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Stretching and relaxation of vesicles

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We study the shape relaxation of spherical giant unilamellar vesicles which have been deformed far from equilibrium into ellipsoids using optical tweezers. The relaxation back to a sphere is determined by elastic constants of the vesicles, and their excess area, parameters that are obtained for each stretched vesicle from shape fluctuations in thermal equilibrium, as well as low Reynolds number fluid flow. The relaxation time could be compared favorably to a simple formula which encompasses the joint effect of membrane rigidity and fluid flow. The time constant of the stretched vesicle is slower than that of its thermal fluctuations, which agrees with a recent theory; however, it is one order of magnitude faster than predicted.

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I. INTRODUCTION

Vesicles are closed lipid bilayer membranes that separate two regions of, possibly different, fluids. In cell biology such closed lipid bilayer membranes are ubiquitous from the plasma membrane—the outer shell of cells—to the membranes that enclose many compartments within cells [1].

Cell membranes are not simply containers for cellular activity, but contain (on their surface or embedded within the membrane) many active biomolecules [2,3]. One key activity of membrane localized biomolecules is that they actively push or pull on the membrane and deform its shape. For example, on the plasma membrane, the outer shell of a cell, proteins such as WASP can induce a pushing force against the membrane through polymerization of the cell's actin scaffold. This leads to protrusions of the membrane which allow a cell to change its shape and migrate.

In addition, detachment from the scaffolding can allow pressure from inside the cell to push localized regions of the membrane outward in the initial stage of a process called blebbing [4]. This swelling process, which is purely physical and does not involve active biomolecular components, reflects the intricate interplay between membrane bending energy and fluid flow. The importance of blebbing for certain types of cell migration has only recently been appreciated [5,6].

Thus, cell-scale changes in membrane shape under active physical or biochemical forcing are of great interest. In particular, it is of interest to understand how both membrane mechanics and fluid dynamics on the inside or outside of the cell contribute to cell shape changes. Here we investigate the shape relaxations of vesicles of size comparable to many cells (of order 15 μm). In both a steady and time-dependent shear flow, different dynamical regimes have been observed, such as tumbling, tank-treading, and wrinkling [7–10].

In this article we discuss experiments in which vesicles are deformed into ellipsoids using optical forces from a holographic optical trap and their relaxation back to their equilibrium shape is measured. Our purpose is to understand the relaxation dynamics and to characterize it based on direct measurement of the relaxation, as well as the equilibrium shape

and shape fluctuations. Indeed, shape fluctuations in thermal equilibrium allow us to determine the physical parameters of the vesicles. The relaxation time is compared to a simple formula which encompasses the joint effect of membrane rigidity and fluid flow.

The vesicle membrane is characterized by the Helfrich bending rigidity κ , the elasticity σ , and its viscosity ζ . The parameters κ and σ were measured from an analysis of unforced thermally fluctuating vesicles. The membrane viscosity ζ could not be measured and it was assumed to be zero in the comparison between experiment and theory.

The excess area is a key parameter of a vesicle. A vesicle with a nonelastic impermeable membrane can only be deformed when it has a nonzero excess area. The excess area is defined such that the vesicle area A is $A = (4\pi + \Delta)r_0^2$, with r_0 defined by the vesicle volume $V = \frac{4}{3}\pi r_0^3$. The excess area Δ is non-negative and vanishes for a sphere. In the absence of thermal fluctuations, it can be proven that vesicles with constant excess area minimize their free energy by taking on the shape of a prolate uniaxial ellipsoid [9].

Quite recently, an interesting closed analytical theory was formulated for the deformation of near-spherical vesicles in shear flow [8,9]. Depending on the shear rate, the excess area, and the viscosities of the fluid inside and outside, these vesicles exhibit intriguing dynamical behavior, such as tumbling, trembling, and tank treading [7,11,12]. These various regimes are predicted by the theory [8–10], which also identifies the appropriate dimensionless parameters to characterize these modes and qualitatively describes the various regions in the experimental phase diagram [13] and in numerical simulations [14]. The theory embodies the low-Reynolds number hydrodynamics of the flow inside and outside the vesicle, the elastic deformation of the membrane, and the viscosity of the membrane itself. The theory also provides the appropriate framework to describe vesicle relaxation and gives a prediction for the relaxation rates observed in our stretching experiments.

A. Fluctuations

The analysis of the fluctuations of an unforced vesicle (“flicker spectroscopy”) is a well-established way to infer its physical parameters from a time series of microscope

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FIG. 1. Geometric arrangement of the experiments. (a) Vesicles are pulled to roughly elliptical shape at their poles. (b) A wrinkled vesicle which is spherical on average. In the experiment the meridional circle $r(\theta, \phi = \pi/2)$ is viewed. The deformation is characterized by $u(\theta)$, with a wave number $q = \theta/2\pi r_0$.

images [15]. However, the use of two-dimensional information and the finite spatial and temporal resolution of microscope images are complicating factors.

The magnitude of the height fluctuations of a membrane is in thermal equilibrium determined by its bending rigidity κ and elasticity σ , with the Fourier amplitudes

$$\langle |u(q)|^2 \rangle = E(q) = \frac{k_B T}{\sigma q^2 + \kappa q^4}, \quad (1)$$

where $u(x, t)$ is the deviation of the membrane from its equilibrium shape, T is the temperature, and k_B the Boltzmann constant. The (imaginary) frequency at wave number q is

$$\omega(q)^{-1} = i \frac{4\eta q}{\sigma q^2 + \kappa q^4}. \quad (2)$$

The averaged spectrum $\langle |u(q)|^2 \rangle$ can be determined from measured vesicle contours, and Eq. (1) can then be used to obtain a value for σ and κ . This procedure has been described excellently by Précréaux *et al.* [15], but it is appropriate to highlight a few details.

Equations (1) and (2) describe the fluctuations of a planar membrane in a viscous fluid with hydrodynamic self-interaction. For a vesicle, the volume of the incompressible fluid inside cannot change, while we assume that the total membrane area also remains constant. Then, the surface tension σ is a fictitious parameter, and is determined by the temperature and the excess area Δ [16]. The key experimental quantity of interest is the cross-spectral density $C_e(q, \tau)$,

$$C_e(q, \tau) = \langle |u(q, t)| |u(q, t + \tau)| \rangle - \langle |u(q, t)|^2 \rangle, \quad (3)$$

which should be compared to

$$C(q, \tau) = k_B T \frac{\tau_f(q)}{4\eta q} \exp[-\tau/\tau_f(q)], \quad (4)$$

with the fluctuation time $\tau_f(q) = -i\omega(q)^{-1}$. We notice that $C(q, \tau = 0) = E(q)$ [Eq. (1)], but that the correlation function of the measured fluctuations $C_e(q, \tau)$ must be normalized as in Eq. (3). According to Eq. (2), large wave numbers correspond to short time scales, which are increasingly affected by the finite camera integration time τ_{int} . For a typical bending rigidity $\kappa \approx 10^{-19}$ J and $\tau_{\text{int}} = 8 \times 10^{-3}$ s, this would affect wave numbers $q \approx 2 \times 10^6 \text{ m}^{-1}$. Consequently, for comparison

to the experiment, $C(q, \tau)$ must be corrected for temporal averaging of the camera.

The geometry of our experiments is sketched in Fig. 1. The surface of a vesicle is viewed projected on the y, z plane, and due to the phase-contrast technique we only see the vesicle perimeter along a meridional circle $r(\theta, \phi = \pi/2)$ in the y, z plane.

As in [15], the connection with the fluctuations of a planar membrane is made by wrapping the membrane as a cylinder along the meridional circle with (mean) radius r_0 , measuring q_y along the circle as $q_y = \theta/2\pi r_0$, and integrating Eqs. (1) and (4) over q_x to obtain the one-dimensional cross-spectral density:

$$C_{1D}(q_y, \tau) = \frac{1}{2\pi} \int_{-\infty}^{\infty} C(q, \tau) dq_x, \quad q^2 = q_x^2 + q_y^2. \quad (5)$$

From now on, we drop the suffix 1D, while the wave-number argument of C is understood to be q_y . Clearly, the planar approximation only applies if $q \gg r_0^{-1}$. The time-dependent cross-spectral density $C(q, \tau)$ now becomes a superposition of time constants τ_f and is no longer simple exponential. Although the proper fluctuation modes are the spherical harmonics [17, 18], the planar mode description has greatly simplified the application of flicker spectroscopy.

In our experiment we compare the cross-spectral density $C(q, \tau)$ to the experiment and determine the parameters κ and σ in a least-squares procedure. From Eq. (1) it follows that the most sensitive dependence on both σ and κ occurs at wave numbers $q \approx (\sigma/\kappa)^{-1/2} \approx 10^6 \text{ m}^{-1}$. For smaller wave numbers the stretch elasticity dominates; for larger wave numbers the bending rigidity.

At wavelengths of the order of the radius of the vesicle, shape relaxation is determined by long-range hydrodynamic interaction, and Eqs. (1) and (2) no longer apply. Long-wavelength deformation is considered in the next section.

B. Relaxation

The relaxation of vesicles in a shear flow through long-range hydrodynamic interactions has recently been considered by Lebedev *et al.* [8, 9]. In the absence of shear flow, it takes on a particularly attractive form,

$$\tau_t \frac{d\Theta}{dt} = \cos(3\Theta), \quad (6)$$

where the dynamical variable Θ gauges the shape of the vesicle and where all physical properties of membrane and fluid(s) are embodied in the time constant τ_t [8, 9].

Let us now describe the variable Θ and the time constant τ_t . The theory assumes an ellipsoidal vesicle deformation that has rotational symmetry around the z axis, $r(\theta, \phi) = r_0[1 + u(\theta)]$, with the deformation $u(\theta)$ expressed in a second-order Legendre polynomial,

$$u(\theta) = \frac{5^{1/2}}{4\pi^{1/2}} [u_1 (1 - 3 \cos^2 \theta) + u_4 3^{1/2} \sin^2 \theta]. \quad (7)$$

The coefficients u_1 and u_4 gauge the shape of the ellipsoid and define the angular variable Θ in Eq. (6) as $\Theta = \tan^{-1}(u_1/u_4)$. For a pulled vesicle which relaxes back to a sphere, u_4 tends to zero faster than u_1 , so that $\Theta \rightarrow -\pi/2$. The theory assumes

constant excess area Δ , with the deformation $u = \mathcal{O}(\Delta^{1/2})$, but ignores thermal fluctuations.

The time constant in Eq. (6) is

$$\tau_t = \frac{7\pi^{1/2}}{12 \cdot 10^{1/2}} \frac{a \eta r_0^3}{\kappa \Delta^{1/2}}, \quad (8)$$

with $a = \frac{16}{3} \left(1 + \frac{23}{32} \frac{\tilde{\eta}}{\eta} + \frac{\zeta}{2 \eta r_0} \right),$

with κ the bending rigidity of the membrane and η the dynamical viscosity of the fluid outside the vesicle, and where the constant a contains the viscosity $\tilde{\eta}$ of the fluid inside the vesicle and ζ is the viscosity of the membrane itself.

The relaxation time constant of a pulled and released vesicle reflects the balance of the membrane elastic forces and fluid friction. When deforming a sphere by squeezing it at its poles by dr_0 , the curvature energy change is approximately $\pi \kappa dr_0/r_0$, which corresponds to a force $\pi \kappa/r_0$. Conversely, the fluid friction force scales with the relaxation velocity u and r_0 as $\eta u r_0 \propto \eta r_0^2/\tau$, and the crude estimate follows:

$$\tau_d = \frac{\eta r_0^3}{\pi \kappa}. \quad (9)$$

Equation (9) is a mere dimensional argument and overestimates the relaxation time constant because the deformations of the vesicle are smaller than its size.

The relation with the theoretical τ_t of Eq. (8) is $\tau_t = \tau_d 7\pi^{3/2}a/(12 \cdot 10^{1/2} \Delta^{1/2})$, where the appearance of the numerical factor and the fluid viscosity results from the analysis of low Reynolds number flow. The emergence of the excess area in Eq. (8) is due to a degeneracy of the third- and second-order expansion in u of the free energy and is connected with the requirement that Δ is constant during vesicle dynamics [9]. Ignoring the membrane viscosity ζ , we notice that $\tau_t \approx 9.4 \tau_d \Delta^{-1/2}$. In our experiments $\Delta = \mathcal{O}(0.1)$, and τ_t would be more than one order of magnitude larger than τ_d . The additional slowness of the forced low-order modes is a striking feature of the theory [9].

The parameters Θ , Δ , and τ can be extracted from our experimental data and the question is if our experiments can be compared favorably with this theory, and in particular, whether the measured time constants can be compared to τ_t .

As a first approach, we express the decay of the vesicle toward the circular cross section in our experiments in terms of the $l = 2$ Legendre polynomial,

$$r(\theta, t) = r_0[1 + b(t) P_2(\cos \theta)]. \quad (10)$$

It turns out that the function $b(t)$ decays approximately exponentially; in fact, this is already embodied by Eq. (6), as for near-spherical vesicles, $\Theta \approx -\pi/2$, with the deviation $-\Theta - \pi/2$ relaxing as

$$-\Theta - \pi/2 \sim \exp(-3t/\tau_t). \quad (11)$$

II. EXPERIMENT

Giant unilamellar vesicles were made using electroformation [19,20]. The lipid compositions used were dioleoyl phosphatidylcholine (DOPC) and +1% *N*-cap biotinylated phosphatidyl ethonolamine (biotin PE). The fluid inside the

vesicles is a 0.3 M sucrose solution, while the outside fluid is a 0.37 M glucose solution. For the fluid viscosities we took $\eta = \tilde{\eta} = 1.217 \times 10^{-3} \text{ kg m}^{-1} \text{ s}^{-1}$. The vesicles are placed in a closed chamber to prevent evaporation and possible fluid flow. Due to the larger density of the inside fluid the vesicles have a slightly negative buoyancy, while the associated contrast of the index of refraction across the lipid bilayer results in an effective force on the membrane in the focused laser traps. By adjusting the glucose concentration outside the vesicles, and thus osmotic pressure difference across the membrane, vesicles with finite excess area could be created. Due to their formation process, a large natural range of vesicle radii can be found in a sample volume. We chose vesicles that were sufficiently large and sufficiently fluctuating.

The experimental setup is sketched in Fig. 2. All experiments are performed on a Bioryx 200 from Arryx, which is a Nikon inverted microscope. A 60 $\times$ oil immersion lens provides a magnification that is near the diffraction limit when imaged using a FastCam CMOS camera. It was run at a frame rate of 125 frames per second with image-pixel size 0.2 μm .

The microscope images show the perimeter of the center-plane horizontal cross section in phase-contrast. It is deformed to an ellipse by two diametrically placed optical traps that are moved apart slowly. Since the hydrodynamic friction force is proportional to r_0^{-2} , the trap separation velocity must be smaller for larger vesicles.

FIG. 2. Experimental setup and geometry. (a) Vesicle pulled by two optical traps indicated by the white circles. (b) Vesicle long after release from the traps. (c) The setup involves a microscope equipped with a holographic optical trap (Bioryx 200 from Arryx, Inc.). Two diametrically placed traps are slowly moved apart and deform the vesicle to an ellipsoid.

A. Image analysis

After the release from the optical traps, a time series of snapshots was registered. First, time-dependent contours $\mathbf{x}(s, t)$, where s is the chord length, were extracted from the movie of the relaxing vesicle using the technique of active contours. Briefly, an active contour in an image is a loop, endowed with physical properties (such as elasticity), which is evolved to find a best fit of the corresponding image object. Technically, this is done by turning the image into a potential energy surface with an energy minimum at the sought image object, that is, the vesicle perimeter [21,22]. In our experiments we used the gradient of the image, identifying the vesicle perimeter as the point where the image gradient is largest. The technique of active contours provides the vesicle contour with subpixel resolution and is superior over pixel-based methods that are much more sensitive to noise.

The center of mass of each contour, $\mathbf{x}_{\text{cm}} = \frac{1}{L} \oint \mathbf{x}(s, t) ds$, with L the contour length, was placed in the origin. The centered contours were turned into cylindrical coordinates $r(s, t) = |\mathbf{x}(s, t)|$, $\theta(s, t) = \tan^{-1}[z(s, t)/y(s, t)]$, with Fourier modes

$$u_m(t) = \frac{1}{2\pi} \int_0^{2\pi} r(\theta, t) \exp(im\theta) d\theta.$$

The linear Fourier modes needed for the fluctuation analysis then follow as

$$u(q, t) = (2\pi r_0)^{1/2} u_m(t), \quad \text{with } q = m/r_0.$$

For large deformations, spherical harmonics provides the appropriate framework. We restrict the expansion of the vesicle contour deformation to the second-order $P_{l=2}$ [Eq. (10)], with the coefficient $b(t)$ obtained by angular integration of the measured contour. The parameters u_1, u_4 in Eq. (7) were determined analogously. Notice that in our case the $l = 2$ spherical and $m = 2$ linear modes are equivalent such that their time dependence can be compared. Let us now dwell on the measurement of two other key parameters, namely, the mean radius r_0 and the excess area Δ .

B. Measuring the excess area Δ

In our experiment, the vesicle is imaged using phase-contrast microscopy, which shows a contour in the meridional plane. Assuming an ellipsoidal vesicle oriented with its long major axis pointing in the z direction, and assuming rotational symmetry around this axis, the volume V and the area A follow from the measured meridional contour $\mathbf{x}(s, t)$ in the y, z plane as

$$V = \frac{\pi}{2} \oint y^2 \left| \frac{dz}{ds} \right| ds \quad (12)$$

and

$$A = \pi \oint |y| ds. \quad (13)$$

Here, s is the chord length and the geometry refers to that shown in Fig. 1. The integration over the entire contour, where in principle its right half $y > 0$ would suffice, improves statistical accuracy. From the measured area and volume, the

excess area is estimated as $\Delta_e = A/r_0^2 - 4\pi$, with the mean radius r_0 defined as $V = \frac{4}{3}\pi r_0^3$.

In an unforced vesicle, the excess area is taken up by thermally excited wrinkles. Although the vesicle is now a sphere on average, rotational symmetry as in Eqs. (12) and (13) can no longer be assumed and the excess area is severely underestimated. Equation (13) misses the wrinkles in the azimuthal direction, and because the wrinkles are isotropic in the polar and azimuthal direction, it is tempting to assume that for a wrinkled sphere the true excess area Δ is twice the measured one Δ_e . This simple rule was verified by constructing randomly wrinkled spheres as the real part of

$$r(\theta, \phi) = 1 + \epsilon \sum_{l=2}^L \sum_{m=-l}^l \xi_{lm} Y_{lm}(\theta, \phi),$$

where Y_{lm} are the spherical harmonics, $\epsilon = \mathcal{O}(10^{-2})$ is a small number and ξ_{lm} are complex numbers uniformly randomly picked from the square $[(-\frac{1}{2}, \frac{1}{2}), i(-\frac{1}{2}, \frac{1}{2})]$. For such spheres the excess area scales as $\Delta \propto \epsilon^2 L^4$, and we verified the rule $\Delta \approx 2.2\Delta_e$. Without doubt such a rule can also be obtained from analytical arguments. Summarizing, for smooth rotationally symmetric ellipsoids Eqs. (12) and (13) provide a correct experimental estimate of the excess area, but for a wrinkled (average) sphere, we should approximately double it.

III. RESULTS

For the experiments, vesicles were selected that had a sizable excess area, and thus could be deformed relatively easily by pulling on their membrane with optical tweezers. Prior to pulling and relaxation the vesicles were observed for typically 40 s (≈ 4500 frames) and the vesicle properties were obtained from analyzing the thermal fluctuations. Then the tweezers were turned on, the vesicle was stretched, held for a while (3–27 s), and released. After relaxation the thermal fluctuations were measured again.

A. Fluctuation analysis

Depending on their preparation, the elasticity, bending rigidity and excess area of vesicles may vary. An example is

FIG. 3. Time-dependent contours of two fluctuating vesicles which have approximately the same size, but with different physical properties: (a) $\kappa = 8.7 \times 10^{-20}$ J and $\sigma = 1.1 \times 10^{-7}$ Nm $^{-1}$ and excess area $\Delta = 0.02$; (b) $\kappa = 4.9 \times 10^{-20}$ J and $\sigma = 0.5 \times 10^{-7}$ Nm $^{-1}$ and excess area $\Delta = 0.04$.

FIG. 4. Analysis of the fluctuations of an unforced vesicle. (a) Normalized cross-spectral density $C_e(q_m, \tau) / C_e(q_m, \tau = 0)$ at discrete wave numbers $q_m = m/r_0$ for $m = 2, 4, 6, 8, 10$ and $r_0 = 13.7 \mu\text{m}$. At $m = 2$ the fluctuations approximately decay as $\exp(-\tau/\tau_f)$, with $\tau_f = 0.25$ s. (b) $C(q, \tau)$ computed for $\kappa = 8.7 \times 10^{-20}$ J and $\sigma = 0.98 \times 10^{-7}$ Nm^{-1} . Because of the projection on the meridional plane, the cross-spectral density deviates from an exponential. (c) Dots connected by lines show the measured $C_e(q, \tau) / C_e(q, 0)$ at times $\tau = i \times 0.016$ s, $i = 1, \dots, 5$; lines indicate the fit. (d) Dots show the measured $\langle |u(q_m)|^2 \rangle$; the full line indicates $C(q, \tau = 0)$ for $\kappa = 8.7 \times 10^{-20}$ J and $\sigma = 0.98 \times 10^{-7}$ Nm^{-1} . The extent of the fitted temporal behavior is shown shaded. The cross-spectral density at $m = 1$ should vanish as it corresponds to a translation of the vesicle. It is not 0 because the center of mass is defined with respect to the chord length s , and not with respect to the angle ϕ . In our application of flicker spectroscopy, we fit the time dependence of the cross-spectral density (c) rather than its absolute value (d).

shown in Fig. 3 for two vesicles of approximately the same size, one of which has an excess area which is twice that of the other one. The elasticity and bending rigidity were inferred from observing the thermal fluctuations of isolated vesicles. The cross-spectral density $C_e(q_m, \tau)$ [Eq. (3)] at discrete wave numbers $q_m = m/r_0$ was computed from a large number of contours.

There are two ways to use the information contained in the measured cross-spectral density. In most applications of flicker spectroscopy, the cross-spectral density at zero time delay is fitted to the theoretical spectrum. However, the absolute value of $C(q, \tau)$ is affected by the finite temporal and spatial resolution of the experiment. The time dependence of $C(q, \tau)$ is less affected by resolution problems. For example, if $C(q, \tau)$ is simply exponential, $C(q, \tau) = A \exp[-\tau/\tau_f(q)]$, temporal averaging will affect the magnitude A , but not the decay time $\tau_f(q)$.

In our experiments both spatial and temporal resolution is limiting, and we use the time- and wave-number-dependence of the normalized $C_e(q, \tau) / C_e(q, 0)$ to determine κ and σ in a least-squares procedure. Ideally, both methods should give the same values for κ and σ [18]. An advantage of our method is that it uses experimental information at all times, not just that

at $\tau = 0$. A problem is that at large q both the absolute value of $C(q, \tau)$ and its decay time become small, so that only small time intervals are available for the fit.

In our fit procedure the minimum value of $C_e(q, \tau)$ considered is 10^{-22} m^3 , as is set by the noise level of the measured vesicle contour, which was determined in a separate experiment. In order to apply the planar version of the fluctuation spectrum Eq. (5), only mode numbers $m \geq 5$ were taken into account. A typical result is shown in Fig. 4(a) and compared to the analytical expression Eq. (5) in Fig. 4(b), with κ and σ determined from a least-squares fit. It is seen that the overall time dependence is represented well; this even includes the $m = 2$ mode, which is outside the fit interval. The fit is further illustrated in Fig. 4(c), which also shows that the quality of the measured data at large wave numbers and long times rapidly deteriorates.

As was argued earlier, because the time dependence of the computed cross-spectral density is near exponential, it was not corrected for the finite temporal resolution. The time scale decreases for increasing mode m (wave number $q = m/r_0$), and for this vesicle equals the camera integration

FIG. 5. A typical relaxation experiment. (a) Vesicle contours relaxing from an approximate ellipse to a circle after release from the optical traps. (b) Full line, time dependence of the coefficient $b(t)$ of the second-order Legendre polynomial of the vesicle contour [Eq. (10)]; dashed line, fit $b(t) \sim \exp(-t/\tau_e)$, with $\tau_e = 1.06$ s. (c) The excess area relaxes from $\Delta \approx 0.11$ when pulling to $\Delta_e \approx 0.02$ after relaxation. The gray line indicates the corrected excess area of the relaxed vesicle, which is approximately twice Δ_e .

time at $m = 28$ ($q \approx 2 \times 10^6 \text{ m}^{-1}$). Remarkably, the temporal information of the cross-spectral density is rarely used in flicker spectroscopy.¹

With the found values of κ and σ , the spectrum $C(q, \tau = 0)$ can be computed and compared to the experiment. Figure 4(d) shows that the fit is not perfect and that the measured fluctuations are underestimated. Because the camera integration was accounted for in the comparison, the discrepancy must be due to the finite spatial resolution of our images. Indeed, the root-mean-square fluctuation $\langle [u(s, t) - \langle u(s, t) \rangle]^2 \rangle^{1/2} \approx 0.13 \mu\text{m}$ [$\approx \int C_e(q, 0) dq$], which is less than a pixel.

The properties σ, κ, r_0 , and Δ of our vesicles were determined before and after the stretching experiment. The difference between the two measurements sets the uncertainty, which is represented by the error bars in Figs. 6 and 8. The measured κ ranged between $(0.4, \dots, 1.9) \times 10^{-19} \text{ J}$ with average $9.1 \times 10^{-20} \text{ J}$, which is close to the value for DOPC vesicles documented in the literature [24,25].

B. Vesicle relaxation

Figure 5 shows the result of a typical relaxation experiment. We project the vesicle contour on the second-order Legendre

FIG. 6. Comparing the measured shape decay time to the simple dimensional estimate τ_d [Eq. (9)]. The error bars are computed from the variation of σ, κ , and r_0 before and after the relaxation experiment, if available. Dashed line, $\tau_d = 8\tau_e$.

¹An recent exception is [23]; however, the consistency with the amplitude spectrum was not checked. Surprisingly, this article also documents the time constant of the nominally zero $m = 1$ mode.

FIG. 7. (a) Contours of the relaxing vesicle of Fig. 5, but now the deviation from a circle is projected on the second-order Legendre polynomial. (b) Time dependence of $\cos[3\Theta(t)]$ [Eq. (6)]. Because $\Theta(t)$ is determined by the ratio of two measured quantities, both of which tend to zero, the noise in Θ becomes excessive at times $t \gtrsim 5$ s, and no data are shown. Dashed line, $\cos[3\Theta(t)] \sim \exp(-t/\tau_e)$, with $\tau_e = 1.0$ s.

polynomial $P_2(\cos \theta)$ [Eq. (10)], with the time dependence of the coefficient $b(t)$ shown in Fig. 5(b), which appears approximately exponential, $b(t) \sim \exp(-t/\tau_e)$. We notice that the relaxation time $\tau_e = 1.06$ s is much larger than the time constant $\tau(q_{m=2}) \approx 0.25$ s of the $m = 2$ fluctuations shown in Fig. 4(a). In Fig. 5(c) we show the time dependence of the excess area; it relaxes from $\Delta_e \approx 0.11$ when the vesicle is pulled to $\Delta_e \approx 0.02$ after release from the optical traps. As argued in Sec. II B, the true excess area should then evolve from $\Delta \approx 0.11$ to $\Delta \approx 0.04$.

In our pulling experiments the excess area is not constant; it roughly decreases by a factor of 2–4 after release. Several explanations are possible. First, in our images we may miss the smallest wrinkles that absorb the excess area of the relaxed vesicle. Those wrinkles are fastest and may be integrated by the camera. Second, these wrinkles may fall below the spatial resolution of the camera. Another explanation may be that a relaxed vesicle sits on the bottom of the test cell (the y, z plane) with a slightly flattened contact area, which may take up a relatively large excess area.

Figure 6 compares the measured relaxation time τ_e to the dimensional estimate τ_d which was computed from the vesicle parameters. These two relaxation times are approximately proportional, but the relaxation after stretch is much faster than τ_d . Finally, let us confront the theory of Sec. I B with the experiment. To this aim we computed the angular variable $\Theta(t)$ from the relaxing vesicles, with a typical result shown in Fig. 7. As the theory expresses the vesicle deformation in terms of the second-order spherical harmonics, the reconstructed contours are also shown in Fig. 7(a). They should be compared to the unfiltered contours of Fig. 5. As anticipated in Eq. (11), $\cos(3\Theta)$ decays approximately exponentially, $\cos(3\Theta) \sim \exp(-t/\tau_e)$, with $\tau_e \approx 1.0$ s; consequently, the decay time constant of Θ is $\tau_{e\Theta} \approx 3.0$ s. Taking the initial $\Delta \approx 0.11$, we can now compute the prediction of the theory Eq. (8), $\tau_t = 95$ s, which is more than one order of magnitude larger than the observed $\tau_{e\Theta}$.

IV. CONCLUSION

Our main point is that the relaxation time of a pulled and released vesicle is the consequence both the membrane rigidity κ and fluid flow; the measured time constant scales with the dimensional estimate τ_d which involves both aspects. In agreement with the theory of [8–10], we observe an additional

slowness of the relaxation of the stretched and released vesicle compared to the unforced thermal fluctuations. However, the predicted time constants are one order of magnitude larger than those observed. The predicted time constant τ_t depends on the excess area Δ , and the bending rigidity κ as $\tau_t \propto \kappa^{-1} \Delta^{-1/2}$. Both measured quantities may have a sizable error; however, we believe that the observed discrepancy can not be explained by these uncertainties.

The membrane surface tension σ is a fictitious quantity. It serves to maintain constant surface area and volume of the vesicle. For a relaxed vesicle, the membrane excess area is stored in thermal fluctuations; for the stretched vesicle it is mainly contained in the $l = 2$ deformation mode. For the excess area stored in thermal wrinkles, the source of σ is entropic, which is the case if $k_B T / 2\kappa \ll \Delta \ll k_B T \ln l_{\max}$, where $l_{\max}$ is the largest spherical mode number, $l_{\max} \propto r_0/d = \mathcal{O}(10^4)$, with d the membrane thickness [16]. Taking the average bending rigidity of our vesicles $\kappa \approx 10^{-19}$ J, we have $0.02 \ll \Delta \ll 0.4$, which holds approximately, and we conclude that thermal fluctuations are important for the relaxation dynamics.

For the relaxed vesicle this implies that $k_B T / 2\kappa \Delta$ should be a function of the dimensionless surface tension $r_0^2 \sigma / \kappa$.

FIG. 8. Relation between the excess area and surface tension of relaxed vesicles. The error bars reflect the reproducibility of the measured κ , σ , Δ , and r_0 before and after the stretching experiment, if available.

This relation is illustrated for our vesicles in Fig. 8, which shows that σ is large when Δ is small, but with large errors. There is a large factor between the dimensionless surface tension and $k_B T / 2\kappa \Delta$, which for entropic surface tension should be of the order of the mode area $l_{\max}^2$. For the stretched ellipsoidal vesicles, where almost all of the excess area is stored in the $l = 2$ deformation, the surface tension may turn negative. Therefore, the explanation of the relaxation of stretched vesicles must involve an additional dynamical equation of the surface tension. The absence of it in the model leading to Eq. (8) may explain the large discrepancy that we find with the predicted relaxation time constants. Recently, the dynamics of σ was considered in [10] in an attempt to explain wrinkling transients of vesicles in time-varying shear flow.

Its bending rigidity and elasticity constitute the simplest membrane characteristics. A more refined description allows for an area difference between the two layers that make the bilayer membrane. The effect of the associated area-difference elasticity (ADE), similar to spontaneous curvature, can lead to spontaneous shape changing and membrane budding [26]. The differential stretching of the membrane layers results in an effective nonlocal bending modulus which can be of the same

order as κ [26]. The effect of ADE shows in the low-order fluctuation modes $q_m, m = 2, 4$, which were disregarded in our analysis which assumed a planar membrane. Advanced fluctuation spectroscopy, involving ensembles of numerically simulated vesicle shapes, has been proposed to cure this problem [27]. All our vesicle contours are circular on average before and after pulling, so that ADE is probably not a large effect. However, ADE may come into play when deforming the vesicles in the optical trap, and the effective bending modulus in Eq. (8) could become as much as twice the value obtained from large- q spontaneous fluctuations, which would halve the discrepancy with the experiment.

Quantitative experiments on pulled vesicles are now possible, which may inspire new theory for the relaxation in the presence of thermal fluctuations.

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