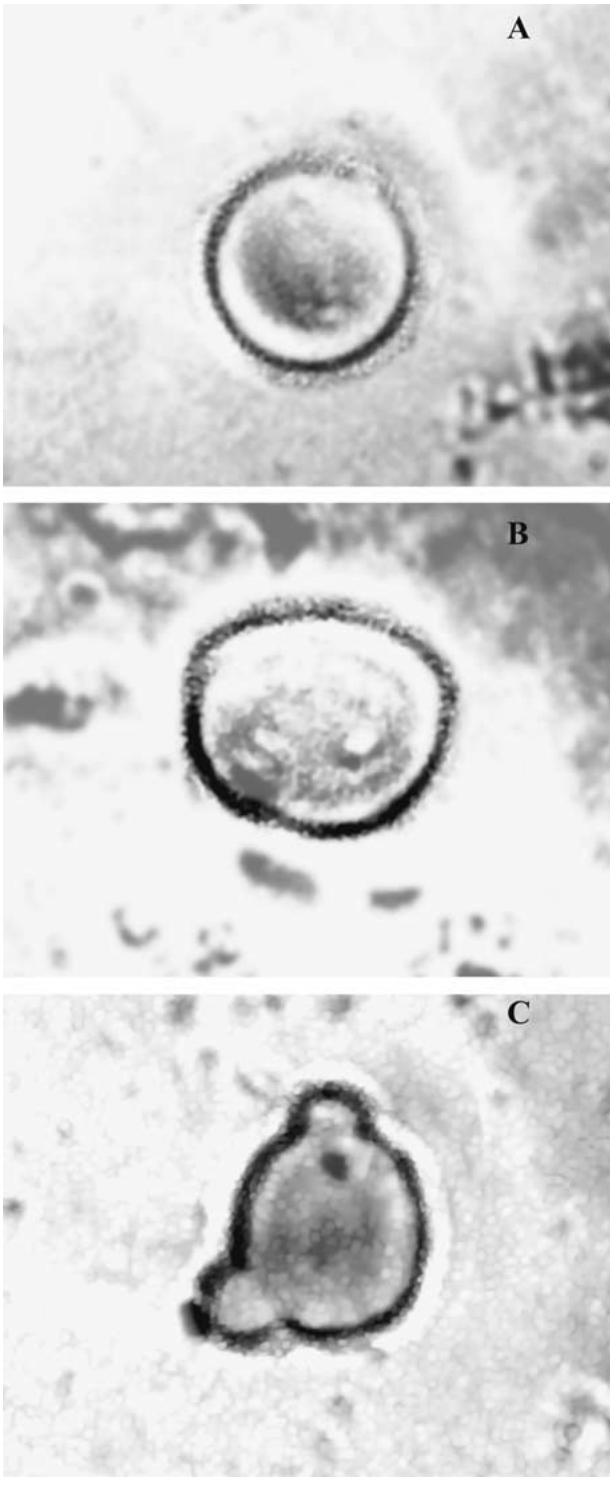

Fig. 2. Images of a DMPC-PS-DOPE vesicle during a high pressure cycle. The vesicle is spherical at atmospheric pressure and keeps this shape during compression. [(*A*) 150 MPa, pressure loading] and pressure-holding time. The shape changes during decompression. [(*B*) 150 MPa pressure release]; deformation persists after complete decompression. [(*C*) 0.1 MPa].

pected, at 60°C the Lα was more compressible and the volume decrease reached 26% although phase transition was not completed.

However these results should be interpreted with care due to the poor statistical significance of these measurements: only 1 to 2 vesicles were taken into account each time.

The calculated water outflow showed that at 40°C, a pressure of 200 MPa was likely to induce an exit of about 10% of initial water content with or without DOPE. This exit took place before the vesicles were in gel phase. In gel phase, compression of the vesicle was less than water compressibility. At 60°C, the long and highly compressible Lα phase allows a theoretical outflow of double the amount.

Comparison with Thermal Phenomena

Our work can be compared to the work of Bagatolli and Gratton (1999) on GUVs made with pure DTPC, DMPC or DPPC phospholipids. In that study, a similar shape hysteresis was obtained after a temperature cycle, which consisted of a cooling phase (from 40°C to about 12°C for DMPC) crossing the main transition temperature of phospholipids (Tm = 24°C for DMPC), followed by a symmetric heating phase. Shape change was observed in passing the phase transition during the heating phase.

During the cooling phase, a rapid decrease in diameter of about 7–8% was also measured when the vesicles reached the gel phase, which represented a decrease of about 25% in vesicle volume. This shrinkage was attributed to the structural change of membrane phospholipid from L_α to P_β. Before this main transition, a pretransition was associated with a first slight increase in diameter of 1%.

Our results resemble this work especially for GUVs made at 40°C. At this temperature, the shape change was observed on GUVs during the pressure phase transition from gel to liquid crystal after a compression phase, which led to a significant decrease in vesicle volume (about 15%). However, in working at 60°C, we have shown that shape hysteresis could be induced apparently without phase change during the pressure cycle. At this temperature, the membrane compression level was enough to induce a shape change back to that at atmospheric pressure.

In contrast to Bagatolli and Gratton (1999), the studies of Melear (1998) and Needham and Evans (1988) on a similar temperature cycle have shown a thermoelastic stress followed by vesicle rupture. Vesicle ruptures were also observed in our laboratory on some DMPC vesicles obtained with another preparation method. In contrast to shape hysteresis, the disruption took place during compression at the pressure transition, but this phenomenon has not been reproduced with the protocol used in this work. We suggest that this variation could be related to the oligo-lamellarity of such GUVs, which could change mechanical properties of the membrane, rather than due to a problem of surface contact. Indeed, in order

Table 2. Compression values of different vesicles during pressure loading as given by image analysis

	Apparent vesicle compression % of initial volume			Theoretical pressure-induced water exit % of initial mass	
	0.1 to 100 MPa	0.1 to 200 MPa	Phase transition pressure La → Pβ′	0.1 to 100 Mpa	0.1 to 200 MPa
DMPC/PS at 40°C	16%	16%	70 → 120 MPa	12.3%	10.7%
DMPC/PS at 60°C	13%	26%	150 → (>250) Mpa	9.4%	20.2%
DMPC/PS/DOPE at 40°C	10%	15%	90 → 160 Mpa	6.3%	9.7%

to prevent surface contact, only floating vesicles were taken into account. This precaution explains why only 1 or 2 vesicles could be observed during each experiment.

Because of the initial spherical shape of vesicles, volume shrinkage implies water exit through the membrane. Bagatolli and Gratton (1999) suggested that this flux was made possible by temporary pore formation in the membrane during the phase transition. In fact, numerous authors have described defects in the membrane due to the existence of phospholipid domains with different density and structure. The same authors in further work (Bagatolli & Gratton, 2000) have also directly observed with the two-photon fluorescence technique the existence and the growth of large domains on DMPC/DMPE phospholipid vesicles. The "pores" situated in the boundary layer may facilitate the leakage of small molecules. Such leakage has also been identified with thermotropic transition. Indeed, numerous authors have shown that the permeability of phospholipid vesicles was clearly enhanced at phase transition pressure (Macdonald, 1984). This phenomenon should be more important with complex membrane composition. However, the water permeability of the membrane suffices to allow volume shrinkage imposed by membrane compression in a very short time (less than 1 ms). Moreover, in this work, at 60°C the water transfer occurred far from transition pressure and with a simple lipid composition. Thus we propose that pore formation can facilitate volume decrease of phospholipid vesicles without rupture only in the presence of solutes. Otherwise, in pure water, pore formation was not necessary to observe shape change on vesicles.

Domain formation could also lead to a local change of surface curvature, which, in turn, could modify vesicle shape. The transition of vesicles to a polygonal shape was observed by Bagatolli and Gratton (1999) just before shape change. Plate domains are assumed to be structured as gel phase, while corner surfaces correspond to liquid domain. This modification was only observed during the heating cycle. This polygonal shape supports the hypothesis of domain formation and could prefigure the mechanism of the shape change observed. The formation of a boundary in the membrane between domains of different lipid composition and thermodynamic phase is energetically unfavorable. It results in a tension line that allows a decrease in curvature of each domain. This situation could lead to a budding mechanism (Lipowsky, 1991; Döbereiner et al., 1993). The formation of these clusters would also imply the presence of interfacial regions, which would induce a significant mechanical fragility and a local energy bending modification, which create membrane anisotropy, inducing a budding mechanism during the S/V ratio modification.

Although we did not observe in our experiments such a remarkable (polygonal) shape, a contribution of domain spontaneous curvature could be envisaged in the shape change and particularly in the orientation of the budding-like expansion observed.

However, experiments at 60°C showed that shape change is mainly due to the increase of S/V ratio during compression, while phase separation and spontaneous curvature effects are considered as less important. The shape modification was induced by a differential compression between vesicle membrane and intravesicular content (water in this case). The phospholipids' compaction induced by pressure during the liquid-crystalline phase was important and thus, the longer the LC phase is stable under pressure, the more important was the modification of S/V ratio and then shape change. We had expected that vesicles formed in gel phase would not be deformed by a pressure cycle.

Further work is necessary to clearly show the water outflow induced by surface compression and the influence of impermeant solutes in this transfer. If pore formation is responsible in this transfer, solutes would accompany the water outflow and an osmotic phenomenon would not be involved.

It would also be interesting to verify if such a phenomenon could also be identified with vesicles made from biological membrane or with alive cells. Such pressure-induced transfer would certainly be damaging for a cell. It would lead, for example, to an osmotic imbalance or to the inactivation of pressure-sensitive ion channels (MacDonald, 2002). The effect of phase transition of the cell membrane and temperature processing on pressure-induced inactivation

has been recently investigated on *Lactobacillus plantarum* (Ulmer et al., 2002) and seems to confirm our observation. These results are also of medical interest and especially for deep-sea-diving medicine. The effect of pressure on the human cell membrane is already known and especially the reverse action of pressure regarding anesthetics addition (Bartlett, 2002). It would be of great interest to test the effect of anesthetics on the pressure-induced shape transition of phospholipid vesicles.

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