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# Osmotic shrinkage and reswelling of giant vesicles composed of dioleoylphosphatidylglycerol and cholesterol

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## Abstract

The osmotic shrinkage of giant unilamellar dioleoylphosphatidylglycerol (DOPG) vesicles in a hypertonic osmotic solution is investigated. The volume reduction for given membrane area leads to a vesiculation of the bilayer into the interior of the giant. The size of the daughter vesicles that appear inside the giant is uniform and an increasing function of the cholesterol content, but independent of the osmotic gradient applied. The radius of the daughter vesicles increases from 0.2  $\mu\text{m}$  to 3.0  $\mu\text{m}$  when the cholesterol content is changed from 0 to 40%. It is argued that the size of the daughter vesicles is regulated by the membrane persistence length, which is an exponential function of the mean bending modulus. From the kinetics of shrinkage it follows that approximately 14% of the daughter vesicles remain attached to the mother giant. This is in reasonable agreement with osmotic swelling experiments which show that approximately 11% of the daughter vesicles is available for area expansion. © 2008 Elsevier B.V. All rights reserved.

**Keywords:** Dehydration; Vesiculation

## 1. Introduction

Living cells that are subjected to drought, freezing, or media of elevated osmolarity, lose water. This dehydration directly reduces volume, which gives rise to an excess of membrane surface area. In some systems, the area to volume ratio is adjusted by vesiculation of the plasma membrane, for example, during plasmolysis in onion epidermal cells [1] and in the Gram-negative bacterium *Escherichia coli* [2]. In guard-cell protoplasts, shrinkage causes vesicular retrieval of the plasma membrane into the cytoplasm [3], and in the axis of dried desiccation-tolerant soybean seeds [4] and the radicals of dried cowpea seeds [5] a clustering of vesicles has been observed along the margins of the plasma membrane, which has not been found in the hydrated state. Dehydration-induced vesiculation

may result in serious damage to the plasma membrane upon subsequent rehydration, when area expansion is required. This is illustrated by the differential reaction of protoplasts from cold-acclimated and non-acclimated rye leaves. In the acclimated protoplast, vesicles remain continuous with the plasma membrane upon freezing-induced contraction [6], whereas the non-acclimated protoplasts vesiculate without the vesicles remaining attached to the plasma membrane [7]. Cold-acclimated protoplasts are able to survive cycles of freezing and thawing while non-acclimated protoplasts die during thawing. The loss of contact of the daughter vesicles with the plasma membrane results in lysis of the protoplast during thawing, which has been linked to lethal damage. When rye protoplast are volumetrically contracted in a medium of elevated osmolarity, its membrane loses material and re-expansion to the original size leads to lysis of the plasma membrane if the membrane cannot incorporate sufficient new material fast enough [8,9]. The inability to reincorporate vesicles into the plasma membrane of the rehydrating cell has also been suggested to be the cause of imbibitional damage in desiccation-tolerant organisms [4].

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Membrane vesiculation upon osmotic contraction appears not to be unique for biological membranes or their lipid extracts [10]. Giant egg-PC vesicles retain their spherical shape during osmotic shrinkage [11]. Recently, it was reported that the volume reduction of a giant vesicle in hypertonic osmotic solution is accompanied by the formation of daughter vesicles inside the shrinking giant [12]. The mode of shrinkage has been observed for vesicles of varying composition, but not for pure dipalmytoylcholinephosphatidylcholine (DPPC) vesicles. This gave rise to the suggestion that the vesiculation is driven by a non-homogeneous distribution of lipids in the bilayer. A non-homogeneous distribution of lipid can in principle result in a non-vanishing spontaneous curvature [12]. However, a spontaneous curvature as introduced by Helfich [13] occurs only in very specific systems, [14,15] and is not necessary to explain the reported observations. Instead, we propose that the membrane persistence length controls the physical behavior of the phospholipid bilayer during osmotic shrinkage. Here we report on the behavior of unilamellar giant DOPG vesicles containing different amounts of cholesterol under hypertonic osmotic stress. The formation of daughter vesicles inside the shrunken giants is followed as function of both lipid bilayer composition and osmotic gradient. We show that the radius of the daughter vesicles can be estimated from the initial and equilibrium size of the mother giant and from the osmotic gradient applied. Furthermore we estimate both the membrane permeability for water and the fraction of vesicles that stay attached to the giant vesicle during osmotic shrinkage from the dehydration kinetics. To verify the estimated values for the fraction of daughter vesicles that remain attached, the reversibility of the shrinkage process is analyzed.

## 2. Materials and methods

Di-oleoylphosphatidylglycerol (DOPG) is obtained from Avanti Polar Lipids, cholesterol from Sigma. To produce giant (unilamellar) vesicles the lipids are dissolved in chloroform and subsequently dried under a stream of nitrogen, leaving a thin lipid film on the wall of a glass tube. Remaining traces of solvent are removed from the lipid film by drying under vacuum for at least 4 h. The dried lipid films are rehydrated using a 50 mM sucrose solution and allowed to

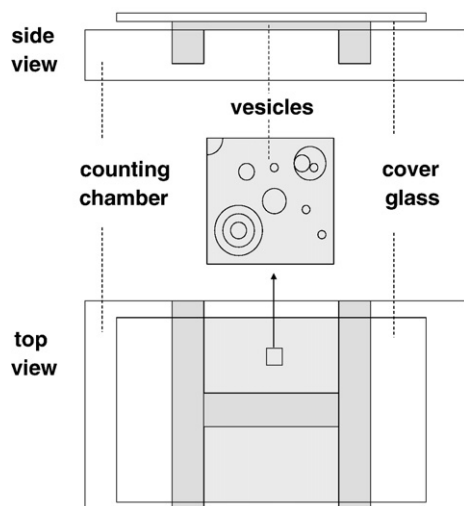


Fig. 1. Schematic representation of the experimental setup.

equilibrate for 48–72 h. This procedure results in a heterogeneous population of spherical vesicles, some of which are unilamellar and large enough for analysis with an optical microscope. Giant DOPG vesicles are produced at a lipid concentration of 0.6  $\mu\text{M}$ . Mixed DOPG/cholesterol vesicles are made at the same concentration with molar ratios of DOPG:cholesterol of 8:2 and 6:4.

The response of the giant vesicles to osmotic gradients of 50, 100, and 200 mM are followed with an Olympus BX60 optical microscope by direct observation of the vesicle radius as a function of time. All observations are made at room temperature which is above the gel-to-liquid-crystalline phase transition temperature of the lipid (mixtures) used.

To subject the vesicles to osmotic gradients, a counting chamber is covered with a normal cover glass and the sucrose solution containing the giant vesicles is injected into one of the grooves (Fig. 1). Capillary forces fill the area under the cover glass with a thin layer of vesicle-containing solution. Lipid bilayers are relatively impermeable to sugars. An osmotic gradient over the vesicle membrane is established by removing the original solution from the grooves and replacing it by a glucose solution of different molarity. The difference in refractive index between sucrose improves the contrast of the vesicle sample. During solvent replacement, the thin layer of vesicle-containing solution between the cover glass and counting chamber remains intact. The convection that results from the osmotic gradient is relatively slow in the thin layer, which makes it possible to study both the osmotic contraction and reswelling of the vesicles.

## 3. Results and analysis

The gentle rehydration of the lipid film gives a suspension containing a mixture of giant vesicles that is heterogeneous in both size and lamellarity. From this population of vesicles, unilamellar specimens are selected by looking for minimal contour contrast and absence of internal structure. Such unilamellar vesicles are subsequently followed during osmotic shrinkage and rehydration.

During osmotic shrinkage, all vesicles tested remain approximately spherical, and no large shape fluctuations or shape instabilities are observed. Fig. 2 shows the relative size decrease of giant vesicles of different composition with an initial radius  $R_0$  at time  $t=0$  of  $\sim 20 \mu\text{m}$  during exposure to an osmotic gradient of 200 mM. Similar experiments are performed using differently sized vesicles ( $20 \mu\text{m} < R < 40 \mu\text{m}$ ) and osmotic gradients of 50 mM and 100 mM. For these conditions a similar behavior of the giant vesicle radius as a function of time  $R(t)$  is observed.

Initially, the vesicle radius  $R(t)$  decreases with time  $t$ . The rate of size decrease is reproducible for vesicles composed of the same lipid species, but is different for vesicles of other composition. The more cholesterol the vesicle membrane contains the slower the observed rate of size decreases. After approximately 40 min the decrease in size ceases for vesicles of all compositions. Osmotic equilibrium between the interior and exterior of the vesicles has been reached and the vesicle radius remains constant with time (Fig. 2). The final  $R/R_0$  is a function of the lipid bilayer composition (Fig. 2). DOPG vesicles containing cholesterol always shrink less than pure DOPG vesicles upon osmotic dehydration.

During osmotically induced shrinkage small daughter vesicles appear in the interior of the giant vesicles. In vesicles composed of DOPG and 40% cholesterol, daughter vesicles have an  $r$  of approximately  $3.0 \mu\text{m}$  (Fig. 2). The radius of the daughter vesicles decreases with decreasing amounts of cholesterol in the bilayer. Daughter vesicles always appear inside the giant vesicles. Outward extrusions are never observed. In giants composed of pure DOPG daughter vesicles are never seen, probably because they are too small to be detected by light microscopy.

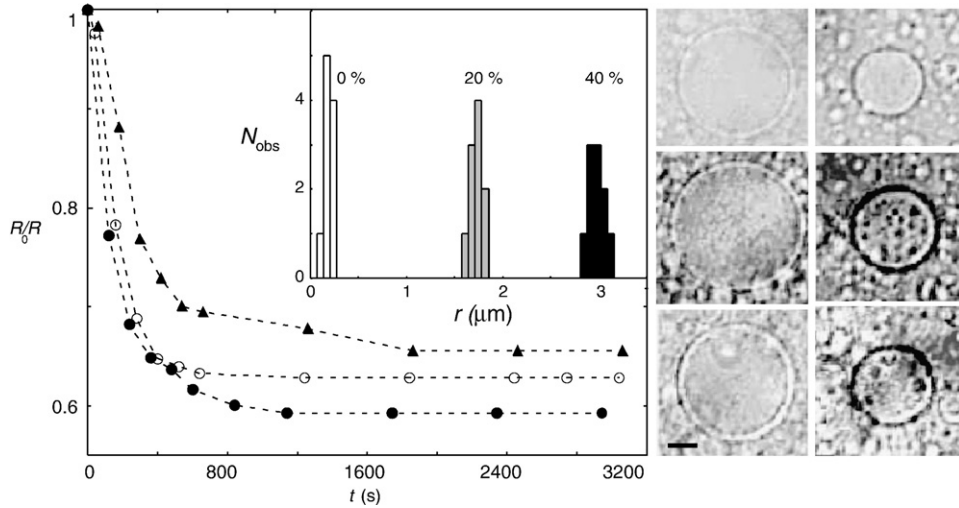


Fig. 2. Relative shrinkage of vesicles with an initial radius  $R_0$  of approximately 20  $\mu\text{m}$  subjected to an osmotic gradient of 200 mM glucose. The vesicles are composed of DOPG (●), DOPG-20% cholesterol (○), or DOPG-40% cholesterol (▲). Inset: Histogram of daughter vesicle radii for DOPG vesicles containing 0%, 20% or 40% cholesterol. The daughter vesicles radii are calculated from the initial concentration gradient over the membrane and giant vesicle radii before and after osmotic shrinkage. On the right: DOPG vesicles containing 0%, 20% and 40% cholesterol before ( $t=0$  s) (left) and after (right) osmotic contraction ( $t=3600$  s). The shrunken vesicles contain visible daughter vesicles that have a radius of approximately 1.8  $\mu\text{m}$  (20% cholesterol) and 3.0  $\mu\text{m}$  (40% cholesterol). The bar indicates 10  $\mu\text{m}$ .

Assuming that the total surface area is conserved by the formation of  $n(t)$  daughter vesicles of radius  $r$  the equilibrium area of the giant vesicle after shrinkage  $A(\infty)$  is given by:  $A(\infty) = 4\pi R(\infty)^2 = 4\pi R_0^2 - 4\pi n(\infty)r^2$ . This gives the following expression for the number of daughter vesicles  $n(\infty)$  at the end of the shrinkage process:

$$n(\infty) = \frac{R_0^2 - R(\infty)^2}{r^2} \quad (1)$$

During osmotic shrinkage daughter vesicles entrap the hypertonic solution from the outside. In our model calculations we therefore consider them to be effectively osmotically inactive. At equilibrium, the concentration of osmoticum in the interior of both the giant and daughter vesicles is supposed to have reached that of the outside solution. Because the membrane is (almost) impermeable to glucose and sucrose, the osmotic gradient determines how much water the vesicle has to lose before this osmotic equilibrium is reached. The volume of the shrunken vesicle at osmotic equilibrium or its final volume  $V(\infty)$  is therefore given by:  $V(\infty) = \frac{c_0}{\Delta c + c_0} \left( \frac{4}{3} \pi R_0^3 \right) = V(\infty) = \frac{4}{3} \pi \left( R(\infty)^3 - n(\infty)r^3 \right)$  where  $c_0$  is the initial sucrose concentration inside the giant and  $\Delta c$  the osmotic gradient over the lipid bilayer. This results in the following expression for the radius of the daughter vesicles inside the giant:

$$r = \frac{R(\infty)^3 - \frac{c_0}{c_0 + \Delta c} R_0^3}{R_0^2 - R(\infty)^2} \quad (2)$$

Using Eq. (2) the radius of the DOPG daughter vesicles is calculated to be  $r=0.2 \pm 0.1$   $\mu\text{m}$ , which is indeed below the resolution of the optical microscope. For DOPG vesicles containing cholesterol, much larger radii for the daughter vesicles are obtained. The size of the daughter vesicles appears to depend

strongly on composition;  $r$  increases to  $1.7 \pm 0.1$   $\mu\text{m}$  for bilayers composed of 80% DOPG and 20% cholesterol and  $3.0 \pm 0.1$   $\mu\text{m}$  for vesicles containing 40% cholesterol (Fig. 2 inset). The values calculated for  $r$  are very close to the radii of the daughter vesicles observed using optical microscopy (Fig. 2).

The water permeability of the bilayer can be obtained from the decrease in the vesicle radius with time (Fig. 2). The hypertonic solution at the outside withdraws water from the vesicle. The rate of water loss through the bilayer is determined by the bilayer permeability coefficient  $P$  and the difference in sugar concentration between in and outside,  $\Delta c$ .

$$\frac{1}{A} \frac{dV}{dt} = P v_w \Delta c \quad (3)$$

Where  $v_w$  is the molar volume of water. The loss of water from the vesicle takes place over a certain membrane area  $A$ . This area is not necessarily given by  $4\pi R(t)^2$ , some area might be hidden in vesicles that remain attached to the mother giant. The area fraction of vesicles that remain attached to the bilayer is given by  $\alpha$ . The area that is accessible for water transport at any time is now given by:

$$A(t) = 4\pi \left[ R(t)^2 + \alpha \left( R^2 - R(t)^2 \right) \right] \quad (4)$$

During osmotic shrinkage  $\Delta c$  decreases. Because  $c(t) = c_0 (V_0/V(t))$ , and  $\Delta c = c(t) - c_0$  can be calculated at any time, Eq. (3) can be rewritten to

$$\frac{dR}{dt} = \frac{P v_w}{R(t)^2 + \frac{2}{3} r R(t)} \left( \frac{c_0 R_0^3}{\left( R(t)^3 + r R(t)^2 - r R_0^2 \right) - c_b} \right) \times \left( (1 - \alpha) R(t)^2 + \alpha R_0^2 \right) \quad (5)$$

in which  $c_b$  is the final sugar concentration in- and outside of the vesicle. From Eq. (5) it follows that besides the membrane permeability  $P$ , the amount of vesicles that remain attached to the bilayer  $\alpha$  can in principle be estimated from the change of the vesicle radius with time. The initial slope of the  $R$  versus time curves appears to be sensitive to  $P$ , and the value of  $\alpha$  can be found from the  $R(t)$  at higher values of  $t$ . We note that Eq. (5) can be solved analytically, this results, however, in a lengthy, complicated, implicit relation between  $R$  and  $t$ . Therefore, Eq. (5) is fitted directly to the derivative of the observed size decrease with time (Fig. 3). Unfortunately, our experiment has too low a time resolution to obtain a reliable value for the permeability coefficient  $P$  of pure DOPG vesicles. The decrease of  $R$  with time is just too fast for these vesicles (Fig. 2), and therefore  $P$  is underestimated. For sufficiently high amounts of added cholesterol the process of shrinkage is slow enough to obtain more reliable data. For vesicles composed of DOPG with 20% cholesterol  $P=15.3\pm 3.4$   $\mu\text{m/s}$ , whereas DOPG vesicles containing 40% cholesterol had a  $P$  of  $6.6\pm 1.5$   $\mu\text{m/s}$ . The more cholesterol the DOPG bilayer contains the less permeable it becomes for water. The values found for  $\alpha$  do not differ much between vesicles of different composition. According to our calculations,  $14\pm 4\%$  of the daughter vesicles remain attached to the mother giant.

The daughter vesicles that remain attached should be easily reincorporated into the membrane during osmotic inflation. To test whether some vesicles indeed remain continuous with the giant, as the values found for  $\alpha$  suggest, shrunken vesicles are subjected to a hypotonic glucose solution. The outside solution is replaced by a 50 mM glucose solution, forcing the vesicles to grow in size. None of the vesicles is able to regain its initial radius; the size increase is limited to approximately 21–28% of the membrane surface area after shrinkage. The maximum area increase depends slightly on composition. Vesicles composed of DOPG alone are able to increase their surface area by  $28\pm 1.9\%$ , whereas the vesicles composed of DOPG with 40% cholesterol can increase their surface area by  $21\pm 1.5\%$  before they lyse.

When fresh, unstressed unilamellar vesicles are subjected to a hypotonic solution ( $\Delta c=50$  mM) they are able to increase

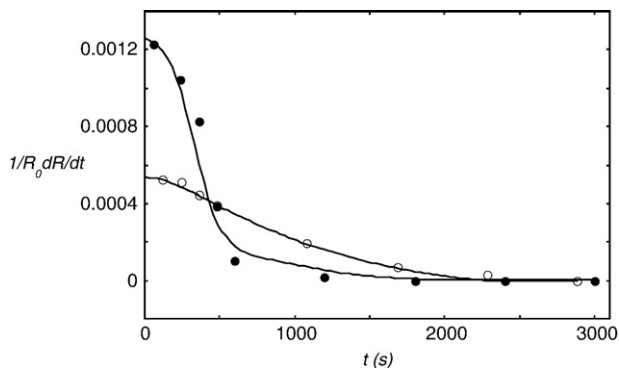


Fig. 3. The measured  $\frac{1}{R_0} \frac{dR}{dt}$  as a function of time for giant vesicles composed of DOPG-20% cholesterol (●) and DOPG-40% cholesterol (○) in an initial osmotic gradient of 200 mM. The data are fitted with Eq. (5), which results in an average  $P=15.3\pm 3.4$   $\mu\text{m/s}$  for DOPG vesicles containing 20% cholesterol and in  $6.6\pm 1.5$   $\mu\text{m/s}$  for those containing 40% cholesterol. The average value for  $\alpha$  is  $14\pm 4$  and does not change with the membrane composition.

their surface area by 4–9%, depending on composition, before they lysed. The more cholesterol the DOPG bilayer contained, the smaller the area increase before lysis. For bilayers composed of DOPG an area increase of  $9.0\pm 1.2\%$  is measured, whereas DOPG bilayers containing 40% cholesterol are able to increase their surface area by only  $4.1\pm 1.3\%$ .

#### 4. Discussion

In a certain lipid composition range giant unilamellar vesicle bilayers, consisting ternary mixtures of a sterol, an unsaturated and a saturated lipid, phase separate into coexisting liquid-ordered and liquid-disordered phases [16–19]. These laterally phase separated membranes exhibit an interesting shape behavior that includes bud-formation and vesicle fission [19,20]. The sign of the curvature of such protrusions depends strongly on the sterol species in the bilayer membrane [20]. However, liquid–liquid phase separation has not been observed in bilayer vesicles composed of binary lipid mixtures or single phospholipid species [18] and this mechanism of daughter vesicle formation is therefore probably not related to the shape transitions described here.

Earlier experiments on osmotic shrinkage of vesicles gave rise to the suggestion that the formation of daughter vesicles is associated with micro-segregation of the membrane components and the existence of a spontaneous bilayer curvature [12]. Our experiments show that the radius of vesicles composed of DOPG decreases during osmotic deflation (Fig. 2). During shrinkage the visible contours of the DOPG vesicles remain spherical. Although using bright field microscopy we never observe any daughter vesicles inside the shrunken DOPG giants, further analysis of the data (Eq. (2)) suggests that the one-component giant DOPG vesicles must also vesiculate. The radius of the daughter vesicles is calculated to be 0.2  $\mu\text{m}$ , which is too small for observation by the optical microscope. The appearance of equally sized daughter vesicles inside one-component giant vesicles has been observed before, when excess area is created by increasing the temperature: both palmitoyl-oleoylphosphatidylcholine (POPC) and stearyl-oleoylphosphatidylcholine (SOPC) giants split off small vesicles [21,22]. However, this kind of vesiculation behavior has not been observed for dimyristoylphosphatidylcholine (DMPC) membranes. This has been explained in terms of transmembrane lipid transfer rate, which is faster for DMPC than for POPC or SOPC [22]. A slower transfer rate of lipids between the monolayers may well result in a stronger tendency for budding, whereas a redistribution of lipids between the monolayers may favor other shape transitions [21,22,20].

The observation that one-component vesicles are able to vesiculate makes the argument that the observed shrinkage is the result of a local spontaneous membrane curvature [12] very unlikely. An alternative explanation is suggested by our experiments in which cholesterol was added to the DOPG bilayer. Substances, such as cholesterol, are expected to increase the mean bending modulus  $k_c$  of the DOPG bilayer. Literature reports large increases in  $k_c$  upon the incorporation of varying percentages of cholesterol in DMPC bilayers [23]. With the incorporation of cholesterol into the DOPG bilayer, daughter vesicles become visible inside the mother giant after exposure to a hypertonic solution (see inset in

Fig. 2). The size of the daughter vesicles increases with increasing cholesterol content. Furthermore, the size of the daughter vesicles appears to be independent of the applied osmotic gradient. Hypertonic osmotic solutions, which imposed gradients of 50, 100, or 200 mM glucose over the vesicle membrane, all generated daughter vesicles of the same size. The size of the daughter vesicles could only be influenced by changing the bilayer composition. It is therefore likely that the radius of the daughter vesicles is controlled by the persistence length  $\xi_p$ , and therefore the  $k_c$  of the bilayer membrane. The bilayer has no resistance against shape fluctuations over length scales larger than  $\xi_p$ . This means that the effective mean bending modulus of the bilayer  $k_c^{\text{eff}}$  is dependent on the vesicle radius  $R$ . The renormalized mean bending modulus  $k_c^{\text{eff}}(R)$  is given by:

$$k_c^{\text{eff}}(R) = k_c - a \frac{k_B T}{4\pi} \ln \left[ \frac{R}{l} \right]$$

in which  $l$  is a length scale proportional to the size of the molecules,  $k_B$  the Boltzmann constant,  $T$  absolute temperature and  $a$  a numerical constant of order unity. As  $\xi_p \propto l e^{\frac{4\pi k_c}{k_B T}}$ ,  $k_c^{\text{eff}}$  becomes zero when  $R \approx \xi_p$ . The same argument holds for the Gaussian bending modulus  $\bar{k}$ , and therefore the curvature energy given by  $E_{\text{ves}} = 4\pi(2k_c + \bar{k})$  will become zero at length scales much larger than  $\xi_p$ . Moreover for bilayer membranes  $k_c$  and  $\bar{k}$  are opposite in sign and for charged lipid bilayers at low ionic strength  $k_c \leq -\bar{k}$  [24,25]. This means that it will cost no curvature energy to form vesicles inside the giant provided that these vesicles are at least of the same order of magnitude as the persistence length and/or  $k_c \leq -\bar{k}$ . This implicates that substances that increase  $k_c$ , such as cholesterol [23], will increase the size of the daughter vesicles. Although the scaling relation will, in reality, be complicated by interactions between the (charged) vesicles [26], an increase in  $r$  with increasing cholesterol content is observed.

In the dehydration experiments presented here, daughter vesicles always appear at the inside of the mother giant; during osmotic shrinkage no outward extrusions are observed. The main reason for this might be the under-pressure inside the vesicle. Moreover, the volume of the vesicle decreases faster with the same decrease in area when vesicles bud to the inside instead of to the outside. However, development of outward extrusions during dehydration (by freezing) is observed for giant vesicles composed of plasma membrane lipid extracts from cold-acclimated rye leaves [10]. Giant vesicles made of the lipid extracts from non-acclimated rye leaves vesiculate to the inside upon freezing-induced dehydration. This difference in reaction on dehydration can only be explained by the difference in lipid composition between these two giant vesicles. Although it is unlikely that there are large differences in lipid composition between the two monolayers of the bilayer some degree of asymmetry in lipid composition is needed to explain the differences in behavior of the giant unilamellar vesicles made of lipid extracts of acclimated and non-acclimated rye leaves upon dehydration. The asymmetry in monolayer composition must be a result of the special tendencies of some components or interactions between different lipids. Asymmetry in composition between the individual monolayers of the membrane is less likely in the simple two-component bilayers

studied here. Alternatively the change in sign of the extrusions might be related to the drastic change in sterol composition and content observed during cold acclimation [27]. Model studies on lipid rafts have shown that the molecular structure of different sterols not only determines the ability to induce fluid-fluid phase separation but also affects the sign of the curvature of the liquid-ordered phases [20].

The incorporation of cholesterol into the bilayer does not only change  $k_c$ . Cholesterol fluidizes gel phase membranes, and increases the packing order of liquid-crystalline bilayers. This increased packing order decreases the water permeability of the bilayer. The incorporation of cholesterol in the DOPG bilayer does indeed affect the permeability of the lipid bilayer as can be seen from the slowed kinetics of size decrease (Fig. 2). With increasing cholesterol content it becomes more difficult for water molecules to cross the membrane and the bilayer permeability coefficient decreases. Qualitatively, the decrease in  $P$  with increasing cholesterol content has been observed before for DOPG bilayers [28]. The permeability constants for cholesterol containing membranes obtained here somewhat are smaller than the literature values for the water permeability of pure phospholipid bilayers [11,29], the order of magnitude is however, comparable.

From the literature [12] it is not clear whether the daughter vesicles remain attached to the mother giant during and after osmotic shrinkage. By fitting Eq. (5) to the data it is possible to estimate the fraction of vesicles that remain attached to the mother giant. Independent of the osmotic gradient approximately 14% of the vesicles is found to stay attached to the mother membrane.

When unstressed vesicles are subjected to a hypotonic osmoticum they are able to increase their surface area by 4–9%. This is in agreement with the  $\delta A/A = 3\%$  reported for giant lecithin vesicles [30] and the  $\delta A/A = 4\text{--}6\%$  [31] or the  $\delta A/A = 10\%$  [32], reported for small DOPG vesicles. The  $\delta A/A$  values measured decrease with increasing cholesterol content of the DOPG bilayer. This trend in maximal area increase before lysis of vesicles in a series of cholesterol contents agrees with the results obtained for cholesterol containing DMPC vesicles [33] and is usually ascribed to the increased bilayer cohesion by the addition of cholesterol. The fact that the vesicles always lysed at the same relative area increase suggests that critical fluctuations in lipid density within the bilayer may be the origin of the failure, agreeing with the idea of a critical rupture tension above which long term survival of the vesicle becomes very unlikely [34,35].

When the same osmotic inflation experiments are performed with vesicles that have first been dehydrated by a hypertonic osmoticum, an area increase of 21–28% is possible. This is significantly more than the 4–9% area increase observed before lysis of fresh untreated vesicles. The larger surface area increase suggests that some of the daughter vesicles remain attached to their mother and are readily available when an area increase is required.

The differences in  $\delta A/A$  values between shrunken vesicles of different composition are comparable to those of  $\delta A/A$  values obtained for untreated unilamellar vesicles. This suggests that these differences are caused by the differences in elasticity of the lipid bilayer. The amount of membrane material incorporated during osmotic inflation is thus comparable for all lipid

compositions. It amounts to approximately 11% of the area split off during shrinkage. This percentage is in good agreement with the fraction of vesicles that remained attached during shrinkage estimated from fitting the decrease of  $R$  in time (14%).

Although the membrane composition seems to have no influence on the percentage of material that can be reincorporated into the expanding vesicle, experiments on rye protoplasts suggest that the lipid composition probably plays an essential role in the behavior of the membrane during surface area to volume changes [36]. No plasma membrane component is unique in either acclimated or non-acclimated protoplasts, but enrichment of the plasma membrane of non-cold-acclimated rye protoplasts with mono- or di-unsaturated species of phosphatidylcholine transforms the cryo behavior of the plasma membrane of non-acclimated protoplast into that of cold-acclimated ones [36].

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