Time scales of membrane fusion revealed by direct imaging of vesicle fusion with high temporal resolution

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Membrane fusion is a vital process of life involved, for example, in cellular secretion via exocytosis, signaling between nerve cells, and virus infection. In both the life sciences and bioengineering, controlled membrane fusion has many possible applications, such as drug delivery, gene transfer, chemical microreactors, or synthesis of nanomaterials. Until now, the fusion dynamics has been elusive because direct observations have been limited to time scales that exceed several milliseconds. Here, the fusion of giant lipid vesicles is induced in a controlled manner and monitored with a temporal resolution of 50 μ s. Two different fusion protocols are used that are based on synthetic fusogenic molecules and electroporation. For both protocols, the opening of the fusion necks is very fast, with an average expansion velocity of centimeters per second. This velocity indicates that the initial formation of a single fusion neck can be completed in a few hundred nanoseconds.

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embrane fusion is a ubiquitous process of life. A prominent example is provided by the fusion of synaptic vesicles to the outer membranes of nerve cells. This fusion results in the release of neurotransmitters into the synaptic cleft and is thus responsible for nerve cell communication. Likewise, fusion is essential for other secretion processes based on exocytosis, for the intracellular traffic of transport vesicles, and for the infection by membrane-enclosed viruses. Furthermore, control of lipid bilayer fusion is desirable in a number of applications in the life sciences. Fusion of functionalized lipid vesicles with cell membranes could be used for advanced drug delivery and gene transfer. Fusion of vesicles containing different reactants provides chemical microreactors with rather small volumes in the picoliter range (1, 2). Fusion of membranes that differ in their composition could also be used to elucidate the character and size of intramembrane domains and rafts (3). Although threecomponent lipid membranes exhibit large intramembrane domains (4-7) that can grow up to many micrometers, the size and character of analogous domains within biological membranes is still controversial.

The fusion of two bilayer membranes is believed to proceed via several stages (8, 9): membrane proximity and contact, local perturbation of bilayer structure, formation of fusion pores or necks, and subsequent expansion of these necks. Snapshots of single fusion necks with a diameter of 50–100 nm have been obtained by electron microscopy (10) and corroborated by atomic force microscopy (11). The time period between local bilayer perturbation and completion of a single fusion neck can be rather short as follows from electrophysiological methods applied to the fusion of small vesicles with cell membranes (12–14). The time evolution of the observed membrane area, indicates that the formation of the fusion neck is presumably faster than 100 μ s. However, for both biological and biomimetic

model membranes, direct imaging of the fusion neck dynamics with this temporal resolution has not been reported so far. Even though electron microscopy produces detailed images of fusion products, it gives only single snapshots of frozen or stained samples (10, 15). Likewise, scanning force microscopy (11) and x-ray diffraction (16) can provide only static images of structures immobilized on a substrate surface. The most promising technique for direct imaging of the dynamics of lipid bilayer fusion is optical video microscopy (17). Until recently, this technique has been limited to conventional video frequencies or millisecond resolution. In the present study, we used a fast digital camera to record the fusion process directly with a time resolution of 50 μ s, which is only feasible if fusion is induced in a controlled manner. We used two different protocols of controlled fusion, one based on synthetic fusogenic molecules (18) and the other on electroporation (19-21).

In the living cell, the fusion process is controlled and regulated by many proteins such as membrane-anchored soluble *N*ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) (22–25) and protein phosphatase (26). The conformational changes of these proteins are believed to induce and assist the formation of a fusion pore or neck (8) by bringing the membranes into close proximity and locally perturbing the two adjacent lipid bilayers. Recently, the action of fusion proteins has been mimicked by synthetic ligand molecules that are connected to lipid-like membrane anchors (18, 27). In the present study, we used amphiphilic ligands with a β -diketone head group that can be cross-linked by Eu³⁺ ions (28).

Results

To induce fusion of the lipid bilayer in a controlled manner, two lipid vesicles functionalized with the β -diketone ligand molecules (the chemical structure is provided in Scheme 1, which is published as supporting information on the PNAS web site) were isolated and brought into close proximity by using two micropi-

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Abbreviation: fps, frames per s.

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Fig. 1. Several series of snapshots for the fusion of two vesicles. (a) Fusion of two functionalized vesicles (of radii 25.4 and 16.6 μ m) held by micropipettes (only the right pipette tip is visible on the snapshots). A third pipette (bottom right corner) is used to inject a small volume (few tens of nanoliters) of 50 μ M solution of EuCl₃. The first image corresponding to the starting time t = 0 represents the last snapshot before the adhesion zone of the vesicles undergoes detectable changes. (b) The behavior of a single vesicle (first image) and a vesicle couple (remaining images) when exposed to a dc pulse in the absence of salt. The amplitude of the dc pulse was 90 V (1.8 kV/cm), and its duration was 150 μ s. (c) Behavior of a single vesicle (first image) and a vesicle couple (remaining images) in the presence of 1 mM NaCl in the exterior solution (the vesicle radii are 29.0 and 26.5 μ m). In this case, the amplitude of the dc pulse was 150 V (3 kV/cm), and its duration was 150 μ s. (c) Behavior of a minus (–) sign. The arrows in the first images indicate porated parts of the membrane, which lead to the leakage of enclosed liquid. For both *b* and *c*, the starting time t = 0 corresponds to the beginning of the dc pulse. In the last two snapshots of the sequence (b), the fused vesicles contain an array of internal vesicles (bright spots) as indicated by the arrows. The image acquisition rate was 20,000 fps. For more details, see Movies 1–4.

pettes. To do so, we first prepared a large number of vesicles by using electroformation as described in *Methods*. From this preparation, we carefully selected two unilamellar vesicles, which had no visible defects, and brought them together via the micropipettes. Then a third micropipette was used to inject a small volume of EuCl₃ solution into the contact zone between the two vesicles (see Fig. 1*a*). This procedure was applied to ≈ 100 vesicle couples, half of which ruptured within a few seconds after the ion injection. For the remaining 50 couples, we observed five successful fusion events, three of which have been recorded by a CCD camera with 28 frames per s (fps) and two by the fast digital camera with 20,000 fps (see Movies 1 and 2, which are published as supporting information on the PNAS web site).

The delay time between the injection of EuCl₃ and the beginning of the fusion process was governed by a broad distribution of times between 1 and 5 min. It is not feasible, at present, to record such a time period with 50- μ s resolution because it would require between 120 and 600 gigabytes of on-board memory storage in the digital camera. We used a camera with 4 gigabytes on-board memory, which allows storing an image sequence of 2 s consisting of 40,000 frames. Thus, to capture a fusion event with the fast digital camera, we had 2 s to visually notice it and stop the camera from erasing its memory.

For comparison, we also studied the dynamics of electrofusion with the same temporal resolution (see Fig. 1 b and c). In the latter case, the delay time between the applied electric pulse and the beginning of the electrofusion process is only a fraction of a millisecond, much shorter than for the ligand-mediated case, and thus, easier to record with the fast camera (see Movies 3 and 4, which are published as supporting information on the PNAS web site).

Applying both ligand-mediated fusion and electrofusion, we identified two different dynamic regimes. During the first $300 \ \mu s$,

the fusion necks opened very rapidly, expanding with an average velocity of centimeters per second for both protocols. This result was surprising because the initial molecular rearrangements were expected to be rather different for the two types of fusion protocols (see Fig. 2). After 300 μ s, the expansion of the fusion neck slowed down by approximately two orders of magnitude. Despite the differences between the two fusion protocols, the



Fig. 2. Molecular mechanisms of membrane fusion. (*a*) The fusion process as observed on the micrometer scale (compare Fig. 1). (*b* and *c*) The cartoons in *b* and *c* are hypothetical molecular rearrangements for ligand-mediated fusion (*b*) and electrofusion (*c*), which we might see if we were able to zoom into the contact zone with molecular resolution.

dynamics of the fusion neck opening was, in fact, found to be quite similar as explained in the following sections.

First Protocol: Ligand-Mediated Fusion. In the first fusion protocol, we used giant unilamellar vesicles made of conventional lipidegg phosphatidylcholine (egg-PC) or lecithin. We functionalized these egg-PC vesicles by incorporating lipid-like molecules with β -diketone "head groups" (28). A micropipette was used to inject a solution of EuCl₃ locally over a population of vesicles or directly into the area of contact between two giant vesicles that were trapped and displaced by two additional micropipettes (see Fig. 1*a*). When injected over vesicles in contact, $EuCl_3$ induced adhesion. In addition, Eu³⁺ is known to form a coordination complex with β -diketone groups in a 1:2 ion-to-ligand ratio (28). When the complex of one europium ion and two or more ligands is formed between two adjacent membranes, fusion is triggered (a possible mechanism for this interaction on the molecular level is shown in Fig. 2b). In contrast, in the absence of the β -diketone groups, nonspecific interaction between the lipid molecules in the membrane and Eu³⁺ induced adhesion but no fusion. No significant dependence on the ligand concentration, the EuCl₃ concentration, or the membrane tension applied with the pipettes was observed (see *Methods* for details).

Second Protocol: Electrofusion. The second fusion protocol is based on electroporation. Initially, the ac field can be used to bring two vesicles into contact and align them in the field direction [an effect similar to the one observed with cells where the latter align in the field direction in pearl chains (19)], after which a dc pulse is applied. The effect of dc pulses on single vesicles has been reported in detail (29). Briefly, the vesicle shapes are deformed by the transmembrane potential, which arises from the applied pulse. In the absence of salt, the vesicles attain a prolate shape (see the first snapshots in Fig. 1b and Movie 3). When the solution outside of the vesicles contains a small amount of salt (\approx 1 mM NaCl), unusual short-lived shape deformations, mainly cylindrical, are observed (see the first snapshots in Fig. 1c). Although intriguing, the nature of these morphological changes is not the main focus of the present study and is discussed elsewhere (30). When the applied transmembrane potential exceeds some critical value, it porates the membranes (see arrows on the first snapshots in Fig. 1 b and c). The minimal pore radius that can be observed in this way is $\approx 0.5 \ \mu m$.

Applying the dc pulses to a couple of vesicles brought into contact leads to fusion (see the series of snapshots in Fig. 1 b and c and Movies 3 and 4). On the molecular scale, the formation of the fusion neck is likely to proceed via the steps shown in Fig. 2c. Membrane poration is a necessary condition for vesicles in contact to fuse. Thus, pulses, which did not result in reaching the critical transmembrane potential, led only to pressing the vesicles together but did not cause fusion. For different pulse parameters (field strength and pulse duration) no qualitative difference in the fusion dynamics was observed.

In the absence of salt, the fusion typically occurs at several contact points as one can deduce by inspection of the optical micrographs (Fig. 1b). When the two vesicles fuse at more than two contact points and form more than two fusion necks, the coalescence of these fusion necks can lead to small, contact zone vesicles, which are visible as bright spots in the two last snapshots in Fig. 1b. Consider, e.g., three fusion necks that expand and touch each other in such a way that they enclose a roughly triangular segment of the contact zone. If the three necks are circular and have grown up to a diameter L_1 , the enclosed contact zone segment will form a contact zone vesicle of radius $R_{czv} = 0.08 L_1$ as follows from simple geometric considerations.

In the presence of salt (\approx 1 mM NaCl) in the solution outside of the vesicles, the dc pulse induces cylindrical deformations as observed for single vesicles (30) (see first snapshot in Fig. 1c). The



Fig. 3. Opening of the fusion neck. The fusion neck diameter, *L*, as a function of time, *t*, is plotted semilogarithmically for ligand-mediated fusion (a) and electofusion (b). The different symbols correspond to different vesicle couples (see *Methods* for details). The data set corresponding to the full squares in a represents a fusion event in which one of the two fusing vesicle ruptures before it completely merges with the second vesicle, which is why we show only data corresponding to the early stage of the fusion (before the membrane ruptures). The first dynamic regime that is characterized by rapid expansion of the fusion neck extends up to ~300 μ s for ligand-mediated fusion and up to ~1 ms for electrofusion (compare with Fig. 5). (*Insets*) Snapshots with a magnified section of the fusion neck covering the time period from 0 to 400 μ s.

two vesicles are pushed together and form a flat contact zone in between (see Movie 4). In this case, no bright spots corresponding to enclosed vesicles are observed, from which we conclude that only one fusion neck or a small number of such necks has been formed initially. This conclusion is consistent with the observation that both membrane area and vesicle volume are conserved during these fusion processes and is confirmed by additional measurements using fluorescence microscopy (data not shown). All fusion events discussed in the following were obtained in the presence of salt, and for these events, no contact zone vesicles were observed. When the expanded fusion neck was located below or above the focal plane, the error in the determination of the neck diameter in the initial 300 μ s was approximately $\pm 2 \ \mu$ m as indicated in Fig. 3b, but with further expansion, the fusion neck was in focus, thus bringing down the error to typical optical resolution of $\pm 0.25 \ \mu$ m.

Temporal Evolution of the Fusion Neck Diameter. For both ligandinduced fusion and electrofusion, we were able to record the opening of the fusion neck with an acquisition rate of 20,000 fps (Fig. 3). Detailed image analysis of the fusion zones shows that the opening of the fusion neck with a radius larger than $\approx 2 \ \mu m$ takes place within a couple of frames, e.g., within 100 μ s (see Fig. 3 Insets and Methods for details on the data). A first inspection of Fig. 3 shows that the data obtained by the two different protocols exhibit a similar functional form for the fusion dynamics, i.e., for the fusion neck diameter, L, as a function of time, t. The time dependence of L already indicates two different dynamic regimes for both fusion protocols (see Fig. 5, which is published as supporting information on the PNAS web site).

A more detailed comparison of the data reveals that the range of neck diameters observed during the electrofusion process (Fig. 3b) is larger than during the ligand-mediated fusion (Fig. 3a). Likewise, the electrofusion data involve six orders of magnitude in time, whereas the ligand-mediated fusion data cover only three orders of magnitude in time. However, we will now show that the different ranges of neck diameters primarily reflect the difference in vesicle sizes, and all data sets for the earlier time regime collapse onto a single curve provided one rescales the neck diameter in an appropriate way. In addition, we show that the different behavior of the fusion neck diameter at later times is a result of the different tensions that the membranes experience during the later stage of the opening of the fusion neck.

The first dynamic regime corresponds to the early stage of the fusion neck opening and extends up to $\approx 300 \ \mu s$ for the ligandmediated fusion process (see Fig. 3a) and up to $\approx 1 \ ms$ for the electrofusion process (see Fig. 3b). In the second dynamic regime, the neck opening slows down and the expansion velocity of the fusion neck, dL/dt, decreases by two orders of magnitude (note that this expansion velocity does not correspond to the slope of the curves in Fig. 3 because these plots are semilogarithmic).

To analyze the early stage of the fusion neck opening, we found it useful to plot the data in a variety of different ways. Because the fusion of larger vesicles would lead to the expansion of the fusion neck to larger diameter, in Fig. 4*a* the neck diameter *L* is rescaled by $(R_1 + R_2)$, where R_1 and R_2 are the radii of the two vesicles before they were brought into contact. Inspection of Fig. 4*a* shows that the data from the two fusion protocols collapse between 50 μ s and \approx 3 ms, i.e., during the early stage of the fusion neck opening.

The later stage of the expansion of the fusion neck covers the time evolution after a few milliseconds. In this regime, the expansion of the fusion neck proceeds much faster for ligand-mediated fusion than for electrofusion. This time shift is understandable if one considers the different constraints imposed on the vesicles during the two fusion protocols. For ligand-mediated fusion, each of the two vesicles is aspirated by a micropipette and the pressure is kept constant during the whole process, which implies that the membranes experience a large and essentially constant tension. After the fusion neck has been formed, it opens rapidly because the rim of the neck is pulled by the large membrane tension. On the other hand, after electrofusion, the tension within the membranes relaxes as the rim of the neck opens up.

It is instructive to use dimensional analysis to find an appropriate time scale, τ , for the later stage of the expansion of the fusion neck, i.e., after a few milliseconds. The driving force for this expansion is provided by the membrane tension, σ , whereas the hydrodynamic or Stokes friction is governed by the viscosity, η , of the aqueous solution to be displaced (we implicitly assume that Stokes friction dominates interbilayer friction as appropriate for length scales that exceed 0.5 μ m). Our system is characterized by two well separated length scales, the membrane thickness, ℓ , and a typical vesicle size, R. We will choose R = $(R_1 + R_2)/2$, where R_1 and R_2 are the initial radii of the two vesicles as described. The only time scale, which one can obtain from a combination of the four variables σ , η , ℓ , and R, is given by $\tau = (\eta R/\sigma)f(\ell/R)$ with the dimensionless function $f(\ell/R)$. Because $\ell \ll R$, we can replace $f(\ell/R)$ by f(0) and, thus, ignore corrections of order (ℓ/R) .

For the electrofused vesicles relaxing at the bottom of the observation chamber, typical tensions should be in the range 0.05



Fig. 4. Comparative data analysis of ligand-mediated fusion and electrofusion. (a) Rescaled fusion neck diameter $L/(R_1 + R_2)$ as a function of time t is plotted semilogarithmically. Note that the data sets collapse onto a single curve between 50 μ s and 3 ms. (Inset) The rescaled fusion neck diameter as a function of the rescaled time t/τ (see text for definition of τ). The normalized data collapse onto a single curve for the later stages of the expansion of the fusion neck corresponding to $t/\tau > 10^3$. (b) Double logarithmic plot of the fusion neck diameter L as a function of time t. The solid line for $t < 50 \ \mu s$ corresponds to an average expansion velocity of 5 cm/s as estimated from the first frames for the four fusion processes. Linear extrapolation of all data with $50 < t < 300 \ \mu s$ leads to the dashed line that corresponds to an expansion velocity of 4 cm/s. The shaded stripe around this line indicates the fitting error as estimated by least-squares fitting. For comparison, simulation data from ref. 29 are also included. The latter data describe the opening of a single fusion neck for a small vesicle with a diameter of 28 nm and are consistent with the experimental data obtained here for the fusion neck expansion of giant vesicles.

to 0.1 mN/m as one can conclude from the visible shape fluctuations of the vesicle membranes (31). In contrast, the vesicle produced via ligand-mediated fusion is still aspirated by the micropipettes. The suction pressure applied by the micropipettes leads to a membrane tension of the order of 5 mN/m. For an effective vesicle radius $R \approx 20 \ \mu m$, e.g., we obtain the time scales $\tau = 4.4 \ \mu s$ for the aspirated vesicles with $\sigma = 5 \ mN/m$, and τ = 0.44 ms for the electrofused vesicles with σ = 0.05 mN/m. For each vesicle couple and tension conditions, we calculated the corresponding value of τ , which was used to define the rescaled time t/τ [see Fig. 4a Inset where we plot the rescaled fusion neck diameter, $L/(R_1 + R_2)$, as a function of t/τ]. This rescaling leads to a collapse of the two types of data sets for the later stage of the expansion of the fusion neck with $t/\tau > 10^3$. Thus, we conclude that both fusion protocols lead to essentially the same fusion dynamics.

Let us now go back to the early stages of the opening of the fusion neck. For the four fusion events displayed in Fig. 3, the fusion neck has attained an average diameter of $\approx 2.5 \,\mu$ m already after the first 50 μ s. Let us first assume that the recorded fusion events have started from a single fusion pore or neck with an initial diameter of the order of 10 nm (i.e., somewhat larger than twice the membrane thickness). Because the measured neck diameter at $t = 50 \ \mu s$ is much larger than 10 nm, it would imply an average expansion velocity of ≈ 5 cm/s during the first 50 μ s. The main error for this estimate comes from the uncertainty for the choice of the initial frame, which represents the limit of our time resolution. To improve this estimate, we also use an extrapolation of the four data sets between 50 and 300 μ s, which correspond to the first six frames. As shown in Fig. 4b, all four data sets have the same slope when plotted in a doublelogarithmic manner. Because this slope is close to one, these data imply a roughly constant expansion velocity for the fusion neck between 50 and 300 μ s, which is found to be \approx 4 cm/s. The latter six-frame estimate is fairly close to, but somewhat smaller than, the first frame estimate of 5 cm/s. This finding is consistent with the general observation that the expansion velocity of the fusion neck decreases monotonically with time for all times.

It is important to note that essentially the same order of magnitude for the average expansion velocity is deduced if we assumed that the fusion process started with N > 1 fusion necks. These fusion necks would grow until they start to touch and coalesce. They would then create a coalesced neck of diameter L if each neck had grown up to L/\sqrt{N} . This implied an average expansion velocity of about $(5/\sqrt{N})$ cm/s, which is still of the same order of magnitude even if N were as large as 10.

Linear extrapolation of the data between 50 and 300 μ s to smaller times leads to the shaded stripe in Fig. 4b. This extrapolation predicts that the formation of the fusion neck with a diameter of ≈ 10 nm should occur within a time period of ≈ 250 ns if the process started with a single neck or 250 \sqrt{N} ns if it started with N such necks. Likewise, the first frame estimate leads to a time scale of $\approx 200 \sqrt{N}$ ns for the initial formation of the fusion necks. It is quite remarkable that time scales of the order of 200 ns were also obtained from computer simulations for the formation of the initial fusion neck between a 28-nm vesicle and a 50-nm membrane segment under relatively large tension (32). In a somewhat speculative vein, we have included the simulation data for the time evolution of one such fusion event in Fig. 4b. Even though the size of the simulated vesicles is much smaller than the size of the giant vesicles studied here, the simulation data are quite consistent with both the first-frame and the six-frame estimate of our microscopy data.

In summary, we have been able to control and observe the fusion of lipid vesicles with a temporal resolution of 50 μ s. We have used two different fusion protocols: fusion mediated by membrane-anchored β -diketone groups, which are cross-linked by europium ions, and fusion triggered by electroporation. In both cases, we found two different dynamic regimes for the expansion of the fusion neck. During the later stage of the fusion process, the neck expansion velocity slowed down by two orders of magnitude. Here, the dynamics were governed mainly by the displacement of the volume of liquid around the fusion neck between the fused vesicles. This conclusion is confirmed by the dimensional analysis provided in *Supporting Text*, which is published as supporting information on the PNAS web site.

The rapid initial expansion explains why it is so difficult to directly image the fusion process. If the fusion processes start with a single fusion neck, the average expansion velocity of the neck during the first 50 μ s is found to be \approx 5 cm/s, whereas the data points between 50 and 300 μ s imply an expansion velocity of \approx 4 cm/s. If the fusion processes started with *N* fusion necks, the average expansion velocity of the necks would be about $(5/\sqrt{N})$ cm/s.

The experimental approach used here can be applied to other fusion protocols as well. A particularly interesting example is provided by soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-induced fusion.

Methods

Preparation and Observation of Giant Unilamellar Vesicles. Giant unilamellar vesicles of L- α -phosphatidylcholine from egg yolk (Sigma, St. Louis, MO) were grown by using the electroformation method (33). The procedures used here are as in ref. 29. The membranes used for micropipette manipulation contained a small fraction ($\leq 0.5 \text{ mol}\%$) of amphiphilic β -diketone ligands (28). The vesicles were swelled in 0.2 M sucrose and subsequently diluted 40 times into 0.2 M glucose solution (some of the vesicle samples used for electrofusion were diluted in glucose solutions containing NaCl). The glucose/sucrose asymmetry creates a refraction index difference between the interior and the exterior of the vesicles. The latter enhances the contrast of the microscopy images (vesicle images appear dark on a bright background; see, e.g., Fig. 1). The observations were performed with an Axiovert135 microscope (Zeiss, Jena, Germany) equipped with $\times 20$ and $\times 40$ phase-contrast objectives and three micromanipulators (Sutter Instruments, Novato, CA and Narishige, Tokyo, Japan). The fusion events were recorded with a fast digital camera HG-100K (Redlake, Tucson, AZ) mounted on the microscope and connected to a personal computer. Image sequences were acquired at a frequency of up to 20,000 fps. The length of recording at this frequency did not exceed 2 s because of the limitation of the on-board memory of the camera as described. The illumination of the observation chamber was achieved with a mercury lamp HBO W/2. Sample heating caused by illumination was measured to be \approx 2°C, and, thus, did not significantly affect the membrane properties. All measurements were performed at room temperature.

Fusion Protocol for Functionalized Membranes. The vesicle solution was placed in a specially designed chamber, which allows accommodation of three micropipettes: two for vesicle manipulation and one for ion injection (see *Supporting Text* for details). EuCl₃ was injected over a population of vesicles or directly into the area of contact between two giant vesicles being isolated by micropipettes. The concentration of EuCl₃ injected was varied between 1 μ M and 1 mM, whereby all solutions were adjusted with glucose to be isoosmolar.

With either functionalized or pure lipid membranes, EuCl₃ was observed to induce adhesion between adjacent vesicles for the whole concentration range. Europium chloride concentrations <1 μ M did not promote the fusion of the functionalized membranes. Concentrations >1 mM were not explored because they caused the membranes to rupture. The latter observation is intriguing because it suggests that the adsorption of Eu³⁺ on the membrane induces some tension. At higher concentrations this tension would reach the critical value of membrane lysis (~7 mN/m), causing the vesicles to rupture. It is interesting to note that the injection of Ni²⁺ ions also leads to the adhesion of the functionalized vesicles, and sometimes to their rupture, but not to bilayer fusion.

The adhesion was found to be reversible once the concentration of EuCl₃ in the vicinity of the membranes decreased because of diffusion. The injected EuCl₃ was observed to induce fusion between adjacent membranes only in the case of functionalized vesicles. The range of β -diketone ligand concentrations that was explored in this study lies between 0.01 mol% (below this concentration no fusion was achieved) and 5 mol% (incorporation of more ligands in the bilayer was not possible). The fusion statistics were found to be essentially independent of the ligand concentration. Likewise, no dependence of the fusion statistics on the EuCl₃ concentration was detected.

The successful fusion events consisted of a prefusion stage, the

formation of a fusion neck, and the subsequent opening of this neck. During the prefusion stage, which lasted between 1 and 5 min after the injection of EuCl₃, the vesicles first adhered to each other, forming an extended contact area, and then the contact area slowly decreased until it was difficult to resolve with optical microscopy. During this prefusion stage, there was no exchange of lipid molecules between the two vesicles as demonstrated by fluorescent labeling of one of the membranes, implying that no hemifusion occurred. As explained before, it is currently not feasible to record the relatively long prefusion stage with a temporal resolution of 50 μ s because of limitations imposed by the memory on the camera chip. Indeed, using the highest temporal resolution corresponding to 20,000 fps, one can record only ≈ 2 s.

When EuCl₃ was injected directly over a population of vesicles (>50 vesicles) fusion events were always observed. Repeating this observation on different preparations led to the statistics of having on average 30% of the vesicles fuse. The probability to induce a successful fusion event between two isolated vesicles using micropipettes is found to be reduced and of the order of 10% for those vesicle couples that do not rupture within a few seconds after the ion injection. As mentioned, we studied ≈ 50 such vesicle couples, 5 of which fused. We recorded three of these successful fusion events with a CCD camera and two with the fast digital camera. The data set presented with open blue squares in Fig. 3a ($R_1 = 25.4 \,\mu m$, $R_2 = 16.6 \ \mu m$) is obtained from one of these fast recordings (Movies 1 and 2 are from this fusion event). The second ligandmediated fusion data set displayed with filled blue squares in Fig. $3a (R_1 = 14.2 \ \mu\text{m}, R_2 = 8.5 \ \hat{\mu}\text{m})$ is limited in duration because one of the vesicle ruptured $\approx 250 \ \mu s$ after fusion.

In the micropipette experiments, we monitored the membrane tension (34, 35) as obtained from the aspiration pressure and the Laplace equation. This tension varied between 0.01 and 7 mN/m but again was not observed to affect the fusion statistics in an essential way.

Electrofusion Protocol. The experimental chamber and the electroporation procedure have been described in detail (29); additional information is contained in *Supporting Text*. The applied field creates a transmembrane potential, which has its maximal value at the vesicle poles facing the electrodes. In these areas, macropores of diameter up to $\approx 5 \mu m$ are observed after $\approx 1 ms$ as demonstrated by the fluid that leaks out of the vesicles (see arrows on the first snapshots in Fig. 1 *b* and *c*). This leakage can be directly observed because the interior and the exterior

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solutions contain sucrose and glucose, respectively, and have sufficiently different refractive indices.

Applying the dc pulses (pulse strength from 1 to 4 kV/cm and pulse duration from 50 to 250 μ s) to a couple of vesicles brought into contact leads to fusion. In the absence of salt, the fusion occurs at several contact points (see Movie 3). The coalescence of these necks can lead to small contact zone vesicles encapsulating glucose solution external to the two initial vesicles. As a consequence, these small vesicles appear as bright spots in the microscopy images (see the two last snapshots in Fig. 1b). One expects that these vesicles are interconnected by thin tethers because pinching the membrane off completely would require some additional energy input. It is interesting to note that this fusion-induced vesicle formation resembles the membrane processes during cell division when we look at them in a time-reversed manner. Indeed, during the initial stages of the division process, the cell accumulates membrane in the form of small vesicles that define the division plane and transform into two adjacent cell membranes.

In the presence of salt (\approx 1 mM NaCl) in the solution outside of the vesicles, the dc pulse induced cylindrical deformations as observed for single vesicles (30) (see the first snapshot in Fig. 1c). The two vesicles were pushed together and formed a flat contact zone in between (see Movie 4). In this case, no bright spots corresponding to enclosed vesicles were observed from which we conclude that only one fusion neck or a small number of such necks had been formed initially.

The probability of successful fusion was $\approx 90\%$. In this case, the delay time between the applied electric pulse and the beginning of the electrofusion process is only a fraction of a millisecond, and it was, thus, easier to record this process with a fast camera. The acquired data on the neck diameter evolution were normalized by the average vesicle diameter as explained above. The fusion dynamics of 10 different vesicle couples was recorded and analyzed. The data set presented with filled red circles in Fig. 3b ($R_1 = 26.5 \ \mu m$, $R_2 = 29.0 \ \mu m$) is the one that is closest to the mean trend averaged from all of the data sets. The second electrofusion data set displayed with open red circles in Fig. 3b ($R_1 = 20.3 \ \mu m$, $R_2 = 22.6 \ \mu m$) represents one of the farthest deviations observed.

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Supporting Text

Fusion Protocol for Functionalized Membranes

After electroformation, the vesicle solution was diluted in glucose and placed in a specially designed chamber consisting of two glass slides and Teflon spacers. The chamber was open to provide space for the insertion of several micropipettes. The pipettes were prepared from borosilicate capillaries (World Precision Instruments, Sarasosta, FL, internal diameter of 1 mm) using a horizontal pipette puller (Sutter Instruments). The internal radius of the pipettes used to manipulate the vesicles was ~5 μ m. They were connected with Teflon tubes to a hydrostatic pressure system, which creates a small suction pressure of a few Pa inside the glass capillary. This underpressure is sufficient to aspirate and hold a vesicle. Two vesicles without visible defects were selected and brought into contact by using two micropipettes. A third pipette with a smaller radius of ~1 μ m was used for local injection of EuCl₃. The injection volumes were controlled by using a picoinjector PLI100 (Harvard Apparatus, Holliston, MA). Using glucose at different concentrations, the osmolarity of the EuCl₃ solutions was adjusted (with an osmometer Osmomat030 Gonotec, Berlin, Germany) to avoid osmotic swelling or shrinking of the vesicles. The concentration of the injected EuCl₃ was varied between 1 μ M and 1 mM.

Electrofusion Protocol

The electroformed vesicles were diluted in glucose solution containing NaCl of concentration between 0 and 1 mM and placed in an observation chamber (Eppendorf, Hamburg, Germany) with two electrodes spaced at a distance of $475 \pm 5 \,\mu\text{m}$. The vesicles stayed at the bottom of the chamber because the interior sugar solution had a somewhat larger density. An alternating electric field was switched on for a short time period of ~10 s before the dc pulse was applied. The ac field aligns the vesicles along the direction of the field and brings them into contact. To some extent, this initial ac field plays the role of the micropipettes in the first protocol since it is used to position the vesicle couples. After two

vesicles had come into contact, a short rectangular dc pulse was applied, which perturbed the vesicles in the contact area and induced their fusion. The dc pulse was generated by using an electric field generator Multiporator (Eppendorf). Its amplitude was varied between 50 and 200 V (1 ± 0.2 to 4 ± 0.4 kV/cm) and its duration varied between 50 and 250 µs.

The vesicles were observed to drift laterally, which indicated that they did not adhere to the glass surface.

The salt concentrations used in this work were substantially below physiological conditions. Concentrations higher than those used here are expected to shorten the time of membrane charging, τ_{charg} , because the media conductivity would be increased. Indeed, the charging time is given by (1)

$$\tau_{\text{charg}} = R C_{\text{m}} \left[\frac{1}{\lambda_{\text{in}}} + \frac{1}{(2\lambda_{\text{out}})} \right], \qquad [1]$$

where $C_{\rm m}$ is the membrane capacitance, and $\lambda_{\rm in}$ and $\lambda_{\rm out}$ are the conductivities inside and outside the vesicle, respectively. Shorter charging times would lead to an increase in the transmembrane potential $V_{\rm m}$, which depends on time *t* according to (1)

$$V_{\rm m} = 1.5R \left| \cos \theta \, \mathrm{E} \right| \left(1 - e^{-t/\tau_{\rm charg}} \right), \tag{2}$$

where *R* is the vesicle radius, E the applied electric field, and θ the angle between the electric field and the vesicle surface normal.

Dimensional Analysis for the Early and Later Stages of Fusion Neck Expansion

The two stages of the fusion process, a very fast early stage and a slower later stage, were detected for both fusion protocols. This was clearly observed when the diameter of the fusion neck was plotted versus time by using a linear time scale as shown in Fig. 5. Fig. 5 contains the same data as in Fig. 3 where a logarithmic time scale is used.

During the early stage, the fusion neck opened very rapidly: the expansion velocity of the rim of the neck was of the order of 4 cm/s. The fusion pore has opened up to micrometers within a hundred microseconds. Intuitively, one would relate this time, τ_{early} , to fast relaxation of the membrane tension. The tension of the vesicles achieved before

fusion was in the stretching regime of the membrane. Thus, τ_{early} should be primarily governed by the relaxation of membrane stretching. The viscous dissipation can be associated with two contributions: in-plane dilatational shear as the fusion neck expands and intermonolayer slip between the two leaflets of the bilayer in the zone of the fusion neck. The second is negligible for diameter of the fusion neck *L* larger than half a micrometer (2). Thus, $\tau_{early} \sim \eta_s / \sigma$, where η_s is the surface dilatational viscosity of the bilayer ≈ 0.35 N.s/m (3) (η_s has units [bulk viscosity] × [membrane thickness]). For membrane tensions of the order of 5 mN/m, which should be close to the tension of rupture, one obtains $\tau_{early} \sim \eta_s / \sigma \sim 100$ µs in agreement with the experimental observations from which we deduced 300 µs as the appropriate time scale to complete the early stage of fusion.

During the later stage of the fusion process, the neck expansion velocity slowed down by two orders of magnitude. Here the dynamics was mainly governed by the displacement of the volume ΔV of fluid around the fusion neck between the fused vesicles. The restoring force was related to the bending elasticity of the lipid bilayer. The corresponding decay time in this later stage can be presented as $\tau_{\text{late}} \sim \eta \Delta V/\kappa$, where η is the bulk viscosity of sucrose/glucose solution, $\Delta V \sim R^3$, and κ is the bending elasticity modulus of the membrane. For egg-PC $\kappa \approx 10^{-19}$ J (4, 5). Thus for a typical vesicle size of $R = 20 \,\mu\text{m}$, we obtained $\tau_{\text{late}} \sim 100$ s, which is the time scale that we measured for complete fusion neck opening.

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