Evolution of the Hemifused Intermediate on the Pathway to Membrane Fusion: Supporting Material

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Proof of Relation between HD Expansion Rate and Leaflet Velocities, $dR_{\rm hd}/dt = (\Delta v)_{r=R_{\rm hd}}$

As the HD grows mass conservation at its rim determines a relationship between the HD growth rate, the leaflet velocity difference $\Delta\mathbf{v} = \mathbf{v}_{\rm out} - \mathbf{v}_{\rm in}$ and the leaflet densities at the rim. Now eq. **7** shows that $\bar{\rho} = \rho_0$ to leading order in the small quantity γ^0/K. Since $\Delta\rho$ is first order in $f_{\rm therm}$ (and in $A^{\rm eq}_{\rm hd}/A_{\rm ves}$) to leading order the leaflet densities equal the initial density, $\rho_{\rm in} = \rho_{\rm out} = \rho_0$. This simplifies mass conservation since only velocities need be considered. Consider the HD expanding with speed $dR_{\rm hd}/dt$. Now as the HD grows the inner leaflets are brought into contact to form new HD area (see fig. 2). This requires that the inner leaflet velocity at the HD rim be $\mathbf{v}_{\rm in} = dR_{\rm hd}/dt\,(1-\cos\theta\ ,\ -\sin\theta)$ where the two components of this vector are with respect to the HD radial direction and the direction normal to the HD, respectively. Meanwhile the outer leaflets move outward with the HD rim, $\mathbf{v}_{\rm out} = dR_{\rm hd}/dt\,(1\ ,\ 0)$ (see fig. 2). Taking the difference of these two vectors gives $\Delta\mathbf{v} = dR_{\rm hd}/dt\,(\cos\theta\ ,\ \sin\theta)$ which is tangent to the non-HD surface at the rim as it must be. Hence the magnitudes are equal, $dR_{\rm hd}/dt = (\Delta v)_{r=R_{\rm hd}}$, as stated in the "Model" section of the main text.

Hemifusion with Vesicle-Substrate Adhesion: Decay of Bilayer Tension during HD Growth (eq. 9 of main text)

In the experiments of ref. [1] to which we compared detailed model predictions the hemifusing vesicles were adhered to a substrate. In this case the decay of tension during HD growth is buffered somewhat by vesicle-substrate adhesion and the linear relationship between tension and HD area of eq. **8** in the main text is no longer valid. This vesicle tension features in the boundary condition eq. **S3** for the diffusive-like dynamics governing evolution of the density difference field $\Delta\rho$. Let us now calculate the decay in this case.

Now the bilayer tension decays with mean lipid density $\bar{\rho}$ according to eq. **7**. $\bar{\rho}$ can be expressed as $\bar{\rho} = \widehat{\rho}_{\rm in} + \widehat{\Delta\rho}/2$ where hat symbols denote spatial averages over the non-HD regions. This relation allows $\bar{\rho}$ to be obtained without need for detailed knowledge of the spatial variations of $\rho_{\rm in}$.

Now in the absence of substrate adhesion the decrease of $\widehat{\rho}_{\rm in}$ would be only second order in $A_{\rm hd}/A_{\rm ves}$ since the inner leaflet is not displaced by HD growth. Hence the origin of its

change is primarily increase in vesicle area resulting from adhesion with the substrate. Using the geometrically determined relation between surface area and substrate contact angle θ_s [2] leads to

$$\widehat{\rho}_{in} = \rho_0 \left(1 - \frac{\theta_s^4}{16}\right) \tag{S1}$$

with second order corrections in the small quantity A_{hd}/A_{ves} and sixth order corrections in θ_s. Using this expression in the relation $\bar{\rho} = \widehat{\rho}_{in} + \widehat{\Delta\rho}/2$ together with the $\widehat{\Delta\rho}$ values determined directly from the $\Delta\rho$ profile tracked by our numerical calculations allows $\bar{\rho}$ to be expressed as a function of substrate contact angle. But the contact angle in turn is a function of bilayer tension and adhesion energy W through Young's equation which implies $\theta_s = \cos^{-1}(1-W/\gamma)$. This gives $\bar{\rho} = \rho_0[1-(\cos^{-1}(1-W/\gamma))^4/16 + (\cos^{-1}(1-W/\gamma^0))^4/16] + \widehat{\Delta\rho}/2$. Inserting this result into the bilayer tension relation, eq. **7**, gives the equation determining bilayer tension during HD growth,

$$\gamma = \gamma^0 + K\left\{\frac{1}{16}\left(\cos^{-1}(1-\frac{W}{\gamma(t)})\right)^4 - \frac{1}{16}\left(\cos^{-1}(1-\frac{W}{\gamma^0})\right)^4 - \frac{\widehat{\Delta\rho}}{2\rho_0}\right\} \tag{S2}$$

Note this result applies to each vesicle. In our numerical calculations $\widehat{\Delta\rho}$ was determined for each vesicle after each time step and eq. **S2** was then solved for the bilayer tension value of each vesicle. The boundary conditions of eq. **S3** were thus updated.

Now eq. **S2** above is general and applicable regardless of vesicle symmetry. In the symmetric case, there is no net flow of outer leaflet lipids between the vesicles, and the situation is somewhat simpler. The mean density difference is $\widehat{\Delta\rho} = A_{hd}/A_{ves}$ to leading order and thus density fields need not be calculated to determine the boundary condition of eq. **5** of the main text. Inserting this into eq. **S2** give eq. **9** of the main text.

HD Growth Kinetics for Asymmetric Hemifusion

Here we generalize the kinetic equations for symmetric hemifusion, **4** and **5** of the main text, to the case of hemifusing vesicles with different areas and tensions. The diffusion-like equation, **4**, remains applicable in each of the two non-HD regions, one in each vesicle. However, the boundary condition and HD growth velocity condition of eq. **5** are different.

Boundary conditions at HD rim. The boundary conditions for the density differences $\Delta\rho_1$ and $\Delta\rho_2$ in vesicles 1 and 2 are determined by the condition of local equilibrium at the HD rim and eq. **5** applies for each vesicle (see ref. [3]):

$$\left(\frac{\Delta\rho_1}{\rho_0}\right)_{r=R_{hd}(t)} = \frac{\gamma_1}{2k_\Delta} + \epsilon^{cation}\ , \qquad \left(\frac{\Delta\rho_2}{\rho_0}\right)_{r=R_{hd}(t)} = \frac{\gamma_2}{2k_\Delta} + \epsilon^{cation}\ . \tag{S3}$$

Relation between HD growth rate and density gradient. As for the symmetric case (see "Proof of Relation between HD Expansion Rate and Leaflet Velocities, $dR_{\rm hd}/dt = (\Delta v)_{r=R_{\rm hd}}$" above) the HD expansion rate $dR_{\rm hd}/dt$ is related to leaflet velocities by mass conservation, again simplified by the fact that leaflet densities equal ρ_0 to leading order. Now HD growth entails the non-HD region inner leaflets being pinched together at the HD rim. Thus, using the same coordinate system as in the analogous symmetric discussion we have

$$\mathbf{v}_1^{\rm in} = \frac{dR_{\rm hd}}{dt}(1-\cos\theta_1\ ,\ -\sin\theta_1)\ , \qquad \mathbf{v}_2^{\rm in} = \frac{dR_{\rm hd}}{dt}(1-\cos\theta_2\ ,\ \sin\theta_2)\ , \tag{S4}$$

where the leaflets make contact angles θ_1 and θ_2 to the HD tangent surface at the rim.

Whereas in the symmetric case the outer leaflet velocity matched the HD boundary velocity, in the asymmetric situation the outer leaflets have differing velocities $\mathbf{v}_1^{\rm out}$ and $\mathbf{v}_2^{\rm out}$ and lipids flow between outer leaflets. The condition of no outer leaflet lipid flux across the HD rim demands that the sum of their velocity components in the radial direction equals $2\,dR_{\rm hd}/dt$. The definition of interleaflet velocity difference is used to relate outer and inner leaflet velocities, $\mathbf{v}_1^{\rm out} = \mathbf{v}_1^{\rm in} + \Delta\mathbf{v}_1$ and $\mathbf{v}_2^{\rm out} = \mathbf{v}_2^{\rm in} + \Delta\mathbf{v}_2$. Using these relationships we sum the radial components of outer velocities using eq. **S4** for inner velocities and the fact that interleaflet velocities must be tangent to their respective membrane surfaces to obtain

$$2\,\frac{dR_{\rm hd}}{dt} = \frac{dR_{\rm hd}}{dt}\ (1-\cos\theta_1) + \Delta v_1\ \cos\theta_1 + \frac{dR_{\rm hd}}{dt}\ (1-\cos\theta_2) + \Delta v_2\ \cos\theta_2\ \ . \tag{S5}$$

From this we obtain $dR_{\rm hd}/dt = (\Delta v_1\ \cos\theta_1 + \Delta v_2\ \cos\theta_2)/(\cos\theta_1 + \cos\theta_2)$. For small HDs we use $\cos\theta_1 \approx \cos\theta_2 \approx 1$ giving

$$\frac{dR_{\rm hd}}{dt} = \frac{1}{2}(\Delta v_1 + \Delta v_2)\ \ . \tag{S6}$$

Using this with eq. **4** of the main text relating Δv to gradients of the density difference yields

$$\frac{d\mathbf{R}_{\rm hd}}{dt} = -\frac{D}{2}\ \left[\nabla\left(\frac{\Delta\rho_1}{\rho_0}\right) + \nabla\left(\frac{\Delta\rho_2}{\rho_0}\right)\right]_{r=R_{\rm hd}(t)}\ \ . \tag{S7}$$

Thus, for asymmetric hemifusion our procedure was to solve the diffusion-like dynamics of eq. **4** on the vesicle surfaces using the boundary conditions of eq. **S3** and use eq. **S7** to continuously update the HD boundary location.

Tension release by vesicle leakage in experiments of Nikolaus et al, ref. [1]

Here we present evidence that vesicle leakage is not an important effect over the short timescales followed in ref. [1] and thus presumably the other experiments of Table 1 under similar conditions. We tracked leakage in the three kinetic data sets of fig. 3 by measuring

vesicle dimensions in Figs. 1,S1 of ref. [1]. Vesicle pair 1 (blue in Fig. 3) and pair 2 (red) showed no leakage before fusion. However the smaller vesicle of pair 3 (green) did show significant leakage during HD extension. This is consistent with our kinetic analysis which suggested that pair 3 had higher bilayer tensions than pairs 1 and 2 (see Fig. 3D,E,F). This is also consistent with the fact that pair 3 ruptured during HD growth, likely from very high tension able to cause leakage and rupture.

Consistent with this, other experiments at similar conditions appear to be unaffected by leakage. In micropipette manipulations of aspirated PG/PC and PA/PC GUVs in 10 μM Ca^{2+}, linear stress-strain relationships were reported up to GUV rupture at tensions 8 mN/m and negligible hysteresis was found between cycles of increasing and decreasing aspiration pressure [4]. This suggests these GUVs were not leaky under tension conditions similar to those in Nikolaus et al. Rand and Reese [5] noted that in electron microscopy DOPS LUV sizes were indistinguishably different with and without 5 mM Ca^{2+}; this suggests leakage was not severe in their experiments even at these cation levels which exceed the 2 mM Mg^{2+} used in the equilibrium studies by Nikolaus et al.

Estimation of Cation Shrinkage Factors and Bilayer Tension for the Experiments of Ref. [1]

Cation shrinkage factors. In ref. [1] Nikolaus et al hemifused and fused GUVs of lipid composition 60% DOPC, 20% DOPS, 20% DOPE. Let us estimate the cation shrinkage factor ϵ^{cation} for the studies at 6 mM Ca^{2+}. (The procedure is the same as that in ref. [3]). We name the shrinkage factors for one-component membranes of each lipid species $\epsilon_{PC}, \epsilon_{PS}$ and ϵ_{PE} respectively. DPPC bilayers contract by 6.4 % in 6 mM Ca^{2+} [6] and DOPC may be expected to respond similarly to cations as they possess the same headgroup, that part of the lipid interacting with solution cations. Thus we take $\epsilon_{PC} \approx 6.4\,\%$. We estimate the DOPS shrinkage factor at 6 mM Ca^{2+} is the monolayer tension 9.5 mN/m induced in these conditions [7] divided by the monolayer stretch modulus (taken as half the bilayer stretch modulus $K = 265$ mN/m of DOPC [8]). This gives $\epsilon_{PS} \approx 7.1\,\%$. Since pure DOPE does not form vesicles and DOPE data to the best of our knowledge are not available we take $\epsilon_{PE} = \epsilon_{PC}$. Assuming linear composition dependence the weighted sum of these values yields the overall shrinkage factor $\epsilon^{\text{cation}} = 6.5\%$.

Bilayer tension before hemifusion. To estimate the initially induced bilayer tension when only one leaflet contacts cations as in ref. [1] we use the monolayer tension under similar conditions. DOPS monolayer tension increased by 9.5 mN/m when 6 mM Ca^{2+} was added [7]. Estimating the DOPC monolayer modulus as half the value of 265 mN/m measured for

bilayers [8] and using $\epsilon_{PC} = 6.4\,\%$ (see above) yields a tension 8.4 mN/m per pure DOPC monolayer in 6 mM Ca^{2+}. Approximating again DOPE properties by those of DOPC and assuming linear composition dependence yields pre-hemifused tension γ^0 =8.7 mN/m for the experiments of ref. [1]. Note this value though larger than the rupture tension 8 mN/m but it is known that GUVs can withstand such tensions for a few seconds [9].

Procedure for Fitting Experimental Data of Ref. [1] for the Interleaflet Friction Coefficient λ

In the experiments of Nikolaus et al, HDs were nucleated in the time interval between camera exposures, $t_{\rm frame}$=250 ms. Thus HD area had already grown of order $\sim 5\mu{\rm m}^2$ in the delay time $t_{\rm delay}$ before it was detected. Note the delay time is an experimental parameter relating to the finite time resolution of the camera and is not a model parameter. In our fitting procedure we allowed each vesicle pair data set to shift by a delay time $t_{\rm delay}$ in the range $0 < t_{\rm delay} < t_{\rm frame}$. The best fit for $t_{\rm delay}$ for each data set was used in Fig. 3 of the main text.

Our fitting procedure was as follows. For the red and blue vesicle pairs, the model equations were solved to produce $A_{\rm hd}(t)$ curves. Many curves were produced over a wide range of λ values and delay times $t_{\rm delay}$ in the range $0 < t_{\rm delay} < t_{\rm frame}$. The best fit value for λ reported in the main text was that which minimized the sum of the errors between the predicted curve $A_{\rm hd}(t)$ and the experimental measurements. The best fits for the delay times for the blue and red pairs were 0.15 s and 0.20 s, respectively.

For the green vesicle pair, we used a similar procedure but instead fit for the initial tension γ^0 reported in the main text which was significantly higher than the other pairs. The best fit delay time for the green pair was 0.225 s.