Rapid Note

Multiple stalk formation as a pathway of defect-induced membrane fusion

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Abstract. We propose that the first stage of membrane fusion need not be the formation of a single stalk. Instead, we consider a scenario for defect-induced membrane fusion that proceeds cooperatively via multiple stalk formation. The defects (stalks or pores) attract each other via membrane-mediated capillary interactions that result in a condensation transition of the defects. The resulting dense phase of stalks corresponds to the so-called fusion intermediate.

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When two bilayer membranes approach each other, they may fuse to form a single bilayer membrane. This process (and its reverse) are of great importance for many processes in a living cell. Nevertheless, the mechanism by which membranes fuse is still a matter of debate [1–3].

The most widely used description of membrane fusion assumes that it involves several steps. It has been argued [4–12] that the initial connection between the membranes is formed either through a stalk or through a pore [7,12]. In fact, it is now commonly accepted that the initial inter-membrane contact is, most likely, a stalk [2] (see the schematic representation of Fig. 1a). This initial state is called the hemifusion [13]. A recent theoretical analysis of the free-energy cost of the hemifusion state [10] predicts that, depending on the magnitude of the spontaneous splay of the lipids, this free energy F can either be positive ($F \approx 45\,k_{\mathrm{B}}\,T$ for the common case of DOPC lipids) or negative ($F \approx -30\,k_{\mathrm{B}}\,T$ for DOPE lipids that have a large and negative spontaneous splay).

The second step of the fusion reaction is the expansion of the hemifusion zone (diaphragm) [11]. The analysis of reference [11] predicts that the expansion of the hemifusion diaphragm is energetically favorable only if the spontaneous splay modulus of lipids is large and negative (*e.g.*, for DOPE lipids) while it costs energy for membranes composed of other types of lipids (*e.g.*, DOPC). It is believed that in the case when the expansion of the hemifusion diaphragm is energetically unfavorable, fusion proteins generate the additional driving force needed to expand the diaphragm. The fusion process is then completed by the subsequent formation and expansion of a fusion pore.

A direct experimental conformation of the above scenario is still lacking after more than two decades of investigations. Only recently [14,15] Yang and Huang have succeeded in crystallizing a stable phase of membrane stalks in a multi-lamellar system, and verified the predicted structure of the stalk intermediate. No similar experiments have been reported in the case of two membranes.

Our principal hypothesis in this paper is based on the observations that i) two stalks or two pores attract each other, ii) the translational entropy associated with the formation of a single stalk or pore is sufficiently large to allow the spontaneous emergence of a dilute gas of such defects, even if the elastic free energy of a defect is positive.

We argue below that the defects that are thus formed, will attract each other and self-assemble into a structure that has the characteristics of a hemifusion zone (see Fig. 1c). This is a cooperative effect, similar to conventional order-disorder transition. We emphasize that the predicted aggregated phase of stalks constitutes an *intermediate* and not a final stage of membrane fusion. The final stage can proceed *e.g.*, either through stalk coalescence and a subsequent pore formation, or through the expulsion of stalks via budding. This final stage of fusion is beyond the subject of the present work.

We stress that the assumption of multiple stalk formation is not as farfetched as it may seem. In fact, the

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transitions from the lamellar to the inverted hexagonal phase [16,17], and from the lamellar to sponge phase [18] are both examples of a similar effect, where the lamellar phase is transformed into a highly connected structure. In any event, the proposed mechanism for the formation of the intermediate state in the fusion process is not restricted to the case of spontaneous (passive) multiple stalk formation. Our conclusions also apply to the case where fusion proteins actively facilitate stalk formation [1,19,20].

To model the condensation of defects during membrane fusion, we exploit the close analogy between the current process and the interaction between mobile receptors that are responsible for the adhesion between cell membranes [21–34]. In what follows, we consider the scenario proposed by Bruinsma, Goulian, and Pincus in reference [22].

Consider two bilayer membranes separated by an equilibrium distance H. This equilibrium separation can be a minimum of the inter-membrane interaction potential $V(h)$ [35], where h is the inter-membrane separation, or an optimal distance maintained by an external force. In the case of biological fusion, H is determined by inter-membrane proteins and by the glycocalyx coating (see Ref. [1] for a review). We are interested in the self-assembly of junctions (*i.e.*, stalks or/and pores) between the membranes (see Fig. 1). Each junction represents an elementary defect.

We assume that each defect imposes a local membrane spacing H_0 that is different from H. In the present case of fusion, H_0 is simply zero, as implied by the geometry of a stalk, or a pore [10,11]. This imposes the boundary conditions on the inter-membrane spacing at the point of a junction $\boldsymbol{\rho}_0$ [22]: $h(\boldsymbol{\rho}_0) = 0$, and $\boldsymbol{\nabla} h(\boldsymbol{\rho}_0) = 0$. The free energy of the system can then be written in the form [22]:

$$F = \int \mathrm{d}^2\boldsymbol{\rho} \left[\frac{\kappa}{2}(\nabla^2 h)^2 + \frac{V''(H)}{2}(h - H)^2\right], \quad (1)$$

where the first term is the Helfrich bending energy [36] with the bending modulus κ, the second term is the interaction energy between the membranes; $h(\boldsymbol{\rho})$ depends on the lateral coordinates $\boldsymbol{\rho} = (x,y)$, and $V''(H) \equiv \frac{\partial^2 V(H)}{\partial h^2}$ (the deviations of the inter-membrane separation $h(\boldsymbol{\rho})$ from the equilibrium value H are assumed to be small, and thus the inter-membrane interaction potential $V(h)$ is expanded to quadratic order in $(h - H)$).

The minimum of equation (1) is given by

$$\nabla^4 h + \frac{h - H}{\lambda^4} = 0, \quad (2)$$

where $\lambda = [\kappa/V''(H)]^{1/4}$ is the capillary length —the characteristic length of the perturbation decay. This length is analogous to the stalk width R in the recent model of the stalk by Kozlovsky and Kozlov [10]. The membrane profile modified by the presence of a junction located at $\boldsymbol{\rho} = 0$ is given by the solution of equation (2) with the corresponding boundary conditions, $h(0) = 0$, and $\boldsymbol{\nabla} h(0) = 0$ [22]:

$$h(\boldsymbol{\rho}) = H + \frac{4}{\pi} H \, \mathrm{kei}(\rho/\lambda), \quad (3)$$
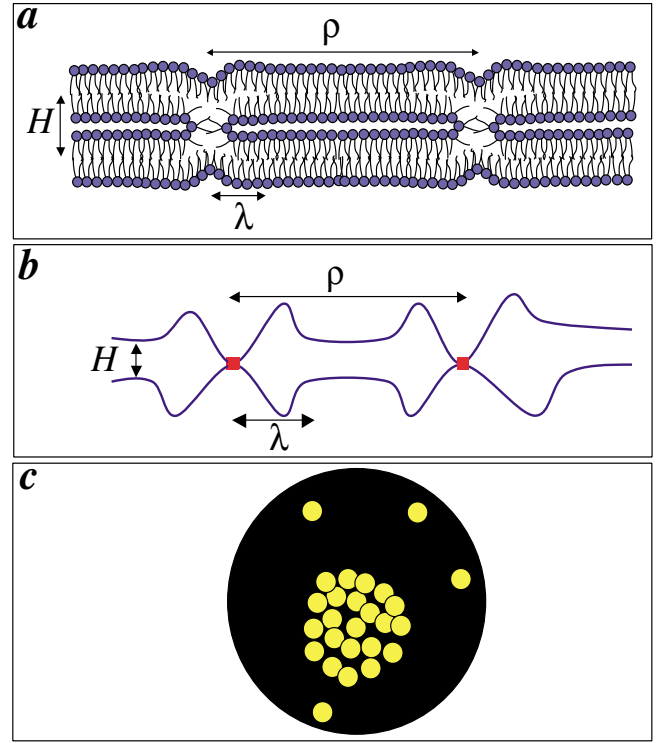

Fig. 1. Schematic representation of the cooperative formation of the fusion intermediate state via condensation of the self-assembled defects (stalks). The stalks represent elementary defects which interact via an effective, membrane-induced mechanism. (a,b): two bilayer membranes with two interacting stalks. (b): within the framework of the model, the membranes have an equilibrium separation H. The stalks constrain undulations of the membranes. The membranes overshoot the equilibrium separation H in the vicinity of the stalks. This is the driving factor for the attraction between the stalks. When the stalks are close to each other, they can share the common overshoot and thus reduce the total bending energy. (c): sketch of the two-dimensional (2D), phase-separated (condensed) phase of stalks in the contact zone of two membranes. The dense phase of stalks is equivalent to a 2D liquid of defects. The stalks are not the only type of possible defects —pores, or pores coexisting with stalks can be also formed.

where $\mathrm{kei}(x)$ is the Kelvin function. An interesting property of this profile is that it overshoots the equilibrium inter-membrane separation, H. This effect is even more pronounced if the non-linear contribution to the interaction potential is taken into account [37,38]. The effect of strong overshooting of membrane profiles when pinched together by optical tweezers has been observed experimentally by Bar-Ziv *et al.* [37] and analyzed theoretically by Menes, Safran, and Kessler [39,38].

The free energy of a single junction is obtained by substituting the membrane profile $h(\boldsymbol{\rho})$ into the free energy equation (1):

$$F = 4\kappa \frac{H^2}{\lambda^2}. \quad (4)$$

This free energy can be directly related to the free energy of the stalk [10]. The dimensionless parameter $\chi \equiv \frac{H^2}{\lambda^2}$ can thus be obtained for a given set of the inter-membrane distance H and the stalk width λ (R in the notations of Ref. [10]). For example, the free energy of the unconstrained stalk in the case of DOPC lipids was estimated in reference [10] to be $F \approx 43 \, k_B T$. This gives $\chi \approx 1$, for a typical value of the bending modulus $\kappa \approx 10 \, k_B T$. This implies that $H/\lambda = \mathcal{O}(1)$. This finding is consistent with the values for H and λ which follow directly from the calculation of the stalk profile in reference [10]: $H \approx 6.2 \, \text{nm}$ and $\lambda \approx 8.7 \, \text{nm}$, leading to the estimate $\chi \approx 0.7$. In other words, the present, simple model allows us to interpret the results of the sophisticated computation of the stalk energy of reference [10] in terms of a single parameter χ, that follows directly from the two intrinsic parameters (H and λ) of the stalk model. In fact, it is argued in reference [10] that the stalk energy is minimal when the stalk width λ is of the order of the inter-membrane separation H. With that information, it follows that the free energy of a single stalk $F \approx 4 \kappa$.

We stress that within our model, a stalk can only have a positive, elastic energy. This is because we assume that the only effect of a stalk is the constraint on the inter-membrane separation, and we neglect the topological change upon the stalk formation (see Fig. 1). To take this into account, each monolayer of membranes must be treated separately [40]. This topological change can lead to a negative stalk energy for lipids with sufficiently large and negative spontaneous splay [10]. Within the framework of our model this can be taken into account phenomenologically by considering the stalk free energy ϵ_s as a sum of the elastic contribution F and the core free energy F_{core}: $\epsilon_s = F + F_{\text{core}}$, where F_{core} can be either positive or negative.

The existence of the attraction between the junctions follows from the analysis of the free energy of a lattice of junctions [22]. In the limit $\rho \gg \lambda$, the interaction free energy between two junctions has the form [41]

$$F_{12} = -C \frac{H^2}{\lambda^2} \frac{\kappa}{\sqrt{\rho/\lambda}} \exp\left(-\frac{\rho}{\sqrt{2}\lambda}\right) \sin\left(\frac{\rho}{\sqrt{2}\lambda}\right), \quad (5)$$

where, $C = 32\sqrt{2/\pi} \approx 25.5$. The larger the bending rigidity κ, the stronger the effective attraction, and the longer the range of this attraction. The characteristic range of the interaction, λ is of the order of 10–20 nm for an artificial phospholipid membrane under physiological conditions [23,29] and thus the long-distance limit equation (5) should be accurate if the junctions are separated by more than 20 nm. We stress that in the opposite limit $\rho \ll \lambda$, the effective interaction remains attractive [22]. Hence, all qualitative conclusions hold irrespective of the value of ρ.

To estimate the phase behavior of the stalks, we use a simple mean-field model analogous to the ones used to describe the aggregation of adhesion molecules or patches within the adhesion zone of two biological or biomimetic membranes [23,29,30,39].

The mean-field free energy of the self-assembling junctions can be constructed with the effective inter-junction potential, equation (5), contributing to the second virial coefficient:

$$f = k_B T \, \phi \ln \phi + k_B T \, (1 - \phi) \ln (1 - \phi) \qquad (6)$$
$$+ J \, \phi (1 - \phi) + \epsilon_s \, \phi,$$

where f is the free energy per elementary surface cell, ϕ is the surface fraction of the junctions, ϵ_s is the stalk free energy (ϵ_s acts as a chemical potential of defects), and J is the effective, thermodynamic interaction potential between the junctions. J is obtained on the level of the linearized second virial coefficient:

$$J = C_1 \, \kappa \, \frac{H^2}{\lambda^2}, \qquad (7)$$

where $C_1 = 16\sqrt{2} \, \pi \, \sin(3\pi/8) \approx 65.7$. Note that the coefficient C_1 is remarkably large. Taking into account that a typical value of the membrane bending modulus is $\kappa \sim 10\text{–}20 \, k_B T$, the large value of C_1 implies that the onset of the phase separation of defects occurs at a very low value of the fusion control parameter, $\chi \equiv \frac{H^2}{\lambda^2}$. Indeed, it follows from the analysis of f that the critical point of the "liquid-gas" phase separation of junctions is $J_c/k_B T = 2$ and $\phi_c = 0.5$. This implies, if we adopt $\kappa \approx 10 \, k_B T$, that the onset of the phase separation occurs at $\chi_c \approx 0.003$. Comparing χ_c with the value of $\chi \approx 1$, estimated above, one concludes that the phase separation occurs already at a vanishingly small concentration of defects. The important message is that the higher the stalk energy, the higher the strength of the effective, inter-junction attraction, and thus the smaller concentration of defects induces the phase separation.

The above arguments indicate that stalk condensation should be possible for reasonable values of the parameters characterizing biological membranes. Yet, the key question is: does it happen in practice? In fact, there is experimental evidence that supports the present scenario: very recently, Yang and Huang [14,15] reported X-ray scattering experiments that show the spontaneous formation of an ordered, dense multiple-stalk structure in a multi-lamellar system of bilayer, diphytanoyl phosphatidylcholine (DPhPC) lipid membranes. From their scattering data, Yang and Huang were able to reconstruct both the global multiple-stalk structure of the fusing membranes, and the structure of the individual stalks. The latter shape turned out to be consistent with the classical stalk structure [10,11,7,12]. We suggest that this kind of multiple-stalk structure should be present in practically all fusion experiments with artificial membranes, provided that the membranes or vesicles have large enough area of inter-bilayer contact (see, e.g., Ref. [42]).

There are also recent computer simulations studies [43, 44], that report either a simultaneous formation of two adjacent fusion zones [44] even in a fairly small fusing vesicles, or a coexistence of fusion stalks and pores [43].

In summary, we propose a possible mechanism of membrane fusion, via a multiple stalk formation. We predict that the intermediate stage of membrane fusion represents a phase-separated phase of self-assembled defects (stalks

or pores, or both stalks and pores). Multiple defect generation is a mechanism alternative to a hypothesis of a single stalk or pore formation and subsequent expansion of the fusion diaphragm. The physical origin of the proposed mechanism is the membrane-induced, effective attraction between fusion defects, similar to the attraction between adhesion receptors. Our conclusions apply both in the case of spontaneous stalk formation and in the case of protein-assisted stalk formation.

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