New optical method for measuring the bending elasticity of lipid bilayers

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Abstract. The knowledge of the elasticity of lipid bilayer structures is fundamental for new developments in biophysics, pharmacology and biomedical research. Lipid vesicles are readily prepared in laboratory conditions and employed for studying the physical properties of lipid membranes. The thermal fluctuation analysis of the shape of lipid vesicles (or flicker spectroscopy) is one of the experimental methods widely used for the measurement of the bending modulus of lipid bilayers. We present direct phase measurements performed on dilute vesicular suspensions by means of a new optical method exploiting holographic microscopy. For the bending constant of phosphatidylcholine bilayers we report the value of $23k_BT$ in agreement with values previously measured by micropipette aspiration, electrodeformation and flicker spectroscopy of giant lipid vesicles. The application of this novel approach for the evaluation of the bending elasticity of lipid membranes opens the way to future developments in the phase measurements on lipid vesicles for the evaluation of their mechanical constants.

1. Introduction

The bending rigidity of lipid membranes is studied in order to quantify their ability to deform, which is relevant to many biomedical applications [1-4]. The deformability of red blood cells, for example, can be affected by various pathologies. It is of major importance in blood circulation and is closely related to the mechanical properties of the cells. In water, lipid bilayers spontaneously form closed structures, which in the case of unilamellar objects with characteristic sizes of the order of tens of micrometers are called giant unilamellar vesicles (GUVs). During the last decades GUVs have been used to probe the membrane properties [5] and cell hydrodynamics in flows [6]. By the application of elaborated experimental protocols, GUVs are reproducibly prepared in laboratory conditions with a good control of the chemical composition of membranes and bathing solutions. Due to the considerable progress so far, the application of advanced experimental methods allows quantifying the membrane bending rigidity [4, 7, 8]. Several decades ago, Schneider et al. [9] have proposed a method for the measurement of the membrane bending constant based on the analysis of the thermal shape fluctuation analysis (or flicker spectroscopy) applied on GUVs. They registered and analyzed the fluctuations of the vesicle diameter in two mutually perpendicular directions. The progress in this direction continued...
with the contributions by Helfrich [10] and Milner and Safran [11], followed by the development of an approach based on the Fourier decomposition of the thermal fluctuations of the vesicle radius [12, 13]. The fluctuation spectroscopy method has been further refined with the introduction of Legendre analysis of the autocorrelation function of the vesicle contour and accounting for the white noise contribution to the calculation of the membrane bending modulus [14]. In the fluctuation spectroscopy, a particularly important step in the image treatment is precisely locating the position of the vesicle contour representing the equatorial cross section of the vesicle with the focal plane of the microscope in each image from the recorded sequence of snapshots [7, 8]. Optical techniques providing information about the fluctuations of vesicle membranes are expected to increase the precision of the bending rigidity measurements. The work presents an experimental evaluation of the bending elasticity modulus of stearoyl-oleoyl phosphatidylcholine membranes by means of a holographic tool that allows performing quantitative phase measurements and reconstituting the vesicle shape.

2. Materials and experimental set-up

Giant unilamellar vesicles (GUVs) were prepared from the lipid 1-stearoyl-2-oleoyl-sn-glycero-3-phospho-choline (Avanti Polar Lipids Inc., AL, USA). A high yield of GUVs with radii of tens of micrometers and without visible defects was obtained by the electroformation method [15, 16]. Methanol and chloroform (for analysis grade) used for dissolving the lipid were provided from Fluka Inc. (Germany).

Prior to the bending elasticity measurements, vesicular suspensions were prepared in the manner that the vesicle membranes enclose 0.2 mol/l of sucrose (Sigma Ultra) in water, while the suspending medium was the iso-osmolar aqueous solution of glucose containing 1 wt% concentration of the biocompatible polymer of glucose, dextran. Sucrose and dextran were provided by Sigma-Aldrich Chemie (Gremany), while glucose was purchased from Merck (Germany). Double distilled water with pH~5.5 was used for the preparation of sucrose and glucose solutions. The measurements of the membrane bending rigidity were performed at room temperature (~22°C).

![Figure 1](image.png)

Figure 1. Schematic view and a photograph of the experimental set-up for the observation of vesicular suspensions: M1, M2 and M3 – mirrors; L1 and L4 – objectives; L2, L3 and L5 – optical lenses; RGG – rotating ground glass; S – sample (for further detail see [17]).

Vesicle fluctuations were observed and registered using a microscopic holographic system described in details elsewhere [17]. Its schematic presentation and photograph are shown in figure 1.
Objectives (Olympus, Japan) with magnification $\times 20$ and numerical aperture NA 0.3 were implemented in the set-up in order to achieve the appropriate optical resolution demanded for the purposes of our study. The experimental parameters of our set-up included as follows: laser wavelength of 633 nm; pixel size of 208 nm; frame rate of 24 frames per second with exposure time of 200 $\mu$s.

3. Results and discussion

Digital holographic microscopy [17] takes benefit of the knowledge of the optical complex amplitude (amplitude and phase) to refocus numerically each slide of an experimental volume. In the experimental protocol, the vesicles were slightly defocused for a correct visualization. Consequently, the optical phase map of the vesicles might be distorted because they don’t correspond to the focus plane of the vesicle. The first step of the processing is thus the determination of the focus plane of each vesicle.

The focus plane of a vesicle was determined by scanning, in depth, a region of interest around the object under investigation and by computing for each plane the gradient of the amplitude modulus. The figure 2 shows an example of the focus curve. Once the focus plane for the object determined, we computed the amplitude and the phase map in that plane.

![Figure 2. Focus determination; amplitude modulus gradient (arbitrary unit) vs. depth reconstruction distance (µm).](image)

Phase maps were unwrapped in order to remove the discontinuities (phase jumps) with a period of $2\pi$. Examples of wrapped and unwrapped phase maps are shown in figure 3. The background phase (phase obtained when no object is present in the field of view) was approximated by a bi-dimensional quadratic phase map. For every studied vesicle the background phase map was fitted on the basis of the phase map in a neighborhood region where no objects were present. The fitted map is then subtracted from the phase image resulting in a flat and constant background.

The measured phase is related to the vesicle diameter and the refractive index difference between the fluid, enclosed by the vesicle membrane and the surrounding aqueous solution. In the present study only the fluctuations of the vesicle diameter laying on the optical axis of the microscope were extracted and analyzed. Therefore, a segmentation process is performed on the vesicle to extract its contour and determine its center. The value of the phase in the center of the vesicle, proportional to the vesicle radius, is then extracted. Its time autocorrelation function is related to the bending modulus and the lateral tension of vesicle membrane. The experimentally acquired data for the fluctuations of the vesicle diameter with time was fitted with the theoretical expression for the autocorrelation function. The fit yielded the values of the two free parameters, namely the bending modulus and the lateral membrane tension for every analyzed vesicle.
Figure 3. Examples of (a) wrapped and (b) unwrapped phase map.

The ensemble of vesicles with goodness-of-fit (see e.g. [18], Chapter 15) higher than 0.1, used for the evaluation of the membrane bending modulus, contained 9 vesicles out of more than 140 recorded and processed. On the basis of this ensemble we calculated for the membrane bending constant and its error the value of $(23 \pm 1) k_B T$, where $k_B$ is the Boltzmann constant and $T$ is the absolute temperature as described in detail in [19]. This experimental finding is in quantitative agreement with our previous results from micromanipulation experiments for the bending elasticity of stearoyl-oleoyl phosphatidylcholine bilayers in aqueous solutions with comparable concentrations of sucrose [20]. It corresponds also to the values of the bending constant of phosphatidylcholine membranes published in the literature so far [21, 22].

4. Concluding remarks
We reported on the determination of the bending rigidity of phosphatidylcholine lipid membranes in aqueous solutions containing carbohydrate molecules by means of a new experimental set-up. It is based on quantitative phase measurements performed on fluctuating giant unilamellar vesicles and the subsequent analysis of the fluctuations of the vesicle diameter along the optical axis of the microscopic imaging system. The complete list describing the advantages of the above-presented approach for the determination of the bending constant for lipid bilayers goes beyond the fully automated data treatment and the potential to reconstitute the tri-dimensional shape of lipid vesicles. Further experimental investigations will shed light on the applicability of the method with other lipid compositions and various aqueous media. Its development and application would be of use in studying the relevance of the physicochemical parameters of the aqueous surroundings to the membrane mechanics.

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