

## Using Stroboscopic Illumination to Improve the Precision of the Bending Modulus Measurement\*

J. Genova<sup>1</sup>, V. Vitkova<sup>1</sup>, L. Aladgem<sup>1</sup>, P. Méléard<sup>2</sup>, M. D. Mitov<sup>1</sup>

<sup>1</sup>Laboratory of Liquid Crystals, Institute of Solid State Physics,  
Bulgarian Academy of Sciences, Sofia 1784, Bulgaria

<sup>2</sup>UMR-CNRS 6052, ENSC-Rennes, avenue du General Leclerc,  
35700 Rennes, France

Received 5 April 2003

**Abstract.** A new experimental set-up for measuring the bending elastic modulus,  $k_c$ , by the analysis of thermally induced shape fluctuations of quasi spherical giant vesicles is proposed. 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 so obtained can be completely interpreted using only two model parameters,  $k_c$ , and the dimensionless membrane tension,  $\bar{\sigma}$ .

PACS number: 87.16.Dg

### 1 Introduction

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

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

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 bending and saddle bending elastic modules of

---

\*This work is dedicated to Professor Alexander Derzhanski, DSci., Corresponding Member of the Bulgarian Academy of Sciences, on the occasion of his 70<sup>th</sup> anniversary.

### Stroboscopy and Bending Modulus Measurement

lipid bilayer, respectively. The spontaneous curvature of a symmetric membrane vanishes,  $c_0 = 0$ .

Bending elastic modulus,  $k_c$ , is an important characteristic of lipid bilayers. That is why, since 1976 many authors try to measure it using different methods. The first attempt was done by Servuss *et al.* [3] using thermally induced shape fluctuations of tubular vesicles. Latter on, more attempts were made using either tubular vesicles [4] or quasi spherical ones [5–7]. In this paper we will concentrate on the methods based on giant quasi spherical vesicles only.

After the first detailed theoretical model of thermally induced shape fluctuations has been proposed by Milner and Safran [8], the researchers had already the theoretical background to develop experimental procedures leading to precise measurements of the bending elastic modulus [9, 10]. The fundamental expression used by the authors is [8]:

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

where:  $\langle |U_n^m(t)|^2 \rangle$  is the mean squared amplitude of the spherical harmonic  $Y_n^m(\theta, \varphi)$ ,  $k_B$  is the Boltzmann's constant,  $T$  is the absolute temperature,  $n$  is the mode number and  $\bar{\sigma} = \sigma R^2/k_c$  (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's model the fluctuation autocorrelation function is monoexponential [8]:

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

with a correlation time,  $\tau_n^m$ , for the amplitude,  $U_n^m(t)$ , of the spherical harmonic  $Y_n^m(\theta, \varphi)$  [8]:

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

where  $\eta$  is the viscosity of the surrounding medium and  $R$  is the vesicle radius. The correlation time decreases as  $n^{-3}$  with the mode number  $n$ . While for a tension free vesicle,  $\bar{\sigma} = 0$ , of radius  $R = 10 \mu\text{m}$  with bending modulus of  $k_c = 10^{-19} \text{ J}$ , suspended in pure water the second harmonic correlation time is  $\tau_2^m = 3.8 \text{ s}$ , the 20<sup>th</sup> harmonic correlation time is as low as  $\tau_{20}^m = 5 \text{ ms}$ .

Comparing equations (2) and (4) one finds the relation:

$$\tau_n^m = \frac{4\pi\eta R^3}{k_B T} \left(2 - \frac{1}{n(n+1)}\right) \frac{2n+1}{4\pi} \langle |U_n^m(t)|^2 \rangle \quad (5)$$

This means that practically the same information can be obtained measuring the  $n$ -mode's correlation time,  $\tau_n^m$ , (see Méléard *et al.* [11] and Pott and Méléard [12] for example) or its mean squared amplitude,  $\langle |U_n^m(t)|^2 \rangle$ , if the Milner and

Safran’s model [8] holds. But some recent theoretical and experimental works show [12–15] that this simple relation (5) breaks if the intermonolayer friction is taken into account.

In fact what is believed to be measured in an experiment of fluctuating quasi spherical giant vesicle is the equatorial cross section radius. It is shown in [9] that its angular autocorrelation function is a sum of Legendre polynomials with amplitudes  $B_n$ , related to the mean squared amplitudes of spherical harmonics:

$$B_n = \frac{2n + 1}{4\pi} \langle |U(t)_n^m|^2 \rangle \quad (6)$$

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

In most of the experiments the observation of the giant vesicle is made by video microscopy. Unfortunately, the video 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 time between two successive frame scans, which for the European TV standard is  $t_s = 40$ ms (25 frames per second). Thus, the fast movements are smeared out and instead of the theoretical model amplitudes,  $B_n$ , one obtains from the experiment,  $B'_n = f_n^{corr} B_n$ , where the correction factor,  $f_n^{corr}$  is calculated in [9] to be:

$$f_n^{corr} = 2 \left( \frac{\tau_n^m}{t_s} \right)^2 \left[ \exp \left( -\frac{t_s}{\tau_n^m} \right) - \left( 1 - \frac{t_s}{\tau_n^m} \right) \right] \quad (7)$$

For the same vesicle of radius  $R = 10 \mu\text{m}$  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  $20^{th}$  mode correlation time  $\tau_{20}^m = 5$  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. As far as the correlation time increases as  $R^3$  with the increase of the vesicle radius the correction factor for the  $20^{th}$  mode of a  $R = 20 \mu\text{m}$  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:

1. The dimensionless membrane tension  $\bar{\sigma}$ . It should be taken into account while fitting the measured mean squared amplitudes of spherical harmonics to the theoretical expressions (6).

## *Stroboscopy and Bending Modulus Measurement*

2. The effect of video camera integration time. This apparatus effect 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 [9].

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 to mention that the correction factors can be calculated by an iterative procedure using the measured mean squared amplitudes,  $B'_n$ , (see equations (5), (6), (7) and work [9] for details). The procedure can be applied only when  $B'_n$  are available from the experiment, when 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 more usable and one sticks with measured values highly deformed by the integration time. One way to overcome this apparatus artifact is to apply stroboscopic illumination as done by Meleard *et al.* [16]. In their work the authors used laser light and had to overcome different problems inherently due to the laser light coherency. In this work we propose a stroboscopic illumination using xenon flash lamp, that is free of all the problems encountered while using laser light. The stroboscopic illumination cancels the artifact due to the video camera integration time and presents an instant picture of the system to the observer. It can be used equally well for static measurements of bending modulus,  $k_c$ , as well as for dynamic measurements of the correlation times,  $\tau_n^m$ .

## **2 Equipment**

The samples of the fluctuating giant vesicles were observed under phase contrast microscope (Axiovert 100 or Axiovert 135, Zeiss, Germany) using either a  $63\times$  (NA 0.9) water immersion objective or  $63\times$  (NA 0.7) long working distance one. For the stroboscopic illumination the original illumination lamp (together with the holder) was replaced by a home made illuminator that was adapted to fit exactly on the place of the original one. The central part of the stroboscopic illuminator is a 60W xenon flash lamp (L6604 or L7684, Hamamatsu, Japan) mounted in a cooling jacket (E6611, Hamamatsu, Japan) and powered by its power supply (C6096, Hamamatsu, Japan). To get the full power from the flash lamp an external main discharge capacitor (E7289-01, Hamamatsu, Japan) was used. The xenon lamp model L7684 has a built-in reflective mirror that makes it 1.5 times brighter than L6604, otherwise they are drop-in replacements of each other. The power supply works in externally triggered mode synchronized with the vertical sync pulses coming from the CCD video camera (C2400, Hamamatsu, Japan). The synchronization is done by a home made TTL circuitry that can synchronize the light pulses to either the odd or even fields of the video camera frame. According to the Hamamatsu data sheet, the light pulses are less

than  $3 \mu\text{s}$  long (full width at half maximum) at 1 J input energy. This is more than sufficient for the purposes of our experiment because the illumination time is almost 3 orders of magnitude less than the fastest correlation time used in our experiments,  $\tau_{20}^m \approx 5 \text{ ms}$ . The corresponding correction factors for the fastest modes are now close to unity,  $f_{20}^{corr} \approx 0.999$ , and can be neglected because the statistically achieved precision in the experiment is not better than 1–5%.

The disadvantage of the stroboscopic illumination is that the pulsed light is irritating for the eyes, so the samples should be observed on an attached TV monitor. Due to the “sample and hold” effect of the CCD matrix the picture on the monitor is not flickering like in the case of a continuous illumination. The video signal from the camera was fed also to a frame grabber board (DT3155, Datatranslation, USA) mounted in a PCI slot of a computer for a proper digitization (768x576 8-bit pixels) and simultaneously recorded on a S-VHS video type for archiving. The obtained digital data was further recorded on the hard disk drive of the PC. Every second an image was acquired and recorded till the total number of images reaches a preliminary given value (about 400 or so). After finishing the data accumulation the 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 CCD camera) and the pixel acquisition clock (in the frame grabber) by a digital interpolation and resampling. The value of the scale factor was determined by  $x$  and  $y$  calibration using an object micrometer rule oriented in the respective directions. 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.* [9].

### 3 Materials and Methods

The 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 method (the gentle hydration method) [17] (see [18] for more details). In order to obtain giant lipid vesicles, 2 mg of the lipid were dissolved in 3 ml 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 lipid film was fully hydrated by the addition of 25 ml of deionized water and held at room temperature for at least 60 hours. The experimental cell used was completely sealed in order to minimize the water evaporation and to avoid the appearance of convective flows in the cell that lead to a difficult follow-up and recording of the observed vesicles.

## Stroboscopy and Bending Modulus Measurement

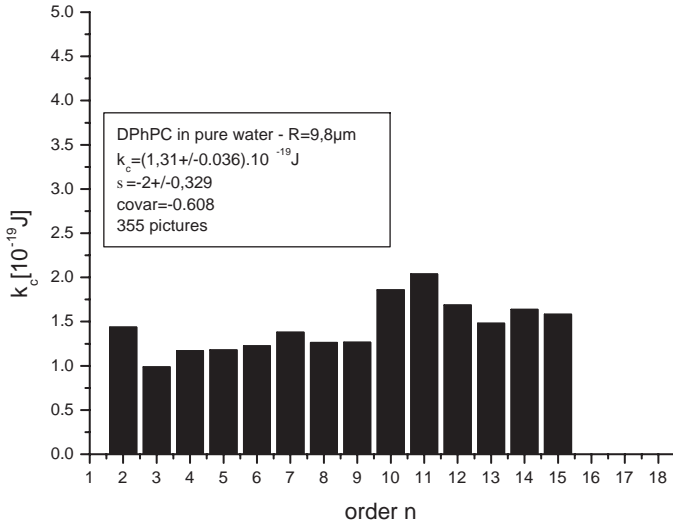


Figure 1. A typical dependence of the membrane bending modulus,  $k_c$ , on the mode number  $n$ . DPhPC vesicle, radius  $R = 9.8 \mu\text{m}$ ,  $k_c = (1.31 \pm 0.036) \times 10^{-19} \text{ J}$ ,  $\bar{s} = -2.0 \pm 0.329$ ,  $\text{covar} = -0.608$ . These data are obtained analysing 355 different images of the observed vesicle.

## 4 Results and Discussion

We have tested the stroboscopic illumination on a bunch of 10 different vesicles. A typical result is shown in Figure 1. The vesicles were selected on the criterion to be fluctuating, to have no visible defects, or to be far from visible defects due to dust particles on the cover slip.

On Figure 2 the continuous line shows a sum of normal distributions each one having the mean value and the standard deviation of a respective vesicle as determined by the fitting procedure. One clearly sees three groups of picks, which can be attributed to vesicles with different number of bilayers. We find 5 vesicles with  $k_c$  between  $(1-1.6) \times 10^{-19} \text{ J}$ , 3 vesicles with  $k_c$  between  $(2-2.5) \times 10^{-19} \text{ J}$  (may be two-lamellar) and 2 vesicles with  $k_c$  between  $(3-3.6) \times 10^{-19} \text{ J}$  (may be three-lamellar). With dashed line we present the sum of normal distributions with means divided by the hypothetical number of bilayers. We see that they fit well together. The weighted value of  $k_c$  over these 10 vesicles (accounting for the number of membranes as explained above) is:  $k_c = (1.23 \pm 0.06) \times 10^{-19} \text{ J}$ . If only the hypothetically single layered vesicles are taken into account (we have 5 like that) the weighted value of bending

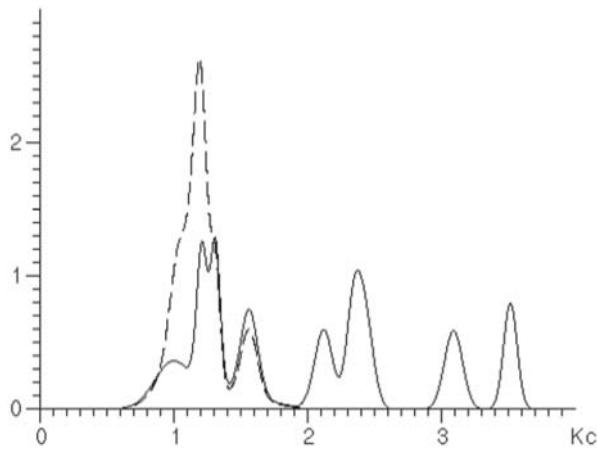


Figure 2. Sum of normal (Gaussian) distributions, each one with a mean and standard deviation of the corresponding vesicle: continuous line means as given by the fitting procedure; dashed line means divided by the hypothetical number of bilayers.

modulus is:  $k_c = (1.30 \pm 0.08) \times 10^{-19}$  J. Both values are in a very good agreement with a previously measured value [19] for the bending elastic modulus of a DPhPC bilayer,  $k_c = (1.17 \pm 0.10) \times 10^{-19}$  J, using continuous illumination and applying a correction factor.

#### Aknowledgments

This work was performed in French-Bulgarian Laboratory, CNRS, France, Bulgarian Academy of Sciences, Bulgaria. One of us (M.D.M.) thanks CNRS and Bulgarian National Fund (contract F-823) for the financial support.

#### References

- [1] W. Helfrich (1973) *Z. Naturforsch.* **28c** 693.
- [2] E.A. Evans (1973) *Biophys. J.* **13** 926.
- [3] R.M. Servus, W. Harbich, W. Helfrich (1976) *Biochim. Biophys. Acta* **436** 900.
- [4] M.B. Schneider, J.T. Jenkins, W.W. Webb (1984) *Biophys. J.* **45** 891.
- [5] M.B. Schneider, J.T. Jenkins, W.W. Webb (1984) *J. Phys. France* **45** 1457.
- [6] H. Engelhardt, H.P. Duwe, E. Sachmann (1985) *J. Phys. Lett. France* **46** L-395.
- [7] I. Bivas, P. Hanusse, P. Bothorel, J. Lalanne, O. Aguerre-Chariol (1987) *J. Phys. France* **48** 855.
- [8] S.T. Milner, S.A. Safran (1987) *Phys. Rev. A* **36** 4371.
- [9] J.F. Faucon, M.D. Mitov, P. Méléard, I. Bivas, P. Bothorel (1989) *J. Phys. France* **50** 2389.

### *Stroboscopy and Bending Modulus Measurement*

- [10] M.D. Mitov, J.F. Faucon, P. Méléard, P. Bothorel (1992)in: *Advances in Supramolecular Chemistry*, vol. 2, ed. G.W. Gokel; Jai Press, Greenwich, p. 93.
- [11] P. Méléard, M.D. Mitov, J.F. Faucon, P. Bothorel (1990) *Europhys. Lett.* **11** 355.
- [12] T. Pott, P. Méléard, *Europhys. Lett.* **59**(2002)87.
- [13] A. Yeung, E. Evans, *J. Phys. II* **5**(1995)1501.
- [14] I. Bivas, P. Méléard, I. Mircheva, P. Bothorel (1999) *Colloids Surf. A* **157** 21.
- [15] L. Miao, M.A. Lomholt, J. Kleis (2002) *Eur. Phys. J. E* **9** 143.
- [16] P. Méléard, J.F. Faucon, M.D. Mitov, P. Bothorel (1992) *Europhys. Lett.* **19** 267.
- [17] J.P. Reeves, R.M. Dowben (1969) *J. Cell Physiol.* **73** 49.
- [18] I. Bivas, V. Vitkova, M.D. Mitov, M. Winterhalter, R. Alargova, M. Méléard, P. Bothorel (2000) *Perspectives in Supramolecular Chemistry, Giant Vesicles*, vol. 6, Eds. P. L. Luisi and P. Walde, John Willey & Sons, 207.
- [19] V. Vitkova (2002) *Ph.D. Thesis*, University of Rennes I, Rennes, France.