
CHAPTER 11

Lipid Acrobatics in the Membrane Fusion Arena

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I. OVERVIEW

In this review, we describe the recent contribution of computer simulation approaches to unravel the molecular details of membrane fusion. Over the past decade, fusion between apposed membranes and vesicles has been studied using a large variety of simulation methods and systems. Despite the variety in techniques, some generic fusion pathways emerge that predict a more complex

picture beyond the traditional stalk–pore pathway. Indeed the traditional pathway is confirmed in particle-based simulations, but in addition alternative pathways are observed in which stalks expand linearly rather than radially, leading to inverted-micellar or asymmetric hemifusion intermediates. Simulations also suggest that the first barrier to fusion is not the formation of the stalk, but rather, the formation of a lipid bridge consisting of one or two lipids only. Fusion occurring during the fission process involves other intermediates, however, and is not just fusion reversed. Finally, recent progress in simulations of peptide and protein-mediated fusion shows how fusion proceeds in a more biologically relevant scenario.

II. INTRODUCTION

The fusion and fission of membranes is an essential process in cell biophysics, occurring during exo- and endocytosis, intracellular trafficking and enveloped virus infection. Membrane fusion is also important in a range of biomedical applications such as in gene or drug delivery. A wide range of regulatory protein complexes exists *in vivo*. Due to the wide variety and complexity of fusion protein arrays, the molecular picture of protein-mediated fusion and fission is largely unclear. The basic mechanism, however, is believed to be primarily determined by the physics of lipid–lipid interactions (Chernomordik & Kozlov, 2008; Jahn & Grubmuller, 2002; Lentz, Malinin, Haque, & Evans, 2000).

This basic mechanism seems simple enough: two vesicles touch and merge to form a single vesicle. This process is understood to follow various discrete stages. It all starts with two separate vesicles that somehow come into close contact. An initial contact is formed involving lipid mixing of the contacting monolayers, leaving the remainder of the vesicle intact, a stage called hemifusion. Once the vesicle contents mix, full fusion is reached. These stages are macroscopic phenomena, relatively easy to discern. However, the structures of these stages and their transitions on a microscopic scale are hard to verify experimentally and have therefore been subject of theoretical and simulation studies. Theoretical studies have traditionally been applied using continuum models, providing insight into the energetics and nature of possible fusion intermediates (for reviews see Kozlovsky, Chernomordik, & Kozlov, 2002; Muller, Katsov, & Schick, 2006). Whereas continuum models depend on the assumption of fusion pathways rather than to be able to predict them, the simulation approach is essentially free of any assumptions about the character and sequence of fusion intermediates. Moreover, once properly parameterized, computer simulations can facilitate the direct visualization of the fusion process and may be used to estimate energy barriers. In the past decade, simulation studies have contributed valuable insights to the fusion field; these include the

prediction of alternative fusion pathways, determination of the rate-limiting steps, and, most recently, exploration of novel roles for fusion proteins.

This review aims to give an overview of the current computational perspective on membrane fusion. We consider particle-based simulations of apposed bilayers, bilayers and vesicles, or between vesicles. We do not try to be comprehensive, but rather give a representative overview of the field. Additional information can be found in a number of other reviews (Marrink, De Vries, & Tieleman, 2009; Muller et al., 2006; Shillcock & Lipowsky, 2006). Our review is organized as follows. We set out with a short overview of the historical background of fusion-related simulation studies, introducing the main particle-based simulation techniques used. We proceed with a detailed description of the various fusion pathways that are observed in these simulations, and the energetics involved. Special attention is devoted to discuss the accumulating evidence that the first barrier to fusion is in fact the splaying of a single lipid tail. Subsequently, we describe the process of vesicle fission, showing that it is not just fusion reversed. The final section deals with the growing body of simulations probing the effect of fusion peptides and proteins on the fusion process. A short outlook section concludes this review.

III. HISTORICAL BACKGROUND

The simplest geometry for simulating membrane fusion seems to be provided by the fusion of two planar membranes. As simulations of flat membranes only require a relatively small simulation box using periodic boundary conditions, they are computationally least demanding and have thus been used in the earliest studies on fusion relevant events, such as lamellar to nonlamellar phase transitions (Knecht, Mark, & Marrink, 2006; Marrink & Mark, 2004), pore formation (Leontiadou, Mark, & Marrink, 2004; Tieleman, Leontiadou, Mark, & Marrink, 2003; Tolpekina, den Otter, & Briels, 2004a), disorientation of lipid tails in closely apposed bilayers (Ohta-Iino et al., 2001), and the evolution of hydrophobic defects (Tieleman & Bentz, 2002). An early simulation of full fusion of two flat membranes was by means of lattice Monte Carlo (MC) (Muller, Katsov, & Schick, 2002, 2003), where lipids were described as amphiphilic diblock copolymers and the surrounding solvent as a melt of hydrophilic homopolymers, each representing a group of solvent molecules.

Simulations of fusion of two planar membranes, however, have an implicit problem in that the boundary conditions may influence the simulations; the volume of solvent between the two fusing membranes remains constant until a fusion pore opens to allow the solvent to flow into the spaces behind the fusing membranes. This may influence the dynamics and require the two membranes to be initially already so close together that they are effectively dehydrated along

their whole surface. Fusing two vesicles or a vesicle with a planar membrane avoids this difficulty as the solvent can flow around the vesicle in response to any local fluctuation of its shape. However, because of the larger system sizes required and the concomitant increase in number of molecules involved, the simulation of complete vesicles, and their fusion, comes at the expense of much higher computational cost and is thus only feasible using less detailed models and/or very small vesicles. The first simulation study of the fusion of two vesicles was performed by Noguchi and Takasu (2001). They studied the fusion pathway of two lipid vesicles using Brownian dynamics (BD) simulations. In these simulations no solvent molecules are present and the amphiphilic molecules were modeled as small rigid rods, built up of three particles, one hydrophilic and two hydrophobic.

Although solvent-free models have a computational advantage through not having to simulate the bulk solvent, the absence of solvent is a disadvantage as the solvent will influence possible membrane conformations as well as the dynamics of the membrane. These disadvantages were overcome by using another simulation technique, dissipative particle dynamics (DPD), which includes explicit solvent particles. This technique has been shown to reproduce the correct hydrodynamic forces appropriate to a fluid, and is capable of exploring the phase behavior of lipid molecules and simulating vesicle formation (Li & Liu, 2005; Shillcock & Lipowsky, 2005). Li and Liu (2005) used DPD to study the fusion of two flat membranes consisting of linear lipids and Shillcock and Lipowsky (2005) used this technique to study the fusion of a flat membrane with a small (28 nm diameter) vesicle. Tensionless vesicles and membranes did not fuse but adhered to each other, whereas fusion could be induced by stretching the membranes. Although their lipid model was already more detailed in having two hydrophobic chains, they did not attempt to represent specific lipid molecules. Instead, they aimed to capture the essential features of tension-induced fusion, arguing that membrane fusion is a ubiquitous process observed in diverse amphiphilic systems, having quite distinct microscopic interactions.

The final technique used, which at the expense of higher computational cost can provide a more accurate chemical representation, is molecular dynamics (MD). Because of the computational cost coarse-grained (CG) models were introduced into the fusion field by different groups, for example, Marrink (Marrink & Mark, 2003), Stevens (Stevens, Hoh, & Woolf, 2003), and Smeijers (Smeijers, Markvoort, Pieterse, & Hilbers, 2006). The use of MD allowed, for instance, to discriminate between different types of lipids. Interesting differences were found in the fusion pathways between, for example, PC and PE lipids, or lysolipids. More recently also simulations at atomistic detail have been reported of full vesicle fusion. The first such study was performed by Knecht and Marrink (2007) on a highly fusogenic mixture of

DPPC and palmitic acid. Because of the high computational cost of such atomistic MD simulations, they considered the self-fusion of a very small ~ 15 -nm vesicle with its periodic image. Noteworthy is also the introduction of distributed computing to the fusion arena by Kasson et al. (2006). The availability of thousands of processor nodes allows for systematic studies on fusion pathway statistics.

IV. FUSION PATHWAYS AT THE MOLECULAR LEVEL

The protein-free fusion process has been studied using a variety of simulation techniques, that not only vary in the coarseness of the representations of both the lipids and the solvent but also in the geometry of the fusing membranes and in the way membranes are brought into sufficiently close juxtaposition to initiate fusion. Despite all differences in the simulation techniques, similar processes and fusion intermediates appear, suggesting that they are robust against details of the simulation methods, thus providing strong evidence of their universality. Based on these simulations we identify three fusion pathways: one traditional pathway that we denote the *symmetric stalk expansion pathway*, and two novel, alternative pathways, the *inverted micelle pathway* and the *stalk-pore complex pathway*. Details of these pathways are presented below, together with an evaluation of their composition dependency. Representative snapshots from simulations, illustrating these pathways, are shown in Fig. 1.

A. *Symmetric Stalk Expansion Pathway*

A widely observed pathway is the *symmetric stalk expansion pathway* that has been suggested from continuum-elasticity models (Kozlovsky et al., 2002) and was first described in Kozlov and Markin (1983). In this pathway, first an hourglass-like connection is made between the outer (*cis*) monolayers of both membranes. This early hemifusion connection is referred to as the fusion stalk. At this stage the outer monolayers can start mixing whereas the two inner (*trans*) monolayers are still completely separated. This stalk then expands radially (axially symmetric), either resulting in the direct formation of a fusion pore or in the formation of a –symmetric– hemifusion diaphragm (HD). The HD is denoted symmetric as it is formed by the two *trans* monolayers of the fusing membranes. The *trans* monolayers still do not mix until a pore is formed in this HD. Upon formation of this pore full fusion is obtained and the inner monolayers as well as the vesicle contents can start mixing.

In this pathway there is thus, apart from usual flip-flop (which, however, is too slow to be observed in realistic simulation models), no exchange of lipids

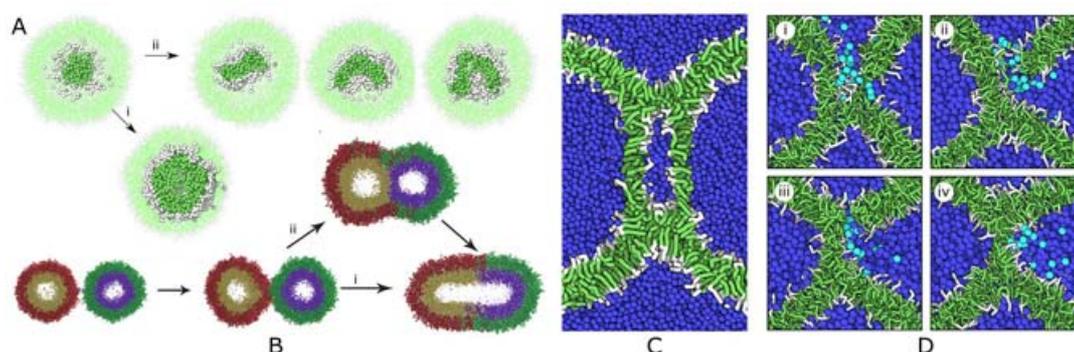


FIGURE 1 Traditional and alternative fusion pathways. (A) All fusion pathways start with the formation of a stalk. This stalk can then expand either radially (i) or anisotropically (ii) (Smeijers, Markvoort, et al., 2006). Based on the fusion intermediates we discern three fusion pathways. (B) In the first pathway, the traditional symmetric stalk expansion pathway, radial stalk formation is followed by direct pore formation (i) or proceeds via a hemifusion diaphragm intermediate (ii) (reproduced with permission from Kasson & Pande, 2007). (C) In the second pathway, the inverse micelle pathway, anisotropic stalk expansion results in formation of an inverse micellar intermediate (adapted from Marrink et al., 2009). (D) In the third pathway, the stalk–pore complex pathway, the stalk nucleates a pore in its vicinity which the stalk subsequently encircles by expanding anisotropically, resulting in the formation of an asymmetric hemifusion diaphragm (reprinted with permission from Smeijers, Markvoort, et al., 2006). Lipids in panels A, C, D are represented with white head groups and gray tails; in panel B lipids are depicted with different shades of gray between *cis* and *trans* monolayers, and between vesicles. Water is depicted either as spheres or omitted for clarity. All snapshots show cross sections through two fusing vesicles, either perpendicular (A) or parallel (B–D) to the fusion coordinate. (See Color Insert.)

between the *cis* and *trans* leaflets of the vesicles and there is no mixing between the vesicle interior and its exterior. The direct pore formation was first observed in BD simulations (Noguchi & Takasu, 2001), which used rigid lipids and implicit solvent. Later this direct pore pathway was also observed with more detailed CG models using DPD (Gao, Lipowsky, & Shillcock, 2008; D. W. Li & Liu, 2005) and MD (Kasson et al., 2006) as well as in atomistic (Kasson, Lindahl, & Pande, 2010) simulations (see Fig. 1A and B). The stalk–HD–pore transition was first observed by Marrink and coworkers, both in CG (Marrink & Mark, 2003) and later in atomistic (Knecht & Marrink, 2007) MD simulations. Using CG MD simulation of over 10,000 fusion events (Kasson et al., 2006) showed that both pathways can happen, randomly, in the same system.

B. Alternative Pathways

Many simulations also demonstrate other fusion pathways, which never have been suggested and analyzed by continuum approaches. A similarity

between these fusion pathways is that they are much less symmetric than the priorly described traditional mechanism. Although the first stage, the stalk formation, is the same once the stalk is formed instead of growing radially, it undergoes anisotropic growth forming an elongated (linear) connection (see Fig. 1A).

In the *inverted micelle pathway* this expansion proceeds in a bent manner, forming into a banana-shaped stalk. Upon closing on further bending, a circular stalk is formed, giving rise to a fusion intermediate in which an inverted micelle is trapped in between two vesicles. Such an inverted micellar intermediate (IMI) has for example been reported in the fusion of two membranes by Li and Liu (2005) in DPD simulations in case of lipids with sufficiently negative spontaneous curvature, and also by Marrink, Fuhrmans, Risselada, and Periole (2008) in CG MD simulations of mixed PC/PE vesicles (Fig. 1C). Formation of a pore through rupture of one of the membranes separating the vesicle interiors from the water bubble, then leads to the hemifused state. Note that, in contrast to the traditional pathway, in this case the HD is asymmetric, consisting of the *cis* and *trans* monolayer of only one of the fusing membranes (Muller, Katsov, & Schick, 2003). A similar linear stalk expansion is also seen in simulations where two vesicles, or a vesicle and a membrane, do not fuse at the central location, that is, the initial point of closest approach, but where first a (large) flattened contact is formed. This is seen both in some CG MD (Stevens et al., 2003) as well as DPD (Grafmuller, Shillcock, & Lipowsky, 2007, 2009) simulations. The stalk is formed and expands along the strained membrane at the contact edge; this is attributed to the high curvature at the edge with lipids already in a tilted or splayed conformation. Via a bent stalk an asymmetric HD is also formed here.

The third pathway, that is, *the stalk-pore complex pathway*, also starts with the stalk expanding linearly. However, in this mechanism a key feature is the destabilization of the contacting membranes by the elongated stalk, as a result of which pores appear in the fusing bilayers in its vicinity. The stalk then starts encircling these pores. When pores nucleate in both membranes the bent stalk aligns them, forming an incomplete fusion pore, and the fusion process is completed by propagation of the stalk along the edges of the aligned pores to produce a fusion pore. This pathway was first observed in the BD simulations at high temperatures (Noguchi & Takasu, 2001) and lattice MC simulations (Muller et al., 2002, 2003), and later also seen in CG MD simulations (Smeijers, Markvoort, et al., 2006). If, on the other hand, the stalk encircles a pore in only one of the membranes, an asymmetric HD is formed again (Fig. 1D). Full fusion is then completed by the formation of a fusion pore upon rupturing of this HD as in the traditional pathway. This pathway was also observed in the before-mentioned MC simulations (Muller et al., 2003) as well as in CG MD simulations (Marrink & Mark, 2003; Smeijers, Markvoort, et al.,

2006) and later confirmed by atomistic simulations (Knecht & Marrink, 2007). Somewhat analogous is the pathway observed by Gao et al. (2008) using DPD, where high tension caused rupture of one of the membranes in the contact zone, also resulting in an HD consisting of the two monolayers of the still intact bilayer.

Thus, simulations of a large number of fusion events show some common fusion pathways, which involve much more disordered and less symmetric intermediate states than is typically assumed (continuum models routinely assume that the fusion intermediates have axially symmetric shapes). Moreover, simulations reveal that the conformations of individual lipids play a crucial role in membrane processes, which although at a very small scale cannot be neglected. Surprisingly, a number of simulations showed that a given system can follow several pathways. A first example is the before-mentioned study by Kasson et al. (2006) where systems of exactly the same size and composition follow the stalk–pore pathway at random with or without the HD intermediate. Even stronger, a given system can fuse following both radial and anisotropic stalk expansion, porous and nonporously (Marrink & Mark, 2003; Smeijers, Markvoort, et al., 2006), where the pathway again seems to be chosen rather stochastically.

How to distinguish, experimentally, between these different pathways? As stated before, the HDs in these linear stalk expansion pathways are not classical ones in the sense that they are comprised of both leaflets of one intact bilayer instead of by the two *trans* monolayers. This implies mixing between *trans* lipids from one membrane and *cis* lipids from the other. Incorporation of lipids with sufficiently slow flip–flop rates allows one, in principle, to probe this mechanism. Linear stalk expansion also results in a partially confined solvent cavity between the two fusing membranes. Upon full encircling this originally external solvent is thus internalized. As suggested in Risselada, Knutzer, and Grubmuller (2011), reasonably large fluorescent molecules, that are too large to diffuse through the leakage pores, may get encapsulated this way offering a route to experimental testing. Moreover, when (transient) holes emerge in the vesicles before the stalk completely encircles the contact site, the contents of the vesicles may mix with the exterior phase. The pathways with linear stalk expansion are thus a leaky way to fuse vesicles, offering a molecular explanation for those effects seen in experimental fusion assays (Frolov, Dunina-Barkovskaya, Samsonov, & Zimmerberg, 2003; Muller et al., 2003) and providing the possibility of lysis, which the standard pathways do not. At the same time, leaky fusion appears not advantageous for living cells as content separation constitutes the primary purpose of vesicle transport. This suggests another potential role for fusion proteins, that is, to avoid the stalk from expanding linearly.

C. *Composition Dependence*

Although simulations show that the choice of fusion pathway is stochastic, that is, the same system can follow multiple pathways, membrane composition does play a role in the prevalence of one pathway over the other. As evidenced from experimental data, the formation of different fusion intermediates can be correlated to the lipids, spontaneous curvature (reviewed in Chernomordik & Kozlov, 2003). This dependence is also apparent from various simulation studies. For instance, DPD simulations (Li & Liu, 2005) showed a stalk–pore complex or IMI for lipids possessing a small head group versus traditional stalks for lipids with larger head groups. Using MD simulations the compositional dependence was studied in more detail. An increased speed of stalk formation on increased PE over PC content is observed by Marrink and Mark (2004) in a study of the lamellar to inverted hexagonal phase transition, a transition that proceeds through stalk formation and subsequent stalk elongation. This study also shows that for a narrow composition/hydration range the stalks do not elongate but remain stable instead. Such a phase, denoted rhombohedral or stalk phase, has also been observed experimentally (Yang & Huang, 2002). Concerning the fusion between small vesicles, Marrink and Mark (2003) found that for a pure POPE system the hemifused state is stable, whereas for mixed PC:PE systems a fusion pore appears quickly and for pure DPPC formation of a HD was never observed. By systematically changing the PC:PE ratio, Kasson and Pande (2007) showed the dependence on the lipid composition of the relative prevalence of a stalk–HD–fusion pore pathway over a less frequent direct transition from stalk to fusion pore. Increased PC content causes increased activation energies for formation of the initial stalk-like intermediate for fusion and of hemifusion intermediates, resulting in many unfused vesicles and an increased utilization of the direct fusion pathway. Increased PE content, on the other hand, not only increases the speed of stalk formation but also stabilizes hemifused states, such that a 1:1 PC:PE ratio offered the fastest rate to full fusion. Stabilization of the HD by PE lipids is confirmed by recent CG MD simulations of Nishizawa and Nishizawa (2010b). These authors showed that DOPE accumulates at the high curvature regions of a preformed HD.

For vesicles with asymmetric lipid composition the rate of fusion not only depends on the overall lipid composition but also on the distribution of lipid types over the monolayers. Marrink and Mark (2003) for instance showed that PE, having a negative spontaneous curvature, increases the fusogenicity when present in the outer monolayer, and stabilizes hemifused states when present in the inner monolayer. Oppositely, lysoPC, having a high positive spontaneous curvature, decreases the likeliness to fuse when present in the outer monolayer,

whereas only present in the inner monolayer, it is found to accelerate fusion by strongly destabilizing the HD. However, more simulation data are needed to get a better understanding of the relationship between lipid composition and fusion intermediates. Clear is that the propensity to fuse is strongly dependent on lipid composition, consistent with evidence from a large number of experimental studies (e.g., reviewed in Chernomordik & Kozlov, 2003). This fusion dependence is usually explained based on the effective molecular shapes of lipids (spontaneous curvature), reflecting the propensity to bend into fusion intermediates. But, as we will discuss in more detail in Section V, simulation studies show that there are more ways in which lipids can increase the fusion rate, not so easily understood in terms of the curvature concept. We hope to provide some insight by taking a closer look at the energy landscape of the fusion arena.

V. ENERGY LANDSCAPE ALONG THE FUSION PATHWAY

In many simulation studies, it is apparent that both the stalk and the HD can be metastable states. In general, high energies of these intermediates are avoided by tilting of the tails, avoiding empty voids, although regions of lower density can be observed. The major energy barriers in these pathways that thus can be identified are (i) in the transition from unfused membranes to a stalk, (ii) in the transition from the stalk to the HD, and (iii) in the initiation of a pore in this HD. Initially, energy estimates for these various intermediate fusion stages and the associated energy barriers were obtained from continuum or field theoretical models (Katsov, Muller, & Schick, 2004; Katsov, Muller, & Schick, 2006; Kozlovsky et al., 2002; Kuzmin, Zimmerberg, Chizmadzhev, & Cohen, 2001; Markin & Albanesi, 2002). Nowadays, exploration of the energy landscape is amenable to more detailed calculations using molecular simulation techniques. In this section, we first discuss recent estimates pertaining the stalk and hemifused states, and then focus on the initial fusion steps involving some true lipid acrobatics. In the last part of this section, we present a schematic overview of the energy landscape emerging from *in silico* studies.

A. Stalk and Hemifusion Diaphragm Intermediates

Because of the height of the energy barriers most membranes do not fuse spontaneously in the (submillisecond) time scales reachable with molecular simulations. Experimentally it has been uncovered that the fusogenicity of liposomes depends on their size and the membrane tension (e.g., Finkelstein, Zimmerberg, & Cohen, 1986; Ohki, 1984). Most simulations are, because of the limited system sizes, on very small vesicles with a size, 15–30 nm in diameter,

which is at the lower limit of experimentally producible vesicles. Although such highly curved membranes already fuse more easily than flat bilayers, in order to obtain fusion within the reachable time frame, fusion often still needs to be induced by initial placement of the membranes/vesicles in close vicinity, which might include partial dehydration of the lipid head groups, the addition of a (polyethylene glycol) cross-linker to keep the membranes together, pushing the membranes toward each other, elevating the temperature, stretching a membrane, or pulling a selected lipid across the inter-bilayer space. This procedure eventually results in stalk formation and possible formation of the HD. Noteworthy, simulations show that the high energy barriers are not formed by the stalk and HD intermediates themselves, but by transitions between such different intermediates.

Using their CG MD simulations and their large-scale distributed computing approach, Kasson et al. (2006) for instance looked at over 10,000 separate fusion events, which allowed them to construct a Markovian state model by which free energies of fusion intermediates as well as rates could be determined. In this way, they obtained decreasing free energies for unfused, stalk, hemifused, and fully fused states in small (14 nm in diameter) POPE vesicles, with a difference in free energy between the stalk and the unfused state of $6 k_B T$. The free energy of stalks has been calculated from some other simulations as well. Norizoe, Daoulas, and Muller (2010) for instance derived, using a thermodynamic integration method, an excess free energy for the stalk of $16 k_B T$ for a solvent-free, CG model of two apposed planar bilayers. A similar energy difference of $10 k_B T$ was derived from self-consistent field calculations of a polymeric diblock system by Katsov et al. (2004). Using CG MD simulations, Smirnova, Marrink, Lipowsky, and Knecht (2010) derive a free energy of $3 k_B T$ for the stalk between two planar DOPC bilayers at low hydration, with an energy barrier from the unconnected state of $20 k_B T$ as will be discussed in Section V.B. Precise values of the stalk free energy thus depend on the lipid composition and the level of dehydration of the unfused reference state; the stalk free energy can be negative for lipids with negative spontaneous curvature (e.g., PEs), and positive for lamellar forming lipids (e.g., PCs). However, simulations indicate that the stalk is not a transition state, but metastable or on the downhill slope of the fusion process.

In the pathways with an HD, full fusion is obtained by the formation of a pore in the HD. Pores can be formed in membranes by several agents, but can also form spontaneously or triggered by electric fields or membrane tension. Such pore formation has been studied extensively in simulations of periodic membranes and has been reviewed recently for instance in Marrink et al. (2009) and Gurtovenko, Anwar, and Vattulainen (2010). It is generally found that the shapes of the pores are toroidal, with the lipid head groups lining the pore wall, quite independent of the details of the models used. Using MD simulations,

Tolpekina et al. (2004a) report extensive free energy calculations on pores, showing an energy barrier of 15–20 $k_B T$ for pore opening, while no barrier to pore closure was found although a small barrier has been predicted from atomistic MD simulations (Marrink, Lindahl, Edholm, & Mark, 2001). The same authors (Tolpekina, den Otter, & Briels, 2004b) also stretched bilayers to study the stability of pores establishing a phase diagram of pores, identifying regions where pores are stable, metastable, or unstable. The HD being metastable, with the fully fused state having the lowest energy, the formation of a local defect is the rate-limiting step in the decay of the HD. The energy barrier may, however, be lower than in a periodic bilayer as in the HD these defects appear most often close to the rim (Gao et al., 2008; Smeijers, Markvoort, et al., 2006) as the membrane is already distorted there (cf. Fig. 3, snapshot of the pore formation barrier).

B. Lipid Splaying as First Step

There is a growing body of evidence from simulations that the first, and often rate-limiting, step in the fusion process is the splaying of a single lipid tail rather than the formation of the stalk. Already in some of the first MD simulations of vesicle fusion (Marrink & Mark, 2003; Stevens et al., 2003) it was observed that stalk formation was preceded by the movement of the tails of just one or two lipids. The process was described in most detail in Stevens et al. (2003). The authors found that, upon pushing two small vesicles toward each other, the bilayers dilate at the contact edge producing a tilting of the individual molecules. Fusion is initiated when some of these tilted lipids splay their aliphatic tails, such that the molecules are shared between the opposing leaflets. Multiple splayed lipids subsequently associate with their aliphatic tails in contact, which produces a new hydrophobic core leading to the stalk state as described in Section IV. Later the initiation of fusion by the splaying of a single lipid tail has also been observed in unconstrained vesicles that fused spontaneously without having tilted lipids at the edge of a flattened contact zone (Smeijers, Markvoort, et al., 2006).

Quite recently, a number of more quantitative studies have been performed in order to elucidate the energetic barriers of the initial fusion event(s), confirming the importance of the splayed-tail intermediate. In a study by Smirnova et al. (2010), free energy calculations were performed for apposed POPC membranes, using the tail of a single lipid as reaction coordinate. Pulling the tail out of the one leaflet toward the other eventually leads to spontaneous stalk formation, as shown in Fig. 2. Stalk formation is initiated by the establishment of a localized hydrophobic contact between the bilayers. This contact is either formed by two partially splayed lipids or a single fully splayed one leading to the formation of a (metastable) splayed lipid bond intermediate. Whereas the stalk structure has

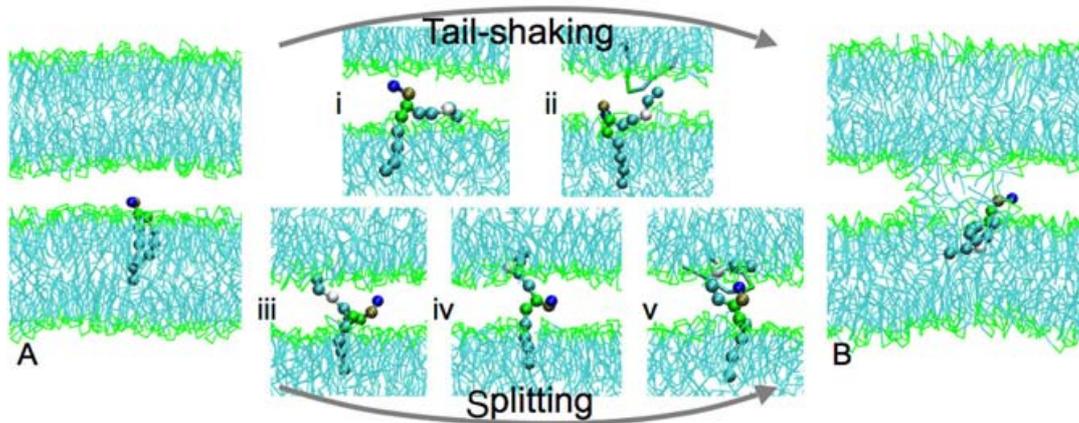


FIGURE 2 Lipid acrobatics during the initial fusion event. Snapshots are taken from a CG simulation of stalk formation between POPC bilayers (Smirnova et al., 2010). (A) Initial state with two closely apposed bilayers; (B) final state in which a stalk has formed. (i–ii) Tail-shaking pathway in which lipid splaying (i) is followed by the establishment of a hydrophobic contact with a lipid tail from the other membrane (ii). (iii–v) Splitting pathway, again initiated with splaying of a single lipid (iii) that subsequently inserts its tail into the other membrane adopting a metastable, split, conformation (iv), followed by the attraction of another lipid tail (v). Lipid tails and glycerol groups are shown in stick representation. Lipid heads and water are not shown for clarity. The lipid triggering the initial fusion event is displayed in sphere representation. (See Color Insert.)

only a small free energy difference, of $3 k_B T$, with respect to the initial state of two unconnected bilayers, the intermediate states were found to have a significant free energy of $20 k_B T$. These findings indicate that, at least under conditions of low hydration, early membrane fusion kinetics is not determined by the stalk energy but by the energy of prestalk transition states involving solvent-exposed lipid tails. A similar conclusion was drawn from Mirjanian, Dickey, Hoh, Woolf, and Stevens (2010) based on CG simulations using a similar force field. In this study, fusion between pairs of liposomes was simulated for four systems: DPPC, DOPC, a 3:1 mixture of DPPC/DPPE, and an asymmetric lipid tail system in which one tail of DPPC was reduced to half the length. The most prominent molecular detail of barrier crossing in all cases examined was, again, the splaying of lipid tails. It was further concluded that the tail splay appears to be closely connected to the energetics of the process. For example, the high barrier for the asymmetric lipid is the result of a smaller distance between terminal methyl groups in the splayed molecule. The shortening of this distance requires the liposomes to be closer together, which significantly increases the cost of water removal and bilayer deformation. Before tail splay can initiate fusion, contact must occur between a tail end and the external water. In the work of Grafmuller, Shillcock, and Lipowsky (2009), the fusion of vesicles with planar lipid bilayers was studied with DPD simulations. Again, it was found that the fusion process starts with individual lipids assuming a splayed tail

configuration with one tail inserted in each membrane. To determine the corresponding energy barrier, the average work to displace one lipid tail from one bilayer to the other was measured. This energy barrier is estimated to lie in the range of 8–15 $k_B T$, depending on the strength of the interaction between the lipid tail and lipid head. Furthermore, it was found that the energy barrier for flipping of the lipid tails decreases with increasing tension of the membrane.

The studies cited above are all based on CG representations of lipids. The obvious question is whether the neglect of the atomistic degrees of freedom results in an overestimation of the importance of splayed-lipid intermediates. First evidence that this is not the case comes from fully atomistic studies of Ohta-Iino et al. (2001) already a while ago. These authors simulated two DMPC bilayers separated by only a thin water layer, in the range of 0–5 waters/lipid. Under these conditions, lipid tails were observed to penetrate into the interbilayer region on a subnanosecond time scale. A more elaborate study on fusion was performed by Kasson et al. (2010). Here, million-atom MD simulations of vesicle fusion were performed using distributed computing. Committor analysis was used to identify a transition state for fusion stalk formation. This transition state was found to occur when the bulk properties of each lipid bilayer remain in a lamellar state but a few hydrophobic tails bulge into the hydrophilic interface layer and make contact to nucleate a stalk.

Thus, both CG and atomistically detailed simulations point at the key roles of lipid tail dynamics at the onset of membrane fusion. The transition state toward stalk formation appears to be either a single lipid splayed between the two fusing membranes (for which we propose the name “*splitting*”), or the merging of the tails of two lipids, one from each membrane (coined as “*tail-shaking*”). See Fig. 2 for illustrative snapshots of these states. Note that the importance of the splitting conformation in triggering membrane fusion was already recognized in the work of Kinnunen and Holopainen (2000), based on indirect experimental evidence. The *in silico* confirmation of the importance of lipid tail splaying has a number of important implications for the role of lipid composition during the initial stages of fusion, namely (i) only the *cis*-monolayer lipid composition plays a role, and (ii) lipid tails, rather than lipid headgroups, are important. In this light, one would predict that especially polyunsaturated lipids accelerate the initial fusion step toward stalk formation, provided they are present in the contacting monolayers. Polyunsaturated tails are both more soluble and are easier to splay compared to saturated ones, favorable conditions for the splayed intermediate. There is experimental evidence that polyunsaturated lipids or fatty acids are indeed important for fusion (e.g., Glaser & Gross, 1994; Stillwell & Wassall, 2003). Likewise, oxidized lipids are expected to trigger fusion, as the oxidized tails have a high propensity to bend toward the water phase where the oxygen atoms form hydrogen bonds with water and the lipid

headgroup (Wong-Ekkabut et al., 2007). Lipids with three tails, for example, triglycerides, could also favor tail splaying due to the large tail volume compared to their head group size. Lysolipids, on the other hand, are unable to adopt a splitting conformation but may still form the tail-shaking intermediate. According to the simulations of Smirnova et al. (2010), both intermediates have a similar free energy. However, the tail-shaking conformation might result in the actual desorption of the single tail lipid, and therefore the presence of lysolipids in the contacting monolayer is predicted to impede the very first step of fusion. Lipids that are unable to form any of the splayed intermediates are cyclic lipids (also denoted tetraether, or bola-lipids); indeed there is experimental evidence that vesicles composed of such lipids do not easily fuse (Relini et al., 1994).

The importance of splayed lipid intermediates may also suggest possible roles for other agents which are known to promote fusion. One example is Ca^{2+} which promotes fusion both *in vivo* and *in vitro* (Papahadjopoulos, Nir, & Duzgunes, 1990). Simulations (Issa, Manke, Jena, & Potoff, 2010) of apposed, dehydrated, membranes in the presence of Ca^{2+} provide some relevant insight. Whereas Ca^{2+} induces an overall ordering of the lipid tails due to the in-plane binding of the PC headgroups, local disordering is observed in regions where the ion bridges lipids from the opposing membranes. In other words, in addition to its role in keeping membranes bound together, Ca^{2+} might be able to destabilize the very lipids that are being bridged, facilitating the formation of splayed lipid intermediates. As we will see in Section VII, fusion peptides might operate in a similar way.

Note that the importance of splayed lipid intermediates at the onset of membrane fusion will be strongly dependent on the level of hydration. Simulations of stalk formation in bilayer stacks at different hydration levels, for instance, show that the rate of stalk formation decreases with increasing width of the hydration layer (Marrink & Mark, 2004). Most of the studies cited above probe conditions of low hydration (~ 5 waters/lipid) for which the splayed lipid constitutes the main barrier. The relative free energy of the stalk state with respect to the splayed lipid state will depend on the separation distance; it is not clear that membrane bridging by a splayed lipid molecule would lead to stalk formation in case there is no strong dehydration over an extended region or strong membrane stress through, for example, bending, both favoring the stalk formation. Whether or not these strongly dehydrated conditions resemble the fusion process *in vivo* is not known.

C. Many Barriers to Cross

Based on the combined data of particle-based simulation studies on membrane fusion, we composed a schematic energy landscape, Fig. 3, presenting the main intermediates and barriers involved. The main intermediates separating the unfused and fused states are (i) the adsorbed state, (ii) the stalk state, and

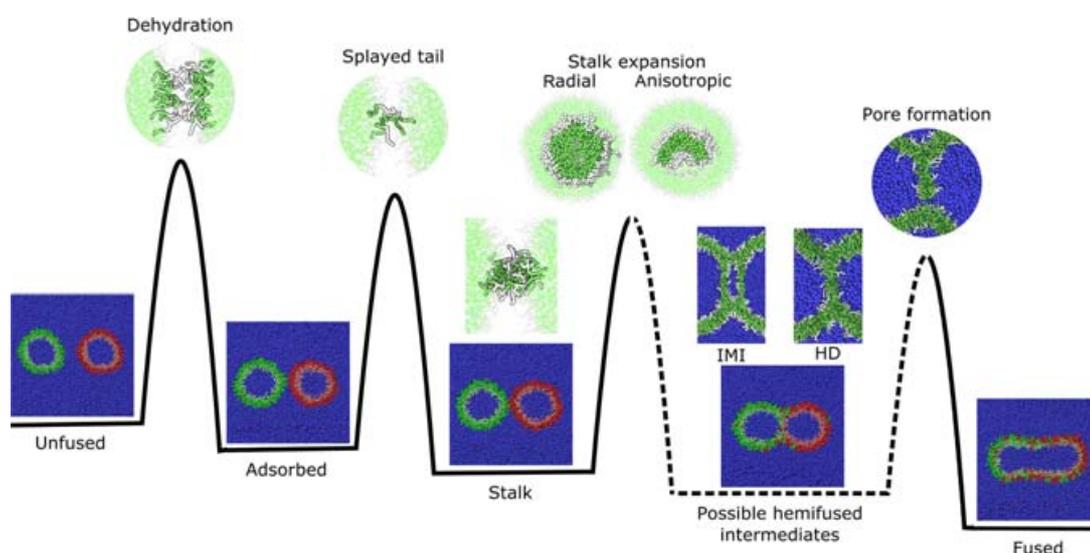


FIGURE 3 Schematic representation of the fusion energy landscape. Stable or metastable intermediate states leading from the “unfused” to the “fused” membranes are indicated, namely the “adsorbed,” “stalk,” and a number of “hemifused” states including the inverted micellar intermediate (IMI) and hemifusion diaphragm (HD). These states are separated by energy barriers involving subsequently “dehydration,” lipid tail “splaying,” either radial or anisotropic “stalk expansion,” and “pore formation.” Snapshots, obtained from CG MD simulations (Smeijers, Markvoort, et al., 2006) show cross-sections through two fusing vesicles, either perpendicular or parallel to the fusion coordinate. Color scheme as in Fig. 1. Note that the relative heights of the barriers and intermediate states have no meaning in this figure, as they will depend strongly on the fusion conditions. (See Color Insert.)

(iii) the IMI and HD states. These last two states are not always observed, that is, fusion may proceed directly from the stalk to the fused state without forming a stable intermediate, or only form the HD state but not the IMI (see Section IV.A). Whether the first, adsorbed, state is (meta)stable seems to depend on the contact area of the fusing membranes; for small vesicles it is not observed but for larger patches the dehydrated, bound state appears as a real intermediate. The barriers in between of the intermediate states differ substantially in their nature, reflecting the complexity of the fusion process. The first barrier is encountered upon bringing the membranes in close proximity, and involves overcoming the undulatory and hydration forces arising from the collective interactions of the lipids in the contact patch (see recent simulation studies on hydration forces, Eun and Berkowitz (2009); Gentilcore, Michaud-Agrawal, Crozier, Stevens, and Woolf (2010)). On the contrary, the second barrier involves splaying of just one or a few lipid tails, as discussed in Section V.B. The third barrier, stalk elongation, involves multiple lipids, and is dominated by the ability of lipids to pack in nonlamellar geometries. The final barrier is the nucleation of the fusion pore, again a localized process involving few lipids only.

Naturally, the relative stabilities of the states depend entirely on the state conditions, that is, composition, temperature, ionic strength, hydration, etc. The energy levels drawn in Fig. 3 do not have any quantitative meaning; for instance, the overall fusion process might be downhill, as observed for some systems *in vitro* which spontaneously fuse, but require substantial energy input (e.g., by means of a protein machinery) under *in vivo* conditions. The *in silico* studies point to some generic mechanisms by which the energy landscape can be modulated. This is summarized in Table I. Here we consider the effect of changing lipid composition, either modifying the head group (PC vs. PE), or the tails (saturated vs. unsaturated lipids and lysolipids), or the effect of adding fusion peptides or fusion proteins. The anticipated effects are only indicated in a qualitative sense, that is, either as stabilizing or destabilizing a particular (transition) state. Even then, Table I should be considered with care as the combined lipid/peptide fusion process is much too complicated to be captured in such a simplistic way. Nevertheless, we hope that Table I may provide some guideline for the understanding of fusion experiments, be it *in silico*, *in vitro*, or *in vivo*. In the following paragraph, we briefly discuss the data in Table I.

The *dehydration* of the interface is expected to be facilitated by lipids that do not swell much, such as PEs. Adsorption of peptides at the interface also lowers the hydration repulsion. An increase in temperature increases the undulation repulsion, and therefore makes it harder for membranes to come into close contact. An increase in membrane tension, on the other hand, suppresses undulations and results in the opposite effect. The stability of the *adsorbed state* is likely governed

TABLE I
Effect of State Conditions on Energy of Fusion Barriers and Intermediates, as Predicted by Simulation Studies^a

	PC	PE	Sat	Unsat	Lyso	Pep	Prot	Temp	Tens
Dehydration	+	–	o	o	o	–	–	+	–
Bound	+	–	o	o	o	–	–	+	–
Splaying	o	o	o	–	+	–		–	–
Stalk	+	–	o	–	+			–	+
Elongation						–		–	
Hemifused	+	–	o	–	+			–	+
Poration	–	+	o	+	–	–	–	–	–

^aAn increase in the energy is denoted by “+,” a decrease by “–.” A “o” indicates that no effect is expected, and a blank that the effect is unknown. The following conditions are considered: increased ratio of PC and PE lipids, lipids with saturated (sat) or unsaturated (unsat) tails, and lysolipids (lyso), or presence of fusion peptides (pep) or fusion proteins (prot). For the proteins, the effect beyond that of the fusion peptide is considered. The effect of increased temperature (temp) or membrane tension (tens) is also listed (see text for details).

by the same factors. Especially proteins are required at this step to bring and keep the membranes close together. *Lipid splaying* is controlled to a large extent by the lipid tails; notably the presence of unsaturated tails will lower this barrier. In addition, fusion peptides are able to facilitate lipid splaying as will be discussed in Section VII.A. Increased temperature and tension result in more tail disorder and are therefore also predicted to favor tail splaying. The *stalk state* is stabilized by lipids with negative spontaneous curvature, such as PEs and unsaturated lipids. Lipids with positive curvature, such as single tail lipids (lysolipids) or lipids with large head groups (PC), destabilize the stalk. The effect of fusion peptides, or proteins, is actually less clear. An increase in temperature induces more negative curvature (through disordering of the tails), and therefore results in lowering of the stalk energy. Membranes under tension, however, are less likely to form (meta) stable stalks as the lipids will be sucked back into the membrane. *Stalk expansion*, subsequently, can proceed via different pathways as discussed in Section IV.A; it is not yet clear from simulation studies which conditions favor this step. There are some indications that fusion peptides may have a role here promoting bending of the stalk or formation of stalk–pore complexes (see Section VII.A). Both *hemi-fused states*, the IMI and HD, still have an overall negative curvature. Indeed simulations point to a stabilization of these states by the same type of lipids that stabilize stalks. Finally, *pore formation* is triggered by lipids with positive curvature, that is, PCs rather than PEs, and saturated rather than unsaturated tails. Short, single tail lysolipids are especially efficient at this stage. Fusion peptides may also play a role in formation of the fusion pore, inducing positive curvature stress due to their interfacial adsorption (see Section VII.A). Both increased temperature and tension also favor pore formation, destabilizing the lamellar state. Possibly also proteins are involved in this step putting the HD under additional tension.

VI. FISSION PATHWAYS IN MOLECULAR DETAIL

The opposite process of fusion, where two membranes fuse to a single membrane, is the division of a single membrane into two. This process, which is called membrane fission, is a crucial stage during exo- and endocytosis as well as in the formation of all kinds of intracellular carriers, for instance by the Golgi apparatus. Like fusion, the fission process can also be simulated using particle-based methods. The fission process is here described in two stages. First, the membrane has to be deformed, forming a narrow neck to allow it to self-fuse. This process of curvature generation, that is often denoted as budding, can be triggered by a number of mechanisms as described below. Second, the actual fission takes place as a self-fusion of the membrane in the neck according to a pathway that could, in principle, be the same as observed in regular fusion. We will see that this is, however, not the case.

A. Budding/Neck Formation

Whereas for fusion two separate membranes have to come into close juxtaposition, an important prerequisite for fission is the deformation of a membrane or vesicle into a shape that can easily be divided into two parts, like a budded shape where a spherical vesicle is connected to a flat membrane or another vesicle by a narrow neck. As the undeformed state has the lowest free energy, a driving force for such membrane budding is needed. This driving force can arise from a number of different (biological) mechanisms, which may involve some kind of protein machinery, but which may also be completely lipid based.

A first example of a protein-based mechanism is the deformation mediated by proteins binding to the membrane and functioning as a template. Atomistic MD simulations for instance showed how so-called BAR domains can induce a strong local curvature upon binding to a negatively charged membrane (Blood & Voth, 2006), whereas multiscale methods (Arkhipov, Yin, & Schulten, 2008; Ayton, Blood, & Voth, 2007) can show the cooperative nature of such BAR domains in inducing global membrane bending. Another nice example of how curved proteins can imply their curvature to an initially flat membrane, finally resulting in a vesicle budding off, is shown by Reynwar et al. (2007). A second mechanism is membrane deformation by a cytoskeletal element capable of either pushing or pulling on the membrane. An example of a simulation study mimicking an external force on a membrane is a BD study (Noguchi & Takasu, 2002b) where a force is applied on the membrane by pulling on nanoparticles inside the vesicle, like in an optical tweezer experiment, which resulted in the deformation and finally fission of the vesicle. A third mechanism comprises the enwrapping of a (extra)cytoplasmic particle, driven by the adhesion of the membrane to an already curved particle. An example of such fission by adhesion to nanoparticles is shown by Noguchi and Takasu (2002a) as well. A final mechanism is asymmetric redistribution of lipids by flippases. Ramachandran, Kumar, and Laradji (2008) and Ramachandran, Laradji, and Kumar (2009) for instance showed bud formation in a flat membrane upon asymmetric flip–flop, where the asymmetric flip–flop was driven by such flippase proteins.

Fission may, however, also occur without such external interaction or presence of proteins. A first example of a lipid-based deformation mechanism is given by membranes formed from multiple lipid components, which undergo lateral phase separation into coexisting liquid phases, or domains, with distinct compositions (Fig. 4A). As already demonstrated before experimentally as well as using continuum models (Baumgart, Hess, & Webb, 2003; Kumar, Gompper, & Lipowsky, 2001; Lipowsky, 1992), minimization of line tension between these domains may then result in membrane deformations and, depending on membrane rigidity and volume constraints, even in parts of the membrane

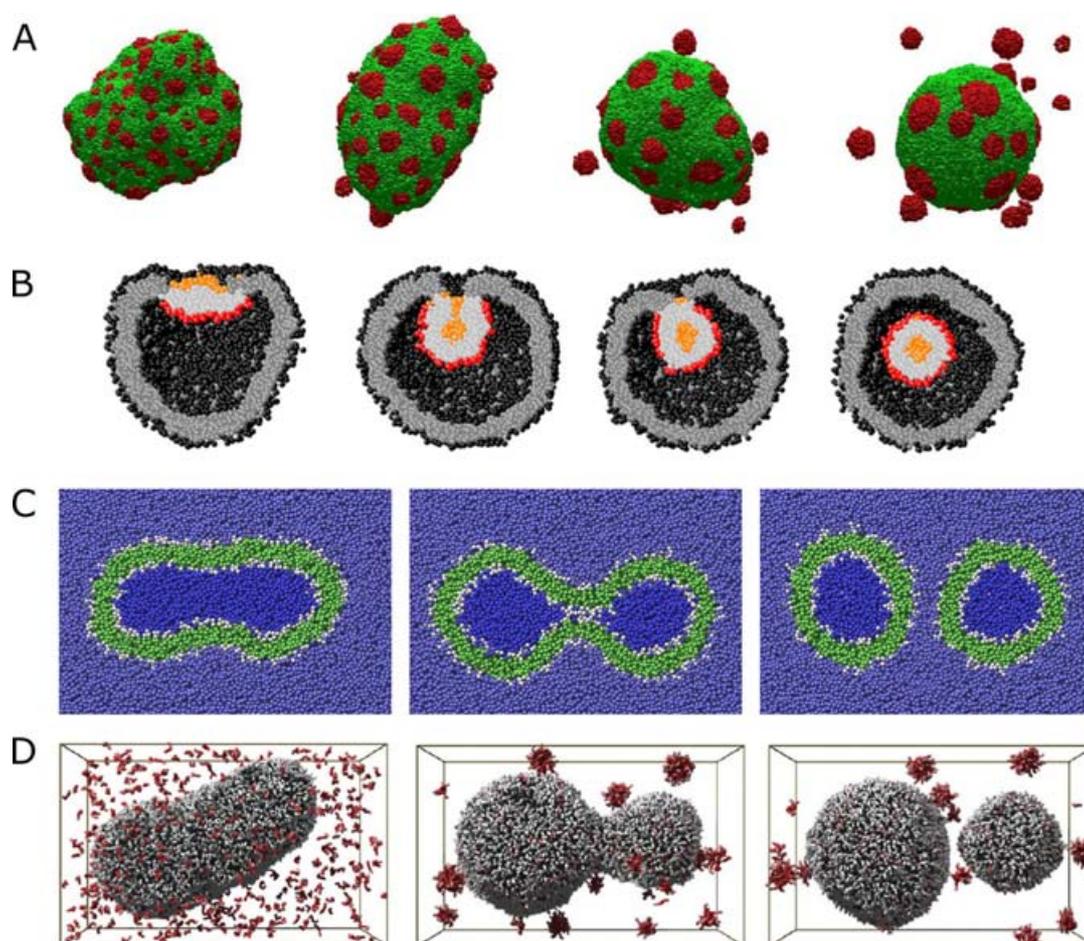


FIGURE 4 Four lipid-based fission driving mechanisms. (A) Phase separation driven budding (reprinted with permission from Laradji & Kumar, 2004). The two lipid types are shown in different shades of gray. (B) Inward budding by phase separation in combination with an asymmetric distribution of lipids over the leaflets (reprinted with permission from Yang et al., 2009). Cross-section, lipids in the bud are shown with light tails, other lipids with darker tails. (C) Fission induced by changing hydration of lipids in outer leaflet (Markvoort, Smeijers, Pieterse, van Santen, & Hilbers, 2007). Cross-section, lipids shown in stick representation with white heads, gray tails, solvent as spheres. (D) Fission induced by asymmetric membrane growth upon uptake of new membrane material in outer leaflet (reprinted with permission from Markvoort et al., 2010). (See Color Insert.)

budding off. Cooke, Kremer, and Deserno (2005) used an implicit solvent model to investigate the kinetics of domain formation and the budding process in membranes made of two lipid types. In their model, the two types of lipids have exactly the same shape, only experiencing a different mutual interaction that drives the phase separation. Laradji and Kumar (2004, 2005, 2006) used DPD with explicit solvent and found that area-to-volume ratio determined final shapes, something that cannot be studied with the implicit solvent models as in such models the vesicle interior volume can freely adapt. Such budding was

also shown with other DPD studies by a number of groups (Fuchslin, Maeke, & McCaskill, 2009; Hong, Qiu, Zhang, & Yang, 2007; Illya, Lipowsky, & Shillcock, 2006; Yamamoto & Hyodo, 2003; Yang, Shao, & Ma, 2009; Zheng, Liu, Li, & Zhang, 2010), where phase separation is obtained by changing interaction parameters between two types of lipids and/or using lipids with different tail lengths (Stevens, 2005). Drawback of these DPD models is that the phase behavior exhibited cannot be mapped directly to that of real lipids. Studies using more detailed models capable of domain formation (e.g., Risselada & Marrink, 2008) are warranted.

Apart from phase separation, a second lipid-based membrane deformation mechanism comprises of an asymmetric lipid distribution over the two leaflets, something that is well known to be the case in biological membranes. Yamamoto and Hyodo (2003) for instance found that if line tension alone is not sufficient for a domain to bud off, an asymmetric transversal distribution of the lipid types can favor budding and fission. The basis behind this mechanism is the notion of a membrane as two monolayers that can slide over each other. In order to align the edges of the two unequally sized domains in the two leaflets, thus minimizing the interaction energy between unlike lipids, the membrane may bend to comply with this area difference (as shown earlier theoretically (Miao, Seifert, Wortis, & Döbereiner, 1994; Seifert, Berndl, & Lipowsky, 1991)). In such a way, the domain may bud off even in case of relatively low edge tension. The same effect was shown by Laradji and Kumar (2006), who also found that such an asymmetry tremendously slows down growth of the curved domains, and Yang et al. (2009), who showed that with a combination of phase separation and an asymmetric distribution of lipids also budding to the inside (endocytic fission) can occur (Fig. 4B).

Also in absence of phase separation, asymmetry between the two leaflets may still drive a vesicle to fission. Markvoort, van Santen, and Hilbers (2006) for instance showed, using their CG MD simulations, that slightly changing the hydration of the lipid head groups in one of the leaflets can result in dramatic shape deformations from prolate to oblate ellipsoid vesicles as well as to cup-shaped, pear-shaped and budded vesicles, where these budded vesicles could eventually split into two, more or less, equal daughter cells (Markvoort, Smeijers, Pieterse, van Santen, & Hilbers, 2007). The influence of the change in hydration can either be explained as a change in area difference between the two leaflets, which is relaxed by (slightly) bending the whole membrane, that is, deforming the vesicle, or as a change in the membranes spontaneous curvature. As the change in head group hydration for instance can be the result of a changing pH or ion concentration outside the vesicle, this mechanism can even drive single component vesicles to fission (Fig. 4C). Li, Liu, Wang, Deng, and Liang (2009) showed fission in a similar fashion in DPD simulations of triblock copolymers.

A final way to introduce an asymmetry between the leaflets (in a single component vesicle) is by the addition of new material to one of the leaflets. The area difference between the two monolayers can, for example, grow by addition of new membrane constituents from the outside (or inside) only (Markvoort et al., 2010; Yang & Ma, 2009), when the rate of addition is fast compared to relaxation by means of flip–flop. The resulting area difference can then again be released by vesicle deformation to budded states, continuing to full fission into two, more or less, equal daughter cells. This mechanism thus provided a good explanation for the so-called matrix effect (Blochliger, Blocher, Walde, & Luisi, 1998) in the reproduction of fatty acid vesicles (Fig. 4D).

B. Fission not Just Fusion Reversed

After the membrane is deformed, by means of one of the above driving mechanisms, in such a way that a narrow neck is formed, the fission process continues with further narrowing of this neck. Abandoning of internal solvent from this neck provides a division of the internal solvent into two parts. As already predicted using a continuum model (Kozlovsky & Kozlov, 2003), at this point the inner monolayer self-fuses. As the lipid head groups in the neck follow the two separating solvent compartments, this self-fusion results in a hemifused state with a continuous outer leaflet and two separate inner leaflets. The outer membrane then forms a circular stalk and breakage of this stalk completes fission. Although fusion and fission are each others inverse processes, the observed pathways are thus not always simply each others reverse. In the first place, a driving mechanism is needed to deform the membrane such that a sufficiently narrow neck is formed. In the second place, whereas the fusion process can follow a variety of both leaky and nonleaky pathways, the fission process follows a nonleaky mechanism that is most like the reverse of the direct stalk–pore fusion mechanism of the symmetric stalk expansion pathway (see Section IV.A). Cross-sections of fission intermediates are for instance shown in Markvoort et al. (2007, 2010). Common fusion intermediates as extended HDs and anisotropically extended stalks are, at least to date, not observed in the simulated fission processes.

Another difference is in the possible shapes of the stalk. In case of phase-separation driven budding the fission process may be triggered by cleavage along the domain boundary, hardly resulting in a stalk. In absence of phase separation, on the other hand, the stalk that is formed can be longer than in case of fusion. Whereas in case of fusion the stalk is limited in length as it is only formed when two membranes are already close, the fission stalk can grow much longer. This can arise especially in case of a relatively long neck, when the inner

leaflets follow the solvent parts that separate when becoming more spherical in reducing their surface tension, or in case the two daughter vesicles are pulled apart (Noguchi & Takasu, 2002b). In this respect also simulations of micelle fission are of interest, mimicking the final stage of the fission of a membrane where a stalk between the two daughter membranes still needs to be broken. Micelle fission has for instance been studied using CG MC simulations (Pool & Bolhuis, 2006, 2007), but, contrary to complete membrane fission, also using atomic scale MD (Sammalkorpi, Karttunen, & Haataja, 2008). The main observation in these atomistic resolution simulations is that micelle fission progresses through a dumbbell-like morphology involving the formation of a long and narrow stalk in which the surfactants are highly interdigitated. The rod-like interdigitating structure allows the fission stalk to elongate over a much longer distance while providing partial shielding of the hydrocarbon tails from the solvent and leading to a stable intermediate.

VII. PEPTIDE MODULATED FUSION

Fusion may be intrinsic to lipids, but peptides and proteins are required to control and/or facilitate the process. They might act passively to bring (and hold) the bilayers together, or have a more active role in stabilizing any of the intermediate steps. This is clear from *in vivo* as well as *in vitro* studies (e.g., reviewed in Jahn & Grubmüller, 2002; Sapir, Avinoam, Podbilewicz, & Chernomordik, 2008), but now also from *in silico* studies.

A. *The Role of Fusion Peptides*

Fusion peptides are moderately hydrophobic segments of viral and nonviral membrane fusion proteins that enable these proteins to disrupt and connect two closely apposed biological membranes (Epanand, 2003; Tamm & Han, 2000). Most fusion peptides are located at the extreme N-termini of the transmembrane subunits of the fusion proteins. Deletion of the fusion peptide and, in many cases, even relatively conservative single amino acid changes in the fusion peptide completely abolish the ability of fusion proteins to fuse membranes. *In vitro* assays furthermore show that even isolated fusion peptides alone can support membrane fusion in model systems. The combined experimental evidence (e.g., Tamm & Han, 2000) points to three effects of the fusion peptides on membrane structure, namely changes in hydration, curvature, and lipid chain order. Furthermore, fusion peptides are observed to induce inverted lipid phases, suggesting stabilization of the stalk intermediate which has a dominant negative curvature. The relation between peptide-induced membrane changes and peptide-induced membrane fusion at the molecular level remains controversial, however.

Most simulation studies to date have concentrated on the hemagglutinin (HA) fusion peptide and its mutants. Full fusion assays are computationally still very challenging, therefore, most of these studies address the binding of a single fusion peptide or analogue, either to a lipid bilayer or micelle. Atomistic simulation studies along these lines have been reported by many groups (Huang, Chen, & Herrmann, 2004; Lague, Roux, & Pastor, 2005; Li, Das, & Zhou, 2010; Nishizawa & Nishizawa, 2010a; Vaccaro et al., 2005; Volynsky, Polyansky, Simakov, Arseniev, & Efremov, 2005). Despite the simplicity of the set-up, a number of key points are revealed: (i) the HA fusion peptide adopts a kinked conformation. The kinking is in agreement with the NMR structure of the peptide in detergent micelles (Han, Bushweller, Cafiso, & Tamm, 2001), revealing a boomerang shape. (ii) The peptide inserts obliquely into the lipid membrane, in line with experimental data (Tamm & Han, 2000). Insertion into the interface is mainly with the N-terminal helix. (iii) The peptide locally induces disorder, increasing the chain gauche population and area of adjacent lipids in the same binding leaflet. Thus, it induces local thinning of the bilayer and disordering of acyl chains of lipids in close proximity to the binding site. A more quantitative analysis of the disordering effect of the HA fusion peptide was performed in the study of Kasson et al. (2010). Lipid tail protrusions were monitored for a POPC bilayer patch at a peptide:lipid ratio of 3:500. Lipids within 5 Å of the peptides exhibited significantly increased protrusion frequencies (approximately fourfold) compared to lipids more than 20 Å away.

The membrane binding of nonfusogenic mutants of the HA fusion peptide was also investigated (Li et al., 2010; Nishizawa & Nishizawa, 2010a; Vaccaro et al., 2005). Compared to the wild type, Vaccaro et al. (2005) observed that most of them equilibrate parallel to the interface plane and do not adopt a tilted conformation. In Li et al. (2010), a total of six single point mutants, all with no fusion or hemifusion activity, were examined systematically. All mutants show a strong tendency toward a linear alpha-helix conformation, with the initial kink structure in the wild-type broken. One of the key hydrophobic residues around the initial kink region, Phe-9, is found to flip away from the membrane surface in most of these mutants. Also in the simulation study of Nishizawa and Nishizawa (2010a), comparison among the mutants supports a view that the oblique orientation of the wild type is required to facilitate perturbation of the lipid-water interface. Moreover, these authors show that in closely apposed bilayers, the wild-type fusion peptide is able to trigger stalk formation under conditions where the pure bilayer is stable. However, when the peptide is forced to adopt a conformation characteristic of some of the mutants, this ability disappears.

Other fusion peptides have received less attention. The structures of the 16-residue fusion peptide of the HIV gp41 fusion protein, two of its mutants, and a shortened peptide were studied by MD simulation in an explicit POPE bilayer (Kamath & Wong, 2002). The simulations reveal that the active wild-type peptide inserts into the bilayer at an oblique angle, whereas the inactive mutants

and the shortened fragment lie on the bilayer surface. Furthermore, the simulations show the structure of the wild type to be remarkably similar to that of the HA fusion peptide. Likewise, the gp41 fusion peptide also has a disruptive effect on the lipid chains. This effect was not observed for the mutants. A comparative study of a number of type 1 viral fusion peptides was performed by Taylor and Sansom (2010). In their study, the physical properties of the fusion peptide surface were related to the tilt angles determined both experimentally and by means of MD simulations. It was found that the relationship between the distribution of lipophilic potential over the peptide surface and the peptide geometry control the tilt angle of the peptide in a DMPC bilayer. The depth of penetration into the bilayer appears to be determined by the electrostatic potential and hydrogen bonding at the C-terminus.

A different approach to assess the effect of fusion peptides was taken by Fuhrmans, Knecht, and Marrink (2009). The use of a CG model allowed for a systematic exploration of the effect of the peptide on the lipid phase diagram. In a spontaneous aggregation approach, starting from random mixtures of DOPE lipids, HA fusion peptides and water, a new phase was found forming exclusively in the presence of the fusion peptide. This new phase is in fact morphologically equivalent to the single diamond cubic phase. In light of the role of the fusion peptide, it is insightful to describe the single diamond phase in terms of pores and stalks; one finds that there is a tight balance of stalks and pores with the network of stalks defining the pores and vice versa. The phenomenon of stalks and pores in close proximity is not uncommon and it was actually predicted to be energetically favorable to form a pore in the presence of a stalk in a field theoretic study (Katsov et al., 2006). Stalk–pore complexes are also observed as intermediate states during fusion along the stalk–pore complex pathway (see Section IV.B), pointing to a potential role of the HA fusion peptide. The peptides' boomerang structure appears to entail a preference to locate between the bases of two emerging stalks with its helical arms neatly lining the surface, possibly reducing the Gaussian curvature elastic energy associated with the stalk–pore structure. See Fig. 5A for a close-up of the location of the peptides at the membrane–water interface. In addition, the peptides seeming ability to stabilize stalks and pores might account for its observed effects on hemolysis and the stabilization of inverted cubic phases (Epanand, 2003). Additional simulation studies (Fuhrmans & Marrink, 2011) probing the ability of mutants of the HA fusion peptide to perturb the lipid phase diagram show much weaker effects compared to the wild type. Again, this is attributed to the unique boomerang shape of the HA fusion peptide.

As mentioned before, full fusion assays are computationally demanding and therefore the simulation studies discussed in this section use indirect measures to assess the role of fusion peptides. Nevertheless, simulation studies suggest that the fusion peptides may be important in a number of ways. First, the presence of the peptides at the membrane–water interface may lower the hydration repulsion, and

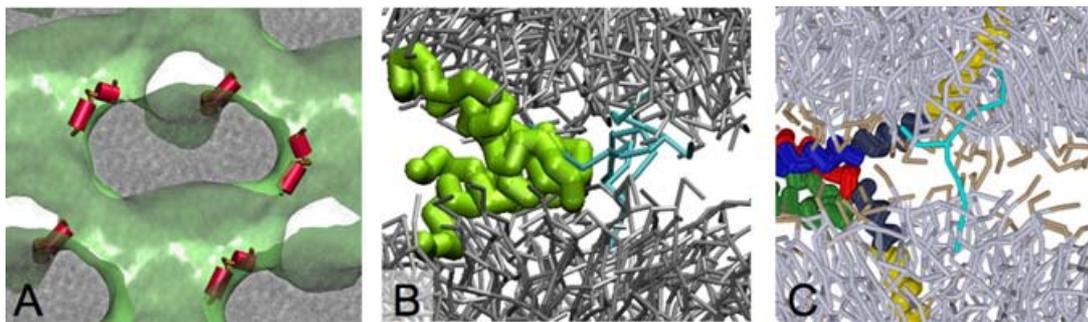


FIGURE 5 Protein-mediated membrane fusion intermediates. (A) Stalk–pore complex stabilized by HA fusion peptides (Fuhrmans et al., 2009). The snapshot shows a cut through the stalks, looking on top of the adjacent pores. The lipid–water interface is shown as a surface, the lipid tail beads as spheres, and the peptides as rods. (B) Fusion of liposomes triggered by lung surfactant protein B (Baoukina & Tieleman, 2010). A snapshot of the initial stage of the fusion process is shown, in which the protein binds the two liposomes together allowing lipids to bridge. (C) Liposomal fusion by the SNARE complex (Risselada et al., 2011). The initial stage of the formation of a splayed lipid intermediate is shown. (See Color Insert.)

thus helps bringing the bilayers together. Second, the peptides induce local disorder and facilitate the protrusion of lipid tails. Assuming this to be the initial energy barrier, as discussed in Section V, the rate of stalk formation is increased. Third, the peptides cause an expansion of the interfacial area and therefore positive curvature stress. Positive curvature helps to form the final fusion pore, or to stabilize the stalk–pore complex along the fusion pathway. The simulation studies also show that the typical V-shape, or boomerang shape of the fusion peptide (most notably for the HA fusion peptide) is optimal in these respects, in line with experimental findings (e.g., Lai, Park, White, & Tamm, 2006). Interestingly, the simulations predict that the stalk state itself is not favoured by the fusion peptides, only its formation rate by lowering of the initial barrier. The effect of peptides on stabilization of inverted phases should then be understood in terms of the positive curvature component that is induced by the peptide, allowing phases with saddle-splay curvature such as the inverted cubic phases. Further evidence for the role of fusion peptides in the formation of pores is found in the combined experimental and simulation study performed by Donald et al (2011). Here it is shown that the fusion peptide of the parainfluenza virus 5 can form a stable hexameric trans-membrane bundle, at least partly filled with water. In fact the structure resembles that of a closed mechano-sensitive channel which may gate in response to membrane tension during the final stage of fusion, opening the fusion pore.

B. Protein-Induced Fusion

Both in viral fusion and in eukaryotic fusion pathways, complex protein machineries exist that regulate and control the fusion process. Given the already

complex energy landscape of lipid-mediated fusion, elucidating the precise role of proteins is an enormous challenge. In general, the proteins will be involved in bringing the fusing membranes together, and by lowering the various intermediate states, to guide the system through a specific fusion pathway (Chernomordik & Kozlov, 2008; Jahn & Grubmuller, 2002). Simulation studies are now probing the interplay between lipids and proteins during fusion, both at a generic level (protein mimetics) and also recently using detailed models.

A study of Smeijers, Pieterse, Markvoort, and Hilbers (2006) explored the effect of small CG protein mimetics on the process of fusion. Especially interesting is their finding that a so-called scramblase, that is, a transmembrane protein with a hydrophilic strip which allows rapid lipid flip–flopping, strongly promotes vesicle fusion. The authors conclude that it does so by providing a way to relieve membrane tension that results from crowding of the outer monolayer due to growth of the stalk. After the fusion process has completed, the presence of the scramblase proteins also helps the vesicles to relax to a spherical shape. Additionally, one of the proteins promotes fusion by inducing pore formation. Incorporation of these proteins allows even flat membranes to fuse spontaneously. Another example of the possible mode of action of fusion proteins is provided by the simulations of Noguchi and Takasu (2002a). They showed that small nanoparticles that adhere to the membrane surface can promote fusion by bending of the stalk (i.e., facilitating the alternative pathways, see Section IV.B).

The first simulations of spontaneous fusion induced by realistic proteins are reported by Baoukina and Tieleman (2010). The fusion of small unilamellar vesicles mediated by lung surfactant protein B (SP-B) was studied, using the MD method and a CG force field in which near-atomic detail is preserved. The fusogenic activity of SP-B protein is known from *in vivo* studies (Oosterlaken-Dijksterhuis, van Eijk, van Golde, & Haagsman, 1992), and is believed to play a role in the lung surfactant secretory pathway (Ryan et al., 2005). In the simulations, an SP-B monomer triggers spontaneous fusion events by anchoring two vesicles and facilitating the formation of a lipid bridge between the proximal leaflets. A snapshot of this stage is shown in Fig. 5B. Once a lipid bridge is formed, fusion proceeds via the previously described stalk–HD–pore pathway (see Section IV.A). In the absence of protein, fusion of vesicles was not observed in either unbiased simulations or upon application of a restraining potential to maintain the vesicles in close proximity. The authors conclude that the particular shape of the SP-B protein is crucial, enabling it to bind to two vesicles at once and forcing their proximity. It is plausible that the protein surface subsequently acts as a hydrophobic scaffold, which is used by the lipid tails to adopt a splayed conformation triggering the stalk formation.

Key player in eukaryotic fusion is the SNARE (Soluble *N*-ethylmaleimide-sensitive fusion protein Attachment Protein (SNAP) REceptor) complex. Assembly of the SNARE proteins into a so-called “*trans*” complex likely

bridges the fusing membranes, inducing their fusion. The core SNARE complex is a four- α -helix bundle, where one α -helix is contributed by syntaxin-1, one α -helix by synaptobrevin and two α -helices are contributed by SNAP-25. Based on the stability of the resultant *cis*-SNARE complex, it has been postulated that energy released during the assembly process serves as a means for overcoming the repulsive forces between the membranes. Because SNARE proteins are anchored in the membranes, and these anchors have been shown to act as force transducers (McNew et al., 2000), a possibility is that the SNARE complex induces a local tension in the membranes allowing them to fuse. Simulation studies of Knecht and Grubmuller (2003) showed that the transmembrane anchors can in principle provide the required mechanical stiffness for them to act as force transducers. The importance of global membrane tension was already shown in the simulation studies of Shillcock et al., discussed in Section IV. In an extension of that work, Shillcock and Lipowsky (2006) tested the hypothesis that SNARE proteins facilitate fusion via local tension. The authors considered a system consisting of a 28 nm diameter vesicle fusing with a planar membrane patch of 100 nm linear dimension. The tension in the planar membrane was generated by pulling six artificial barrel proteins, mimicking the action of the SNARE proteins. Although full fusion could indeed be observed, the work required to induce formation of the fusion pore was quite large, approximately 90 $k_B T$ per protein. Another DPD simulation study (Wu & Guo, 2009) has elaborated on this theme by including the extracellular piece of the SNARE proteins as well as their transmembrane anchors. By explicitly adding attractive forces between the model proteins, the formation of the fusion complex was mimicked. Binding of the proteins resulted in bringing the membranes together, creating enough tension in the center of the protein ring to open a fusion pore.

The power of simulation studies is further evidenced from the recent work of Risselada et al. (2011). Here, the SNARE-mediated fusion of two vesicles was simulated, using the same near-atomistic CG model as in the study of lung surfactant protein discussed above. The set-up consisted of two small POPE liposomes with either one or two preassembled *trans* SNARE complexes, in close resemblance to *in vitro* fusion assays (Weber et al., 1998). The model of the SNARE complex was based on the X-ray resolved structure (Stein, Weber, Wahl, & Jahn, 2009). Fusion, defined as content mixing, was observed to occur spontaneously in 4 out of 10 simulations in case of 2 complexes, and in 2 out of 10 with only 1 complex present. Control simulations without proteins did not show any fusion events. In all successful cases, the fusion process proceeds via four stages. In the first stage, the transmembrane SNARE receptors (TMRs) are able to destabilize individual lipids, leading to the formation of a stalk via a splayed-lipid intermediate as illustrated in Fig. 5C. In the second stage, the stalk

expands in a linear fashion leading to the inverted-micellar intermediate (see Section IV.B). The third and fourth stage comprise the rupture of the membranes to form an asymmetric HD and full fusion pore. The simulations suggest that the formation of the fusion pore is mediated by the charged C-terminal of the TMR. Insertion of this charged end into the membrane interior is forced by the mechanical stress stored in the SNARE complex; by doing so the charge opens up the fusion pore to avoid becoming dehydrated. Remarkably, the final structure of the SNARE complex resembles the postfusion structure suggested from the X-ray data (Stein et al., 2009).

Taken together, the *in silico* experiments on protein-mediated membrane fusion point to two key events for which the proteins are important: lipid splaying and pore formation. Concerning lipid splaying, direct evidence for the inducement of splayed intermediates comes from the simulations on lung surfactant protein B- (Baoukina & Tieleman, 2010) and SNARE- (Risselada et al., 2011) mediated fusion. Indirect evidence is provided by the numerous studies on the lipid disturbing effects of fusion peptides in general, as discussed in Section VII.A. The role of fusion proteins in the pore formation is evident from the simulated SNARE-mediated fusion (Risselada et al., 2011), and, more indirectly, from the ability of fusion peptides to induce positive curvature and to stabilize stalk–pore complexes (Fuhrmans et al., 2009). Note that, both lipid splaying and pore formation are favored by the presence of membrane tension, which may also be induced by the fusion proteins (Shillcock & Lipowsky, 2006). Additionally, simulations show that protein mimetics could have an effect on, for example, stalk bending (Noguchi & Takasu, 2002a) or lipid flip–flopping (Smeijers, Pieterse, et al., 2006).

VII. OUTLOOK

The progress in particle-based simulation studies of membrane fusion has been quite substantial. *In silico* modeling of fusion is becoming a valuable tool nowadays, completing the large range of experimental methods. Simulation studies have proven most powerful in revealing detailed pathways for membrane fusion and fission. The many ways in which lipids can change their conformations to adopt to, or give rise to, the fusion intermediates is rather impressive. On top of this sits the whole protein fusion machinery, adding another layer of complexity. Simulation studies are now beginning to explore their role as well, with recent highlights the spontaneous protein-mediated fusion of vesicles observed in Baoukina and Tieleman (2010) and Risselada et al. (2011).

What is needed now is a more systematic exploration of fusion pathways as a function of system parameters such as lipid composition, membrane curvature,

membrane separation, system size, tension, temperature, salt conditions, etc., in order to extract statistical information about which pathways are observed under which conditions, and what are the associated energies along the way. Especially, we anticipate a significant increase in studies on peptide and protein-mediated fusion, probing the fascinating lipid/protein interplay. With the enormous growth in number of processor nodes foreseen for the next 10 years, such systematic studies can become very powerful. As an example might serve the work of Kasson et al. (2006) who used distributed computing to explore the fusion of small vesicles over thousands of trajectories. Another development of interest is the use of multiscale modeling techniques (Ayton, Noid, & Voth, 2007; Nielsen, Bulo, Moore, & Ensing, 2010) in which detailed, atomistic resolution is combined with the effectiveness of CG models. One could think of, for example, fusion or fission of large vesicles in which only the contacting parts are simulated in full detail and the remainder is treated at a reduced level of resolution.

From the experimental side, the challenge is to detect some of the pathways or intermediates that are predicted *in silico*. Most notable in this respect are the pathways involving a linear expansion of the stalk, seen in many different simulation studies. Direct observation of the inverted-micellar intermediate would lend strong support for this mechanism. However, the absence of this intermediate does not disprove the linear stalk expansion pathway, as pores might form before the water droplet is trapped. Mixing of *trans* and *cis* lipids from the two fusing membranes is another signature of the alternative pathways, which proceed via an asymmetric hemifusion state. Although probably hard to detect experimentally, the initial fusion events are predicted to be dominated by the ability of single lipids to splay and cross the inter bilayer space, at least when fusion involves strongly dehydrated membranes. Indirect indications could come from the expected fusogenicity of polyunsaturated lipids when present in the outer leaflets, or the decreased ability to fuse for membranes enriched in cyclic lipids, for instance. Finally, the combined simulation data point to novel roles of fusion peptides, facilitating lipid tail splaying and promoting pore or pore–stalk complex formation by inducing positive curvature stress; knowledge which can aid in the interpretation of experimental measurements.

In conclusion, simulation studies on membrane fusion point to a large variety of fusion pathways, revealing a more complex energy landscape beyond the traditional stalk–HD–fusion pore mechanism. Truly, lipids are remarkably versatile molecules that give rise to some amazing acrobatics in the fusion arena.

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