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Fluorescence Microscopy to Study Pressure Between Lipids in Giant Unilamellar Vesicles

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Summary

The authors developed a technique to apply high hydrostatic pressure to giant unilamellar vesicles and to directly observe the consequent structural changes with two-photon fluorescence microscopy imaging using high numerical aperture oil-immersion objectives. The data demonstrate that high pressure has a dramatic effect on the shape of the vesicles, and both fluidity and homogeneity of the membrane.

Key Words: Microscopy; pressure; vesicles.

1. Introduction

Lipid membranes are the envelopes that separate the interior of the cell and its organelles from external media. Extensive research in the past two decades has shown that the lipids forming the matrix of the cell membranes do not merely play a structural role but have a crucial function in regulating many vital processes *(1,2)*. Recently, much attention has been paid to the dynamics of lipid interaction and organization in the membrane. In particular, the lipid phase determines the fluidity of the membrane, and thus, regulates the diffusion of membrane proteins. Many studies have pointed out that microdomains of different fluidity and lipid organization (rafts) exist under physiological conditions in biomembranes. For this reason, the direct visualization of membrane heterogeneity has received particular attention in the past few years. Fluorescence microscopy has proved to be a powerful tool to directly study biological membranes noninvasively under physiological conditions.

Among different artificial membranes, giant unilamellar vesicles (GUV) are particularly good models of cell membranes because of their similarity to cells in terms of their dimensions (10–100 μm), radius of curvature, and lamellarity. Moreover, GUV can be visualized individually under the microscope, allowing researchers to study the local properties of the membrane. The study of the physics involved in lipid–lipid interactions requires perturbation of the system and the eventual observation of how the system reacts to the perturbation. In this chapter, a technique is presented that allows to visualize the local changes in GUV membranes caused by high-pressure perturbation.

The response of biomembranes to pressure has been studied for a variety of reasons: from the need to understand the adaptation of deep-sea organisms to high pressures (up to a 1 Kbar) to the effects of pressure on anesthesia *(3–5)*. Moreover, pressure offers a very powerful means to perturb the biophysical conditions of the membranes, and therefore, can be used to investigate the physics of the interactions of biomolecules contained in the bilayer. Many

studies have been performed to study the response of lipid vesicles to pressure *(6–8)*. However, to the knowledge of the authors no high-pressure study has been conducted on individual vesicles.

As a general observation, membranes in the liquid-crystalline state transform to the gel phase under pressure. Chong et al. put forward the idea of some compensation existing between temperature and pressure effects. As temperature increases, lipid–lipid interactions tend to decrease. On the other hand, high hydrostatic pressure tends to put the lipids closer together, which results in compensation between increasing the temperature and increasing the pressure. In the gel phase, the total volume and surface area occupied by lipids in the membrane is reduced, and water is expelled from the membrane. However, in the case of vesicles a change in the surface area will cause a change in the volume of the entire vesicle. However, unless the internal vesicle volume can adapt to the reduced surface area by expelling the internal water, the membrane will be under stress. Although water will slowly permeate across the membrane, rapid changes in volume cannot be rapidly compensated, and changes in shape are needed to account for the difference in volume-surface area caused by the change in pressure.

An interesting observation is that if the initial pressure is high and the pressure is decreased up to values in which the gel-like membrane becomes fluid, the surface area increases. If the internal volume remains the same, then the shape of the vesicle must change, breaking the spherical symmetry of the vesicle. This departure from the spherical shape will produce regions of different curvature. These regions would have particular properties and determine the local phase state of the membrane. However, these phenomena are transient, because water will eventually leak through the membrane. Therefore, a cycle of increasing–decreasing pressure will show different behavior depending on whether the pressure is increased or decreased. In this chapter a technique is presented that couples high pressure with two-photon fluorescence microscopy. In the setup, hydrostatic pressure is applied to GUVs in aqueous solution contained in a thin-fused silica capillary. The capillary is filled with the GUVs in buffer, sealed and connected to a high-pressure pump. The vesicles inside the capillary can be visualized with high numerical aperture immersion objectives under the microscope. This microscopy method allows the study of local membrane heterogeneities caused by pressure.

2. Materials

1. The experiments reported in this contribution were performed using palmitoyl-oleoyl-3-glycerol-phosphoatidilcholine GUVs, labeled with LAURDAN.
2. Palmitoyl-oleoyl-3-glycerol-phosphatidilcholine (both in powder and in chloroform, 99% pure) was purchased from Avanti Polar Lipids (Alabaster, AL).
3. LAURDAN was purchased from Molecular Probes (Eugene, OR).
4. The pressure is applied by means of a high-pressure pump connected to a cylindrical fused silica capillary with an outer diameter of 360 µm and an inner diameter of 50 µm, as described in **ref. *9***. The thickness of the wall of the capillary (155 µm) enables the use of high numerical aperture oil-immersion objectives. The capillary was purchased from Polymicro technologies (Phoenix, AX).

3. Methods

1. The spectral behavior of LAURDAN at lipid interfaces has been extensively described (*see* **ref. *10*** for a review). LAURDAN emission spectrum is sensitive to the degree of water penetration in the membrane, and therefore, to the degree of lipid packing in the membrane. In the gel phase, the spectrum emission maximum is at 440 nm. In the liquid-crystalline phase, the spectrum shifts to approx 490–500 nm.

2. To quantify the spectral shift of LAURDAN, and thus, the lipid phase, the GP function has been introduced *(11)*, which is defined as:

$$\mathrm{GP} = \frac{I_b - I_r}{I_b + I_r}$$

where I_b and I_r are the intensities at 440 and 490 nm, respectively. High values of GP correspond to highly ordered lipids (gel phase), whereas low values correspond to the disordered, liquid-crystalline phase.

3. GUVs are grown at room temperature following the electroformation method previously described by Angelova et al. *(12)*. The chamber used for the GUV formation was previously described by Bagatolli et al. LAURDAN in dimethyl sulfoxide is added to the lipids dissolved in chloroform in a ratio of 4:100 molar dye:lipid. The mixture is then dried to remove the solvent, and dissolved again in chloroform to a concentration of 0.2 mg/mL. On each of two (2-cm long) platinum electrodes 6 μL of lipid solution is spread. To the chamber 300 μL of nanopure water is added. An AC field with a frequency of 10 Hz and amplitude of 3 V field is applied to the electrodes for 40 min.

4. The vesicles are then detached from the electrodes by gradually decreasing the frequency and increasing the amplitude of the applied field to 0.1 Hz and 8 V. The vesicle solution is then transferred to an Eppendorf tube using a pipet. The capillary is filled up with the vesicle solution applying suction with a syringe. One end of the capillary is sealed using a blow torch. The capillary is then connected to the pressure pump, and secured to a custom-made stage on the microscope. The experimental setup is schematically shown in **Fig. 1**.

5. A home-built, two-photon, two-channel fluorescence microscope is used to image the GUVs in the capillary. Two-photon microscopy allows high-spatial resolution, whereas minimizing out-of-focus photo damage. The design was described in **ref. 13**. A Ti:Sapphire (Mira 900, Coherent Palo, Alto, CA) optically pumped by a Nd:Vanadate laser (Verdi; Coherent) is used as the light source. An excitation wavelength of 780 nm is used. The laser is driven into the microscope by a couple of galvanometric mirrors (Cambridge Technology, Watertown, MA), which allow the scanning of the beam in the *x* and *y* directions. A 63X Zeiss objective (PLAN-apochromat) is used to image the vesicles in the capillary.

6. The fluorescence light is detected by two photomultipliers (Hamamatsu R-5600, Hamamatsu city, Japan). A dichroic filter in the emission path reflects the light with a wavelength smaller than 470 nm and transmits the rest. Two additional interference filters centered about 440 and 490 nm, before the two detectors minimize cross talk and detection of scatter.

7. Intensity images are acquired for both the blue and the green channels. Then, they are processed point by point during data acquisition to yield the GP image. The obtained images are analyzed with the same acquisition software. The background is subtracted, and the average GP for each vesicle in the image is calculated together with the standard deviation.

8. Once a GUV is localized in the capillary, the pressure is increased and images of the GUV at constant pressure intervals are acquired. With this setup, the same vesicle can be imaged as a function of pressure throughout the whole experiment. The GUV does not move significantly during the acquisition of each image; however, in a sequence of images changes in shape and dimension of the GUV can be seen clearly.

4. Notes

1. The data shown were acquired every 200 bars. **Figure 2** shows the series of images taken from a GUV at an atmospheric pressure up to 2500 bars. The top part of the image shows the intensity images in one channel, whereas the bottom part shows the changes in GP. As the pressure is increased, an increase in the average GP value and a decrease in the diameter of the vesicle can be clearly observed, whereas the vesicle maintains its spherical shape. A volume reduction of about 40% was estimated.
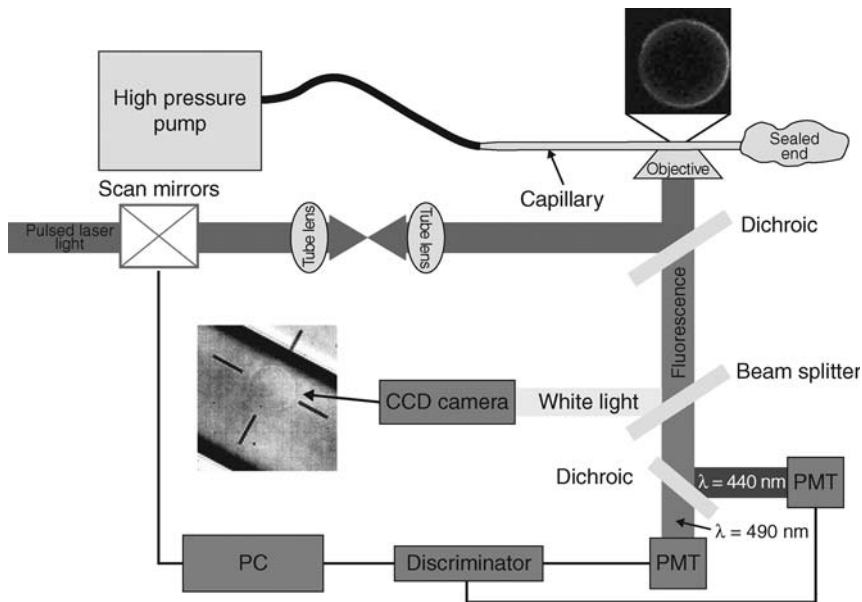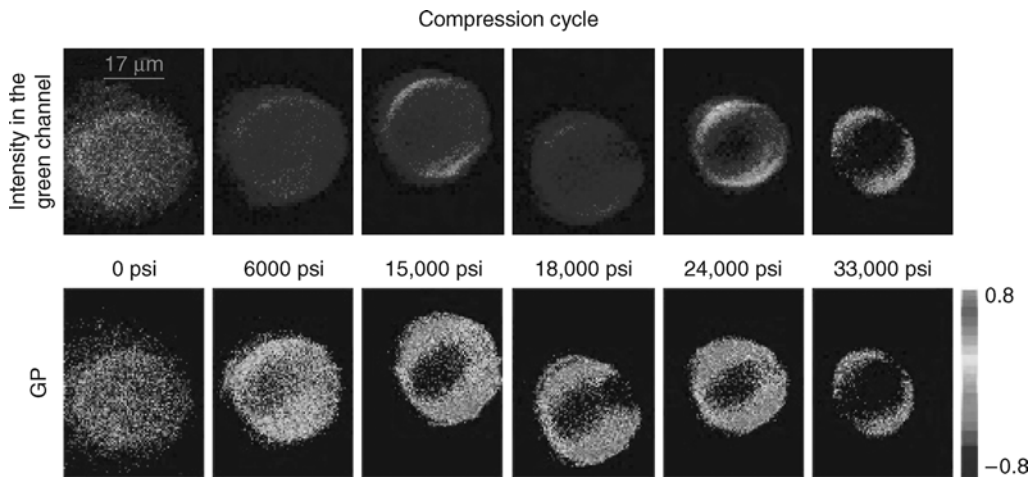
Fig. 1. Experimental setup.

Fig. 2. Effects of compression on the GUV. The top series of images show the intensity in the green channel. It is evident that the vesicle shrinks drastically when pressure increases. On the bottom part the GP images are shown. Notice how the GP average value increases with pressure.

2. **Figure 3** shows the effects of decompression on the vesicle. It was noticed that GUV looses its spherical shape around 1500 bars. This is consistent with the observation of shape changes in temperature-driven phase transitions reported in **ref. *14***. As the pressure is further released, it was observed that the vesicle abruptly looses its tension and becomes flabby at a pressure of about 1500 bars.
3. The GP is not homogeneous throughout the whole vesicle. In particular, it can be seen in **Fig. 4** that parts of the membrane with high curvature radius tend to have a higher value of GP.
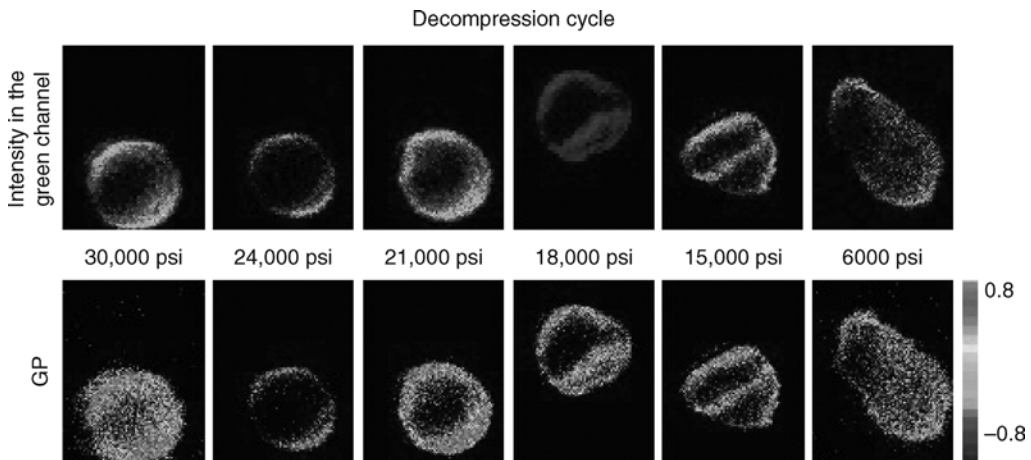
Decompression cycle

Fig. 3. Effects of decompression on the GUV. The most striking effect of decompression is the loss of membrane tension and spherical shape of the vesicle. As the pressure is released, the GUV starts wobbling and becomes very dim. Another interesting observation is that the GP is not homogeneous throughout the vesicle.
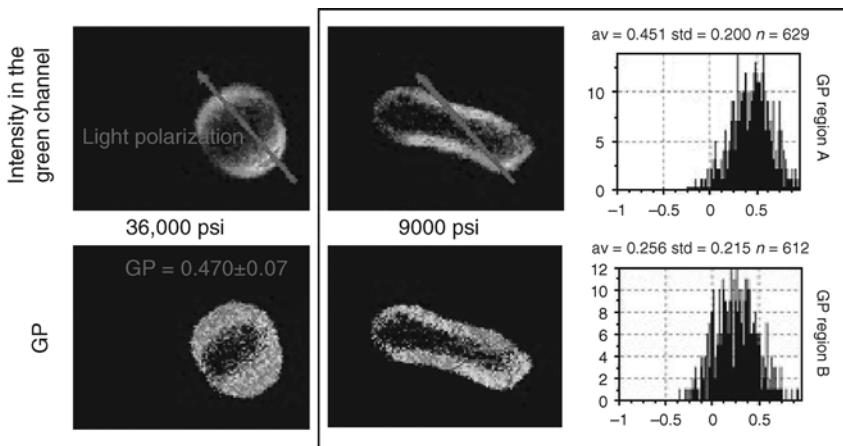

Fig. 4. Inhomogeneity of the GP. At high pressure, the vesicle GP appears to be homogeneous; the variations in intensity are because of the nonperfect circular polarization of the excitation light. The arrow in the intensity images indicates the direction of the polarization of the light. Notice that the high GP regions are not in the direction of the polarization of light for the vesicle at 9000 psi during the decompression cycle. Moreover, it is interesting that the high GP regions appear to be flat. This shows that the technique can be useful to study the correlation between the morphology of the membrane and the degree of lipid hydration and fluidity.

4. A graph of changes in the vesicle GP as a function of pressure clearly shows a transition at a pressure of about 1300 bars (*see* **Fig. 5**). The curve relative to the decompression of the vesicle is very noisy, which reflects the shape hysteresis that the vesicle undergoes and the inhomogeneity of GP values across the membrane.
5. In the experiments, a drastic reduction in the volume of the vesicle was observed. The change in volume of the vesicle is more than the change in volume of water because of the compression of
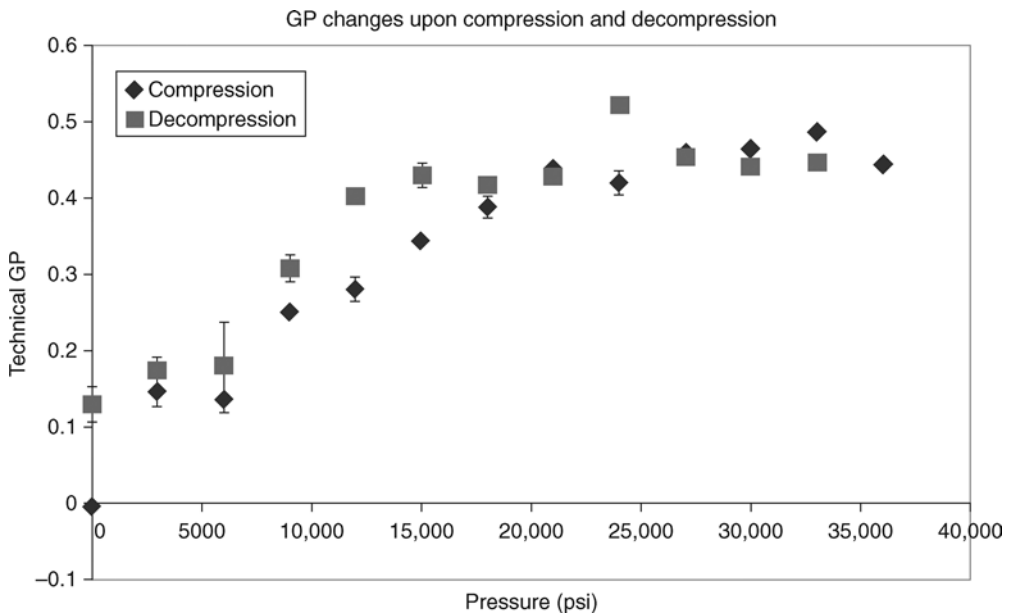
Fig. 5. GP graph. In this graph, the GP value calculated for each pressure is shown. The hysteresis in the curve indicates that the process is not reversible.

water (the change of volume for water is of ~5% at 1000 bar). From this, it is concluded that water is expelled from the GUV during the compression.

6. The increase in the GP value clearly indicates a reduction in the degree of water penetration in the membrane, and therefore, a tighter packing membrane lipids.

7. When the pressure is released, the vesicle starts to "crack" as the packing of the lipids becomes less tight. As the pressure is further released, the lipid surface area increases, and the membrane regain its fluidity. However, the vesicle cannot absorb the amount of water that is ejected during the compression cycle, and therefore, the membrane of the GUV appears to have lost its tension.

5. Conclusions

1. The microscope setup here described allows researchers to directly observe the effect of high pressure on the structure and fluidity of model membranes. The ability to visualize individual vesicles during the compression and decompression cycles enabled the study of the changes in structure and fluidity of the membrane on a local scale. This type of information is lost in steady-state bulk measurements, which is the result of averaging over many vesicles.

2. By observing the GP value locally, the curvature of the membrane can be related with its fluidity. Moreover, the setup here presented allows also the use of other techniques, such as fluorescence correlation spectroscopy, which can give the information about the diffusion of particles in the membrane as a function of pressure.

3. Using the setup here described the authors intend to study the dynamics of phase transition on single lipid membranes as well as on membranes made of lipids mixtures displaying phase separation at given temperatures. This method can also be used to study the behavior of membrane proteins under pressure, and finally, that of live cells.

Acknowledgment

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