Insights into the Molecular Mechanism of Membrane Fusion from Simulation: Evidence for the Association of Splayed Tails

Mark J. Stevens
Sandia National Laboratories, P.O. Box 5800, MS 1411, Albuquerque, New Mexico 87185-1411, USA

Jan H. Hoh and Thomas B. Woolf
Department of Physiology, Johns Hopkins University, School of Medicine, Baltimore, Maryland 21205, USA
(Received 8 July 2003; published 30 October 2003)

We present coarse-grained simulations of fusion between two liposomes from which a detailed picture of lipid movements emerges. In these simulations the bilayers dilate at the contact edge, and the resulting increase in the area per lipid produces a tilting of the individual molecules as predicted. 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. As the tails extend into a more parallel conformation the two outer leaflets become contiguous to produce a hemifused structure. The results have interesting implications for biological membrane fusion and suggest new possibilities for designing molecules that control fusion.

DOI: 10.1103/PhysRevLett.91.188102 PACS numbers: 87.16.Dg, 87.14.Cc, 87.15.Aa

The molecular details of the fusion between two lipid bilayers is one of the most vexing problems in membrane biophysics today [1,2]. Fusion models based on continuum mechanics predict initial fusion of the outer leaflets to form a stalklike structure and the subsequent merger of the two sides [2–4]. Here we present coarse-grained simulations of fusion between two liposomes pushed into contact, from which a detailed picture of lipid movements emerges. The results have interesting implications for membrane fusion and suggest new possibilities for designing molecules that control fusion.

The process of membrane fusion is central to biology and plays a role in events such as vesicular trafficking, fertilization, viral entry, and mitosis [5,6]. While fusion between two biological membranes is normally a process mediated by proteins, many pure lipid bilayer systems will spontaneously fuse implying important similarities in the underlying physical chemistry [1,2,7]. In addition, mechanisms of fusion between pure lipid systems have a number of technological applications such as liposome based drug delivery [8,9] and the formation of supported lipid bilayer biosensors and other devices [10]. As a result, fusion between pure lipid membranes has been the subject of a wide range of experimental, computational, and theoretical studies. These efforts have produced a number of models, typically involving the local deformation of a membrane, the formation of a hemifused inverted hexagonal phaselike structure as an intermediate, and the subsequent complete merger of the two sides [2–4,11]. In the hemifused intermediate the outer leaflets are fused while the inner leaflets remain separate to form a connection between membranes referred to as a stalk. However, the molecular aspects of membrane fusion are not treated by the continuum models and have yet to be determined experimentally. Here we have performed molecular dynamics (MD) simulations that suggest new processes in membrane fusion and reveal novel molecular details of lipid movement.

Experimental measurements of fusion suggest a process that occurs beyond the time scale that is available to traditional MD methods [1,12]. Two aspects of our simulations of liposome fusion are critical for overcoming this time-scale difficulty. First, we coarse grain the system using bead-spring models for the lipid molecule. This approach has been extensively used in polymer simulations, where these models have been shown to capture central physical and chemical features [13]. More recently the approach has been applied to lipids, where it has been shown to reproduce the spontaneous process of lipid self-assembly into a bilayer [14]. Simulations of trimer amphiphiles without solvent have produced vesicle fusion [15]. However, even when using coarse-grained lipid models, the time for two liposomes in near contact to spontaneously fuse is prohibitive and to overcome that barrier we push the liposomes together. This will bias the simulations with respect to spontaneous fusion, but methods exist to compensate for this biasing [16,17]. Moreover, the function of some fusion proteins and other fusion molecules may be to act in lowering the fusion barrier in a similar manner. There are both qualitative and quantitative aspects to a full understanding of the process. Here we focus on qualitative mechanistic insights into the geometry of membranes and the conformational changes of the individual lipids during fusion and leave a quantitative analysis for future publication.

The lipid model used for the simulations is composed of 11 beads, four for each aliphatic tail (T beads) and three for the headgroup (H beads). The solvent (S beads)

 © 2003 The American Physical Society

is represented by a single bead. Beads interact through the Lennard-Jones (LJ) potential

$$u_{\mathrm{LJ}}(r) = 4\epsilon[(\sigma/r)^{12} - (\sigma/r)^{6}]. \quad (1)$$

The size of a single bead is given by the LJ diameter σ which is approximately 0.5 nm [18]. The TT, HH, HS, and SS interactions have the LJ potential with a cutoff $r_c = 2.5\sigma$. The HT and TS interactions are purely repulsive with cutoff $r_c = 2^{1/6}\sigma$. The bond potential [19] is the sum of the attractive finitely extensible, nonlinear elastic potential and the purely repulsive LJ potential u_{LJ} with cutoff equal to $2^{1/6}\sigma$,

$$U_{\mathrm{bond}}(r) = -\tfrac{1}{2}k_0R_0^2\ln(1 - r^2/R_0^2) + u_{\mathrm{LJ}}(r), \quad (2)$$

where $k_0 = 30\epsilon/\sigma^2$ and $R_0 = 1.5\sigma$. Angle terms are included via the harmonic potential

$$U_{\mathrm{bend}}(\theta) = k_\theta(\theta - \theta_0)^2, \quad (3)$$

where θ is the angle between neighboring bonds and $k_\theta = 2\epsilon/\mathrm{rad}^2$ and $\theta_0 = 180°$ give a persistence length of about 2σ for the tails. Other angle terms are included for the headgroup geometry. This model system spontaneously self-assembles into a lamellar bilayer when started from a random configuration of lipids and solvent at appropriate lipid concentrations (see also Ref. [14]). Massively parallel MD simulations using a Verlet integrator with a time step of 0.005τ were performed on a CPlant cluster [20]. Simulations were performed at constant temperature $T = 1\epsilon$ using the Langevin thermostat.

To form the starting state, we create a spherical liposome by placing lipids on a sphere with their tails in contact. For our calculations, we created liposomes with outer diameters D of 30σ and 50σ. The number of lipids is determined by the area per lipid A which is chosen to be $4.0\sigma^2$, slightly larger than the values obtained in simulations of lamellar bilayers. Solvent particles are placed randomly in the unoccupied volumes inside and outside the liposome. The effective osmotic pressure is controlled through our choice of the amount of solvent placed in these two regions. A nonzero osmotic pressure was found to speed up the fusion process. However, zero osmotic pressure simulations yielded the same qualitative dynamics. For a fusion simulation, this single liposome is first equilibrated for about 300 μs to ensure stability. The liposome is copied and two equilibrated liposomes are placed with their outer diameters about 10σ apart along the axis of approach (x axis). For liposomes with $D = 30\sigma$, the total system has $N = 330\,000$ beads. Two liposomes are brought together by applying small biasing forces in the range 0.0025–$0.001\epsilon/\sigma$ in the x direction to each bead in the lipid molecules. Once a few lipids have been exchanged between the two liposomes, the force is released and the process allowed to proceed. Once contact occurs the work by the biasing forces goes into deforming the liposome and promoting fusion as well as translation. The total work by the biasing force from contact to initial fusion is the upper bound of the fusion barrier. For the smallest biasing force the total work is 41 kT which is consistent with calculations [21].

In a typical fusion simulation, the process begins after the liposomes contact and a flattened contact forms between the liposomes [Fig. 1(a)]. This causes membrane bending at the contact edge bringing two strained points on the membranes into close proximity. Fusion between the outer membrane leaflets initiates at the edge of the contact surface [Fig. 1(b)]. There are several factors that promote fusion at the contact edge, including strain that is relieved by fusing with the neighboring liposome and an increase in area per lipid giving more mobility to molecules in this region. Recent calculations show that edges in the internal leaflets of membrane greatly reduce the free energy cost of the stable intermediate [22]. Similar calculations should show that the cost of forming the external edge is within reason. While this finding of the edge originated fusion is intuitive, it offers a contrasting view to that described in existing models. For example, Kuzmin and co-workers suggested that formation of a nipple shaped protrusion in opposing bilayers serves as a point of initiation for fusion and leads to the formation of a stable intermediate [21]. The present work suggests a second although not mutually exclusive mechanism in which first a membrane-membrane contact forms (here through an initially applied force). This causes membrane bending at the contact edge bringing two strained points on the membranes into close proximity where fusion initiates. Hence the point of fusion can be significantly distal to the initial point of closest approach.

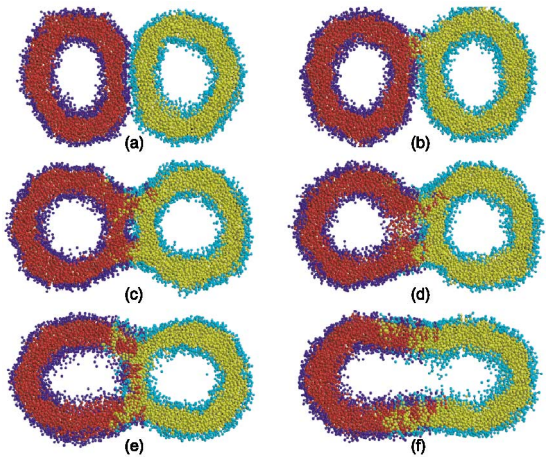

FIG. 1 (color). Cross sections (10σ thick) of fusing liposomes showing a sequence of events to complete fusion. (a) Flat interface at $t = 55$ μs; (b) initial stalk at $t = 94$ μs; (c) growth of stalk to other side with solvent cavity at $t = 140$ μs; (d) dissolution of one connector and solvent cavity $t = 149$ μs; (e) intermediate fusion state at $t = 204$ μs; (f) complete fusion at $t = 231$ μs [18]. Coloring scheme: right (left) liposome has H blue (cyan) and T red (yellow). Solvent is not displayed. The system is under osmotic pressure with internal solvent density 20% larger than external density.

The simulations also provide direct insight into the dynamics of individual lipids during the early stages of fusion. To begin with there is a tilting of the lipids at the presumptive point of fusion, similar to that proposed for the modified stalk model [4,23] which appears to be facilitated by the local increase in the area per lipid. The first exchange between the membranes occurs when an aliphatic tail rotates out of the parent membrane and inserts into the opposing membrane, resulting in a tilted lipid with the aliphatic tails in a splayed (trans) conformation that is shared between the two fusing bilayers (Fig. 2). This immediately suggests a mechanism for accommodating curvature strain on a molecular level. The possibility of splayed lipid conformations has been considered in some models for membrane fusion [24,25], however the picture that emerges here is distinctly different. In our simulations the splayed lipids tend to orient such that their aliphatic tails contact each other, thus creating the beginnings of a new hydrophobic core. As additional (tilted) lipids associate with the splayed bridging lipids, the aliphatic tails of these molecules extend into a more cislike conformation, establishing a hydrophobic core and eventually forming a classical stalk.

A somewhat surprising finding is that following the initial formation of the stalk, this structure expands highly asymmetrically along the strained membrane at the contact edge (Fig. 3). In current fusion models the stalk forms in a central location and growth occurs radially outward from the center with cylindrical symmetry. A consequence of fusion along the contact edge is the formation of a partially confined solvent cavity between the two liposomes [see Fig. 1(c)]. Some of the confined solvent joins the external solvent, and some empties into the left liposome as the inner bilayer dissolves and an extended hemifused structure forms [Fig. 1(d)]. We also note that in the simulation of the larger system, multiple, independent stalks initially formed as one might expect for a system large enough to allow well-separated initiation sites. The dynamics become more complex in this case as stalks merge and dissolve. We present here the simpler, single stalk case whose dynamics forms the basis of the more complex possibilities contained in larger systems.

While many of the general features of our simulations agree well with models based on a continuum mechanics framework, the present work offers significant insights into the molecular details of the early stages of fusion. In particular the simulations suggest a model for specific molecular rearrangements that results in the formation of a stalk (Fig. 4). This model builds on the notion of tilted lipids in a strained prestalk structure [4,23], which here facilitates the splaying of lipids to connect the two fusing membranes. A critical event is then the association of the aliphatic tails of splayed lipids, which nucleates a new hydrophobic core that spans the gap between the original membranes. This core grows as the chains extend and resolves completely the fusion between the outer leaflets. In addition, the importance of mechanisms that accommodate changes in membrane curvature is highlighted. Continuum models of the stalk do not provide a clear molecular view of how the stresses are resolved during the fusion process [22].

A number of interesting considerations arise from these results, including new ways in which proteins might

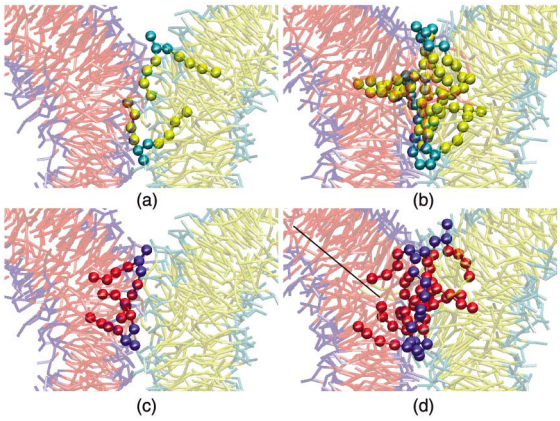

FIG. 2 (color). Lipid conformations and associations in the early stages of fusion. System and coloring scheme is the same as in Fig. 1. The left side is at $t = 58$ μs and the right side at $t = 66$ μs. (a) and (c) show splaying of lipids that bridges the two outer leaflets. There is a tendency for the aliphatic tails of the splayed lipids to oppose each other. (b) and (d) show association of splayed lipids and the beginning of a hydrophobic core that spans the space between the two liposomes. The line in (d) is a guide to the eye to indicate the ordering of the upper bilayer to become part of the stalk. For evolution beyond $t = 66$ μs the external force was turned off.

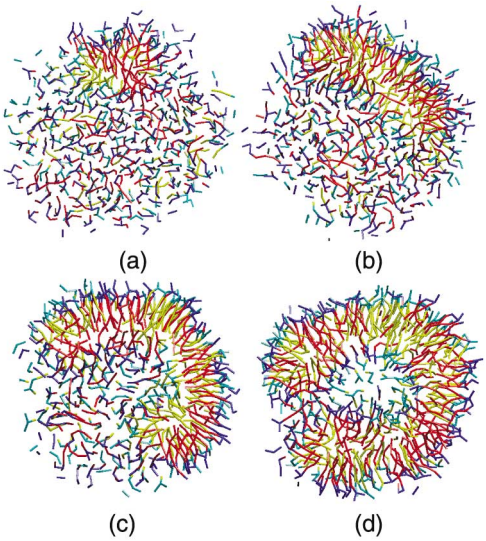

FIG. 3 (color). Cross sectional images of fusion in the plane of fusion (yz): (a) initial stalk at time $t = 69$ μs; growings stalk at (b) 86 μs; (c) 108 μs; and (d) almost complete fusion of outer leaflets at $t = 140$ μs. Only lipids in the outer leaflets are shown and the slice is about 10σ thick.

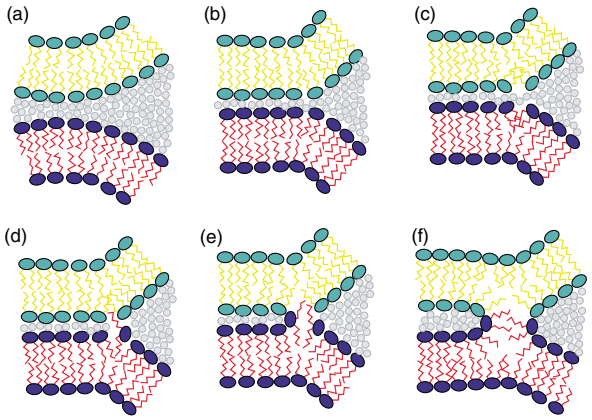

FIG. 4 (color). Schematic of the early stages of fusion and association of splayed lipids suggested by the simulations. (a) Two liposomes are brought together and (b) a flat contact forms where, at the edge, the area per lipid in the outer leaflet increases as the membrane is strained. (c) Lipids tilt at strained contact and (d) subsequently the aliphatic tails of some molecules begin to splay. Although not shown here, lipid exchange occurs early in the process. (e) Splayed lipids then associate by their tails to form a new hydrophobic core, (f) which expands as the tails extend to form a classical stalklike structure.

mediate the fusion process. For example, proteins might act directly to facilitate splaying of lipid tails or stabilize bridging lipid molecules. Proteins might also act more indirectly as adhesion molecules that promote the formation of a flat contact that produces a strained contact edge. In such a picture, proteins would serve a relatively passive function by keeping membranes apposed, while fusion occurs away from the actual protein mediated contact. The results also suggest that conformational properties of lipids that have not previously been closely examined play an important role in fusion. In that context, it is interesting to consider the possibility of rationally designing lipids with an improved ability to adopt splayed conformations, or lipids that can controllably be switched between a cis and a trans conformation of the tails, or other novel molecules that might be used to facilitate or control membrane fusion.

This work was supported at Sandia (M. J. S.) by the DOE National Nuclear Security Administration under Contract No. DE-AC04-94AL85000. Sandia is a multiprogram laboratory operated by Sandia Corporation, a Lockheed Martin Company, for the DOE. The work at Johns Hopkins was supported by a grant from the American Cancer Society (ACS-RSG-01-048-01-GMC) to T. B. W.

[1] J. Lee and B. Lentz, Proc. Natl. Acad. Sci. U.S.A. **95**, 9274 (1998).
[2] J. Zimmerberg and L. Chernomordik, Adv. Drug Deliv. Rev. **38**, 197 (1999).
[3] D. Gingell and L. Ginsberg, in *Membrane Fusion*, edited by G. Poste and G. Nicholson (Elsevier, Amsterdam, 1978), p. 791.
[4] M. M. Kozlov and V. Markin, Biofizika **28**, 242 (1983).
[5] B. Lentz, V. Malinin, M. Haque, and K. Evans, Curr. Opin. Struct. Biol. **10**, 607 (2000).
[6] A. Mayer, Ann. Rev. Cell Dev. Biol. **18**, 289 (2002).
[7] R. Jahn and H. Grubmuller, Curr. Opin. Cell Biol. **14**, 488 (2002).
[8] C. Dass, Drug Delivery **9**, 169 (2002).
[9] D. Lasic, Trends Biotechnol. **16**, 307 (1998).
[10] E. Sackmann, Science **271**, 43 (1996).
[11] L. V. Chernomordik, G. Melikyan, and Y. Chizmadzhev, Biochim. Biophys. Acta **906**, 309 (1987).
[12] A. Spruce, A. Iwata, and W. Almers, Proc. Natl. Acad. Sci. U.S.A. **88**, 3623 (1991).
[13] *Monte Carlo and Molecular Dynamics Simulations in Polymer Science*, edited by K. Binder (Oxford, New York, 1995).
[14] R. Goetz and R. Lipowsky, J. Chem. Phys. **108**, 7397 (1998); R. Goetz, G. Gompper, and R. Lipowsky, Phys. Rev. Lett. **82**, 221 (1999).
[15] H. Noguchi and M. Takasu, J. Chem. Phys. **115**, 9547 (2001).
[16] D. Frenkel and B. Smit, *Understanding Molecular Simulations* (Academic Press, San Diego, 1996).
[17] In full generality, various force biasing methods can be used to calculate the free energy changes along a reaction coordinate. Here we employ a computational biasing of our reduced complexity system that brings the liposomes in contact and then removes the biasing force. While there is no calculation of the free energy throughout the transition, there is a significant improvement of sampling for the transition event.
[18] The conversion of the LJ units for the coarse-grained model to metric values is not unique as the real structure and dynamics will map in more than one way to the coarse-grained model. For hydrocarbon tails, one map involves equating the persistence length of the flexible coarse-grained model to that of a true lipid. For polyethylene this yields $\sigma = 0.5$ nm. The energy scale is set by the temperature T and the relation $\epsilon = kT$. By equating the simulation value of the diffusion constant for a lipid molecule in a lamellar bilayer to a measured value of 10^{-8} cm^2/s, the LJ time unit τ is mapped to 11 ns. We use this value of τ in the rest of the discussion.
[19] K. Kremer and G. S. Grest, J. Chem. Phys. **92**, 5057 (1990).
[20] R. Brightwell, L. Fisk, D. Greenberg, T. Hudson, M. Levenhagen, A. Maccabe, and R. Riesen, Parallel Computing **26**, 243 (2000).
[21] P. Kuzmin, J. Zimmerberg, Y. Chizmadzhev, and F. Cohen, Proc. Natl. Acad. Sci. U.S.A. **98**, 7235 (2001).
[22] S. May, Biophys. J. **83**, 2969 (2002).
[23] Y. Kozlovsky and M. Kozlov, Biophys. J. **82**, 882 (2002).
[24] R. Corkery, Colloids Surf. B **26**, 3 (2001).
[25] P. Kinnunen and J. Holopainen, Biosci. Rep. **20**, 465 (2001).