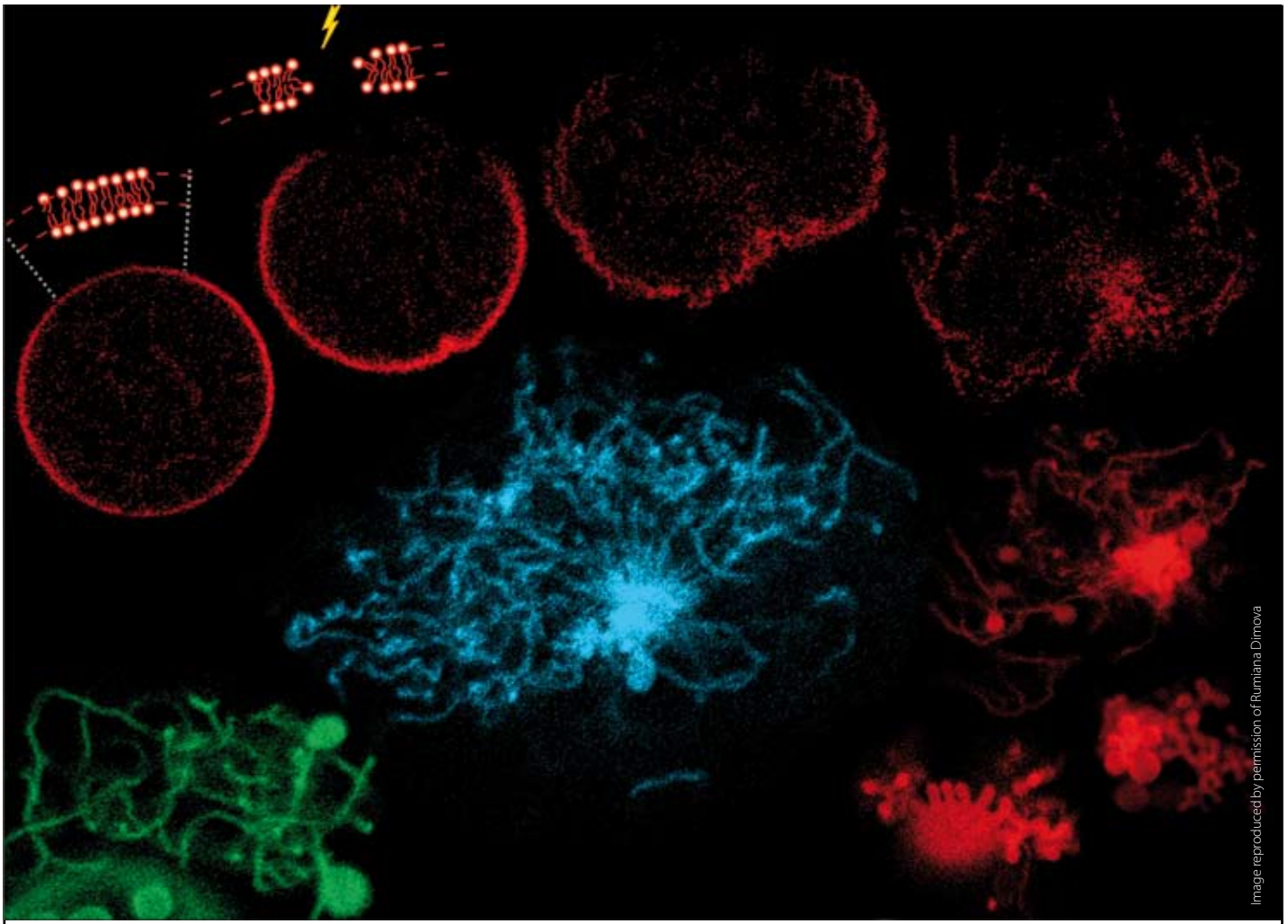

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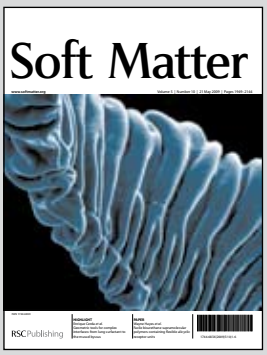
Showcasing research from the Dimova lab, Max Planck Institute of Colloids and Interfaces.

Title: Bursting of charged multicomponent vesicles subjected to electric pulses

When exposed to electric pulses above the poration threshold, neutral lipid vesicles typically rupture but reseal within milliseconds. In contrast to this poration-resealing behaviour, negatively charged spherical vesicles become unstable and rupture irreversibly. After bursting, the membrane rearranges into unusual tubular structures.

As featured in:



See Karin A. Riske, Roland L. Knorr and Rumiana Dimova, *Soft Matter*, 2009, **5**, 1983.

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Bursting of charged multicomponent vesicles subjected to electric pulses†

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Strong electric pulses applied to phosphatidylcholine (PC) giant vesicles induce the formation of pores, which reseal within milliseconds. Here, we study the response of vesicles containing PC and negatively charged lipids like phospatidylglycerol (PG) to such pulses. Vesicles composed of 1:1 PG:PC in a buffered solution of Hepes and EDTA exhibit the same behaviour as observed with PC membranes. Surprisingly, when the medium is changed to a non-buffered solution with or without salt, the vesicles burst and disintegrate to tubular structures after the pulse is applied. A fast digital camera and confocal microscopy were used to observe the dynamics of vesicle rupture and the membrane reorganization after the applied pulse. The nature of this instability and structural rearrangement is poorly understood. The experiments reported here suggest that the membrane charge plays a significant role. Vesicles made of lipid extract from human plasma membranes behave in the same fashion, implying that the reported bilayer reorganization may also occur to a certain degree in the membrane of electroporated cells.

Electroporation of membranes – the event of breakdown of the lipid bilayer of cells or vesicles when subjected to strong electric pulses – is widely used in biotechnological applications like gene therapy and cancer treatment. Despite the great potential for application, there is still lack of understanding about the physical mechanisms of electropermeabilization and, in particular, about the related structural changes in the bilayer. In the past, the use of simple model systems such as single-component phosphatidylcholine vesicles significantly contributed to understanding the behaviour of cell membranes in response to electric fields. For example, the lifetime of pores in electroporated membranes was found to depend on material properties such as membrane viscosity and pore-edge tension.[1,2] A significant amount of theoretical work was developed, in the majority of which the membrane detail was ignored.[3] Theoretical predictions were either followed or preceded by experimental studies mainly on single-component phosphatidylcholine membranes. Among them, those performed on giant unilamellar vesicles (GUVs) – cell-size membrane envelopes – allowed for direct microscopy observation of the bilayer response.[2,4] The pores in phosphatidylcholine GUVs were visualized[2] and characterized as having a lifetime in the millisecond range, after which the pores resealed completely.

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Altogether, using single-component model membranes has been very beneficial for identifying and characterizing various factors influencing the behaviour of membranes in electric fields. In the attempt to better mimic biological membranes, the small step of rendering this system more complex, whether by including another lipid component, or introducing salt or buffered solution, can evoke a range of unexpected phenomena. The aim of this work is to report such a phenomenon, apparently caused by the presence of other lipids and/or variation in the medium. In particular, vesicles containing a fraction of negatively charged lipids burst when subjected to pulses, *i.e.* they entirely rearrange, losing integrity. Alternatively, such electric pulses cause only transient poration and subsequent resealing in neutral membranes.

Using the electroformation method, we prepared GUVs composed of mixtures of palmitoyloleoylphosphatidylcholine (PC), palmitoyloleoylphosphatidylglycerol (PG) and palmitoyloleoylphosphatidylethanolamine (PE), or of lipid extract from the plasma membrane of red blood cells. The details on lipid extraction, vesicle formation and the observation chamber are given in the ESI†. Three types of solutions for the vesicle preparation were used: water, 1 mM Hepes (pH 7.4) with 0.1 mM EDTA, and 0.5 mM NaCl (which provides the same ionic strength as the buffered solution). Throughout the text, we will refer to these solutions as non-buffered, buffered and salt solutions, respectively. To ensure good optical contrast, the vesicles in all preparations also contained 0.2 M sucrose inside and isotonic glucose solution outside.

In all of the above solutions, PC vesicles transiently porate when subjected to electric pulses, but reseal afterwards. PC vesicles containing PG up to 1:1 molar ratios in buffered solution exhibit the same behavior. One example is illustrated in Fig. 1a, where vesicle poration is visualized by the leakage of the enclosed darker sucrose solution. The observations were done with a fast digital camera at acquisition speed of 20 000 frames per second (fps). Macropores with diameter up to about 10 μm are observed to reseal within ~50 ms. Note that nanopores are not optically detectable due to the microscopy resolution.

It is interesting to note that it can take significantly longer (~20 s) for the perturbed membrane to fully relax. Due to leakage, the created excess area (as compared to the area of a spherical vesicle enclosing the same volume) can participate in budding, as recorded seconds after the pulse; see the snapshot at 5 s in Fig. 1a and the ESI†. Such morphological changes were observed on vesicles only in the presence of oppositely charged ions with very different sizes, *e.g.* $Hepes^-$ and Na^+. The bud-like protrusions always faced the anode.

The origin of the buds is probably related to a temporary asymmetric and local change in the concentration of ions in the membrane vicinity, which alters the bilayer's spontaneous curvature. Buds facing the cathode were not observed, presumably because of the finite source of $Hepes^-$ ions inside the vesicle. Furthermore, strong curving

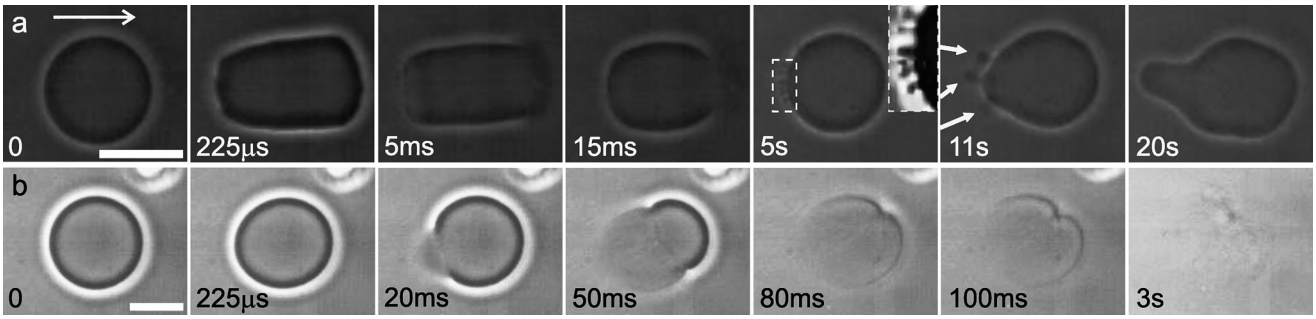

Fig. 1 Snapshots from fast camera observation of 1:1 PG:PC vesicles in two different solutions subjected to electric pulses. The time after the beginning of the pulse is marked on each image. The field direction is indicated in the first snapshot. (a) GUV in buffered solution subjected to a pulse of 4 kV/cm, 200 μs. The vesicle porates and reseals (see also the ESI†). The inset with enhanced contrast (5 s) and the arrows (11 s) show the sprouting of buds, which relax back to the vesicle body after several seconds. (b) GUV in salt solution subjected to a weaker pulse (1.2 kV/cm, 200 μs). The vesicle bursts and disintegrates. All scale bars correspond to 20 μm.

of the bilayer was observed in simulations of membranes subjected to pulses.[5] Presumably, such curved states (with dimensions on the nanometer scale as limited by the size of the simulation box) can proceed to form microscopic buds as observed in this work. The time characterizing the relaxation of a bud with volume V_b can be expressed as $\eta V_b^3/\kappa$, where η is the medium viscosity and κ is the membrane bending stiffness. Buds with typical size 3–5 μm can take 1–6 s to relax; larger buds will relax even slower. We emphasize that this long membrane relaxation should be considered when studying the response of cells subjected to a sequence of short-spaced pulses. Such sequences are typically applied in gene transfer studies. Although the budding phenomenon is interesting, we will not focus on it here but in a following work.

The conventional poration–resealing behaviour was observed for PG:PC vesicles also in non-buffered and salt solutions, but only at low PG content (1:9 and 2:8, and in pure PC vesicles). Surprisingly, 1:1 PG:PC vesicles in non-buffered or salt solutions displayed very different behaviour: they disintegrate after electroporation. Typically, one macropore formed and expanded in the first 50–100 ms at a very high speed of approximately 1 mm/s, releasing the entire vesicle content out (darker sucrose solution). Fig. 1b shows one example of such a vesicle bursting. This membrane response was found independent on the electrode polarity. To better resolve the vesicle reorganization after rupture, we used fluorescent labelling and confocal microscopy, as shown in Fig. 2a (see the ESI† for images with higher resolution). The bursting was followed by restructuring of the membrane into what seemed to be interconnected bilayer fragments in the first seconds, and then a tether-like structure in the first minute. In the following minutes, the membrane stabilized into interconnected micron-sized tubules and small vesicles.

These observations suggest that the vesicle bursting and membrane instability is related to the large amount of PG in the bilayer and to the medium. No such effect was observed in buffered solution and for lower content of PG. To rule out that membranes composed of any lipid mixture are destabilized by electroporation, experiments with 1:1 PC:PE vesicles were performed. PE lipids are zwitterionic and have smaller headgroups and weaker hydration than PC. Electric pulses induced the opening of macropores in PC:PE vesicles, which after ~15 ms resealed. Vesicle disintegration was never observed.

Thus, we considered the hypothesis that vesicle bursting and membrane instability is related to the charged state of the bilayer. Since cell membranes have a significant amount of negatively charged lipids, we performed experiments on vesicles prepared from lipid extract (LE) from plasma membranes of human red blood cells. Interestingly, LE vesicles behaved in the same way as synthetic 1:1 PG:PC vesicles. Conventional poration–resealing was observed in buffered solution, whereas the unusual bursting occurred in non-buffered and salt solutions. Let us also emphasize that the media conductivity (and, correspondingly, the membrane charging time) does not affect the vesicle behaviour significantly. Tests performed in solution conditions similar to those applied for *in vitro* gene transfer experiments[6] showed also vesicle bursting, suggesting that the only necessary condition for the bursting of the LE vesicles is the formation of a pore in the membrane. One example of a bursting LE vesicle recorded with the fast camera is given in Fig. 2b. These results suggest that the bursting is not specific to PG but to the charged state of the membrane. The LE membranes contained approximately 10 mol% anionic lipids (the majority being phosphatidylserines, PS), which was enough to induce membrane destabilization. In the synthetic membranes 50 mol% of PG was needed to lead to the same effect. In addition to the small fraction of charged lipids, the LE membranes contain up to about 50 mol% of cholesterol, which may also influence their behaviour.

The amount of PG in the bilayer is not the only factor triggering bursting in the synthetic membranes. In particular, vesicles with the same high content of PG (50%) do not burst in buffered solution. Even though the main difference between buffered solution and the non-buffered and salt solution seems to be the pH, significant protonation of PG[7] and PS should occur only for pH lower than 5.5, which is below the working pH values in this study. Thus, with respect to pH the solutions are not very different.

As already mentioned, the ionic strength of the buffered and the salt solutions is identical. Then, strictly speaking, the only composition difference between the two solutions is the presence of Hepes (1 mM) and EDTA (0.1 mM) in the buffer. Hepes is not known to interact specifically with PG or PS headgroups. The remaining candidate responsible for the vesicle bursting is the absence of the chelating agent EDTA, *i.e.*, the presence of small amounts (impurities) of multivalent cations in the salt and non-buffered solutions. Experiments on vesicles in non-buffered solutions show that the addition of 0.1 mM EDTA prevents vesicle bursting. On the other hand, vesicles in 1 mM Hepes without EDTA burst as in non-buffered solutions. Thus we conclude that the component that can prevent vesicle bursting is indeed EDTA. Since EDTA is generally

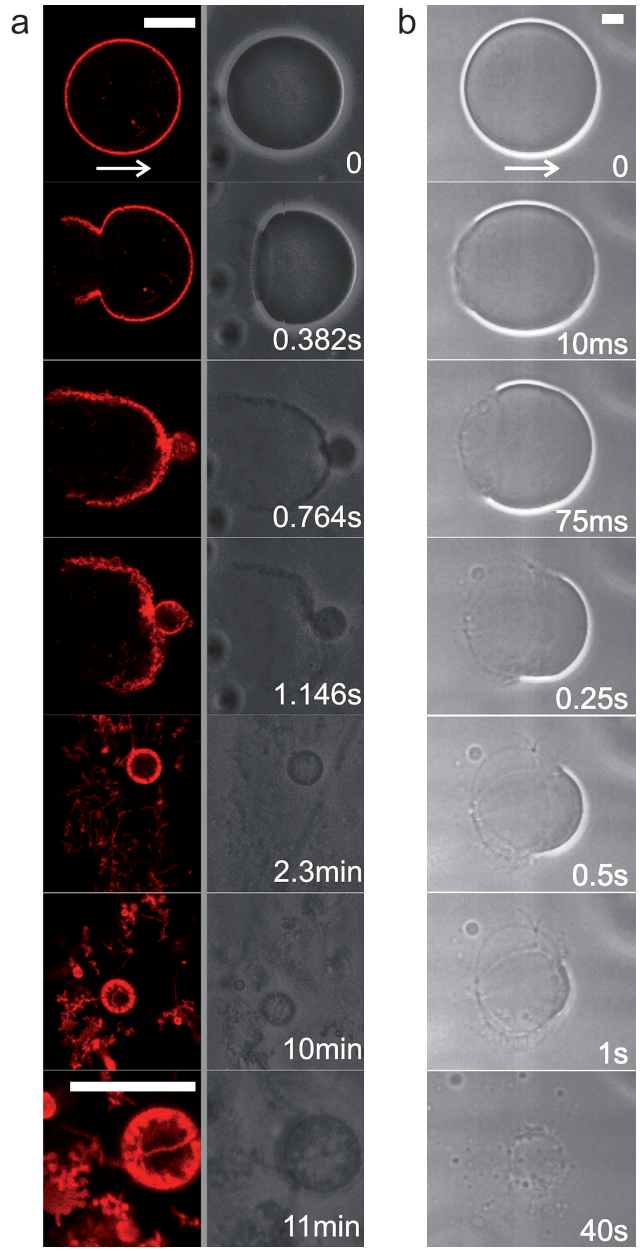

Fig. 2 Bursting vesicles. (a) Confocal microscopy sections (left) and phase contrast images (right) of a GUV (1:1 PG:PC) in salt solution subjected to a pulse (1.4 kV/cm, 200 μs). The approximate time after the beginning of the pulse is marked; note that the scanning speed is 2.62 fps. The last image is a magnified fraction (refocused). (b) Fast camera images of a bursting LE vesicle in salt solution subjected to a pulse (2 kV/cm, 300 μs). All scale bars correspond to 15 μm. In each case, the field direction is indicated on the first snapshot with an arrow.

added in solutions to bind possible multivalent ions present as impurities in the solution, like calcium,[8] we examined the vesicle behaviour after adding 0.5 mM $CaCl_2$ to the 0.1 mM EDTA vesicle solution. The bursting behaviour was not observed in this case. However, the vesicle poration appeared to be facilitated by Ca^{2+} ions: in the presence of calcium, vesicles porate but this does not happen for the same pulse conditions and vesicles of similar sizes in the absence of calcium. This effect might be related to the increased ionic strength of the solutions. Further studies are required to clarify the vesicle response to electric field in the presence of multivalent ions, and to reveal the cause and mechanism of vesicle bursting. The latter will be subject of future work. Here we restrict our discussion to the phenomenon of irrevocable membrane rupture as observed in the charged vesicles.

When subject to electroporation or significant osmotic stress, cell membranes should exhibit similar bursting behaviour as that of LE vesicles, since their lipid composition is alike. Indeed, stable holes and disruption leading to vesiculation has been observed in red blood cell ghosts.[9] This phenomenon was later interpreted as instability due to the charge in the membrane.[10] However, we emphasize that membranes of cells are subjected to internal mechanical constraint imposed by the cytoskeleton, which is why cells do not disintegrate even if their membranes are prone to disruption when subjected to pulses. Instead, the pores in the membrane are stabilized for a long time,[11] and can either lead to cell death by lysis or resealing depending on the media.[12] The latter is the key to the efficiency of electroporation-based protocols for drug or gene transfer in cells. The results reported here suggest that membrane charge as well as minute amounts of molecules like EDTA as pore stabilizers might be important but not yet well-understood regulating agents in these protocols.

The aim of this communication has been to report that even a tiny variation in the environment of charged bilayers can cause a dramatic change in the behaviour of membranes subjected to electric pulses. Membrane composition and degree of charging plays a significant role. The origin of bursting in charged vesicles is curious. Revealing the processes involved requires a detailed study of the lipid bilayer behaviour in various conditions, which is currently being pursued in our lab. This will shed light on the underlying mechanisms of structural reorganization involved in cell membranes and tissue electroporation.

Acknowledgements

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Bursting of charged multicomponent vesicles subjected to electric pulses
Supplementary Information

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Lipid extraction from red blood cells

Fresh human red blood cells (RBC) (courtesy of Nina Geldmacher from the Max Planck Institute for Infection Biology, Berlin) were purified according to the method described in (1). The RBC were washed 4 times in cold isotonic buffer (145 mM NaCl, 5 mM KCl, 5 mM Hepes, pH 7.4 at 4 °C) and centrifuged 10 min at 2000 × *g* at 4 °C. Then, the cells were lysed by shaking 10 min on ice in hypotonic solution (15 mM KCl, 0.01 mM EDTA, 1 mM EGTA, 5 mM Hepes, pH 6.0) to reduce premature, spontaneous resealing of the ghosts. EDTA, EGTA and Hepes were purchased from Sigma-Aldrich, Germany. The ghosts were washed once in hypotonic solution without EGTA and twice in hypotonic solution without EGTA but with 2 mM Mg^{2+} and centrifuged for 10 min at 4°C and 12000 × *g*. To remove peripherally associated proteins (and remaining hemoglobin, which is known to contaminate lipid extracts (2)) from the membrane of the open RBC ghosts, they have been shaken for 30 min on ice in 10 mM NaOH and centrifuged. The lipids were extracted from the obtained pellet using the method of Bligh and Dyer (3). Briefly, the pellet was diluted with bidistilled water to a volume of 240 µl, followed by stepwise addition and vortexing of the following solutions: 300 µl chloroform, 600 µl methanol, 300 µl chloroform, 300 µl bidistilled water. After 5 min centrifugation of the obtained solution at 2000 × *g*, the chloroform-rich phase was separated, stored at −20°C and used for vesicle preparation.

The lipid extract obtained according to the protocol of Bligh and Dyer (3) is known to contain about 10 % phosphatidylserines, 14 % phosphatidylcholines, 14 % phosphatidylethanolamines, 11 % sphingomyelines, and 50 % cholesterol (4).

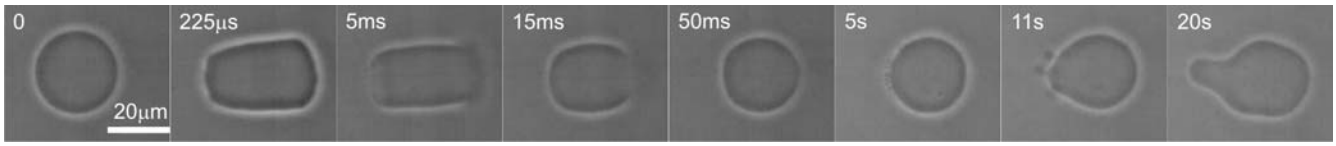
Vesicle preparation and observation

Giant unilamellar vesicles were prepared from palmitoyloleoylphosphatidylcholine (PC), palmitoyloleoylphosphatidylglycerol (PG) and palmitoyloleoylphosphatidylethanolamine (PE) (Avanti Polar Lipids, Alabaster, AL) or lipid extract (LE) from the RBC plasma membrane using the electroformation method (5). PG and PC were mixed in molar ratios 1:9, 2:8 and 1:1. Only the 1:1 molar ratio was explored for mixtures of PE and PC. For the samples used for confocal microscopy, up to 0.2 mol % of dipalmitoylphosphatidylethanolamine-Rhodamine (Rh-DPPE) or 0.5 % of 1-Oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-Glycero-3-[Phospho-rac-(1-glycerol)] (Ammonium Salt) (NBD-PG) from Avanti Polar Lipids were incorporated in the membrane. No change in the vesicle behavior was observed depending on the type of dye and compared to the dye-free vesicles.

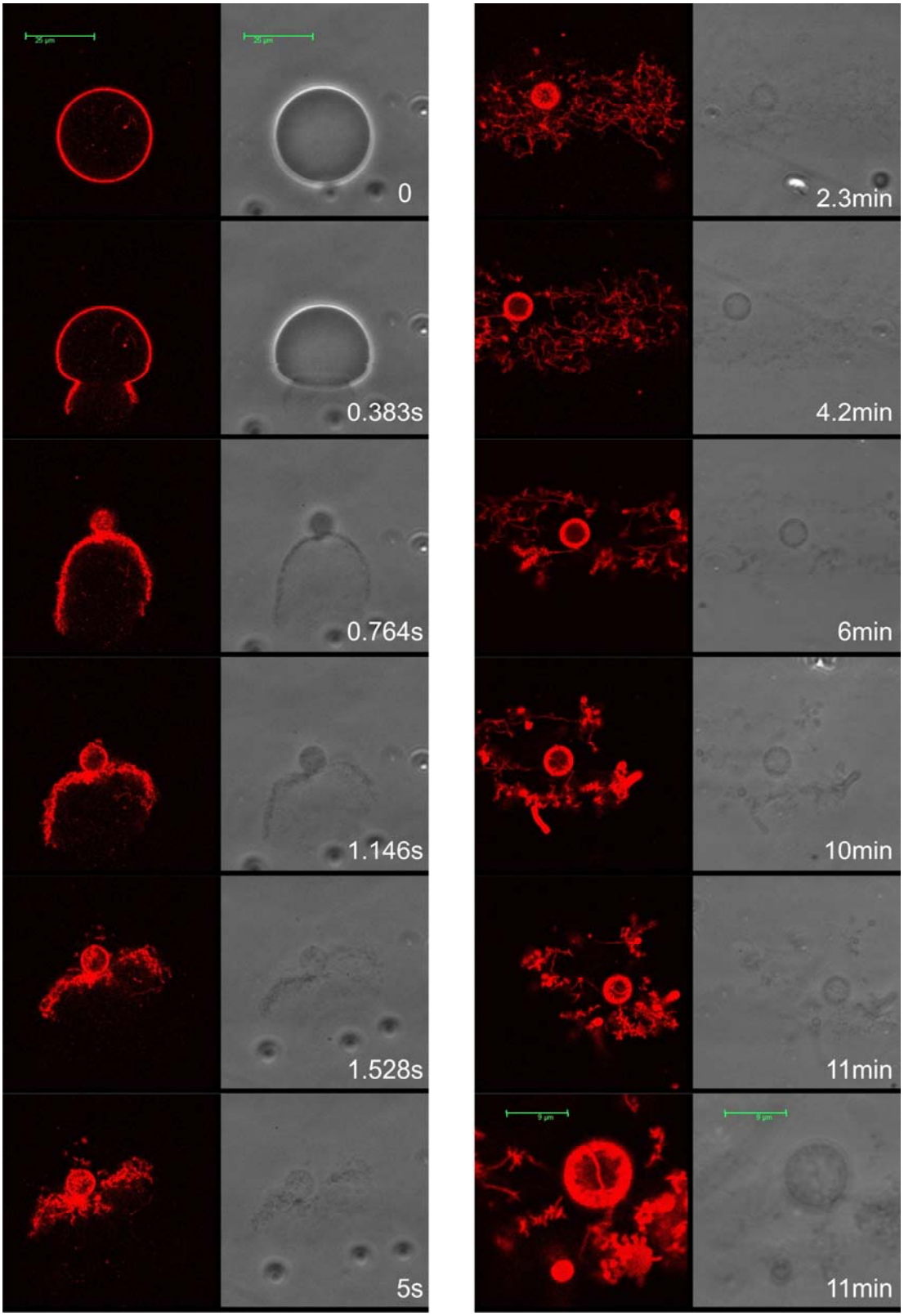
The procedure for vesicle electroformation is described in detail in (6). Briefly, 15 µl of a 2 mg/ml lipid solution in chloroform were spread on the surfaces of two conductive glasses (coated with Indium Tin Oxide). To prepare vesicles to be observed with confocal microscopy 0.2 mol% fluorescent dye was included in the lipid chloroform solution. The glasses with the lipid film were kept under vacuum for about 2 h to remove all traces of the organic solvent. The two glasses separated by a 2 mm thick Teflon frame were assembled to form a chamber sealed with silicon grease. The glass plates were connected to an AC field function generator. The chamber was filled with 0.2 M sucrose solution (referred to as non-buffered solution in the article). In some samples, we added 1mM Hepes and 0.1 mM EDTA, pH 7.4 (buffered solution). In others, we added 0.5 mM NaCl (salt solution), which provides the same ionic strength as the buffered solutions. An alternating current of 1 V at 10 Hz frequency was applied for 1 h. Vesicles with an average diameter of 50 µm and a large polydispersity were observed to form. The vesicle suspension was diluted 20 times into 0.2 M glucose solution (again in certain cases containing the above-mentioned buffer and salt solutions), thus creating sugar asymmetry across the vesicle membrane. The osmolarities of the inner and the outer solutions were measured with a cryoscopic osmometer Osmomat 030 (Gonotec, Germany) and carefully matched to avoid osmotic stress.

After dilution, the vesicles were transferred to a commercially available electrofusion chamber (Eppendorf, Hamburg, Germany). The latter consist of two parallel cylindrical electrodes of about 92 mm radius with 500 µm gap distance. Due to the density difference between the sucrose and glucose solutions, the vesicles settled at the bottom of the observation chamber where they were easy to locate. The refractive index difference produced good optical contrast for phase contrast observation: the vesicles appear as dark objects on a light gray background. The leakage through micrometer pores could be visualized by the dark sucrose leaving the vesicles. Electric pulses were generated by a Multiporator (Eppendorf). Videos were recorded with fast imaging camera HG-100 K (Redlake, San Diego, CA) or confocal SP5 system (DMI 6000, Leica Microsystems Heidelberg GmbH, Germany) equipped with a 40 × HCX Plan APO objective (NA 0.75). Laser sources at 561 nm and 488 nm were used to excite Rh-DPPE and NBD-PG, respectively. The acquisition speed using the fast camera was 20 000 frames per second (fps), while with the confocal microscope it was approximately 3 fps. Because of the slow scanning speed for the confocal microscopy observations, snapshots immediately after the pulse may be distorted in the field direction due to the slow scanning in lines perpendicular to it.

The following figure provides the snapshot sequence in Fig. 1 in the main text, here supplemented with an additional image demonstrating that the formed pore is closed within the first 50 ms:



Below we include more images with better resolution of the vesicle from Fig. 2 in the article. The membrane was labeled with Rh-DPPE. The scale-bar on all but the last images corresponds to 25 μm. The last image is refocused and magnified with scale-bar corresponding to 9 μm.



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