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The modified fluorescence based vesicle fluctuation spectroscopy technique for determination of lipid bilayer bending properties



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ABSTRACT

Lipid bilayer is the main constitutive element of biological membrane, which confines intracellular space. The mechanical properties of biological membranes may be characterized by various parameters including membrane stiffness or membrane bending rigidity, which can be measured using flicker noise spectroscopy. The flicker noise spectroscopy exploits the spontaneous thermal undulations of the membrane. The method is based on the quantitative analysis of a series of microscopic images captured during thermal membrane fluctuations. Thus, measured bending rigidity coefficient depends on the image quality as well as the selection of computational tools for image processing and mathematical model used. In this work scanning and spinning disc confocal microscopies were used to visualize fluctuating membranes of giant unilamellar vesicles. The bending rigidity coefficient was calculated for different acquisition modes, using different fluorescent probes and different image processing methods. It was shown that both imaging approaches gave similar bending coefficient values regardless of acquisition time. Using the developed methodology the effect of fluorescent probe type and aqueous phase composition on the value of the membrane bending rigidity coefficient was measured. Specifically it was found that the bending rigidity coefficient of DOPC bilayer in water is smaller than that determined for POPC membrane. It has been found that the POPC and DOPC bending rigidities coefficient in sucrose solution was lower than that in water. Fluorescence imaging makes possible the quantitative analysis of membrane mechanical properties of inhomogeneous membrane.

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1. Introduction

The mechanics of biological structures is a critical property for proper functioning of any biological system. [1–3] Whereas most of research is devoted to the mechanics of protein networks alone or together with associated cellular membranes, the mechanics of lipid bilayer component of biological membranes is less frequently studied as it is widely viewed as a passive and mechanically irrelevant element of biological systems. There are, however, circumstances where the lipid bilayer mechanics is of critical importance. The flow and redistribution of intracellular membranes requiring continuous shape and topology transformations, accompanied by the lipid and membrane protein sorting, depend on the flexibility of the lipid bilayer alone and/or the lipid bilayer combined with associated proteins [4–7]. Similarly the

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control of cell volume or signal transduction facilitated by mechanosensors requires involvement of mechanically well-defined lipid bilayers [8]. Lipid phase and topology as well as functioning of membraneassociated proteins are all affected by intra-membrane pressure that is manifested by the extent of the bilaver undulation dynamics [9,10]. Membrane thermal fluctuations are necessary element of cell mobility and protein fibre-based trafficking of endo-membranes [11]. The mechanics of lipid bilayer is a complex material property that can be described by different theoretical models used for the identification of quantitative parameters, which experimentally determined values depend on the quality of technical infrastructure and type of methodology used [12]. The conceptual perception of the biological membrane used to formulate a quantitative biophysical theory is a direct consequence of model first proposed by of the Singer and Nicolson (fluid mosaic model [13]). In this model the lipid bilayer is a scaffold in which various proteins are immersed in or associated with. Consequently, the most simple theoretical model of the biological membrane assumes that its mechanical properties can be reduced to these of the lipid bilayer [14]. In most of the cases the lipid bilayer can be considered as a two-dimensional surface, since its thickness (about 5 nm) is orders of magnitude smaller than the size of closed a biological membranes

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like cells or giant unilamellar vesicles $(3-100 \,\mu\text{m})$. In this representation the elastic energy of the membrane can be described by Helfrich Eq. (1).

$$E_{el} = 2\kappa (H - C_0)^2 + \kappa_C K \tag{1}$$

where κ is the bending rigidity constant, κ_{G} is Gaussian bending constant, related to the resistance to deformation during membrane topological transformation, c₀ is the spontaneous curvature of the membrane and H and K are the mean and Gaussian curvatures of the membrane surface, respectively [14,15]. To measure mechanical properties of the lipid bilayer, the membrane deformation, caused by various stimuli, needs to be visualized. To perform such experiments the giant unilamellar vesicles (GUV) have been used [16-18]. Typically, the vesicle topology is altered by external stimulus, followed by acquisition of microscopic images of GUVs and image analysis allowing for the determination of membrane mechanical properties [17,19,20]. When thermally induced membrane fluctuations are analysed (the flicker noise spectroscopy technique [21,22]) a large number of rapidly acquired images is required. These images are usually produced with the phase contrast microscopy [17,23-28] or occasionally with the light sheet fluorescence microscopy [29]. Phase contrast images of membranes can be rapidly acquired, contrary to the fluorescence confocal laser scanning microscopy (CLSM), where the image acquisition is slow. However, the CLSM has the capacity to visualize membrane inhomogeneity or its intrinsic biophysical properties (charge, fluidity) making possible the correlation between membrane mechanics and other membrane affecting factors [30,31]. In the paper two fluorescence imaging techniques, several image processing procedures and calculation methods, have been evaluated with the respect to their capacity to produce reliable values of the bending rigidity coefficient. Specifically, the slow but delivering high-contrast CLSM was compared with the fast but producing low-contrast images spinning disk confocal microscopy (SDM). The latter delivers almost instantaneously the images of the entire vesicle, while the former produces images, which are smeared in time. The aim of presented studies was to estimate the effect of an image acquisition technique and image processing method on the experimentally determined bending rigidity coefficient of POPC and DOPC lipid bilayers. In addition, the effect of sucrose of the bending rigidity coefficient of the two membranes was measured.

2. Materials and methods

2.1. Materials

Lipids (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC)), NBD-PC (1-acyl-2-(6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl)sn-glycero-3-phosphocholine) and Rhodamine PE (18:1 Liss Rhod PE 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) ammonium salt) were purchased from Avanti Polar Lipids (USA). β -BODIPY FL DHPE and Atto 488 DOPE were purchased from Life Technologies (USA) and AttoTech (Germany), respectively. Stocked solutions of lipids with fluorescent probes in chloroform were analysed with HPLC (Knauer, Germany) equipped with ELSD detector (Grace, USA) before each experiment. [32,33] Concentrations of fluorophores were determined with UV/VIS spectroscopy using the excitations wavelengths and the extinction coefficient from probe specification sheets. The 18 M Ω deionized water was used in all experiments (ELGA system, Poland).

2.2. Preparation of giant unilamellar vesicles (GUV)

A modified electroformation method, originally developed by Angelova and Dimitrov, [16] was used for model lipid membranes formation. Specifically, $10 \ \mu$ l of POPC in chloroform (0.75 mM) mixed with 1 mol% or 0.5 mol% of fluorescent probe (for SDM or CLSM measurements, respectively) was deposited in small quantities (as 2 µl droplets) onto platinum electrodes. The organic solvent was evaporated with a stream of nitrogen and the electrodes covered with lipid film were kept for 1 h under reduced pressure to remove traces of organic solvents. Next, the electrodes were immersed in aqueous solution and exposed to the AC 1 Hz electrical field for 24 h in a PTFE (Polytetrafluoroethylene) electroformation chamber (Lipid Systems, Poland). Finally, vesicles were transferred into a PTFE observation chamber (Lipid Systems, Poland). The observation chamber design prevents the convection of liquids, therefore enabling extended microscopic observations. Typically, the average diameter of liposomes was equal to 13 ± 6 µm.

2.3. Acquisition of microscopic images

Two confocal microscopic modalities were employed in this study: spinning disk microscopy (SDM) and confocal laser scanning microscopy (CLSM). The Cell Observer SD spinning disk confocal microscope (Zeiss, Germany) was equipped with α Plan-Apochromat 100×/1.46 oil immersion objective (Zeiss, Germany). 512×512 pixels images were recorded with an EMCCD camera (iXon3 885, Andor, UK) using 2×2 binning with 0.133 µm pixel size at a rate of 33 frames per second (fps) with a video integration time of 30 ms. Samples were illuminated with 488 nm (NBD-PC, Atto 488 DOPE and β -BODIPY FL) and 561 nm (Rhodamine-PE) lasers and emitted light was passed through 527/54 and 629/62 filters, respectively. The LSM 510 Meta laser-scanning microscope (Zeiss, Germany) was equipped with a $40 \times /1.2$ water immersion objective (Zeiss, Germany) and PMT detectors. 300×300 pixel images were acquired with 0.13-0.25 µm pixel size at a rate of 3-4 fps with a video integration time of 229-343 ms. Samples were illuminated with excitation wavelengths equal to 477 nm (NBD-PC), 488 nm (Atto 488 DOPE) and 514 nm (Rhodamine PE and B-BODIPY FL) and fluorescence emission was detected using appropriate cut-off filters. All samples were measured at 24 \pm 1 °C. All measurements have been performed in dedicated observation chamber to reduce the effect of uncontrolled vesicle movements. SDM has the value of depth of focus equal to 0.85 µm, while the depth of focus of CLSM equals to 1.38 µm. To improve further the quality of analysis the radius of vesicle was calculated for each image and when the fluctuations of radius was unacceptable large the series of images were discarded from further analysis.

2.4. Fluorescence-based vesicle fluctuation spectroscopy method

The flicker noise spectroscopy is based on the analysis of a vesicle shape fluctuations in time. The reliability of the method depends on the image quality, acquisition time, probing frequency and number of images collected. Whereas phase contrast microscopy is capable of delivering a large number of instantaneous, good quality images the CLSM produces limited number of smeared images due to extended in time acquisition and photo-bleaching effect. The acquisition speed and number of available images can be radically increased by application of SDM. However, due to shorter exposure times the SDM requires application of higher laser power causing photo-destruction of fluorescent probes. Using the SDM series of 5000-10,000 images were recorded with 33 Hz probing frequency, while 1000-2500 images were acquired with 2-3 Hz probing frequency using the standard CLSM. Example of such an image is presented in Fig. 1a. Prior any membrane fluctuation analysis images were pre-processed to remove background noise using the rolling ball algorithm (radius 10) with disabled smoothing using the Image] software. [34] Extraction of the membrane fluctuation spectrum required the determination of the membrane contour location. Typically, this is done by detecting contour extremes and defining contour location as equivalent to the half-height position of the sigmoidal-shape of the radial fluorescence intensity profile, [20,35] by the location of a largest gradient in the signal intensity profile, [23] or by fitting the intensity profile with the Lorentzian



Fig. 1. The main steps of image processing leading to the vesicle contour determination. Panel a shows an example of a fluorescence image of a vesicle. Panel b demonstrates the processed image from panel a prior to the vesicle contour centre determination. Panel c shows linear intensity profiles on vesicle image that are used to determine vesicle cross-section centre location.

function. [36] In the paper, initially the grey-scale images were smoothed using a mean filter matrix of size 5. Next, images were binarized using either Otsu or Triangle algorithms (Fig. 1b). The approximate position of the vesicle contour was determined with the procedure based on the algorithm proposed by Suzuki. Threshold and contour determinations were performed using a software written in C + + with OpenCV (image processing library) [37]. A circle was fitted to predetermined initial vesicle contour using an algebraic procedure based on the Taubin method [38]. This provided the location of the vesicle centre. Next, using the initial non-smoothed image radial intensity profiles (typically 300) were collected and distances between the contour and the vesicle centre were calculated using the Bresenham algorithm as illustrated in Fig. 1c. Specifically, the intensity maximum along the radius of the circle was identified and the Lorentzian function was fitted to pixel intensity values around the intensity maximum. The fit was performed using the Levenberg–Marguardt algorithm [39]. Despite application of well establish image-processing methods, occasional local errors in contour detection may occur. This is due to the low level of the image/background ratio, especially frequent in images generated using the SDM. In order to eliminate such events three image processing procedures were tested. For that purpose 9 β-BODIPY-labelled POPC vesicles suspended in 75 mOsm sucrose solution were used and series of images collected using the SDM. Fig. 2 shows plots of the contour radial position as a function of the angle for DOPC vesicle, when three different image-processing methods were used. The first (smoothing) method is based on the calculation of the firstdegree polynomial regression for the contour fragment. Intensities, which differ by more than six standards deviations from the mean intensity value, were eliminated from further analysis. In the second (polynomial) method the intensity value at each point was extrapolated with polynomial function (of 9th degree) fitted to the whole contour. In cases where the intensity value differed significantly from the extrapolated value it was replaced by the value of the polynomial function. In the third (quadratic) method the quadratic interpolation of the two neighbouring contour points was used to determine contour value at a given radial angle. The three image processing methods were tested and compared on the same large set of images. Selected image of vesicles, represented as distances between the membrane contour and the vesicle centre in polar coordinates, are presented in Fig. 2. To calculate the bending rigidity coefficient from a set of time-lapsed images a correlation between the two dimensional images and three dimensional membrane elasticity model had to be established [14]. This was achieved by means of the angular autocorrelation function $\xi(\gamma, t)$ defined by Eq. 2 [21,36].

$$\xi(\gamma, t) = \frac{1}{2\pi^2 R^2} \int_0^{2\pi} [\rho(\phi + \gamma, t) - \rho(t)] \cdot [\rho(\phi, t) - \rho(t)] d\phi$$
(2)

Cross-sectional radius $\rho(\phi, t)$ is the position of vesicle bilayer at a given angle ϕ and at a given time t; $\rho(t)$ is an averaged vesicle radius of a given image recorded at time t using Eq. 3 as proposed by Pecreaux et al. [20] $R = \langle \rho(t) \rangle$ is the vesicle radius.

$$\rho(t) = \frac{1}{4\pi} \sum_{i=1}^{N} (\rho_i + \rho_{i+1}) \cdot (\phi_{i+1} - \phi_i)$$
(3)

Selected autocorrelation curves for the three image processing method are presented in Fig. 3. Thermally driven membrane undulations were then used to calculate mechanical parameters, the bending rigidity coefficient κ and membrane tension σ of the lipid membrane. All calculations were performed using the MATLABTM 7 software.



Fig. 2. The radial positions of three fluorescently labelled POPC membranes in 75 mOsm sucrose solution recorded using SDM were selected from a large (more than 5000) set of time-lapse images. Panels a, b and c show results of the smoothing and polynomial extrapolation, and quadratic interpolation image processing method, respectively.



Fig. 3. Examples of autocorrelation functions calculated for selected images of POPC vesicles in 75 mOsm sucrose solution treated with (a) smoothing, (b) polynomial extrapolation and (c) quadratic interpolation image processing methods. The images were obtained using SDM.

3. Results and discussion

3.1. The optimization of the image processing procedure

Membrane undulations have been quantified using two procedures: average-based and statistical approaches. [21,24,27,35] In the averagebased approach the angular autocorrelation curve is represented as the Legendre polynomials according to Eq. 4, where P_n are Legendre polynomial of nth degree, n_{max} is an upper mode cutoff defined as a square root of the number of lipid molecules in a membrane of a single vesicle N_{lip} (this value is usually limited to 30 due to smearing of the membrane contour in recorded images [36]) and coefficients $B_n(t)$ are interpreted as amplitudes derived in the decomposition of the angular correlation function in the Legendre polynomial series.

$$\xi(\gamma, t) = \langle B_0 \rangle \cdot P_0(\cos\gamma) + \sum_{n=2}^{n_{max}} B_n(t) \cdot P_n(\cos\gamma)$$
(4)

Since only positive values of $B_n(t)$ had physical meaning, $\langle B_n(t) \rangle$ is an average over positive $B_n(t)$. The numerical values of coefficients $\langle B_n(t) \rangle$ extracted from experimental images are related to physical events such as changes of the vesicle radius $\rho(t)$ (n = 0) or changes of the position of the vesicle centre of mass (n = 1). Thermal fluctuations were assigned to second and higher modes. Recorded GUV images have been averaged over the image acquisition time t_s , therefore, it was not possible to obtain the exact values of B_n coefficients, instead, only the averaged values were available $B'_n = f_n^{corr} B_n$. [24] The correction factor f_n^{corr} is defined by Eq. 5, where t_s is the image acquisition time, a value specific for each type of microscope (for the SDM $t_s = 30$ ms), and τ_n^m is the correlation time defined by Eq. 6, where η is the viscosity of the medium surrounding the vesicle membrane, k is Boltzmann constant and T is temperature. By combining Eq. 5 with definition of B_n the Eq. 7 is obtained. The Eq. 7 can be used to calculate the correlation time τ_n^m . For images obtained using the SDM correction factors, for all modes and all image processing methods, were equal or higher than 0.999 indicating the high quality of the recovered vesicle contour.

$$f_n^{corr} = 2\left(\frac{\tau_n^m}{t_s}\right)^2 \left[exp\left(-\frac{t_s}{\tau_n^m}\right) - \left(1 - \frac{t_s}{\tau_n^m}\right)\right]$$
(5)

$$\tau_n^m = \frac{4\pi\eta R^3}{k_b T} \left(2 - \frac{1}{n(n+1)}\right) B_n(\kappa,\overline{\sigma}) \tag{6}$$

$$B'_{n} = 2\left(\frac{t_{n}^{m}}{t_{s}}\right)^{2} \left[exp\left(-\frac{t_{s}}{\tau_{n}^{m}}\right) - \left(1 - \frac{t_{s}}{\tau_{n}^{m}}\right) \right] \cdot \left(\frac{k_{b}T\tau_{n}^{m}}{4\pi\eta R^{3}}\right) \cdot \left(2 - \frac{1}{n(n+1)}\right)^{-1}$$
(7)

Fitting Eq. 8 to experimental values of amplitudes using the Levenberg–Marquardt algorithm produced the bending rigidity κ and membrane tension σ values. In the fitting procedure the first few modes were omitted since their high level of uncertainty would distort the value of the calculated bending rigidity. High modes (n > 30) were also discarded due to their low detection levels. In Eq. 8 reduced membrane tension $\overline{\sigma}$ defined by Eq. 9 was used to maintain the same magnitude of numerical values. This has reduced the occurrence of the 'machine epsilon' effect in computations.

$$B_n(\kappa,\overline{\sigma}) = \frac{2n+1}{4\pi} \cdot \frac{kT}{\kappa(n+2)(n-1)[\overline{\sigma}+n(n+1)]} \text{ for } n > 1$$
(8)

$$\overline{\sigma} = \frac{\sigma R^2}{\kappa} \tag{9}$$

Values of $\langle B_n(t) \rangle$ coefficients plotted as a function of mode, along with fitted theoretical curves, are presented in Fig. 4. For contours, determined using the smoothing method, the calculated value of the bending rigidity coefficient (determined for 9 POPC vesicles in sucrose) equalled to $(175 \pm 210) \cdot k_b T$ (corresponding to $\kappa = (72 \pm 86) \cdot 10^{-20} J$) and the value of the reduced membrane tension $\overline{\sigma}$ equalled to 1.7. For the contour of vesicles cross-sections, processed using the polynomial extrapolation method, the bending rigidity coefficient equalled to $(31 \pm 11) \cdot k_b T$ (corresponding to $\kappa = (13.0 \pm 4.5) \cdot 10^{-20} J$) and the value of the reduced membrane tension, $\overline{\sigma}$, equalled to 1.4. When contour was determined using the quadratic interpolation method, bending coefficient was equal to $(48 \pm 38) \cdot k_b T$ (corresponding to $\kappa =$ $(20.0 \pm 15.7) \cdot 10^{-20}$ J) and reduced membrane tension, $\overline{\sigma}$, equalled to 1.8. Only contours treated with extrapolation method provided the measured bending coefficient values within the range expected for POPC vesicles

The statistical approach, proposed by Meleard et al., [36] took into account overall distribution of shape fluctuations, which were used for the determination of the bending rigidity coefficient κ . [40,41] In this approach autocorrelation curves represented by the Eq. 10 were represented as a Fourier series, in which sine components were omitted (curves are even functions).

$$\xi(\gamma, t) = \sum_{0 < m}^{n_{max}} \chi^m(t) \cos(m\gamma)$$
⁽¹⁰⁾



Fig. 4. The dependence of B_n coefficients on the mode number along with the fit of the Eq. 8. Panels a, b and c show experimentally determined values of coefficients B_n for contours determined using smoothing, polynomial extrapolation and quadratic interpolation methods, respectively. Images of POPC vesicles in 75 mOsm sucrose solution were rescored using SDM.

Amplitudes of cosine functions for each frame of a given mode m, $\chi^m(t)$, were histogrammed and fitted by mono-exponential distributions $\Gamma^m(\chi^m)$ (Eq. 11, Fig. 5). Frequencies in the distribution were adjusted with a normalization factor equal to the number of counts of the largest frequency.

$$\Gamma^{m} = a \cdot \exp\left(-R^{m}\left(\frac{\kappa}{kT},\overline{\sigma}\right) \cdot \frac{\chi^{m}}{2}\right)$$
(11)

The mono-exponential character of distributions suggests that the model adequately describes the thermal fluctuation of the membrane. Fig. 5 shows frequency distributions Γ^{m} of shape fluctuations for selected amplitudes when images were treated with different image processing methods. The figure shows that the polynomial extrapolation method produced results that were best fitted with the mono-exponential function. For that reason the polynomial extrapolation method was used for further analysis. To determine bending rigidity coefficient from distributions, decays were fitted using mono-exponential function. Fits were carried out for Γ^{m} ranging from 0.6 to 0.08 as suggested by Meleard et al. [36]. Amplitudes bigger than 0.6 were omitted since they are statistically insignificant due to the low probability of occurrence. Amplitudes smaller than 0.08 are too close to the resolution

limit therefore they are also omitted. The quality of fitting was evaluated with residual values calculated according to Eq. 12.

$$\Delta\Gamma^{m} = \frac{R^{m}}{\sqrt{N}} \cdot \sqrt{\sum_{i} \left(\ln \left[\Gamma^{m} \left(\frac{\chi_{i}^{m}}{2} \right) \right] - \ln \left(a \right) + R^{m} \cdot \frac{\chi_{i}^{m}}{2} \right)^{2}}$$
(12)

In order to derive Eq. 12, Eq. 11 was linearized as $\ln(\Gamma^m) = \ln(a) - R^m(\frac{\kappa}{kT}, \overline{\sigma}) \cdot (\chi^m/2)$. The part of Eq. 12 under square root is the linearized distribution residuals while R^m/\sqrt{N} is related to uncertainty of Γ^m slope.

The bending rigidity coefficient can be determine by fitting the Eq. 13 to experimentally determined \mathbb{R}^m values, where σ_{wh} is a superposition of white noise, defined by the optical resolution of microscope, and the electronic noise, generated by the video camera, as defined by H. Bouvrais [42]. \mathcal{P}_n^m are, normalized according to Eq. 14, Legendre polynomials \mathbb{P}_n^m . Finally, $\lambda_n(\overline{\sigma})$ is the function dependent on the reduced membrane tension as defined by Eq. 15.

$$R^{m}\left(\frac{\kappa}{kT},\overline{\sigma},\sigma_{wh}\right) = \frac{1}{\frac{\kappa}{kT}\left(\sum_{n\geq m}^{n_{max}}\frac{\left[\mathcal{P}_{n}^{m}(\mathbf{0})\right]^{2}}{\lambda_{n}(\overline{\sigma})}\right) + \sigma_{wh}^{2}}$$
(13)



Fig. 5. Distributions Γ^m of shape fluctuations for selected mth orders acquired from contours derived using (a) smoothing, (b) polynomial extrapolation and (c) quadratic interpolation methods. The images of POPC vesicles in 75 mOsm sucrose solution were recorded using SDM.



Fig. 6. The dependence of R^m parameter (Eq. 11) as a function of mode m. Best fits of the theoretical curve (Eq. 13) are also presented. Plots in panels a, b and c were derived from contours extracted from images using smoothing, polynomial extrapolation and quadratic interpolation image processing methods, respectively. The images of POPC vesicle in 75 mOsm sucrose solution were recorded using SDM.

$$\mathcal{P}_{n}^{m}(x) = (-1)^{m} \sqrt{\frac{(2n+1)(n-m)!}{4\pi(n+m)!}} P_{n}^{m}(x)$$
(14)

$$\lambda_n(\overline{\sigma}) = (n+1)(n+2)[\overline{\sigma} + n(n+1)] \tag{15}$$

The R^m values plotted as a function of mode, along with fitted Eq. 13, are presented in Fig. 6. The calculated value of the bending rigidity coefficient depends on the method used to process vesicle images. Specifically, when the liposome image was processed using the smoothing method the value of bending rigidity coefficient of 9 analysed vesicles was equal to $(5\pm3)\cdot k_bT$ ($\kappa = (1.9\pm1.3)\cdot10^{-20}$ J) and the value of the reduced membrane tension $\overline{\sigma}$ equalled to 1.9. When vesicle image was treated with the polynomial extrapolation method the derived value of bending rigidity coefficient was equal to $(37 \pm 15) \cdot k_b T$ $(\kappa = (15.1 \pm 6.3) \cdot 10^{-20} \text{ J})$ and the value of the reduced membrane tension $\overline{\sigma}$ was equal to 1.9, but when the interpolation method was used the value of the bending rigidity coefficient was equal to $(19 \pm 9) \cdot k_b T$ $(\kappa = (8 \pm 4) \cdot 10^{-20} J)$ and the value of the reduced membrane tension $\overline{\sigma}$ was equal to 2. In both, interpolation and smoothing, image processing methods values of bending rigidity coefficient calculated using the average-based and statistical approaches were differed. Only the polynomial image processing method delivered consistent results therefore it has been selected for further analysis.

3.2. The effect of geometrical uncertainty of vesicle image reconstruction on the determined values of bending rigidity coefficient

The accuracy of the bending rigidity coefficient measurements depends mainly on the shape of autocorrelation curve, which is related to recurrence of fluctuations of given mode. This depends on the precision of the reconstruction of the vesicle geometry. It has been demonstrated previously by Loftus et al. [29] that certain alterations of membrane images did not affect much the value of the bending rigidity coefficient. The effect of image imperfections on autocorrelation curve and consequently the value of the bending rigidity coefficient was tested using controlled image distortion procedure. Specifically, the contour fragment was substituted with a set of random intensity values drown from the range of all intensities of the contour to simulate either poorly illuminated vesicle fragment or miss-detection of the bilayer edge. The increasing fraction of the vesicle contour was altered and the bending rigidity coefficient calculated. The dependence of the bending rigidity coefficient value on the size of the image distortion, calculated using both, average-based and statistical approaches, are presented in Fig. 7. Results show that the statistical approach is more resilient to contour distortion than averaged-based approach since distorted contour length of 0.13 rad (2% of the whole contour length) was required to alter significantly the value of the bending rigidity coefficient. The average-based approach was more sensitive to the contour



Fig. 7. The effect of the size of the vesicle contour distortion on the value of the calculated bending rigidity coefficient when average-based (a) or statistical (b) method was used. Inset in panel a shows a magnified part of the curve. The images of POPC vesicles were recorded using SDM.

distortion since 0.06 rad distortion (1% of the whole contour) was sufficient to alter bending rigidity coefficient value.

Since radial distances, used to evaluate the membrane fluctuation, may be affected by the precise location of vesicle centre we assessed the dependence of the value of the bending rigidity coefficient on the distortion of the centre positioning. Fig. 8 shows the effect of the vesicle centre repositioning on the determined value of the bending rigidity coefficient calculated with both; average-based and statistical approaches. We found that the value of bending rigidity coefficient, determined using the statistical method, was not sensitive to changes in the location of the vesicle centre. The variation in the vesicle centre location resulted only with an increase of an uncertainty value of the bending rigidity coefficient (Fig. 8b). The average-based approach, on the other hand, was very sensitive to the repositioning of the vesicle centre being detectable when the centre position was changed by as little as 3 pixels (Fig. 8a). The presented results demonstrate the importance of the image processing method and the model of membrane elasticity used for the evaluation of bending rigidity coefficient of the membrane.

3.3. Effects of imaging acquisition method and fluorescent probe type on the determined bending rigidity value

The evaluation of the lipid bilayer mechanical properties using the flicker noise spectroscopy requires acquisition of a series of a large number of images. To increase the probing frequency, which is low for the standard CLSM, the SDM was used. Improved sampling rate, resulting from the increased acquisition speed, was accompanied by the decreased image quality and elevated photo-damage of the fluorophore. Surprisingly, the determined value of the bending rigidity coefficient has not depended on the image acquisition time showing that the probing frequency of the fluctuating membrane is insignificant, as long as enough images were collected so the distribution functions would be correctly determined. The other source of experimental uncertainty may result from the construction of the experimental system itself. All images used for the analysis were based on the fluorescence of a probe incorporated into or associated with the membrane. The fluorescence intensity is sensitive to variety of factors such as photobleaching, polarity of the immediate vicinity of the fluorescent moiety or properties of the aqueous phase, which may affect the probe location with respect to the membrane surface [43,44]. It has been demonstrated previously that fluorescent probes at concentrations up to 2 mol% do not affect mechanical properties of the lipid bilayer [36]. To confirm this observation in our experimental setups POPC liposomes in 75 mOsm sucrose solution were labelled with four different fluorescent moieties (Atto-DOPE 488, B-BODIPY FL DHPE, NBD-PC and RhodaminePE). All tested fluorophores were covalently attached to lipid molecules albeit at different locations with respect to the membrane surface. Specifically, B-BODIPY and NBD moieties are located within the interface of the lipid bilayer [45,46]. Hydrophobic Atto moiety is located in the membrane hydrophobic core, [47,48] whereas hydrophilic rhodamine is located in the aqueous phase adjacent to the membrane surface [49]. Such diversified set of fluorescent probes enabled testing the effect of their physicochemical and optical properties on the determined value of the bending rigidity coefficient. The fluorescent images were acquired using both, the SDM and CLSM as described in the Methods section. The values of the bending rigidity coefficient for individual vesicle as well as their averaged values are presented in Fig. 9. The determined value of bending rigidity coefficient did not depend on calculation method used or on the fluorophore type. The only exception was the sample where membranes were labelled with NBD-PC fluorophore and when images were acquired using the SDM. This is likely due to photobleaching of the dye, since NBD is prone to oxidation and the energy of laser used was much higher in the spinning disk system.

3.4. The effect of aqueous phase composition on the bending rigidity coefficient of DOPC and POPC membranes

DOPC and POPC lipid bilayers have been frequently used as models in biophysical studies, therefore numerous experimental data are available in the literature [17]. These data were used to evaluate the consistency of fluorescent-based image acquisition technique with the traditional, phase contrast-based method. There is an excellent agreement between experimental values of bending rigidity coefficient determine for POPC bilayer using phase contrast microscopy and fluorescence confocal microscopies. In the other experiment images of POPC and DOPC vesicles, labelled by fluorescent probe, were acquired for vesicles formed in water or 75 mOsm sucrose solution using both SDM and CLSM. The bending rigidity coefficients were calculated using both; the average-based and statistical methods. For each sample ten randomly selected vesicles were analysed. The obtained values of bending rigidity coefficients are summarized in Table 1.

Data presented in Table 1 indicates that the bending rigidity coefficients of membrane formed from DOPC in water is smaller than the bending rigidity coefficient of POPC bilayer, which is in agreement with the data presented by others [17,50]. The effect of sucrose on the bending rigidity coefficient of POPC membrane was similar to that observed by others for l-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine (SOPC) bilayers [51,52]. Specifically the presence of sucrose has reduced the POPC membrane stiffness. Identical effect has been observed for DOPC membranes by us and others [53]. Those data show that the



Fig. 8. The effect of the repositioning of the vesicle centre on the bending rigidity coefficient when calculated using the average-based (a) and statistical (b) methods. Dotted lines represent the lower and upper uncertainty level as defined in the text. The images of POPC vesicle were recorded using SDM.



Fig. 9. The effect of fluorescent probe used for POPC vesicle labelling on the bending rigidity coefficient. Vesicles were labelled with β-BODIPY (◄), Rhodamine (▲), Atto (►) and NBD-PC (▼) probes. Images were collected using SDM (top panels) and CLSM (bottom panels). The bending rigidity coefficient was calculated using average-based approach (panels a and c) or statistical approach (panels b and d). The left part of each plot shows values of bending rigidity coefficients obtained for single vesicles whereas the right part shows averaged values.

mechanical properties of the lipid bilayer are the result of a complex interdependences between physical parameters of the sample (temperature), lipid organization and local deformation of lipids (depending on the type of lipid used) and/or interface state (for instance organization of water adjacent to the membrane surface) [17,54–57].

Conclusions

The flicker noise spectroscopy is a method used for the determination of mechanical properties of lipid membranes. The method is based on the quantification of membrane thermal fluctuations from a series of images acquired by phase contrast microscopy. The phase contrast microscopy is an experimental technique performing well when the analysed membrane is homogeneous. The experimental procedure presented in the paper is a modified version of the standard flicker noise spectroscopy, which uses the fluorescence as a contrast for the vesicle contour determination. The fluorescence labelling makes the visualization of any membrane heterogeneities possible. However, the image acquisition using CLSM used for the bending rigidity coefficient determination is inherently slow. To overcome this limitation SDM was used. Despite differences in image acquisition the measured values of the bending rigidity coefficient were similar for all imaging techniques used. This shows that fluorescence-based slow imaging techniques have no effect on the determined value of the bending rigidity coefficient. In addition the sensitivity of the determined value of the bending rigidity coefficient to various image imperfections, image acquisition time and fluorescence probe used was evaluated. When the membrane lipid was changed from POPC to DOPC the value of the bending rigidity coefficient decreased, showing the direct correlation between membrane mechanical properties and molecular organization of lipids. When the POPC and DOPC membranes were immersed in sucrose solution their rigidity decreased in a matter similar to that reported previously for membranes formed from SOPC. Nevertheless, the experimental protocol described in the paper opens the possibility for studies of membranes having intrinsic tendency for domain formation or where its inhomogeneity is induced by the membrane modifications with surface-active compounds (amphiphiles, peptides or proteins).

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Table 1

The effect of the lipid and aqueous phase composition on the value of the bending rigidity coefficient []]. The bending rigidity coefficient of POPC and DOPC lipid bilayers were determined by using the average-based and statistical approaches from images collected by a spinning disk microscope and CLSM.

| Sample (T = 24 $^{\circ}$ C) | SDM — Average-based | SDM — Statistical | CLSM — Average-based | CLSM — Statistical |
|--|---|---|--|---|
| POPC in sucrose POPC in water DOPC in sucrose DOPC in water | $\begin{array}{c} 13.0\pm 4.5\cdot 10^{-20}\\ 22.3\pm 4.2\cdot 10^{-20}\\ 14.1\pm 5.4\cdot 10^{-20}\\ 17.5\pm 9.8\cdot 10^{-20}\end{array}$ | $\begin{array}{c} 15.1\pm 6.3\cdot 10^{-20}\\ 21.2\pm 9.7\cdot 10^{-20}\\ 13.3\pm 4.9\cdot 10^{-20}\\ 17.8\pm 8.2\cdot 10^{-20}\end{array}$ | $\begin{array}{c} 10.5\pm5.8\cdot10^{-20}\\ 20.4\pm5.7\cdot10^{-20}\\ 13.3\pm5.8\cdot10^{-20}\\ 16.6\pm5.6\cdot10^{-20} \end{array}$ | $\begin{array}{c} 11.7\pm7.0\cdot10^{-20}\\ 19.5\pm5.7\cdot10^{-20}\\ 13.8\pm5.7\cdot10^{-20}\\ 17.1\pm5.6\cdot10^{-20}\end{array}$ |

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Appendix A. Supplementary data

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References

- A. Anishkin, S.H. Loukin, J.F. Teng, C. Kung, Feeling the hidden mechanical forces in lipid bilayer is an original sense, Proc. Natl. Acad. Sci. USA 111 (2014) 7898–7905.
- [2] C.J. Miller, L.A. Davidson, The interplay between cell signalling and mechanics in developmental processes, Nat. Rev. Genet. 14 (2013) 733–744.
- [3] E. Moeendarbary, A.R. Harris, Cell mechanics: principles, practices, and prospects, WIREs Syst. Biol. Med. 6 (2014) 371–387.
- [4] J. Fels, P. Jeggle, I. Liashkovich, W. Peters, H. Oberleithner, Nanomechanics of vascular endothelium, Cell Tissue Res. 355 (2014) 727–737.
- [5] H. Mohammadi, C.A. McCulloch, Impact of elastic and inelastic substrate behaviors on mechanosensation, Soft Matter 10 (2014) 408–420.
- [6] J. Oldenburg, J. de Rooij, Mechanical control of the endothelial barrier, Cell Tissue Res. 355 (2014) 545–555.
- [7] N.V. Bukoreshtliev, K. Haase, A.E. Pelling, Mechanical cues in cellular signalling and communication, Cell Tissue Res. 352 (2013) 77–94.
- [8] H. Sprong, P. van der Sluijs, G. van Meer, How proteins move lipids and lipids move proteins, Nat. Rev. Mol. Cell Biol. 2 (2001) 504–513.
- [9] R.S. Cantor, Lipid composition and the lateral pressure profile in bilayers, Biophys. J. 76 (1999) 2625–2639.
- [10] R.S. Cantor, The influence of membrane lateral pressures on simple geometric models pf protein conformational equilibria, Chem. Phys. Lipids 101 (1999) 45–56.
- [11] J.W. Shaevitz, D.A. Fletcher, Load fluctuations drive actin network growth, Proc. Natl. Acad. Sci. USA 104 (2007) 15688–15692.
- [12] K.J. Van Vliet, G. Bao, S. Suresh, The biomechanics toolbox: experimental approaches for living cells and biomolecules, Acta Mater. 51 (2003) 5881–5905.
- [13] S.J. Singer, G.L. Nicolson, The fluid mosaic model for the structure of cell membranes, Science 175 (1972) 720–731.
- [14] W. Helfrich, Elastic properties of lipid bilayers: theory and possible experiments, Z. Naturforsch. 28 (1973) 693–703.
- [15] S.A. Safran, Curvature elasticity of thin films, Adv. Phys. 48 (1999) 395–448.
- [16] M. Angelova, D.S. Dimitrov, A mechanism of liposome electroformation, Progr.
- Colloid Polym. Sci. 76 (1988) 59–67. [17] R. Dimova, Recent developments in the field of bending rigidity measurements on
- membranes, Adv. Colloid Interf. Sci. 208 (2014) 225–234.
- [18] D. Boal, Mechanics of the Cell, Cambridge Univer. Press, New York, 2012.
- [19] F.L.H. Brown, Continuum simulations of biomembrane dynamics and the importance of hydrodynamic effects, Q. Rev. Biophys. 44 (2011) 391–432.
- [20] J. Pecreaux, H.-G. Dobereiner, J. Prost, J.-F. Joanny, P. Bassereau, Refined contour analysis of giant unilamellar vesicles, Eur. Phys. J. E 13 (2004) 277–290.
- [21] J.F. Faucon, M.D. Mitov, P. Meleard, I. Bivas, P. Bothorel, Bending elasticity and thermal fluctuations of lipid membranes. Theoretical and experimental requirements, J. Phys. France 50 (1989) 2389–2414.
- [22] S. Penic, A. Iglic, I. Bivas, F. M., Bending elasticity of vesicle membranes studied by Monte Carlo simulations of vesicle thermal shape fluctuations, Soft Matter (2015).
- [23] J. Henriksen, A.C. Rowat, J.H. Ipsen, Vesicle fluctuation analysis of the effects of sterols on membrane bending rigidity, Eur. Biophys. J. 33 (2004) 732–741.
- [24] J. Genova, V. Vitkova, L. Aladgem, P. Meleard, M.D. Mitov, Using stroboscopic illumination to improve the precision of the bending modulus measurement, Bulg. J. Phys. 31 (2004) 68–75.
- [25] H. Bouvrais, P. Meleard, T. Pott, K.J. Jensen, J. Brask, J.H. Ipsen, Softening of POPC membranes by magainin, Biophys. Chem. 137 (2008) 7–12.
- [26] H.P. Duwe, E. Sackmann, Bending elasticity and thermal excitation of lipid bilayer vesicles: modulation by solutes, Phys. A 163 (1990) 410–428.
- [27] J. Henriksen, J.H. Ipsen, Thermal undulations of quasi-spherical vesicles stabilized by gravity, Eur. Biophys. J. 9 (2002) 365–374.
- [28] R.S. Gracia, R.L. Knorr, R. Lipowsky, R. Dimova, Effect of cholesterol on the rigidity of saturated and unsaturated membranes: fluctuation and electrodeformation analysis of giant vesicles, Soft Matter 6 (2009) 1472–1482.

- [29] A.F. Loftus, S. Noreng, V.L. Hsieh, R. Parthasarathy, Robust measurement of membrane bending moduli using light sheet fluorescence imaging of vesicle fluctuations, Langmuir 29 (2013) 588–594.
- [30] R. Parthasarathy, J.T. Groves, Curvature and spatial organization in biological membranes, Soft Matter 3 (2007) 24–33.
- [31] L. Bagatolli, P.B.S. Kumar, Phase behavior of multicomponent membranes: experimental and computational techniques, Soft Matter 5 (2009) 3234–3248.
- [32] N.C. Megoulas, M.A. Koupparis, Twenty years of evaporative light scattering detection, Crit. Rev. Anal. Chem. 35 (2005) 301–316.
- [33] D.G. Watson, C. Atsriku, E.J. Oliveira, Review role of liquid chromatography-mass spectrometry in the analysis of oxidation products and antioxidants in biological systems, Anal. Chim. Acta 492 (2003) 17–47.
- [34] J. Schindelin, I. Carreras-Arganda, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J.-Y. Tineves, D.J. White, V. Hartenstein, K. Eliceiri, P. Tomancak, A. Cardona, Fiji: an open-source platform for biologicalimage analysis, Nat. Methods 9 (2012) 676–682.
- [35] I. Bivas, P. Hanusee, P. Bothorel, J. Lalanne, O. Aguerre-Chariol, An application of the optical microscopy to the determination of the curvature elastic modulus of biological and model membranes, J. Physique 48 (1987) 855–867.
- [36] P. Meleard, T. Pott, H. Bouvrais, J.H. Ipsen, Advantages of statistical analysis of giant vesicle flickering for bending elasticity measurements, Eur. Biophys. J. E 34 (2011) 1–14.
- [37] G. Bradski, The OpenCV Library, Dr. Dobb's Journal of Software Tools, 2000.
- [38] G. Taubin, Estimation of planar curves, surfaces and nonplanar space curves defined by implicit equations, with applications to edge and range image segmentation, IEEE Trans. PAMI 13 (1991) 1115–1138.
- [39] D.W. Marquardt, An algorithm for least-squares estimation of nonlinear parameters, J. Soc. Ind. Appl. Math. 11 (1963) 431–441.
- [40] H. Bouvrais, T. Pott, L.A. Bagatolli, J.H. Ipsen, P. Meleard, Impact of membraneanchored fluorescent probes on the mechanical properties of lipid bilayers, Biochim. Biophys. Acta 1798 (2010) 1333–1337.
- [41] H. Bouvrais, M. Holmstrup, P. Westh, J.H. Ipsen, Analysis of the shape fluctuations of reconstitued membranes using GUVs made from lipid extracts of invertebrates, Biology Open 0 (2013) 1–6.
- [42] H. Bouvrais, Bending Rigidities of Lipid Bilayers: Their Determination and Main Inputs in Biophysical Studies, Elsevier Inc., Oxford, UK, 2012.
- [43] G.B. Behera, B.K. Mishra, P.K. Behera, M. Panda, Fluorescent probes for structural and distance effect studies in micelles, reversed micelles and microemulsions, Adv. Colloid Interf. Sci. 82 (1999) 1–42.
- [44] A.P. Demchenko, Y. Mely, G. Duportail, A.S. Klymchenko, Monitoring biophysical properties of lipid membranes by environmental-sensitive fluorescent probes, Biophys. J. 96 (2009) 3461–3470.
- [45] R. Sachl, I. Boldyrev, L.B.A. Johansson, Localisation of BODIPY-labelled phosphatidylcholines in lipdi bilayers, Phys. Chem. Chem. Phys. 12 (2009) 27–34.
- [46] D. Huster, P. Muller, K. Arnold, A. Hermann, Dynamics of membrane penetration of the fluorescent 7-nitrobenz-2-Oxa-1,3-diazol-4-yl (NBD) group attached to an acyl chain of phosphatidylocholine, Biophys. J. 80 (2001) 822–831.
- [47] A.S. Klymchenko, R. Kreder, Fluorescent probes for lipid rafts: from model membranes to living cells, Chem. Biol. 21 (2014) 97–113.
- [48] T. Tabarin, A. Martin, R.J. Forster, T.E. Keyes, Poly-ethylene glycol induced superdiffusivity in lipid bilayer membranes, Soft Matter 8 (2012) 43–51.
- [49] A. Kyrychenko, A molecular dynamics model of rhodamine-labeled phospholipid incorporated into a lipid bilayer, Chem. Phys. Lett. 485 (7 December 2009) 95–99.
- [50] N. Kucerka, S. Tristram-Nagle, J.F. Nagle, Structure of fully hydrated fluid phase lipid bilayers with monounsaturated chains, J. Membr. Biol. 208 (2005) 193–202.
- [51] J. Genova, A. Zheliaskova, M.D. Mitov, The influence of sucrose on the elasticity of SOPC lipid membrane studied by the analysis of thermally induced shape fluctuations, Colloids Surf., A 282–283 (2005) 420–422.
- [52] J. Genova, A. Zheliaskova, M.D. Mitov, Monosaccharides (fructose, glucose) and disaccharides (sucrose, trehalose) influence the elasticity of SOPC membranes, J. Optoelectron. Adv. Mater. 9 (2006) 427–430.
- [53] J.F. Nagle, M.S. Jablin, S. Nagle-Tristram, A. Kiyotaka, What are the true values of the bending modulus of simple lipid bilayers? Chem. Phys. Lipids 185 (2015) 3–10.
- [54] L.A. Bagatolli, D. Needham, Quantitative optical microscopy and micromanipulation studies on the lipid bilayer membranes of giant unilamellar vesicles, Chem. Phys. Lipids 181 (2014) 99–120.
- [55] D.D. Lasic, D. Needham, The "stealth" liposome: a prototypical biomaterial, Chem. Rev. 95 (1995) 2601–2628.
- [56] A. Velikonja, S. Perutkova, E. Gongadze, P. Kramar, A. Polak, A. Maček-Lebar, A. Iglič, Monovalent ions and water dipoles in contact with dipolar zwitterionic lipid headroups – theory and MD simulations, Int. J. Mol. Sci. 14 (2013) 2846–2861.
- [57] E. Gongadze, A. Velikonja, Š. Perutkova, P. Kramar, A. Maček-Lebar, V. Kralj-Iglič, A. Iglič, Ions and water molecules in an electrolyte solution in contact with charged and dipolar surfaces, Electrochim. Acta 126 (2014) 42–60.