# Bending rigidity of SOPC membranes containing cholesterol

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ABSTRACT Bilayer membranes in the fluid state exhibit a large resistance to changes in surface area, negligible resistance to surface shear deformation, and a small but finite resistance to bending. The presence of cholesterol in the membrane is known to increase its resistance to area dilation. In this report, a new method for measuring bilayer membrane bending stiffness has been used to investigate the effect of cholesterol on the bending rigidity of SOPC (1,stearoyl-2,oleoyl-phosphatidylcholine) membranes. The curvature elasticity ($k_c$) for membranes saturated with cholesterol was measured to be $3.3 \times 10^{-19}$ J, ~3-fold larger than that the modulus for cholesterol-free SOPC membrane. These findings are consistent with previous measurements of bending stiffness based on thermal fluctuations, which showed a similar ~3-fold increase in the modulus with cholesterol addition (Evans and Rawicz, 1990, *Phys. Rev. Lett.* 64:2094) and provide further substantiation of the important contribution that cholesterol makes to membrane cohesion and stability.

## INTRODUCTION

On a macroscopic scale, bilayer membranes above the main phase transition temperature exhibit the characteristics of a two-dimensional fluid. They strongly resist changes in surface area but offer negligible resistance to in-plane shear deformation. In addition, they exhibit a small but finite elastic resistance to changes in curvature. This curvature elasticity is important in determining the mechanical stability of unsupported bilayer membrane, and is the major determinant of bilayer membrane flexibility, for example, in thermally driven fluctuations of the membrane contour. A number of investigators have taken advantage of the relationship between the magnitude and frequency of thermally driven surface fluctuations to deduce values of membrane bending stiffness from measurements of surface fluctuations (1–3) or membrane tensions generated by these fluctuations (4). However, interpretation of these measurements may be problematic either because of technical limitations of the measurements or to shortcomings of the analytical models used to interpret the observation. Values of the bending modulus estimated using this approach for lecithin membranes have ranged from $0.5–3.0 \times 10^{-19}$ J. Measurement of thermal tensions in membranes circumvents many of the difficulties of trying to measure the fluctuations directly, and this approach has yielded a value of $0.9 \times 10^{-19}$ J for SOPC (1-stearoyl,2-oleoyl phosphatidylcholine). Recently, a novel approach using a direct mechanical method to measure the bending modulus of bilayer membrane has been developed (5–7). This new approach involves the formation of thin cylindrical membrane strands (called microtubes or tethers[1]) from large thin-walled phospholipid vesicles. Using this approach, a value for the bending modulus of SOPC bilayer of $\sim 1.2 \times 10^{-19}$ J was obtained, in good agreement with the value obtained from measurements of thermal tensions (4).

Cholesterol plays an important role in modulating the mechanical behavior of bilayer membranes. Needham and Nunn (8) performed an extensive study of the effects of cholesterol on the cohesive properties of bilayers composed of different lipids. The surface compressibility is markedly reduced by cholesterol addition, the magnitude of the change depending on the type of lipid composing the membrane. SOPC bilayers exhibited an increase in resistance to area dilation of 3–5 fold (8, 9). This increase in resistance to area dilation is expected to produce similar increases in bending resistance (10). In the present report we use tether formation to measure the bending resistance of SOPC bilayers containing saturating amounts of cholesterol, and confirm the expected increase in stiffness for membranes containing cholesterol.

## METHODS

The procedures for tether formation from giant lipid vesicles and calculation of the bending stiffness from the experimental measurements are described in detail in previous reports (7). Cholesterol, SOPC and POPS (1-palmitoyl,2-oleoyl phosphatidylserine) were obtained from Avanti Polar Lipids (Birmingham, AL). A stock solution of lipids and cholesterol was prepared (~1 mg/ml in chloroform:methanol (2:1)). The lipids were 98% SOPC (mol/mol) and 2% POPS, and these were combined in a ratio of 2:3 (mol:mol), lipid to cholesterol. (These proportions should produce membranes saturated with cholesterol.) The mixture was stored at −10°C under nitrogen. The lipid mixture was dried onto a teflon surface, then rehydrated in 100 mM sucrose to form large thin-walled vesicles over a period of 2–6 days. The vesicles were diluted into ~110 mM glucose and placed in a chamber for viewing via a microscope constructed with its optical axis oriented horizontally. The vesicles were captured in a micropipette (inside diameter, 6.0–10.0 μm) and allowed to adhere to a glass bead of known density.[2] Reducing

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[1] The term tether is the traditional name for these strands (thin bilayer cylinders) and should not be confused with the term "tethered membranes" used to describe two-dimensional entropic networks.

[2] This adhesive interaction occurred spontaneously between lipid and glass for vesicle preparations between 2 and 6 days after rehydration.


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the aspiration pressure holding the vesicle allows the adherent bead to fall away from the vesicle forming a thin, cylindrical membrane strand (tether) between the body of the vesicle and the bead. The force on the tether can be calculated from the bead density and volume. The diameter of the tether can be calculated from the geometric constraints that the surface area and volume of the vesicle are constant and measurements of the change in the length of the vesicle projection in the pipette as the tether length increases. The aspiration pressure needed to establish equilibrium was also measured as a function of the tether length.

The calculation of the bending modulus from these data assumes that the vesicle is in equilibrium when the tether growth rate is zero. The bending stiffness is calculated by:

$$k_c = \frac{(f_{eff} - \gamma L_t)^2}{4\pi^2 \Delta p}\left(\frac{1}{R_p} - \frac{1}{R_v}\right), \quad (1)$$

where,

$$f_{eff} = -2\pi \Delta p R_p^2 \left(\frac{dL_p}{dL_t}\right) \quad (2)$$

$$\gamma = \frac{X f_{eff}}{\Delta p + X L_t} \quad (3)$$

and

$$X = \frac{f_{eff} R_p}{16\pi R_v^3 (R_v - R_p)} - \frac{1}{2}\frac{d\Delta p}{dL_t}. \quad (4)$$

Note that the aspiration pressure $\Delta p$, the pipette radius $R_p$, the vesicle radius $R_v$, the change in projection length with tether length $dL_p/dL_t$, and the change in equilibrium pressure with tether length $d\Delta p/dL_t$ are all measurable quantities. Thus, the effective force $f_{eff}$ is readily calculable from measured quantities, and the parameter $\gamma$ can be calculated from $f_{eff}$ and measurable quantities. Knowing these two quantities the bending stiffness $k_c$ can be calculated via Eq. 1. Details of the derivation of these parameters and their physical significance are described in (7). In addition, the radius of the strand can be calculated according to:

$$R_t = R_p\left(1 - \frac{R_p}{R_v}\right)\left(-\frac{dL_p}{dL_t}\right)\left(1 - \frac{\gamma L_t}{f_{eff}}\right). \quad (5)$$

## RESULTS

The dependence of the vesicle projection length $L_p$ and the equilibrium aspiration pressure $\Delta p$ on tether length are illustrated in Fig. 1 *a* and *b*. These data along with measurements of the pipette radius and vesicle diameter form the basis for calculating the membrane bending stiffness. Multiple measurements (2–5 "pulls") were performed on a total of nine different tethers. The mean values for the bending stiffness determined for the nine tethers ranged from 2.6–4.0 $\times 10^{-19}$ J, with a mean value of 3.3 $\times 10^{-19}$ J, and a standard deviation of 0.4 $\times 10^{-19}$ J. The age of the vesicles tested ranged from 2 to 6 days, and no systematic variation in membrane properties with increasing age was observed during this period. These data were consistent with data obtained in preliminary experiments (11). In those early studies, the change in equilibrium pressure with tether length was not measured, and the bending modulus was estimated assuming that the equilibrium pressure was independent of tether length. (Based on measurements of the dependence of equilibrium pressure on tether length in subsequent experiments, this assumption is expected to lead to an overestimation of the modulus by ~0.4 $\times 10^{-19}$ J.) The measured values of the tether radius as a function of equilibrium aspiration pressure and tethering force in those experiments are indistinguishable from the measurements made in the present study, and the estimated value of the modulus (~3.6 $\times 10^{-19}$ J) was consistent

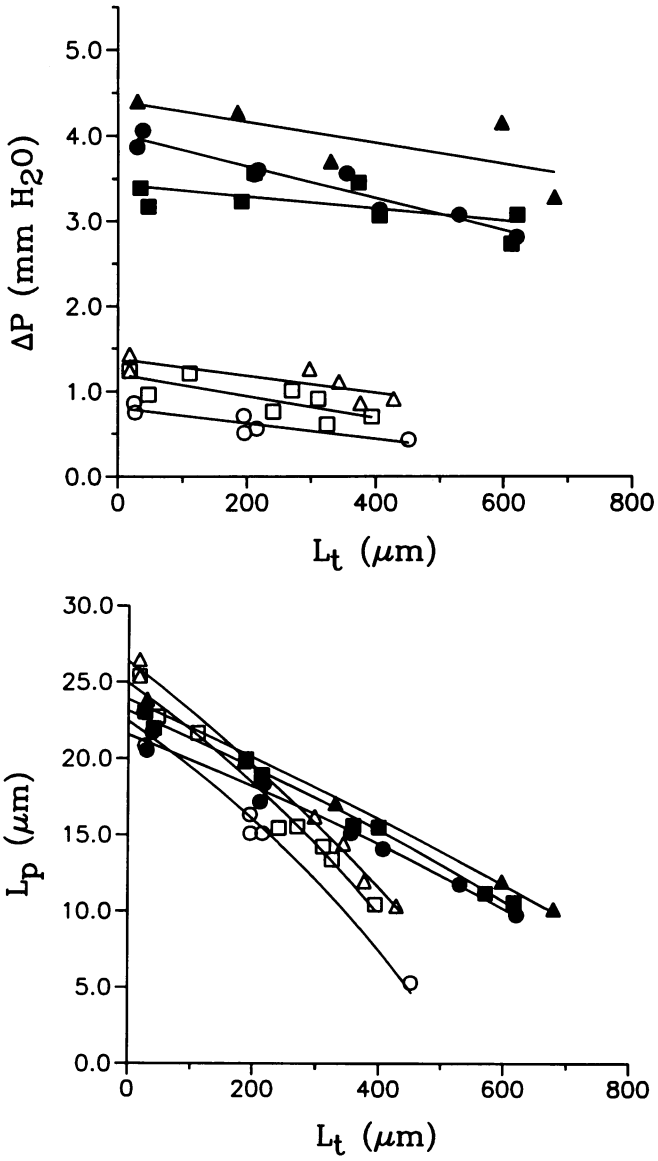


FIGURE 1 Aspiration pressure at zero tether growth rate (*a*) and projection length (*b*) as functions of tether length. Data are shown for 3 successive "pulls" on each of two different vesicles, data for vesicle 1 indicated by solid symbols, for vesicle 2 by open symbols. The tether force on the vesicle 1 was 5.5 $\times 10^{-11}$ N, and on vesicle 2, 3.0 $\times 10^{-11}$ N. The corresponding tether radii were ~40 and ~80 nm, respectively. The bending stiffness determined from these data was ~3.2 $\times 10^{-19}$ J for both vesicles.

The mechanism of the adhesion is not known, but it probably involved electrostatic interactions between the vesicle and the bead.

with the value reported here. An important observation made in the early study was that the value of the modulus was not different when the cholesterol content of the lipid mixture used to form the vesicles was either 43% (a total of 20 tethers measured, $k_c = \sim 3.6 \times 10^{-19}$ J) or 67% (17 tethers measured, $k_c = \sim 3.7 \times 10^{-19}$ J).

## DISCUSSION

The present results show that the addition of cholesterol to lipid bilayer membranes increases the membrane bending rigidity nearly 3-fold. Our findings are consistent with those of other investigators who have documented increased membrane rigidity as a result of cholesterol incorporation. Evans and Rawicz (4) used measurements of thermally generated membrane tension to estimate the bending modulus of SOPC membrane containing 50% cholesterol and found $k_c = 2.5 \times 10^{-19}$ J, nearly 3-fold greater than the value they obtained for $k_c$ of SOPC membrane without cholesterol ($0.9 \times 10^{-19}$ J). Our value for $k_c$ ($3.3 \times 10^{-19}$ J) is slightly larger than theirs, but considering the widely different approaches of measurement, the agreement is quite good. Most interestingly, the ratio of the moduli for membrane with and without cholesterol (2.8) is identical for the two methods.

These findings are also consistent with measurements of changes in membrane area expansivity and the expected relationship between expansivity and membrane thickness. Several investigators have recognized that the bending stiffness of a fluid membrane should be proportional to the product of the area expansivity modulus ($K$) and the square of the membrane thickness ($h$) (10, 12):

$$k_c \propto Kh^2.$$

Measurements of membrane thickness by x-ray diffraction show little effect of cholesterol on membrane thickness (13), and so the bending stiffness is expected to change in proportion to the expansivity modulus. Consistent with our finding of a 3-fold increase in $k_c$ with saturation concentrations of cholesterol, the expansivity modulus increases from a value of ~200 mN/m for SOPC membranes without cholesterol to ~600 mN/m for SOPC membranes plus 40–50 mol% cholesterol (4,8,9). Needham and Nunn (8) observed even larger increases in the expansivity modulus of cholesterol-containing SOPC membranes when the nominal cholesterol concentration was pushed as high as 60% ($K \approx 1{,}000$ mN/m). Thus, our findings that there is no increase in the membrane bending stiffness above a cholesterol concentration of 43% is somewhat surprising. It is interesting that our findings are consistent with differential scanning calorimetry measurements of membrane phase transitions that show that SOPC membranes become saturated when the cholesterol content reached ~40% (14), and neutron scattering measurements that show saturation of fluid dimyristoyl phosphatidylcholine membranes at a mole fraction of cholesterol of ~0.5 (15). On the other hand, Needham and Nunn's data are in exact agreement with recent findings by Fralix et al. (16), that the interaction of water with egg PC membranes containing cholesterol (as assessed by magnetization transfer) exhibits marked changes in the range of 40–60% cholesterol, and does not become saturated until the cholesterol concentration approaches 60%. Taken together these results suggest that although $k_c$ and $K$ are related, the relationship may not be as straightforward as the one given above, and that $K$ may be more sensitive to changes in interactions at the membrane-water interface, whereas bending stiffness may be more sensitive to the state of the lipid acyl chains.

An alternative explanation for the difference between bending stiffness in the vesicle tether and the reported compressibility of the vesicle membrane might be a compositional difference between the tether and the membrane from which it was formed. Consideration of the energies involved in such a separation, however, makes this explanation seem unlikely. We base our estimate on the assumption that the molecules of the tether are exchangeable with the molecules on the vesicle body, and that the tether is formed slowly enough that the process takes place at or near chemical equilibrium. If the compositions of the two regions are different, there must be a contribution to the tethering force equivalent to the work required to move a molecule of lipid from a region in which its mole fraction is $X_1$ to a different region in which the composition is $X_2$. If the membrane consists of an ideal mixture, this contribution is given by:

$$f = \frac{2\pi R_t kT}{\tilde{A}} \ln \frac{X_2}{X_1}.$$

Taking the tether radius $R_t = 30$ nm, $kT = 4 \times 10^{-21}$ J, and the area per molecule $\tilde{A} = 0.5$ nm$^2$, we find that a force on the order of 1 $\mu$dyn ($10^{-11}$ N) could produce a difference in mole fraction of less than 1.0%. Thus, significant differences in composition between the tether and the vesicle body appear unlikely, unless there are discontinuous phases on the vesicle surface, and tethers are formed selectively from only one phase.

A surprising but potentially interesting aspect of our results is that the nonlocal contribution to the bending stiffness (contained in the parameter $\gamma$) was not different from the nonlocal contribution in cholesterol-free membranes (7). The parameter $\gamma$ may represent any recoverable, length-dependent change in the equilibrium state of the tethered vesicle. Although its value might be affected by multiple bilayers associated with the vesicle surface, under ideal conditions it is expected to reflect the differential expansion/compression of the outer/inner membrane leaflets as the tether is formed. Thus, the magnitude of $\gamma$ is expected to increase in proportion to the membrane rigidity, $K$ (7). The fact that $\gamma$

does not change, even though $K$ and $k_c$ have increased 3-fold, might be explained by the facile exchange of cholesterol between the two leaflets. Unlike phosphatidylcholine, which exchanges very slowly between the membrane leaflets (17), there is evidence that the exchange of cholesterol between leaflets is rapid (18, 19). Transmembrane movement of cholesterol would alleviate the differential expansion/compression of the membrane leaflets and thus reduce $\gamma$ below expected values. Thus, the effects of cholesterol on the nonlocal and dynamic resistance of the membrane to bending (i.e., on the resistance due to expansion/compression and lateral displacement of the two constituent leaflets of the bilayer) is likely to be complex, and a detailed understanding of these effects must await further study.

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