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A Novel Assay to Detect Fusion Pore Formation: Implication for Fusion Mechanism

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Abstract

Membrane fusion is broadly envisioned as a two- or three-step process proceeding from contacting bilayers through one or two semi-stable, non-lamellar lipidic intermediate structures to a fusion pore. A true fusion event requires mixing of contents between compartments and is monitored by the movement of soluble molecules between trapped compartments. We have used Poly(ethylene glycol) to rapidly generate an ensemble aggregated state A that proceeds sequentially through intermediates (I_1 and/or I_2) to a final fusion pore state (FP) with rate constants k_1 , k_2 , and k_3 . Movement of moderately sized solutes (*e.g.*, Tb^{3+} /dipicolinic acid) has been used to detect pores assigned to intermediate states as well as to the final state (FP). Analysis of ensemble kinetic data has required that mixing of contents occur with defined probabilities (α_i) in each ensemble state, although it is unclear whether pores that form in different states are different. We introduce here a simple new assay that employs fluorescence resonance energy transfer (FRET) between a FAM (donor) and TAMRA (acceptor), which are covalently attached to complementary sequences of 10-base pair oligonucleotides. Complementary sequences of fluorophore-labeled oligonucleotides were incorporated in vesicles separately, and FRET increased in a simple exponential fashion during PEG-mediated fusion. The resulting rate constant corresponded closely to the slow rate constant of FP formation (k_3) derived from small molecule assays. Additionally, the total extent of oligonucleotide mixing corresponded to the fraction of content mixing that occurred in state FP in the small molecule assay. The results show that both large “final pores” and small (presumably transient) pores can form between vesicles throughout the fusion process. The implications of this result for the mechanism of membranes fusion are discussed.

Keywords

Membrane fusion; membrane fusion assay; pore formation; fluctuation pore

INTRODUCTION

Membrane fusion is a ubiquitous event in eukaryotic cells, critical to organelle membrane trafficking, synaptic transmission, fertilization, enveloped viral infection, and myoblast syncytia formation. Nearly two decades of research has produced fairly wide agreement that membrane fusion proceeds in both model membranes and protein-laden biological membranes *via* rearrangement of lipids (1, 2) during a multi-step process proceeding from contacting bilayers through one or two semi-stable, non-lamellar intermediate structures to a fusion pore, as envisioned in the “modified stalk hypothesis” (3, 4). Because these structures

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form on a very small length scale (several nm) and evolve on a very fast time scale (several millisecond (5)), it is not possible to test the modified stalk hypothesis by directly observing intermediate structures during single fusion events, although single event measurements do confirm the existence of two or more steps in the process (6, 7). Distributions of average “wait-times” between content-mixing or lipid-mixing single events (revealed by fluorescence methods) (8) show bi-exponential behavior, consistent with at least a two-step process. Finally, analysis of ensemble kinetic measurements in terms of a two- or three-step model (9, 10) yield rate constants comparable to the exponential constants derived from “wait-time” distributions, which also supports the “modified stalk hypothesis”. While multi-exponential behavior is detected by both ensemble kinetics and “wait-time” analysis of single events, two studies, one using ensemble kinetics (11) and other analyzing “wait-time” distributions of single events (12), were able to resolve not just two exponentials but two processes with intervening lag phases, providing even more dramatic evidence for the “modified stalk hypothesis”.

Until recently (7), both approaches (single-event and ensemble) failed to address whether pores between vesicles detected at different times during the fusion process (*i.e.*, in different intermediate states) are similar or different. Early electrophysiological experiments on patch-clamped exocytic cells reported “flickering” or transient, small pores early in the fusion process (13). This phenomenon was also reported in model membrane fusion (14) and was suggested by reports based on ensemble fusion kinetics (11), although it is impossible to unambiguously detect transient or reversible pores using fluorescent probes, as pore opening results in an irreversible event, mainly rapid diffusion of fluorescent molecules between trapped compartments. It would clearly be useful to know whether pores formed early in the fusion process as monitored by ensemble kinetics are smaller than pores formed near the end of the process, as might be implied by “flickering pore” observations. Here we introduce a new class of content mixing probe (fluorescently labeled 10-base oligonucleotides) that monitor content mixing only through large pores. Measuring content mixing using these new oligo probes in combination with small molecule (Tb^{3+} /DPA) probes allowed us to parse for the first time by our ensemble kinetic methods the coexistence of two classes of pores throughout the fusion process.

MATERIALS AND METHODS

Materials

Chloroform stock solutions of 1,2-dioleoyl-3-sn-phosphatidylcholine (DOPC), 1,2-dioleoyl-3-sn-phosphatidylethanolamine (DOPE), 1,2-dioleoyl-3-sn-phosphatidylserine (DOPS) and bovine sphingomyelin (SM) were purchased from Avanti Polar Lipids (Birmingham, AL) and used without further purification. The concentration of the stock lipids was determined by phosphate assay. Cholesterol (CH) was purchased from Avanti Polar Lipids and was further purified by published procedures (15). Fluorescence labeled oligonucleotides (5-FAM/TGGAGAAGGC and GCCTTCTCCA/3-TAMRA) were purchased from Integrated DNA Technologies, USA. Poly(ethylene glycol) of molecular weight 7000–9000 (PEG 8000) was purchased from Thermo Fisher Scientific (Waltham, MA) and purified as previously reported (16). All other reagents were of the highest purity available.

Vesicle Preparation

Vesicles were prepared either from a 35/30/15/20 molar mixture of DOPC/DOPE/SM/CH or a 32/25/15/20/8 DOPC/DOPE/SM/CH/DOPS. Lipids at this molar ratio in cyclohexane/methanol mixed solvent were freeze-dried under high vacuum overnight. The dried lipid powders were hydrated at 23°C for 1 hour in a sonication buffer contained 10 mM TES, 100

mM NaCl, 1 mM EDTA and 1 mM CaCl₂ at pH 7.4. Then small unilamellar vesicles were prepared using probe sonication as documented previously (17). All contents mixing, leakage, and lipid mixing experiments were performed at 0.2 mM lipid.

Preparation of Vesicles Containing gp41 Fusion Peptide and Synaptobrevin Trans-Membrane Domain (TMD) Peptide

The gp41 fusion peptide from HIV, sequence **AVGIGALFLGFLGAAGSTMGARS**, was chemically synthesized and purified by the peptide synthesis laboratory at University of North Carolina, Chapel Hill. The peptide was synthesized by the standard solid phase method using Fmoc chemistry (18, 19). *Trans*-membrane domain of synaptobrevin (SB TMD), sequence **KLKRKYWWKNLKMMLGVICAILIIIVYFST**, was purchased from Invitrogen, USA and used without further purification. Purity of all the peptides was checked with mass spectrometry. For gp41 fusion peptide, stock peptide solutions were prepared in DMSO and small aliquots of these solutions were added to vesicle suspensions at a peptide/Lipid ratio of 1/200. DMSO was always less than 1% of the buffer volume, and control experiments showed that this amount of DMSO had no detectable effect on either fusion or membrane structure. For SB-TMD, peptide was added to the chloroform solution of lipids at a peptide/lipid ratio of 1/900 and co-lyophilized with the lipids.

Initiation of fusion reaction

The fusion process was initiated by adding poly(ethylene) glycol (PEG): 5 wt% ($\pm$ gp41 fusion peptide) for PC/PE/SM/CH SUVs and 6 wt% for phosphatidylserine (PS)-containing SUVs. Signals were recorded within 6 sec after adding PEG, such that vesicle aggregation was well completed before fusion events were recorded.

Lipid Mixing Assay

Fluorescent lipid probes with fluorophores attached to their acyl-chains, BODIPY500-PC and BODIPY530-PE (Molecular probes, Eugene, OR), were used for measuring lipid transfer during PEG-mediated vesicle fusion, with a probe-containing/probe-free vesicle ratio of 1:4, as described in detail elsewhere (20, 21). Ratio of labeled and unlabeled lipids in the probe-containing vesicles is 1:200. Here we normalized the F_D/F_A fluorescence intensity with respect to the F_D/F_A in presence of the detergent, ideally a situation in which all lipids are mixed. In this method, the fraction of lipid mixing is:

$$LM(t) = \frac{1}{n} \exp \left(\left\{ \frac{k [(F_D/F_{DA})_t - (F_D/F_{DA})_0]}{(F_D/F_{DA})_{\text{detergent}}} \right\} - 1 \right)$$

where n is the ratio of probe-free to probe-containing vesicles in a sample, F_D is the fluorescence of donor taken at a wavelength (520 nm) at which acceptor does not contribute, F_A is the fluorescence of acceptor taken at 560 nm (where there is little contribution from donor), F_{DA} is the fluorescence of donor in presence of acceptor and k is a calibration constant obtained for each experimental system (for SUVs fusing with SUVs, it ranges between 3.5 and 3.8). This somewhat unconventional quantity is related to but not equal to FRET efficiency, but is ideally adapted to measurements in real time on a T-format fluorometer (21).

Tb³⁺/DPA content mixing and leakage assays

Mixing and leakage of the trapped contents of SUVs was monitored by the Tb³⁺/DPA assay (15). Vesicles were prepared in sonication buffer containing either 8 mM Tb³⁺ or 80 mM DPA and used in a 1:1 ratio in content mixing experiments. PEG was added as noted to

trigger fusion, which was then monitored with time at 23°C. Formation of a Tb^{3+} -DPA complex protects Tb^{3+} from quenching by water, producing an increase in fluorescence intensity. Content leakage was detected using vesicles containing co-encapsulated Tb^{3+} and DPA at concentrations of 4 mM and 40 mM, respectively, and measuring the loss of fluorescence with time (15). All experiments were carried out at pH 7.4.

Fluorophore-labeled Oligonucleotide based content mixing and leakage assay

6-carboxyfluorescein (FAM) and tetramethylrhodamine (TAMRA) are a classic example of a FRET pair, where FAM is the donor and TAMRA is the acceptor. The absorption maximum of FAM (donor) is 494 nm with an emission peak at 518 nm. TAMRA (acceptor) absorbs light emitted by FAM and emits at 580 nm. In our assay, FAM and TAMRA are tethered either at the 5' or 3' ends of two 10-mer complementary sequence of oligonucleotide (Figure 1). Small unilamellar vesicles (SUVs) were prepared in buffers containing either 5 μM of FAM-oligonucleotide or 5 μM of TAMRA-oligonucleotides, with excess oligonucleotide probes removed using a Sephadex G-75 column. Membrane fusion was initiated as described by either 5wt% or 6wt% PEG in a mixture of 100 μM (total lipid) TAMRA SUVs and 100 μM FAM SUVs. As large fusion pores form, fluorophore-labeled oligos move from one compartment to the other and the complementary oligos hybridize so as to bring the FRET pairs in proximity, producing an increase in FRET efficiency ($1 - F_D/F_{DA}$), or quenching of the donor fluorescence. Thus the measurement of FRET efficiency as a function of time represents the kinetics of large fusion pore formation. This overall process required recording of two time courses. In the first, we monitored donor (FAM) fluorescence intensity (at 518 nm) in a mixture of 100 μM (lipid concentration) of FAM-oligo-containing vesicles and 100 μM (lipid concentration) of vesicles containing no fluorophore, with fusion triggered by PEG, this provides time course of F_D . In the second, we monitored donor fluorescence intensity (at 518 nm) when 100 μM of FAM (donor)-oligo containing vesicles were triggered to fuse with 100 μM of TAMRA (acceptor)-oligo containing vesicles using 5 or 6 wt% PEG; this provided the time course of F_{DA} . The time course of FRET efficiency ($1 - F_D/F_{DA}$) increase is the time course of large pore formation. For content leakage experiments, 5 μM of FAM- and 5 μM of TAMRA-containing oligonucleotides were co-encapsulating in small unilamellar vesicles (SUVs), and excess probes in external buffer were removed using a Sephadex G-75 column and measuring the loss of FRET. Inside the vesicles, all the complementary strands are hybridized and we observed the maximum FRET. When vesicles were disrupted with detergent (C_{12}E_8), the FRET efficiency dropped precipitously because the concentrations of oligonucleotide in the external buffer were then less than femtomolar, which is much lower than the binding K_d of the two complementary sequences of 10-mer oligos. Note that the probe pair characterization shown in Figure 1 was performed probe concentrations above the pair's K_d .

Data Analysis—We analyzed Tb^{3+} /DPA content mixing and leakage along with BODIPY500-PC and BODIPY530-PE lipid-mixing data in terms of a sequential, 3-state (two step) fusion process (see following Diagram as described in detail elsewhere (10)). Each “state” represents a thermodynamic ensemble of similar molecular arrangements (“microstructures”) (9), each of which is related to the structures proposed by Siegel and others (3), although it is envisioned that the range of molecular microstructures contributing to each thermodynamic state is quite broad. Although not permitted by the structural model of Siegel, our data require that there are non-zero probabilities of content mixing (α) and lipid mixing (β) throughout the time evolution of the ensemble, consistent with the proposal that each state represents a multitude of different microstructures, some of which would be expected to contain reversible or “flickering” pores (13) between trapped compartments. The ensemble kinetic model is illustrated by the accompanying diagram, ‘A’ represents vesicles in contact within aggregates, ‘I’ is semi-stable intermediate state, and ‘FP’ is the

final fusion pore state (FP). The rate constants for conversion between ensemble states are k_1 for $A \rightarrow I_1$, k_2 for $I_1 \rightarrow I_2$ and k_3 for $I_2 \rightarrow FP$ (9). For the particular system examined here, only a single intermediate is detected. Since fusion is likely to occur from the I_2 geometry, we assumed that I_1 and I_2 are rapidly interchangeable in these instances (22) and relabeled I_2 as I (see diagram).

The model can account for the time courses of five observables associated with PEG-mediated fusion (9), although the time courses of three basic observables, lipid mixing (LM), contents mixing (CM), and leakage (L), are sufficient to uniquely define rate constants that account for the other two observables (light scattering, and formation and disappearance of non-lamellar intermediates (23)) in all cases examined thus far (9, 10, 20). In addition to rate constants, the β_i 's and α_i 's are probabilities and λ_i 's are leakage rates required to describe the contribution of each state to particular observables (lipid mixing, content mixing, and content leakage). While this might seem an excessive number of parameters, experimental observations allow for elimination of some of these. The probabilities of content and lipid mixing in the "A" state were negligibly small (*i.e.* $\alpha_0 = \beta_0 = 0$), and a very small amount of lipid mixing takes place in the FP state (β_3 is assumed to be close to zero). Along with normalization conditions for the probabilities, this leads to two intrinsic rate constants (k_1 and k_3) and just five extensive parameters ($\alpha_1, \beta_1, \lambda_0, \lambda_1, \lambda_3$) to describe three independent time courses that in most cases must be represented by double exponentials. A useful feature of this method is that the transitions are between thermodynamic states, so the temperature dependence of ensemble rate constants can yield activation thermodynamics of each step.

RESULTS

Probe Pair Characterization

Because of the cooperative nature of oligonucleotide dimerization, it is critical to design an oligonucleotide FRET pair to match exactly the details of the planned experiment. We designed the oligonucleotide sequence for our probe pair using OligoAnalyze 3.1 (Integrated DNA Technologies, USA) so that the individual oligonucleotide strands did not form hairpin structures and did not undergo homo-dimerization at experimental temperatures. We also confirmed that the melting temperature of the oligonucleotide duplex is higher than our experimental temperature (23°C). Figure 2 shows donor (FAM) fluorescence intensity in a mixture of 0.5 μ M of each oligos, as a function of temperature. The melting temperature (T_m) for the pair is about 35°C, but at 23°C, where we have performed our fusion experiments, the oligo nucleotide pair was nearly all involved in forming duplex that quenched donor fluorescence *via* FRET and increased FRET efficiency. A similar method using a 22-residue oligo-nucleotide was recently proposed and applied to single-event measurements in a model synaptic vesicle fusion process (7, 24). The difference between these two assays is that ours relies on the designed formation of the inter-nucleotide hybrid at 23°C to trigger an increase in FRET efficiency, while the other relies on designed loss of

intra-oligo-nucleotide pairing (as triggered by a lipid linked oligo in one vesicle population), resulting in loss of FRET efficiency (24).

Comparison of Small and Large Pore Formation in Four Model Membrane Systems

PEG-mediated model membrane fusion has been extensively used to examine the molecular mechanism of membrane fusion (25). Here we test the applicability of this assay to four model membrane systems. Appropriate controls have already demonstrated that the low concentrations of PEG used (5 or 6 wt%) do not alter bilayer or peptide structure (19) (Tarafdar & Lentz, unpublished observations).

Figure 3A shows the time course of content mixing between DOPC/DOPE/SM/CH (35/30/15/20) SUVs using the oligonucleotide-based assay at pH 7.4 in the absence and in presence of gp41 fusion peptide (FP) at 23°C and at a lipid to peptide ration of 200:1. These conditions are optimal for observing the influence of the gp41 fusion peptide on fusion (Chakraborty, Lentz et. al. personal communication). This peptide promotes the rate of pore formation and increases the probability of content mixing to the earlier in the fusion process, as reflected by increasing α_1 (Table 1). Figure 3B(ii) shows similar time courses using the common Tb³⁺/DPA probe pair, while Figure 3B (i) and 3B (iii) show lipid mixing and content leakage time courses, respectively, for this vesicle system. We measured leakage of the oligo probes by encapsulating both the probes in one set of vesicles and then following the time course of loss of FRET efficiency (see Methods). We detected no more than 1.5% leakage for any of the systems described here (data not shown). It is immediately obvious from Figures 3A and 3B(ii) that these two types of assays for content mixing report very different events occurring on different time scales. The three time courses recorded in Figures 3B(i-iii) all required description by double exponential curves, but together allowed us to analyze the data obtained with the Tb³⁺/DPA content mixing/leakage assay in terms of our ensemble kinetic treatment as described in Methods (9, 10). This treatment determines a rate constant k_1 and for converting the aggregated state (A) to the initial intermediate state (I), which then converts to a final fusion pore (FP state) with a rate constant k_3 (9, 10, 22). These rate constants along with other parameters defined by the ensemble kinetics analysis, α_1 (probability of content mixing in state I) and f_{CM} (total extent of contents mixing), are collected in Table 1. By contrast, the time courses in Figure 3A were appropriately described by a single exponential equation (rate constant k_O), and thus likely represent a single kinetic process. The extent of total possible oligonucleotide content mixing ($f_{CM,O}$) was determined using vesicles containing co-encapsulated FAM and TAMRA oligonucleotides. The parameters k_O and $f_{CM,O}$ for PEG-mediated fusion of PC/PE/SM/CH SUVs are given in Table 1. The rate of oligo-nucleotide movement between compartments (*i.e.*, the rate of large pore formation; $k_O = 1.9 \pm 0.04 \cdot 10^{-3} \text{ s}^{-1}$) was quite similar to the rate, k_3 ($1.7 \pm 0.01 \cdot 10^{-3} \text{ s}^{-1}$), of final pore formation obtained using the Tb³⁺/DPA content mixing assay. In addition, the amount of total Tb³⁺/DPA content mixing occurring in the FP (final pore) state ($\alpha_3 \cdot f_{CM} = 0.18 \pm 0.04$) was also similar to amount of total content mixing ($f_{CM,O}$) obtained from the oligo-nucleotide assay (0.14 ± 0.04). These similarities suggest that formation of state FP (final fusion pore) is related to formation of large pores detected by oligonucleotides.

The kinetics and activation thermodynamics of PEG-mediated fusion vary considerably with the lipid composition of SUVs (9, 10, 22, 26). In order to test whether the results in the first row of Table 1 might be specific to a given lipid composition, we examined PEG-mediated fusion of DOPC/DOPE/SM/CH/DOPS (32/25/15/20/8) SUVs. The presence of DOPS alters fusion behavior significantly (26). Row 3 of Table 1 summarizes the results of these experiments. Again, we note the close correspondence of k_3 ($0.53 \pm 0.08 \cdot 10^{-3} \text{ s}^{-1}$) to k_O ($0.42 \pm 0.02 \cdot 10^{-3} \text{ s}^{-1}$) as well as the similarity of $\alpha_3 \cdot f_{CM}$ (0.11 ± 0.01) to $f_{CM,O}$ (0.12 ± 0.03),

strongly supporting the hypothesis that FP formation corresponds to large pore formation. Phosphatidylserine (PS) is required to see the catalytic influence of the *trans*-membrane domain of the SNARE protein synaptobrevin (Tarafdar, Lentz, *et al.* personal communications). In PS-containing membranes it enhances both the rates of initial intermediate and final pore formation as well as the probability of pore formation early in the fusion process α_1 (Table 1).

Results from experiments with both catalytic peptides examined, as incorporated into their two optimal SUVs compositions, showed that both increased both k_3 from the Tb^{3+} /DPA assay as well as k_O from the oligo-nucleotide assay to roughly the same extents.

DISCUSSION

Pore Formation Assays

While it would seem to be obvious that oligonucleotides should be useful for following large pore formation during fusion, we knew of only one paper that has taken this approach when we started this work (27). This work used radioactive nucleotides, so it was not very useful in following rates of content mixing. The use of fluorescently labeled oligo-nucleotides as “molecular beacons” to detect oligo sequences was suggested in 1966 (28). A very recent paper used the molecular beacon concept to detect large pore formation by chemically linking oligonucleotides to lipids in target vesicles (7). The current work introduces a unique probe that allows continuous monitoring of content mixing *via* FRET but without requiring oligonucleotides chemically linker to lipids. We estimated the size of the probe to be ~2 nm in rotational diameter when unfolded (29). A folded 22-residue stem-loop oligo-nucleotide probe is similarly estimated to be ~ 2 nm in diameter by MD simulations (7). Tb^{3+} has an ionic radius of 1.2Å, but is also likely to have a hydration shell of perhaps 1Å, leaving it with a hydrated diameter of ~ 4-5Å. The chemical structure of dipicolinic acid suggests that it should have a rotational diameter of 6-8Å. Thus, both Tb^{3+} and DPA are very small molecules capable of passing through pores that are roughly ~5 times smaller than those available to oligonucleotide probes.

Two Classes of Pores

Estimates of the size of fusion pores vary widely. Electron micrographs can resolve continuities between synaptic vesicles and synaptic space of ~ 5-10 nm in diameter (30). However, conductance and capacitance measurements during secretory vesicle release suggest that initial pores open very quickly ($\lesssim$ 1ms and are very small, $\lesssim$ 1nm (31)). It is also clear that these initially very small pores can reclose or expand to form much larger pores (5-10nm) (31). A consequence of this appears to be observation of “flickering pores” during mast cell granule and chromaffin cell release that precede final release (13, 32). Flickering pores have also been reported to form during osmotically triggered fusion of vesicles with black lipid membranes (14). The existence of very small (capable of passing H^+ only) pores early in PEG-mediated fusion of very “non-leaky” 45 nm dilinolenoylPC/DOPC (15/85) vesicles has been reported (11). Finally, correlation of leakage rate to probability of fusion in intermediate (I_1 or I_2) *versus* FP states previously was suggested to support two classes of pores during PEG-mediated fusion: early pores associated with leakage and later pores less correlated with leakage (9). Schick and co-workers (33) proposed, based on coarse-grain Monte Carlo simulations that both leakage and inter-vesicle pores are associated with “stalks” structures (I state) presumed to exist very early in the fusion process but then growing to larger structures.

The results reported here are consistent with the existence of early transient pores and later larger pores and offer a more precise definition of “final pores” that form during PEG-

mediated fusion. Of course, results supporting two pore sizes cannot show that the smaller pores are “flickering” or transient, although this is consistent with the electrical and other measurements discussed above. The results summarized in Table 1 strongly support the hypothesis that “final pores” are equivalent to the large pores defined by oligonucleotide mixing between SUVs, *i.e.*, pores with diameters of ~ 2 nm. Since the diameter of an SUV is ~ 23 - 25 nm, it is unlikely that more than one such large pore could form between SUVs at one time and one point of contact. A pore of this size would require lipid rearrangements substantially larger than the correlated movements of only a few lipids that has been suggested to constitute the transition state to pore formation (10). Such substantial lipid rearrangements are reported to occur at rates comparable to that of final pore formation. At the PEG threshold concentration for observing fusion, lipids redistribute from outer to inner leaflets of dilinolenoylPC/DOPC:15/85 SUVs at a slow rate of $\sim 4.6 \times 10^{-3} \text{ s}^{-1}$, which is comparable to that of inner-leaflet lipid-mixing ($\sim 3.7 \times 10^{-3} \text{ s}^{-1}$) or content mixing ($\sim 5.8 \times 10^{-3} \text{ s}^{-1}$) for this system (34). This paper also reported that the polarity at the methyl amine of TMA-DPH (trimethylamine diphenylhexatriene) decreased exponentially at an even lower rate ($\sim 2 \times 10^{-3} \text{ s}^{-1}$) (34), implying that remnant curvature stress continued to slowly dissipate even after lipids redistributed sufficiently to form a large pore. Taken with the results reported here, we suggest that slow content mixing events reported here correspond to formation of large final pores, and that this predictably requires *trans*-bilayer and other lipid rearrangement. The faster content and lipid mixing rates can reasonable be assigned to transient pores, for which less dramatic lipid rearrangements should be required.

Membrane-contacting regions (*trans*-membrane domains and “fusion peptides”) of fusion proteins catalyze both steps of the fusion process in the two lipid systems examined and promote pore formation earlier in the process (Chakraborty, Tarafdar, Lentz *et al.*, personal communications), so the fact that both these membrane-contacting regions promote the rate of large pore formation as judged by both the oligo-nucleotide and as judged by the rate of final pore formation using a small-molecule content-mixing assays (Table 1) is strong support for our conclusions. Interestingly, while both peptides increase α_1 , they decrease α_3 so that the extent of content mixing that occurs though large pores ($\alpha_3 x f_{CM} = f_{CM,O}$) remains unchanged (Table 1).

Large Pores are Observed throughout the Fusion Process

A potentially surprising aspect of our results is that both final pores and small pores form throughout the fusion process. The sequential model of fusion posits that the initial aggregated state of closely contacting SUVs proceeds through intermediates to the final fusion pore state. In this picture, the rate k_1 of intermediate formation corresponds to the rate of minor lipid rearrangements (lipid mixing) that lead to content-leakage or -mixing pores (33). If intermediate must form prior to FP, we expect to see bi-exponential kinetics in which the two exponentials may or may not be clearly resolved depending on how similar are k_1 and k_3 (9). We nearly always resolve one fast component (lipid mixing and transitory pore formation) and a 10-times slower component (final large pore formation; see Table 1). In a recent single event analysis of Ca^{2+} triggered fusion between tethered vesicles, the distribution of dwell times for content mixing events was also reported to be a double exponential, with the slow exponential having a decay constant roughly an order of magnitude smaller than the fastest (8). Thus, both ensemble kinetic and dwell-time analyses of multiple single events support coexistence of slow and fast content and lipid mixing components, with our current results attributing the slow component to formation of large pores.

A more recent dwell time analysis compared movement of small molecules and DNA oligonucleotides between docked vesicles as stimulated by Ca^{2+} and synaptotagmin (7).

This study reported a very small amount of large pore formation, but, unlike our ensemble results, the dwell time distribution was biphasic, with a very slow phase preceding a faster phase. As we point out, ensemble kinetic measurements and dwell time analyses of individual events should yield similar results, since they describe averages over many vesicle pairs or over many vesicle pair events, respectively. It is difficult to say with certainty the reason for the disagreement between our results and those of Lai et al. (7). One possibility could be that the greater time resolution of the dwell time analysis (200 ms) allows detection of the initial lag time. However, another recent dwell-time analysis with a similar time resolution (200 or 500 ms) reported only two time constants, differing by roughly an order of magnitude, for Ca^{2+} -triggered content mixing between SNARE- and synaptotagmin-linked vesicles, an observation consistent with our ensemble kinetic measurements on PEG-triggered fusion (8). Another possibility is that both Ca^{2+} and synaptotagmin were needed to complete molecular rearrangements of the syntaxin/v-SNARE complex (7), so the slow process could correspond to completion of the appropriate close-contacted state required for fusion. Fusion rate and extent vary with how closely vesicles are forced into contact (35), so if this inter-bilayer distance changes as inter-vesicle complexes mature, this could cause a delay in initiation of the fusion process. Finally, Lai *et al.* and we have studied very different systems: Ca^{2+} -triggered fusion of SNARE and synaptotagmin-linked 100 nm vesicles and PEG-triggered fusion of PEG-aggregated 23 nm SUVs; with both vesicle preparations having different compositions. A strong argument against this final possible explanation is our observation that the phenomenon reported here ($k_3 \approx k_0$) holds for two very different lipid compositions and for SUVs in the presence or absence of membrane-contacting catalytic sequences from two types of fusion proteins. We conclude that it is likely that large pores appear throughout the fusion process at least as detected by ensemble kinetic analysis but likely also as detected by dwell-time analysis of many independent fusion events (8).

Is the Fusion Process Sequential as Proposed by the Modified Stalk Hypothesis?

If the process is sequential, how is it that the FP state appears throughout the fusion process? The answer lies in the ensemble nature of our experiments and analysis (9, 10, 20, 26) and in the similar ensemble nature of dwell-time analysis of single events (8). The modified stalk hypothesis (3), as applied to fusion of a single pair of highly curved vesicles, predicts that the free energy of intermediate structures from the initial stalk (I_1) to the final pre-fusion intermediate (I_2) evolves continuously as the geometry and structure of the pair of hemi-fused vesicles evolves (36). This is a *sequential structural model*. When translated to an ensemble model, there will be many different structural species of closely apposed SUV pairs in any intermediate ensemble state that contribute to the evolving ensemble process as well as to the transition states (TS) between semi stable states. Analysis of ensemble kinetic experiments performed at different temperatures provides estimates of the activation thermodynamics of each step of the ensemble fusion process in which one ensemble state evolves to another (10). According to this analysis, the transition state between intermediate I (the intermediate) and FP, which is termed TS3 (10, 36), is very similar to the I state, but differs in the average radii (termed “stalk radius”) of single-bilayer diaphragms between the trapped compartments in the “hemi-fused” I state and the transition state TS3. The circumference of this diaphragm is predicted to be quite unstable (33, 36), supporting the possibility that “correlated fluctuations” of multiple lipids in the diaphragm might account for the activation thermodynamics of pore formation (10). We suggest that these fluctuations, being random events, can occur at any time during the evolution of I through TS3 to FP but should increase in number with increasing average stalk radius, which increases as the system approaches the FP state (36). This ensemble kinetic model is thus consistent with our observation that both small and large pores can occur at any time during the process. However, the slow component of content mixing [$\alpha_3\text{-FP}(t)$] increases ten times

slower than the fast component, which largely levels out at longer times (larger stalk radii), while the slow component is still growing. In this picture, every vesicle pair can evolve towards a large final fusion pore by a sequential structural mechanism according to the model of Siegel, while the entire ensemble can evolve such that large and small pores can occur at any time, but with large pores contributing increasingly to content mixing at long times. If k_1 and k_3 were sufficiently different, or if a sufficiently slow event separated these intermediate and pore formation events, it might be possible to resolve the sequential nature of the process from ensemble kinetics, as we have reported (11), but in most instances, this is not the case.

SUMMARY

We introduce a new assay for the mixing of large solutes (oligo nucleotides) between trapped fusion compartments and use this to demonstrate that large and small pores form throughout the fusion process, as detected by ensemble methods. The relevance of these results to single-event studies of membrane fusion is discussed, as is the relation of an ensemble model to a single event structural model.

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Figure 1. Illustration of the fluorescence labeled oligonucleotide based content mixing and leakage assays

Fluorescently labeled oligos 5-FAM/TGGAGAAGGC and GCCTTCTCCA/3-TAMRA were used to detect the content mixing and leakage. Oligo probes move from one membrane to other only through the expanded pore and that leads to hybridization of oligonucleotide. 6-carboxyfluorescein (FAM; Donor) comes into close contact to tetramethylrhodamine (TAMRA; Acceptor), leading to FRET.

Fig 2. Melting profile of the complimentary oligomers designed for content mixing and leakage assay

At low temperature, equimolar (0.5 μ M, above the K_d of the hybridized oligomeric complex) mixture of 5-FAM/TGGAGAAGGC (donor) and GCCTTCTCCA/3-TAMRA (acceptor) hybridize and donor fluorescence is quenched due to FRET. The oligo duplex breaks down with increasing temperature and donor fluorescence increases. These data demonstrate that the designed pair of oligonucleotides will be a useful probe pair 30 ° C.

A

B

Fig 3. Comparison of time courses of content mixing using the oligonucleotide-based assay and small molecule based assay for vesicles with and without the HIV gp41 fusion peptide
 (A) Content mixing was carried out for DOPC/DOPE/SM/CH (35/30/15/20) SUVs at pH 7.4 in absence (red) and in presence of gp41 fusion peptide (green) in a peptide and lipid ratio 1:200 using oligonucleotide based assay. The time course was best described by a single exponential equation, $y = y_0 + a(1 - e^{-bx})$, with the resulting parameters tabulated in Table 1.

(B) Traces of (i) lipid mixing, (ii) content mixing and (iii) content leakage for the PC/PE/SM/CH SUVs at pH 7.4 using conventional assays (10) in absence (red) and in presence of gp41 fusion peptide (green) at a peptide-to-lipid ratio 1:200. Membrane fusion was induced with 5% PEG at 23 °C. F is the fluorescence intensity at time ' t ', F_0 is the fluorescence intensity at time ' $t=0$ '. Measurements were carried out in 10 mM MES, 100 mM NaCl, 1 mM CaCl_2 , 1 mM EDTA, pH 5.0 at a total lipid concentration was 0.2 mM. The smooth curves drawn through the data represent 3-state sequential model global fit.

A

B

Fig 4. Comparison of time courses of content mixing using the oligonucleotide-based assay and small molecule based assay for vesicles with and without the synaptobrevin transmembrane domain peptide

(A) Content mixing was carried out for DOPC/DOPE/SM/CH/DOPS (32/25/15/20/8) SUVs at pH 7.4 in absence (red) and in presence of synaptobrevin *trans*-membrane domain (green) in a peptide and lipid ratio 1:900 using oligonucleotide based assay. The time course was

best described by a single exponential equation, $y = y_0 + a(1 - e^{-bx})$, with the resulting parameters tabulated in Table 1. (B) Traces of (i) lipid mixing, (ii) content mixing and (iii) content leakage for the PC/PE/SM/CH SUVs at pH 7.4 in absence (red) and in presence of synaptobrevin trans-membrane domain (green) in a peptide and lipid ratio 1:900 using small molecule based assay. For all cases membrane fusion was induced by 6% PEG at 23 °C. Measurements were carried out in 10 mM MES, 100 mM NaCl, 1mM CaCl₂, 1 mM EDTA, pH 5.0 at a total lipid concentration was 0.2 mM. The smooth curves drawn through the data represent 3-state sequential model global fit.

Table 1

Summary data sheet from analysis of LM, CM and leakage experiments in absence and in presence of peptide using 3-state sequential model. Table also includes the rate of pore formation and extent of content mixing using oligonucleotide based assay. Experiments were carried out at 23°C and at pH 7.4. k_1 and k_2 are rate constants of intermediate and final pore formation, α_1 and α_3 are the probabilities of content mixing (in Tb/DPA assay) at the intermediate and final pore state, f_{CM} is the fraction of content mixing, $\alpha_3 f_{CM}$ is the fraction of content mixing through the final pore using Tb/DPA assay, k_O is the rate constant of pore formation using oligonucleotide based assay, $f_{CM,O}$ is the fraction of content mixing using oligonucleotide based assay.

System (Peptide/Lipid)	$k_1 \times 10^3$	$k_2 \times 10^3$	α_1	α_3	f_{CM}	$\alpha_3 f_{CM}$	$k_O \times 10^3$ From Oligo Assay	$f_{CM,O}$ from Oligo Assay
PC/PE/SM/CH	16.0 ± 0.5	1.69 ± 0.01	0.28 $\pm$ 0.010	0.72 ± 0.1	0.25 ± 0.2	0.18 ± 0.04	1.90 ± 0.04	0.14 ± 0.04
gp41 FP (1/200) in PC/PE/SM/CH	18.0 ± 0.22	1.86 ± 0.04	0.44 ± 0.02	0.56 ± 0.02	0.27 ± 0.03	0.15 ± 0.02	2.3 ± 0.05	0.15 ± 0.03
PC/PE/SM/CH/PS	5.78 ± 0.31	0.53 ± 0.08	0.25 ± 0.01	0.75	0.15 ± 0.2	0.11 ± 0.01	0.42 ± 0.02	0.12 ± 0.03
SB TMD (1/900) in PC/PE/SM/CH/PS	8.83 ± 0.47	0.98 ± 0.09	0.34 ± 0.01	0.66	0.16 ± 0.3	0.11 ± 0.03	1.56 ± 0.02	0.12 ± 0.03