The Influence of High Pressure on the Bending Rigidity of Model Membranes

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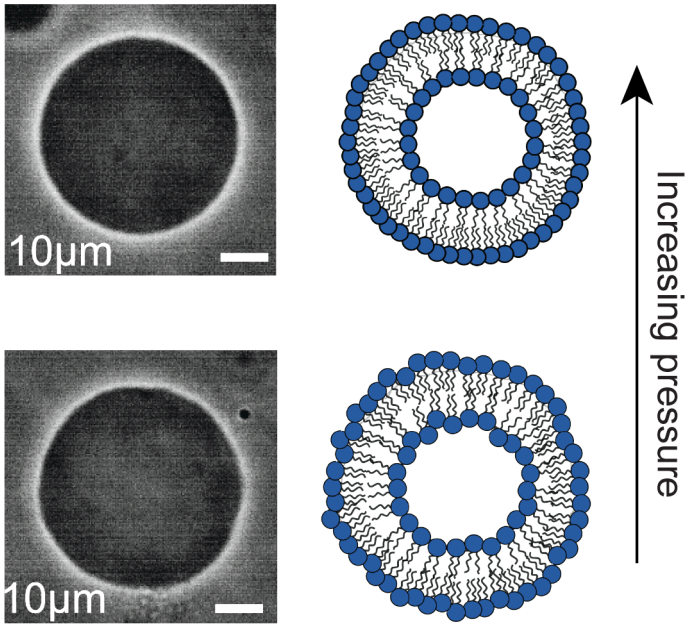
Abstract

Curvature is a fundamental lipid membrane property that influences many membrane-mediated biological processes and dynamic soft materials. One of the key parameters that determines the energetics of curvature change is the membrane bending rigidity. Understanding the intrinsic effect of pressure on membrane bending is critical to understanding the adaptation and structural behavior of bio-membranes in deep-sea organisms, as well as soft material processing. However, it has not previously been possible to measure the influence of high hydrostatic pressure on membrane bending energetics and this bottleneck has primarily been due to a lack of technology platforms for performing such measurements. We have developed a new high pressure microscopy cell which, combined with vesicle fluctuation analysis, has allowed us to make the first measurements of membrane bending rigidity as a function of pressure. Our results show a significant increase in bending rigidity at pressures up to 40 MPa. Above 40 MPa, the membrane mechanics become more complex. Corresponding small and wide angle X-ray diffraction shows an increase in density and thickness of the bilayer with increasing pressure which correlates with the micro-mechanical measurements and these results are consistent with recent theoretical predictions of the bending rigidity as a function of hydrocarbon chain density. This technology has the potential to transform our quantitative understanding of the role of pressure in soft material processing, the structural behavior of bio-membranes and the adaptation mechanisms employed by deep-sea organisms.

TOC Graphic



Keywords: Membranes, bending rigidity, pressure, lipids, fluctuation analysis

Introduction

Lipid membranes are vital in maintaining cellular integrity and play an active role in a wide range of biological functions, including cell signaling, endo- and exo-cytosis, intracellular cargo delivery and generally modulating activity of membrane associated proteins. Many of these processes are facilitated by, or coupled to, changes in the curvature of lipid bilayers.[1] Over 70% of the earth is covered by oceans, and life has been found down to the bottom of the deepest ocean trenches at pressures up to 110 MPa (1100 bar). At these pressure, the membranes of many deep sea organisms contain high proportions of poly-unsaturated fatty acids[2] and increases in lipid chain unsaturation are known to significantly reduce the bending rigidity of model membranes at constant pressure,[3] however the biophysical feedback mechanisms that underpin these membrane composition changes are not well understood. In addition, pressures of up to 40 MPa are known to have a significant effect on general anesthesia,[4] first reversing anesthetic effect at around 20 MPa and then reinstating it by 40 MPa; while this effect is still poorly understood, it has been closely linked with membrane structural properties. Understanding the variation of membrane bending rigidity with pressure is vital both to probe the adaptation mechanisms employed by deep sea organisms in response to their high pressure environment[2] and fully understand the membrane structural changes that occur during anesthetic reversal. Lipid membrane assemblies are also increasingly being employed in biotechnical and soft material applications such as drug delivery,[5] small molecule encapsulation and membrane protein crystallization.[6] The micromechanics of such systems are critical to their function and many of them will be exposed to high pressures during production, processing or use.

Despite the crucial role of membrane curvature both in nature and bio-technology, it has not previously been possible to measure the influence of hydrostatic pressure on the bending rigidity of membranes. A major barrier to probing the curvature properties of soft materials

and model membranes at high pressure has been a lack of technologies that allow such samples to be subjected to pressure while measuring their micromechanical properties. We have addressed this by developing a temperature controlled high-pressure microscope cell that allows giant unilamellar vesicle (GUV) model membranes to be imaged while being held at pressures up to 400 MPa. This is coupled to a microscopy system that allows high speed recording of the GUV thermal fluctuations, which are subsequently analyzed to extract the membrane bending rigidity.

Using this system, we have performed the first measurements of the micromechanical properties of lipid membranes at high pressure. The bending rigidity and membrane tension of dioleoyl-phosphatidylcholine (DOPC) model membranes were investigated at pressures up to 80 MPa (800 bar). Up to 40 MPa, both the membrane bending rigidity and the membrane tension increase significantly with increasing pressure. This is consistent with changes in bilayer structure, probed using high pressure X-ray scattering. Above approximately 40 MPa, the mechanical behavior of the membrane becomes more complex and this is discussed further below.

High Pressure Microscopy System

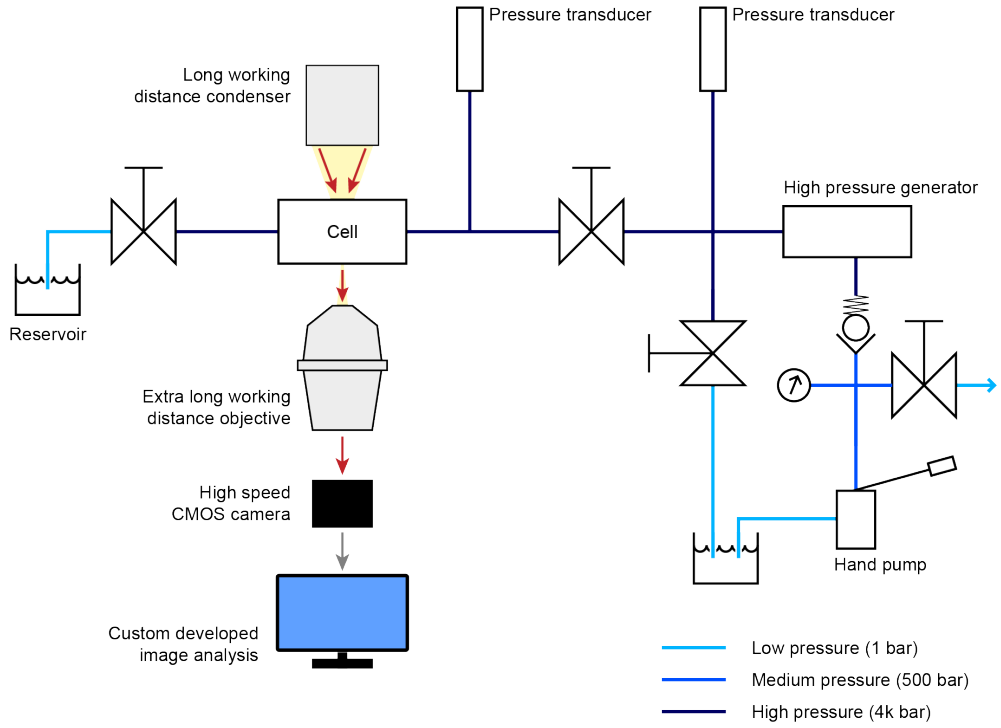


Figure 1. Schematic of the high pressure microscopy system used to image GUVs.

A schematic of the high pressure microscopy system is shown in Figure 1. The pressure cell consists of a high tensile strength stainless steel body with a central bore that contains the sample. The sample chamber is sealed on either side with 2 mm thick, 5 mm diameter fused silica windows which are suitable for use up to 150 MPa (sapphire or diamond windows can be used to access higher pressures). The windows are mounted on metal window holders and sealed with O-rings. Pressures of up to 400 MPa are transmitted to the chamber via a water filled pressure network similar to that described previously.[7] A water circulating channel within the periphery of the cell is connected to a circulating water bath to control the temperature of the cell within the range -10 to 120 °C and the temperature is measured using a PT100 sensor mounted in the cell body close to the sample position.

In addition to the experiments described here, this system has been used to image membrane phase separation at high pressure[8] and we hope that it will facilitate a wide range of measurements of membrane structure and dynamics in the future.

Effect of pressure on the membrane bending rigidity

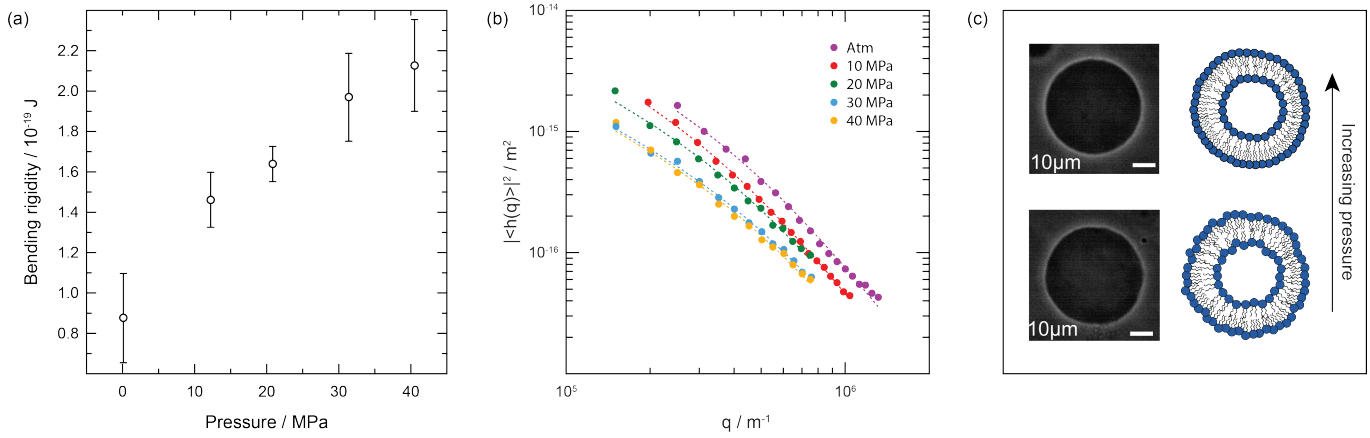


Figure 2. (a) Average bending rigidity of four independent DOPC GUVs, (b) power spectra from the thermal fluctuations of a single GUV and (c) schematic showing the pressure induced reduction in membrane fluctuation.

The thermal fluctuations in GUVs composed of dioleoyl-phosphatidylcholine (DOPC) were recorded at 10 MPa intervals between atmospheric pressure and 80 MPa at a temperature of 24 °C. The recorded fluctuations were used to calculate the membrane bending rigidity as a function of pressure (Figure 2b and see methods section). Due to the stability of the high pressure and imaging systems, it was possible to measure the change in bending rigidity for the same individual vesicles, as the pressure was increased and decreased. Additionally, high pressure small and wide angle X-ray diffraction (SAXS / WAXS)[7] were used to probe the bilayer structure and determine the changes in molecular organization that contribute to the variation in membrane bending rigidity.

The trend of bending rigidity (average of 4 independent vesicles) is shown in Figure 2a: As the pressure increases, the bilayer bending rigidity rises significantly, increasing by a factor of approximately 1.9 between atmospheric pressure and 40 MPa.

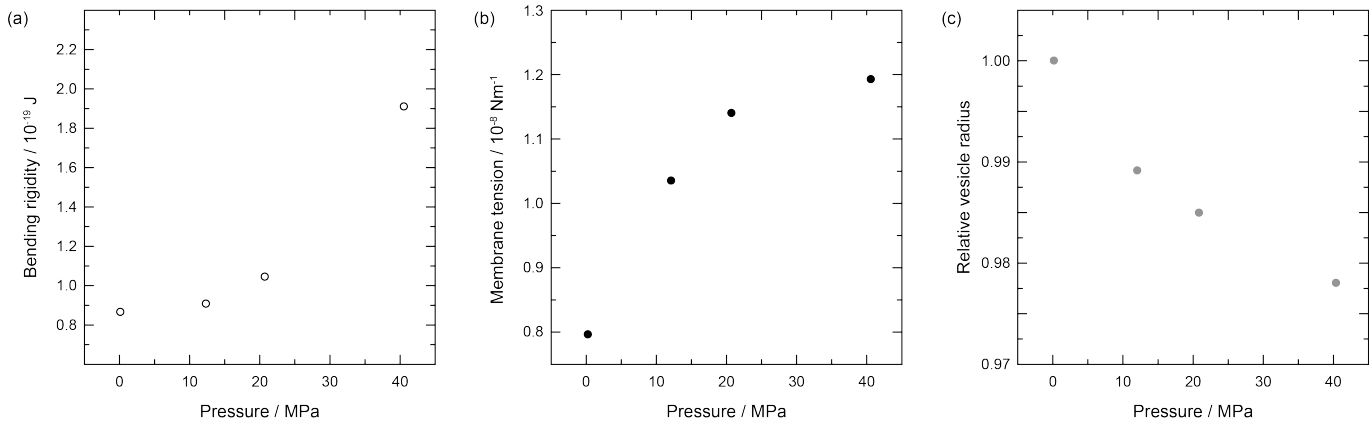


Figure 3. The (a) bending rigidity, (b) membrane tension and (c) relative radius of a single DOPC GUV model membrane at pressure between atmospheric pressure and 40 MPa at 24 °C.

Individual vesicles show similar trends in their bending rigidity with increasing pressure (Figure 3a) and in addition there is a reduction in the vesicle radius (Figure 3c) due to isotropic compression of the water core and an increase in membrane tension (Figure 3b) because the lipid bilayer compresses laterally at a greater rate than the reduction in surface area of the aqueous core.

Pressure induced structural changes in uncharged (or zwitterionic) lipid systems tend to be dominated by changes in the hydrocarbon chain region.[9] The interactions between lipid hydrocarbon chains are a complex combination of Van der Waals, steric, and entropic or fluctuation forces,[10] however hydrostatic pressure will always drive a reduction in volume. The net result is restriction of the hydrocarbon chain motion and lateral compression of the bilayer (i.e. a reduction in the area per lipid molecule, and thickening of the bilayer).[11] These structural changes in the lipid hydrocarbon chains can be probed using WAXS: the position of the broad WAXS peak (characteristic of a fluid bilayer) can be related to the inter-chain spacing. High pressure WAXS data (Figure 4a) show significant lateral compression of the

lipid chains on increasing pressure, with a reduction in the average chain spacing from 4.58 to 4.48 Å (approximately 2 %) between atmospheric pressure and 80 MPa.

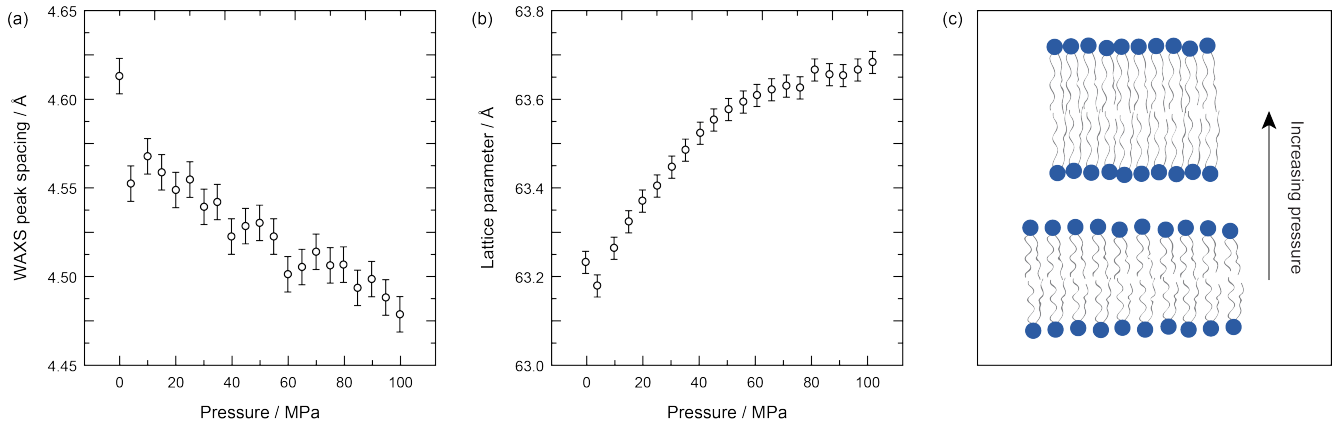


Figure 4. (a) The position of the WAXS peak for DOPC and (b) the inter-bilayer spacing of the DOPC fluid lamellar phase measured using SAXS at pressures between 0.1 and 100 MPa. (c) Schematic showing the lateral compression and thickening of a bilayer under pressure.

The bilayer thickness is difficult to probe directly while ensuring full hydration of the lipids, however SAXS can be used to determine the bilayer repeat distance (lattice parameter) in stacked bilayer lamellar structures under pressure.[12] Figure 4b shows a relatively small but significant increase in the lattice parameter of DOPC in excess water with increasing pressure, from around 63.20 Å at atmospheric pressure to 63.65 Å at 80 MPa. The volume of the aqueous layers between the lipid bilayers will decrease by approximately 4.5% between atmospheric pressure and 100 MPa due to isotropic compression of the water, which corresponds to a 1.5 % reduction in the thickness of this layer. So while the change in lattice parameter can not be directly related to the change in bilayer thickness, there must be a significant increase in the bilayer thickness with pressure.

Simulations of bilayers that consider surfactant molecules as dangling rigid rods with Leonard Jones interactions have shown that decreasing the area per chain can cause a large increase in the membrane bending rigidity[13] which suggests that the reduction in chain

spacing observed here with increasing pressure will contribute to the increase in bending rigidity. Additionally, the bending rigidity of membranes is known to be highly sensitive to the membrane thickness, with at least a thickness squared dependence depending on the complexity of the model used to describe the membrane and coupling between the monolayers,[14] hence the bilayer thickening observed here must also contribute to the increase in bending rigidity.

Comparison of pressure and temperature effects on membrane bending rigidity

The effect of increasing pressure on the structure of lipid bilayers is known to be qualitatively similar to that of decreasing temperature.[9] Increasing temperature has previously been observed to cause a decrease in the bending rigidity of membranes[10, 15] and this effect has been attributed to an increase in the area per lipid molecule and thinning of the bilayers, which corresponds closely to the effects observed here with pressure.

It is worth noting that while the observed relationship between temperature and pressure qualitatively corresponds to the Clausius–Clapeyron relation, this cannot necessarily be quantitatively applied because the system is not at a phase boundary and therefore the free energy for the structural change is not zero.

Change in behavior at higher pressures

The mechanical behavior of the GUV membranes appears to change significantly above approximately 40 MPa. This behavior is rather variable, but generally the vesicle rigidity *decreases* to approximately that observed at atmospheric pressure and the membrane tension reduces significantly, while the vesicle radius does not show any discontinuity. We believe that this may be due to local loss of membrane integrity driven by the highly increased membrane tension, and may be related to budding previously observed in vesicles under pressure.[16]

Conclusions

By developing a novel high pressure microscopy system we have been able to make the first measurement of the effect of high hydrostatic pressure on the bending rigidity of model membranes. Increasing hydrostatic pressure causes the bending rigidity of DOPC bilayers to increase. SAXS / WAXS shows that increasing pressure causes a decrease in the area per lipid chain and an increase in the bilayer thickness, which in turn leads to an increase in membrane bending rigidity. Significantly, this is direct experimental evidence consistent with theoretical predictions of the relationship between bending rigidity and area per molecule for surfactant bilayers.

Lipid membranes are crucial for mediating protein interactions and so the dramatic effect of pressure on the bending mechanics of model lipid membranes observed here is likely to have significant implications for the regulation and adaptation of bio-membranes in deep sea environments. Interestingly, the decrease in bending rigidity known to result from increased lipid hydrocarbon chain unsaturation[3] opposes the intrinsic increase in lipid membrane bending rigidity with pressure found here at constant composition. This offers the intriguing possibility that the increased concentration of polyunsaturated fatty acids found in the membranes of deep sea organisms[2] may counteract the increase in bending rigidity that would otherwise be caused by their high pressure environment.

Additionally these results may open up new possibilities in smart pressure-responsive vesicle delivery systems. The technology that has facilitated these experiments is relatively accessible and easy to use, and we hope that it will catalyze a wide range of experiments on the high pressure behavior of biological and bio-mimetic membrane constructs in the future.

Experimental methods

GUV imaging

The pressure cell (Figure 1) was mounted on a Nikon TE-2000E inverted microscope equipped with phase contract optics that allows clear visualization of the GUVs. All experiments were performed using a 20X magnification extra-long working distance objective lens (ELWD 20X, NA 0.40, WD 11mm, Nikon). A 200 μm thick flat PTFE O-ring was mounted on objective lens side window of the pressure cell using adhesive tape, this was then filled with approximately 2 μl of the diluted GUV suspension and sealed using a round cover slip (60 μm thick, 6 mm wide, VWR, UK).

A Lumenera Infinity-1 CMOS based camera (Media Cybernetics, UK) was used to image thermal fluctuations in the GUV membrane at 60 frames per second with an exposure time of 1 ms.

Calculation of the membrane bending rigidity

To allow quantitative analysis of the vesicle shape, the GUV contour was extracted from each video frame with sub-pixel resolution. The GUV membrane gives a distinct optical signature when imaged using phase contrast optics. The maximum intensity of this profile was taken as the membrane position and was found in approximately 1 degree arcs to sub pixel resolution by radial intensity integration and subsequent cubic interpellation of the intensity profile. This process was developed and parallelized using National Instruments LabVIEW to achieve an analysis rate of approximately 20 frames per second.

Phase contrast microscopy was used to view a two dimensional image of a plane through the vesicle and the microscope was focused to image the vesicle equator.

Membrane bending rigidities were calculated by analyzing the thermal fluctuations exhibited by giant unilamellar (GUV) membranes.[17] We assume a planar approximation of the membrane since all the vesicles studied were 13 μm or larger in diameter. For small

membrane fluctuations, variations in the distance from the membrane to the center of the vesicle can be Fourier transformed to give a fluctuation power spectrum and the mean square amplitude ($\langle h^2 \rangle$) of each bending mode (q_x) at the vesicle equator (y = 0) is given by:

$$\langle h(q_x, y = 0)^2 \rangle = \frac{1}{L} \frac{k_B T}{2\sigma} \left(\frac{1}{q_x} - \frac{1}{\sqrt{\frac{\sigma}{k_c} + {q_x}^2}} \right) \qquad \text{Equation 1}$$

where L is the average circumference of the vesicle contour.[17]

Experimentally, $h(q_x)$ is calculated by taking the Fourier transform of the membrane fluctuations about the mean radius of the vesicle. The vesicle position obtained from each image is represented in polar coordinates as $r_n(\theta)$. The amplitude of the membrane fluctuations about the mean radius ($\langle r \rangle$ taken over all frames) is then given by:

$$f_n(\theta) = r_n(\theta) - \langle r \rangle \qquad \text{Equation 2}$$

The discreet Fourier transform (DFT) of these fluctuations is calculated using the fast Fourier transform (FFT) function built into LabVIEW which calculates a series of complex Fourier coefficients. The real mode amplitudes ($h(q_x)^2$) are then calculated as the squared modulus of the complex Fourier coefficients. Finally, the integer mode number from the FFT are related to the continuous Fourier transform and so q_x by:

$$q_x = \frac{m}{\langle r \rangle} \text{ with } m = 1, 2, \ldots, N/2 \qquad \text{Equation 3}$$

Where N is the total number of arcs integrated around the vesicle circumference.

The amplitudes of the lowest modes are not described well by the planar expression of equation 1, which has been shown to work well for modes $n > 5$.[17] In general, low modes are dominated by the membrane tension, and high modes by the bending rigidity. There are limits at very high modes that come from both spatial and temporal resolution of the measurement. We find here that the amplitudes of the intermediate fluctuation modes (between modes 6 and 20) are represented well by equation 1, which is fitted to extract the tension and bending rigidity. A detailed description of fluctuation mode analysis is available in a number of papers.[17-20]

GUV preparation

GUVs were prepared by electro-formation as described previously.[21] Briefly; approximately 3 μl of 0.5 mg/ml dioleoyl-phosphatidylcoline (DOPC) (Avanti Polar Lipids, AL) in chloroform (VWR, UK) was spread on an indium tin oxide (ITO) coated glass slide (Sigma Aldrich, UK). The solution was allowed to dry in air to form a thin film of lipid and then and kept under vacuum for at least one hour to remove any residual solvent. A sample chamber for formed by placing a1 mm thick polydimethoxysilane (PDMS) frame between the coated ITO plate a second ITO plate without lipid deposited. The chamber was filled with 100 mM sucrose solution (Sigma Aldrich, UK) and a 10 Hz AC potential of 2.6 V (rms) was applied between the ITO coated plates for 3 hours. The potential was then increased to 4.4 V (rms) and the frequency was reduced to 4 Hz for further 45 minutes to allow vesicles to detach from the surface. The GUV preparation system was incubated at 25°C throughout the process.

The GUV suspension was then diluted with a 125 mM glucose solution (1:4 GUV suspension : glucose solution). The higher density of the sucrose inside the vesicles causes them to sink to the bottom of the imaging chamber which makes visualization significantly easier. Additionally, the refractive index difference between the sucrose solution inside the

vesicles and sucrose / glucose solution in the bulk enhances the contrast when imaging the vesicles and the higher osmotic strength of the bulk solution draws water out of the GUVs, reducing the membrane tension and allowing thermally induced fluctuations to be visualized.

Acknowledgments

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Supporting Information Available

(1) Sample images, contours and fluctuation power spectra from an individual vesicle at varying hydrostatic pressure. (2) Schematic diagram of the high pressure microscopy system components. This information is available free of charge via the Internet at http://pubs.acs.org

References

1. McMahon, H. T.; Gallop, J. L., Membrane Curvature and Mechanisms of Dynamic Cell Membrane Remodelling. *Nature* **2005,** *438*, 590-596.

2. Yano, Y.; Nakayama, A.; Ishihara, K.; Saito, H., Adaptive Changes in Membrane Lipids of Barophilic Bacteria in Response to Changes in Growth Pressure. *Appl. Environ. Microbiol.* **1998,** *64*, 479-485.

3. Rawicz, W.; Olbrich, K. C.; McIntosh, T.; Needham, D.; Evans, E., Effect of Chain Length and Unsaturation on Elasticity of Lipid Bilayers. *Biophys. J.* **2000,** *79*, 328-339.

4. Chau, P. L., New Insights into the Molecular Mechanisms of General Anaesthetics. *Br. J. Pharmacol.* **2010,** *161*, 288-307.

5. Kohli, A. G.; Kierstead, P. H.; Venditto, V. J.; Walsh, C. L.; Szoka, F. C., Designer Lipids for Drug Delivery: From Heads to Tails. *J. Controlled Release* **2014,** *190*, 274-287.

6. Cherezov, V., Lipidic Cubic Phase Technologies for Membrane Protein Structural Studies. *Curr. Opin. Struct. Biol.* **2011,** *21*, 559-566.

7. Brooks, N. J.; Gauthe, B. L. L. E.; Terrill, N. J.; Rogers, S. E.; Templer, R. H.; Ces, O.; Seddon, J. M., Automated High Pressure Cell for Pressure Jump X-Ray Diffraction. *Rev. Sci. Instrum.* **2010,** *81*, 064103.

8. McCarthy, N. L.; Ces, O.; Law, R. V.; Seddon, J. M.; Brooks, N. J., Separation of Liquid Domains in Model Membranes Induced with High Hydrostatic Pressure. *Chem Commun (Camb)* **2015,** *51*, 8675-8.

9. Brooks, N. J.; Ces, O.; Templer, R. H.; Seddon, J. M., Pressure Effects on Lipid Membrane Structure and Dynamics. *Chem. Phys. Lipids* **2011,** *164*, 89-98.

10. Pan, J.; Tristram-Nagle, S.; Kucerka, N.; Nagle, J. F., Temperature Dependence of Structure, Bending Rigidity, and Bilayer Interactions of Dioleoylphosphatidylcholine Bilayers. *Biophys. J.* **2008,** *94*, 117-124.

11. Bonev, B.; Morrow, M. R., Effects of Hydrostatic Pressure on Bilayer Phase Behavior and Dynamics of Dilauroylphosphatidylcholine. *Biophys. J.* **1996,** *70*, 2727-2735.

12. Winter, R.; Jeworrek, C., Effect of Pressure on Membranes. *Soft Matter* **2009,** *5*, 3157-3173.

13. Wurger, A., Bending Elasticity of Surfactant Films: The Role of the Hydrophobic Tails. *Phys. Rev. Lett.* **2000,** *85*, 337-340.

14. Bermudez, H.; Hammer, D. A.; Discher, D. E., Effect of Bilayer Thickness on Membrane Bending Rigidity. *Langmuir* **2004,** *20*, 540-543.

15. Niggemann, G.; Kummrow, M.; Helfrich, W., The Bending Rigidity of Phosphatidylcholine Bilayers - Dependences on Experimental-Method, Sample Cell Sealing and Temperature. *J. Phys. II* **1995,** *5*, 413-425.

16. Nicolini, C.; Celli, A.; Gratton, E.; Winter, R., Pressure Tuning of the Morphology of Heterogeneous Lipid Vesicles: A Two-Photon-Excitation Fluorescence Microscopy Study. *Biophys. J.* **2006,** *91*, 2936-2942.

17. Yoon, Y. Z.; Hale, J. P.; Petrov, P. G.; Cicuta, P., Mechanical Properties of Ternary Lipid Membranes near a Liquid-Liquid Phase Separation Boundary. *J Phys-Condens Mat* **2010,** *22*, 062101.

18. Pecreaux, J.; Dobereiner, H. G.; Prost, J.; Joanny, J. F.; Bassereau, P., Refined Contour Analysis of Giant Unilamellar Vesicles. *Eur Phys J E* **2004,** *13*, 277-290.

19. Faucon, J. F.; Mitov, M. D.; Meleard, P.; Bivas, I.; Bothorel, P., Bending Elasticity and Thermal Fluctuations of Lipid-Membranes - Theoretical and Experimental Requirements. *J Phys-Paris* **1989,** *50*, 2389-2414.

20. Gracia, R. S.; Bezlyepkina, N.; Knorr, R. L.; Lipowsky, R.; Dimova, R., Effect of Cholesterol on the Rigidity of Saturated and Unsaturated Membranes: Fluctuation and Electrodeformation Analysis of Giant Vesicles. *Soft Matter* **2010,** *6*, 1472-1482.

21. Angelova, M. I.; Dimitrov, D. S., Liposome Electroformation. *Faraday Discuss.* **1986,** *81*, 303-311.