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The Hemifused State on the Pathway to Membrane Fusion

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Fusion of compartments enclosed by membrane bilayers enables secretion and other vital cellular processes and is widely studied in model synthetic membrane systems. Experiments suggest the fusion pathway passes through a hemifused intermediate where only outer monolayers are fused. Here we show membrane tension and divalent cations drive vesicles to hemifused equilibrium with expanded hemifusion diaphragms (HDs) where inner monolayers engage. Predicted HD sizes agree with recent measurements of ref. [1]. The fusion pathway is completed by HD lysis provided HD tension is sufficiently high.

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Fusion of membrane-enclosed compartments to mix or release contents is essential for intracellular trafficking, secretion, fertilization and development [2] and is a critical step during cell invasion by enveloped viruses [3]. The driving forces are provided by specialized fusion proteins, for example the SNARE [2] and hemagglutinin (HA) [3] proteins in intracellular and influenza viral fusion, respectively. A large body of research has explored the underlying physics of fusion in these systems by study of protein-free synthetic lipid membranes [1, 4–7]. Synthetic vesicle fusion is commonly driven by divalent cations or osmotically-generated membrane tension [4–7].

The pathways to fusion remain controversial. However, considerable evidence suggests a critical intermediate *hemifused* state whose maturation is required for fusion (Fig. 1A). In this state only outer (proximal) leaflets of the apposing membranes are fused while the inner (distal) leaflets engage in a new bilayer region called the hemifusion diaphragm (HD) [3, 6, 8–10]. It is established that calcium or magnesium cations hemifuse vesicles at lower concentrations [1] but trigger fusion above a threshold [1, 5, 7]. It has been proposed that the initial connection is a minimally sized HD, termed a stalk [11, 12] (see Fig. 1A), and stalk-like connections have been identified in multilamellar lipid phases [13]. However, HDs directly visualized in fusion systems have been expanded, with sizes ranging from ~ 5 -nm in vesicles at the neural synapse [9] to microns in HA-mediated whole-cell fusion [10] systems. Recently, Nikolaus et al quantitatively documented the hemifused state in vesicle-vesicle systems for the first time [1]. In 2 mM Mg^{2+} , synthetic GUVs formed hemifusion connections with stable HDs $\sim 10 \mu\text{m}$ in diameter visualized by labeled bilayer-spanning peptides excluded from the hemifused regions. The HD area for a given hemifusing pair was proportional to the mean area of the GUVs [1].

Many theoretical studies have examined fusion in protein-free membrane systems, with focus on hemifusion, the pathway to fusion and the role of tension. Field theoretic methods were used to study stalk structure and energetics [11, 12] and the tension-dependence

of membrane pore formation [14, 15]. Computer simulations with explicit representation of lipids exhibited stalk-like and HD-like intermediate states on the pathway to vesicle-vesicle fusion [16], while in refs. [17] hemifused end states were observed in simulated vesicle-planar membrane systems at high enough membrane tensions, with fusion or membrane rupture the outcome at yet higher tensions. In the simulations of ref. [18] the predominant pathway to fusion was via an expanded HD while a second pathway involved a stalk intermediate.

These works have provided important insights, but the mechanisms of hemifusion and fusion remain unclear and quantitative relations are lacking. Direct comparison of simulations with experiment has not been possible as computer resources limit the accessible ranges of membrane tension and vesicle size, and divalent cations, the most widely employed experimental fusogens, have not been addressed. Divalent cations are known to augment membrane tension [5] and to condense negatively charged lipid membranes electrostatically; e.g., Ca^{2+} reduces phospholipid leaflets areas by factors $\epsilon_{\text{cation}} = 5\% - 7\%$, depending on concentration and lipid composition [19]. However, it has not been established how these effects may relate to hemifusion and fusion.

In this Letter we present a systematic theory of tension- or divalent cation-driven hemifusion of protein-free membranes that quantitatively relates the final hemifused state to the tension and the cationic driving forces. We show that an equilibrium hemifused state exists whose physics stem from its unusual membrane leaflet topology, and our predictions show close quantitative agreement with the recent HD measurements by Nikolaus et al [1]. We find membrane tension, which tends to reduce membrane area, drives HD expansion because the HD constitutes a shared vesicle boundary and thus reduces total bilayer area (Fig. 1B). Divalent cations drive hemifusion both by increasing tension and by tending to shrink charged monolayers. The net driving force for HD expansion, due to tension and cations, is opposed by a second type of membrane tension we call interleaflet

tension, arising due to areal mismatch between the inner and outer vesicle membrane leaflets that accompanies HD growth. The balance of these forces determines the HD size and tension in the equilibrium hemifused structure.

Lipid membranes have 2 leaflets and the equilibrium behavior depends both on aggregate properties and asymmetry between the leaflets (Fig. 1B,C). Thus, in addition to the chemical potential $\bar{\mu} \equiv \partial F / \partial \bar{N}$ and the familiar membrane tension $\gamma \equiv \partial F / \partial A$, asymmetry is described by the interleaflet tension $\gamma_\Delta = \rho_0 (\partial F / \partial \Delta N)$. Here $F(A, \bar{N}, \Delta N)$ is the free energy, A is the projected area, $\bar{N}$ and ΔN are the mean and difference of the numbers of lipids in the two leaflets and ρ_0 is the leaflet density in a symmetric reference state. We will see γ_Δ is central to hemifusion as the outer (inner) leaflets are compressed (expanded) in the hemifused state, and generally interleaflet tension grows when leaflet density differences $\Delta\rho = \Delta N / A$ are imposed, $\gamma_\Delta = k_\Delta (\Delta\rho / \rho_0)$. The interleaflet tension modulus k_Δ has been measured by extraction from GUVs of microscopically narrow membrane tubes with high differential leaflet area (e.g. $k_\Delta = 23$ mN/m in ref. [20]).

A few relations involving these forces will prove useful. First, the membrane chemical potentials are closely related to the those of the individual inner and outer leaflets, $\bar{\mu} = \mu_{\text{out}} + \mu_{\text{in}}$ and $\gamma_\Delta = \rho_0 (\mu_{\text{out}} - \mu_{\text{in}}) / 2$. Second, the Gibbs Duhem relation reads $d\gamma = -\bar{\rho} d\bar{\mu} - (\Delta\rho / \rho_0) d\gamma_\Delta$. Changes in the mean lipid density $\bar{\rho} \equiv \bar{N} / A$ are typically small ($\sim 5\%$ variation over a large tension range $\gamma \sim 10^{-3} - 10$ mN/m [15]) and $\gamma_\Delta, \Delta\rho$ are first order in HD size (see below). Thus to leading order

$$d\gamma = -\rho_0 d\bar{\mu} . \quad (1)$$

Our goal is to determine the fate of a localized hemifusion connection, assumed to have been nucleated between 2 vesicles at bilayer tension γ . Does a HD grow, and if so what is the HD size and tension in the equilibrium state? This depends on competition between the HD and vesicle bilayer tensions tending to close down and expand the HD, respectively (Fig. 1C). For a small change in the HD area A_{hd} , the net work done by these forces gives the free energy change

$$dF = (\gamma_{\text{hd}} - 2\gamma \cos \theta) dA_{\text{hd}} , \quad (2)$$

where θ is the vesicle contact angle.

To proceed, we must compute the HD tension γ_{hd} in this expression. From eq. 1, conditions in the HD region (subscript HD) and in the non-HD vesicle regions (no subscript) are related by $\gamma_{\text{hd}} - \gamma = -\rho_0 (\bar{\mu}_{\text{hd}} - \bar{\mu})$. The defining property of the hemifused state is that the chemical potentials of the inner vesicle and HD leaflets are equal due to their connectivity, $\mu_{\text{in}} = \mu_{\text{hd}}$. Expressing the aggregate chemical potentials in terms of leaflet chemical potentials one then finds

$$\gamma_{\text{hd}} = \gamma + 2\gamma_\Delta . \quad (3)$$

This is the fundamental relation between the HD tension and the vesicle tensions. It shows that HD growth, by increasing the vesicle interleaflet tension γ_Δ , tends to increase the HD tension.

There are two contributions to the interleaflet tension. First, by displacing the outer leaflets HD growth creates interleaflet density difference $\Delta\rho = \rho_0 (A_{\text{hd}} / A_{\text{ves}})$ where A_{ves} is the vesicle area (Fig. 1B). Second, in typical fusion experiments such as those of Nikolaus et al Ca^{2+} or Mg^{2+} cations are present in solution but not in the vesicle interior. Thus cations contact the *outer* vesicle leaflets only and their tendency to condense lipids by the factor ϵ_{cation} favors a non-zero density difference. The net effect on interleaflet tension is

$$\gamma_\Delta = k_\Delta \left(\frac{A_{\text{hd}}}{A_{\text{ves}}} - \epsilon_{\text{cation}} \right) . \quad (4)$$

In equilibrium the free energy of eq. 2 is a minimum, $dF = 0$, whence $\gamma_{\text{hd}} = 2\gamma \cos \theta$. Using eq. 4 in eq. 3 to obtain the HD tension then leads to the following results for the equilibrium HD area and tension, valid to first order in HD area (to this order the contact angle vanishes):

$$\gamma_{\text{hd}} = 2\gamma , \quad A_{\text{hd}}^{\text{eq}} = A_{\text{ves}} \left(\frac{\gamma}{2k_\Delta} + \epsilon_{\text{cation}} \right) \text{ (symmetric)} . \quad (5)$$

We have generalized the above results for the symmetric case (identical vesicles) to hemifusion between vesicles with different areas and tensions (see [21]). A new feature is that outer leaflet lipids flow from the less to the more tense vesicle, to maintain chemical potential equality. We find the equilibrium HD bilayer tension and area are

$$\gamma_{\text{hd}} = \sum_i \gamma^i , \quad A_{\text{hd}}^{\text{eq}} = \frac{1}{2} \sum_i A_{\text{ves}}^i \left(\frac{\gamma^i}{2k_\Delta} + \epsilon_{\text{cation}} \right) , \quad (6)$$

where $i = 1, 2$ labels the two vesicles.

In Supplementary Material [21] we show that this equilibrium is stable with respect to membrane shape changes, e.g. the formation of membrane blebs, that could partially accommodate the interleaflet area difference created by the expanded HD. We find that bilayer tension and bending energy costs render such blebs free energetically unfavorable.

Comparison of theory with experiment of ref. [1]. Nikolaus et al [1] measured equilibrium HD areas in 2 mM Mg^{2+} for 50 hemifused GUV pairs with lipid composition 60% DOPC, 20% DOPS, 20% DOPE. To compare with, eq. 6, we must first estimate the area reduction and membrane tension induced by Mg^{2+} . Here we summarize our procedures (for a detailed account, see SM). (1) Assuming linear composition dependence and using literature values for cationic shrinkage factors of one-component membranes [5, 19], we estimated $\epsilon_{\text{cation}} = 5.8\%$ for the lipid composition and

Mg^{2+} concentration used by Nikolaus et al. This is consistent with Ca^{2+} -driven condensation factors of 3–9% measured in simulations of PC:PS bilayers [22]. (2) In ref. [1] the GUVs were adhered to the polylysine substrate, and the mean vesicle-substrate contact angle was $\theta_s = 31 \pm 14^\circ$ for unhemifused vesicles but increased to $\theta_s = 82 \pm 9^\circ$ for hemifused vesicles [1, 21]. To estimate the unhemifused bilayer tension γ^0 for this situation where cations contact only one leaflet, we used measured monolayer tensions under similar conditions [5, 19] and assumed linear composition dependence, giving $\gamma^0 = 7.7$ mN/m. Then Young’s equation $W = \gamma_{\text{ves}}(1 - \cos \theta_s)$ with $\gamma_{\text{ves}} = \gamma^0$ yields the adhesion energy W . Applying Young’s equation to the hemifused state gives the mean tension of hemifused GUVs, $\gamma = 1.3$ mN/m.

Using these values for the tension and cationic shrinkage factor, we determined the equilibrium HD areas from eq. 6. As the correspondence between contact angle and HD area for individual hemifused pairs was not measured, we used the mean tension computed as above. This amounts to assuming equal tensions within a hemifusing pair, when eq. 6 simplifies to $A_{\text{hd}}^{\text{eq}} = \bar{A}_{\text{ves}}(\gamma/2k_\Delta + \epsilon_{\text{cation}})$ with $\bar{A}_{\text{ves}}$ the mean of the vesicle areas. (From the measured spread in contact angles, we estimate actual tension variations 1.1-1.6 mN/m; from eq. 6, the equal tension approximation then gives only $\sim 4\%$ errors in predicted HD sizes.) The predicted HD areas are plotted in Fig. 2 versus mean area of the hemifusing GUVs for a range of vesicle area ratios. The predicted linear relation is closely obeyed by the experimental data, and a best fit yielded $k_\Delta = 19 \pm 5$ mN/m (95% confidence interval) for the interleaflet modulus, close to the value 23 ± 9 mN/m reported from membrane tether experiments [20].

While the linear relation collapses most data, HD areas for the most asymmetric vesicle pairs (Fig. 2, open circles) clearly do not correlate with the mean vesicle area. This is as expected since eq. 6 is valid only when the predicted HD size is less than that of the smaller vesicle. Using our estimates for γ and ϵ_{cation} , this excludes area ratios $\gtrsim 4.4$. For such situations we find A_{hd} saturates at one half the area of the smaller vesicle and the HD is approximately hemispherical. In ref. [1], the measured HD areas were in fact projected onto the vesicle center plane. Thus, we predict the reported areas are one quarter the area of the smallest vesicle, clearly obeyed by the most asymmetric pairs (Fig. 2, inset). Overall, the model predictions are in agreement with hemifusion equilibrium measurements of ref. [1] for all vesicle area ratios.

Finally, we remark that our estimate of tension in these experiments ($\gamma = 1.3$ mN/m) was based on literature measurements on planar membranes and hence neglects vesicle curvature stress contributions $\sim 2\kappa/R_{\text{ves}}^2$, where R_{ves} is the vesicle radius [11]. Taking bending modulus $\kappa = 20 kT$ [15], for the GUVs employed this gives negligible corrections. (Even for very small vesicles, e.g. $R_{\text{ves}} = 25$ nm, curvature stresses ~ 0.2 mN/m would

be well below typical cation-induced tensions; thus, for equal vesicle tensions, at fixed cation concentration eq. 6 implies a linear relation between HD area and mean vesicle area for all realizable vesicle sizes.)

Relation of the stalk to the equilibrium hemifused state. How does the expanded equilibrium HD relate to the collapsed stalk (Fig. 1A)? At such scales the effective line tension T_{hd} due to the high curvature HD perimeter is relevant (Fig. 1A) [11, 12]. Adding the term $2\pi R_{\text{hd}}T_{\text{hd}}$ to F of eq. 2 one finds the stalk is metastable, and beyond a radius $R_{\text{crit}} = T_{\text{hd}}/(\gamma + 2k_\Delta \epsilon_{\text{cation}})$ will spontaneously expand to the equilibrium HD of eq. 6. (The concept of a critical HD radius was proposed in a model of PEG polymer-driven hemifusion between supported bilayers [23]). For the lipid compositions of ref. [1] we find $T_{\text{hd}} = 1.8 \times 10^{-11}$ N using the line tension model of ref. [12], giving a free energy barrier $F_{\text{crit}} = 27 kT$ and $R_{\text{crit}} = 2$ nm. We find the equilibrium HD then lies in a deep well, $\Delta F \approx 10^9 kT$, over 10^3 -fold greater than the line tension energy. This validates our neglect of line tension in deriving eqs. 5, 6 and shows that a powerful thermodynamic force drives HD expansion, consistent with the $\sim 10 \mu\text{m}$ sized HDs observed (Fig. 2). Similar conclusions would apply to smaller vesicles, e.g. for 50-nm SUVs the energy barrier and critical radius are unchanged and the equilibrium well $\Delta F \approx 10^4 kT$.

HD lysis and completion of the pathway to fusion. Nikolaus et al reported stable HDs in 2 mM Mg^{2+} , but in 6 mM Ca^{2+} growing HDs ruptured to yield fusion [1] (Fig. 1A). What determines if the hemifused state evolves to fusion? Previous experiments suggested fusion pores may evolve within extended HDs [6] and models of pore formation support such a mechanism as energetically feasible for high enough HD tension [14, 24]. The present work is consistent with this picture and provides a quantitative framework. A key output of our theory is the HD tension γ_{hd} , which in equilibrium is the sum of the vesicle tensions (eq. 6). Thus, the HD is particularly susceptible to pore formation and lysis as membrane tension is greatest there. Since tension increases with salt concentration [5], thus fusion occurs only above the threshold cation concentration where γ_{hd} equals the lysis tension (e.g. ~ 8 mN/m for DOPC GUVs [15]). This accords with the findings of Nikolaus et al and many others that fusion requires a sufficiently high cation concentration [5, 7] or osmotically-generated membrane tension [6]. A more quantitative analysis of the transition to complete fusion, beyond the scope of this Letter, must account for the evolving vesicle and HD tensions during HD growth and the ability of membranes to tolerate super-lysis tensions for limited time periods [15].

In summary, we elucidated the key intermediate in membrane fusion, the hemifused state. The fusion pathway in protein-free systems driven by Mg^{2+} , Ca^{2+} and osmotic gradients has been studied over many years [1, 4–8]. Our model describes this pathway as centered

on the equilibrium hemifused state, downstream of the metastable stalk and upstream of the fused state (Fig. 1A). We showed that HD expansion is driven by cations and membrane tension but opposed by interleaflet tension, and the balance leads to predicted HD sizes in quantitative agreement with measurements of ref. [1]. For typical experimental conditions we predict HD area is $\sim 5 - 10\%$ of the vesicle area, i.e. micron-sized HDs for GUVs or $\sim 20 - 30$ -nm HDs for SUVs. The HD either transits to fusion if its evolving tension is high enough to cause HD rupture (high salt) or terminates in hemifusion equilibrium (low salt). These outcomes were observed in ref. [1] at, respectively, 6 mM Ca^{2+} and 2 mM Mg^{2+} .

Finally, our work may illuminate mechanisms of biological fusion, a major motivation for studies of protein-free fusion. Qualitatively consistent with eq. 6, measured HD sizes in biological systems are greater when the membranes involved have greater area [9, 10]. Moreover, many cellular fusion pathways are regulated by Ca^{2+} spikes that may induce high tensions and condensation locally. For example, similarly to our procedure for ref. [1] described above, we estimated tension and condensation factors for the 25-300 μM Ca^{2+} spikes during exocytosis [25] and found relative driving forces somewhat less than but similar to those in typical protein-free membrane experiments (see [21]). Such forces could locally promote HD expansion. It will be of interest to establish the implications of these calcium-induced driving forces for the mechanisms employed by biological fusion machines.

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FIG. 1: The hemifused state is a key intermediate on the pathway to lipid membrane fusion. (A) Considerable evidence suggests the pathway to vesicle fusion is multistage. (1) Hemifusion nucleation: fusion of outer monolayers (red). The initial connection may be a stalk. (2) Expansion of hemifusion diaphragm (HD) to equilibrium. Inner monolayers (green) engage in the HD. (3) HD lysis and (4) fusion. Divalent cations and membrane tension drive HD expansion to equilibrium (dead-end hemifusion) or fusion if the HD tension is sufficient for HD rupture. (B) Hemifused vesicles of area A_{ves} (symmetric case shown). Expansion of the HD (area A_{hd}) compresses outer lipid leaflets and expands inner leaflets, generating interleaflet tension γ_{Δ} . (C) Blow-up of box in (B). HD expansion is driven by the vesicle tensions γ but opposed by HD tension γ_{hd} . The tensions γ , γ_{hd} , γ_{Δ} obey the fundamental relation eq. 3 due to the hemifused topology where only fused outer leaflets share lipids.

FIG. 2: Equilibrium HD areas versus mean area of hemifused vesicles: comparison of model predictions to experiments of ref. [1]. Each symbol denotes a different vesicle area ratio. Red line: model prediction, eq. 6, fitted to experiment with area ratios < 4.4 . Best fit for interleaflet tension modulus yields $k_{\Delta} = 19 \pm 5$ mN/m ($R^2 = 0.92$). The predicted linear relation describes all data except large area ratios > 4.4 (open circles). Inset: Equilibrium HD area versus smallest vesicle area A_{ves}^2 for vesicle pairs with area ratios > 4.4 . Green line (slope $1/4$): model prediction.

Figure 1

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Figure 2 LF13596 13Dec2011