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**Supporting Material**

**Flip-Flop Induced Relaxation Of Bending Energy: Implications For Membrane Remodeling**

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## Supplementary Information

### I. Calculation of membrane outer leaflet area change following deflation

Vesicles were assumed to begin as spheres with radius from the vesicle center to the mid-point of the membrane interior equal to 50 nm. Membranes were assumed to be 3 nm thick. Initial outer leaflet area was then calculated assuming a radius of 51.5 nm, while initial volume was calculated assuming a radius of 48.5 nm (summarized in Fig. S2).

The extent of deflation was calculated using values for solution osmolalities obtained by measurement on a vapor pressure osmometer. Because the extent of deflation is dominated by the osmotic energy (with negligible contribution from the elastic membrane energy) the deflated volume is equal to the initial volume times the ratio of the osmolality of the encapsulated volume to the osmolality of the outside solution after an equal volume of hypertonic solution has been added (i.e.  $V_{\text{final}} = V_{\text{initial}} [\text{Osm}_{\text{in}}/\text{Osm}_{\text{out}}]$ ). Deflated vesicle shape was assumed to be a prolate spheroid as this is the shape that minimizes the area change.

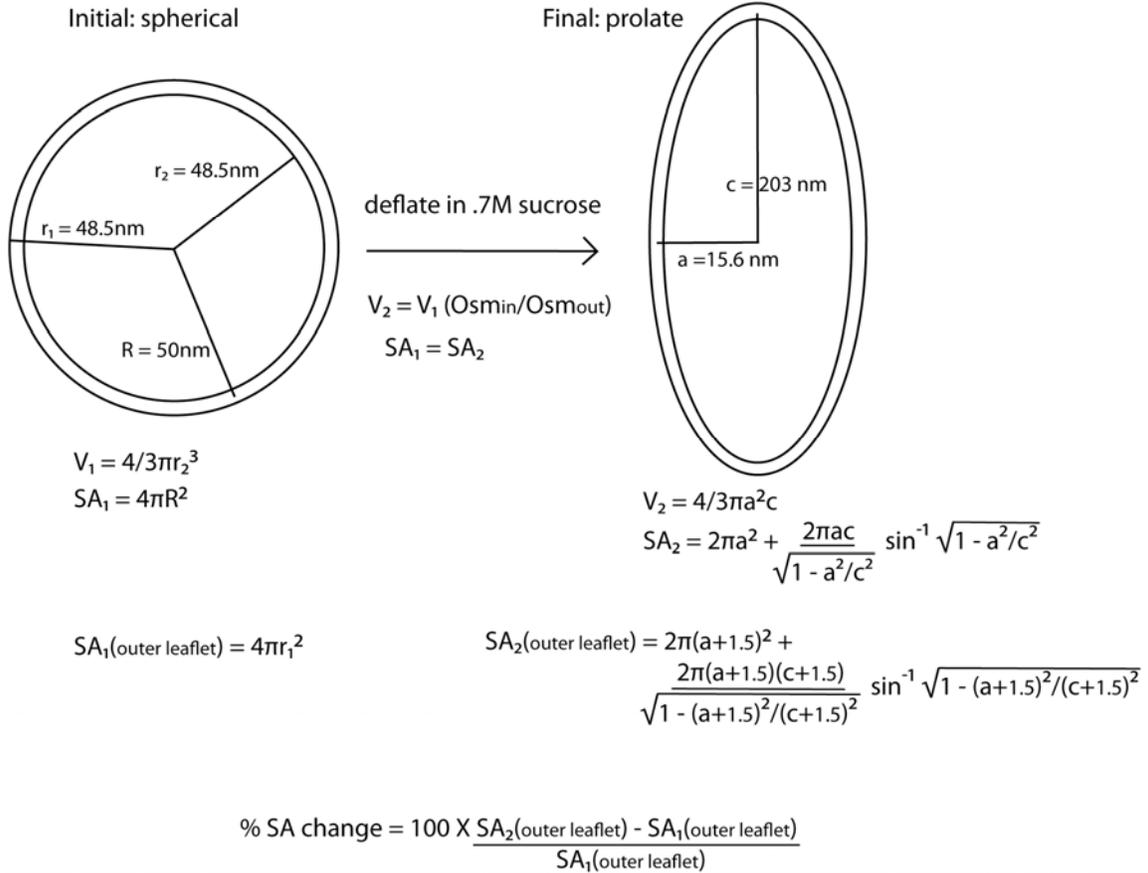
In the deflated state, membrane area was taken to be constant at the mid-point of the membrane interior. The expression for the surface area of a prolate spheroid,

$$SA = 2\pi a^2 + \frac{2\pi ac}{\sqrt{1 - \frac{a^2}{c^2}}} \sin^{-1} \sqrt{1 - \frac{a^2}{c^2}}$$

where  $a$  is the spheroidal semi-minor axis, and  $c$  is the semi-major axis, was solved for  $a$  and  $c$  such that both (1) the area at the membrane interior mid-point was equal to the area calculated at the mid-point for the initial spherical shape, and (2) that the values of  $a$  and  $c$  satisfied the volume constraint calculated above (i.e.  $V_{\text{prolate}} = 4/3 \pi a^2 c$ ).

The area of the prolate outer leaflet was then calculated by solving the above equation for SA with the values  $a + 1.5$  nm and  $c + 1.5$  nm. Percent area expansion was calculated by comparing this area to the initial area of the spherical outer leaflet.

Figure S1



## II. Theoretical Considerations

The various experimental approaches that we employed do not yet provide a quantitative picture of the decay of membrane energy during the observed flip-flop of fatty acids or cholesterol. However, our experiments do imply that the energy required for membrane deformation depends on the time scale of the deformation if the membranes contain molecules that can flip from one leaflet to the other. Here we attempt to put these observations into the context of longstanding theoretical modeling of the bilayer membrane. Membrane bending has been treated as a dynamic process in the past. Seifert

and Langer<sup>1</sup> considered the small spontaneous deformations of a bilayer that result in local regions of compression and expansion within each leaflet. Lateral lipid diffusion from compressed to expanded regions provides a relaxation pathway, the effectiveness of which depends upon the time-scale of the deformation. Fast fluctuations cannot be damped by this pathway, since the lipids do not have time to redistribute, while slower fluctuations relax in part by lateral lipid diffusion. Our experiments address the dynamic nature of membrane remodeling in a different context: instead of in-plane viscosity, we are concerned with trans-bilayer viscous flow. Elastic membrane models such as the Spontaneous Curvature model<sup>2</sup> and the ADE<sup>3</sup> model employ the term  $C_0$  to quantify initial geometric and chemical asymmetries between the two leaflets of a bilayer. Typically,  $C_0$  has been treated as a constant<sup>3</sup>: chemical asymmetries between the two leaflets are maintained over the course of a simulated or experimental membrane deformation. The observation of trans-bilayer lipid diffusion during membrane remodeling can be understood by recognizing that  $C_0$  is not necessarily constant. As fatty acids or cholesterol migrate from one leaflet to another, they change the chemical make-up of the two monolayers. Seifert has taken a different approach to this problem in considering the shape transformations of two-component vesicles<sup>4</sup>. In this scenario, the value of  $C_0$  at a given point on the vesicle surface is a function of local lipid concentrations. This model could be a useful theoretical framework for modeling the dynamic consequences of cholesterol flip-flop, once quantitative data are available concerning the fraction of cholesterol that flips in response to a given deformation.

Despite the qualitative nature of our detection of cholesterol flip flop, we may nevertheless characterize the trans-bilayer diffusion process as we understand it. Here we focus on the simplest case where we assume that the vesicle is made of phospholipids, which cannot flip, and cholesterol which can flip in response to changes in their concentration until the inner and outer leaflet areal concentrations have become equalized (or more generally until the chemical potential is stationary). To characterize this effect, we consider the difference in the spatially averaged areal density of lipid molecules between the leaflets  $\Delta\rho(t)$  which vanishes for a naturally flat membrane. However, when the membrane is bent into a spherical cap, the effective density of lipids on the

compressed leaflet is slightly more than that in the stretched leaflet, so that it is entropically favorable for the lipids to flip from the compressed to the tensed side. Symmetry dictates that at leading order, the density difference is proportional to the difference between the instantaneous membrane curvature  $C(t)$  and its externally imposed curvature, by say osmotic deflation  $C_o$  as well as the length of the lipid molecules  $b$ , so that we may write  $\Delta\rho(t) = \alpha b(C_o - C(t))$ , where  $\alpha^{-1}$  is the effective projected area of a single lipid molecule. Thus when the natural curvature of the membrane imposed by the osmotic deflation is equal to the actual curvature of the membrane, the density difference between the leaflets vanishes. A non-zero value of this difference leads to lipid flipping via a thermally activated process with a rate determined by a Kramers rate  $\tau^{-1}$  for the dynamics of the hydrophilic lipid head to rotate past the hydrophobic core between the leaflets.

At a scaling level, we may thus write  $\tau \sim \left(\frac{k_B T}{U}\right)^{1/2} \frac{\gamma}{k} \exp[U / k_B T]$  where  $U$  is the hydrophobic barrier energy,  $\gamma$  is the viscous drag coefficient and  $k$  is the elasticity associated with lipid rotation through the leaflet. We note that  $\gamma / k$  is dependent on the lipid length  $b$  and is in fact an increasing function thereof, so that the characteristic rate of flipping decreases for longer lipids as expected. We may thus write a simple equation for the dynamics of the differential density of lipids driven by the sudden osmotic deflation (whose time scale is assumed to be much smaller than  $\tau$  so that the dynamics of deflation is dynamically decoupled from that of lipid flipping)

$$\frac{d\Delta\rho}{dt} = -\frac{\alpha b}{\tau}[C_o - C] = -\frac{\Delta\rho}{\tau},$$

so that  $\Delta\rho(t) = \alpha b \Delta C(0) \exp[-\frac{t}{\tau}]$ , with  $\Delta C(0) = C_o - C(0) = C_o$  being the imposed curvature at the start of the osmotic deflation experiment. We see that as the density equilibrates over long times,  $\Delta\rho(\infty) \sim \Delta C(\infty) \rightarrow 0$  so that the instantaneous curvature of the membrane approaches the imposed curvature, i.e.  $C(\infty) \rightarrow C_o$ . This is qualitatively

consistent with the results of our experiments on fatty acid membranes that show a simple exponential behavior for the relaxation induced by fatty acid flip flop, which can and does lead to the rapid relaxation of membrane bending stress.

## REFERENCES

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