Negative Tension Induced by Lipid Uptake

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Membrane fusion is an important process in cell biology. While the molecular mechanisms of fusion are actively studied at a very local scale, the consequences of fusion at a larger scale on the shape and stability of the membrane are still not explored. In this Letter, the evolution of the membrane tension during the fusion of positive small unilamellar vesicles with a negative giant unilamellar vesicle has been experimentally investigated and compared to an existing theoretical model. The tension has been deduced using videomicroscopy from the measurement of the fluctuation spectrum and of the time correlation function of the fluctuations. We show that fusion induces a strong decrease in the effective tension of the membrane which eventually reaches negative values. Under these conditions, we show that localized instabilities appear on the vesicle. The membrane finally collapses, forming dense lipid structures.

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The physical properties of membranes at equilibrium are now well understood [1]. Yet, a generic understanding of nonequilibrium membranes would be very useful to allow us to discriminate between specific and nonspecific behavior in biology. Using giant liposomes containing ion pumps, physicists recently succeeded in investigating theoretically and experimentally the consequences of nonequilibrium protein activity on the membrane fluctuations [2]. Another class of nonequilibrium membranes has also been considered: membranes exchanging surface with a surrounding medium. In particular, the consequences on the deformation and stability following the addition (or the removal) of a large number of lipids have been recently investigated theoretically [3,4]. These papers predict that the surface tension sign change, induced by fusion events, may lead to an instability similar to the buckling instability of Langmuir films [5]. The goal of this Letter is to study experimentally the consequences of nonequilibrium membrane fluxes on a fluid model membrane in the large scale limit.

We study the consequences of lipid uptake, through fusion processes, on membrane stability and morphology. We have used a simple system consisting of a giant unilamellar vesicle (GUV) interacting with sonicated small unilamellar vesicles (SUVs) through electrostatic interactions. The SUVs have a size similar to cellular transport intermediates (i.e., typically from 50 to 100 nm in diameter [6]) and are positively charged with cationic 1,2-dioleoyl-3-trimethylammonium-propane (10% w/w) mixed with neutral 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC). The negatively charged GUV contains 10% 1,2-dioleoyl-*sn*-glycero-3-[phospho-*L*-serine] and 90% DOPC. Using the classical electroformation technique [7], GUVs are generated with a size ranging between 10 and 50 μm in diameter. A single GUV is injected with a micropipette in a chamber containing about 10^{11} SUVs/ml in a 170 mM sucrose solution, and the evolution of its shape is immediately recorded using phase contrast microscopy. The interesting aspect of electrostatic interaction is that it can be tuned by changing the surface charge density, more easily than specific protein interactions. In our experiments, we have chosen to keep the charge density of the GUVs constant and to vary that of the SUVs. Similarly to what was previously shown by Lei and MacDonald [8] for two GUVs of opposite charges, at low charge density, the electrostatic interaction is not strong enough to fuse the vesicles and the SUVs adhere on the surface of the GUV, whereas at high charge density, the interaction between membranes is strong enough to induce fusion. This fusion has also been theoretically predicted [9], and the conditions under which two oppositely charged membranes show a dynamic, attractive instability facilitating fusion were calculated. In Ref. [4], we predict the dominant instability modes and time scales and show how these are controlled by the relative charge and membrane viscosities. These dynamic instabilities may be the precursors of membrane fusion in systems where artificial vesicles are engulfed by biological cells of opposite charge.

In our system, an efficient and important fusion occurs when the charge density of SUVs is larger than 7%. We observe then that the GUV becomes more and more flaccid in a few seconds typically after injection and that the amplitudes of the fluctuations rapidly increase (see Fig. 1 and movie 1 in Ref. [10]); the shape of the vesicle is not spherical anymore but strongly deformed [Fig. 1(c)]. Moreover, some vesicles connected to the membrane and a few instabilities with thin tether shapes grow on the GUV, as expected from Refs. [3,4]. A further transition is observed after these spectacular membrane deformations: The vesicle contracts and recovers its spherical shape, while dark aggregates form on the surface of the membrane [Fig. 1(d)]. Similar lipid aggregates have also been ob-

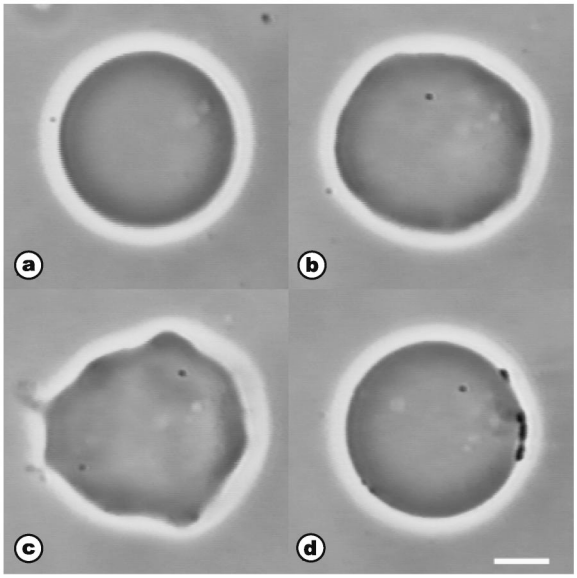

FIG. 1. Transformation of the shape of a negatively charged giant vesicle in contact with positive small vesicles. (a) At $t = 0$, the vesicle is optically tense. (b) At $t = 10$ s, the amplitude of the fluctuations increases rapidly, and the vesicle is strongly deformed. (c) At $t = 15$ s, thin tubular instabilities grow around the vesicle. (d) At $t = 45$ s, the vesicle recovers its initial spherical shape and tension but with dense lipid aggregates (catons) on its surface (bar, 10 μm).

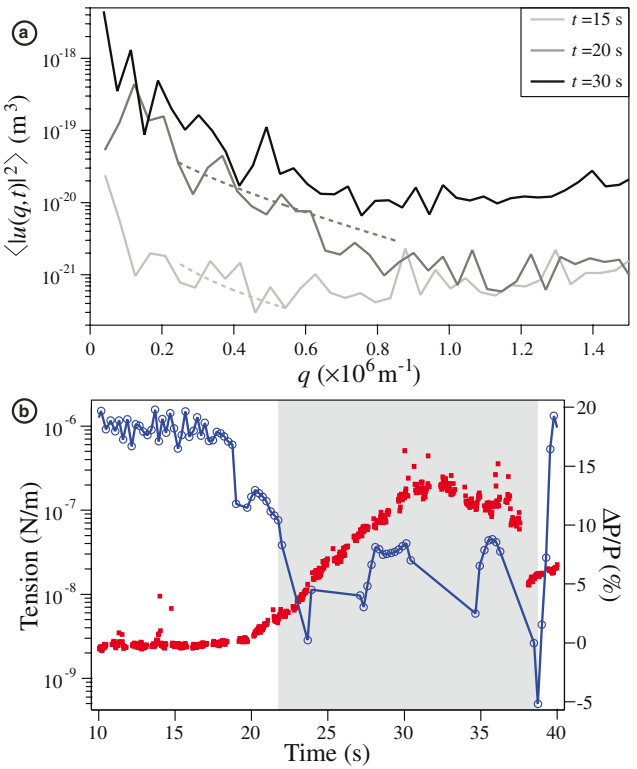

FIG. 2 (color online). (a) Evolution of the fluctuation spectrum of a vesicle during the fusion. The effective tension was fitted (dotted line) using a procedure described in our auxiliary material [10] with $\kappa = 1.1 \times 10^{-19}$ J: $\sigma^e = 1.6 \pm 0.2 \times 10^{-6}$ N/m and $1.4 \pm 0.2 \times 10^{-7}$ N/m for $t = 15$ and 20 s, respectively. For $t = 30$ s, σ^e was too low to be accurately fitted. (b) Variation of the membrane tension (○) and of the perimeter (■) as a function of time. After 22 s, the tension measurement is no longer valid because of the lack of sensitivity of the fit (gray zone).

served when a giant vesicle is submitted to a strong osmotic shock or to heating [11] or after fusion of GUVs [8,12].

The details of the GUV shape transformations depend strongly on the charge density and on the SUV concentration (see Tables I and II in Ref. [10]). The above sequence is fully consistent with the existence of fusion events with the GUV and cannot be explained if the SUVs only adhere on its membrane. Classical tests for fusion, such as dynamic light scattering, fluorescence microscopy, and fluorescence resonance energy transfer, are inadequate in the present situation, because of the size difference between the fusing objects and/or because of the relative rapidity of vesicle fusions. However, the size variation of the GUV can be measured using a real-time contour analysis technique developed in our lab [13]. A rapid variation of the equatorial perimeter, from a few percent up to 40% typically within 10 s, can be detected, confirming the size increase of the vesicle [Fig. 2(b)]. As the vesicle loses its spherical shape, the fusion rate cannot be simply deduced from the perimeter variation. In the late stage of the sequence, the vesicle recovers its initial quasispherical form with dense lipid aggregates present on its surface; in the case of Fig. 2, the perimeter is a few percent larger than the initial value. This is an additional proof that lipids have been incorporated into the GUV membrane. Note that the relative surface increase $\delta S/S \simeq Nr^2/R^2$ (where N is the total number of fused SUVs, r the SUV radius, and R the GUV radius) over the relative volume increase $\delta V/V \simeq Nr^3/R^3$ is equal to $R/r \simeq 10^3$. As a result, the area increases much faster than the volume, which necessarily leads to a tension decrease together with fluctuation amplification.

The contours of the vesicle can be represented in polar coordinates (r, θ) as $r(\theta) = R(1 + \sum_{n=1}^{\infty} a_n \cos(n\theta) + b_n \sin(n\theta))$. The amplitude corresponding to the fluctuation mode number n is given by $c_n = \sqrt{a_n^2 + b_n^2}$. The fluctuation spectrum $\langle |u(n, t)|^2 \rangle$ at a given time t can then be deduced following the expression [13]:

$$\langle |u(n, t)|^2 \rangle = \langle c_n^2(t) \rangle - \langle c_n(t) \rangle^2. \quad (1)$$

Figure 2(a) shows the fluctuation spectra of a GUV at different times during the fusion process. These data represent an example of a single vesicle, but the reproducibility of the process has been tested on tens of vesicles. We observe on all of them an amplification of the membrane fluctuations by 1–2 orders of magnitude. For a vesicle fluctuating at equilibrium, the fluctuation spectrum is directly related to the tension σ and to the bending modulus κ of the membrane [14]. In our case, the vesicle is no longer at equilibrium, but Girard *et al.* [4] have shown that,

in a stationary state, the fluctuation spectrum of a membrane exchanging lipids with the bulk by a fusion process remains similar to the usual equilibrium thermal correlator with an effective tension defined by $\sigma^e = \sigma - f(\{p\})$. $f(\{p\})$ is a function dependent on the parameters $\{p\}$ which contain the molecular details and the dynamics of the fusion events, such as the generalized force acting on the membrane to add lipids into the membrane. In our experiments, considering that on a short time scale (during 1 s) the vesicle is in a quasisteady state, an effective membrane tension σ^e can be deduced from the fluctuation spectrum:

$$\langle |u(q)|^2 \rangle = \frac{kT}{\sigma^e q^2 + \kappa q^4}, \tag{2}$$

with $q = n/R$, with a procedure described in details in Ref. [13] and recalled in our auxiliary material [10]. The use of thermal energy as the main noise source may look surprising but is justified in our auxiliary material. In our experiments, the fusion events add less than 1% of positive charges and do not strongly modify the bending modulus of the GUV membrane [15]. Under these conditions, Eq. (2) allows us to measure the evolution of the effective tension of the membrane during the process. The fluctuation spectra $\langle |u(q, t)|^2 \rangle$ are averaged over 1 s. Equation (2) for $\langle |u(q)|^2 \rangle$ has been corrected for limitations due to the integration time of the camera [13] and can be fitted using σ^e as a single free parameter. The following sequence is then observed: The tension remains constant at about 10^{-6} N/m over typically 15–20 s, and then a strong decrease of 2 orders of magnitude of the tension is measured in typically 5 s. Simultaneously, we observe a rapid increase of the perimeter [Fig. 2(b)]. The switchlike behavior implies that fusion is probably a two-step process, the initial step being slow compared to the second one. Subsequently, a wave vector independent contribution to $\langle |u(q, t)|^2 \rangle$ shows up for $n \gtrsim 20$. It reveals the presence of nonspatially correlated structures of lateral size smaller than about $\pi R/40 \approx 1$ μm. This likely corresponds to the onset of tubelike structure formation. This interpretation is supported by the appearance of elongated structure at later stages [Fig. 1(c)]. When the tension is below 10^{-8} N/m, the tension can no longer be determined properly by the contour analysis [gray region in Fig. 2(b)]. However, it can be deduced from the correlation time of the fluctuations τ_m, which has a similar form in the nonequilibrium case as at equilibrium

$$\tau_m^{-1} = \frac{q}{4\eta}(\sigma^e + \kappa q^2), \tag{3}$$

where η is approximately equal to the fluid viscosity [4]. We measured the autocorrelation function $c_n(\tau, t)$ for each mode n over a 5 s sliding window, defined as: $c_n(\tau, t) = (\langle c_n(t) \times c_n(t+\tau) \rangle - \langle c_n(t) \rangle \langle c_n(t+\tau) \rangle)/\langle c_n(t)^2 \rangle$. The window is shifted by 33 ms, corresponding to the acquisition frequency of our camera. The operation is repeated in order to measure the variation of the fluctuation correlation time during the fusion process. In particular, we have measured the correlation time for mode 10 during fusion [Fig. 3(b)]; if σ^e was positive, this mode should be too fast to be detected [Fig. 3(a)]. Indeed, at equilibrium, this time would be of the order of 100 ms or shorter with our typical experimental tensions, which is also of the order of the integration time of our camera. At the beginning of the process, in agreement with this remark, the correlation time is very short and smaller than 100 ms. It increases dramatically in 25 s concomitantly with the vesicle area increase. It reaches more than 1 s, 1 order of magnitude larger than the longest possible correlation time at equilibrium. Similar correlation times have been measured for lower modes (see Fig. S1 in Ref. [10]); they are probably underestimated due to the limited size of the sliding window. According to Eq. (3), such long correlation times are possible only if the effective tension of the membrane is negative. In fact, if the fusion activity $f(\{p\})$ is positive and strong enough, the effective tension can decrease during the fusion and negative values of the effective tension can be expected [4]. In case of the mode $n = 10$, a correlation time of 1 s corresponds to a negative effective tension $\sigma^e \sim -10^{-8}$ N/m. When the effective tension becomes negative, the membrane is no longer stable, and a mode of wavelength $\lambda \sim \pi\sqrt{3\kappa/|\sigma|}/2 \sim 0.5$ μm is expected to grow [4]. This instability is followed by the appearance of dense lipid aggregates on the membrane [Fig. 1(d)] and a restoration of the tense spherical shape. We interpret this last transition as a collapse of the bilayer. On a Langmuir monolayer, collapse or buckling occurs at a high compression rate, and multilayer aggregates are formed on top of the monolayer [5]. As a test, we have injected the same

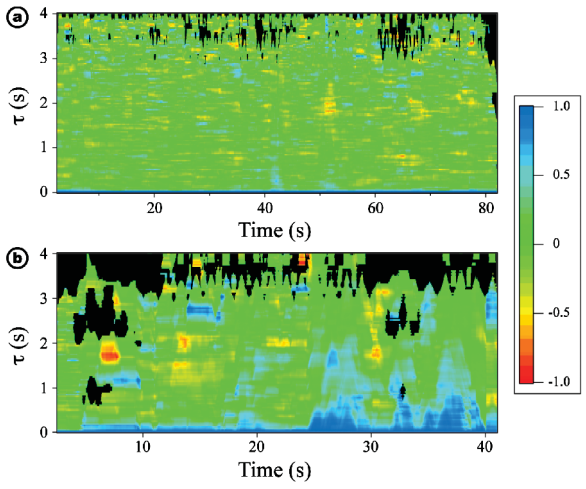

FIG. 3 (color). Evolution of the time autocorrelation functions of the fluctuations $c_n(\tau, t)$ (in color levels) for $n = 10$ with τ the correlation time. (a) For a passive fluctuating vesicle (with $\sigma = 1.8 \times 10^{-7}$ N/m). (b) During the fusion process (for the same vesicle as Fig. 2). For (a), the time correlation is below the resolution of the experiment. For (b), time correlations of about 1 s (blue level) are measured. The black points correspond to nonmeasured points because of low statistics. (See [10]).

SUVs under a dense fluorescent monolayer (23 mN/m) with a composition similar to the GUV membrane: The surface pressure first increases by about 2–3 mN/m and then decreases slowly by 5 mN/m over 15 min. Highly fluorescent aggregates appear during this surface pressure decrease (see Fig. S2 in Ref. [10]). Similarly, we suggest that a more stable state of the vesicle is obtained if the bilayer collapses forming multilamellar structures, additionally stabilized by electrostatic interactions. The formation of these multilamellar structures (that we name ''caton'') can be understood as a nucleation process. The energy E of a lipid island of radius r growing on a bilayer is

$$E = 2\pi\gamma r - \pi r^2(-\sigma_{cc} + p_{2D}), \tag{4}$$

where the first term is the energy cost to create a caton with a line tension γ, and the second term is the energy gained in creating a multilayer structure. σ_{cc} is a layer-layer interfacial tension and p_{2D} corresponds to the pressure release in the membrane resulting from the decrease in the number of lipids. Note that σ_{cc} is probably small because no caton is observed at equilibrium. During caton growth, this energy increases up to a critical radius $r_c = \gamma/p_{2D}$ defining an energetic nucleation barrier $W_b = 2\pi\gamma^2/p_{2D}$ and then decreases. The pressure release is given by $p_{2D} \sim \chi\delta a/a^3$, where a is the area per molecule, δa its variation due to excess membrane, and χ the membrane compressibility [4]. Estimating $\chi \simeq kTa$ and $\gamma = kT/\sqrt{a}$, where kT is the thermal energy, we get, assuming a Kramer's nucleation rate $\nu = \nu_0 e^{-W_b/kT}$,

$$\frac{\delta a}{a} = \ln\left(\frac{\nu_0}{\nu}\right), \tag{5}$$

where ν is the experimental nucleation rate and ν_0 the attempt rate. ν_0 is a molecular vibration frequency per unit area on the order of 10^{30} s^{-1} m^{-2}; ν is experimentally found on the order of 10^9 s^{-1} m^{-2}. We obtain thus $\delta a/a \simeq 2\%$, which is reasonable. Note that the exact values of ν_0 and ν are not very important, as the dependence is logarithmic. This model shows that a few percent of excess area $\delta a/a$ can induce the caton formation as seen in our experiments.

Although the origin of the fusion is not biological in our experiments, the consequences are general: A high flux of lipids is able to create instabilities and to strongly destabilize a membrane. To our knowledge, it is the first time that such nonequilibrium shape instabilities have been observed and analyzed. They contrast with the shape transformations observed at equilibrium [16]. Some elongated shape may also be observed when a force is applied to a membrane [17] or when the curvature is modified by polymers [18], but they correspond to stable shapes. We also have shown that these fusion events induce a substantial decrease of an effective tension σ^e down to negative values. As a result of the bilayer collapse, a positive membrane tension is eventually restored. In a cell, the flux of lipids is regulated and lipids are often simultaneously removed from the membrane by the ejection of small vesicles or tubules associated with exo- and endocytosis machineries. Tubular structures are observed in some internal cellular compartments, such as sorting endosomes [19] or multivesicular bodies [20], where intense lipid exchange takes place. In general, these tubes are pulled by molecular motors. We have shown the existence of a membrane tension threshold above which the tube formation is inhibited [21]. An interesting hypothesis would be that the addition of lipids following the fusion of vesicles reduces membrane tension enough to allow for the extraction of membrane tubes by motors and, thus, trigger internal membrane transport. Membrane tension, modulated by fusion events, could be an internal switch for intracellular trafficking.

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[1] *Structure and Dynamics of Membranes*, edited by R. Lipowsky and E. Sackmann (North-Holland, Amsterdam, 1995).
[2] S. Ramaswamy and M. Rao, C.R. Acad. Sci., Ser. IV **2**, 817 (2001); P. Girard, J. Prost, and P. Bassereau, Phys. Rev. Lett. **94**, 088102 (2005), and references therein.
[3] M. Rao and R. Sarasij, Phys. Rev. Lett. **87**, 128101 (2001).
[4] P. Girard, F. Jülicher, and J. Prost, Eur. Phys. J. E **14**, 387 (2004). In principle, a term proportional to q^3 should be included in the denominator of Eq. (2) and, similarly, a q^2 term in Eq. (3). In a quasisteady state, and in the conditions defined in this reference, this term is probably small and is not needed in our analysis.
[5] W. Lu *et al.*, Phys. Rev. Lett. **89**, 146107 (2002).
[6] T. Kirchausen, Nat. Rev. Mol. Cell Biol. **1**, 187 (2000).
[7] M. Angelova and D. Dimitrov, Faraday Discuss. Chem. Soc. **81**, 303 (1986).
[8] G. Lei and R. C. MacDonald, Biophys. J. **85**, 1585 (2003).
[9] G. Hed and S. Safran, Phys. Rev. Lett. **93**, 138101 (2004).
[10] See EPAPS Document No. E-PRLTAO-97-066635 for supplementary figures and movies. For more information on EPAPS, see http://www.aip.org/pubservs/epaps.html.
[11] W. Häckl, U. Seifert, and E. Sackmann, J. Phys. II (France) **7**, 1141 (1997).
[12] J. Heuvingh, F. Pincet, and S. Cribier, Eur. Phys. J. E **14**, 269 (2004).
[13] J. Pécréaux *et al.*, Eur. Phys. J. E **13**, 277 (2004).
[14] W. Helfrich, Z. Naturforsch. **28C**, 693 (1973).
[15] Y. W. Kim and W. Sung, Europhys. Lett. **58**, 147 (2002).
[16] H.-G. Döbereiner *et al.*, Phys. Rev. E **55**, 4458 (1997).
[17] I. Derényi, F. Jülicher, and J. Prost, Phys. Rev. Lett. **88**, 238101 (2002).
[18] I. Tsafrir *et al.*, Phys. Rev. Lett. **91**, 138102 (2003).
[19] S. Mukherjee, R. N. Gosh, and F. R. Maxfield, Physiol. Rev. **77**, 759 (1997).
[20] M. Kleijmeer *et al.*, J. Cell Biol. **155**, 53 (2001).
[21] C. Leduc *et al.*, Proc. Natl. Acad. Sci. U.S.A. **101**, 17 096 (2004).