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Rapid report

Raspberry vesicles

Anne-Laure Bernard[a,1], Marie-Alice Guedeau-Boudeville[a], Ludovic Jullien[b], Jean-Marc di Meglio[c,*]

[a] *Laboratoire de Physique de la Matière Condensée (CNRS URA 792), Collège de France, 11 place Marcelin Berthelot, 75231 Paris Cedex 05, France*
[b] *Département de Chimie (CNRS UMR 8640), École Normale Supérieure, 24 rue Lhomond, 75231 Paris Cedex 05, France*
[c] *Institut Charles Sadron (CNRS UPR 022), Université Louis Pasteur and Institut Universitaire de France, 6 rue Boussingault, 67083 Strasbourg Cedex, France*

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Abstract

We present a method to control the osmotic stress of giant unilamellar vesicles (GUV) and we report an original shrinkage mode of the vesicles: the volume reduction is accompanied by the formation of inverted daughter vesicles which gives the shrunk vesicles the appearance of raspberries. We analyze this peculiar shrinkage and we propose some physical origins for the observed phenomena.

Keywords: Vesicle; Osmotic shock; Shrinkage

Vesicles constitute simple and convenient experimental models for the study of important phenomena occurring in actual biological cells such as curvature, stretching and permeability of the cytoplasmic membrane [1–5]. Small vesicles (or liposomes) are also of great practical importance since they can be used for drug delivery purposes. During their processing, and particularly for encapsulation, vesicles are submitted to osmotic stresses. The phospholipid bilayer, in spite of its hydrophobic aliphatic core, is permeable to water: a vesicle shrinks in the presence of an external hypertonic solution [6–8]. An important and sudden change of the osmotic pressure often results in the destruction of the vesicle (the so-called osmotic shock), especially for large vesicles. We present in this report a method developed to control the shock endured by giant unilamellar vesicles (GUV) and that surprisingly results in very original vesicular shapes reminiscent of raspberries.

GUV have been prepared according to the electrical method originally described by Angelova and Dimitrov [9]: thin films are obtained by evaporation on conducting plates (ITO-coated glass slides) of freshly prepared chloroform/methanol (9:1) solutions of phospholipids and are subsequently swollen in 50 mM sucrose aqueous solutions in the presence of an alternating electrical field. We have experimented on different phospholipid systems: (i) 70% EPC (egg phosphatidylcholine), 20% dicetyl phosphate, 10% cholesterol, (ii) EPC, (iii) 90% EPC, 10% cholesterol and (iv) DPPC (dipalmitoylphosphatidylcholine). All chemicals have been purchased from Sigma-Aldrich and used as received. To induce sedimentation by gravity, vesicles are transferred at constant osmolarity into a less dense aqueous solution (50 mM glucose). The experimental set-up designed to change the osmotic pressure is presented in Fig. 1: the vesicles, initially in an isotonic solution (the external concentration of glucose c_0 is equal to the internal concentration of sucrose c_0 with $c_0 = 50$ mM in all experiments), are compartmented by an *Anopore* membrane (a membrane made of aluminum oxide with pore size of 0.2 μm, commercially available from Whatman International or SPI Supplies for instance). A solution of glucose at concentration $c = c_0 + \Delta c > c_0$ is added in the top compartment of the cell. The membrane filter breaks the convection resulting from thermal and concentration gradients. The cell has a diameter of 22 mm, the lower compartment that contains the vesicles has a volume of

* Corresponding author.
E-mail address: dimeglio@ccr.jussieu.fr (J.-M. di Meglio).
[1] Present address: L'Oréal, Laboratoire de Physico-chimie, 66 rue Henri Barbusse, 92117, Clichy Cedex, France.

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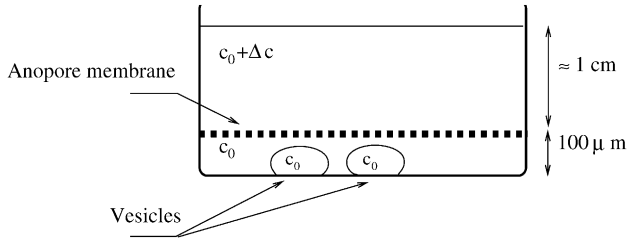

Fig. 1. Schematic experimental set-up.

4.2×10^{-2} cm^3 while the upper compartment with the hypertonic solution has a volume of 3.8 cm^3. The process of osmotic shrinkage is observed at room temperature in phase contrast or epifluorescence using an inverted microscope.

Fig. 2 shows the evolution of giant vesicles from (A) to (B) under osmotic stress. Smaller objects (daughter vesicles) with a characteristic size of 4 μm cover the interior of the (mother) vesicle as evidenced in (C, D and E). A schematic representation of a daughter vesicle connected to its mother is shown in F. Fig. 3 shows the shrinkage dynamics of a giant vesicle submitted to different osmotic stresses. This dynamic allows to determine the permeability P of the membrane as reported in Ref. [7]. We assume that during shrinkage, the *total* area of the vesicle membrane remains constant and equal to $4\pi R_0^2$ with R_0 the initial radius. If J is the molar flux of water through the membrane and $\Delta c(>0)$ the difference of molar concentrations across the vesicle membrane, P is defined by:

$$J = P\Delta c$$

The molar flux of water is given by:

$$J = -\frac{1}{4\pi R^2}\frac{\mathrm{d}(V(t)/v_\mathrm{m})}{\mathrm{d}t}$$

where $V(t)$ is the volume encapsulated by the vesicle and v_m the water molar volume; the water exchange occurs through the external surface of area $4\pi R^2$. $V(t)$ can be expressed as a function of R, r (the radius of the daughter vesicle assumed to be constant) and $N(t)$ the number of daughter vesicles: $V(t) = 4/3\pi R^3 - N(t)4/3\pi r^3$. We define the adimensional variables $\alpha = R/R_0$, $\beta = r/R$. The conservation of the membrane area then reads:

$$1/\alpha^2 = 1 + N(t)\beta^2$$

which leads to:

$$\frac{\mathrm{d}\alpha}{\mathrm{d}t}(1 + 2/3\beta) = -\frac{v_m P\Delta c}{R_0}$$

We have generally observed that $\beta = r/R \ll 1$ and we thus obtain (for short times):

$$\alpha = 1 - \left(\frac{v_\mathrm{m} P\Delta c}{R_0}\right)t$$

This initial linear behaviour is well observed (full lines in Fig. 3) and yields an average value for P equal to $(1.0 \pm 1)\ 10^{-3}$ cm s^{-1} for the EPC/dicetyl-phosphate/cholesterol system[2] in reasonable agreement with the values reported in Ref. [7] (4.1×10^{-3} cm s^{-1}) or in Ref. [10] (3.7×10^{-3} cm s^{-1}) for EPC systems. This value also suggests that the water transport occurs at a molecular scale [11] and does not involve the formation of macroscopic pores yielding much larger values (10^4 m s^{-1}) [12]. We have assumed in our derivation of the permeability P that the vesicle is in contact with the hypertonic solution (with $c = c_0 + \Delta c$) right from the beginning of the shrinkage process which is certainly not the case because of the presence of the *Anopore* membrane; this might explain why our permeability values are smaller than previous determinations [7,10], and also the smaller value obtained for $\Delta c = 10$ mM.

The final radius $R(t \to \infty)$ of the vesicle can be predicted from geometrical arguments. The adjustment of the internal concentration to c imposes:

$$1/\alpha^3 = \epsilon(1 - N\beta^3)$$

with $\epsilon = c/c_0 = (c_0 + \Delta c)/c_0$. Using the conservation of the membrane area, we obtain:

$$\begin{cases} \beta = \dfrac{\epsilon\alpha^3 - 1}{\epsilon\alpha(1 - \alpha^2)} \\ N = \dfrac{\epsilon^2(1 - \alpha^2)^3}{(\epsilon\alpha^3 - 1)^2} \end{cases}$$

N diverges for $\alpha = \epsilon^{-1/3}$, i.e., $\alpha = 0.55$ for $\epsilon = 6$, $\alpha = 0.79$ for $\epsilon = 2$, $\alpha = 0.87$ for $\epsilon = 3/2$ or $\alpha = 0.94$ for $\epsilon = 6/5$, in agreement with experiments (Fig. 3). In fact, N could be limited by the compact piling of the daughter vesicles on a sphere of radius $R - r$; when $\beta \ll 1$, N should then be equal to $2\pi/\sqrt{3}\left(\frac{1-\beta}{\beta}\right)^2$. This yields a prediction for α and β that is not obeyed by experiments: it is difficult from our microscopy observations to check whether we do have a compact layer of daughter vesicles and we have evidence that daughter vesicles also form in the very interior of the mother vesicle after the initial wrinkling of the membrane. The value of measured radius r of the daughter vesicles has to be given another non-geometrical origin. Boroske et

[2] We found $P \simeq 0.5 \times 10^{-3}$ cm s^{-1} for $\Delta c = 10$ mM.

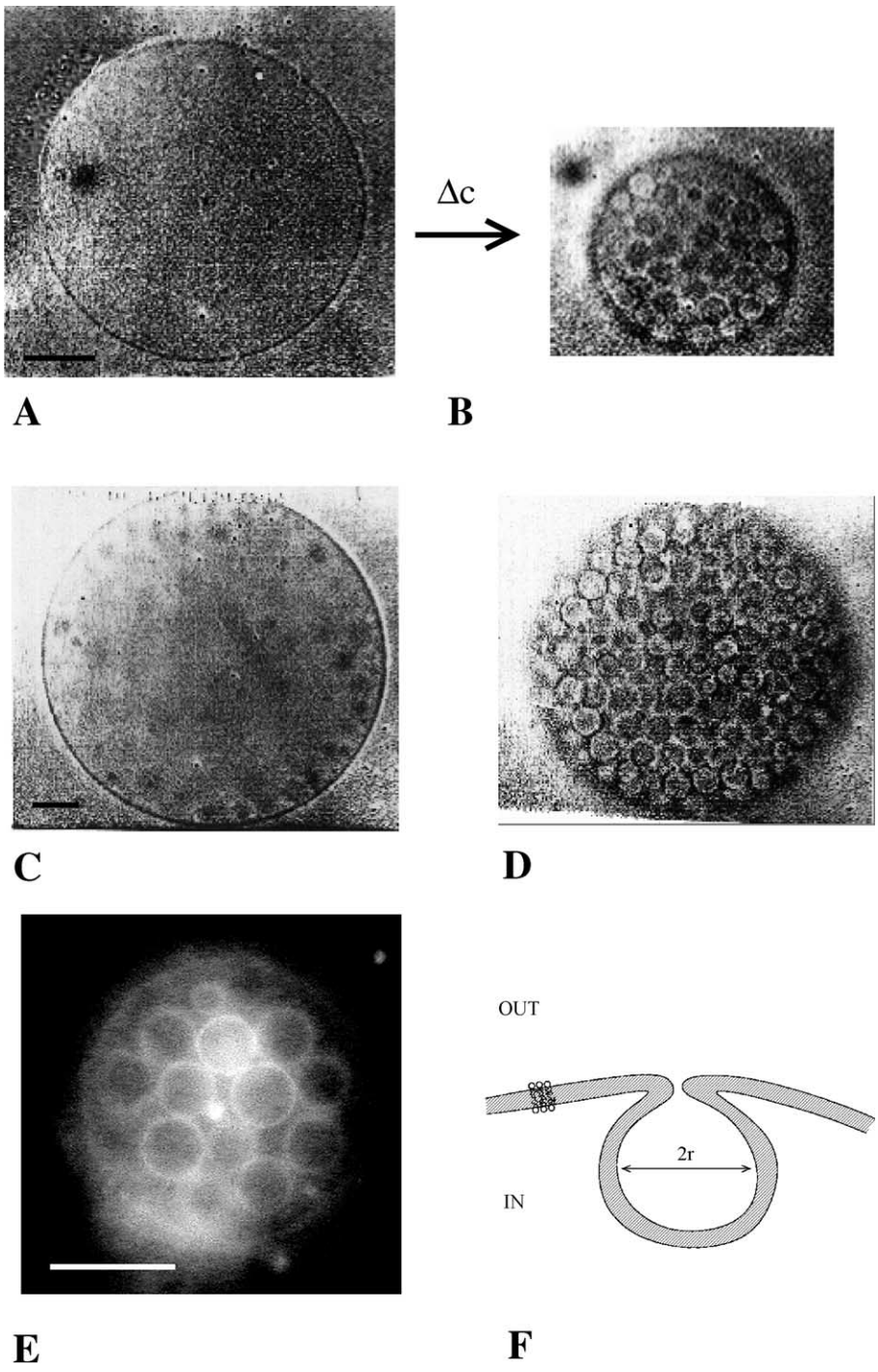

Fig. 2. (A) A giant unilamellar vesicle in isotonic solution; (B) same vesicle as in A, 15 min after a controlled osmotic shock of glucose with $\Delta c = 250$ mM. (C) Shrunk vesicle, focus plane at the equatorial plane of the vesicle; (D) same vesicle as in C, focus plane at the apex. (E) Micro-photograph of a shrunk vesicle where the bilayer has been dyed (1% w/w) with a fluorescent probe (β-BODIPY® 530/550 C_{12}-HPE, Molecular Probes), the daughter vesicles appear clearly *inside* the mother vesicle; (F) sketch of a daughter vesicle (of radius *r*) inside its mother (of radius *R*). A few phospholipid molecules have been represented. All scale bars are 10 μm. Vesicles made from the EPC/dicetyl phosphate/cholesterol mixture.

al. [7] proposed that the shrinkage of the vesicle induces a non-vanishing spontaneous curvature. This homogeneous spontaneous curvature gives different surface tensions for the mother and the daughter vesicles. Assuming that the Laplace pressures of the mother and the daughter vesicles are identical (mechanical equilibrium), an estimation of the surface tensions of the membrane could be given [7]. This approach is perfectly sound when the daughters are connected by a filament to the mother vesicle and *outside* the mother vesicle then leading to budding and pearling

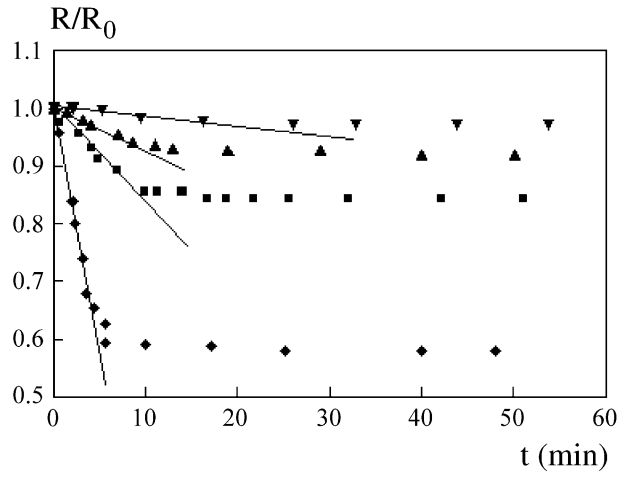

Fig. 3. Shrinkage dynamics of giant vesicles submitted to different osmotic stresses, R_0 is the initial radius of the vesicle at $t=0$, R its radius at time t. ▼, $\Delta c=10$ nM; ▲, $\Delta c=25$ mM; ■, $\Delta c=50$ mM; ◆, $\Delta c=250$ mM.

phenomena [13–15]. In our observations, we have always found that the daughter vesicles form *inside* the mother. The mechanical equilibrium implies that the spontaneous curvature is *non-uniform* over the membrane as pointed out by Rauch and Farge [16] in a recent work where a *local* endocytosis of membrane is reported. In the following, we give some simple scaling arguments to estimate the size of the daughter vesicles.

The onset of a buckling phenomenon has been studied experimentally for rigid vesicles (containing micro-tubules) by Elbaum et al. [17] or theoretically by Moldovan and Golubovic [18]. This buckling phenomenon is reminiscent of the stress of aqueous gels by osmotic pressure changes that gives well-defined shrunk structures [19]. The shrinkage of giant vesicles through the polymerization of their membrane has also lead to membrane-wrinkled shapes [20]. Initially, the vesicle is a flask object which is characterized by its rigidity constant κ_c (with dimension of energy); it is then submitted to an external stress $\Delta c\ k_BT$ assuming a van't Hoff ideal expression for the osmotic pressure. This gives a scale λ for the deformation of the stressed vesicle:

$$\lambda = \left(\frac{\kappa_c}{\Delta c\ k_BT}\right)^{1/3} \quad (1)$$

This would give $\lambda=4$ nm for instance for $\Delta c=250$ mM, assuming $\kappa_c=10\ k_BT$, clearly off of the observation of Fig. 2. We can still argue that the excess of glucose *progressively* increases from 0 to Δc (and smoothly, because of the presence of the *Anopore* membrane) and that there exists a threshold in concentration leading to the buckling instability (the threshold can be estimated: for $r=4$ μm, we get a critical Δc equal to 2.5×10^{-7} mM, a very low value indeed). A *natural* buckling length (that does not depend on the osmotic pressure excess) can be introduced for a stretched bilayer from the rigidity constant κ_c and the surface tension Σ_c by:

$$\lambda = \left(\frac{\kappa_c}{\Sigma_c}\right)^{1/2} \quad (2)$$

Σ_c is the tension of the membrane needed for the nucleation of pores and the permeation of water. The membrane tension values of flask liposomes are in the range of $0–3\times10^{-5}$ mN m^{-1} [21] and it has been shown in Ref. [12] that a tension of 10^{-3} mN m^{-1} induced by adsorption leads to the creation of macroscopic pores. We can then reasonably estimate that $\Sigma_c\simeq10^{-4}$ mN m^{-1}. Taking $\kappa_c=10\ k_BT$, this gives $\lambda\simeq1$ μm, to be compared with the average observed value (4 μm).

In fact, as suggested previously, we have experimental evidence that the observed wrinkling is associated with a micro-segregation of the components of the vesicle mem-

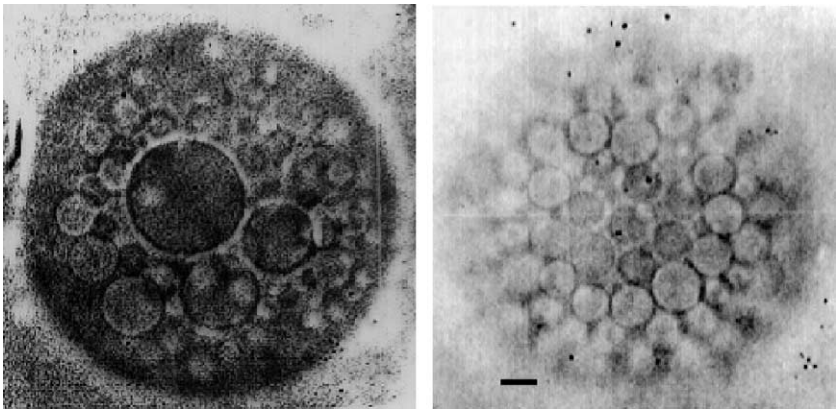

Fig. 4. Shrunk vesicles. Left: pure EPC membrane, right: EPC + cholesterol membrane. The scale bar represents 4 μm.

brane. We have observed spontaneous rearrangements of the distribution of the daughter vesicles that could be driven by a *heterogeneous* distribution of the surface-active molecules of the membrane. The size distribution of the daughter vesicles also seems to depend on the membrane composition as shown in Fig. 4 (note that EPC membranes cannot be considered as one-component membranes since EPC is a mixture of lipid components among which some are negatively charged [22]). It is interesting to notice that the addition of cholesterol leads to a decrease of the average size of daughter vesicles, likely because it increases the membrane tension, reinforcing then the validity of Eq. (2). Finally we have found that pure DPPC vesicles do not exhibit the raspberry-mode shrinkage.

In conclusion, we have shown that a *gentle* osmotic shock on giant vesicles induces a peculiar folding: the presence of a small-pore membrane in our experimental set-up suppresses convection that usually leads to the rupture of the vesicles; the over-concentration of glucose then solely reaches the vesicle bilayers by diffusion and gives time to vesicles for a novel and original rearrangement of their membranes. The final size of the vesicle can be simply obtained from the strength of the osmotic (controlled) shock and we have given some scaling arguments for the prediction of the size of the daughter vesicles; our experiments also suggest that the distribution of molecules in the bilayer is affected by the additional osmotic pressure.

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References

[1] W. Helfrich, Z. Naturforsch., C 28 (1973) 693–703.
[2] E. Evans, W. Rawicz, Phys. Rev. Lett. 17 (1990) 2094–2097.
[3] R. Lipowsky, Curr. Opin. Struct. Biol. 5 (1995) 531–540.
[4] K. Berndl, J. Käs, R. Lipowsky, E. Sackmann, U. Seifert, Europhys. Lett. 13 (1990) 659–664.
[5] R. Lipowsky, Nature 349 (1991) 475–481.
[6] C. Taupin, M. Dvolaitzky, C. Sauterey, Biochemistry 14 (1975) 4771–4775.
[7] E. Boroske, M. Elwenspoek, W. Helfrich, Biophys. J. 34 (1981) 95–109.
[8] M. Kummrow, W. Helfrich, Chem. Phys. Lipids 79 (1996) 147–156.
[9] M. Angelova, D. Dimitrov, Mol. Cryst. Liq. Cryst. 152 (1987) 89–104.
[10] R. Fettiplace, Biochim. Biophys. Acta 513 (1978) 1–10.
[11] E. Disalvo, A. Campos, E. Abuin, E. Lissi, Chem. Phys. Lipids 84 (1996) 35–45.
[12] A.-L. Bernard, M.-A. Guedeau-Boudeville, L. Jullien, J.-M. di Meglio, Langmuir 16 (2000) 6809–6820.
[13] L. Miao, B. Fourcade, M. Rao, M. Wortis, R. Zia, Phys. Rev., A 43 (1991) 6843–6856.
[14] R. Bruinsma, J. Phys., II (France) 1 (1991) 995–1012.
[15] R. Bar-Ziv, T. Tlusty, E. Moses, S. Safran, A. Bershadsky, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 10140–10145.
[16] C. Rauch, E. Farge, Biophys. J. 78 (2000) 3036–3047.
[17] M. Elbaum, D. Fygenson, A. Libchaber, Phys. Rev. Lett. 76 (1996) 4078–4081.
[18] D. Moldovan, L. Golubovic, Phys. Rev. Lett. 82 (1999) 2884–2887.
[19] E. Matsuo, T. Tanaka, Nature 358 (1992) 482–485.
[20] M. Dvolaitzky, M.-A. Guedeau-Boudeville, L. Léger, Langmuir 8 (1992) 2595–2597.
[21] P. Méléard, PhD, Université Bordeaux I, 1990.
[22] F. Pincet, S. Cribier, E. Perez, Eur. Phys. J., B 311 (1999) 127–130.