Membrane Fusion: the Emergence of a New Paradigm

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Abstract Theoretical and experimental work over the last ten years has led to the emergence of a new paradigm for the pathway by which fusion of biological membranes occurs. The path results from the great stability of lipid vesicles, a necessary attribute if they are to function properly. As a consequence, fusion is not easily initiated. The new pathway differs significantly from the conventional one which has framed the picture of fusion for the last thirty years.

Keywords Membrane fusion · Field theory of membranes

1 Introduction

Membrane fusion is crucial for many biological functions; vesicular transport of cargo within the cell, transport of material out of, and into, the cell (i.e. exocytosis and endocytosis), the fusion of cells themselves, and fertilization. It is also a necessary step in the pathway by which foreign and hostile entities, such as viruses, invade the cell. For intracellular vesicular transport, it is important that the cargo go to its intended destination. Therefore it is not surprising that an elaborate, and energetically expensive, machinery exists to bring this about [1]. At the heart of this machinery are complementary SNARE proteins, one affixed to the vesicle, the other to its target. The proteins recognize one another, form a coil-coiled structure, and pull target and vesicle together. This takes a great deal of energy because the lipids comprising each membrane have dipolar headgroups which interact favorably with water, and this water must be removed in order for the membranes to be brought sufficiently close to one another to fuse. Removal of the water causes an increase in the free energy per unit area, or equivalently, the surface tension. The response of the system to this increased tension is either to fuse or to lyse [2–4], two processes which reduce the bilayer surface area. For viral fusion, the machinery is certainly very different, although it is unknown whether the basic fusion pathway differs. The machinery is different because viral fusion is expected

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to be far more promiscuous. The target is well defined, and it does not matter a great deal whether one particular virion makes it into the target or not because there are so many others that are trying. Hence there is no elaborate SNARE mechanism, but rather a small fusogen, hemmagglutinin in the case of influenza, which provides the energy needed to bring about local dehydration.

The pathway by which fusion occurs is not very well understood. Much experimental investigation has been carried out on protein-free membranes, with the necessary energy being provided by some external force. An example is the use of polyethylene glycol, a large water-soluble polymer which, being excluded because of its size from the region between bilayers, causes a depletion force which pushes the bilayers together. Fusion is indeed observed in these protein-free systems. Sometimes leakage and/or lysis is also observed [5, 6]. Fusion peptides like hemagglutinin (influenza) and gp41 (HIV) can also be added to these systems. Again, leakage is often observed along with fusion [6–9].

Finally, there is a conundrum at the heart of fusion, which is the following. In order to be biologically useful, vesicles must be stable, and therefore the probability that a hole should form in them must be exceedingly low. On the other hand, for fusion to occur successfully, the probability of a hole to form in them must be rather high. The conundrum is how these opposing requirements can be reconciled. It can be argued that the same tension which results when the bilayers are locally dehydrated is sufficient to cause the probability of hole formation to increase dramatically. This may indeed be the case, but is not obvious because one does not know the relative magnitudes of the barriers to dehydration and hole formation. All that one can conclude is that, because bilayers should be stable, their fusion should not be easy.

2 Theory

Theoretical understanding has, for a long time, been dominated by the stalk, hemifusion diaphragm hypothesis [10, 11], which is some thirty years old. It has become, literally, the textbook explanation [12]. This idea posits an axially-symmetric process which is shown schematically in Fig. 1. The initial stalk, formed by lipid tails, is a contact between the two opposing, inner, cis, leaflets and is shown in (b). The hypothesis is that the stalk expands axially as the tension causes the inner leaflets to withdraw leaving, in (e), a hemifusion diaphragm which consists of the two outer, trans, leaves of the opposing bilayers [13]. Note that the bilayer area in the hemifusion region has been reduced by half. The formation of a hole in this diaphragm brings about a fusion pore, which can continue to expand. One point to be stressed about this hypothesized pathway is that fusion is not leaky; that is, there is no way in which the contents of either vesicle can leak out into the surrounding medium. Similarly it does not suggest why lysis should be a competing pathway.

The stalk mechanism, originally investigated by phenomenological methods which treat the membranes as elastic sheets [14–16], has been revisited many times particularly with the aim of reducing the estimate for the free energy of the stalk itself. These methods now yield [17] a minimum of $45k_BT$.

About ten years ago, an alternative, symmetry-broken path, was proposed by two groups who investigated the fusion of two planar bilayers. They studied different models and employed different simulation methods, but observed the same pathway. Noguchi and Takasu [18] utilized a Brownian dynamics simulation on a model of amphiphiles consisting of one hydrophilic head bead and two hydrophobic tail beads. The solvent was not modeled explicitly. Müller, Katsov, and Schick [19] performed a Monte Carlo simulation on a system in

Fig. 1 Density profiles of the stalk-like structures shown in the r, z plane of cylindrical coordinates. (**a**) Two bilayers in solvent. There is no stalk between them. (**b**) Unstable transition state to the formation of the initial stalk. (**c**) The metastable stalk itself. (**d**) The unstable transition state between the metastable stalk and the hemifusion diaphragm. (**e**) A small hemifusion diaphragm. Figure from [33]
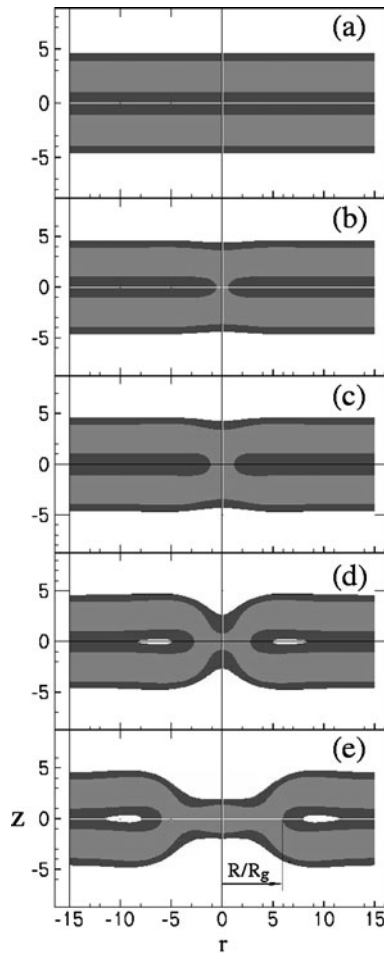
which the amphiphiles more closely resembled block copolymers containing 32 segments, of which 10 were hydrophilic and 12 hydrophobic. The solvent was represented by homopolymers of 32 segments. The pathway observed was as follows: Initially a stalk between the opposing bilayers was formed. However instead of expanding axially, the stalk elongated linearly and began to wander. The presence of the stalk tended to destabilize the membranes causing a hole to appear near the stalk in at least one of the bilayers. This was attributed [20] to the fact that the line tension of the edge of a hole is reduced if it is created near a stalk, as can be intuited from Fig. 2. More succinctly, the probability of hole formation is proportional exponentially to the square of the line tension [21] so that even small reductions in the line tension can have a large effect of the occurrence of holes. Because of the reduction of the free energy of the hole, it is not surprising that the stalk encircles the hole thereby forming a hemifusion diaphragm which consists of the other intact bilayer; i.e. one cis and one trans leaf. This is in contrast with the hemifusion diaphragm in the standard mechanism which consists of the two trans leaves, one from each bilayer. Hole formation in the diaphragm completes the process of pore formation. One variant on this pathway is obtained if the characteristic time for holes to appear is shorter than the time for the stalk to close on

Fig. 2 Schematic explanation of the line tension reduction near the stalk
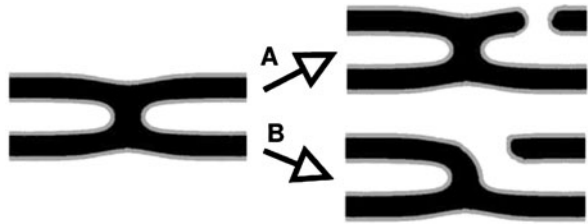
Fig. 3 Density profile of an inverted micellar intermediate (IMI). The architecture of the amphiphiles is characterized by $f = 0.3$, and the tension is zero. Figure from [21]
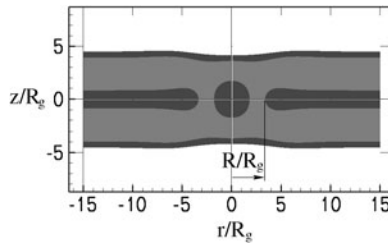
itself, in which case the stalk must encircle holes in each of the bilayers in order to form the fusion pore. It was emphasized by Müller et al. [20], and it should be clear from Fig. 2, that either of these pathways permit the possibility of leakage and lysis. While different in realization, the connection between leakage and fusion pore formation is probably closest to earlier speculations by Bonnafous and Stegmann [8]. Another variant of the pathway occurs if the free energy of hole formation is sufficiently large that the characteristic time for one to appear is longer than the time it takes for the stalk to close on itself. In this case, the stalk provides a smooth closed contact between the intact bilayers, enclosing a portion of both of them and perhaps some solvent entrained between them. The resulting structure, shown in cross-section in Fig. 3, was denoted an inverted micellar intermediate (IMI) after a different structure of the same topology [22]. There is *no* hemifusion diaphragm until a hole appears in one of the two bilayers, and then it consists of the other intact bilayer. The appearance of a hole in the remaining bilayer completes the fusion pore. One would not expect leakage, or lysis, to result from this structure.

The free energy of the inverted micellar intermediate was analyzed by Katsov et al. [21]. In particular they analyzed the conditions under which one should expect the stalk to close upon itself forming an IMI before a hole appears. If a hole does appear before the stalk closes on itself, a portion of the circumference of the hole will be "bare", that is, will not have the stalk next to it. Hence if the free energy of a bare, or isolated, hole is large compared to $k_B T$, that will favor the formation of an IMI. Given that bilayers are very stable, one expects that the energy of an isolated hole is in fact large. Hence the formation of an IMI is probable. Katsov et al. went on to consider the system after the stalk had formed, comparing the free energy barriers encountered in the standard, axially-symmetric, fusion pathway to those that would be encountered in the asymmetric pathway, which includes the possibility of IMI formation. They found that for almost all likely architectures and surface tensions, the asymmetric pathway was favored. Again, if a hole forms before the stalk closes upon itself, leakage and or lysis could occur, while if the stalk closes on itself before a hole forms, they would not occur.

Since the simulations of Noguchi [18] and of Müller et al. [19, 20] observed a non-standard pathway, many others have been carried out. They have been reviewed recently [23]. To the question of whether the pathway to fusion is the standard one or a non-standard

one like that presented above, their answers are surprisingly mixed. One of the earliest [24], simulating the fusion of two small, lipid, vesicles, clearly observed the formation of an IMI. The stalk formed, not on the axis of contact, but rather at the periphery of contact where the two vesicles bent away from the flat region of contact. It then wandered around this periphery to form an IMI. Fusion followed. Some later simulations observed either the standard pathway [25, 26], or the standard pathway on some runs, and the non-standard one on others [27–29]. In particular, formation of an IMI was observed in some runs, again on small, lipid, vesicles [28, 30].

Of great interest is the recent simulation by Risselada and Grubmüller [31] of liposome vesicle fusion mediated by one or more SNARE proteins. They observed fusion to proceed by the non-standard pathway. The vesicles are pulled toward one another by the SNARE proteins and a flattened area of contact between the vesicles is produced. A stalk forms, and expands along the perimeter of the membrane-membrane contact interface. The stalk closes upon itself forming an inverted micelle intermediate and entrains some solvent. A hole forms in one of the two bilayers which leaves a hemifusion diaphragm consisting of the remaining intact bilayer. This diaphragm is under considerable tension, and a hole forms in it to complete the fusion pore. The presence of many SNARE complexes can hinder the second step of the closure of the stalk upon itself and permit the first hole to appear before the IMI is complete, leading to transient leakage. However the stalk completes the closure and seals this first hole before the second one appears.

In addition to the above observations, Risselada and Grubmüller also observed that without the SNARE proteins, stalks only appeared when the vesicles were strongly pressed together, and the process did not continue on to fusion. When stalks appeared, they did so at the edge of the contact surface. In the presence of SNARES, the stalks were initiated near the trans-membrane region of the proteins. The authors attribute the stalk formation there to the distortion in the lipid packing induced by the trans membrane region of the SNARE.

3 Conclusions

From the above calculations and simulations, one concludes the following;

  (i) Fusion is not a process easily initiated. This is, again, due to the requirement that, to be useful, vesicles must be stable. In accord with this, it seems that for fusion to occur, it is not sufficient to simply bring membranes close together [32]. It is this stability which appears to make unlikely the realization of the standard fusion mechanism by which a hemifusion diaphragm forms from the axial expansion of the stalk.

 (ii) Because of this stability, the mechanism which drives the bilayers together causes a flattened region of contact between the still-intact bilayers. A stalk eventually forms in response either to a distortion in the lipid packing brought about by a non-lipid element, such as a protein, or the stress at the circumference of the contact region. Once formed, the stalk wanders around the periphery of the contact zone thereby sewing together the two bilayers and initiating the formation of an inverted miceller intermediate.

(iii) With the formation of the inverted micellar intermediate, there are still two intact bilayers in apposition. However the presence of the stalk at the periphery of the contact area decreases the energy of hole formation with the consequence that a hole forms in one of the two bilayers leaving behind a hemifusion diaphragm which consists of the remaining intact bilayer.

(iv) Formation of a hole in the remaining bilayer brings about the fusion pore.

(v) Because the presence of the stalk decreases the energy of hole formation, a hole in one of the two bilayers can appear before the stalk closes upon itself to form the IMI. In this case there can be leakage. This would cease once the stalk closes upon itself.

In conclusion, the above paradigm is one that has emerged from a fruitful combination of experiment, simulations, and theoretical analysis. It is not yet in final form, but even now it differs substantially from the view commonly held not very long ago. Further work, both theoretical and experimental, will presumably lead to a greater understanding of this important biological process.

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