RESEARCH NOTE

REVISED Observations of membrane fusion in a liposome dispersion: the missing fusion intermediate? [v2; ref status: indexed, http://f1000r.es/56t]

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v2 **First published:** 08 Jan 2015, **4**:4 (doi: 10.12688/f1000research.6003.1)
Latest published: 18 Mar 2015, **4**:4 (doi: 10.12688/f1000research.6003.2)

Abstract

Early intermediate structures of liposome-liposome fusion events were captured by freeze-fracture electron microscopic (EM) technique. The images show the morphology of the fusion interface at several different stages of the fusion event. One of the intermediates was captured at a serendipitous stage of two vesicles' membranes (both leaflets) merging and their contents starting to intermix clearly showing the fusion interface with a previously unseen fusion rim. From the morphological information a hypothetical sequence of the fusion event and corresponding lipid structural arrangements are described.

Open Peer Review

Referee Status: ✓ ✗ ✓

	Invited Referees 1	2	3
REVISED version 2 published 18 Mar 2015	✓ report	✗ report	✓ report
version 1 published 08 Jan 2015	? report	✗ report	? report

1 **James McNew**, Rice University USA

2 **M Joseph Costello**, University of North Carolina USA

3 **Jesse Hay**, The University of Montana USA

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Comments (0)

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How to cite this article: Foldvari M. **Observations of membrane fusion in a liposome dispersion: the missing fusion intermediate? [v2; ref status: indexed, http://f1000r.es/56t]** *F1000Research* 2015, **4**:4 (doi: 10.12688/f1000research.6003.2)

Grant information: The author(s) declared that no grants were involved in supporting this work.

Competing interests: No competing interests were disclosed.

First published: 08 Jan 2015, **4**:4 (doi: 10.12688/f1000research.6003.1)
First indexed: 11 May 2015, **4**:4 (doi: 10.12688/f1000research.6003.2)

REVISED **Amendments from Version 1**

While tempered with the limitation of observing one lipid vesicle composition and one fusogenic agent providing a limited number of fusion events, the reviewers recognized the noteworthy attribute of the lipid vesicle fusion intermediate in this study.

The scarcity of visual information on lipid vesicle interactions captured at different time-points leads us to present these images as possible additional information. Literature published in the past two decades indicates that capturing a fusion event at the most revealing time point is indeed a challenge.

It is recognized that alternative interpretations for fusion intermediate structure and differences between different lipid systems could also be contemplated. However, due to the rarity of such image, it is important to present this to the scientific community. As we indicate the serendipity of this finding, it is recognized that this paper can only be a small piece of information that stimulates further studies.

We have revised the paper to clarify that the morphological observation and the schematic interpretation are from a single examination and limited to one lipid system and fusogenic agent combination that will require other studies and confirmation in the future.

See referee reports

Introduction

Phospholipid vesicles (liposomes) are suitable models for studying biological membrane behaviour. Liposomes can be made from a single lipid or lipid mixtures that can provide opportunities to investigate membrane-related events under different conditions and in the presence of additives. The fusion process has been investigated in various artificial membrane systems using electron microscopic, small-angle X-ray scattering (SAXS), nuclear magnetic resonance (NMR) and fluorescence-based kinetic techniques[1–5]. Despite the efforts, complete understanding of the molecular structural and kinetic details of the fusion event is still lacking.

The first fusion mechanism from early studies proposed 'lipidic particles' (intermediates between lamellar and H_{II} hexagonal phases of the phospholipids or inverted micelles) as the possible intermediate in the fusion process of model lipid vesicles[6–8], since these particles seemed to be present at attachment sites of lipid vesicles and could be visualized by freeze-fracture EM. These intermediates could also be detected in fusion processes of biological membranes (exocytosis, myoblast fusion, protoplast fusion). For example, Satir *et al.*[9] observed small particles arranged in rosettes at the site where fusion of the mucocysts in Tetrahymena pyriformis was initiated. Other authors questioned the existence of lipidic particles as dynamic fusion intermediates[10,11] because these particles could not be observed every time at fusion interfaces, and suggested that 'lipidic particles' develop subsequent to the fusion process. However, it was suspected that some type of a non-bilayer structure formed in the fusion event. Bearer *et al.*[10] specifically suggested the existence of an "'elusive' intermediate" that has not been visualized in published morphological studies and suggest that the absence of lipidic particles or other intermediate structures may not mean that some dynamic process is taking place and that fusion intermediates could be unstable and convert to different polymorphic forms.

Another mechanism proposed was the 'stalk mechanism'[12–14], which involved the formation of a trilaminar structure between the closely apposed bilayers such that the outer monolayers bend to the side to allow joining of the inner monolayers (trans-monolayer contact (TMC)[15]), which form a stalk at the attachment site of the two membranes[12,15–17].

The theoretical sequence of events in model lipid membrane fusion can be summarized as follows: 1) close apposition of the two bilayers (<1 nm); 2) local dehydration of phosphorus head groups; 3) destabilization of bilayers; and *either* 4a) formation of inverted micelle intermediates (IMI) at the attachment site; *or* 4b) formation of stalk and TMC); and 5) completion of fusion, i.e. the leakless mixing of contents of two vesicles.

Most of these stages of membrane fusion were described and indirectly measured or modeled, but direct visual evidence is still lacking. Siegel[18] and Cullis *et al.*[19], and more recently Lentz *et al.*[20,21] speculated that the reason why only some of the actual intermediate structures were detected or visualized is the short lifetime (1 msec or less[18]) of any given fusion intermediate, making the capture very challenging even with rapid freezing, ^{31}P-NMR or SAXS techniques.

In this study, we have observed some fusion intermediate structures in a liposome system in the presence of glycerol by freeze-fracture EM. One of these intermediates, the 'fusion rim intermediate' may provide new structural/morphological information on membrane fusion events.

Methods

Liposomes (small unilamellar vesicles, SUVs) were prepared with soybean lipids (Centrolex P; Central Soya, Fort Wayne, IN). The liposomes were prepared by high shear dispersion using Microfluidizer M110 (Microfluidics Inc. Newton, MA). The liposomes were freeze-fractured without glycerol or after preincubation in 30% v/v glycerol for 30 minutes at room temperature. A drop of the liposome suspension was placed on a gold specimen stub and rapidly frozen in liquid nitrogen cooled Freon 22 (-158°C). All samples were fractured at -105°C in a Balzers 360 freeze-fracture unit. The fracture surfaces were shadowed at 45° angle with a thin layer of platinum-carbon followed by vertical deposition of a carbon layer for replica support. The replicas were floated onto the surface of distilled water and subsequently cleaned with sodium hypochlorite (5% chlorine) and 60% sulfuric acid. After the final washing in distilled water the replicas were picked up on 200 mesh copper grids and examined in a Phillips 200 EM and photographed on Kodak fine grain positive film.

Results

The fusion of liposomes was induced by glycerol and several fusion intermediates were captured in the replicas (Figure 1). Without glycerol, there was no liposome fusion (Figure 1A). The relatively low concentration of glycerol provided slow dehydration at the phospholipid head group regions which made it possible to observe vesicles still in the fusion process. The micrographs captured vesicles (assumed to be) at various stages of the membrane fusion event (Figure 1A–C). Figure 1B (large arrow) depicts the initial

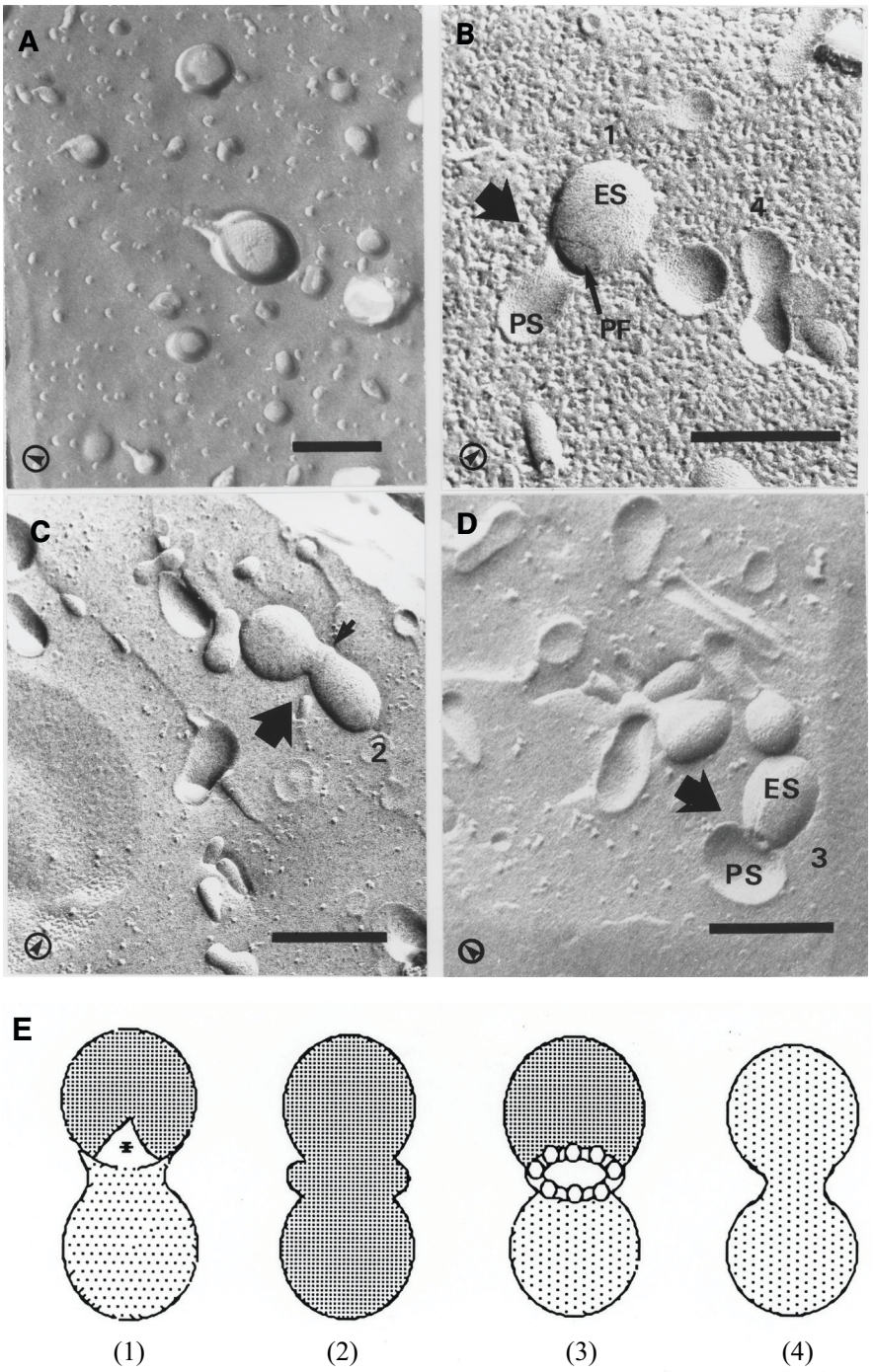

Figure 1. Fusion events in a model liposome dispersion induced by glycerin. Various stages of liposome-liposome fusion event were captured by rapid freezing. **A**) Liposomes were freeze-fractured without the addition of glycerol. **B–D**) Liposomes were preincubated with 30% v/v for 30 minutes before freezing. **B**) The first stage of liposome-liposome fusion: the joining of the outer monolayers of the liposomes (large arrow) is apparent without communication between their internal spaces. **C**) Early liposome-liposome fusion intermediate (large arrow) fractured at the exterior surface of the membrane. The rim between the two vesicles (small arrow) is indicative of non-bilayer intermediate structures. **D**) Late liposome-liposome fusion intermediate (large arrow). One of the liposomes was fractured on the external surface, the other on the internal surface with its internal space is being visible and exposing the fusion interface. The presence of small (10–12 nm) particles can be distinguished at the fusion rim. **E**) Schematic representation of the identified fusion intermediates. Numbers on micrographs correspond to the numbers of the diagrams. Freeze-fracture nomenclature[27]: ES - external surface; PS - cytoplasmic surface (in this case interior surface of liposome); PF and * - fractured face of the lipid monolayer adjacent to the interior space of the vesicle; arrows in left corners of micrographs indicate the shadowing direction. Bars 250 nm.

contact between vesicles – their external monolayers fused but no communication between the two aqueous compartments started. Figure 1D shows a previously unseen moment of liposome-liposome fusion. During the freeze-fracturing procedure one of the liposomes was fractured on the outside surface (it shows the E face), while the other shows the cytoplasmic (P) face (the interior surface of liposome). The leakless intermixing of the aqueous contents of the liposomes had started. At the perimeter of the fusion interface small, 10–12 nm diameter, particles can be distinguished, which probably correspond to the inverted micellar intermediates, so called, lipidic particles. This fusion rim intermediate structure is depicted in the insert of Figure 2. The presence of these lipidic particles could be suspected from another micrograph (Figure 1C, large arrow), which may represent a preceding intermediate state of fusion.

On the basis of this morphological evidence we constructed a schematic set of drawings to represent a modified model for phospholipid vesicle fusion (Figure 2). When the bilayers of two separate vesicles are in close apposition (Figure 2 I), an initial fusion product will form. The outer leaflets of the bilayers of the two vesicles fuse, while their inner leaflets form one common bilayer at the attachment site (Figure 2 IIa). This stage is followed by the formation of inverted micelles (lipidic particles) around the attachment site (Figure 2 IIb). The organization of the next fusion intermediate (Figure 2 IIc and Figure 1D) involves the formation of a fusion orifice. At the perimeter of this orifice, the fusion rim can be seen (Figure 2, insert), which contains inverted micelles all around. The development of an intermediate like this appears feasible from both energetic and morphological viewpoints, if we take into consideration that the excess phospholipid molecules cleared from the attachment site at this particular stage should be accommodated somewhere, until incorporated into the expanded bilayer of the single larger liposome.

Discussion and conclusions

Glycerol-induced fusion seen in this study may bear similarities to polyethylene glycol (PEG)-induced fusion[20,22] with dehydration at the liposome attachment site contributing to close contact between the bilayers. It is recognized that fusion is a very dynamic event and certain stages of the fusion are easier to demonstrate than others. Due to the low frequency of vesicle collisions and short lifetime of the actual fusion event and the fact that fusion of vesicles in an aqueous medium is not a synchronous event, visualization of intermediary fusion features on all liposomes in a sample is difficult.

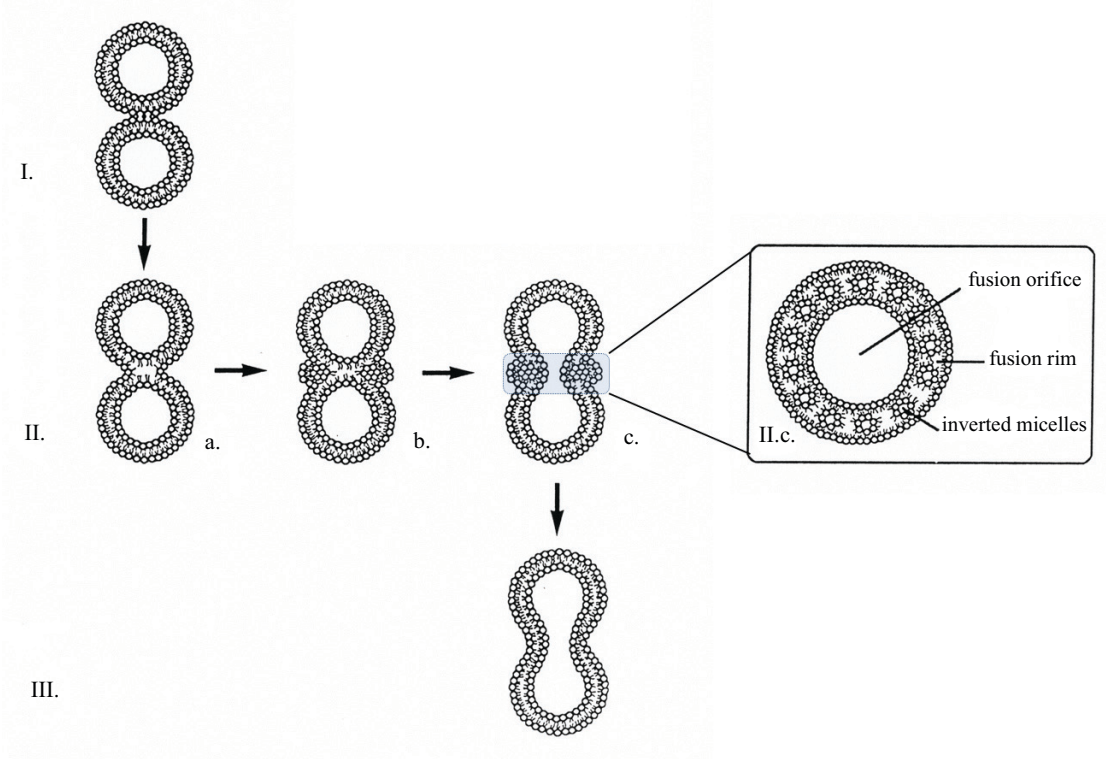

Figure 2. Possible fusion mechanism in a model liposome dispersion induced by glycerin. I. Close apposition of two bilayers and formation of an aggregation site when bilayers touch each other. **II.** Merging of two bilayers. **a**) formation of the initial fusion product: the outer leaflets of the bilayers of both vesicles join (fuse), while their inner bilayer leaflets form one common bilayer at the attachment site. **b**) phospholipid molecules from the outer leaflet of the vesicle bilayers, ie. From the attachment site are pushed sideways and form inverted micelles; at the attachment site transient bilayer form composed of the inner bilayer leaflets of the vesicles. **c**) formation of the fusion orifice: the phospholipid molecules from the attachment site are used for the formation of the outer monolayer of the inverted micelles. Inverted micelles are situated all around the fusion orifice. Mixing of the contents of the vesicles has started. Boxed insert shows a horizontal section of the fusion area viewed from above. **III.** Expansion of the bilayer to form a single larger liposome.

Most of the morphological freeze-fracture studies in the literature show liposomes just before fusion or at the stage already well undergoing fusion. It would be important to clarify events at the stage, where bilayers of the two vesicles are merging and communication between their aqueous spaces begins. The freeze-fracture results in this work supplement those previously reported in the literature and potentially add a new visual image of an intermediate structure to the model of membrane fusion. The initial fusion product (Figure 2 I) must be very similar to the one proposed by Kozlov and Markin[12] on the basis of theoretical considerations. The molecular arrangement of lipids in the 'fusion rim intermediate' (Figure 1D and Figure 2 IIc) could provide an alternative interpretation of the IMI[8,23] or could be considered the next step after the previously described stalk and TMC intermediate[15,17] (this latter may correspond to the image on Figure 1B), and may also be similar to membrane hemifusion events involving proteins[21,24–26]. However, this study has limitation in that the morphological observation and the schematic interpretation are from a single examination and limited to one lipid system and fusogenic agent combination that will require other studies and confirmation in the future.

Competing interests

No competing interests were disclosed.

Grant information

The author(s) declared that no grants were involved in supporting this work.

Acknowledgements

This paper is dedicated to the memory of Dr Michael Mezei, my mentor and friend. I specifically thank Dr Michael Mezei for providing the liposome dispersion and inspiring the study. I am also grateful to Dr Gary T. Faulkner for guidance with the freeze-fracturing and useful discussions of the micrographs.

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Current Referee Status:   

Version 2

Referee Report 11 May 2015

doi:10.5256/f1000research.6725.r8030

James McNew
Department of Biosciences, Rice University, Houston, TX, USA

The author correctly acknowledges the limitation of the current approach and has tempered interpretation accordingly.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Referee Report 29 April 2015

doi:10.5256/f1000research.6725.r8028

M Joseph Costello
Department of Cell Biology and Physiology, University of North Carolina, Chapel Hill, NC, USA

The minimal revision and associated comments did not adequately address the concerns expressed in the review.

I have read this submission. I believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Competing Interests: No competing interests were disclosed.

Referee Report 23 April 2015

doi:10.5256/f1000research.6725.r8029

Jesse Hay
Division of Biological Sciences, The University of Montana, Missoula, MT, USA

This paper reports essentially a single finding with a single system and therefore it is important that it be viewed as motivation for further investigations rather than as strong support for a particular membrane fusion model. However, I believe the authors have adequately conveyed these limitations in their response to the earlier reviews and in the revised version of the paper.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Version 1

Referee Report 30 January 2015

doi:10.5256/f1000research.6424.r7233

Jesse Hay
Division of Biological Sciences, The University of Montana, Missoula, MT, USA

This submission uses freeze-fracture electron microscopy to observe fusion intermediates in glycerol-induced fusion of unilamellar liposomes. Though fusion in this system is asynchronous, several images are presented that presumably correspond to different sequential intermediates in membrane fusion. One particularly suggestive image is interpreted to indicate that the hemi-fusion to full-fusion transition proceeds through an intermediate containing a circular array of lipidic particles just beneath the junction between the fused outer leaflets. The lipid particle model is proposed to potentially reflect the mechanism of membrane fusion induced by biological fusion protein machinery. The lipid particle model is not a new idea, but has been controversial and lacked direct support.

The paper is interesting and makes a unique contribution to understanding the lipidic intermediates in fusion. However, it was disappointing that only a single image of the key intermediate is shown and therefore the characteristics of the intermediate cannot be confirmed. If more examples were analyzed, it should be possible to estimate quantitative parameters such as the approximate number of lipid molecules in the particles, how uniform they are and how often they occur per unit of membrane, etc., that might allow future energetic understanding of the proposed process. Likewise I found it somewhat disappointing that only glycerol was used to induce fusion. If the proposed intermediate is generalizable to biological membrane fusion, it should be apparent using another fusogen.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

Author Response 13 Mar 2015

Marianna Foldvari, University of Waterloo, Canada

Thank you for the valid comments on the limited number of events in the experiment. In this case we have mostly observed fusion-related events at stages that are not revealing the moment of complete merging of the membranes. Following the field in the past two decades indicate that capturing a fusion event at the most revealing time point is indeed a challenge. Due to the rarity of such image, it is important to present this to the scientific community. As we indicate the serendipity of such finding, it is recognized that this paper can only be a small piece of information that stimulates further studies.

Competing Interests: No competing interests were disclosed.

Referee Report 22 January 2015

doi:10.5256/f1000research.6424.r7364

M Joseph Costello
Department of Cell Biology and Physiology, University of North Carolina, Chapel Hill, NC, USA

Conventional freeze fracture experiments were performed on small unilamellar vesicles prepared from soybean lipids using a microfluidizer. Glycerol was used to induce fusion, which was random producing a few pairs of vesicles in the process of fusion. The images of intermediate states are interpreted to support the presence of a ring of lipidic particles at the critical point of bilayer-bilayer fusion. This approach is promising and the images are interesting; however, there are major concerns with the controls and with the interpretation of the images.

Specific comments:

1. Figure 1A. The image does not display a freeze fracture experiment. There is no fracture plane through the lipid vesicles. If such a fracture plane existed in this region, then vesicles with both convex and concave surfaces would be visible (as in subsequent images). Only convex surfaces are visible. In addition the convex surface of the larger structures have a revealing pattern of density: the four larger vesicles have a dark rim and light center. This is due to the carbon layer that builds up on the curved surface to be thick near the edges but thin enough in the center to be nearly transparent. It appears that a layer of sample, perhaps adjacent to a fractured region, was shadowed to reveal some vesicles but not the expected vesicle size distribution or fracture pattern of a control.

2. Figure 1B. The vesicles exposed in this image are consistent with a normal fracture pattern where both convex and concave vesicles are visible. It should be noted that the fracture step through the monolayer of lipid is present on concave vesicles and, as the authors point out, on the large vesicle where a flap has been fractured away. The difficulty with this image is that the background ice is not smooth as would be expected for samples prepared in 30% glycerol. The authors should explain why the background here is different from those in subsequent freeze fracture images. One possibility is that some etching occurred.

3. Figure 1C. The main emphasis in this image is the mound or bump near the fusion interface of the vesicle pair labeled 2. The interpretation is that the bump represents a fracture through a ring of lipidic particles. The problem is that a single bump is not consistent with a ring of particles and the hydrophobic plane of fracture around a lipid particle may not appear as a bump or a bulge (as a

whole particle might). Fractures from several different viewpoints and proceeding around the fusion site are needed to properly interpret this pattern in terms of fusion intermediate structures. A single image showing a slight protrusion is not convincing even if the interpretation turns out to be correct.

4. Figure 1D. This shows an interesting image of a fusion contact site (labeled 3). The interpretation of this pattern as a fracture through a ring of lipid particles is difficult to justify based on the irregular size of the particles in the ring. More such images are needed showing particles of consistent size and distribution. In addition the image shows some other features that should be explained, such as the large number of oblong (or rod-like) vesicles, up to about five, and the large number of small particles in the background ice (also present in 1C). It is not clear whether these particles are from contaminant proteins in the solution or from artifacts deposited during the fracturing or shadowing steps.

5. Figures 1E and 2. The diagrams and molecular interpretations emphasize the role of lipidic particles in the fusion process. These particles have been implicated in bilayer and membrane fusion for decades without clear resolution about their presence in native fusion events within cells. The use of glycerol to induce fusion implies that glycerol does not significantly interact with the lipid bilayers or induce lipidic particles. At least one report suggests that glycerol (unlike polyethylene glycol) can intercalate within the lipid head groups to the extent of inducing interdigitation of lipid chains in the gel phase (BBA 731:97-108, 1983). Less interaction is seen for fluid phase lipids as used here but glycerol is not a passive inducer of fusion. Furthermore, Bearer *et al.* (ref. 10) suggest that glycerol induces lipidic particles and that such particles are not typically found in the fusion process in their model system. The critical point is that the glycerol-induced fusion of soy lipids may not represent natural lipid fusion and thus is not likely to expose the long sought lipid pore intermediate in natural fusion events.

I have read this submission. I believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Competing Interests: No competing interests were disclosed.

Author Response 13 Mar 2015

Marianna Foldvari, University of Waterloo, Canada

The author thanks the reviewer for the comments and alternative ideas. The freeze-fracture/etching technique used in this experiment utilized a Balzers 360 instrument which has certain limitations related to manual operation and timing of the sample mounting, temperature adjustment, fracturing and shadowing steps, providing some inconsistencies within sample areas. Nevertheless, the vesicles and fusion events can be observed and some new useful information may be gleaned from this liposome system.

Previous synchrotron x-ray scattering studies examined fusion events in various lipid membrane compositions in aligned multilayer lipid deposit samples [Yang and Huang (2002) Science 297:1877-1879; Yang and Huang (2003) Biophys J. 84:1808-1817; Wang et al. (2006) Biophys. J. 91:736-743 and Qian et al (2008) PNAS 105:17379-83.] In these studies the electron density distribution was used to map the merging of apposed lipid bilayers and the presence of transmembrane pores and the stalk structure.

However, the scarcity of visual information on lipid vesicle interactions captured at different time points lead us to present these images as possible additional information.
In the paper by Bearer et al. (1982) the authors have examined PE:PS and PC: CL vesicles by quick freezing in the presence of Ca^{2+} or glycerol and Ca^{2+}. It was stated that no lipidic particles were observed after the addition of Ca^{2+} and very few vesicles showed lipidic-particle-like structures after preincubation with glycerol followed by Ca^{2+} addition and this was only after long incubations of 1-2 h, although fusion-related images were frequently observed. We think that the findings in this study do not exclude the possibility of existence of various fusion intermediates just because they were not captured in the samples viewed. The authors actually suggest that the absence of lipidic particles or other intermediate structures may not mean that some dynamic process is taking place and that fusion intermediates could be unstable and convert to different polymorphic forms.
As the authors state: “This conclusion [ie. “lipidic particles (as defined by their morphology in freeze-fracture electron microscopy) are not involved as an intermediate in the stages of fusion”], however, does not exclude the possibility of a transitory intermediate at the site of membrane fusion which involves a lipid conformation distinguishable from the unmodified stable bilayer configuration. This 'elusive' intermediate, which is not visualized at present in any morphological studies, could be an inverted micellar or some other non-bilayer structure or a small domain of a more condensed or crystalline lipid bilayer. For lack of concrete evidence at this point, this 'intermediate' could be characterized simply as a local perturbation of the lipid bilayer structure, which allows mixing of lipid molecules between the two closely apposed membranes.”

While it is agreed that one image is not sufficient to base final conclusions, this note (or ‘case report’) attempts to report an interesting morphological observation that others in the field may find motivating.
We have revised the paper to clarify that the morphological observation and the schematic interpretation are from a single examination and limited to one lipid system and fusogenic agent combination that will require other studies and confirmation in the future.

Competing Interests: No competing interests were disclosed.

Referee Report 22 January 2015

doi:10.5256/f1000research.6424.r7365

James McNew
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This is an intriguing observation of potential liposomal fusion intermediates. While the method of inducing protein-free lipid mixing and fusion is unconventional, the structures observed by freeze fracture are unique. I think it would be important to confirm these intermediates under conditions that have been studied by other means. The wealth of information on PEG-mediated liposome fusion could be brought to bear if similar conditions were used. Additionally, it would be beneficial to examine the lipid requirements of such intermediates. While the lifetime of these structures are likely short making their identification challenging, altered lipid compositions such as at the inclusion of hexagonal phase ii forming lipids like phosphatidylethanolamine may increase the propensity of observing the types of intermediated like the "fusion rim" or "fusion orifice" depicted in this work. Overall, the proposed intermediate are interesting, but require further experimental evidence to support their role as bona fide fusion intermediates.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

Author Response 13 Mar 2015

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The reviewer's comments are appreciated and will stimulate further work regarding the better understanding of fusion mechanism. It is agreed that the proposed fusion intermediate observed in this liposome system only represents one possible lipid organization at the fusion interface and further studies with the addition of other lipids, proteins or fusogenic agents could provide more clarity of the fusion event.

Competing Interests: No competing interests were disclosed.