Membrane tension lowering induced by protein activity

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We present measurements of the fluctuation spectrum of giant vesicles containing bacteriorhodopsin (BR) pumps using video-microscopy. When the pumps are activated, we observe a significant increase of the fluctuations in the low wavevector region, which we interpret as due to a lowering of the effective tension of the membrane.

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Membranes are self-assembled bilayers of surfactants or phospholipids, which form flexible surfaces. The mechanical properties of membranes are essentially controlled by parameters, such as the membrane tension, bending modulus and spontaneous curvature. These parameters completely characterize the membrane fluctuation spectrum at thermal equilibrium, but they are not sufficient to characterize real biological membranes, such as cell plasma membranes, which are non-equilibrium systems. Biological membranes are in general active in the sense that they are constantly maintained out of equilibrium either by active proteins inside the membrane that are often ATP-consuming enzymes or by an energy flow, for example a lipid flux. Due to the complexity of active biological systems in vitro, recent studies have focused on in vivo biomimetic systems. An example of an active system is a giant unilamellar vesicle (GUV) which is rendered active by the inclusion of light-activated bacteriorhodopsin (BR) pumps. The bacteriorhodopsin pumps transfer protons unidirectionally across the membrane as a consequence of their conformational changes, when they are excited by light of a specific wavelength. Experiments on giant unilamellar vesicles containing bacteriorhodopsin pumps or Ca2+ ATPase pumps have shown that the non-equilibrium forces arising from ion pumps embedded in the membrane are able to significantly enhance the membrane fluctuations. In these experiments, the membrane tension has been fixed by means of micropipet aspiration and the corresponding excess area measured. The slope of such a curve defines an effective temperature, measuring the enhancement of the membrane fluctuations due to activity. The amplification of the fluctuations due to activity has been originally predicted in Ref. 8.

In this Letter, we report the first experimental measurements of an active fluctuation spectrum of GUVs containing bacteriorhodopsin pumps using video microscopy. The details of the technique and of the analysis are given in Ref. 9. These measurements are complementary to previous experiments using micropipets since they probe a range of wavevectors not accessible otherwise. Micropipet experiments provide information on the active fluctuations integrated over all wave vectors which require an independent calibration of the membrane tension. On the contrary, video microscopy gives a direct measurement of the fluctuation spectrum from which the membrane tension can be extracted. In this paper, we analyze theoretically the active fluctuation spectrum using the model of Ref. 10, which is an extension of the hydrodynamic model of Ref. 5 to quasi-spherical vesicles taking into account the active noise of the pumps and localized active forces.

Let us first discuss the passive behavior of giant vesicles. For the sake of simplicity we use notations appropriate to quasi-planar membranes. We consider a quasi-planar membrane surface in the Monge gauge, in which it is defined by its height u(x) at position x. Two density fields are defined on the surface, ψ↑ and ψ↓, corresponding to the surface densities of proteins oriented with the two possible orientations. The membrane free energy is a function of the height field u(x) and of the imbalance of protein densities ψ(x) = ψ↑(x) − ψ↓(x). We expand the free energy to quadratic order in these variables:

\[ F[u, \psi] = \frac{1}{2} \int d^2x [\kappa (\nabla^2 u)^2 + \sigma (\nabla u)^2 + \chi \psi^2 - 2\Xi \psi \nabla^2 u], \]

where \( \kappa \) is the bending modulus, \( \sigma \) the surface tension, \( \chi \) a susceptibility coefficient and \( \Xi \) a coefficient characterizing the coupling between the membrane curvature and the average orientation of the proteins. In the following, we neglect the curvature induced coupling; this is justified for bacteriorhodopsin pumps since the incorporation of proteins does not lead to any measurable renormalization of the bending modulus. Note that for dilute proteins in the membrane, \( \chi = k_B T/n_0 \), where \( n_0 \) is the average density of proteins, of the order of \( 10^{16} \text{m}^{-2} \). In our experiments, only the vesicle contour at the equator corresponding to a slice of the vesicle in the plane \( y = 0 \) is recorded and is used to calculate the fluctuation spectrum: the measured quantity is

\[ \langle |u(q_x, y = 0)|^2 \rangle = \frac{1}{2\pi} \int_{-\infty}^{+\infty} \langle |u(q_x, q_y)|^2 \rangle dq_y, \]
The passive equilibrium value of this fluctuation spectrum is calculated using the equipartition theorem from Eqs. 12

\[ \langle |u(q_x, y = 0)|^2 \rangle = \frac{k_B T}{2\sigma} \left[ \frac{1}{q_x} - \frac{1}{\sqrt{\sigma^2 + q_x^2}} \right] = g(q_x, \sigma, \kappa). \] (3)

Fluctuation spectra are often analyzed in terms of power laws defined by \( g(q_x, \sigma, \kappa) \approx q_x^\nu \). Two regimes can be distinguished depending on the position of the wave vector \( q_x \) compared to the cross-over wavevector \( q_c = \sqrt{\sigma/\kappa} \). For \( q \ll q_c \), membrane tension dominates and \( \nu = -1 \), whereas for \( q \gg q_c \), bending elasticity dominates and \( \nu = -3 \). Our experiments lead to \( \nu \approx -2 \), in the cross-over region between these two regimes.

We now discuss active membrane fluctuations. In Ref. 5, a hydrodynamic theory was developed to calculate the non-equilibrium fluctuations of an active membrane containing ion pumps. This work has stimulated substantial theoretical interest, focused mainly on the general question of the proper description of non-equilibrium effects associated to protein conformation changes 11. More recent developments of a similar hydrodynamic approach have lead to a general theoretical description of active gels 12, 14, 15. In Ref. 10, the active force distribution is modeled as a superposition of dipoles located along the membrane normal \( n \) (not along \( z \) as in previous models), which generalizes Ref. 13. Experiments do not probe this force distribution directly but rather its first two moments. The first moment represents the active contribution to the membrane tension and is denoted by \( \sigma_{dip} \) and the second moment \( Q \) represents the modification of the membrane bending moments by the activity of the force dipoles 10. Both active contributions to the tension and to the membrane curvature are also present in Ref. 16 where the activity is due the the myosin molecular motors in the cortical layer bound to the cell membrane.

The fluctuation spectrum of an active membrane in a quasi-spherical geometry can be calculated using an expansion around a spherical shape with radius \( R_0 \) to first order in the deviations from the sphere using spherical harmonics, with \( R = R_0 + n R_0 \sum_l m u_{lm} Y_{lm} \). The fluctuation spectrum of a quasi-spherical vesicle is characterized by the amplitude of the spherical modes \( \langle |u_{lm}|^2 \rangle \) which are function of \( l \) only for symmetry reasons. In Ref. 9, the fluctuation spectrum of a quasi-spherical vesicle is compared to the corresponding spectrum for a planar membrane, and this comparison showed that for modes \( l > 5 \), the fluctuation spectrum of a quasi-spherical vesicle is very well approximated by that of a planar membrane at a wave vector \( q \), \( \langle |u(q)|^2 \rangle \); at a wavevector \( q = l/R_0 \), the two spectra are related by \( \langle |u(q)|^2 \rangle = R_0^4 \langle |u_{lm}|^2 \rangle \). For the sake of simplicity, we discuss in the following the effect of the activity on the fluctuation spectrum only at the level of planar membranes. We have also simplified the calculation of Ref. 10, by neglecting the shot-noise, which is the intrinsic noise of the pumps 8. This approximation is reasonable because the main effect of the shot-noise is to modify the fluctuation spectrum in the high wavevector region 10 which is not the region where we observe a large modification of the spectrum due to activity in our experiments. We denote here \( F_2 \) the constant entering in \( Q = F_2 \nu \nu \). This generalizes the notation of Ref. 3, where this quantity represented the quadrupole moment of the force dipole. With these three approximations, we obtain the following spectrum

\[ \langle |u(q_x, y)|^2 \rangle = \frac{k_B T}{\kappa q^4 + \sigma q^2} \left\{ 1 + \frac{F_2^2 \nu^2}{4\kappa^2} \frac{q^2}{\kappa q^4 + 4\eta D q + \sigma} \right\}, \] (4)

for \( D \) is the diffusion coefficient of the proteins which is of the order of \( 10^{-14} \text{m}^2 \text{s}^{-1} \) and \( \eta \) is the viscosity of water. Since the tension of the vesicles is at least of the order of \( 10^{-8} \text{N/m} \), we find that the term \( 4\eta D q \) can be neglected with respect to \( \sigma \) in the range of wavevectors relevant to the experiments 17. The effective tension \( \tilde{\sigma} \) is defined as \( \tilde{\sigma} = \sigma + \sigma_{dip} \), where \( \sigma \) is the passive contribution to the tension and \( \sigma_{dip} \) the active part due to BR proteins. After integration as in Eq. 3 we find

\[ \langle |u(q_x, y = 0)|^2 \rangle = g(q_x, \tilde{\sigma}, \kappa) + \frac{F_2^2 \nu^2}{16\kappa^2} \frac{1}{(q_x^2 + \frac{\sigma}{\kappa})^{3/2}}, \] (5)

in which both terms on the right hand side contain active contributions when \( \sigma_{dip} \) or \( F_2 \) do not vanish. Note that Eq. 5 is very similar to the active spectrum given Ref. 3, with the correspondence \( F_2 = 2 \nu P_a \) except for one important difference: the tension contains an active contribution but not the bending modulus whereas in Ref. 3 it is the opposite. Because of this difference the active spectrum of Ref. 3 does not fit our experiments (it would predict a large effect of activity at high wavevectors whereas the effect is observed here mainly at small wavevectors). Another consequence of this difference, is that the active spectrum of Eq. 5 cannot in general be described in terms of an active temperature even in the low wavevector limit 18.

Experiments have been performed with GUVs made of Egg Phosphatidyl-Choline at a molar fraction of 240 lipids per protein, using the BR reconstitution protocol developed in Ref. 19. We have used a PEG passivated substrate and checked that vesicles fluctuate without adhering 3. In Fig. 1 we show the measured fluctuation spectrum for the same GUV containing BR in active and passive states. To ensure reproducibility of the data, three consecutive passive and then, three active spectra were recorded. This figure confirms that activity leads to an enhancement of the fluctuations as expected from previous experimental studies 3, 6. It is important to point out that to observe this effect 1mM of azide must be present in the solution. In the absence of this compound no enhancement of the fluctuation spectrum was observed 20. This observation is consistent with several studies which suggest that azide enhances the proton transfer in BR 21. A large enhancement of the
active as compared to passive fluctuations is observed at low wavevectors, which we attribute to a lowering of the membrane tension due to the activity.

We first fit the passive spectrum using Eq. 3. This leads to \( \sigma = 3.9 \cdot 10^{-7} \pm 3 \cdot 10^{-8} \text{N/m} \) and an apparent bending modulus \( \kappa = 5.6 \cdot 10^{-19} \pm 4.4 \cdot 10^{-20} \text{J} \). The value of the bending modulus deduced from such a fit is overestimated. Several artifacts perturb the measurements at high wavevectors: the pixel noise due to the discrete detection of the images, the integration time of the camera, and the effect of gravity [2]. Taking into account these corrections at high \( q \) becomes very difficult in the active case, we thus have chosen a simple fitting procedure, in which the corrections due to the integration time and to the gravity are neglected for both passive and active spectra. This procedure is justified by the observation that both spectra are superimposed at high \( q \). The determination of the bending modulus \( \kappa \) is therefore delicate with our video microscopy technique while the measurement of the tension is reliable. With the value of the bending modulus kept fixed, we fit the three active spectra of Fig. 1 using Eq. 5. One fit which is shown in Fig. 2 leads to \( \hat{\sigma} = 5.3 \cdot 10^{-8} \text{N/m} \) and \( F_2 = 4 \cdot 10^{-28} \text{Jm} \). Thus the active contribution to the tension \( \sigma_{\text{dip}} = \hat{\sigma} - \sigma \) is negative and of the order of \(-3.3 \cdot 10^{-7} \text{N/m} \) for this particular vesicle (see table I for a summary of the results of the fits for the GUV of Fig. 1). The lowering of the tension is systematic in all our experiments. The fact that \( \sigma_{\text{dip}} \approx -3.3 \cdot 10^{-7} \text{N/m} \) is very different from the estimate of \( 1.6 \cdot 10^{-3} \text{N/m} \) based on Refs. 2, 10 suggests that the modeling of the force distribution of these references is not appropriate to our experiments. In table I, we have also shown the exponent \( \nu \), which is the apparent power law exponent of the spectrum. When the vesicle is active the exponent gets closer to \(-3 \), which is the expected value in the bending dominated regime. This is consistent with a lowering of the cross-over wavevector \( q_c = \sqrt{\sigma/\kappa} \), and thus with a lowering of the tension since in this model \( \kappa \) is not affected by activity. We have ignored any dependence of the protein activity on the local membrane curvature, an assumption justified by the observation that the main effect is a correction to the tension and not to the bending modulus. The value of the parameter \( F_2 \) deduced from our fit is of the same order of magnitude as that estimated in Ref. 2 based on micropipet experiments since the factor 2-3 increase in effective temperature measured in this reference leads to \( F_2 \approx 9 \cdot 10^{-28} \text{Jm} \). To summarize, Eq. 4 successfully describes both the micropipet and the fluctuation spectrum measurements.

We now discuss in more details the lowering of the membrane tension due to activity. We have checked that this effect is compatible with the constraints of constant surface and volume of the vesicle, which must be imposed if permeation is negligible [5]. To do so, we estimate the excess area \( \alpha = \int q^3 dq \langle |u(q)|^2 \rangle /4/\pi \) for passive and active vesicles based on the above fluctuation spectra, by taking the lower bound of integration to be \( q_{\min} = 2\pi / R_0 \), with \( R_0 = 10 \mu m \) the radius of the vesicles; this lower bound corresponds roughly to the lowest measured value in Fig. 1. In the accessible range of \( q \) values, the excess area density \( \alpha q^2 \langle |u(q)|^2 \rangle /4/\pi \) of the active vesicles is always larger than the excess area density of the passive vesicles. At higher \( q \) wavevectors, it should be the opposite; but the \( q \) range where this would happen may be outside the accessible range.

Another important question is the sign of the effect, which can not be fixed by symmetry arguments [5]. Indeed, both orientations of the force dipoles [5, 11], inwards or outwards with respect to the membrane surface, are possible. The present experiments suggest that here the force dipoles must have an inward orientation (corresponding to contractile forces [14]) to produce a lowering of the tension. Another possibility would be to consider lateral dipoles. Here, we propose that the lowering of the tension is a consequence of electrostatic effects. This interpretation is not incompatible with the fact that BR conformational changes also play an important role. We have checked experimentally that a pH gradient builds up in our GUVs when they are activated, which suggests

![FIG. 1: Fluctuation spectrum of a single GUV containing BR in a buffer with 1mM sodium azide. The red (empty) symbols correspond to three consecutive recorded passive spectra, and the green (filled) ones to three consecutive active spectra.](image1)

![FIG. 2: Fit of one active spectra of Fig. 1 using Eq. 5. The parameter apparent \( \kappa \) was fixed to \( 5.6 \cdot 10^{-19} \text{J} \) obtained by fitting the passive spectrum. This fit gives \( \hat{\sigma} = 5.3 \cdot 10^{-8} \text{N/m} \) and \( F_2 = 3.9 \cdot 10^{-28} \text{Jm} \).](image2)
that a significant voltage drop exists across the membrane. Furthermore in membranes containing BR, the activity of the BR can be suppressed by applying an external voltage drop across the membrane [22]. In view of these observations, we propose that Maxwell stresses created by ion transport lead to a renormalization of the tension as shown in a simple model developed by two of us [23]. In this model, the electrostatic correction to the tension is proportional to the square of the electric current going across the membrane. Maxwell stresses are large because of the large mismatch in dielectric constants at the membrane, and they tend to reduce the membrane area, thus producing a negative electrostatic contribution to the tension. A lowering of the membrane tension due to the application of normal electric fields very generally leads to instabilities of the membrane [24, 25].

To summarize, we have presented what are to our knowledge the first measurements of active fluctuation spectra of GUVs containing BR proteins using video microscopy. The experiments show that activity enhances the fluctuations. The effect is strong in the low wavevector region, which we interpret as a lowering of the tension due to activity. This effect could not have been detected in micropipet experiments which are done at constant membrane tension. A possible candidate for explaining the lowering of the tension would be that it is caused by Maxwell stresses due to the transport of ions across the membrane. Further experiments (using for instance patch-clamp techniques with the same GUV or other GUVs containing ion channels rather pumps) are required to confirm these suggestions.

\[ \sigma (x = 10^{-7} \text{ N/m}) F_2 (x = 10^{-28} \text{ J.m}) \nu \]

| Spectrum | $\sigma$ | $F_2$ | $\nu$ |
|----------|---------|-------|-------|
| passive  | 3.9 ± 0.3 | 0 (imposed) | −2.15 ± 0.04 |
| active 1 | 0.53 ± 0.2 | 3.9 ± 1.1 | −2.7 ± 0.04 |
| active 2 | 0.79 ± 0.06 | 9.6 ± 1.1 | −2.78 ± 0.06 |
| active 3 | 0.35 ± 0.1 | 7.2 ± 0.6 | −2.87 ± 0.07 |

TABLE I: Fitting parameters using the fluctuation spectrum of Eq. 5. The passive spectrum is fitted with the condition $F_2 = 0$. Then three consecutive active spectra are fitted with the same vesicle using the value of $\kappa_{ap} = 5.6 \cdot 10^{-19} \pm 4.4 \cdot 10^{-20} \text{ J}$ determined from the fit of the passive spectrum. From this, the membrane tension $\sigma$, $F_2$ and the exponent $\nu$ are deduced.

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