Experimental study of the bending elasticity of charged lipid bilayers in aqueous solutions with pH5

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Abstract. Exposure to high concentrations of contaminations due to air polluting gases, vapours and aerosols and possibly altering the normal pH in the body could lead to undesirable changes in the properties of biological cells. Here, we study experimentally the mechanical properties of synthetic phospholipid bilayers containing increasing molar fractions (up to 0.15) of charged lipid (synthetic phosphatidylserine) in aqueous solutions with controlled ionic strength and at pH 5, which is slightly lower than the physiological values of pH. Our observations in phase contrast and fluorescence testified to the coexistence of two phases in membranes for temperatures below 29°C. Micro-sized inhomogeneities in vesicle membranes were systematically observed at temperatures lower than 29°C and for molar fractions of phosphatidylserine in the bilayer higher than 0.1. For the quantitative determination of the membrane bending rigidity, we applied thermal fluctuation analysis of the shape of quasispherical lipid vesicles. As far as the liquid-crystalline state of the bilayer is a necessary condition for the application of the experimental method, only vesicles satisfying this requirement were processed for determination of their membrane bending rigidity. The value obtained for the bending modulus of bilayers with 0.15 molar content of charged lipid is about two times higher than the bending modulus of uncharged membranes in the same bathing solution. These findings are in qualitative agreement with our previous results for the bending rigidity of charged bilayers, measured by vesicle micromanipulation.

1. Introduction

Air polluting gases, vapors and aerosols are treated as potentially dangerous if their concentration for a certain period of time can become so high as to cause damage to health. Exposure to high concentrations of pollutants influencing the normal pH values (pH 5.5) in the body could lead to undesirable consequences for cells’ properties and functioning [1-3]. Mechanical constants of biological membranes are somehow related to all vital processes in cells [4]. In this respect, the investigation of membrane mechanics and its alteration by external factors is of importance to our knowledge about the impact of emissions of various types on living organisms.

Lipid bilayers model some basic physical characteristics of biomembranes such as their permeability and resistance to bending and stretching. In water, lipid bilayers spontaneously form closed structures, which in the case of unilamellar quasispherical objects with diameters of the order of
tens of micrometers are called ‘giant unilamellar vesicles’ (or GUVs). During the last decades GUVs have been exploited as a rough physical model of simple biological cells and have been used to probe the membrane properties [5] and cell hydrodynamics in flows [6].

Thanks to elaborated experimental protocols, GUVs are easy to prepare in laboratory conditions with a good control of the chemical composition of membranes and bathing solutions. Due to the considerable progress achieved so far, the application of advanced experimental methods allows quantifying the membrane bending rigidity [7, 8]. Phosphatidylserine is one of the major phospholipid components in cellular membranes [9] and its contribution to the membrane bending rigidity has been in the scope of our interest so far [10].

The present work aims at the experimental determination of the bending elasticity modulus of phosphatidylcholine lipid membranes, containing various molar concentrations of synthetic phosphatidylserine, in aqueous solutions with pH 5.

2. Materials and methods
Lipid vesicles were prepared from l–stearoyl–2–oleoyl–sn–glycerol–3–phosphocholine (SOPC, 788.14 g/mol, Avanti Polar Lipids Inc., USA) and molar fraction of 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS (sodium salt) 810.03 g/mol, Avanti Polar Lipids Inc., USA) via electroformation [11, 12] in aqueous solutions of 0.19 M of sucrose (C_{12}H_{22}O_{11}, 342.3 g/mol, Sigma-Aldrich, Germany) and sodium salt (NaCl, 58.44 g/mol, INEOS, UK) with different pH. For the preparation of all aqueous solutions double distilled water, obtained by quartz distiller, was used. Hydrochloric acid (HCl, 36.46 g/mol, Sigma-Aldrich, Germany) with concentration of 0.1 M was added in aqueous solutions of sucrose and sodium salt in order to achieve the desired acidity. The electroformation protocols were adapted to the presence of 0.01 M NaCl in the aqueous phase [13]. All pH measurements were performed by a combined pH-meter with Ag/AgCl electrode Lovibond (Germany). Fluorescent label Lissamine™ Rhodamine B 1,2-Dihexadecenoyl-sn-Glycero-3-Phosphoethanolamine, Triethyl-ammonium Salt (Rh-DHPE, Molecular probes, USA) was used for visualisation of the membrane phase separation. For the quantitative determination of the membrane resistance to bending, analysis of the thermal fluctuations of shapes of quasispherical vesicles was applied [7, 14].

Membrane fluctuations of stroboscopically illuminated [15] quasispherical vesicles without defects were observed and recorded by means of an inverted light microscope by Zeiss, Axiovert 100 (Germany) in phase contrast with a thermo-controlled long-working distance oil-immersed objective (100x, NA 1.25). CCD camera (C3582, Hamamatsu Photonics, Japan) was mounted on the microscope and connected to the video input of a frame grabber board (DT3155, Datatranslation, USA) in a computer for proper digitization (768x576 8-bit pixels, pixel size: 0.106 μm/pix). Fluorescence observation regime of the microscope with image intensifier (M4314, Hamamatsu Photonics, Japan) was exploited to visualize phase separations in lipid membranes.

All measurements of the bending rigidity of charged membranes were performed at constant temperature of (30±0.1)°C using thermo-controlled oil-immersed objective (100x, NA 1.25) and microscope stage (Zeiss, Germany).

3. Results and discussion
Vesicles with inhomogeneous membranes are not appropriate for contour analysis and determination of the bending elasticity modulus due to the limitations of the applied theoretical model [16, 17] assuming liquid state of the vesicle membrane. Flatt non-fluctuating domains in vesicle membranes were systematically observed at temperatures lower than 29°C and for molar fractions of phosphatidylserine in the bilayer higher than 0.1. Recently, it has been shown theoretically that charged membranes show complex phase behavior at various salt concentrations in the aqueous solution [18]. Up to our knowledge the phase diagram of fully hydrated two-component lipid mixtures (SOPC lipid matrix with DOPS charged lipid) in presence of sucrose (0.19 M C_{12}H_{22}O_{11}) and 0.01 M NaCl at pH 5.0 (0.1 M HCl) has not been explored systematically, yet.
Our observations of DOPS/SOPC vesicles in the mentioned-above aqueous surroundings revealed the existence of micro-sized inhomogeneities in the membranes at temperatures below 29°C. This phase separation was detected by means of phase-contrast microscopy (see figure 1) as well as in fluorescence regime (figures 2 and 3). Figure 1a represents two subsequent images of the equatorial cross-section of a giant vesicle with membrane containing 20 mol% DOPS in SOPC matrix in aqueous solution of sucrose (0.19 M C₁₂H₂₂O₁₁) with 0.01 M NaCl at pH 5.0 (0.1 M HCl). Two adjacent contours of a vesicle, whose membrane is composed of 50 mol% DOPS in SOPC in the same aqueous environment, are shown in figure 1b. Stiffer non-fluctuating parts on the membrane contours of fluctuating vesicles were readily observable during experiment. White arrows on figure 1 point at such flat domains positioned at the equator of observed vesicles.

![Figure 1. Phase contrast microscopy: a) Two-component lipid vesicles (SOPC lipid matrix with 20 mol% DOPS charged lipid) in aqueous solution of sucrose (0.19 M C₁₂H₂₂O₁₁) containing 0.01 M NaCl with pH 5.0, temperature 23°C; b) Two-component lipid vesicles (SOPC lipid matrix with 50 mol% DOPS charged lipid) in the same aqueous solution and at same temperature as in (a). The bars correspond to 10 μm. Arrows point at flat lipid domains (see text).](image)

In order to better visualize the flat non-fluctuating domains in vesicle membranes, fluorescence microscopy was applied. Rhodamine dyes have been commonly used in membrane biophysics although some evidences exist for their contribution to the lipid peroxidation related to large-raft formation in membranes [19, 20]. Rh-DHPE was used in our study with 1.5% molar concentration in the membrane. Initially, observations were performed on vesicles with ternary membranes SOPC/DOPS/Rh-DHPE (0.785/0.2/0.015) in aqueous solutions of sucrose (0.19 M C₁₂H₂₂O₁₁) and sodium chloride (0.01 M NaCl) with pH 5.0 (0.1 M HCl) as presented in figure 3. Micrometric dark
domains were systematically observed on vesicle membranes at T<29°C, testifying to phase separation in bilayers.

Figure 2. Fluorescence microscopy of a ternary lipid vesicle (SOPC lipid matrix with 20 mol% DOPS and 1.5 mol% Rh-DHPE) in aqueous solution of 0.19 M C_{12}H_{22}O_{11} and 0.01 M NaCl with pH 5.0 at: a) temperature 23°C; b) temperature 27°C. The bar corresponds to 10 μm.

Figure 3. Fluorescence microscopy of lipid vesicles with three-component membranes (SOPC lipid matrix with 20 mol% DOPS and 1.5 mol% Rh-DHPE) in double distilled water at: a) temperature 23°C; b) temperature 27°C. The bar corresponds to 10 μm.
Control experiments in double distilled water were performed with the same ternary vesicles in attempts to reveal the contribution of the hydrophilic additives on the phase behavior. Images of such vesicle with ternary membrane in double-distilled water are shown in figure 3. Dark domains can be clearly distinguished on vesicle membranes. Phase separation has been observed in similar ternary membranes composed of natural lipids egg-PC/brain-PS/Rh-DOPE with 0.89/0.1/0.01 molar ratios at temperatures around 15°C (G. Staneva, unpublished).

The experimental fact that flat domains were observed even in two-component SOPC/DOPS membranes (figure 1), provides evidence against the hypothesis for a possible Rh-DHPE-related lipid peroxidation promoting phase separation [19]. Future effort must focus on the exploration of the phase diagram of the studied system and the revelation of the contribution of pH and solutes on the membrane phase state [18].

Our experimental observations led to the conclusion that the studied membranes are in their homogeneous liquid state at temperatures above 29°C and consequently, all measurements of the membrane bending elasticity were performed at T=30°C. At least twenty vesicles were recorded for each DOPS content in the membrane (0, 10 or 15 mol %). Less than a half of the registered vesicles, i.e. around ten vesicles for each DOPS molar fraction, satisfied all criteria for goodness during the contour analysis (Bivas et al., to be published) and were used for calculation of the membrane bending elasticity modulus. The experimental results from thermal fluctuation analysis showed that the presence of 15 mol% of phosphatidylserine in the bilayer lead to almost a double increase of the membrane bending rigidity at pH 5 as depicted in figure 4. Points with the error bars represent values of the membrane bending modulus, calculated by taking into account the error of single measurements. This experimental finding is in qualitative agreement with our previous results from micromanipulation experiments for the bending elasticity of SOPC bilayers containing up to 40 mol% brain-PS [10].

Figure 4. Bending elasticity modulus of two-component membranes (SOPC lipid matrix with DOPS charged lipid) in aqueous solutions containing 0.19 M C_{12}H_{22}O_{11} and 0.01 M NaCl with pH5 (0.1 M HCl).
4. Conclusion
In the present work a systematic experimental study was conducted for the determination of the bending rigidity of phosphatidylcholine lipid (SOPC) membranes, containing increasing molar concentrations (up to 0.15) of synthetic phosphatidylserine (DOPS), in aqueous solutions with controlled ionic strength and at pH 5. Two-phase coexistence was observed by phase-contrast and fluorescence microscopy in membranes with molar fractions of phosphatidylserine in the bilayer higher than 0.1 and at temperatures lower than around 30°C. By means of thermal shape fluctuation analysis of nearly spherical vesicles, the bending elasticity was quantified for homogeneous liquid membranes. The value of the bending modulus of bilayers containing 0.15 molar fraction of charged lipid was obtained to be about two times higher than the bending modulus of uncharged membranes in the same surrounding medium.

Further experimental investigations with higher molar concentrations of DOPS in SOPC membranes and at lower pH values will shed light on how the acidity of the bathing solution alters the bending elasticity of charged membranes, thus helping to elucidate the relevance of the physicochemical parameters of the aqueous surroundings to the membrane mechanics.

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