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STROBOSCOPIC ILLUMINATION GIVES NEW OPPORTUNITIES AND IMPROVES THE PRECISION OF BENDING ELASTIC MODULUS MEASUREMENTS

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In this work we propose an improved experimental set-up for the measurement of the bending elastic modulus by the analysis of thermally induced shape fluctuations of quasi spherical GUVs using stroboscopic illumination. The stroboscopic video microscopy has better time resolution than the continuous illumination video microscopy. Consequently, it is no more necessary to use a "correction factor" to account for the artifact due to the finite video camera integration time. The experimental data, acquired under the stroboscopic illumination can be completely interpreted using only two model parameters, the bending elastic modulus and the dimensionless membrane tension.

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1. Introduction

Lipid bilayers are important constituents of living cells. Knowing the mechanical properties of membranes is important for understanding cell resistance to external influences. The first theoretical models of membrane mechanical properties were proposed by Helfrich [1] and Evans [2]. According to these, the elastic energy per unit area of lipid membrane, F_c, is

$$F_c = \frac{1}{2} k_c (c_1 + c_2 - c_0)^2 + \bar{k}_c c_1 c_2 \tag{1}$$

where c_1 and c_2 are the membrane principal curvatures, c_0 is the spontaneous curvature, and k_c and $\bar{k}_c$ are the bending and saddle bending elastic modules of the lipid bilayer, respectively. The spontaneous curvature of a symmetric membrane vanishes, i.e. $c_o = 0$.

Following the first detailed theoretical model of thermally induced shape fluctuations [3], researchers had the theoretical background to develop experimental procedures leading to precise measurements of the bending elastic modulus [4, 5]. The fundamental expression is

$$\left\langle \left| U_n^m(t) \right|^2 \right\rangle = \frac{k_B T}{k_c} \frac{1}{(n-1)(n+2)[\bar{\sigma} + n(n+1)]} , \tag{2}$$

where $\left\langle \left| U_n^m(t) \right|^2 \right\rangle$ is the mean squared amplitude of the spherical harmonic $Y_n^m(\theta,\varphi)$, k_B is Boltzmann's constant, T is the absolute temperature, n is the mode number and $\bar{\sigma} = \sigma R^2 / k_c$

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(or $\bar{\sigma} = \sigma R^2 / k_c + 2c_0 R + c_0^2 R^2 / 2$, if $c_0 \neq 0$) is the dimensionless membrane tension. In the Milner and Safran model [3], the fluctuation autocorrelation function is monoexponential:

$$\left\langle U_n^m(t) U_n^{*m}(t+\Delta t) \right\rangle = \left\langle \left| U_n^m(t) \right|^2 \right\rangle \exp\left(-\frac{\Delta t}{\tau_n^m} \right) \tag{3}$$

with a correlation time, τ_n^m, for the amplitude, $U_n^m(t)$, of the spherical harmonic $Y_n^m(\theta, \varphi)$:

$$\tau_n^m = \frac{\eta R^3}{k_c} \frac{2n+1}{(n-1)(n+2)[\bar{\sigma} + n(n+1)]} \left(2 - \frac{1}{n(n+1)} \right) \tag{4}$$

where η is the viscosity of the surrounding medium and R is the vesicle radius. The correlation time decreases as n^{-3} with the mode number n. For a tension free vesicle, $\bar{\sigma}$ = 0, of radius R = 10 μm with bending modulus of $k_c = 10^{-19}$ J, suspended in pure water, the second harmonic correlation time is, τ_2^m = 3.8 s, and the 20th harmonic correlation time is as low as τ_{20}^m = 5 ms.

In fact what is believed to be measured in an experiment on a fluctuating quasi-spherical giant vesicle is the equatorial cross section radius. Its angular autocorrelation function is a sum of Legendre polynomials with amplitudes B_n, related to the mean squared amplitudes of spherical harmonics [4]:

$$B_n = \frac{2n+1}{4\pi} \left\langle \left| U_n^m(t) \right|^2 \right\rangle \tag{5}$$

where the factor $2n+1$ reflects the $2n+1$ different m-modes for a given n, and 4π comes from the different normalizations of the Legendre polynomials and spherical harmonics.

In most experiments, observation of the giant vesicle is made by video microscopy. Unfortunately, the cameras used (CCDs or vacuum tubes) possess an intrinsic "defect"- the image presented to the observer (on the video monitor or in numerical form after digitalization by a frame grabber) reflects the integral energy accumulated on a given point (pixel) during the exposure time. For a vacuum tube, the exposure time is that between two successive frame scans, which for the European TV standard is t_s = 40 ms (25 frames per second). For a CCD, the exposure time could be between 2 and 20 ms, depending on the electronics controlling the CCD chip. Thus, fast movements are smeared out and instead of the theoretical model amplitudes, B_n, one obtains $B_n^{'} = f_n^{corr} B_n$, where the correction factor, f_n^{corr} is calculated in [4] to be:

$$f_n^{corr} = 2\left(\frac{\tau_n^m}{t_s} \right)^2 \left[\exp\left(-\frac{t_s}{\tau_n^m} \right) - \left(1 - \frac{t_s}{\tau_n^m} \right) \right] \tag{6}$$

For the same vesicle of radius R = 10 μm used as a reference above, the correction factor for the slow second mode is f_2^{corr} = 0.99 and therefore can be neglected. But the 20th mode correlation time, τ_{20}^m = 5 ms, is small compared to the video camera integration time, t_s = 40 ms, and the correction factor drops to f_{20}^{corr} = 0.22. That is an almost five-fold decrease of the experimentally measured mean squared amplitudes, $B_n^{'}$, compared to the theoretically anticipated ones, B_n. Clearly, such a difference cannot be neglected. Since the correlation time increases as R^3 with increasing vesicle radius, the correction factor for the 20th mode of a R = 20 μm vesicle has a better value, f_{20}^{corr} = 0.74, but still cannot be neglected in a precision experiment.

Two factors turn out to be of crucial importance for the precise determination of the bending modulus by the method of shape analysis of fluctuating quasi spherical giant vesicles. One is the dimensionless membrane tension $\bar{\sigma}$. This should be taken into account while fitting the measured mean squared amplitudes of spherical harmonics to the theoretical expressions (4). The second is the

effect of the video camera integration time. This results in a dramatic reduction of the measured mean squared amplitudes of higher spherical harmonics, and can lead to a severe overestimation of the bending modulus if not properly accounted for [4].

While the first factor has its strongest influence on the low order modes, the second one has its biggest influence on the fastest - the higher order ones. It is worth mentioning that the correction factors can be calculated by an iterative procedure using the measured mean squared amplitudes, $B_n^{'}$, (see equation (5) and reference [4] for details). The procedure can be applied only when $B_n^{'}$ are available from an experiment in which the bending elastic modulus, k_c, is measured. If one is interested in the dynamics of vesicle fluctuations and measures the time correlation function, the correction factor is no longer usable, and one must stick with values highly deformed by the integration time. One way to overcome this apparatus artifact is to apply stroboscopic illumination, as done by Méléard *et al*. [6]. These authors used laser light and had to overcome different problems inherently due to the light coherency. In the present work, we propose stroboscopic illumination using a xenon flash lamp. This removes all problems encountered with laser light, and yields an instant picture of the system. It can be used equally well for static measurements of bending modulus, k_c, as for dynamic measurements of the correlation times, τ_n^m.

2. Experimental equipment

Samples of the fluctuating giant vesicles were observed with a phase contrast microscope (Axiovert 100 or Axiovert 135, Zeiss, Germany) using either a 63× (NA 0.9) water immersion objective or a 63 × (NA 0.7) long working distance one. Stroboscopic illumination, was supplied by a 60W xenon flash lamp (L6604 or L7684) in a E6611 cooling jacket, powered by a C6096 supply. To get the full power from the flash lamp, an E7289-01 external main discharge capacitor was used (all items from Hamamatsu, Japan). The L7684 lamp had a built-in mirror that made it 1.5 times brighter than the L6604, otherwise they were identical. The power supply worked in externally triggered mode, synchronized with the vertical sync pulses from the CCD video camera controller (Hamamatsu C2400-60). Synchronization could be made to either the odd or even fields of the camera frame. The light pulses were less than 3 μs long (fwhm) at 1 J input energy. This was more than sufficient for our experiments because the illumination time was almost 3 orders of magnitude less than the fastest correlation time used, $\tau_{20}^m \approx 5$ ms. The corresponding correction factors for the fastest modes were thus close to unity, $f_{20}^{corr} \approx 0.999$, and could be neglected because the statistically achieved precision in the experiment was not better than 1-5%.

The disadvantage of the stroboscopic illumination was that the pulsed light irritates the eyes, so the samples should be observed on a TV monitor. Due to the “sample and hold” effect of the CCD matrix, the picture on the monitor was not flickering exactly as in the case of continuous illumination. The video signal from the camera was also fed to a frame grabber board (DT3155, Datatranslation, USA) mounted in a computer for a proper digitization (768×576 8-bit pixels). Once per second, an image was acquired and recorded on the PC, until the total number of images reached a specified value (~ 400). The resulting images were corrected for the difference of the scale factors in *x* and *y* directions coming from the mismatch of the CCD's pixel shift clock (in the camera) and the pixel acquisition clock (in the frame grabber) by digital interpolation and resampling. The value of the scale factor was determined by *x* and *y* calibration using a micrometer rule. Further details on the contour determination, mean squared amplitudes calculation and fitting procedure to determine the bending elastic modulus, k_c, and the dimensionless membrane tension, $\bar{\sigma}$, can be found in the article of Faucon *et al.* [4].

3. Materials and methods

1,2-diphytanoyl-sn-glycero-3-phosphatidylcholine (DPhPC) (Avanti Polar Lipids, USA), was used without further purification. Giant vesicles were prepared by the spontaneous swelling (gentle hydration) method [7] (see [8] for more details). To obtain giant lipid vesicles, 2 mg of the

lipid were dissolved in 3 ml of a 2:1 (v:v) chloroform:methanol mixture. A lipid film was formed on the bottom of a glass flask by the evaporation of the organic solvent under vacuum for about 5 hours. After the complete evaporation of the organic solvent, the film was fully hydrated by the addition of 25 ml of deionized water and holding at room temperature for at least 60 hours. The experimental cell used was sealed, to minimize water evaporation and to avoid convective flows which would have led to difficult follow-up and recording of the observed vesicles.

4. Results and conclusions

We tested the stroboscopic illumination on a batch of 10 different vesicles, selected on the criteria to be fluctuating, to have no visible defects, and to be far from visible defects due to dust particles on the cover slip. The continuous line in Fig. 1 shows a sum of normal distributions, each having the mean value and standard deviation of a respective vesicle, as determined by the fitting procedure.

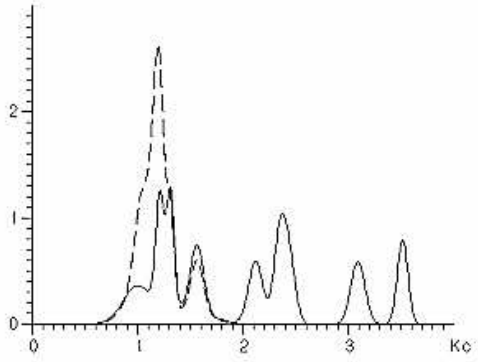

Fig. 1. Sum of normal (Gaussian) distributions, each with a mean and standard deviation of the corresponding vesicle: Continuous line - means as given by the fitting procedure. Dashed line – means divided by the hypothetical number of bilayers.

One clearly sees three groups of peaks, which can be attributed to vesicles with different numbers of bilayers. We found five vesicles with k_c of 1-1.6 $\times$ 10^{-19} J, three with k_c of 2-2.5 $\times$ 10^{-19} J (supposed to be two-lamellar) and two vwith k_c of 3-3.6 $\times$ 10^{-19} J (supposed to be three-lamellar). The dashed line presents the sum of the normal distributions with means divided by the hypothetical number of bilayers. We see that the agreement is good. The weighted value of k_c over the 10 vesicles (accounting for the number of membranes as above) is $(1.23 \pm 0.06) \times 10^{-19}$ J. If only the five hypothetically single layered vesicles are taken into account, the weighted value of the bending modulus is $(1.30 \pm 0.08) \times 10^{-19}$ J. Both values are in very good agreement with a previously measured value[9] of $(1.17 \pm 0.10) \times 10^{-19}$ J for the bending elastic modulus of a DPhPC bilayer, obtained using continuous illumination and applying the correction factor.

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