

# New mechanism of membrane fusion

M. Müller

*Institut für Physik, WA 331, Johannes Gutenberg Universität, D-55099 Mainz, Germany*

K. Katsov and M. Schick

*Department of Physics, University of Washington, Box 351560, Seattle, WA 98195-1560 USA*

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We have carried out Monte Carlo simulation of the fusion of bilayers of single chain amphiphiles which show phase behavior similar to that of biological lipids. The fusion mechanism we observe is very different from the “stalk” hypothesis. Stalks do form on the first stage of fusion, but they do not grow radially to form a single bilayer diaphragm. Instead, stalk formation destabilizes the membranes and results in hole formation in the vicinity of the stalks. When holes in each bilayer nucleate spontaneously next to the same stalk, an incomplete fusion pore is formed. The fusion process is completed by propagation of the initial connection, the stalk, along the edges of the aligned holes.

## I. INTRODUCTION

Fusion of membranes is involved in basic biological processes but its mechanism remains poorly understood. That different proteins trigger different fusion events obscures the possibility of a stage common to them, one which depends only on the properties of membrane bilayers themselves. The existence of such a universality is made plausible by the realization that, in all fusion processes, membranes must merge, and the properties of the membranes will most likely determine the nature of this stage, the fusion intermediate.<sup>1</sup> Time and length scales of fusion events are of the order of microseconds<sup>2</sup> and nanometers,<sup>3,4</sup> so that direct measurements of intermediate structure have not been possible. Limited theoretical treatment of this problem, based on membrane elasticity theory, has focused on the so-called *stalk* mechanism.<sup>5-7</sup> In this scenario, the leaves of the two *cis* membranes, closest to one another, fuse forming a stalk. The stalk expands radially and thins creating a single bilayer diaphragm from the *trans* leaves. Hole formation in this bilayer completes the fusion pore. Due to the lack of direct experimental confirmation, this mechanism, although plausible, remains hypothetical.

To obtain a direct view of the fusion mechanism we have undertaken the first Monte Carlo simulation of membrane fusion, one in which membranes are treated on the molecular level. In contrast to the stalk hypothesis, we find a very different fusion mechanism. Although stalk formation does take place in the initial stage of fusion, it does not result directly in the fusion pore. Instead, the stalk destabilizes the contacting bilayer membranes and promotes formation of transient holes *next* to it. When two holes are nucleated in apposing bilayers in the vicinity of the stalk, the latter grows along the hole edges, sealing their periphery like a zipper, and eventually closes thereby forming the fusion pore.

## II. THE MODEL

Our model describes membranes formed by single-chain amphiphiles, like block copolymers which exhibit the same phases as do biological lipids, and also form vesicles.<sup>8</sup> It has the advantage that it has been well studied, permits detailed analysis of molecular configurations, and is well suited to processes occurring on the small time and length scales characteristic of fusion. The amphiphiles are treated using the bond fluctuation model<sup>9</sup> in which each molecular segment occupies a cube of a three-dimensional lattice. The eight lattice sites defining the cube cannot be occupied by another segment centered on neighboring sites. Segments along an amphiphile are connected by one of 108 bond vectors of lengths  $2, \sqrt{3}, \sqrt{5}, 3$  or  $\sqrt{10}$ , measured in units of the lattice spacing  $a_0$ . Mapping this model onto lipids in solution,<sup>10</sup> we find the lattice spacing to correspond to approximately 1Å. The amphiphilic molecules consist of  $N = 32$  segments, of which 10 are hydrophilic and 22 are hydrophobic. This particular choice of the ratio of hydrophilic and hydrophobic segments results in the diblock system being close to coexistence of the lamellar and inverted hexagonal phases. The solvent is represented by a homopolymer, chains consisting of 32 hydrophilic segments. Like segments attract each other and unlike segments repel each other via a square well potential which comprises the nearest 54 lattice sites. Each contact changes the energy by an amount  $\epsilon = 0.177k_B T$ . The particular choice of the interaction parameter  $\epsilon$  guarantees that the interfacial width between hydrophilic and hydrophobic segments is not too small to be comparable to the lattice spacing, and at the same time results in well-defined bilayers. It is for this same reason that the solvent is chosen to be homopolymers rather than monomers.

The simulation cell is  $L \times L$  in the  $x, y$  directions and of length  $D$  in the  $z$  direction, with  $L = 156a_0$  and  $D = 96a_0$ . Periodic boundary conditions are utilized in all three directions. The monomer density of the system is  $\rho = 1/(16a_0^3)$ , corresponding to 146,016 seg-

ments within the volume, or 2376 amphiphiles and 2187 homopolymers. To encourage the fusion of the bilayers, we prepare them under tension by providing only enough amphiphiles to form bilayers of thickness  $25a_0$ , thinner than their equilibrium thickness of  $25.2a_0$ , which we determined independently. Given that the system is nearly incompressible, this corresponds to a fractional increase of area of less than 0.8%, one easily sustained by biological membranes.<sup>11,12</sup> Two such bilayers are created parallel to the  $x - y$  plane, stacked one upon the other, thereby mimicking the dehydration which permits close bilayer contact, a circumstance known to promote fusion.<sup>13</sup> Sixty-four independent starting configurations were prepared. Monte Carlo simulations were performed in the canonical ensemble. The conformations are updated by local monomer displacements and slithering-snake like movements. The different moves are applied with a ratio 1 : 3. The latter moves do not mimic the realistic dynamics of lipid molecules and we cannot identify the number of Monte Carlo steps with time. However, the density of hydrophilic and hydrophobic segments is conserved and the molecules diffuse. Consequently, we expect the time sequence on length scales larger than a single molecule to resemble qualitatively those of a simulation with more realistic dynamics.

We have also simulated a single bilayer under the above conditions. The system was extremely stable with respect to hole formation, apparently due to a very high energy cost to form the hole's edge, and exhibited the usual capillary wave fluctuations. In contrast, the system with two apposing membranes resulted in a range of structural transformations that eventually led to fusion pore formation. Runs were terminated when at least one pore was observed.

### III. RESULTS

#### A. Qualitative observations

Initially the apposed bilayers formed a number of local connections, the so-called stalks. Formation of these stalks was promoted by the close contact of the bilayers and by system being near bulk coexistence between lamellar and inverted hexagonal phases. In the stalk mechanism, these stalks grow radially. In contrast, we observed anisotropic growth of the stalks, which resulted in the formation of structures whose length was a few times that of their width. These may be precursors of line defects observed during the lamellar to inverted-hexagonal transition.<sup>7</sup>

Formation of the elongated stalks led to local *destabilization* of the bilayers. Holes formed next to these defects. We stress again that single, isolated, membranes were very stable. Thus hole formation in the apposed bilayers is apparently due to inter-bilayer interactions expressed in the stalk formation. It is easily seen that a hole formed next to a stalk has a smaller edge energy, in comparison to an isolated hole due to the reduction in

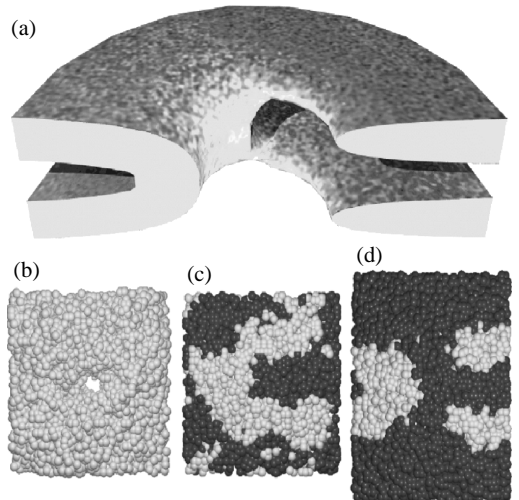


FIG. 1. The fusion intermediate. (a) Schematic sketch of the intermediate. Only hydrophobic portions are shown. (b) Top view of a fusion pore through two membranes. Only hydrophobic segments are shown. (c) Top view of the layer between the two membranes. Hydrophilic segments are dark grey, hydrophobic segments are light grey. (d) Side view of the snapshot.

curvature energy. This reduction is roughly proportional to the length of the edge adjacent to the stalk.

Hole formation was a transient process, *i.e.* numerous holes were observed to open and close reversibly with sufficiently short lifetime to preclude their significant diffusion. Only when two holes, one in each bilayer, nucleated next to, and on the same side of, a given stalk did the final stage of the fusion process commence. The stalk then propagated along the edges of the two holes bringing them together, like a zipper, to complete the fusion pore.

We show what one such partially formed pore looks like in FIG. 1. Panel (b) presents the top view of the two membranes. Only tail segments are shown. The pore through the two bilayers is clearly visible. Panel (c) presents the top view of the layer between the two membranes. Hydrophobic segments are light grey, while hydrophilic segments are dark grey. Connectivity is established along a portion of the pore's rim by the stalk, while the two hydrophobic sheets are still unconnected elsewhere. This is confirmed by a side view through the pore (d). A schematic of this intermediate is shown in panel (a).

#### B. Quantitative analysis

After 187,500 slithering-snake movements per molecule, we find that the initially sharp interfaces of the bilayers have widened, and profiles across the membranes have adopted locally their stationary form. Profiles at a later stage are broad-

ened due to fluctuations of the local membrane position, and to the formation of holes in the membranes. In order to reduce the effect of membrane fluctuations on our observation of the local profiles, we define at each  $x$  and  $y$  a local midpoint,  $z_{\text{middle}}(x, y)$ , of the two-bilayer complex as follows.<sup>14</sup> For each  $x$ ,  $y$  and  $z$ , we determine the total number of amphiphile segments,  $n_a(x, y, z)$ , in a volume centered at these coordinates, of width  $B = 28 a_0$  in the lateral directions, and  $50 a_0$  vertically, the thickness of two bilayers. The value of the coordinate  $z$  which maximizes  $n_a(x, y, z)$  defines  $z_{\text{middle}}(x, y)$ . We have chosen a rather large lateral length scale,  $B$ , so that a small hole in a single bilayer does not significantly alter our estimate for the midpoint of the two bilayers.

To analyze structural changes in the system, we looked not only at the fusion pores traversing both bilayers, but also at the holes in each bilayer which we found to occur about an order of magnitude more frequently than pores. We did this by calculating the local density of hydrophobic tail segments coarse grained over  $4a_0 \times 4a_0$  square columns centered at  $x$  and  $y$ . Holes and pores should have a very low, or vanishing, density of tails compared to the areal density of two normal bilayers. If the local value of the coarse-grained density of tail segments within a column extending through the whole  $z$ -range falls below 25% of the normal areal density of the two bilayers, we define this column as belonging to a pore traversing them. To locate a hole in only one of the bilayers, the integration over the  $z$  coordinate, rather than extending over the whole simulation box width,  $D$ , is limited to either the upper or lower half of it starting at the point between the two bilayers. If the local coarse-grained tail density falls below 25% of the normal areal density of a single bilayer, the column belongs to a hole. Pores and holes are defined as the aggregate of nearest neighbor  $4a_0 \times 4a_0$  plaquettes with low hydrophobic segment density.

The probability of hole sizes in a bilayer in the system with two apposed bilayers or with a single one are presented in FIG. 2. In the time interval investigated, the ratio of the average number of holes formed in the isolated membrane to that in the system with two apposed membranes is small. Further the decay of the probability distribution is much more rapid in the isolated bilayer. For example, this ratio is  $10^{-1}$  for holes of all sizes, but falls to  $10^{-3}$  for those with  $R > 5.5a_0$ , a sufficiently large radius that the hole is open; *i.e.* there is a channel accessible by solvent. This indicates that the edge free energy of holes in the isolated bilayer is larger and corroborates the observation that stalk formation promotes hole formation when two bilayers are in contact. It follows from the above that dehydration is more important than tension for pore formation.

To determine unambiguously whether fusion pore formation involves a stage in which the *trans* leaves make contact, one unavoidable in the standard stalk mechanism, we perform a statistical analysis of the fusion intermediates observed in our simulations. We exam-

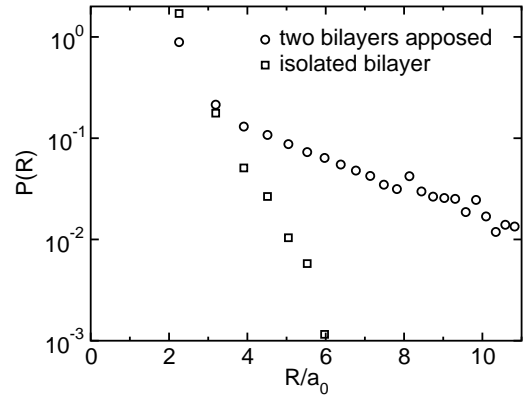


FIG. 2. Normalized probability distribution of the hole sizes in a bilayer. Circles refer to the system of two apposed bilayers, while squares denote the results for a single bilayer. Data for the two apposed bilayers were collected from 64 systems and 750,000 up to 956,250 slithering-snake steps. Hole lifetime is approximately 20,000 such steps. Data for the single bilayer were sampled over 7 configurations but much longer times.

ine in each simulation run the first pore which exceeds  $R_{\text{min}} = 5.5a_0$  (open pores).

Our strategy is to look at the region along the rim, or edge, of the pore in the pore's mid plane. If fusion proceeds *via* a single bilayer diaphragm, this region has to be essentially completely hydrophobic. In contrast, if fusion proceeds through the formation of two transient holes in apposing bilayers, then we may see pores in an intermediate stage in which the holes are aligned, but the rim of the pore has not fully formed. In such a case, the region along the rim of the pore would be at least partially hydrophilic. We measure the density of segments in a series of columns of height  $5a_0$  and base  $4a_0 \times 4a_0$  centered along the rim of the pore, which is defined as the points in the  $x - y$ -mid plane with a distance  $14a_0 \pm 2a_0$  from the center of the pore. This distance corresponds to a sum of radius of the pore,  $5.5a_0$ , and half of the hydrophobic thickness of the bilayer,  $8.5a_0$ .

To quantify the connectivity of the pore's rim, we extract the distribution of the density difference  $\Delta\phi$  between hydrophilic and hydrophobic segments in this region. On average there are  $\phi_0 = 5$  monomers in a column whose volume is  $4a_0 \times 4a_0 \times 5a_0$ . If the connectivity of the hydrophobic regions of the two bilayers were complete, as in the stalk mechanism, then we expect the rim region to be hydrophobic, so that the distribution of  $\Delta\phi$  from all these columns would exhibit a single peak around  $\Delta\phi/\phi_0 = -1$ . This is not at all what we see. Instead, only 12 pores have this single peaked distribution, whereas the rest are either bimodal (37 configurations) with a second peak centered about  $\Delta\phi/\phi_0 = +1$  or have a peak at  $\Delta\phi/\phi_0 = -1$  with a long "tail" (15 configurations) toward positive  $\Delta\phi$ . From this we can conclude that in the majority of cases there is a considerable por-

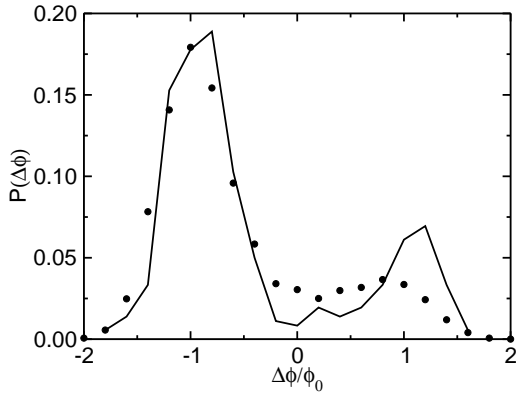


FIG. 3. Probability distribution of the difference between hydrophilic and hydrophobic segment density in the rim. Positive and negative values correspond to hydrophilic and hydrophobic dominated regions respectively. Circles show the average over all 64 independent configurations, while the solid line depicts the result for the snapshot in FIG. 1

tion of the rim which is still hydrophilic; that is, the rims of the two holes are only partially connected. The average of all distributions which we obtain are shown by the solid circles in FIG. 3. The distribution of the values of  $\Delta\phi$  obtained from the series of columns along the rim of the particular pore in FIG. 1 are shown as a solid line.

#### IV. DISCUSSION

The fusion mechanism we propose is similar to that considered for electrofusion,<sup>15</sup> with the addition that we specify the form of the fusion intermediate. It is in accord with the recent observation in molecular dynamics simulations that lipid tails take on a much greater range of configurations, including those sampling the hydrophilic region, when membranes are brought into close contact.<sup>16</sup> Our mechanism is similar in spirit to the initial stages of the mechanism of hemagglutinin-mediated viral fusion recently proposed by Bonnafous and Stegmann<sup>17</sup> who suggest that before fusion can occur, a hole must first form in the target membrane. However they go on to suggest that this hole leads to formation of a single bilayer diaphragm in which a hole is nucleated. In contrast, such an intermediate plays no role in the scenario we propose. Before pore formation, we find strong mixing of amphiphiles in the *cis* leaves which results from the formation of the stalks. Such mixing is observed experimentally to precede complete fusion.<sup>18</sup>

The destabilization of the membranes by the stalks that leads to formation of the transient holes in both bilayers, observed by us, is consistent with the work of Cevc and Richardsen<sup>13</sup> who emphasize the fact that membrane fusion is strongly promoted by *defects* in the bilayer structure. It seems clear that the additional destabilization of bilayers by the defects embedded in the membranes prior

to fusion facilitates formation of the transient holes necessary for fusion.

The destabilizing role of the stalk intermediates, observed in our simulations, is missing from the current phenomenological models of membrane fusion. Although we do not observe the standard stalk mechanism, our simulations do not rule it out under different thermodynamic conditions. Extensions of phenomenological approaches that include the new observed intermediates should clarify this point. We expect the fusion intermediate we have seen in our simulation to be similar to that in biological membranes. Its elucidation should facilitate the control and modification of the fusion process itself.

Note: While preparing this manuscript, we learned that Noguchi & Takasu,<sup>19</sup> studying a very different system consisting of rigid amphiphilic molecules of three atoms and containing no solvent, observed fusion behavior over a limited temperature range which is similar to that we observed in our system. This may indicate a universality to the mechanism we have described.

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