Visualization of membrane loss during the shrinkage of giant vesicles under electropulsation

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Abstract

We study the effect of permeabilizing electric fields applied to two different types of giant unilamellar vesicles, the first formed from EggPC lipids and the second formed from DOPC lipids. Experiments on vesicles of both lipid types show a decrease in vesicle radius which is interpreted as being due to lipid loss during the permeabilization process. We show that the decrease in size can be qualitatively explained as a loss of lipid area which is proportional to the area of the vesicle which is permeabilized. Three possible modes of membrane loss were directly observed: pore formation, vesicle formation and tubule formation.

Key words: giant liposomes; electroporation; vesicles; pores; tubules
**ABBREVIATIONS - LIST OF SYMBOLS**

EggPC: L-α-Phosphatidylcholine (Egg, Chicken)
DOPC: 1,2-Dioleoyl-sn-Glycero-3-Phosphocholine
DiIC$_{18}$: 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
Rhodamine PE: L-α-Phosphatidylethanolamine-N-(Egg Lissamine Rhodamine PE)

$E$: magnitude of the applied electric field

$\Delta \Psi$: transmembrane voltage

$\Delta \Psi_0$: initial transmembrane voltage induced by the first pulse at the poles of the liposomes.

$\Delta \Psi_c$: critical transmembrane voltage

$n$: number of pulses

$R(n)$: radius of the vesicle after $n$ pulses

$R_c$: critical radius

$W(n)$: rescaled radius of the vesicle after $n$ pulses $R(n)/R(0)$

$W_c$: rescaled critical radius $R_c/R(0)$

$\lambda$: fraction of the permeabilized area lost per pulse

$N_c$: number of pulses needed to enter the shrinking regime

$q$: probability that one pulse induces a transition from the pre-shrinking to the shrinking regime

SR: shrinking regime

PR: pre-shrinking regime

$a$: membrane thickness

$a_e$: membrane electrical thickness

$\epsilon_m$: membrane dielectric constant

$C$: constant depending on $R$, $a$ and the various conductivities of the problem

$\theta$: angle on the cell surface with respect to the direction of the applied field
\( \theta_c \): critical angle \\
\( A \): area of the vesicle \\
\( A_p \): permeabilized area \\
\( \Sigma_0 \): initial surface tension \\
\( \Sigma_{el} \): surface tension induced by the electric field \\
\( \Sigma_{lys} \): lysis tension \\
\( p \): dipole moment of the PC headgroup \\
\( l \): length of the hydrocarbon chain \\
\( \rho \): effective radius of the lipid hydrocarbon tail viewed as a cylinder \\
\( \mu \): lipid tail hydrophobic free energy per unit of area

INTRODUCTION

Electropermeabilization is a commonly used physical method where electric pulses are applied to cells and vesicles and has been widely reviewed in the literature (1–7). An effect, of major importance, of the electric pulses is that under certain circumstances they can induce the transient permeabilization of the cell plasma membrane. This permeabilization manifests itself via the crossing of the cell membrane by molecules which would normally not be able to permeate the cell membrane. When subjected to sufficiently large electric fields, vesicle membranes become permeable to small molecules (8, 9) and flat membranes show a marked increase in their electrical conductance (10). Small molecules appear to cross permeabilized membranes via simple diffusion. However complex processes, such as electrophoresis and direct interactions with the membrane, come into play for larger molecules such as DNA. Electropermeabilization is now regularly employed as a delivery method for a large variety of molecules such as drugs, antibodies, oligonucleotides, RNA and DNA (6, 9, 11–13). Initial studies were carried out in vitro on cells in culture, but as the technique has developed an increasing amount of data has been obtained in vivo on tissues (14–16) and the method is being adapted to the clinical context (17, 18). Clearly the method has a huge potential in the fields of cancer treatment and gene therapy, offering, in some cases more efficient, more controllable and safer treatment protocols (when compared to viral transfection methods for example). From a purely physical point of view the application of an electric field to a lipid membrane has two notable effects. The first is a mechanical one where the stresses caused by the field can deform the membrane, for instance causing a spherical vesicle to deform into an ellipsoidal or cylindrical one (19, 22). This deformation can be thoroughly understood in terms of a macroscopic
continuum description of the cell membrane in terms of its bulk electrical and mechanical properties. The second phenomenon of electroporpermeabilization is much less well understood. Despite its increasing popularity as a therapeutic method, there are still many open questions about the underlying physical mechanisms involved in electroporpermeabilization. Indeed, at the simplest level, the basic structural changes induced by the field on the membrane structure are still to be fully understood. A number of physical theories have been put forward to explain the phenomenon of electroporpermeabilization. Historically the first explanations of electroporpermeabilization were based on classical continuum theories which predict dielectric breakdown of the membrane at a critical field strength \( \text{[23, 28]} \). The main problem with such theories is that while predicting a dielectric/mechanical breakdown transition they do not provide a description of the physical state of the permeabilized membrane. Currently, the most popular explanation for electroporpermeabilization is that pores are formed due to a local increase in the surface tension due to the electric field \( \text{[29, 33]} \). This increase in surface tension energetically favors the formation of pores which is otherwise energetically defavored by their line tension, a similar theory was first introduced to explain the rupture of soap films \( \text{[34]} \). In this theory the pores can become stabilized in a hydrophobic to hydrophilic pore transition via the rearrangement of the lipids at the pore edges. Because the permeabilization is explained by the formation of pores, the phenomenon described by this theory is referred to as electroporation. Recently numerical simulations have confirmed that pores can be induced by strong electric fields \( \text{[35, 41]} \), typically the systems simulated are small and no significant lipid loss during pore formation has been reported.

When discussing the phenomenon of electroporpermeabilization we must distinguish between two key stages of the process (i) the physical change induced in the membrane by the field (in the absence of molecules to be transported) and (ii) the interaction of the molecules that are to be transported with the modified membrane. At the simplest level, combination of steps (i) and (ii) can be observed experimentally as a transport phenomenon using marked molecules or via conductivity experiments. In this paper we demonstrate that the step (i) can be indirectly detected via a change in the size of giant liposomes under electropulapsulation and an associated direct visualization of the expulsion of lipids from the liposomes. Concretely we study the effect of a series of permeabilizing pulses, well separated in time, on the size of giant unilamellar vesicles (GUVs). In the experiments the radius of the GUV is measured after each pulse and we find that each GUV studied shows, on average, a decrease in its radius down to a critical radius beyond which its size no longer changes. This decrease in size points to the fact that, during the physical processes leading to electroporpermeabilization, lipids are lost from the vesicle thus leading to a reduction in their size.

Our experiments are not a direct study of permeabilization, however they constitute an indirect method of studying electroporpermeabilization which is relatively straightforward to carry out and interpret in terms of simple physical models which are relatively well established. From an experimental point of view the crucial advantage of using GUVs is that their composition can be varied and controlled and also their membrane is not subjected to internal mechanical constraints as is the case for living cells with cellular cytoskeletons. Furthermore, their size is similar to that of mammalian cells, which allows a direct visualization by an optical microscope.
Naively lipid loss during electroporation seems normal as if, for instance, pores are formed the lipids near the edges of these pores will be subject to strong variations in the local electric potential and the electric field. Charges and dipole moments on lipids will interact strongly with the electric field and variations of the electric field respectively. The forces involved may well be capable of tearing lipids from the membrane structure. However our experiments suggest that the mechanism of lipid loss is a collective one which involves the formation of small structures such as tubules and vesicles as well as pores. A simple comparison of electrostatic (dipole electric field interaction) energy and hydrophobic free energy suggests that individual lipids cannot be removed from the membrane.

The phenomenon of lipid loss due to an applied field has previously been studied in [42] but from quite a different point of view (in this study the effect of single pulses was examined). DOPC vesicles of sizes of the order of 20 µm were subjected to pulsed electric fields of the order of 1 kV/cm and duration 700 µs. The vesicles were observed using a standard fluorescent microscope and at the cathode facing side single pores of the size of about 7 µm were observed. Such pores were however seldom found on the anode facing side. However it was inferred that this side was also permeabilized but that the pores responsible were too small to be observed. In the experiments it was also noted that up to 14% of the vesicle surface could be lost during the process of pore formation/permeabilization.

**EXPERIMENTAL SETUP**

We decided to work with two different lipids. However, we wanted phospholipids with identical head groups in order to obtain the same dipole behaviour. Thus we used DOPC and EggPC, purchased from Avanti Polar Lipids (Alabaster, AL). The formation medium is an aqueous solution with 240 mM sucrose. The pulsation buffer is an aqueous solution of 260 mM glucose that also contains 1 mM phosphate buffer \( KH_2PO_4/K_2HPO_4 \) (Merck, Darmstadt, Germany) in order to impose a physiological pH of 7.4, and 1 mM sodium chloride (Prolabo, Briare, France) in order to achieve an electrical conductivity in the range of a few hundreds of µS/cm. Conductivities of internal and external solutions are measured with a HI 8820 conductimeter (Hanna Instruments, Lingolsheim, France), and have the values \( \sigma_i \approx 15 \mu S/cm \) and \( \sigma_e \approx 460 \mu S/cm \) respectively. The osmolarities are 285 mOsm/kg for the formation medium, and 305 mOsm/kg for the pulsation buffer. These measurements were performed with an Osmomat 030 osmometer (Gonotec, Berlin, Germany). The different refractive indexes of the internal and external media yields a contrast which enables the vesicles to be visualized using a microscope, and the density difference allows the sedimentation of the vesicles on the bottom of the chamber, thus reducing their distance from the objective. EggPC liposomes are visualized by phase contrast, and DOPC liposomes by fluorescence microscopy. We worked with two different dyes (Rhodamine PE (Avanti Polar Lipids) and DiIC\(_{18}\) (Molecular Probes, Eugene, OR)) without any noticeable change in our experimental results. The vesicle formation method employed here is electroformation, as described in [43]. We chose this
technique because it is simple, easily reproducible and has a good yield. Furthermore, a large amount of the produced vesicles is unilamellar, as demonstrated in (44).

**Electroformation**

**Lipid solution**

The lipids are diluted in chloroform, at a mass concentration of 0.5 mg/mL. For DOPC vesicles, the fluorescent probe is added at 0.005 mg/mL. This preparation and the following steps can be performed at room temperature, because the gel phase/liquid phase transition temperature of the lipids used is much lower.

**Formation chamber**

The chamber is made of two glass layers covered with Indium Tin Oxide to ensure the electrical conductivity of the surface. The two layers are separated by an adhesive silicone joint of 1 mm width. The connection with the generator (AC Exact, model 128; Hillsboro, OR) is maintained by two wires, each one soldered on a small copper strip stuck on the ITO slide. Then, 15 µL of lipid solution is deposited on the conducting sides of the glass slides. The deposition is carried out slowly and at constant rate in a chamber held at 4°C to slowly evaporate the chloroform and then the slides are dried under vaccum for a couple of hours in order to entirely remove the remaining solvent molecules.

Finally, the slides are sealed together, and the chamber is filled with the formation medium.

**Voltage application**

We apply a sinusoidal voltage of 25 mV peak to peak at 8 Hz. The voltage is increased by 100 mV steps every 5 minutes, up to a value of 1225 mV. It is maintained under these conditions overnight. Next we apply a square wave of same amplitude at 4 Hz for one hour in order to detach the liposomes from the slides.

**Electropulsation**

**Pulsation chamber**

The chamber where the GUVs are subjected to the electric field is composed of a glass slide and a coverslip. Two parallel copper strips of thickness 70 µm are stuck on the slide at a distance of 1 cm apart. The coverslip is then stuck onto the dispositive with heated parafilm. The chamber is 1 cm long (between electrodes), 2.6 cm wide (width of the coverslip) and 250 µm high (value estimated via measurements with a microscope). We first introduce 60 µL of pulsation buffer between the slide and the coverslip, while taking care of filling the whole chamber so as to ensure the conductivity
of our medium. Next we add 5 µL of our GUV preparation. Capillarity phenomena prevents the solution from leaking out of the chamber.

The electrode thickness is about the size of our biggest liposomes, which represents only a quarter of the chamber height. We could not \textit{a priori} be certain of the homogeneity of the field. However, we solved numerically Laplace’s equation with finite element software Comsol Multiphysics (Comsol, Burlington, MA) for the case of our geometry. We found that the field was almost homogeneous in the bottom part of the chamber between the electrodes, and that the size and shape of the permeabilized area were not significantly different from that computed for a geometry with much bigger electrodes (data not shown).

**Pulsation method**

Electropulsation is carried out using a CNRS cell electropulsator (Jouan, St-Herblain, France) which delivered square-wave electric pulses. An oscilloscope (Enertec, St-Etienne, France) is used to monitor the pulse shape and amplitude. The process of electropulsation is performed directly under the microscope. For the phase contrast visualization we used an inverted epifluorescence microscope Leica DM IRB (Leica Microsystems, Wetzlar, Germany) equipped with a Princeton RTE/CCD-1317-K/0 camera (Princeton Instruments, Trenton, NJ) and a 40× Leica phase contrast objective, and an inverted confocal microscope Zeiss LSM 510 (Carl Zeiss, Jena, Germany) with a 63× Zeiss objective for fluorescence imaging. Excitation at 543 nm was provided by a HeNe laser, and emission filter was a 560 nm longpass. The pulse duration was not set to a few hundreds of µs as in (21, 22, 42), but to 5 ms because this value is commonly used for gene transfer protocols in mammalian cells (9). In most cases, we apply pulses at 0.5 Hz. However, we sometimes have to interrupt the pulse train for a few seconds in order to re-center the image on the liposome of interest. Indeed, the observed vesicle does not always stay immobile. It often experiences a translational motion toward the positive electrode, because of which we sometimes have to modify the centering. This displacement was always directed toward the anode, irrespective of the net electric charge of the fluorescent probe we used (negative for Rhodamine PE and positive for DiIC\textsubscript{18}). As we will see later, the direction of this motion is coherent with the sign of the \( \zeta \) potentials of the vesicles, which does not depend on the type of dye chosen. Due to the need to re-center the image from time to time, the frequency of the pulses is not constant over a whole experiment, but we checked this did not affect our results. The time delay between two consecutive pulses is of the same order of magnitude, ranging from 2 seconds to a few tens of seconds. This duration seems to be much longer than the time needed by the vesicle to relax after one pulse, therefore it does not matter if pulses are separated by 2 or 20 seconds. Direct observation showed that vesicles were distorted rapidly after the pulse application, but as far as the eye could see there was no visible size or shape change between two consecutive pulses. The pulse amplitude is chosen according to the rule \( ED = (4/3)\Delta\Psi_0 = Const \) (see details later for this choice), where \( E \) denotes the amplitude of the electric field, and \( D \) the initial diameter of the GUV. The constant is chosen to be 1.7 V. This choice means that at the beginning of every experiment the potential difference drop, \( \Delta\Psi_0 \), across the
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GUV membrane at the poles facing the electrodes is theoretically (see later) equal to about 1.3 V - this value is well beyond the value of 200 mV typically cited as the permeabilization threshold for Chinese hamster ovary cells (45, 46) and of the order of that cited for artificial vesicles and other cell types (3, 26, 47). In the pulsation chamber the distance between the electrodes is 1 cm and so the potential applied between the electrodes is 1.7/D V, where D is measured in cm or conveniently 17/D kV if we measure D in µm. The idea behind this large choice of initial transmembrane potential ΔΨ₀ is that the field will initially permeabilize the membrane and continue to do so till the vesicle size becomes significantly smaller than the initial one. We note that our protocol yields initial transmembrane potentials which are slightly lower, but of the same order as those in the experiments of (42) (which varied between 1.4-2.5 V).

The experimental strategy is simple. We focus on a liposome and we measure its initial diameter. We then tune the voltage amplitude according to the rule described above, and we apply a pulse train until the GUV does not shrink anymore. We acquire one image between two consecutive pulses (about 1 s after each pulse), so we are sure that the vesicle has experienced an electric pulse between two consecutive values of the diameters we measure. Image processing tasks are performed with ImageJ (National Institutes of Health, Bethesda, MD).

ζ potentials measurements

We measured the average ζ potentials of our GUVs on a Malvern Zetasizer 3000 HS (Malvern, Worcestershire, United Kingdom), using the following method. We diluted 1 mL of the GUV solution obtained after electroformation in 2 mL of a special buffer containing 240 mM sucrose, 1.5 mM phosphate buffer and 1.5 mM sodium chloride. Vesicles are thus suspended in a medium containing 1 mM sodium chloride, 1 mM phosphate buffer and 240 mM sucrose. This composition is the same as that of our pulsation medium, except for the 260 mM glucose replaced by 240 mM sucrose in order to avoid sedimentation of the vesicles which would make the measurement impossible. We then split the 3 mL into two samples, on which we performed two series of ten measurements each.

THEORY

The basic theory which explains electroporation is based on the modeling of the vesicle electrode system in terms of a weakly conductive cell membrane of conductivity denoted by σₘ with external and internal media of much higher conductivities denoted by σₑ and σᵢ respectively. We denote by R the radius of the vesicle which is assumed to be spherical and indeed stays spherical throughout the experiments. In our experiments R lies typically between 10 and 100 µm. The thickness of the vesicle membrane is denoted by a and typically has the value of 4 nm. In the steady state, which is achieved on time scales much shorter than the time over which the pulse is applied, the electric potential Ψ obeys Laplace’s equation and if θ denotes the angle on the cell surface with respect to the direction of the applied field which is of magnitude E then the potential drop across the
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where $C$ is a constant depending on $R$, $a$, and the various conductivities of the problem. In the limits where $\sigma_m \ll \sigma_i$, $\sigma_m \ll \sigma_e$ and $a \ll R$ the constant $C$ becomes very simple and takes the value $C = 3/2$. For the parameters of the experiments carried out here we are close to the limit where $C$ takes this limiting value. The most important point for our analysis here is that $C$ is independent of $R$. We thus find that for a thin membrane the electric field inside the membrane and normal to its surface, denoted by $E_n$, is given by

$$E_n(\theta) = \frac{CRE \cos(\theta)}{a}. \quad (2)$$

Eq. 2 demonstrates that there is a huge amplification of the externally applied field across the membrane. This huge electric field internal to the membrane causes structural changes. Whether this structural change corresponds to the formation of pores, dielectric breakdown or the formation of defects or vesicles is still open to debate. However in experiments where permeabilization is measured either via conductivity measurements of planar membranes or by direct optical observation of the entry of marker molecules a consensus exists that permeabilization occurs locally in the membrane when the magnitude of the potential drop across the membrane $\Delta \Psi$ exceeds a certain threshold $\Delta \Psi_c$ which is estimated to be of the order of 0.25-1.0 V (3, 26, 45–47). This corresponds to a field within the membrane of about 50-250 000 kV/m (for a membrane of thickness 4 nm). This critical threshold is seemingly quite universal, being largely independent of cell and vesicle composition. There is an alternative though largely equivalent physical explanation of field induced breakdown of the membrane. The effect of a local potential drop $\Delta \Psi$ across the membrane is to induce a local electrical surface tension $\Sigma_{el}$ which can be computed via the Maxwell stress tensor and is given by $\Sigma_{el} = \epsilon_m \Delta \Psi^2 a/2a_e^2$ where $\epsilon_m$ is the dielectric constant of the membrane, $a$ is its thickness and $a_e$ its electrical thickness (7, 26). If the initial surface tension of the membrane is $\Sigma_0$ then upon applying the field the total tension is $\Sigma = \Sigma_0 + \Sigma_{el}$. The tension of rupture of a lipid membrane is called the lysis tension $\Sigma_{ly}$ and thus when the local tension $\Sigma$ exceeds $\Sigma_{ly}$, we expect the membrane to be destabilized. This formulation is strictly equivalent to the existence of a critical value of the local electric field in the membrane at which breakdown will occur. However in this formulation we see that $\Delta \Psi_c$ will depend on the initial surface tension of the vesicle $\Sigma_0$. Indeed such a dependence on $\Sigma_0$ has been reported experimentally (21). In terms of the initial and lysis tension the critical potential is given by

$$\Delta \Psi_c = \sqrt{\frac{2a_e^2}{\epsilon_m a} (\Sigma_{ly} - \Sigma_0)}, \quad (3)$$

and thus we see that the value of the applied field required to affect the membrane will depend on the initial tension of the vesicle. In our study we are interested in the mechanism of lipid loss
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and the $\Delta \Psi_c$ that induces lipid loss does not necessarily correspond to that necessary to induce permeabilization, however it is reasonable to expect that the two critical potentials have the same order of magnitude. Studies of electropermeabilization phenomena show that the critical potential depends on the duration of the applied pulse, the critical potential being smaller for longer pulses (11). This means that the underlying physical mechanisms rely on activated processes such as nucleation events for first order phase transitions. This means that an applied pulse may have no effect with some probability, this probability should decrease with the amplitude and duration of the pulse. In our experimental set up the liposomes are visibly under an initial tension and we also expect that there is some distribution of initial tensions even for vesicles of the same composition and similar sizes. The critical potential for each vesicle should therefore be expected to vary.

As we are looking at vesicles we can neglect any possible modification of the transmembrane potential due to cellular activity and thus assume that it is given purely by Eq. (1). Assuming that the mechanical and electric membrane thickness $a$ and $a_e$ remains constant, there is a critical transmembrane potential drop beyond which the membrane becomes permeabilized or susceptible to lipid loss. Clearly, at fixed electric field parameters (amplitude and duration), a cell can no longer be permeabilized when its radius is smaller than a certain critical radius $R_c$ beyond which no part of the cell is permeabilized. We thus expect that the permeabilization and thus vesicle shrinkage will stop once the vesicle has this critical radius. The region where the magnitude of $\Delta \Psi$ is maximal is clearly that facing the electrodes, corresponding to $\theta = 0$ and $\theta = \pi$, and so these are the last points where the membrane is permeabilizable. The value of $R_c$ is thus given by

$$\Delta \Psi_c = CER_c \ .$$

If we are in the situation where $R > R_c$ then about the pole at $\theta = 0$ the region where $\theta$ is between 0 and $\theta_c$ is permeabilized and $\theta_c$ is given by

$$\theta_c = \arccos \left( \frac{\Delta \Psi_c}{C R E} \right) \ .$$

This region gives one half of the total permeabilized area of the vesicle which we denote by $A_p$. We thus find that

$$\frac{1}{2} A_p = 2\pi \int_0^{\theta_c} R^2 \sin(\theta) d\theta = 2\pi R^2 \left( 1 - \frac{R_c}{R} \right) \ .$$

Now we consider how the area loss upon a pulsation can be related to the physical parameters of the system. The simplest idea is to assume that the area lost is simply proportional to the permeabilized membrane area. This does not presuppose the mechanism of lipid loss - we simply assume that in the region where the field exceeds the critical value the membrane structure is altered. This alteration can be interpreted as a form of dielectric breakdown and where it occurs we assume that lipids can be effectively lost from the membrane surface.

If $n$ denotes the number of pulses, treating $n$ as a continuous variable we can write that on average:

$$\frac{dA}{dn} = -\lambda A_p \ .$$
that is to say the average area lost per pulse is simply proportional to the area where the critical
membrane potential (or equivalently surface tension) is exceeded. Note that we should really use
a discrete difference equation rather than the continuous one above, however we have, numerically,
checked that the difference behavior is insignificant when compared to the typical experimental
errors. Now if we assume that $\Delta \Psi_c$ remains constant throughout the experiment, Eq. 7 can be
solved using $A = 4\pi R^2$ to obtain

$$R(n) = R_c + (R(0) - R_c) \exp \left( -\frac{\lambda}{2} n \right).$$  \hspace{1cm} (8)

Thus we expect an exponential decay to the critical value of $R_c$ as given by Eq. 3. If we define the
dimensionless variable

$$W(n) = \frac{R(n)}{R(0)},$$  \hspace{1cm} (9)

then $W(n)$ obeys

$$W(n) = W_c + (1 - W_c) \exp \left( -\lambda \frac{n}{2} \right),$$  \hspace{1cm} (10)

and $W_c$ is the asymptotic value of $W$ after a large number of pulses have been applied and beyond
which the vesicle is no longer permeabilizable; it is given by

$$W_c = \frac{R_c}{R(0)} = \frac{\Delta \Psi_c}{CER(0)}.$$  \hspace{1cm} (11)

Now in the experiments if we choose to apply fields $E$ such that $ER(0)$ is constant, then if $\Delta \Psi_c$
and $C$ are constant we find that

$$W_c = \frac{\Delta \Psi_c}{\Delta \Psi_0},$$  \hspace{1cm} (12)

where $\Delta \Psi_0$ is the initial experimentally imposed potential drop at the poles of the cells and is by
construction (i.e. via the choice of $E$) the same for all vesicles. With this choice of $E$ all plots of
$W$ as a function of the number of pulses $n$ should collapse onto the same curve if $\Delta \Psi_c$ remains
constant during the experiment and if it is the same for all vesicles. All plots will have $W(0) = 1$
and should attain the asymptotic value $W_c$ after the same characteristic number of pulses (as we
have assumed that $\lambda$ is independent of $R$).

We stress here that if $ER(0)$ is taken to be constant then the normal component of the electric
field within the membrane is the same for every vesicle studied at the beginning of each experiment
and thus, independently of any theory used to analyze the results, we are always looking at systems
where the local electric fields in the membranes are the same.

Clearly three sources of additional complexity are neglected in the above analysis (i) the surface
tension will fluctuate during the permeabilization/lipid loss process (ii) the local electric field seen
by the vesicles will fluctuate due to the presence of other vesicles (iii) we shall see in the section
on experimental results that several mechanisms can be involved in the process of lipid loss (pore,
vesicle and tubule formation) and clearly the choice of a single fitting parameter for lipid loss per permeabilized area \( \lambda \) is another simplification. Indeed \( \lambda \) should be interpreted as an average area loss parameter due to the (at least) three visualized mechanisms of lipid expulsion.

The initial surface tension (which will have some distribution about an average value) will also play a role in the initiation of the permeabilization and lipid loss process. To what extent the vesicle retains a memory of this initial tension is an important point. If after each pulse it had the same tension, then the distribution of the values of \( W_c \) would be a direct reflection of this initial surface tension distribution. However it is likely that the tension will vary after each pulse and indeed that the tension is a dynamical variable. Our experimental results imply that the reduction of the radius is due to expulsion of lipid from the main vesicle but that some expelled lipid is still in contact with the main vesicle (as in the case of tubules). These attached lipids will constitute a reservoir which will modify the effective surface tension of the main vesicle and this tension itself will evolve if the system has not had time to equilibriate between pulses. We conclude that in fitting the data with the simple model presented here we should find a scatter in the resulting values of \( \lambda \) and \( W_c \) due to the points (i), (ii) and (iii) mentioned above.

**EXPERIMENTAL RESULTS**

**Observations and data fitting**

The existence of the critical radius \( R_c \) was confirmed by the two following observations. (i) After a sufficiently large number of pulses had been applied, all the vesicles we could find in our sample had sizes lower than the one of the initial liposome of interest. (ii) We noticed that a liposome which had reached its critical radius could experience another shrinkage if the field magnitude was increased. We should mention that we sometimes saw vesicles disintegrating, and thus we could not observe the size stabilization. We only kept data corresponding to shrinking and stabilizing GUVs, and we finally gathered 51 data sets for DOPC and 47 or EggPC. Another fact that must be mentioned is the following. In some cases, the size diminution did not begin immediately after the first pulse. We had to apply several electric pulses before being able to detect radius decrease. A possible explanation for this fact is that, like the permeabilization process, the mechanism for lipid loss requires a change in the physical state of the membrane, the formation of defects or pores for example. The effect of the field is therefore twofold - it allows for the formation of defects and once defects are present the field along with the presence of the defects allow for lipid loss. We may assume that the creation of defects is an activated process and at each pulse the membrane develops defects with some probability \( q \). Note that we assume it is only the defect creation process which has this probabilistic nature (once the vesicle size has begun to decrease, lipids are expelled after each pulse as long as the vesicle radius is greater than \( R_c \)). In order to describe this phenomenon, we suppose that one vesicle can be found either in a pre-shrinking (no defects) or in a shrinking (with defects) regime (PR or SR respectively), the transition to the SR after a pulse being a stochastic event occurring with constant probability \( q \), independent of the number of
pulses applied before. This hypothesis of a random event is legitimate because our model should incorporate the intrinsic stochastic nature of permeabilization processes (3). The fact that \( q \) does not depend on \( n \) is justified if we assume that a vesicle having experienced a “harmless” pulse recovers the same state it had in the PR. Within this modified framework, the former expression of the scaled variable \( W(n) \) (Eq. 10) now reads

\[
W(n) = H(Nc - n - 1) + H(n + 1 - Nc) \left[ W_c^{fit} + (1 - W_c^{fit}) \exp \left( -\frac{\lambda^{fit}}{2} n \right) \right]
\]  

(13)

where \( H \) denotes a Heaviside function taking the value 1 for a positive argument and 0 otherwise, and \( Nc \) the critical number of pulses needed before entrance in the SR. This means that the fitted curve will be constant up till \( Nc \) and then decay exponentially after \( n = Nc \). We have denoted the critical value of \( Wc \) given by the fit as \( W_c^{fit} \) and the effective value of \( \lambda \) estimated from fitting is denoted by \( \lambda^{fit} \). In terms of our theory we expect the average value of \( W_c^{fit} \) to be concentrated about \( Wc \) with fluctuations about this value. All fits were performed with the formula given by Eq. 13 so we obtained values of \( Nc, W_c^{fit} \) and \( \lambda^{fit} \) for each of the 51 DOPC data sets. With assumptions described above, the random variable \( Nc \) should follow a geometric (discrete and memory-less) distribution. We checked this by plotting the normalized histogram of \( Nc \), and as Fig. 1 shows, the values of \( Nc \) are well fitted by a geometric distribution of the form

\[
\text{Probability}(Nc = n) = q(1 - q)^{n-1}
\]  

(14)

The shown fit yields the value \( q = 0.33 \), which means that \( Nc \) has the average value \( \langle Nc \rangle = 1/q = 3 \). In Fig. 2 we present four examples of data sets (× marks) and associated fits (full lines). Diamond marks correspond to the images shown in Figs. 5 and 6 depicting the different mechanisms of lipid loss (see details below). Except for liposome C which immediately starts to shrink, we can clearly identify the PRs, the SRs and the stabilization of sizes. Detailed information about pulse spacing for data from Fig. 2, which is not constant over a whole experiment because of the lateral motion of the vesicles, can be found in Table S1 in the Supplementary Material.

**Quantitative analysis - DOPC**

As a first step in our data analysis, we can take the average of all the experimental curves and then carry out a fit, this yields the values \( \lambda = 0.16 \) and \( Wc = 0.65 \). The fit also yields the number of pulses necessary to put the liposome in the active state, where lipid loss can be induced, to be \( Nc = 1.73 \). The experimental data was also examined to see if there was any correlation between the fitted value of \( Wc \) and \( \lambda \) with the initial vesicle radius \( R(0) \), no appreciable correlation was seen, thus validating our hypothesis that the vesicle shrinkage can be well described in terms of the rescaled (dimensionless) quantity \( W(n) \). A second way to estimate the parameters of the model is to fit \( \lambda \) and \( Wc \) for each curve individually to obtain \( \langle \lambda^{fit} \rangle, \langle W_c^{fit} \rangle \) and \( \langle Nc \rangle \), the average value of the fitting parameters averaged over the individual experiments. The values obtained
were $\langle \lambda^{fit} \rangle = 0.25$, $\langle W^{fit}_c \rangle = 0.58$ and $\langle N_c \rangle = 4.99$. This value of $\langle N_c \rangle$ agrees well with that of 3 estimated by the geometric distribution fit to the histogram of the fitted values for $N_c$.

Figs. 3 and 4 show the histograms of $\lambda^{fit}$ and $W^{fit}_c$ respectively. As mentioned in the theory section, in fitting the data with our simple model we should expect to see variation in the values of $\lambda$ and $W_c$ obtained due to fluctuations of the surface tension (both initial and during the permeabilization process), local electric field and possibly the effective number of defects created after the $N_c$ pulses needed to enter into the permeabilized state. We note that it has indeed been demonstrated, in $^{21,22}$, that the critical potential necessary to induce permeabilization is indeed dependent on the surface tension.

### Quantitative analysis - EggPC

The experiments with EggPC were performed first and at that time we had not yet made the considerations about the PR and the SR. We only kept data sets corresponding to immediately shrinking vesicles, therefore in this section $N_c = 1$ for each liposome. Despite this simplification, we did the same data processing as that described for DOPC. The fit on the average of all experimental curves yields the values $\lambda = 0.27$ and $W_c = 0.77$. The values of the fitting parameters averaged over the individual experiments are $\langle \lambda^{fit} \rangle = 0.31$ and $\langle W^{fit}_c \rangle = 0.69$.

### About the anode-directed motion of the vesicles

The translational motion we observed was always directed toward the anode, suggesting that the GUVs could carry a net negative charge, even with a positively charged fluorescent dye. We checked this by measuring the $\zeta$ potential of the vesicles in a medium with ionic composition equivalent to that of our pulsation medium, the sugar composition being different to avoid vesicle sedimentation making the measure impossible. We examined four different types of vesicles: EggPC alone, DOPC alone, DOPC labeled with Rhodamine PE, and DOPC labeled with DiIC$_{18}$. We did not use EggPC vesicles with a fluorescent dye, because our experiments involving EggPC were performed via phase contrast microscopy, without any probe.

For all four vesicle compositions, we find an average $\zeta$ potential of the order of $-20 \ mV$, a value in agreement with what was found in $^{49}$ for DOPC GUVs, and whose sign is consistent with our observations. This corresponds to a negligible negative surface charge for the GUVs, less than one elementary charge per thousand of lipids. This residual electric charge possibly due to lipid impurities manifests itself only via the anode-directed motion of the vesicles, because of the large magnitude of the applied electric field.

### About the initial pH asymmetry

Internal and external media of our GUVs were not at the same pH conditions (6.6 and 7.4 respectively). It was thus questionnable if this pH asymmetry had any significant influence in our experiments. The answer is no, based on the three following arguments. First, it is true that local
pH gradients can induce the formation of tubular structures \cite{50}. However, such gradients have a magnitude of \(\sim 4\) pH units, much higher than our 0.8 pH units. Then, as can be seen on our phase contrast images (data not shown) GUVs become permeabilized during pulsation, and experience a mixing of their internal and external media. Thus the initial pH asymmetry should disappear after a few permeabilizing pulses. Finally, the observation that the vesicles are stable and do not exhibit any shape changes until the electric field is applied corroborates the fact that the initial pH asymmetry of our GUVs has no significant effect.

**Mechanisms of lipid loss**

One of the most fascinating aspects of the experiments is the wide variety of mechanisms of lipid loss that can be observed. Three different mechanisms of lipid loss are observed when the lipids are fluorescently marked as is the case on our experiments on DOPC liposomes (these observed mechanisms do not show any appreciable dependance on the probe employed). We emphasize here that the term lipid loss implies loss of lipid from the bulk spherical part of the vesicle, the lipid ejected appears in most cases to remain attached to or close to the parent vesicle.

The three basic mechanisms are shown in Figs. 5 and 6. Images were taken with the confocal microscope.

The first and most frequent mechanism is the formation of small vesicles at both the anode and cathode-facing poles. Those vesicles are mainly thrown out of the GUVs, but some of them were also driven inside the GUVs. Liposomes A and C of Fig. 5 lost their lipids in such a manner (Movie S1 available online shows that mechanism for another GUV). Interestingly a similar phenomena has been reported when high frequency alternating electric fields are applied to sea urchin eggs \cite{51}, firstly the cell is deformed and elongated by the field and then this cell splits into two smaller cells and a number of much smaller vesicles.

The second phenomenon we could observe (see photographs for liposome B in Fig. 5) was the creation of lipid tubules on the exterior of the anode-facing hemisphere (Movie S2 available online shows that mechanism for liposome B). DOPC molecules expelled from the membrane rearranged in the form of tubular structures, whose lengths grew with the number of applied pulses. These structures initiated from the pole facing the positive electrode and remained attached to the vesicle. However they then appeared to diffuse away from the pole towards the equator (while remaining attached to the membrane) and appeared to cover most of the anode-facing hemisphere as shown in Fig. 5. We also saw on the cathode facing side of Fig. 6 that tubules can grow on the interior surface of the liposome. These structures also diffuse toward the equatorial regions, the number and size of tubules however being smaller. This mechanism of tubule formation appears to be stronger on the anode-facing hemisphere.

Finally, we also noticed the presence of pores on the cathode-facing hemisphere (as did \cite{42}). This was a quite rare observation, but it is normal because our acquisition times were of a few hundreds of milliseconds, the same order of magnitude than the lifetimes of such pores \cite{42}. Liposome D, which has entered the SR after 16 pulses, is found to have pores after 16 and 18 pulses, as show
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On the next images, we can see the beginning of the formation of the tubular structures described previously. We thus conclude that those two mechanisms could occur together for a same vesicle. The fact that we detected only a few GUVs exhibiting pore formation is certainly due to the too low acquisition speed of our experimental setup. Recently it has been shown that pore formation can be induced in vesicles by solubilizing the membrane (52) and that this process of pore formation is also associated with membrane loss and thus vesicle shrinkage. An animation of the shrinkage of liposome D associated with pores and tubules formation is available online in Movie S3.

The eventual long term evolution of the structures described above (after pulsation has been stopped) varied from one experiment to another. The small vesicles, in most cases, diffused away from the liposome and the vesicle radius stayed constant. However the behavior of the tubular structures exhibited wide variation. Some of the tubules broke away from the GUV and diffused away, sometimes forming vesicles and sometimes not. Other tubules remained attached to the vesicles, exhibiting polymer-like fluctuations. In some cases they were reabsorbed into the GUV membrane after a time of the order of minutes. In fact, the eventual fate of tubules was strongly dependant on their environment, notably on whether other vesicles came in contact with them or not. In the cases where tubules were reabsorbed, the volume of the vesicle they were attached to increased and the final state of the vesicle was often non-spherical and appeared to be under little tension (in agreement with the idea that the attached lipids act as reservoir of lipid for the main vesicle).

DISCUSSION

Giant liposomes subjected to pulsed DC electric fields diminish in size and lose lipids via several observable mechanisms, vesicle ejection, tubule formation and pore formation. This is quite different to what is observed in living cells which tend to swell under electroporation (53–56). The experiments, along with the associated model, provide us with the following picture of lipid loss due to applied pulses. The lipid loss proceeds by a two stage process. First if the applied field is high enough a membrane passes from an inactive state where it has no induced defects to one where defects are present. We have seen that this process is of an exponential or character reminiscent of radioactive decay. Secondly, for DOPC composed vesicles, once defects are present the membrane loss per pulse is of the order of \( \lambda \approx 0.20 \) of the area in which the transmembrane potential exceeds the critical value, denoted here by \( \Delta \Psi_c \). From our estimate \( W_c = 0.65 \) obtained by fitting the average of all curves, we find that on average \( \Delta \Psi_c = W_c \times \Delta \Psi_0 = 0.65 \times 1.3 \text{ V} \approx 0.85 \text{ V} \). If we use the average value of \( \langle W_c^{fit} \rangle \) obtained by fitting the individual curves, we obtain \( \Delta \Psi_c \approx 0.75 \text{ V} \).

These values of \( \Delta \Psi_c \) are to be compared with those reported for certain cell membranes \( \Delta \Psi_c \approx 1 \text{ V} \) (3, 47) and tension free vesicles (1-stearoyl-2-oleoyl phosphatidycholine and dioleoyl phosphatidyglycerol) (26) where \( \Delta \Psi_c \approx 1.1 \text{ V} \). Similar results apply for EggPC but in this case, \( \lambda \approx 0.29 \) and there is thus, with comparison to DOPC, more lipid loss per unit area of where the
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critical transmembrane potential is exceeded. The estimated value of $W_c$ obtained by fitting the average of all curves is 0.77, which gives a critical transmembrane voltage of $1 \text{ V}$. The estimate from the average values obtained over individual fits yields a value of 0.69 for $W_c$, thus leading to a critical transmembrane voltage $\Delta \Psi_c \approx 0.89 \text{ V}$.

Recently numerical simulations have provided much insight into the membrane organization occurring during the membrane permeabilization process (35–41). The picture emerging is one where the strong electric field present in the membrane causes water molecules (via their dipole interaction with the applied field) to penetrate into the membrane. There is an initial formation of so called hydrophobic pores because the water molecules are in proximity to the hydrophobic core of the membrane. Subsequently the lipid head dipoles re-orientate to form hydrophilic pores where the lipid heads line the inside of the pore. The mechanism behind this reorientation involves hydrophobic effects and electrostatic effects. For example dipole moments which are orientated normal to the membrane surface (which is roughly the case for DOPC) are favorably aligned on one side of the membrane but not on the other. This means that on the side where they are well oriented the field keeps them straight toward the normal. However on the side where they are mal-orientated they can lower their energy by turning in toward the core of the membrane. This tendency to turn inside the membrane lowers their electrostatic energy and aids the formation of hydrophilic pores. The same effect is clearly present before water penetration into the bilayer core and helps to form defects which favor penetration by water molecules. This explains why formation appears to be initiated from a particular membrane side in electrically neutral membranes. However in numerical simulations lipid loss from the membrane is not generally observed during pore formation and pore resealing. This could be because the time scales over which the simulations are carried out are too short. Indeed it is difficult to see, if we accept the above image of the pore formation mechanism, how lipid loss to the extent observed in our experiments can be explained by such processes. The main differences between the experiments here and numerical simulations is that the system here is much larger and that the pores formed are an order of magnitude larger than those seen in simulations (which can be interpreted as pre-pores). We have seen that vesicle formation seems to make a major contribution to the observed lipid loss and there is presumably a minimal size that a vesicle can have (for thermodynamic and mechanical reasons), thus if the simulated system contains less lipids than required to build a vesicle of minimal size then lipid loss by vesiculization cannot be observed. Another possible mechanism for lipid loss is that lipid head group dipoles which are mal-orientated, instead of turning into the membrane to be better orientated are simply expelled from the membrane. This expulsion will increase the free energy of the lipid due to hydrophobic interactions but lower the electrostatic energy. The hydrophobic component of the free energy increase could be lowered by the formation of small vesicles into which these expelled lipids could be incorporated. We recall that in smaller vesicles the electrostatic energy of mal-orientated lipid head group dipoles is much smaller due to the scaling with $R$, the vesicle radius, of the potential drop across the membrane. The idea that single lipids can be extracted due to the field turns out to be unrealistic. The dipole moment $p$ of the PC head group is about 20 Debyes (see (57) and references therein), this means that the maximal electrostatic energy of a mal-oriented dipole
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is of the order \( E_D \approx p(\Delta \Psi/a) \), where \( \Delta \Psi \) is the potential drop across the membrane. However the hydrophobic energy of a lipid tail placed in water is given by \( E_{\text{hydro}} \approx 2\pi \rho l \mu \) where \( l \) is the total length of the hydrocarbon chain and \( \rho \) is its effective radius (viewed as a cylinder). Clearly the tail length is approximately related to the membrane thickness by \( l \approx a/2 \). The term \( \mu \) is a hydrophobic free energy per unit of area and takes a value of about \( 40 \text{ mJ/m}^2 \) \((58)\). The effective cylindrical radius of the lipid hydrocarbon tail is estimated at \( 0.8 \text{ nm} \) (there is of course really two tails each of radius approximately \( 0.4 \text{ nm} \) \((58)\)). Equating these two energies yields a critical transmembrane potential beyond which lipids can be torn out directly by the field as

\[
\Delta \Psi^* \approx \frac{\pi a^2 \rho \mu}{p} \approx 24 \text{ V}.
\]

This value of \( \Delta \Psi^* \) is to be compared with the value given typically for the critical potential drop across the membrane necessary to achieve permeabilization which, as previously mentioned, is about \( 200 \text{ mV} \) for a wide range of membrane types. In addition the electric field seen by the lipid heads is only the amplified one if we assume that the head region is of low conductivity having a value close to that cited for the total membrane conductivity. We thus conclude that for permeabilization seen in the range of voltages of our experiments, a simple mechanism of tearing out lipids is unlikely to occur (although this mechanism could conceivably play a role when high intensity short pulses are applied). The conclusion of the above estimation is that lipids must be ejected together in structures that minimize their hydrophobic energy such as micelles, tubules and vesicles as is indeed seen in our experimental results.

There is a clear asymmetry in our observations of lipid loss, in agreement with the observations of \((42)\) when we observed pore formation it was on the cathode facing side of the liposome. However the anode facing side was the one where the formation of tubules was favored. The mechanism of symmetry breaking could well be related to the anisotropic dielectric structure of the membrane due to the behavior of its lipid components.

Another interesting feature of our results is that the vesicle does not always lose lipid material from the first pulse onwards. This implies that the vesicle needs to be in a particular state (induced by the field with some probability) in order to enter into the shrinking regime (SR). The difference between the SR and pre-shrinking regime is unclear, but one could speculate that in the SR the membrane has defects which facilitate the loss of lipids. The number and nature of defects created at the inception of the SR is presumably stochastic in nature and could be responsible for the variations in the parameter \( \lambda \) seen in our experiments. The continued application of pulses then leads to a number of visible modes of membrane loss, vesicle formation, tubule formation and pore formation. In the context of applied DC pulses only pore formation has been previously reported \((42)\). Vesicle formation due to alternating fields has been reported \((51)\) but the underlying physics appears quite different as in the presence of AC fields the formation of small vesicles occurs via the fission of the initial cell into two similar sized daughter cells. Perhaps the most striking phenomenon is that of tubule formation which leads to a hair like structure of tubules around the liposome. Thus repeated application of short DC pulses leads to the shrinkage of artificial vesicles and a
rich phenomenology of lipid structure formation. As a final comment the phenomenon of lipid loss observed here seems to support aspects of the phase transition model of electroporation (59). In this model the electric field can induce a transition from a state where the bilayer is thermodynamically stable to one where smaller units, for example micelles, are thermodynamically preferred. The fact that the lipid loss process is not always immediately initiated, when $N_c \neq 1$, supports the first order nature of the transition.

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Figure Legends

Figure 1. Normalized distribution of the values of $N_c$ obtained after fitting of experimental data for DOPC vesicles. Solid line is a fit to a geometric distribution of the form given in Eq. 14, yielding the value $q = 0.33$.

Figure 2. Examples of experimental data and corresponding fits for DOPC liposomes. Top Left: liposome A; fit results are $N_c = 6$, $\lambda = 0.13$ and $W_{c}^{fit} = 0.35$. Top Right: liposome B; fit results are $N_c = 9$, $\lambda = 0.19$ and $W_{c}^{fit} = 0.69$. Bottom Left: liposome C; fit results are $N_c = 1$, $\lambda = 0.15$ and $W_{c}^{fit} = 0.51$. Bottom Right: liposome D; fit results are $N_c = 16$, $\lambda = 0.30$ and $W_{c}^{fit} = 0.68$. Pulse magnitudes are 290, 360, 235 and 300 V/cm respectively. Pulse duration is 5 ms. Arrows, if present, indicate data just before which we had to re-center the image on the liposome of interest. There is thus a time interval of $\approx 10$ s before the indicated point, instead of 2 s as in all other cases.

Figure 3. Distribution of the values of $\lambda^{fit}$ obtained after data fitting for DOPC liposomes.

Figure 4. Distribution of the values of $W_{c}^{fit}$ obtained after data fitting for DOPC liposomes.

Figure 5. Images of liposomes A, B and C, composed of DOPC and labeled with Rhodamine PE, at times indicated by the diamonds in Fig. 2 corresponding to 0, 12 and 24 applied pulses. Liposomes A and C lose lipids by formation of vesicles, and liposome B by formation of tubules. Scalebars (20 µm length) and positions of the electrodes appear in the first photograph of each vesicle. Pulse magnitudes are 290, 360 and 235 V/cm respectively. Pulse duration is 5 ms. Times in upper right corners indicate when images were acquired, the time origin being the onset of the first pulse. No time indication means that the picture was taken before the first pulse.

Figure 6. Images of liposome D, composed of DOPC and labeled with Rhodamine PE, at times indicated by the diamonds in Fig. 2. Image D1 is acquired after 15 pulses, D2 after 16 pulses, D3 after 17 pulses, etc. We can see pores on pictures D2 and D4 on the cathode-facing hemisphere. Scalebar
(20 µm length) and position of the electrodes appear in the first photograph. Pulse magnitude and duration are 300 V/cm and 5 ms. Times in upper right corners indicate when images were acquired, the time origin being the onset of the first pulse.
Figure 1:

Normalized distribution of $N_c$

geometric fit ($q=0.33$)
Figure 2:
Figure 3: Normalized distribution of $\lambda_{fit}$:

Number of occurrences / number of events

Fraction of permeabilised area lost per pulse estimated from fitting $\lambda_{fit}$.
Figure 4: Normalized distribution of $W_c^{\text{fit}}$. The rescaled critical radius estimated from fitting $W_c^{\text{fit}}$ is shown, with the normalized distribution indicating the number of occurrences per number of events.
Figure 5:
Figure 6: