Overview of a Quest for Bending Elasticity Measurement

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Abstract

We present a brief review of over 30 years of research that led step-by-step to a reproductive method to determine bending elasticity, based on the analysis of thermal shape fluctuations of giant unilamellar vesicles. We also acknowledge the strong contribution of Marin D. Mitov and of a long-lasting French–Bulgarian cooperation in this research. The chapter starts with an introduction of the main theoretical notions necessary to understand this experimental method. Then we review the link between this physical model and the experimental measurable quantities, that is, the amplitudes of the vesicle thermal shape fluctuations. Further, we discuss the technical progress necessary to gradually overcome some principal technical limitations. Finally, we summarize what is currently the most accurate technique for bending elasticity measurements and briefly review published bending elasticity values obtained using thermally induced shape fluctuations of quasi-spherical giant unilamellar vesicles.

1. INTRODUCTION

Biological membranes and their models are examples of soft materials. They are highly deformable systems that can bend quite easily in response to external stresses such as those encountered by red blood cells (RBC) when passing capillaries [1]. These capillaries are indeed so thin that blood cells can

only pass through them in a single file and a deformed state. The mechanical property that characterizes this membrane ability to bend when submitted to such constraints is called bending elasticity (k_c). Yet, biological or model membranes have a thickness of only some nanometers and as such their bending elasticity has to be very small. Therefore, k_c measurement was, and still is, a real challenge for biophysicists. Nowadays, we can look back to over 30 years of progress in this field and review the long story that would not have been possible without the interplay of theoretical, experimental, and technical progresses.

Experimentally, it began in 1975 when Brochard and Lennon in France published the very first estimation of bending elasticity after studying RBC membranes [2]. They were using the speckle patterns observed when light is transmitted through the RBC central and flat region whose thickness was fluctuating in response to Brownian motion. Regrettably, this innovative technique was limited to a single system, the RBC, whose thermal fluctuations are easily seen and measurable using an optical microscope. Hence, it was obvious that it would be necessary to work with model membrane systems rather than with whole cells, especially to study the relationship between membrane compositions and membrane stiffness. So, most of the work published later is based on artificial model membranes, namely, vesicles with a controlled lipid composition (liposomes). Indeed, 1 year after the pioneering work of Brochard and Lennon, the group of Helfrich published a k_c value using large tubular vesicles whose thermal fluctuations can be easily observed using an optical microscope [3]. Subsequently, giant unilamellar vesicles (GUVs) became the favorite model systems for determining the mechanical properties of lipid bilayers instead of tubular liposomes, for several reasons. These cell-sized or even larger structures are relatively easy to produce [4]. Their size makes easy the visualization of lipid bilayer thermal fluctuations using an optical microscope and a contrast enhancement technique (Zernicke phase contrast, differential interference contrast, or Hoffman modulation contrast). Further, the existing approaches of physics could be applied to these quasi-spherical objects [3,5–9].

However, GUVs were initially made from simple one-compound lipid systems, that is, either pure synthetic phospholipid or phosphatidylcholine extracts from egg yolk [10,11]. The method of choice was the "easy to do" swelling method [4,9]. A major drawback of this method was the time necessary to produce GUVs, a process that could take a few days. It was finally shown that this is too long for mixtures containing unsaturated phospholipids that undergo rapid degradation [12]. In contrast, GUV electroformation, initially published in 1986 [13], is a rapid procedure

(typically, in 1–3 h [12]) that allows working with fragile lipid compositions. GUVs obtained by this method are immobilized on the electrodes but can be detached to observe them freely moving in the aqueous environment. In this case, GUVs have a fixed area and a volume controlled mainly by water permeability. Their center-of-mass movement is Brownian and they may show membrane fluctuations analogous to the RBC undulations used by Brochard and Lennon to measure bending elasticity [2]. Freely moving GUVs can be trapped further by a micropipette to determine their membrane-stretching elasticity [10,14]. Additionally, immobilization on the electrodes may be required for some techniques, such as in confocal microscopy [15] or in studies related to membrane budding [16].

The electroformation method was subsequently used to study GUVs of more complex lipid mixtures. Membrane bending elasticity was determined for GUVs containing cholesterol molar fractions similar to those of mammalian cells [17]. Original studies using fluorescent dyes focused on the direct visualization of membrane domains [18–21]. Since then, GUVs with more and more complex membrane compositions have been made and binary or ternary lipid mixtures, as well as natural lipid extracts, studied [17,20,22–26].

However, one main disadvantage of the electroformation method was that the lipid deposit on the electrodes was done from a phospholipid solution in organic solvents [10,17,27]. This prohibited the incorporation of membrane proteins into GUVs as well as the production of GUVs from natural cell membranes. At the same time, such complex compositions that may also contain membrane proteins were routinely used for smaller liposomes (LUV, SUVs, etc.). Our group, therefore, attempted proteo-GUV electroformation from deposits made from proteoliposomes [28]. The method being successful and leading to high GUV formation rates, it was applied by Girard and colleagues [29] following our suggestion, and by others [30]. Thereafter, we generalized the exploration of other vesicle or liposome preparations classically used in a variety of biochemical or biophysical studies [31].

Even so, there were still some shortcomings concerning GUV formation. First, the general belief was that GUV electroformation would work only for low salt concentrations, that is $\leq$10 mM NaCl [27,32], so that studies involving physiological electrolyte concentrations were largely prohibited. Since then, it has become possible to attain high GUV production rates in electrolyte solutions containing buffers at physiologically relevant concentrations using an optimized GUV electroformation protocol [31]. Using this protocol, Bagatolli and colleagues were able to produce GUVs directly from RBC ghosts [25]. The obtained giant ghosts were further shown to maintain the initial asymmetry of the plasma membrane [25].

GUV production methods other than electroformation or the swelling method have been published, for instance, to produce GUV with asymmetric membranes from lipid mixtures with one involving the manipulation of an inverse emulsion [33] and another using chemically induced vesiculation or "blebbing" [34]. One peculiar method developed by Mitov and colleagues is an ingenious technique where sound waves generated by a loudspeaker are used to mechanically agitate phospholipid bilayers [35]. As a matter of fact, GUVs are now a widely used model for biophysicists, biologists, and physicists and without the substantial development to ease up GUV production and to widen its application to different systems, many studies, including some on membrane mechanical properties, would have been impossible.

Quasi-spherical giant unilamellar vesicles (QSGUVs), that is, GUVs that possess an area larger than the corresponding sphere with the same volume, are the most popular system when mechanical properties of synthetic membranes are studied. Indeed, almost all recent methods published for measuring bending elasticity of synthetic membranes use QSGUV thermal fluctuations. One approach is based on the observation of the thermal fluctuations of freely moving QSGUVs [5,7–9,17,36–43]. Other methods are based on the response of the QSGUV membrane to mechanical deformation originating from micropipette aspiration techniques [14,44–47], tether formation [48–50], bead diffusion [51], optical force in a confocal microscope [52–54], or electric field [55–59].

In the following, we review the crucial steps in the development of the method used to determine bending elasticity of model membranes by the observation of thermal shape fluctuations of freely suspended QSGUVs. Theoretical points of view as well as technical ones are discussed including the recent developments we have made to increase the precision in the determination of this mechanical property. Looking back on all the progress that has been made in this field, we also highlight the scientific impact of a strong French–Bulgarian collaboration, where Mitov was one of the best contributors. We conclude this presentation with a review of the main published results on bending elasticity measurements using thermally induced shape fluctuations of QSGUVs as a function of their bilayer composition or physical–chemical parameters of the study.

2. FROM GUV FLUCTUATIONS TO BENDING ELASTICITY MEASUREMENTS

In France, the pioneering work of Brochard and Lennon was pursued in the mid-1980s using GUVs as model systems, the principal contributors being Bivas and Bothorel, as a starting point of the previously mentioned

Bulgarian–French cooperation [8]. In 1987, one of us (PM) continued this initial work with Faucon, in order to understand why it was still impossible to get a precise measurement of bending elasticity of model membranes, a physical property that should depend only on membrane composition and environmental conditions. In this context, two valuable assets should be mentioned. First, a paper published in 1987 by S.T. Milner and S.A. Safran, dealing with microemulsion and liposome thermal shape fluctuations, pointed out the geometric characteristics of the thin layer object, involving for the first time the importance of the surface to volume ratio in this field [60]. Second, Mitov was invited to France, to join this Bulgarian–French cooperation as a senior scientific visitor. He was already well known as a theoretician of liquid crystal properties [61,62] and organization as a function of applied electric fields [63]. He was also fond of applied mathematics, computer programming, and highly skilled in electronics. He took an important place in the development of what is currently known as the most direct method for measuring bending elasticity of model membranes containing single phospholipids, a mixture of lipids, or eventually, some peptides or proteins. His contribution to this work was both technical, theoretical, and occasionally, experimental.

Figure 3.1 shows a QSGUV observed using phase contrast microscopy. Indeed, the thermal shape fluctuations are easily seen. Now, let us define V and S as the inner volume and surface area of the vesicle and R_0 as the vesicle

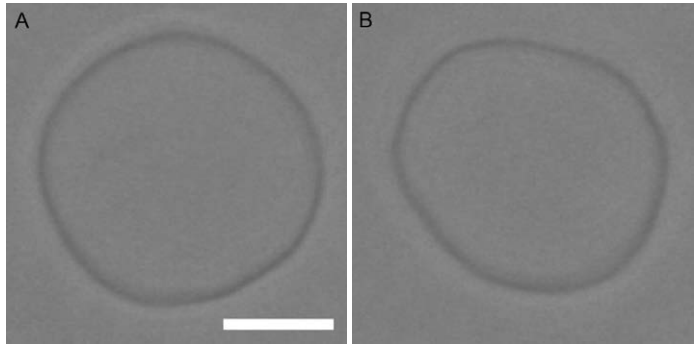

Figure 3.1 A giant unilamellar vesicle observed using an optical microscope equipped with a phase contrast device. The membrane appears as a black line on a medium grey background corrected for optical imperfections (bar length equal 10 μm). As can be seen when comparing the pictures (A) and (B) taken at different times, GUV membrane deformations are easily seen. Their amplitudes are directly related to thermal energy and bending elasticity, taking into account the surface-to-volume ratio (see text for details).

radius according to $V = 4\pi R_0^3/3$. When S is larger than $S_0 = 4\pi R_0^2$, the area of the sphere with the inner volume of the GUV, the vesicle has a relative excess area, $s = (S - S_0)/S_0 \geq 0$, that can be used to produce large deformations of the membrane, as easily seen when comparing Fig. 3.1A and B. Such large deformations are thermally induced, that is, they are enabled by Brownian motions of the surrounding environment (mainly the water molecules) and that of the lipids within the bilayer. These deformations can be measured using homemade software that analyzes the video recording of the vesicle movements [43,64].

Obviously, these deformations are dependent on the one hand on the temperature, the disturbance source, and on the mechanical properties characterizing the membrane bending resistance, namely, the bending elasticity, k_c. On the other hand, another vesicle with the same volume but a smaller area will not show measurable fluctuations when s approaches 0, that is, when its excess area is becoming negligible. Therefore, a physical model describing the relationship between thermal fluctuations and membrane stiffness has to include k_c, the temperature T, and a parameter taking into account the geometric constraints of the vesicle related to the above-mentioned relative excess area, s.

2.1. Toward a model for describing QSGUV thermal fluctuations

This approach involving both k_c, T and a "geometric" parameter of the QSGUVs was effectively presented by Milner and Safran in 1987 [60] and introduced as an experimental and usable notion by Mitov [9,65] shortly after this initial publication.

Stretching k_s and bending k_c elasticities are the main parameters characterizing steady mechanical properties of model membranes [66]. However, due to the relatively high value of k_s together with the poor water solubility of phospholipid bilayers, GUV surface area, S, can be considered constant. Hence, thermally excited membrane undulations do not produce any measurable surface change. At osmotic and hydrostatic equilibrium, the liposome volume should be constant as well. These two geometric constraints, $V = \text{Cst}$ and $S = \text{Cst}$, are therefore limiting the number of shapes a given vesicle can adopt when submitted to thermal agitation.

To describe one of the fluctuating shapes respecting these two geometric constraints, one can use the shape function $r(\theta,\varphi,t)$, which locates the membrane position and depends on the considered spherical angles θ and φ, and the time t. The studied liposome being a QSGUV, we can also use the

already introduced mean radius of the vesicle, R_0, that was defined with the vesicle volume, $V = 4\pi R_0^3/3$, and the relative shape deformation of the vesicle, $u_0(\theta,\varphi) + \delta u(\theta,\varphi,t)$, with respect to the sphere with radius R_0:

$$r(\theta,\varphi,t) = R_0[1 + u_0(\theta,\varphi) + \delta u(\theta,\varphi,t)] \qquad [3.1]$$

In the above equation, $u_0(\theta,\varphi)$ is the static mean deviation with respect to the sphere of radius R_0 (the equilibrium shape) and $\delta u(\theta,\varphi,t)$ is the corresponding time-dependent deviation [9,60,67], the quasi-spherical shape imposing $\|u_0(\theta,\varphi) + \delta u(\theta,\varphi,t)\| \ll 1$. Using the spherical harmonics Y_n^m as base functions [68], one gets easily [9,60,67]:

$$r(\theta,\varphi,t) = R_0\left[1 + \left(A_0^0 + U_0^0(t)\right)Y_0^0 + \sum_{n=2}^{n_{\max}} \sum_{m=-n}^{+n} U_n^m(t)\, Y_n^m(\theta,\varphi)\right] \qquad [3.2]$$

where the only contribution to u_0 is a constant term, $A_0^0 Y_0^0$, when considering quasi-spherical shapes [9,67]. In Eq. (3.2), $U_n^m(t)$ are the complex amplitudes of the time-dependent shape of the vesicle using the spherical harmonics as base functions for the decomposition and $n_{\max}$ is a cutoff corresponding to higher mode deformations [9,67].

As in many thermodynamic systems, the free energy of one of these fluctuating shapes defined by $u_0(\theta,\varphi) + \delta u(\theta,\varphi,t)$ has to be a bit larger than the energy of the static shape only, $u_0(\theta,\varphi)$, that corresponds to a minimum (this is required as u_0 is a static shape or, equivalently, an equilibrium state). This is what we obtain in the case of a quasi-spherical shape where the difference between the free energy of the fluctuating state, $u_0(\theta,\varphi) + \delta u(\theta,\varphi,t)$, and that of the static shape, $u_0(\theta,\varphi)$, is

$$F\{u_0 + \delta u\} - F\{u_0\} = \frac{k_c}{2}\sum_{n=2}^{n_{\max}} \lambda_n(\bar{\sigma}) \sum_{|m|\le n} \left|U_n^m(t)\right|^2 \qquad [3.3]$$

with

$$\lambda_n(\bar{\sigma}) = (n+2)(n-1)[\bar{\sigma} + n(n+1)], \quad \bar{\sigma} \ge -6 \qquad [3.4]$$

As for $n \ge 2$, $\lambda_n(\bar{\sigma}) \ge 0$, the right-hand side of Eq. (3.3) is a sum of quadratic terms, and the equipartition theorem can be applied. Taking the time average of the measured squared moduli, $\langle |U_n^m(t)|^2 \rangle_t$, we obtain:

$$\frac{k_c}{2}\lambda_n(\bar{\sigma})\left\langle \left|U_n^m(t)\right|^2 \right\rangle_t = \frac{k_B T}{2}, n \ge 2, |m| \le n$$

or equivalently

$$\left\langle \left| U_n^m(t) \right|^2 \right\rangle_t = \frac{k_B T}{k_c} \frac{1}{(n+2)(n-1)[\bar{\sigma} + n(n+1)]}, \quad n \geq 2, |m| \leq n, \bar{\sigma} \geq -6 \tag{3.5}$$

The reduced membrane tension, $\bar{\sigma}$, is the parameter that describes the geometric constraints $V = \text{Cst}$ and $S = \text{Cst}$ characterizing the QSGUV mean shape we were looking for earlier in the text. When $\bar{\sigma}$ increases well above 20, the vesicle is becoming more and more spherical, the fluctuation amplitudes decreasing until they are no more visible using an optical microscope. When $\bar{\sigma} \to -6$ on the contrary, the vesicle is fluctuating a lot and may resemble from time to time an ellipsoidal GUV (see Refs. [9,67] for other details). Before, the introduction of this reduced membrane tension, $\bar{\sigma}$, made first by Milner and Safran [60] and experimentally introduced in Ref. [9,65], such different vesicle behaviors were incomprehensible and the material property k_c could not be properly extracted from the measurable thermal fluctuation amplitudes.

The averages $\langle | U_n^m(t) |^2 \rangle_t$ are obtained from fluctuating amplitudes, whose values are varying strongly from one image of the flickering vesicle to the other. To go further and make better use of the data, it is possible to study the dynamics of the fluctuations [64,69] or the shape of the distribution of the fluctuations as recently done by our group [43].

2.2. From the physical model to the experimental measurable quantities

The most direct way to apply the physical model we introduced in Eqs. (3.2) and (3.5) is to make a large number of measurements of the time-dependent shape fluctuations, $U_n^m(t)$. These amplitudes are describing the instantaneous vesicle shape using the base functions adapted to the quasi-spherical state, namely, the spherical harmonics $Y_n^m(\theta,\varphi)$, their determination needing an instantaneous 3D picture. Unfortunately, at the end of the 1980s, there was no technique able to produce such three-dimensional information and even nowadays, confocal microscopy is not fast enough to be applicable in this context.

Video images produced by an optical microscope show only the equatorial cross section of QSGUVs (Fig. 3.1). This limited portion of the vesicle shape should be sufficient if it is feasible to observe many different images of the possible 2D cross sections. Therefore, to get a good (statistically relevant) view of the fluctuations of the GUV, one needs a large number of snapshots giving independent deformation states, that is, the vesicle shape has to be

recorded and analyzed over a long time (see the following section presenting the experimental details).

Calling $\rho(\varphi,t) = r(\theta = \pi/2, \varphi, t)$ the radius of the equatorial cross section of the vesicle shape and $\rho(t)$ and ρ the mean instantaneous contour radius and mean contour radius, respectively:

$$\rho(t) = \frac{1}{2\pi}\int_{\varphi=0}^{2\pi} \rho(\varphi,t)\mathrm{d}\varphi \quad \text{and} \quad \rho = \langle \rho(t) \rangle_t = R_0\left[1 + \frac{A_0^0}{\sqrt{2\pi}}\right] \sim R_0$$

one can define the 2D instantaneous relative fluctuations of the GUV contours as:

$$\delta v(\varphi,t) = \frac{\rho(\varphi,t) - \rho(t)}{\rho} \sim \sum_{n=2}^{n_{\max}} \sum_{m\neq 0, |m|\leq n} U_n^m(t) Y_n^m(\pi/2,\varphi).$$

Using the Fourier decomposition of the contour fluctuations as in Sackman's group [7]:

$$\delta v(\varphi,t) = \frac{1}{\sqrt{2\pi}} \sum_{q=-n_{\max}}^{n_{\max}} V_q(t) \exp^{-jq\varphi} + \frac{U_0^0(t)}{\sqrt{4\pi}} \qquad [3.6]$$

one can also obtain:

$$V_q(t) = \sum_{n=|q|}^{n_{\max}} U_n^q(t) \Theta_n^q(\pi/2) \qquad [3.7]$$

where the function:

$$\Theta_n^q(\pi/2) = (-1)^m \sqrt{\frac{2n+1}{2}\frac{(n-m)!}{(n+m)!}} P_n^m(0)$$

related to the Legendre polynomials, P_n^m, was introduced [68]. The time average of the square of the Fourier amplitudes, Eq. (3.7), has to be related to the bending elasticity k_c and reduced tension $\bar{\sigma}$ according to [7,67]:

$$\left\langle \left| V_q(t) \right|^2 \right\rangle_t = \frac{k_B T}{k_c} \sum_{n=\max(2,|q|)}^{n_{\max}} \frac{\left|\Theta_n^q(\pi/2)\right|^2}{(n+2)(n-1)[\bar{\sigma} + n(n+1)]} \qquad [3.8]$$

Another experimental analysis used the autocorrelation function of the contour fluctuations introduced in this context by Bivas and colleagues [8]:

$$\xi(\gamma,t) = \frac{1}{2\pi}\int_{\varphi=0}^{2\pi} \frac{[\rho(\varphi+\gamma,t)-\rho(t)]}{\rho(t)} \times \frac{[\rho*(\varphi,t)-\rho(t)]}{\rho(t)} \mathrm{d}\varphi$$
$$\sim \frac{1}{2\pi R_0^2}\int_{\varphi=0}^{2\pi} \left[\rho(\varphi+\gamma,t)\rho*(\varphi,t) - \rho(t)^2\right] \mathrm{d}\varphi \qquad [3.9]$$

Taking the time average and using the addition theorem for spherical harmonics [68], one can get the following expression introduced by Mitov in Refs. [9,67]:

$$\xi(\gamma,t)_t = \xi(\gamma) = \sum_{n=2}^{n_{\max}} \left|U_n^m(t)\right|^2 \sum_{m\neq 0} Y_n^m(\pi/2,\gamma)\, Y_n^m(\pi/2,0) \Leftrightarrow \xi(\gamma)$$
$$= \frac{k_B T}{4\pi k_c} \sum_{n=2}^{n_{\max}} \left(\frac{2n+1}{(n+2)(n-1)[\bar{\sigma}+n(n+1)]}\right) P_n^0(\cos\gamma) \qquad [3.10]$$
$$- \frac{k_B T}{4\pi k_c} \sum_{n=2}^{n_{\max}} \frac{\left[\Theta_n^0(\pi/2)\right]^2}{(n+2)(n-1)[\bar{\sigma}+n(n+1)]}$$

the only experimentally interesting term of the last equation being the first sum where the different Legendre polynomials appear as functions of γ.

At the end of the 1980s, the last experimental analysis, that is, the calculation of the autocorrelation function using the contour fluctuations and its decomposition as a sum of Legendre polynomials was more interesting. The principal argument for this affirmation was the direct extraction of independent modes relying on k_c and $\bar{\sigma}$ [9,67]. This property minimized noise contribution coming mainly from the image digitization and gave us the opportunity to correct for image blurring as introduced in the next section.

To go further and better use the large amount of data that can be obtained nowadays from image analysis of QSGUVs as seen by video microscopy, one can study the distributions of the fluctuating amplitudes when the autocorrelation function is decomposed into its Fourier components instead of limiting ourselves to their averages. Using Eq. (3.9), we have:

$$\xi(\gamma,t) = \sum_{0<m}^{n_{\max}} \chi^m(t)\cos(m\gamma) \qquad [3.11]$$

the fluctuating amplitudes $\chi^m(t)$ being characterized by Boltzmann-like distributions:

$$\Gamma^m(\chi^m) \propto \exp\left[-R^m(k_c/k_B T,\bar{\sigma})\frac{\chi^m}{2}\right] \qquad [3.12]$$

where

$$R^m(k_c/k_B T,\bar{\sigma}) = \frac{2\pi k_c}{k_B T} \Big/ \sum_{n\geq m}^{n_{max}} \varsigma_n^m(\bar{\sigma})$$

and

$$\varsigma_n^m(\bar{\sigma}) = \frac{\left[\Theta_n^m(\pi/2)\right]^2}{(n+2)(n-1)[\bar{\sigma}+n(n+1)]}.$$

This approach, presented recently in more detail in Ref. [43], leads to much higher precision in the determination of bending elasticity of GUVs than the other methods previously published in the literature.

2.3. Technical aspects

Phase contrast microscopy produces B&W images that look very simple. After a subtraction of the background, that is, a very simple image treatment designed to correct for imperfections along the optical path of the microscope, one obtains pictures such as those in Fig. 3.1. The contour of the vesicle is seen as a black thick line limiting the inner region of the liposome. Following a radius from the center of the contour to the outside environment of the liposome, the intensity is roughly constant as a middle grey while it becomes very small (darker) when crossing the contour location whose thickness is typically 4–8 pixels wide [67]. The image software had to follow this contour shape and to fit its intensity profile to precisely determine the position of the membrane on this radius located by an angle φ, seen in Fig. 3.2. Reducing the image resolution by a decrease in the number of pixels describing the digitized image or deteriorating the contrast of the video had, naturally, a very large impact on the quality and reproducibility of the bending elasticity measurements. These effects were effectively shown to occur in the case of the analysis of the thermal fluctuations of QSGUVs, using either numerical simulations or experimental procedures as demonstrated in Ref. [67], the consequences on the precision

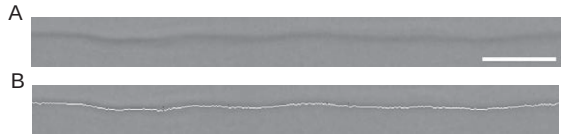

Figure 3.2 Flat representations of the region enclosing the membrane of the vesicle shown in Fig. 3.1A (bar length equal 10 μm), before (up) and after (down) contour extraction (the membrane position is localized by the white thin line).

and reproducibility of bending elasticity measurements being minimized when choosing to decompose the autocorrelation function as a sum of Legendre polynomials instead of direct Fourier analysis. It can be noted that even if the last 10 years have seen a significant improvement in the video resolution, the limiting factor is still the optical resolution of the microscope setup.

At the end of the 1980s, any procedure that involved image treatment needed very expensive tools both for digitization of analogical videos and for image analysis. As a consequence, the published methods for bending elasticity measurements were limited to the use of a small number of images to study and understand thermal fluctuations of QSGUVs. In our case, for example, we were effectively dealing with 400–500 images of a typical video sequence of about 7 min (1 digitized image per second). Associated to highly contrasted images of the GUV contours, this number was most of the time sufficient to get the time average of the autocorrelation function and its corresponding Legendre polynomial amplitudes with reasonable standard errors. Nowadays, digitization problems are directly solved by the acquisition of one of the relatively cheap digital cameras that can be adapted to any optical microscope. Such a digital camera can be attached either to a numerical video recorder or directly connected to a computer equipped with a large RAM whose size has been increased greatly compared to the situation 15 years ago. This is the reason why the number of collected video images for measuring bending elasticity of any fluctuating GUV has increased to 2000 [42], 3000–4000 [41,70], or even 15,000 [43,64] during the past years. For comparison, a typical procedure for evaluating bending elasticity of a QSGUV in the late 1980s needed approximately 2 h from the initial recording of the video images using a phase contrast microscope, including user and computation time of big and highly expensive computers, for a number of video images close to 500. Currently, we need almost the same time in our lab for a typical bending elasticity measurement with 30 times more video images, involving a simple and quite cheap laptop computer.

In addition to these above-mentioned experimental limitations (limited image resolution and image number), a more problematic effect was also shown to have a deep impact on the reliability of bending elasticity measurements. Consider a fluctuating vesicle whose movements are detected by a routine video camera. The time needed to form the image on the camera target is typically 40 ms for European video standards. Any slow enough deformation, say a change of the membrane position of about one pixel per second, will be easily followed using such technology as it needs about 25 images to detect a significant movement of the membrane. On the

contrary, a fast motion similar to what is occurring when trying to rapidly reposition the vesicle in the center of the microscope image will produce a fuzzy image of the contour position, increasing its apparent thickness and decreasing its inherent contrast. In the case of a fluctuating vesicle, a similar blurring effect is occurring but its importance will depend first on the vesicle size and second on the typical membrane movement we consider. Simply speaking, we demonstrated that such a video integration effect is dramatic for measuring bending elasticity if the vesicle is very small or if the video camera integration time is artificially increased [9,67]. To limit its direct influence, we first introduced a correction factor for this video integration effect that can be easily applied if we determine independent fluctuation modes as in the case of the Legendre polynomial decomposition of the autocorrelation function introduced above. Second, we also introduced for the first time the usefulness of a short pulse stroboscopic lighting of the microscope, using high power laser source and a rapid optical switch [69]. But unfortunately, this technical device could not be initially applied routinely. Anyway, it was useful to prove the video correction factor we mentioned above to be valid. Therefore, we could safely use it while no other technical solution was available. Later on, other partial solutions were adopted by different groups, including a reduction of the exposure time of the video camera [71]. Thanks to Mitov, it is now possible to adapt on a microscope, instead of the commonly used halogen bulbs, a stroboscopic lighting whose pulse duration is very small (<10 μs) compared to typical times that characterized most of the membrane movement we are analyzing [43,72,73], making obsolete the video correction factor [9,67].

The very last improvement we have made recently is the statistical treatment of the very large amount of data we get when analyzing the shape fluctuations of a QSGUV. The idea behind this statistical approach to contour fluctuation analysis was not only to gain precision in the determination of k_c but also to identify clear criteria of how perfectly, or imperfectly, a given GUV relates to the theory presented originally by Milner and Safran in 1987 [60] and first used for bending elasticity measurements in Refs. [9,67]. This new method is based on the intrinsic Gaussian distributions of the thermal fluctuation statistics. Their analysis allows proper selection, based on unbiased criteria, of GUVs for which the quasi-spherical model applies objectively. Looking for nice and "ideal" QSGUVs, the user is helped with objective arguments to analyze the behavior of the fluctuating vesicles, dealing both with the size of the vesicle in relation to the optical resolution, the statistical dependence (correlation) in the determination of

k_c and $\bar{\sigma}$, and finally, the statistical significance (quality) of the fitting procedure itself (see [43] for a detailed presentation of these criteria). This new approach leads to a better reproducibility of the bending elasticity measurements obtained with different QSGUVs characterized by the same membrane composition. Together, these improvements yield average bending elasticities of unprecedented high precision (standard error less than 1%) as well as a confident measurement of k_c from individual GUVs. This is crucial to detect small changes in the bending elasticity caused, for instance, by environment alterations such as temperature, pH, or solute concentration or by changes in the bilayer composition. It could help the observation of tiny dependences of bending elasticity with physical or chemical factors [17,39,71,74,75]. A further advantage is that the statistical method can also be used when a GUV system becomes inhomogeneous, a situation that may arise due to a chemical reaction or some slow molecular rearrangement that affects individual GUVs differently, as demonstrated in the case of photo-induced lipid peroxidation triggered by some fluorescent dyes [43,75].

3. CONCLUSION

Bending elasticity measurements have been made on many different systems after the first work by Brochard and Lennon [2], most of them using QSGUV thermal fluctuation measurements as presented in this chapter. The reproducibility of the measurement of this physical constant improved greatly after the initial work involving Mitov [9] and it soon became possible to get very close values from one experimental setup to the other, located in different labs, giving confidence in the published values and leading to possible comparison of different system behaviors. Beginning with the simplest one-component lipid bilayers (Table 3.1), it led, for example, to the detection of an anomalous behavior of bending elasticity close to the main phase transition temperature observed independently by us [39] and a Danish team [74]. Binary lipid bilayers were also studied in the literature, the main focus being on the sterol effect in relation to the transition from liquid-disordered to liquid-ordered phase already identified in saturated phosphatidylcholine membranes when mixed with cholesterol (see Table 3.2 and cited references). In parallel, bending elasticity values of some natural lipid mixtures were reported (Table 3.3). Later on, model membranes containing not only lipids but also membrane proteins or amphiphilic peptides were studied (Table 3.4). All these studies allowed isolating some of the molecular

Table 3.1 Bending elasticity k_c of one-lipid bilayer systems in various environments

System	k_c ($\times 10^{-19}$ J)	References
DLPC		
In water ($T \gg T_m$)	0.92 ± 0.05	[39]
	1.20 ± 0.02	[43]
In buffer (pH 7.4) at RT	0.96 ± 0.08	[76]
DMPC		
In water ($T \gg T_m$)	1.27 ± 0.09	[39]
In water at 30 °C	1.15 ± 0.15	[37]
DPPC		
In water ($T \gg T_m$)	1.50 ± 0.09	[39]
DLPC, DMPC, and DPPC at a function of T close to T_m		
In water		[39]
POPC		
In water at RT	1.58 ± 0.03	[41]
	1.29 ± 0.04	[75]
In Tris/EDTA buffer (pH 7.4) and 100 mM NaCl at 30 °C	1.05 ± 0.01	[31,71]
SOPC		
In water at RT	1.27 ± 0.07	[40]
	1.17 ± 0.10	[77]
	1.26 ± 0.26	[42]
In Tris buffer (pH 7.4) at RT	1.81 ± 0.08	[40]
In Tris/EDTA buffer (pH 7.4) at RT	1.50 ± 0.03	[64]
In 0.19 M sucrose (pH 6.83) at RT	1.10 ± 0.04	[78]
In 0.19 M sucrose (pH 4.28) at RT	0.92 ± 0.09	[78]
In sucrose solutions at RT		[77,79]
In 0.2 M sucrose solutions at RT	1.16 ± 0.01	Unpublished
In 0.2 M glucose solutions at RT	1.30 ± 0.4	Unpublished
In maltose solutions at RT		[80]
DPhPC		
In buffer (pH 7.4) at RT	1.17 ± 0.10	[76]

The symbols T and T_m are used for representing the vesicle temperature and the main phase transition temperature.

Table 3.2 Bending elasticity k_c of bilayers containing one phospholipid and a sterol as a function of temperature (in water)

System		k_c ($\times 10^{-19}$ J)	References
DMPC/chol at 30 °C	90/10[a]	2.00 ± 0.01	[17]
	80/20[a]	2.10 ± 0.25	[37]
	70/30[a]	4.10 ± 0.25	[17]
		4.00 ± 0.8	[37]
	50/50[a]	6.10 ± 0.3	[17]
DMPC/chol at 40 °C	90/10[a]	1.84 ± 0.09	[17]
	70/30[a]	3.07 ± 0.13	[17]
	50/50[a]	3.70 ± 0.3	[17]
POPC/chol at 25 °C	90/10[a]	2.24 ± 0.06	[41]
	80/20[a]	2.89 ± 0.03	[41]
	70/30[a]	3.57 ± 0.06	[41]
POPC/lano at 25 °C	90/10[a]	2.13 ± 0.05	[41]
	80/20[a]	2.51 ± 0.05	[41]
	70/30[a]	2.94 ± 0.02	[41]
POPC/ergo at 25 °C	90/10[a]	1.88 ± 0.05	[41]
	80/20[a]	2.20 ± 0.07	[41]
	70/30[a]	2.25 ± 0.05	[41]
SOPC/chol at RT	50/50[a]	2.96 ± 0.33	[42]

[a]DMPC/chol 90/10 indicates a bilayer with 90 mol% DMPC and 10 mol% cholesterol.

Table 3.3 Bending elasticity k_c of natural lipid mixtures

System	k_c ($\times 10^{-19}$ J)	References
Egg PC in water	0.66 ± 0.06	[12]
	0.42 ± 0.09	[42]
	1.15 ± 0.15	[37]
MGDG in water	$0.15 - 0.40$	[37]
DGDG in water	$0.08 - 0.10$	[40]
RBC lipid extract in water at 37 °C	1.46 ± 0.16	[17]

Table 3.4 Bending elasticity k_c of one-component lipid with another hydrophobic or amphiphilic additive

System	References
EggPC with bacteriorhodopsin	[81]
SOPC with gramicidin D	[40]
DLPC with alamethicin	[76]
DPhPC with alamethicin	[76]
POPC with magainin at 30 °C	[71]

Table 3.5 Symbols and lipid names

Lipid short name	Lipid full name
DLPC	1,2-Dilauroyl-*sn*-glycero-3-phosphocholine
DMPC	1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine
DPPC	1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine
POPC	1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
SOPC	1-Stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
DOPC	1,2-Dioleoyl-*sn*-glycero-3-phosphocholine
DPhPC	1,2-Diphytanoyl-*sn*-glycero-3-phosphocholine
Egg PC	Phosphatidylcholine from egg yolk
DGDG	Digalactosyl diacylglycerol from whole wheat flour
RBC	Red blood cell
Chol	Cholesterol
Lano	Lanosterol
Ergo	Ergosterol

parameters that are responsible for bending elasticity variation, such as the chemical lipid structure or the membrane composition (Table 3.5).

In the future, we can expect a better understanding of the mechanical properties of model or natural membrane systems, especially in the context of strong collaborations between scientists with different specialties, similar to the one we conducted in the past involving Bulgarian physicists and French biophysicists. This will be crucial to gain knowledge and comprehension of the factors influencing membrane mechanical properties of complex systems.

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