

Direct Observation of Post-Sonoporation Membrane Resealing in the Giant Unilamellar Vesicle Model

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Sonoporation is a microbubble (MB)-mediated ultrasonic cavitation, which has attracted much attention in recent years due to its high efficiency and precision in non-invasive drug and gene delivery (Bouakaz et al., 2016; Helfield et al., 2016). However, the cell spontaneous repair process of the membrane after sonoporation, especially the dynamic mechanism of membrane resealing, has not been fully elucidated (Rich et al., 2022). Among them, a key mechanistic question is whether biophysical forces (such as membrane tension) play an important role in the membrane resealing process. However, this issue remains challenging due to the large number of biological processes in cells that may affect the dynamic behavior of membranes, such as cytoskeletal rearrangement (Chen et al., 2014) and endocytosis (Delalande et al., 2015; Zeghimi et al., 2015). To address this issue, this study innovatively used giant unilamellar vesicles (GUVs) composed only of phospholipid bilayers as a minimally simplified membrane research model to explore the membrane reseal mechanism in sonoporation. In the methodology, GUVs were successfully prepared by electroforming. Combined with a coupled ultrasound platform of fluorescence microscopy and high-speed camera, the GUV and microbubble mixed system was exposed in a precise controllable ultrasound field (ultrasonic frequency: 1 MHz, 10% duty cycle, 1Vpp) to simulate the sonoporation. The experimental results showed that GUVs were able to spontaneously reseal their membrane pore after sonoporation, indicating that the phospholipid bilayer has the ability to self-reseal even without the contribution of cell-mediated physiological processes. Further statistical analysis showed that the size of the microbubble had a significant effect on the GUV self reseal results: smaller microbubbles (2–3 μm in diameter) usually formed reversible and easily healed micropores, while larger microbubbles ($>8 \mu\text{m}$) often led to severe holes that were difficult to bridge ($p < 0.01$), ultimately destroying the stability of the vesicles. This discovery demonstrated for the first time the key role of biophysical forces in the membrane reseal stage after sonoporation. This study not only provides a new experimental model and platform based on GUVs, laying the foundation for future studies of the biophysical mechanism of sonoporation, but also provides a new perspective for a deeper understanding of the biophysical nature of membrane reseal.

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The journey was not easy, but along the way, I have been fortunate to meet some wonderful people and discover fascinating things. I am deeply grateful to everyone I have had the opportunity to work with—for tolerating my imperfections. And to myself—thank you for pushing through and persevering. The unknown road ahead is even longer, please walk bravely with the lovely people!

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List of Abbreviations

C ₄ F ₁₀	Perfluorobutane
CAD	Computer-Aided Design
Chol	Cholesterol
DC	Duty Cycle
DIC	Differential Interference Contrast
DPPC	1,2-Dipalmitoyl-sn-glycero-3-phosphocholine
DSPC	1,2-Distearoyl-sn-glycero-3-phosphocholine
DSTAP	1,2-stearoyl-3-trimethylammonium-propane
FDA	Food and Drug Administration
FI	Fluorescence Intensity
GUV	Giant Unilamellar Vesicle
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIFU	High-intensity focused ultrasound
HMC	Hoffman Modulation Contrast
ISPTA	Intensity Spatial-Peak Temporal-Average
ITO	Indium Tin Oxide
L _α	Liquid crystal phase
MB	Microbubble
MI	Mechanical Index
mRNA	Messenger ribonucleic acid
PBS	Phosphate-buffered saline
POPS	Palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine
PRF	Pulse Repetition Frequency
PRP	Pulse Repetition Period
RT	Room temperature
siRNA	Small interfering ribonucleic acid
TI	Thermal Index
T _m	Phase transition temperature
US	Ultrasound

List of Symbols

δ	Duty cycle
τ	Duration of a single ultrasound pulse
d	Electrode spacing
E	Mean electric field strength
f	Center frequency of the transducer
f_{end}	Final applied frequency during GUV electroformation
f_{start}	Initial applied frequency during GUV electroformation
p	Spatial peak pressure
Δt	Duration of transducer operation
V	Peak value of the applied voltage
V_{end}	Final applied voltage during GUV electroformation
V_{start}	Initial applied voltage during GUV electroformation
W	Acoustic power
W_{deg}	Acoustic power required to increase the temperature by 1°C
$W_{Transducer}$	Constant power of the transducer

Chapter 1 Introduction

1.1 Chapter Overview

The synergistic effect of ultrasound (US) and MB holds great promise for non-invasive drug and gene delivery applications. In short, under ultrasound irradiation, MB undergoes acoustic cavitation, thereby inducing sonoporation, forming temporary pores in the cell membrane, allowing drug molecules to penetrate (Bouakaz et al., 2016). However, the clinical application of sonoporation is still limited, and the key obstacle to continued success is that the biophysical mechanism behind it is not fully understood, especially the role of the biophysical force of the membrane in the resealing process is still unclear. The study of this issue is challenging due to the large number of biological processes in cells (such as cytoskeleton rearrangement and endocytosis), which naturally affect the dynamic behavior of the membrane. To this end, this study uses GUV, an "artificial cell membrane model", to construct a new experimental model for the study of sonoporation dynamics, in order to explore the membrane repair process and its key influencing factors in a more pure membrane structure environment. On this basis, it is necessary to first understand the following: What is sonoporation? What is GUV? How does GUV serve as an experimental model? Therefore, in Section 1.2, I will introduce the relevant background knowledge, including: 1) Current drug delivery applications based on sonoporation; 2) Its basic principles and ultrasound experimental settings; 3) The structural characteristics and preparation methods of GUVs. After the background information is clear, Section 1.3 will further explore the key issues of scientific research, including research gaps, research motivations, hypotheses, and research goals.

1.2 Background

1.2.1 Drug delivery and gene therapy applications based on sonoporation

In the field of modern biomedicine and pharmaceuticals, achieving precise and controllable drug delivery has always been the goal pursued by researchers. Ideally, drugs should reach target cells directly and quickly and exert therapeutic effects without damaging normal tissues of the body (non-invasive). However, as a natural barrier between cells and the external environment, cell membranes usually have low permeability to macromolecular drugs, proteins or nucleic acid molecules, which poses a huge challenge to efficient drug delivery. In recent years, the sonoporation based on ultrasound technology has provided a new solution to this problem. In short, this technology refers to the oscillation or collapse of MBs when US waves and MBs act synergistically on target cells, accompanied by strong shear effects such as impact flow, thereby forming temporary holes in the membrane. These holes allow chemotherapy drugs, proteins, and gene fragments (such as siRNA (small interfering ribonucleic acid), mRNA (messenger ribonucleic acid), plasmid DNA) (Miller et al., 2002; Fan et al., 2014) that were originally unable to penetrate the cell membrane to enter the cell in large quantities in a short period of time. Its main advantages include: 1) high localization (Iwanaga et al., 2007); 2) precise and controllable delivery (Fan et al., 2012); 3) applicable to a variety of macromolecular drugs (Helfield et al., 2016). Based on this, the technology has been

initially explored in multiple scenarios in clinical applications and has been shown to be able to efficiently deliver drugs to cancer cells and liver cells. Although its potential application space is broad, the biophysical mechanism (especially the membrane reseal mechanism after perforation) has not been fully clarified, which has become the main obstacle to its large-scale clinical promotion.

1.2.2 Ultrasound and energy transfer into tissue

In the field of medicine and bioengineering, ultrasound is usually generated by the piezoelectric effect of the transducer and transmitted to the target site through a medium (such as water, tissue, solution). During the transmission process, ultrasound is mainly propagated in soft tissue, blood or other body fluids in the form of mechanical waves, and depending on the medium characteristics and ultrasound parameters, part of the sound energy will be absorbed, scattered or reflected in the form of heat or mechanical vibration, thus affecting the actual energy distribution. Generally, the higher the ultrasound frequency, the more limited the penetration depth, but the resolution and local energy deposition are higher; while lower frequency ultrasound can reach deeper tissues, but it is difficult to maintain relatively fine focus and energy control (Leighton et al., 2007; O'Brien et al., 2007).

In drug or gene delivery, pulsed waveform ultrasound is usually used to balance treatment efficiency and safety. If τ represents the duration of a single pulse and PRP (pulse repetition period) is the pulse interval, the duty cycle (δ) is defined as:

Equation 1

$$\delta = \frac{\tau}{PRP}$$

The transducer outputs energy intermittently under pulse excitation to avoid excessive heat deposition caused by long-term continuous irradiation. When δ increases, the energy transmitted to the medium per unit time also increases accordingly. In addition, increasing the pulse repetition frequency (PRF) or exposure duration will also increase the total acoustic energy delivery by increasing the number of ultrasound pulses. In theory, the total energy transmitted to the medium can be approximately quantified as:

Equation 2

$$E = W_{Transducer} \times \Delta t$$

Where W is the constant power of the transducer and Δt is the working time.

Unlike diagnostic ultrasound (such as B-ultrasound), the common clinical application frequency band of therapeutic ultrasound is 0.5 ~ 3 MHz (Miller et al., 2012), of which around 1 MHz is most used for drug delivery and tumor treatment. In the drug delivery scenario, the δ of pulsed ultrasound (Pulsed US) is about 10% to 20% (Pan et al., 2005). If evaluated by spatial peak and time average intensity (ISPTA), the common energy density is about 0.5 to 5 W/cm², but for High-intensity

focused ultrasound (HIFU) tissue thermal ablation, the instantaneous power at the focus can reach hundreds of W/cm², which is mainly used for tissue destruction (Izadifar et al., 2020). The U.S. Food and Drug Administration (FDA) stipulates that the ISPTA exposure limit for ultrasound is 720 mW/cm² (Nelson et al., 2009). If only the sonoporation is pursued, the required intensity is relatively low because it mainly relies on mechanical vibration rather than thermal effects. Therefore, in US research based on drug delivery, multi-parameter coordinated optimization is required to take into account both transmembrane delivery efficiency and safety to surrounding tissues.

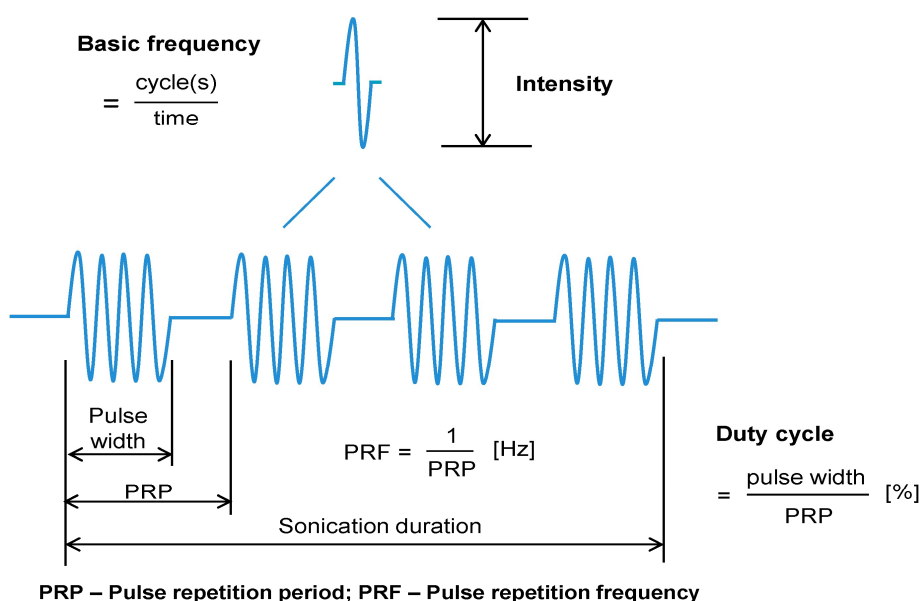


Figure 1 Schematic diagram of ultrasonic pulse parameters: relationship between frequency, duty cycle and pulse.

1.2.3 Sonoporation

1.2.3.1 The ultrasound contrast agent: microbubbles

Ultrasound contrast agents are a type of gaseous microbubbles containing an inert, low-solubility gas core that can enhance the contrast inside blood vessels by scattering ultrasound, thereby improving the quality of ultrasound images. Such microbubbles are usually composed of a phospholipid membrane or a protein membrane and a gas core, and are usually 1-10 μm in diameter (Stride et al., 2020). Microbubbles are usually used clinically in diagnostic ultrasound, and the prepared microbubble contrast agent is injected into the patient's body through intravenous injection or infusion pump, usually 1-2 mL (Sirsi et al, 2009; Roovers et al., 2019). Microbubbles can not only be used as contrast agents for ultrasound imaging but can also be regarded as triggers for sonoporation. When the surface components or shell materials of the lipid envelope of microbubbles are modified, for example, by modifying antibodies, peptides or ligands on the microbubble shell layer (Sirsi et al, 2009; Roovers et al., 2019), the microbubbles can be enriched on specific tissues or target cell receptors, thereby inducing the sonoporation(the specific mechanism of the sonoporation will be described in 1.3.1.3) on the target cells under the action of ultrasound, which

can significantly improve the accuracy of drug delivery. Although it has been widely proven that the microbubble-mediated sonoporation can effectively improve the efficiency and accuracy of drug delivery, especially for immune cells and cancer, the biophysical mechanism behind it is still incomplete and needs to be further elucidated, especially the details of how microbubbles interact with membranes lack a systematic and complete understanding.

1.2.3.2 Ultrasound-induced MB cavitation

Ultrasound waves propagate in the form of mechanical waves, and the molecules of the medium gather and disperse periodically in space and time, thus forming a phenomenon in which high-pressure areas (compression) and low-pressure areas (expansion) appear alternately in the direction of the wave advance. For microbubbles, the negative pressure (expansion) stage makes the bubble volume tend to expand, and the positive pressure (compression) stage makes it shrink, causing it to oscillate, expand or collapse periodically. According to the different oscillation amplitude and energy intensity, cavitation can be divided into stable cavitation and inertial cavitation, both of which will produce significant mechanical effects on the surrounding medium or biofilm. Stable cavitation usually occurs when the sound pressure amplitude is relatively low, and the microbubbles will expand and contract periodically and evenly with the ultrasound. This process can form annular microstreaming around the microbubbles, causing local fluid shear force. When the sound pressure intensity is high enough, or the microbubbles are expanded, the positive pressure period will cause them to collapse or explode sharply in a short time, eventually causing MB destruction and fragmentation, which is inertial cavitation. When the gas core of MB collapses suddenly, the surrounding fluid gathers to the collapse point, forming a microjet with strong destructive power.

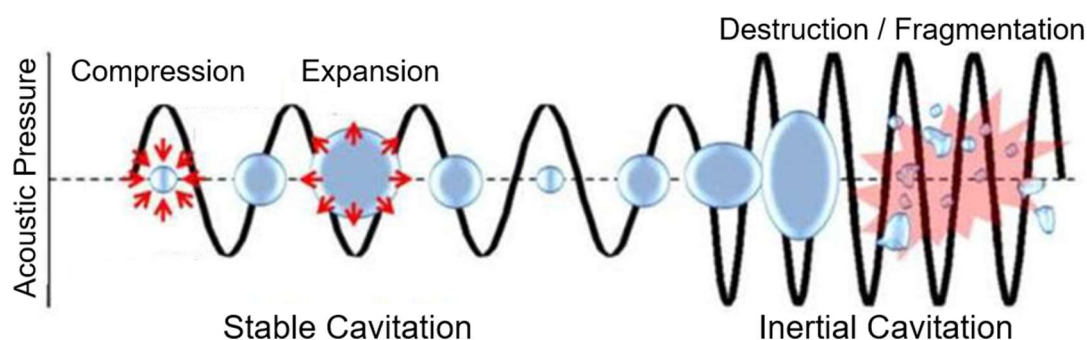


Figure 2 Microbubble cavitation: the formation and difference between stable cavitation and inertial cavitation (Adapted from Kaykanat et al, 2023).

1.2.3.3 Physical interaction between membrane and MB cavitation

In the previous section, two modes of MB cavitation have been discussed, and different cavitation modes correspond to different mechanisms of sonoporation. However, from a biophysical point of view, the essence of cavitation is the interaction between microbubbles and membranes during cavitation, which mainly involves mechanical impact, membrane structural changes, and membrane reseal dynamics (reseal process will be further discussed in 1.2.3.4). If the microbubbles produce

stable cavitation and are located in the vicinity of cells, their symmetrical or slightly asymmetrical oscillations can periodically impact the plasma membrane in a "push-pull" manner (Fan et al., 2014), destroying the integrity of the plasma membrane, similar to the "cell massage" effect. In addition, as mentioned in the previous section, stable cavitation can induce the generation of microfluids, generating additional shear forces on the membrane surface, which may be as high as thousands of kilopascals, enough to tear or loosen the membrane structure at the nano or micro scale, thereby inducing a transient increase in membrane permeability (Alfred et al., 2022). When MB undergoes inertial cavitation, the microbubbles will completely explode in a very short time, and the energy they carry will be transferred to the surrounding membrane structure in the form of shock waves or microjets near the collapse point. High-speed jets or impact forces can often form relatively large pores on the membrane, forming micro-scale ultrasonic pore sites, and even submicron pores, which can cause severe perforation or even cytoskeleton rupture (Alfred et al., 2022).

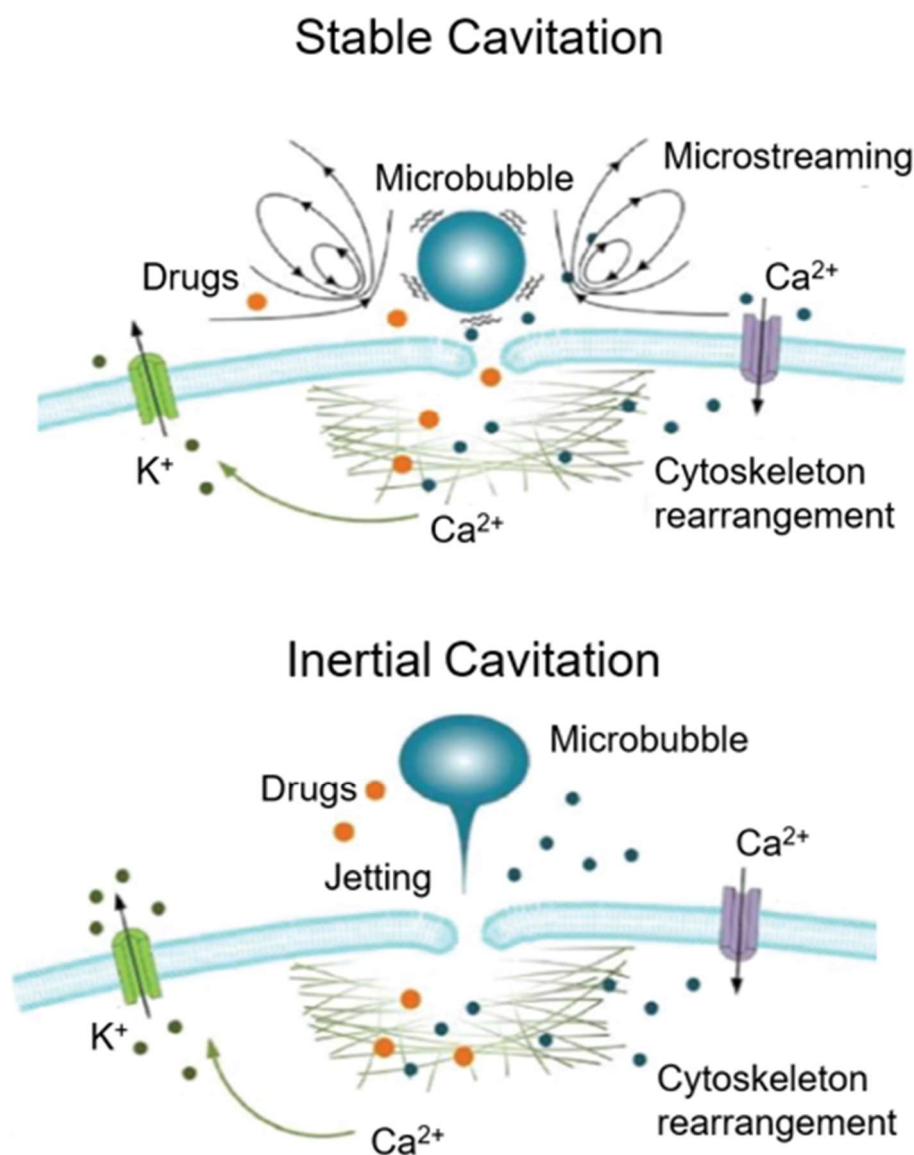


Figure 3 Schematic diagram of different Sonoporation mechanisms corresponding to

different cavitations (Adapted from Alfred, 2022).

1.2.3.4 Biophysical changes of membranes after sonoporation

After the sonoporation, it has been proven that the cell membrane will enter a reseal process, and the membrane structure will reclose the membrane pores and restore integrity within a certain period (Bouakaz et al., 2016). The evolution of the cell membrane from milliseconds to seconds or longer after the end of sonoporation has been recorded in real time by confocal microscopy, proving that the pore size of the cell membrane pores can gradually decrease and recover to the intact membrane surface. During the recovery process, it has been proven that cell biological structures such as actin filaments and membrane skeleton proteins synergistically participate in the dynamic process of cell membrane reseal, accompanied by changes in transmembrane ion channels such as Ca^{2+} influx and K^{+} efflux (Fan et al., 2014). In some cases, the membrane pores will also "swallow" the broken fragments into the cell through endocytosis for reuse or degradation, achieving a one-time complete "suturing" (Li et al, 2021). In addition, although studies have shown that the membrane will produce a local tension gradient during the pore healing process, prompting phospholipid molecules to move to the pore edge and assist in sealing, an important question related to the membrane reseal mechanism is whether biophysical forces (such as membrane tension) play an important role in membrane resealing and the specific role they play in the stage is still unclear. Because there are a large number of intracellular biological processes and subcellular dynamics that naturally affect the dynamic behavior of the cell membrane, such as cytoskeletal rearrangement and endocytosis.

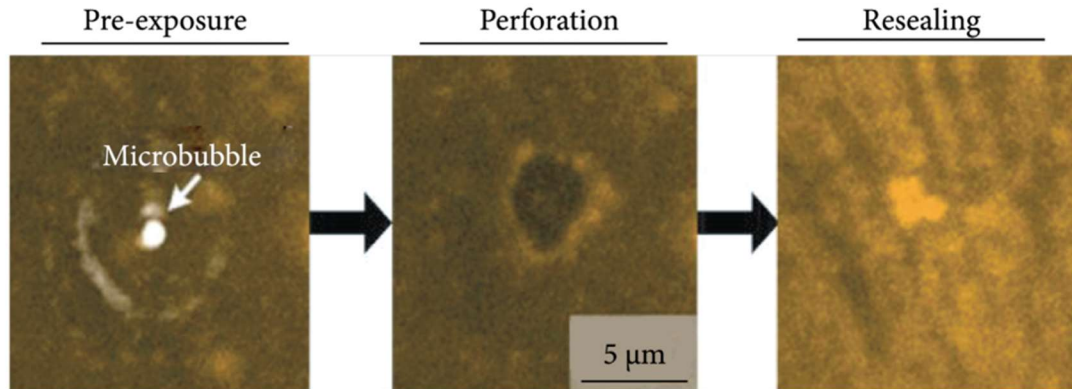


Figure 4 Cell membrane perforation and self-reseal process under sonoporation (Hu et al, 2013).

1.2.3.5 Parameters for sonoporation

1.2.3.5.1 Ultrasound factors

In sonoporation-mediated drug delivery, ultrasound factors such as ultrasound frequency, acoustic pressure, and exposure duration interact with each other to affect the cavitation behavior of microbubbles and the result of cell membrane permeability. Typically, an ultrasound frequency of 1 MHz is often used because it best matches the resonance conditions of most commercial

microbubbles (diameter of about 1–3 μm) (Cosgrove et al., 2009). However, for microbubble solutions with a larger distribution range, lower frequencies (such as 500 kHz) may be more efficient in energy coupling, resulting in stronger cell permeability enhancement but also accompanied by higher cell damage. In terms of acoustic pressure, the peak negative pressure not only determines the size of the membrane pores but also affects the contradictory relationship between cell viability and transfection efficiency: the higher the acoustic pressure, the larger the pores and the higher the drug delivery efficiency, but the cell survival rate is negatively correlated (Coussios et al., 2008). When the acoustic pressure is too high, it may even lead to transient cell lysis and apoptosis. In addition, the exposure duration (which can be adjusted by parameters such as pulse length, PRF, duty cycle and total US exposure time) will further affect the "accumulated acoustic energy", which in turn affects the balance between pore formation and repair: an increase in total time or duty cycle usually increases membrane pore size and transfection efficiency but reduces cell viability (Delaney et al., 2022).

Table 1 Effects of ultrasound parameters on sonoporation transfection efficiency, cell survival rate and repair rate (Adapted from Alfred, 2022).

Impact factors		Sonoporation outcomes		
Category	Range	Transfection efficiency	Cell viability	Repairing rate
Total time↑	0.1-900 s	↑	↓	
Pulse length↑	4-1000 μs	↑	↓	↓
Duty cycle↑	2%-90%	↑	↓	↓
PRF↑	10-3000 Hz	↑	↓	

1.2.3.5.2 Microbubble factors

In ultrasound-mediated sonoporation, microbubbles play a key role as "cavitation nuclei". Changes in microbubbles can also significantly affect the sonoporation, such as the size, concentration and material of microbubbles. As mentioned in the previous section, microbubbles will produce stronger oscillations or collapse under ultrasound drive that matches their resonant frequency. Therefore, under 1 MHz ultrasound, microbubbles of 1~3 μm are more likely to obtain effective cavitation (Cosgrove et al., 2009). For ultrasound fields with lower frequencies, large-sized microbubbles are usually more likely to oscillate with high amplitudes, resulting in stronger cell permeability enhancement. In addition, the increase in microbubble concentration can effectively reduce the cavitation threshold and enhance membrane permeability and transfection efficiency. The reason is that more microbubbles attached to the target cells can cause cavitation synchronously or in relay at the same sound pressure level, increasing the local impact force and the probability of pore formation. However, as the concentration continues to increase, cell viability is bound to be more significantly affected, and even at high concentrations, a large number of cells will be lysed, and the membrane will be irreversibly damaged (Roovers et al., 2019). In addition, the material of MB will also greatly affect the sonoporation effect. The cavitation activity can be enhanced by modifying the microbubble shell or adjusting the gas core. For example, modifying the ligand, antibody or changing the shell surface charge (Luo et al., 2017) on the microbubble shell can not only shorten

the distance between the microbubble and the target cell, but also provide more accurate membrane pore positioning under ultrasound induction, which is crucial to further improve the controllability and stability of the sonoporation.

1.2.3.6 The safety indexes: medical index (MI) and thermal index

(TI):

To balance the production of controlled membrane pores in target cells or tissues and avoid excessive damage, safety indexes are particularly important in the research and application of sonoporation. The two commonly used physical quantities to measure ultrasound safety are the mechanical index (MI) and the thermal index (TI). The mechanical index is mainly used to quantitatively evaluate the ability of ultrasound to induce inertial cavitation. Its relationship with spatial peak pressure (p) and center frequency of the transducer (f) can be expressed by the following formula:

Equation 3

$$MI = \frac{p}{\sqrt{f}}$$

The larger the MI value, the higher the possibility of strong microbubble collapse (inertial cavitation), and the greater the risk of tissue damage. In medical diagnostic ultrasound, $MI \leq 1.9$ is usually considered the upper limit of the safety range (Nelson et al., 2009). The thermal index (TI) is used to estimate the potential temperature rise caused by the absorption of ultrasound energy at the focus or target area. It can be expressed by the following formula:

Equation 4

$$TI = \frac{W}{W_{deg}}$$

Where W is the acoustic power of ultrasound at the target site; W_{deg} is the minimum power required to raise the temperature of the target tissue by 1 °C. In routine diagnosis or low-intensity ultrasound drug delivery, in order to reduce thermal damage to surrounding normal tissues, it is usually hoped that TI will be kept at a relatively low level (within 1~2). According to FDA standards, the maximum Ti is 6.0, except for fetal or ophthalmic applications (Leighton, 2007).

1.2.4 Ultrasound setups

Ultrasound devices are usually regarded as a controllable experimental platform, which can produce predictable biological effects (such as cavitation or thermal effects) in target tissues or cell suspensions by setting the parameters of ultrasound. Whether in vivo or in vitro, the design of the platform focuses on maintaining the controllability and repeatability of experimental conditions and

treatment results. For the research of ultrasound experimental platforms that can be visualized, the platform architecture generally includes the following five core components: 1) Signal generator: responsible for generating signals, setting ultrasound waveform parameters such as pulse form, duty cycle and pulse repetition period, 2) Power amplifier: amplifying the low-power signal output by the function generator to a power level sufficient to drive the ultrasound transducer; 3) Transducer: convert electrical signals into mechanical vibrations (ultrasound waves), and different transducers also determine the focal characteristics, bandwidth and sound field distribution of ultrasound. 4) Sample cavity: a container for placing cell suspensions or samples, generally using transparent films or acoustic window materials (such as polyimide films, rigid glass) to reduce sound attenuation and reflection 5) Imaging and monitoring system: visualize the sonoporation and observe microbubble oscillation or cell membrane deformation in real time. In addition, coupling gel or water is usually used to match the acoustic impedance between the transducer and the sample cavity to facilitate the propagation of ultrasound. Therefore, the transducer and the sample cavity are usually placed in a water tank or connected through a waveguide containing coupling gel, as shown in the figure below. The following are several common visualization in vitro ultrasound devices: 1) The sample is placed in a culture dish in the transparent water tank, the inverted microscope lens images upward from the bottom of the water tank, and the transducer is immersed in the water tank, emitting ultrasound from the side. 2) A specially designed sealed culture chamber is placed on the microscope stage, the sample maintains a stable liquid surface in the chamber, and the transducer is located above to apply sound waves into it with gel or water as the coupling medium. 3) A customized waveguide is used to guide ultrasound to a small area under the focus of the microscope. The sample can be observed in the glass slide, and the tip of the waveguide is close to the sample area. Since it is desired to visualize the sonoporation, in this study, an ultrasound device based on a previously published paper in the laboratory (type 3 waveguide device) was optimized to track the dynamic process of the membrane (Hu et al., 2013). This type of setup helps to realize the pursuit of the sonoporation in two aspects: 1) achieving more localized acoustic energy concentration and higher coupling efficiency in a narrow imaging space; 2) facilitating combination with advanced optical tools such as high-magnification fluorescence imaging or high-speed cameras to capture the microscopic changes of microbubbles and membranes.

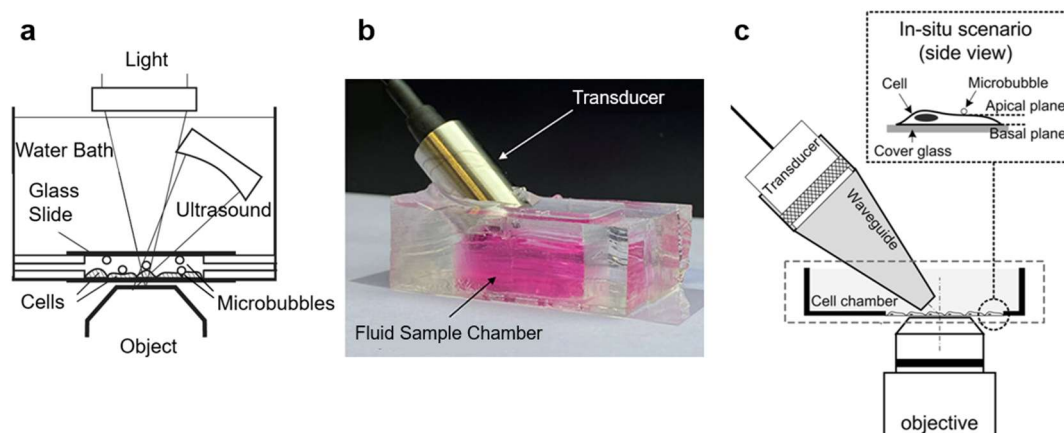


Figure 5 Different ultrasound setup methods for in vitro sonoporation: (a) ultrasound exposure device based on transparent water tank and culture dish, (b) ultrasound exposure

device based on sealed culture chamber, (c) ultrasound exposure device based on waveguide
(Adapted from Kudo et al., 2009; Pattinson et al., 2023; Hu et al., 2010).

1.2.5 GUV - artificial cell membrane model

1.2.5.1 Introduction of GUV

GUVs are liposomes composed of a single phospholipid bilayer encapsulating an aqueous phase. With diameters typically ranging from tens to hundreds of micrometers, GUVs retain the fundamental structural characteristics of a cell membrane while eliminating the complexity of biochemical processes in living cells (Fenz et al., 2012). Their size is comparable to various biological cells, enabling direct morphological and dynamic observations under optical microscopy. By employing specific fabrication techniques, such as electroformation or natural hydration, and incorporating different components into the phospholipid membrane, researchers can precisely regulate the membrane properties and internal environment of GUVs (Boban et al., 2021). This allows for systematic studies on how phospholipid bilayers respond to external stimuli (e.g., temperature fluctuations, mechanical stress) and their self-reseal mechanisms. Additionally, GUVs serve as a model system for investigating membrane dynamics, permeability, and drug carrier behavior (Chan et al., 2007). Due to their highly simplified yet customizable nature, GUVs offer unique advantages in biophysics, membrane science, and drug delivery research. They provide a “pure” experimental platform for studying membrane dynamics and functional properties. For instance, in sonoporation studies, GUVs enable high spatiotemporal resolution observations of pore formation and closure, offering critical insights into membrane perforation and reseal mechanisms.

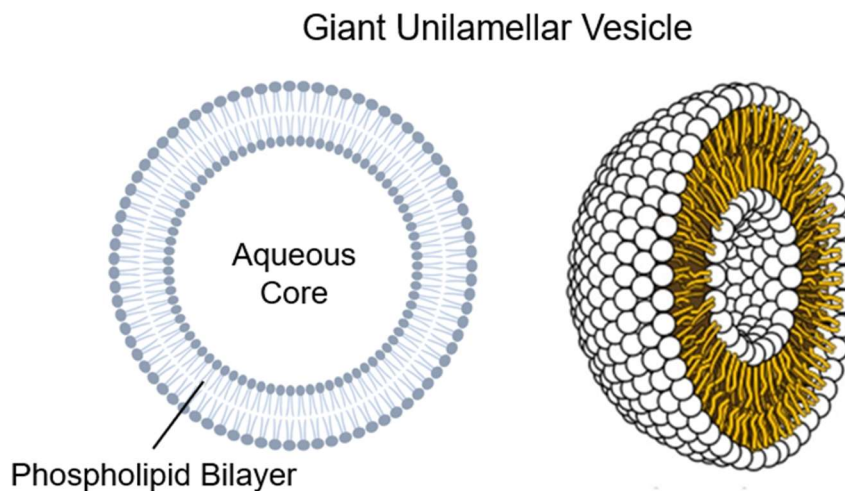


Figure 6 GUV plane and three-dimensional structure.

1.2.5.2 Preparation methods of GUV

Since their introduction, the preparation methods of GUVs have undergone significant technological

advancements. Initially, gentle hydration was used, where a dried lipid film was exposed to an aqueous phase, allowing slow swelling and vesicle formation. However, this method often resulted in multilamellar structures or a broad size distribution (Rideau et al., 2019). As research on the mechanical and thermodynamic properties of phospholipid membranes progressed, various refinements were introduced, such as incorporating osmotic gradients, buffer systems, or temperature control to enhance the unilamellarity and uniformity of GUVs. Despite these improvements, many methods still suffered from low reproducibility or difficulties in producing large, stable unilamellar vesicles.

In recent years, microfluidics-based strategies have enabled precise regulation of lipid concentration, flow rate, and interfacial interactions, achieving greater control and higher throughput in the production of vesicles ranging from the micron to the sub-millimeter scale (Staufer et al, 2021). However, these approaches typically require sophisticated equipment and are not suited for high-throughput production of GUVs in bulk (Robinson et al., 2019). Against this backdrop, electroformation has gained widespread popularity due to its simplicity and high yield. This technique involves applying an alternating electric field across lipid-coated electrodes, where heat and electrostatic forces facilitate the detachment and self-assembly of lipid molecules into large, unilamellar vesicles (the detailed mechanism will be discussed in the next section). Electroformation not only produces GUVs with a relatively uniform size distribution but also minimizes multilamellar contamination, ensuring predominantly unilamellar membranes (Drabik et al., 2018). For these reasons, this study employs electroformation as the primary method for GUV preparation, ensuring a high-yield, reproducible, and structurally consistent vesicle population. This provides an ideal experimental model for subsequent microscopic imaging and membrane biophysics research.

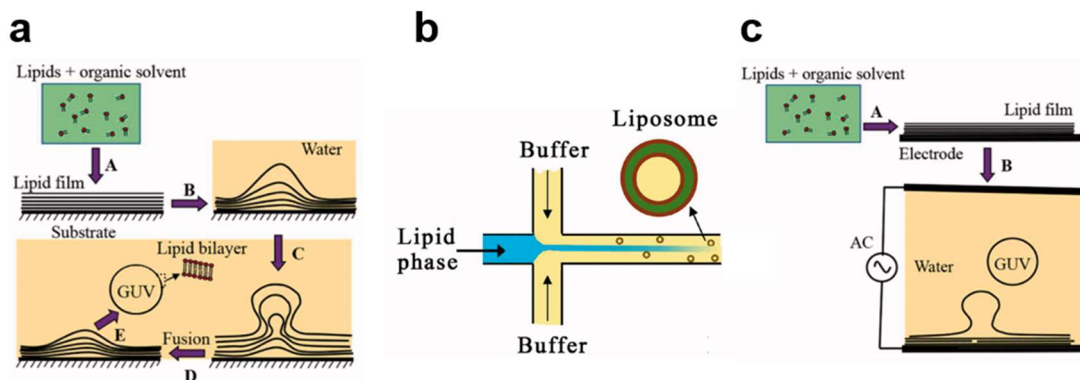


Figure 7 Different methods for preparing GUVs by (a) gentle hydration (b) microfluidic device (c) electroformation (Adapted from Paul et al., 2015).

1.2.5.3 GUV electroformation mechanism

The core concept of electroforming is to apply an alternating electric field between conductive substrates (such as Indium-Tin Oxide (ITO) glass slides, platinum slides) so that the dry lipid film is gradually peeled off and expanded to form a single-layer large bubble in a suitable temperature

and aqueous environment. Specifically, the lipids are initially close to the surface of the conductive glass slide in a dry state. By applying an alternating electric field and keeping the aqueous phase above the membrane at a temperature above the phase transition temperature (T_m), the membrane can gradually absorb water and peel off. The peeled lipid molecules spontaneously assemble into vesicles and absorb external water under the periodic push and pull of the electric field, and the volume increases accordingly. With the gradual change of the electric field parameters (such as high frequency and low voltage first, and then gradually increasing the voltage and decreasing the frequency), the lipids are fully peeled off in the early stage, and then grow steadily at a moderate intensity, eventually forming a single-layer, large-sized GUV.

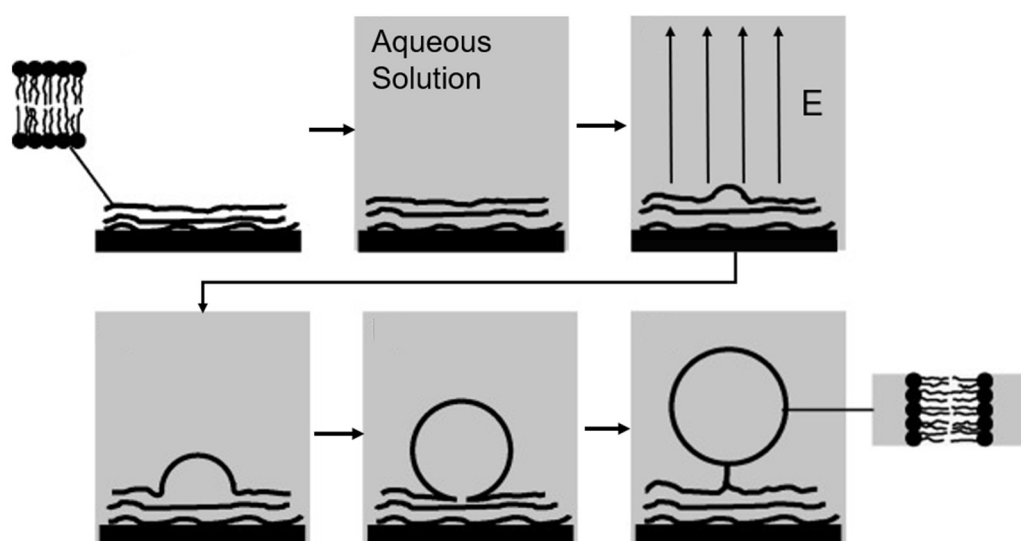


Figure 8 GUV formation process in aqueous solution electric field (Adapted from Walde et al., 2010).

1.2.5.4 GUV electroforming equipment

The design of electroforming equipment has its own characteristics, including the most common "ITO glass slide sandwich" chamber, commercial chamber with liquid exchange function, platinum sheet sandwich chamber, etc. They all apply an AC electric field to the dry lipid membrane coated on a conductive substrate (or copper, platinum and other electrodes) at high temperature to promote the phospholipid layer to peel off and aggregate into large-sized single-bilayer vesicles (GUVs) in sequence. In the classic "ITO glass slide sandwich" chamber method, the lipid membrane is usually evenly dripped on a glass slide with a conductive layer such as ITO glass. After drying, it is sandwiched with another electrode glass slide to form a narrow chamber, and pre-heated high-temperature buffer solution is injected. Then, a function generator is used to output a multi-stage voltage-frequency, from the initial high-frequency and low-voltage loosening of the membrane to the mid-to-late stage of boosting and reducing the frequency to make the vesicles expand stably, and finally a large number of GUVs can be generated. Some studies have chosen to use platinum sheets as the conductive layer. Although platinum sheets can be reused, which reduces costs, metal sheets

are prone to small surface bumps or brushed textures. If they are not finely polished, irregular accumulation is likely to occur during lipid film coating. At the same time, due to its strong reflective properties, it cannot be directly optically observed during electroforming (Pereno et al., 2017; Boban et al., 2021). Commercial integrated electroforming equipment is often too expensive and has low equipment flexibility, which may be limited for experiments that want to customize electrode spacing, gasket thickness, or special power plant frequency gradients. Therefore, the most common "ITO glass slide sandwich" chamber was used in this study to prepare GUV.

1.2.5.5 GUV electroforming parameters

1.2.5.5.1 Electric field strength and frequency

The high efficiency and simplicity of electroformation make it widely favored in the preparation of GUVs. However, during the electroformation process, the vesicle formation efficiency, size distribution and stability are affected by many parameters.

The electroformation process depends on the driving force exerted by the external AC electric field on the lipid layer. If the peak value of the applied voltage is V and the electrode spacing is d , the average electric field strength can be approximately expressed as

Equation 5

$$E = \frac{V}{d}$$

When the electric field strength is too low, lipid stripping is not sufficient and the vesicle yield is low; when it is too high, it may cause partial tearing of the lipid membrane or lead to multilayering. Frequency is also key: it is usually feasible in the range of 5~10 Hz, but some studies have shown that good GUVs can be produced in the range of hundreds of Hz. Low frequencies are more likely to drive lipid layer stripping and expansion, but if the frequency is too low or unsuitable, the vesicle growth rate is also limited. Therefore, initial stripping is usually performed at a higher voltage and higher frequency, and then the voltage and frequency are gradually reduced to allow the vesicles to grow and detach from the electrode surface (Politano et al., 2010).

1.2.5.5.2 Temperature and lipid solution formulation

Electroformation usually takes place in a lab oven because the process of electroformation requires constant heating: ensure that the entire process is carried out at a temperature that is ****higher than the main phase transition temperature (T_m) of phospholipids**** to ensure that the lipid membrane is in the liquid crystal phase (L_α phase), which is conducive to molecular movement and vesicle formation (Boban et al., 2021). If the lipid composition contains cholesterol or specific functionalized lipids, its phase transition temperature and fluidity will also change. Therefore, temperature control is required according to the actual membrane composition to ensure that the lipid sample is in the liquid crystal phase during the electroformation process. In addition, deionized water or an appropriate amount of buffer is usually injected between the electrodes in the chamber,

and solutes such as glucose and sucrose can be added to form a certain osmotic pressure gradient to help the vesicles separate from the electrode substrate and maintain stability after collection and reduce sudden changes in membrane tension (M    ard et al., 2009).

1.2.5.5.3 Electric field and frequency gradient process

Most electroforming processes do not use a fixed single voltage or frequency, but rather a gradual change in steps:

Equation 6

$$f(t) = f_{start} \rightarrow f_{end}$$

$$V(t) = V_{start} \rightarrow V_{end}$$

Where f_{start} and f_{end} are initial and final applied frequency during GUV electroformation, V_{start} and V_{end} are initial and final applied voltage during GUV electroformation. Gradual change in steps is because the kinetic requirements for lipids to peel off from the electrode surface and assemble into vesicles are different at different stages over time. Simply put, sufficient electric field "stimulation" is needed in the early stage to allow lipids to detach quickly, and a slightly lower intensity drive is needed in the later stage to allow the vesicles to expand stably and reduce excessive oscillations or multilayer membrane formation. Therefore, high frequency and low voltage are required in the initial stage. In the first few minutes, the membrane is still tightly attached to the electrode surface, and a faster frequency (such as 10 Hz) is needed to provide periodic alternating forces for lipid molecules so that they can loosen rhythmically and gradually peel off; at this time, if the voltage (i.e., electric field strength) is too high, the vesicle prototype is likely to be torn or expand irregularly before it is formed, resulting in multilayer stacking; therefore, only a low electric field (such as 0.1-0.2 V/mm) is needed in the early stage to allow lipids to "desorb" first and form preliminary vesicles (Politano et al., 2010). The voltage needs to be gradually increased in the middle stage. When lipids begin to peel off, vesicles sprout and gradually grow. At this time, the voltage should be increased (such as 0.7 V/mm or above) to ensure that there is sufficient field strength to help the newly formed vesicles to smoothly detach from the substrate and expand (Politano et al., 2010). In the later stage, the voltage and frequency need to be downgraded in both directions. When the vesicles have reached a considerable size, continuing to maintain high voltage or strong field strength may cause the formed large bubbles to rub against each other or fluctuate excessively, resulting in membrane extrusion or partial multilayering. Therefore, it is necessary to gradually reduce the voltage and continue to slow down the frequency in the later stage, so that the large vesicles can eventually detach from the electrode surface and stay still in the chamber in a low-disturbance environment (Politano et al., 2010).

1.3 Outline of thesis study

1.3.1 Motivation and hypothesis

As mentioned above, MB-mediated sonoporation offers great potential for controlled and targeted delivery of therapeutic agents. However, the fundamental reason for the lack of sustained clinical success is that the biophysics of sonoporation is not fully understood, and thus it is challenging to rationally induce sonoporation without causing lethal damage to cells. To address this significant knowledge gap, it is necessary to determine the basic biophysical details of membrane behavior after sonoporation, such as how the membrane reseals after pore formation. Such a mechanistic understanding will help to elucidate the biophysical factors that affect the resealing outcome and cell viability after sonoporation, thereby facilitating the improvement of the effectiveness of sonoporation-mediated drug delivery.

Regarding sonoporation biophysics, an important mechanistic question is whether biophysical forces (such as membrane tension) play an important role in membrane resealing. However, finding an answer to this question remains challenging because there are a large number of intracellular biological processes that naturally affect the dynamic behavior of cell membranes, such as cytoskeletal remodeling and endocytosis. In addition, membrane resealing exhibits heterogeneity in different cell types, as changes in cellular structure and function further increase the variability of the resealing process. Therefore, previous sonoporation biophysics studies on living cells have only demonstrated that membrane resealing after sonoporation may or may not occur, but it is still unclear whether this resealing process is mechanistically attributed to the biophysical forces acting on the cell membrane.

This research project aims to investigate the above question to find out whether the biophysical forces of the cell membrane contribute to the resealing of the membrane pore. Therefore, in this study, we innovatively used giant unilamellar vesicles as a new experimental model for sonoporation biophysics studies. As discussed in the previous sections, GUVs lack complex intracellular machinery and provide a clean and controllable system for studying membrane dynamics. Our core hypothesis is that in the absence of cellular biochemical machinery, GUV membranes may still exhibit the ability to self-reseal after sonoporation because the molecular forces on the phospholipid bilayer naturally act as the driving force for the autonomous restoration of membrane integrity.

1.3.2 Research objectives

To investigate the role of cell membrane biophysical forces (e.g., membrane tension) in the resealing of membrane pores after sonoporation, we developed an experimental framework based on giant GUVs as a model for sonoporation dynamic investigation, which includes: (i) preparing GUVs and self-assembled MBs by electroforming, (ii) designing an ultrasound-coupled exposure system combined with fluorescence microscopy and high-speed camera, (iii) exposing GUVs to MB-US within the coupled system, and (iv) analyzing membrane dynamics during and after sonoporation. Details of each research objective are described below: 1. Electroforming was used to prepare GUVs with a diameter of typically tens of micrometers, and their lipid membranes were fluorescently stained to ensure the visualization and controllability of their membrane structures. At the same time, through mechanical stirring of lipid solutions and low-solubility gases and ultrasonic oscillation

processes, MBs were self-assembled, and their shell materials and surface charges were modified for subsequent interaction with GUVs. 2. To apply ultrasound while ensuring microscopic imaging, we built an experimental device that integrates an ultrasonic transducer, a waveguide, and a microscope platform, so that GUV and MB can receive precise ultrasonic pulses under visual conditions. Fluorescence microscopy and high-speed camera functions are built into the system: the former monitors the distribution of fluorescent markers and pore formation on the vesicle membrane, and the latter captures transient processes such as MB collapse and vesicle membrane deformation. 3. Expose GUV to MB-US to induce sonoporation. In the experimental process, MB and GUV solution were mixed (~1:1) and then dropped onto a glass slide. 4. Then, the transducer was set with appropriate frequency, sound pressure, pulse duty cycle and other parameters through a programmable function generator and a power amplifier, so that the microbubbles could produce cavitation oscillation or collapse in the ultrasonic field, thereby inducing temporary holes in the GUV membrane. 4. Analyze the membrane dynamics during and after sonoporation: Use a high-speed camera to capture the microbubble oscillation and GUV deformation instantaneously and use a fluorescence microscope to observe the changes in membrane fluorescence intensity and the pore morphology to determine the pore size, distribution and sealing trajectory over time. In addition, focus on the duration of pore self-resealing, the correlation between pore size and MB size and ultrasonic parameters to verify the core hypothesis of "whether the GUV membrane has self-reseal ability under cell-free biochemical intervention".

1.3.3 Significance and contributions

In this study, we used GUVs as a novel sonoporation experimental model, developed a platform and protocol for GUV-based sonoporation membrane dynamic investigation, and thus provided the first evidence for the ability of membranes to self-reseal in the absence of subcellular processes after sonoporation. Our results reveal the dynamic process of pore formation and membrane self-sealing in GUVs under ultrasound exposure. This study provides new evidence for the ability of membranes to recover after sonoporation and provides insights into the underlying mechanisms, while also providing new perspectives for a deeper understanding of the biophysical nature of membrane repair. These findings are of great significance for advancing sonoporation-based therapeutic applications.

Chapter 2 Materials and Methods

2.1 Chapter overview

This chapter introduces the materials and methods required to achieve the four research objectives. The specific methodology for each objective is outlined as follows: Research Objective 1: Preparation of GUVs using electroformation. Section 2.2 describes the experimental setup required for GUV fabrication, including the GUV chamber, the electroformation procedure, and the collection process. Section 2.3 details the preparation of MBs, including the specific protocol for their fabrication and the methods used to modify their shell material and surface charge. Additionally, Section 2.4 investigates whether MBs can precisely adsorb onto the GUV membrane, ensuring stable sonoporation in subsequent experiments. Research Objective 2: Design of a visualized ultrasound coupling exposure system by integrating fluorescence microscopy with a high-speed camera. Section 2.5 introduces the imaging system based on fluorescence microscopy, while Section 2.6 describes the ultrasound equipment (waveguide system) and how it is coupled to the imaging setup. Research Objective 3: Inducing sonoporation by exposing GUVs to MB-US. Section 2.7 provides details on the ultrasound parameters and experimental protocol used to achieve sonoporation in this study. Research Objective 4: Analyzing membrane dynamics during and after sonoporation. Section 2.8 explains how fluorescence microscopy and high-speed imaging are employed to capture the instantaneous oscillations of MBs and the deformation of GUV membranes both during and after sonoporation. In summary, this chapter outlines the sample preparation procedures (GUVs and MBs), the setup of the ultrasound exposure system, and the experimental sonoporation protocol. All specific test results will be presented in the next chapter.

2.2 GUV electroformation and collection

2.2.1 GUV material and chamber

In this experiment, indium tin oxide (ITO) glass slides were used as electrodes, and a sandwich-type chamber structure was employed as the chamber for GUV electroformation. The specific assembly procedure is as follows: First, the lipid solution for coating the inner surface of the chamber was prepared. Lipids (Avanti Polar Lipids, Alabaster, AL) with a molar ratio of 15:1:4 for dipalmitoyl phosphatidylcholine (DPPC, 850355C), 1,2-palmitoyl-oleoyl-sn-glycero-3-phosphoserine (POPS, 840034P), and cholesterol were dissolved in chloroform (C2432; Sigma, Burlington, MA). To fluorescently label the GUV membranes upon their formation, CellMask Orange dye (C10045; Thermo Fisher Scientific, Waltham, MA) was added at 1:1000 Vol% to the lipid solution. Next, the ITO glass electrodes were prepared. The lipid solution was uniformly spread onto the ITO glass slides ($20\text{ }\mu\text{g}/\text{cm}^2$, $70\text{--}100\text{ }\Omega/\text{sq}$; 576352, Sigma) to form a thin and even lipid film. Due to thin-film optical interference, a uniform lipid layer should exhibit distinct rainbow-like fringes under illumination. The slides were then dried with nitrogen gas for 10 minutes to remove residual solvent, followed by vacuum drying for 2 hours to ensure complete dehydration and minimize solvent interference in the electroformation process. This step ensures uniform lipid film distribution and complete drying, allowing smooth detachment of lipid molecules under an applied electric field to form GUVs. Following this, the electroformation chamber was assembled. A 1.5 mm thick silicone

spacer was placed on the ITO slide containing the dried lipid film to create a sealed reaction chamber, thereby defining the space for GUV formation. A second ITO glass slide was then placed on top as the counter electrode, and copper tape was used to connect the conductive surfaces of both slides, completing the electrical circuit. The final setup formed a parallel-electrode enclosed chamber, which allows for controlled solution filling and precise electric field application during GUV electroformation.

Table 2 GUV electroforming material formulae.

Chemical	Molar Percentage	Molecular Mass (g / mol)
DPPC	75.00%	734.039
POPS	5.00%	783.988
Cholesterol	20.00%	386.65
CellMask Dye	0.10%	

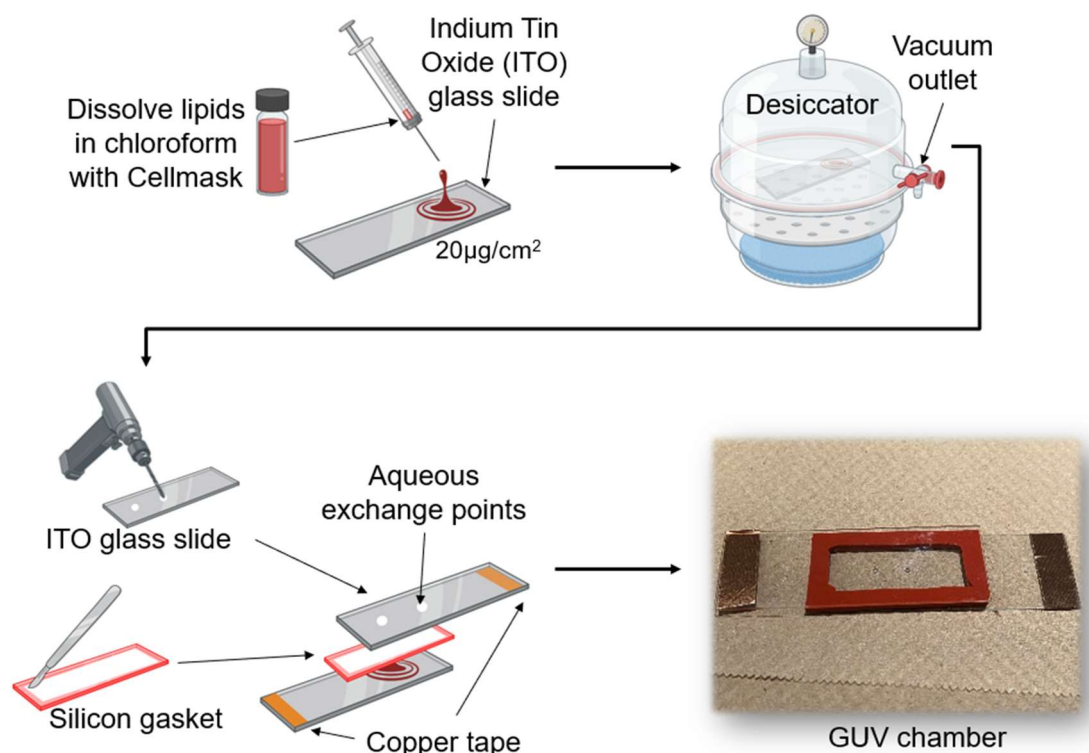


Figure 9 GUV chamber assembly process.

2.2.2 Electroformation protocol

Next, electroformation was performed. To initiate this process, a pH 7.4 sucrose buffer containing 200 mM sucrose and 1 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was

injected into the chamber to provide an optimal hydration environment. The chamber was then placed in a laboratory oven (Model 30; Quincy Lab Inc., Burr Ridge, IL) and heated to 55°C to maintain the lipid membrane in the liquid crystalline phase ($L\alpha$), ensuring the successful formation of GUVs. The two copper strips on the chamber were connected via alligator clip cables to a function generator (SDG2042X; SIGLENT Technologies, Shenzhen, China).

An alternating electric field was applied using the function generator, following a five-stage voltage-frequency gradient strategy to induce GUV formation: 1) Ramp-up phase: The voltage was linearly increased from 200 mV/mm to 700 mV/mm over 5 minutes at 10 Hz to promote the initial detachment of the lipid membrane. 2) Plateau phase: The voltage remained at 700 mV/mm with a frequency of 10 Hz for 2 hours to facilitate the gradual growth of GUVs. 3) First voltage and frequency reduction phase: The voltage was decreased to 350 mV/mm at 5 Hz for 30 minutes. 4) Second voltage and frequency reduction phase: The voltage was further reduced to 175 mV/mm at 2.5 Hz for 15 minutes. 5) Final voltage and frequency reduction phase: The voltage was lowered to 88 mV/mm at 1 Hz for 15 minutes. The last three phases promoted the detachment of mature GUVs from the ITO glass surface. This five-stage electrical protocol has previously been shown to effectively generate stable GUVs.

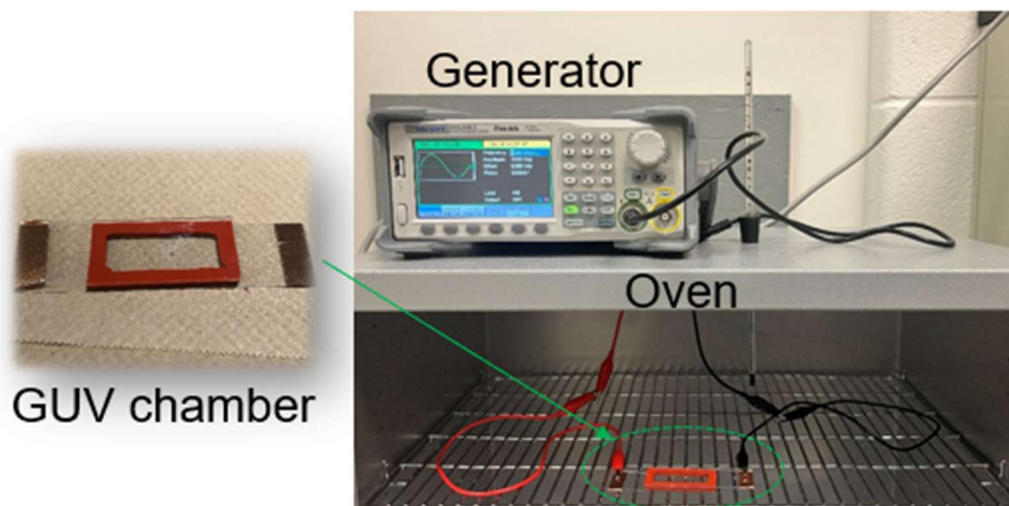


Figure 10 GUV electric field experimental device: generator and heating chamber system.

2.2.3 GUV collection and filtration

After the electroporation step, the GUVs were pipetted out of the chamber housing and placed in an isotonic but less dense glucose buffer (200 mM glucose, 1 mM HEPES, pH 7.4) to allow the GUVs to sink to the bottom of the solution. The collected GUVs were filtered using a Whatman filter (WHA10417506; Sigma) to select GUVs with diameters ranging from 8 to 40 μm , according to previous studies by others (Tamba et al., 2011; Karal et al., 2021). The filtered GUVs were used for our sonoporation experiments. They were counted using a hemocytometer under a phase contrast

microscope (Axio Observer; Zeiss, Oberkochen, Germany). The corresponding concentration was measured to be 78 GUVs/ μL .

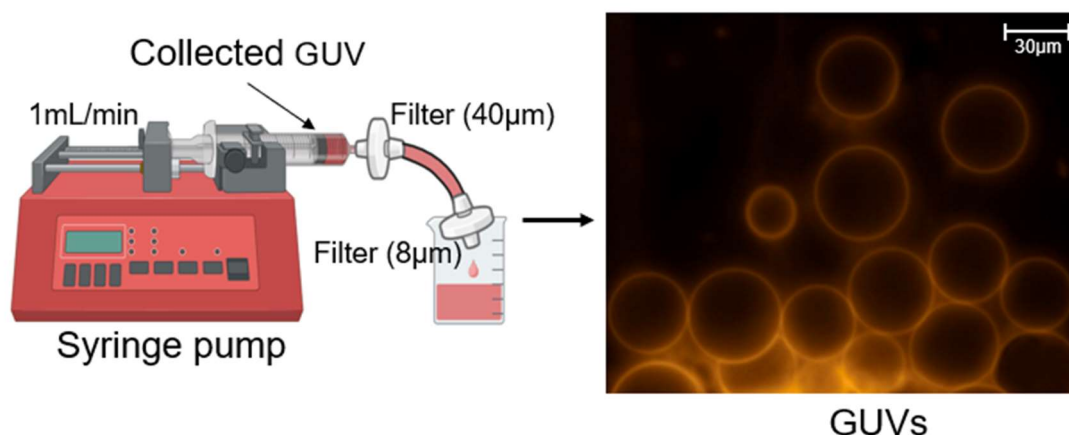


Figure 11 GUVs filtration device and GUV samples.

2.3 Formation of MBs

In this study, phospholipid MBs were prepared using a mechanical stirring–self-assembly method, and their surface charge was modified to produce cationic MBs due to their affinity for binding with GUVs. The MB shell consisted of a 19:1 molar ratio mixture of distearoylphosphatidylcholine (DSPC) (850365C; Avanti Polar Lipids) and 1,2-stearoyl-3-trimethylammonium-propane (DSTAP) (890880C; Avanti Polar Lipids), the latter being a cationic lipid. To fabricate these MBs, DSPC and DSTAP were dissolved in chloroform at a concentration of 2 mg/mL. The solution was then placed under a nitrogen stream for 10 minutes, followed by vacuum drying for 12 hours to completely remove residual solvents, forming a dried lipid film adhered to the bottom of a glass vial. The lipid film was subsequently rehydrated in 10 mL of 1X Phosphate-buffered saline (PBS) (P2272; Sigma), yielding a final concentration of 5 mg/mL. The lipid suspension was then sonicated using an ultrasonic disruptor (SFX 250; Branson Ultrasonics, Brookfield, CT, USA) for 40 minutes until the solution became clear, ensuring complete lipid hydration. For MB formation, perfluorobutane (C_4F_{10}) gas (N2HP-2204E; Avantor, VWR, Radnor, PA) was introduced at the gas-liquid interface of the hydrated lipid solution. The solution was then stirred for 30 seconds using an UltraMix stirrer (Trust BioSonics, Hsinchu, Taiwan) to facilitate C_4F_{10} gas dispersion into the aqueous phase, leading to the self-assembly of lipid-coated MBs. The newly formed MBs floated to the surface, forming an opaque white dispersion layer. The MB concentration was determined using a hemocytometer, yielding a measured concentration of 642 MBs/ μL .

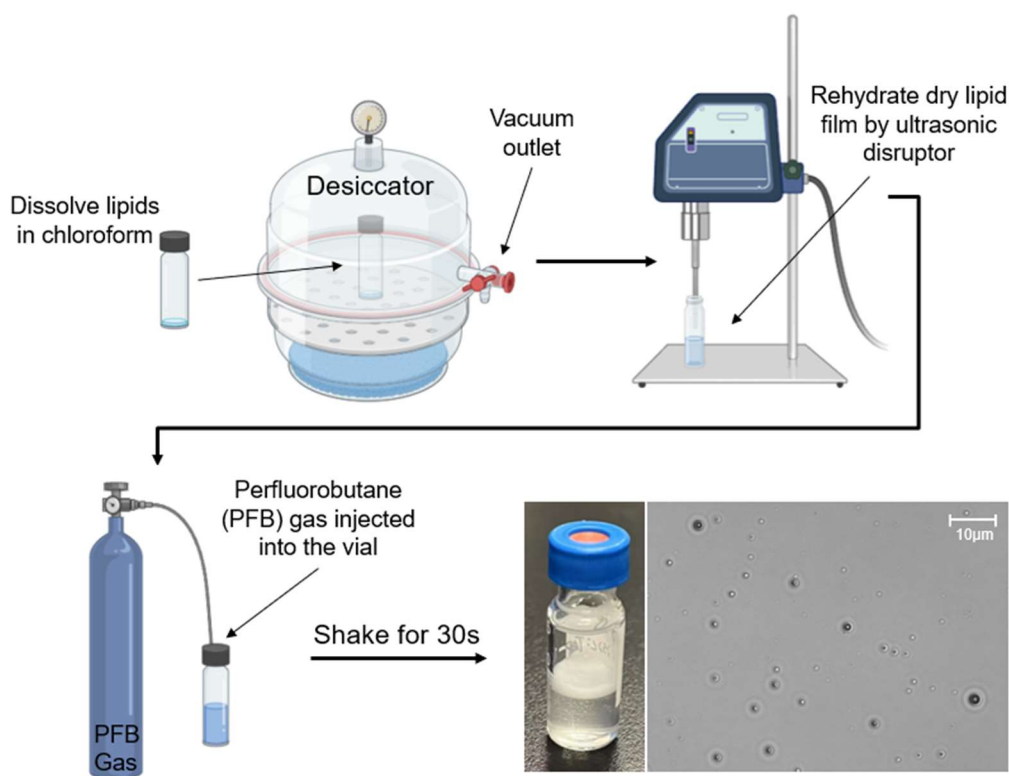


Figure 12 Microbubble preparation process.

2.4 Attachment of MBs to GUVs

In Chapter 1, we discussed how the effectiveness of sonoporation is influenced by the distance between MBs and the target cells. To achieve a stable and efficient sonoporation, it is necessary to ensure that MBs can effectively adhere to the GUV surface. In the previous section, we described the surface charge modification of MBs to render them cationic. Based on this, the present study employs an electrostatic interaction strategy to facilitate the natural binding of GUVs and MBs. To assess binding affinity, the zeta potential of GUVs and MBs was measured using dynamic light scattering (Malvern Panalytical Ltd., Malvern, UK).

2.5 Microscope imaging system setup

To enable real-time and direct observation of the MB-mediated GUV sonoporation process, we designed an imaging platform integrating an ultrasound exposure device, a high-speed camera (Photron Nova S, Tokyo, Japan), and a fluorescence microscope. The microscope used in this platform is an inverted fluorescence imaging system (Axio Observer, Zeiss, Oberkochen, Germany) equipped with a Zeiss Axio camera to capture fluorescence images of GUVs. A 63 $\times$ oil immersion objective lens with a numerical aperture of 1.4 was used. To achieve higher frame rate imaging, an illumination light box (HL250, AmScope, Irvine, CA) was employed as an auxiliary light source. Light was guided through a fiber optic cable and a condenser lens, positioning the light source directly above the objective to maximize intensity and illuminate the sample effectively. Additionally, the high-speed camera was fitted with an extra aperture and lens (105mm, f/2.8,

SIGMA) to enhance the field of view for high-speed imaging.

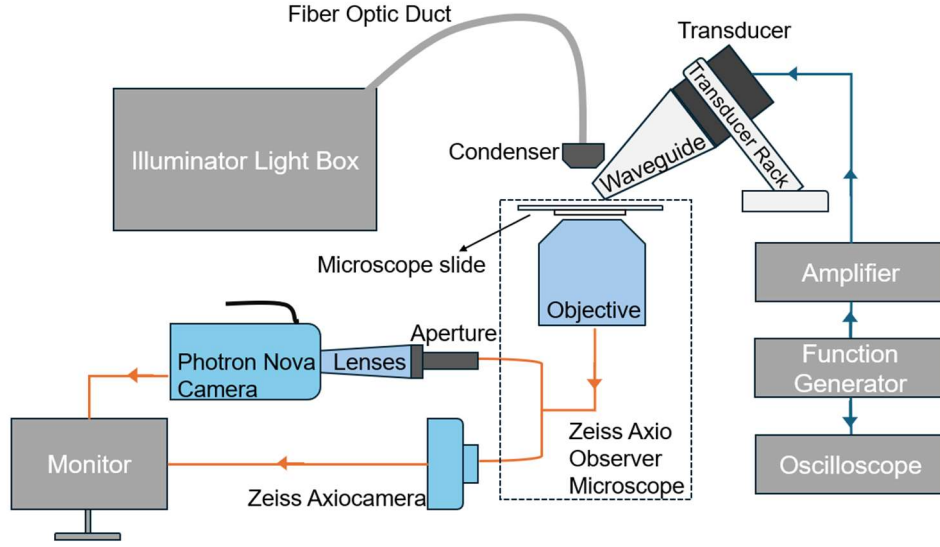


Figure 13 Microscope imaging system setup.

2.6 Ultrasound equipment setup and parameters

2.6.1 Ultrasound equipment setup

Based on previous studies, we designed a custom transducer mount and a conical waveguide with a height of 8 cm, a tip diameter of 0.8 cm, and a base diameter of 3.4 cm to deliver ultrasound at a 45° angle. These components were modeled using computer-aided design (CAD) software (SolidWorks; Dassault Systèmes, Vélizy-Villacoublay, France) according to the specified dimensions. They were fabricated using 3D printing (L3D-4; Elegoo, Shenzhen, China) with resin as the printing material (PRTTO2407C1101; Anycubic, Shenzhen, China). The waveguide was mounted onto a transducer (A304S-SU; Olympus, Tokyo, Japan) with a diameter of 32 mm and a center frequency of 1 MHz. The transducer mount was attached to the back of the transducer, and the assembled system was positioned on the real-time imaging platform. The primary function of this mount is to ensure that, regardless of the movement of the microscope slide sample during image navigation, the waveguide tip remains centered within the microscope's field of view and maintains a consistent 45° contact angle with the slide.

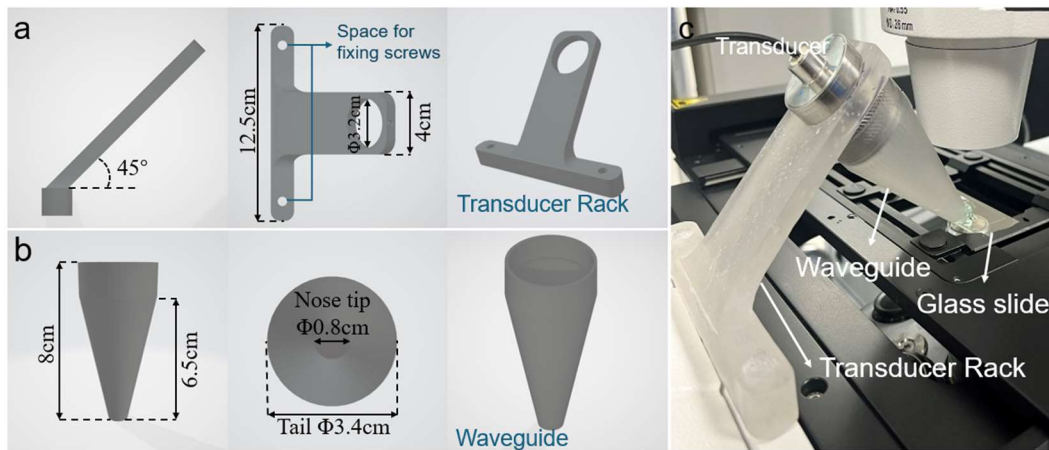


Figure 14 3D printed schematics of (a) the transducer rack and (b) the waveguide. From left to right are their side view, top view, and isometric projection view. (c) Diagram of the US exposure equipment assembled on the microscope.

2.6.2 Ultrasound field calibration

Hydrophone measurements were conducted to characterize the in-situ ultrasound exposure conditions at the sample position. In this study, a capsule hydrophone (HNR-0500; ONDA, Sunnyvale, CA) was mounted on an automated three-axis micropositioner (PGCN23; Sure Gear, Cumming, GA) and placed in a 37°C degassed water bath for precise positioning and scanning along the xy-plane. The waveguide was mounted onto the transducer and fixed at a 45° angle using a custom-designed holder.

Considering the thickness of the microscope slide, the actual distance between the sample and the waveguide tip was 1 mm. Therefore, all pressure field measurements were performed at 1 mm from the waveguide tip. The ultrasound pressure field was found to be relatively uniform within a 250×250 μm^2 central region, which corresponds to the maximum imaging field of view used in this study. The measured peak negative ultrasound pressure was 0.4 MPa. Within the region of interest, the variation in the ultrasound pressure field was found to be less than 1%.

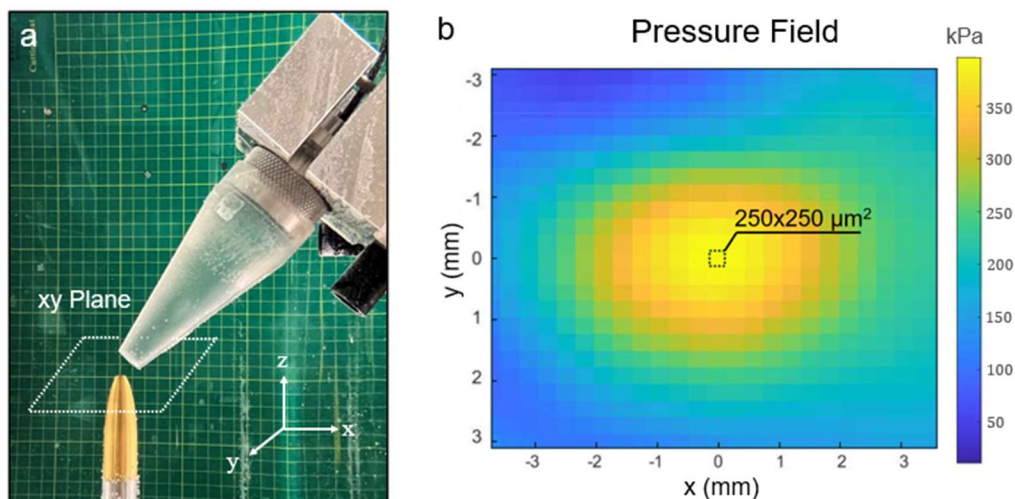


Figure 15 (a) ultrasound field measurement setup and (b) acoustic pressure field.

2.7 Ultrasound exposure protocol and parameters

For the ultrasound experiment, 16 μL of CellMask-stained GUVs was gently mixed with 2 μL of 5% DSTAP MBs (GUV:MB $\approx$ 1:1) for 10 minutes, followed by the addition of 2 μL of calcein (C0875; 100 μM , Sigma). To directly observe sonoporation and ensure that GUVs and MBs were in the same focal plane, the sample was transferred onto a standard microscope slide (48300-026; 25 $\times$ 75 $\times$ 1 mm, Avantor) and covered with a coverslip (12545E; 22 $\times$ 50 $\times$ 1 mm, Fisher Scientific). Both the slide and coverslip were pretreated with bovine serum albumin 1 g/L (A8806; Sigma) to prevent GUV rupture due to surface tension and nonspecific adhesion. The prepared slide sample was then placed on the mechanical stage, ensuring close contact between the slide and the waveguide tip. All gaps were filled with ultrasound transmission gel (Parker Laboratory, INC) to facilitate ultrasound propagation. In the sonoporation experiment, short-pulse waveforms were generated using a function generator. The waveform frequency was set to 1 MHz, with a burst period of 1 ms and a pulse duration of 100 cycles. The generated waveform was amplified by a power amplifier (A075; Electronics and Innovation Ltd., Rochester, NY, USA) to drive a 1 MHz piezoelectric transducer, producing the ultrasound waves required for sonoporation.

2.8 Image acquisition and processing

All bright-field microscopy images were acquired using Hoffman modulation contrast (HMC) or differential interference contrast (DIC). For fluorescence imaging, a 488 nm excitation source and a 515 nm emission filter were used to observe the calcein channel. For the CellMask Orange channel, a 554 nm excitation source and a 567 nm emission filter were used. Microscopy images were analyzed and processed using ImageJ and Zen software (3.6, Zeiss). For high-speed camera imaging, the maximum frame rate was set to 40,000 fps, with a shutter speed of 1/frame per second and a resolution of 1024 $\times$ 1024 pixels. Videos were processed using PFV4 software (PFV4, Photron).

Chapter 3 Experimental Results

3.1 Chapter Overview

This chapter introduces the results that support four research objectives. The specific evaluation criteria for each research objective are as follows: Research Objective 1: Successfully combine fluorescence microscopy and high-speed camera to realize a visualized ultrasound-coupled exposure system. The simplified schematic diagram of the system is shown in Section 3.2 (the detailed system setup has been explained in Section 2.5), and Section 3.2 contains the protocol for the entire ultrasound exposure experiment. Research Objective 2: Successfully prepare GUVs by electroforming through a custom-made chamber, and prepare cationic MBs by mechanical stirring self-assembly, and they can be successfully adsorbed together naturally. Microscope photos of GUV samples, cationic MB samples, and their attachment are shown in Section 3.3. Research Objective 3: Expose GUVs to MB-US and successfully achieve sonoporation. In Sections 3.4.-3.6, fluorescence microscopy and high-speed camera are used to show the process of GUVs and their membranes before and after sonoporation and during sonoporation. Research Objective 4: Based on the different performances of GUVs in sonoporation, the GUVs were divided into a group with self-resealable membrane and a group with non-self-resealable membrane after sonoporation. The temporal changes of the GUV membranes in the two groups, as well as the specific conditions of GUV response to calcein uptake were analyzed.

3.2 Experimental framework for investigating GUV membrane resealing in sonoporation

A new body of experimental evidence on post-sonoporation membrane resealing without the involvement of subcellular processes was acquired by developing an experimental framework that included: (i) preparation of GUVs using an electroformation method (Fig. 15a); (ii) design of an ultrasound exposure setup that was coupled with fluorescence microscopy and high-speed imaging (Fig. 15b); (iii) in-situ application of MB-mediated ultrasound exposure to the GUVs; (iv) observation and analysis of membrane dynamics during and after sonoporation. Research results were acquired using the timeline shown in Fig. 15c. Using this experimental framework, direct observations of the dynamic changes in GUV membranes were acquired during sonoporation episodes. The sonoporated GUVs were accordingly categorized into two distinct groups for analysis: (i) those with successful membrane resealing and (ii) those that failed to reseal. Post-sonoporation membrane resealing kinetics were then analyzed by measuring changes in pore size over time and the probability of successful resealing, as will be presented in the following subsections.

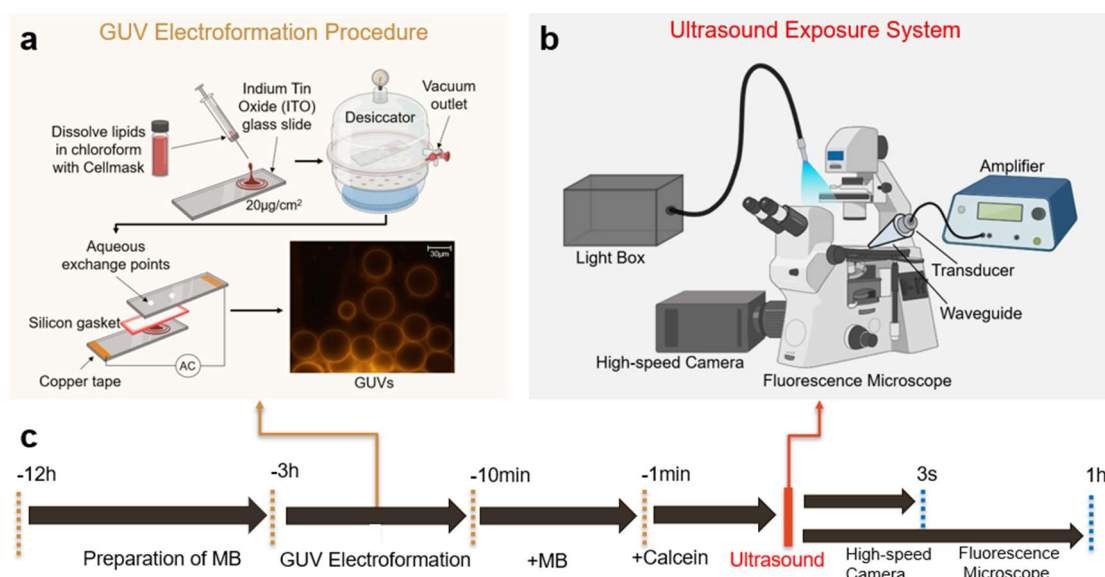


Fig 16 Experimental framework for investigating membrane self-resealing in sonoporation. (a) GUVs with phospholipid bilayer membrane, serving as model cells, were prepared using the electroforming method. (b) Using a calibrated acoustic exposure platform, MB-mediated ultrasound was delivered to the GUV samples at 0 s, and then a high-speed camera and fluorescence microscope were used. (c) GUVs, MBs, ultrasound exposure, and GUV imaging were prepared or performed according to the indicated timeline.

3.3 Pre-Exposure attachment of MBs to GUVs were observed

As an initial result, Fig. 2a shows an ensemble of fluorescent GUVs that were successfully fabricated using our electroformation protocol (see Methods; GUV Fabrication and Collection). These GUVs had a round appearance as expected, and their diameter ranged between 8 to 40 μm . The zeta potential of them is $-11.29 (\pm 3.07)$ mV. The membrane of these GUVs consisted of constituent lipid components similar to those found in eukaryotic cells, including dipalmitoylphosphatidylcholine (DPPC), 1,2-palmitoyl-oleoyl-sn-glycero-3-phosphoserine (POPS), and cholesterol. These three components had a molar ratio of 15:1:4 (DPPC: POPS: cholesterol). The inner core of these GUVs was filled with sucrose. The GUV membrane was fluorescently labeled using the CellMask Orange dye. As a size comparison for our fabricated GUVs, Fig. 2b shows a brightfield image of an ensemble of cationic MBs fabricated and used in this work (see Methods; Formation of MBs). These cationic MBs were of 1-5 μm in size and the zeta potential of them is $8.33 (\pm 4.08)$ mV. They had natural binding affinity to GUVs as their lipid shell was composed of 19:1 neutral-charged distearoylphosphatidylcholine (DSPC) and cationic 1,2-Stearoyl-3-trimethylammoniumpropane (DSTAP). Their inner core was filled with perfluorobutane gas (C_4F_{10}). The natural attachment of MBs to GUVs upon mixing is demonstrated in the series of images shown in Figs. 2c-2e. In this investigation, MBs were added in dilute form so that on average, no more than one MB was attached to a GUV as confirmed in Fig. 2c. In doing so, single site sonoporation episodes were instigated upon ultrasound pulsing. Note that calcein was added as a sonoporation marker before exposure.

Calcein fluorescence was expectedly not observed within the GUVs (Fig. 2d) since their membrane integrity remained intact (Fig. 2e) prior to ultrasound exposure.

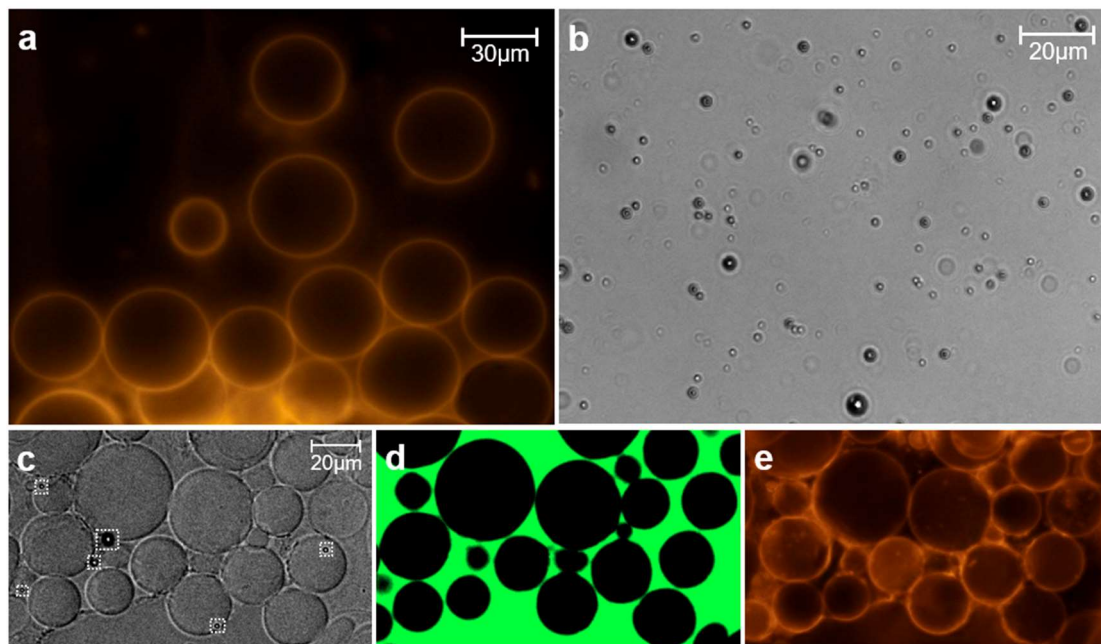


Fig 17 (a) CellMask Fluorescence image of GUVs formed via electroformation. (b) Brightfield image of cationic MBs (c-e) cationic MBs attached to GUVs under different imaging channels: brightfield (dotted boxes highlight MBs adhered to the GUV surface), Calcein channel and CellMask channel. Scale bars are 20 μm .

3.4 MB-Mediated ultrasound triggered single-site sonoporation of GUVs

3.4.1 Temporal evolution of sonoporation and membrane resealing

When 1 MHz pulsed ultrasound (10% duty cycle, 0.45 MPa in situ peak rarefactional pressure) was applied to the sample chamber, single site sonoporation events were induced due to the cavitation activity of MBs adjacent to the GUV membrane. A sequence of time-lapse images under CellMask fluorescence imaging is shown in Fig. 17a-17e, capturing the progression of membrane response at different time points ($t = 100, 200, 300, 400$, and 500 ms).

At $t = 100$ ms, the GUV membrane exhibited a uniform fluorescence distribution, with a clearly defined circular morphology. A yellow dashed box in Fig. 17a marks the initial region of interest (ROI) where sonoporation is expected to occur. Upon ultrasound exposure, fluorescence intensity in this region significantly decreased at $t = 200$ ms, indicating the formation of a transient pore in the membrane. The affected area remained dark at 300 ms, suggesting sustained membrane permeability. By $t = 400$ ms, fluorescence recovery was evident, and at $t = 500$ ms, the fluorescence intensity within the red dashed box (highlighted in Fig. 17e) had nearly returned to pre-sonoporation levels. The yellow dashed box in Fig. 17a corresponds to the initial poration site, while the red dashed box in Fig. 17e marks the final region where fluorescence recovery was assessed. This time-dependent fluorescence evolution demonstrates the gradual resealing process following

sonoporation, with a complete resealing occurring within approximately **500 ms** after the initial membrane disruption.

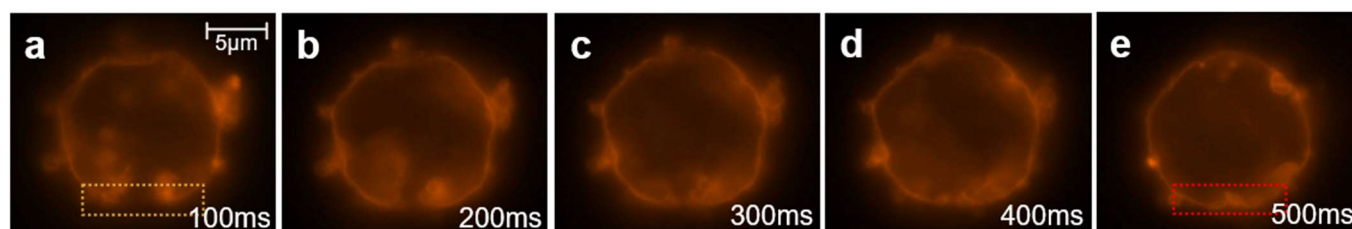


Fig 18 A time-lapse image sequence of GUVs after sonoporation in the CellMask fluorescence channel at different time points ($t = 100, 200, 300, 400$ and 500 ms) is shown. The scale bar is $5 \mu\text{m}$.

3.4.2 Fluorescence intensity dynamics at the poration site

The fluorescence intensity distribution at the **pore site** was analyzed and is shown in **Fig. 18**, where CellMask fluorescence intensity profiles were plotted as a function of lateral membrane position. At the onset of sonoporation, fluorescence intensity at the poration site (**orange curve**) exhibited a sharp decline relative to the surrounding membrane, indicating the formation of a membrane pore. Meanwhile, fluorescence intensity at the periphery of the poration site was elevated compared to the unperturbed regions of the membrane. Over time, fluorescence intensity at the poration site gradually increased, as shown by the post-resealing intensity profile (**red curve**), which closely aligned with the fluorescence levels of the intact membrane regions. The measured **pore region**, highlighted in **Fig. 18**, demonstrated a progressive fluorescence restoration over the course of the sonoporation episode

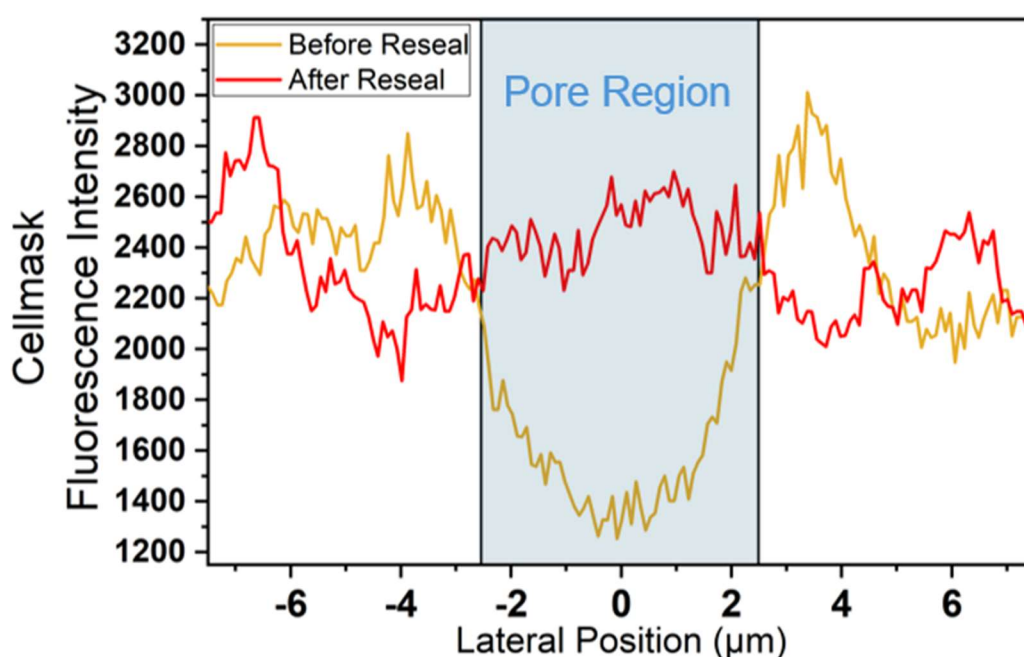


Fig 19 The FI (Fluorescence Intensity) of CellMask at the local poration site is tracked (within the dashed box in Fig. 17a and Fig. 17e), comparing the initial condition at $t = 0$ ms and the state at $t = 400$ ms.

3.4.3 Probability of successful membrane resealing and its dependence on MB size

The outcome of sonoporation events varied depending on the size of the MBs responsible for the cavitation activity. A statistical analysis of the relationship between MB diameter and pore resealing probability is shown in Fig. 19 where MB size distributions were compared between resealable and non-resealable pore formation cases. MBs associated with resealable pores had a median diameter significantly lower than those linked to permanent, non-resealable pores. The boxplot illustrates the measured MB diameter range for each case, with a clear size distinction between the two groups. The statistical evaluation showed a significant correlation between MB size and the likelihood of pore resealing ($N = 21$, $p < 0.01$). MBs with diameters in the lower range primarily resulted in pores that successfully resealed, while larger MBs consistently led to persistent membrane disruptions.

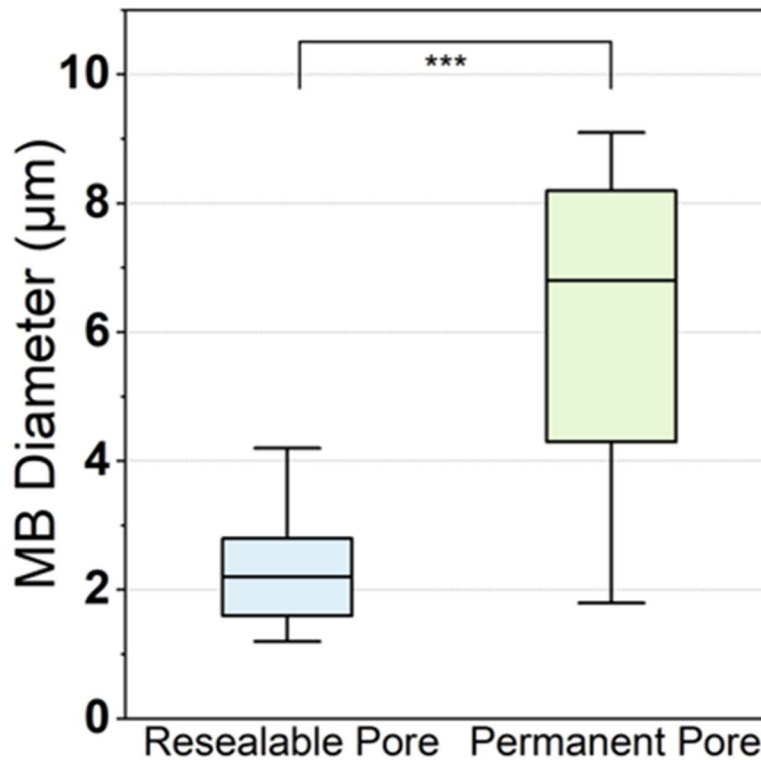


Fig 20 A statistical analysis of pore resealing under different MB sizes, showing a significant correlation between MB size and the likelihood of pore resealing ($p < 0.01$).

3.5 Membrane Morphology and Uptake Kinetics in Resealable Sonoporation

3.5.1 Fluorescence imaging before sonoporation

To substantiate the GUV membrane pore resealing after sonoporation observations obtained in Fig. 17, we tracked the GUV morphology and calcein internalization kinetics in another sonoporation episode whereby a 2.1 μm MB adhered to the GUV surface prior to ultrasound pulsing. Before exposure, calcein fluorescence was expectedly not observed inside the GUV (Fig. 20). The bright-field image shows an intact GUV structure with a well-defined membrane, while the CellMask fluorescence highlights the uniform phospholipid bilayer. The structural integrity of the GUV was confirmed by the absence of any observable fluorescence leakage or deformation in the bright-field and fluorescence images. Additionally, the GUV maintained its spherical morphology without signs of stress-induced distortion, reinforcing the notion that it remained in a stable state before ultrasound exposure. No passive calcein diffusion was detected within the GUV interior, confirming that the membrane barrier was fully intact and preventing any spontaneous molecular transport. The MB positioned adjacent to the GUV membrane remained stationary, indicating a stable interaction before ultrasound activation.

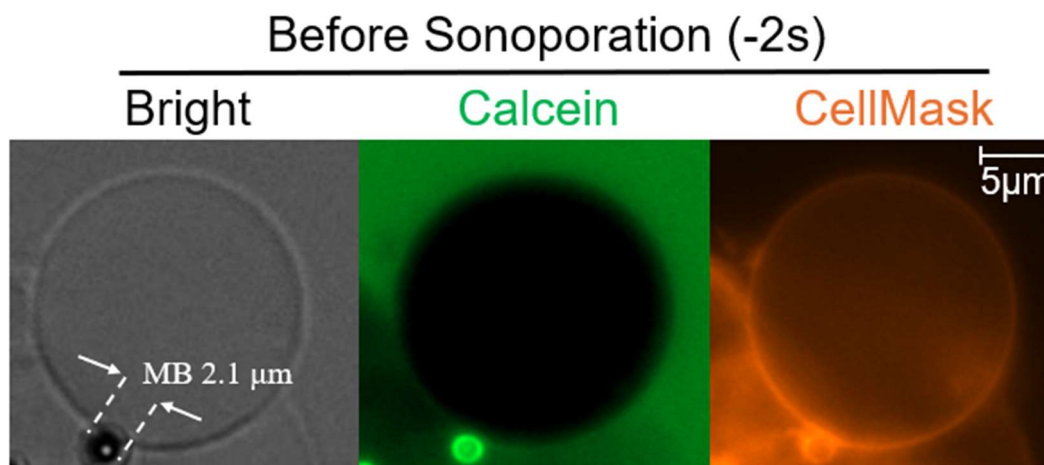


Fig 21 GUV before the application of pulsed US (-2s) in the bright channel, Calcein channel, and CellMask Orange channel (from left to right). Scale bar is 5 μm

3.5.2 High-Speed imaging of sonoporation event

To observe the cavitation process and the ensuing membrane dynamics, we switched on the high-speed camera (Fig. 21). Upon the application of pulsed ultrasound, the MB underwent inertial cavitation, initially expanding at 100 μs and then rapidly collapsing at 200 μs . This process induced a single-site membrane poration episode, with a measured pore size of 2.7 μm . The high-speed imaging sequence captured the rapid oscillation of the MB and the immediate formation of the membrane pore, demonstrating the transient mechanical disruption of the phospholipid bilayer due to the inertial cavitation effects. The sequence of images shows the MB initially maintaining a stable

position against the GUV membrane, followed by a rapid expansion phase due to the rarefactional cycle of ultrasound. At 100 μs , the MB reached its maximum expansion, exerting stress on the adjacent GUV membrane. Subsequently, the MB rapidly collapsed at 200 μs which led to the observed membrane poration. The cavitation event lasted approximately 300 μs , during which the membrane remained visibly deformed. The pore formed at the sonoporation site was transient, as indicated by its gradual reduction in size over subsequent frames. This rapid sequence of events, occurring within a sub-millisecond timescale, showing the dynamic nature of MB-induced sonoporation and the immediate mechanical response of the GUV membrane under cavitation stress.

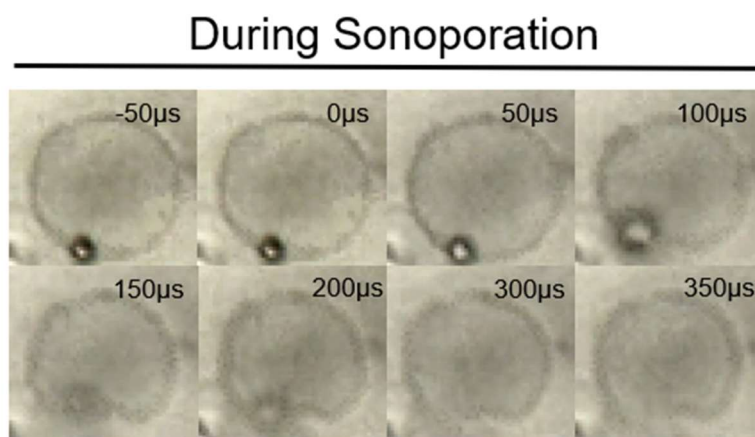


Fig 22 Dynamic process of sonoporation recorded by a high-speed camera at a frame rate of 20,000 FPS.

3.5.3 Fluorescence imaging after sonoporation

Our platform's fluorescence microscope was used to track that GUV sample calcein fluorescence after sonoporation. At 8.5 s post-sonoporation, the fluorescence intensity inside the GUV had stabilized (Fig. 22). The dashed box indicates the original location of the MB, showing no visible pores and only slight membrane deformation. The absence of significant structural disruptions in the bright-field channel further supports the conclusion that the GUV membrane remained intact after resealing. The gradual increase in fluorescence intensity inside the GUV suggests a transient phase of molecular transport before the membrane barrier was fully re-established.

The restoration of CellMask fluorescence at the poration site suggests that the membrane resealing process had taken place, preventing further molecular transport across the bilayer. This is consistent with the time-dependent fluorescence intensity plateau observed in the calcein channel, where no additional accumulation of dye was detected beyond the 8.5 s mark. The final fluorescence intensity of the membrane was comparable to pre-sonoporation levels, confirming the structural recovery of the GUV. Additionally, the uniformity of the fluorescence signal across the GUV membrane indicates that lipid redistribution following sonoporation was completed within this timeframe, further supporting the hypothesis of membrane self-repair without external biochemical factors.

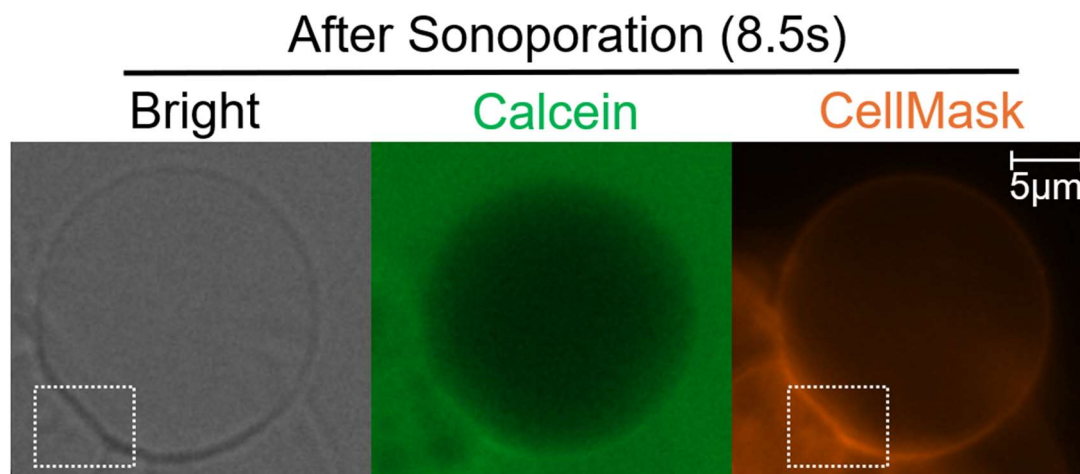


Fig 23 GUV 8.5s after sonoporation. The dashed box indicates the MB original location, showing no visible pores and only slight membrane deformation.

3.5.4 Uptake rate of calcein into GUV

The calcein fluorescence inside the GUV increased immediately after ultrasound pulsing and reached a plateau at 8.5 s, ultimately showing a lower fluorescence than that of the surrounding buffer solution (Fig. 23). The fluorescence intensity rise was initially rapid, suggesting an immediate influx of calcein following membrane poration. The increase slowed as the membrane began resealing, with the final equilibrium reached within the recorded timeframe. No further increase in calcein fluorescence was observed beyond this time point, indicating that the resealed membrane effectively prevented additional dye uptake. The fluorescence intensity inside the GUV was measured to be approximately 51% of that in the surrounding buffer, suggesting that while calcein permeated the vesicle interior during the open-pore phase, the total internal concentration did not reach full equilibrium with the external environment before resealing was complete. This result aligns with the resealing kinetics inferred from CellMask fluorescence recovery, confirming that the membrane regained its integrity within a finite timescale. Additionally, the plateau in calcein intensity further supports the observation that pore closure occurred at a well-defined time point rather than through a prolonged diffusion-limited process. The correlation between fluorescence stabilization and CellMask intensity recovery suggests that molecular transport through the membrane ceases as soon as resealing is complete, effectively restricting any further exchange of solutes. This self-limiting uptake behavior provides a quantitative measure of the transient poration window and highlights the dynamic interplay between sonoporation-induced permeability and membrane repair.

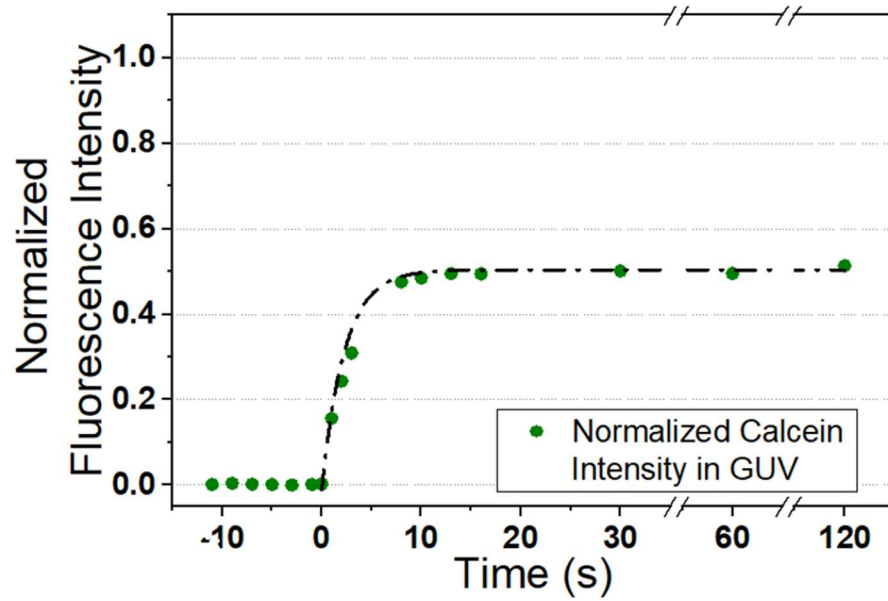


Fig 24 Normalized fluorescence intensity of Calcein inside the GUV after pulsed ultrasound.

3.6 Irreversible sonoporation yielded different membrane dynamics and uptake kinetics

3.6.1 Fluorescence imaging before sonoporation

Prior to the application of ultrasound, a single MB with a diameter of 5.6 μm was adhering to the GUV membrane. The bright-field image confirmed that the GUV maintained a stable and well-defined spherical morphology, with no visible deformations or distortions. The MB appeared to be in direct contact with the outer surface of the phospholipid bilayer, positioned at a stable site along the membrane. No fluorescence signal from calcein was detected inside the GUV (Fig. 24), verifying that the membrane was intact and remained impermeable to external molecules. The CellMask fluorescence image further highlighted a uniform phospholipid bilayer with continuous structural integrity, showing no pre-existing membrane defects or irregularities in lipid distribution. The lipid membrane exhibited consistent fluorescence intensity, confirming that no spontaneous poration had occurred prior to ultrasound exposure. Additionally, the GUV maintained a smooth and symmetrical boundary with no observable lipid aggregation or membrane thinning, suggesting that the membrane was under normal physiological tension. The MB's stable attachment to the membrane indicated that cavitation would occur at a well-defined site, allowing for a controlled and localized assessment of membrane response.

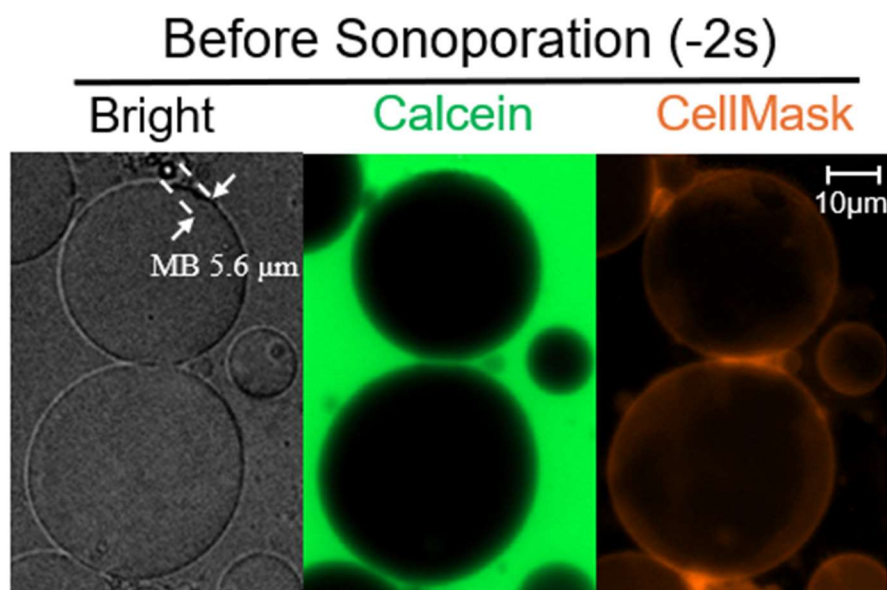


Fig 25 GUV before the application of pulsed US (-2s) in the bright channel, Calcein channel, and CellMask Orange channel (from left to right). The scale bar is 10 μ m

3.6.2 Fluorescence imaging after sonoporation

Upon ultrasound pulses, the MB collapsed immediately, causing the GUV membrane to deform. After 1 second, the GUV membrane was observed to be no longer intact, with a pore size of 6.9 μ m (Figure 25 a). At the same time, the surrounding calcein solution infiltrated into the interior of the GUV, where calcein fluorescence was detected, resulting in calcein fluorescence detected within the vesicle. The influx of calcein indicated rapid molecular exchange between the internal and external environments, confirming that the pore remained open within this time frame. Brightfield images showed continued deformation of the membrane at the site of pore formation, with a clear loss of structural continuity. In this irreversible sonoporation, after 100 seconds, the calcein fluorescence inside and outside the GUV reached equilibrium, resulting in the GUV being undetectable in the calcein fluorescence image (Figure 25 b). At this point, the sonoporation-generated pore size was 7.2 μ m, which was still detectable. Unlike the resealable sonoporation case, no evidence of membrane recovery was observed within the imaging time frame.

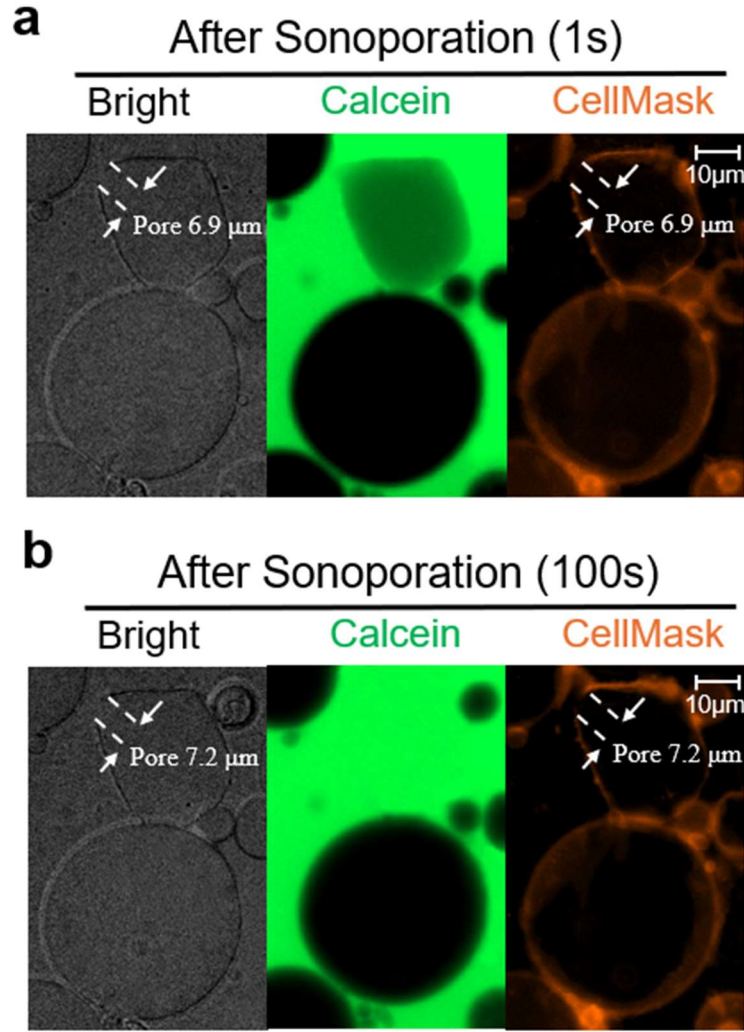


Fig 26 GUVs at different time points during the sonoporation process: (a) 1, and (b) 100s, respectively. Each figure includes the bright channel, Calcein channel, and CellMask Orange channel (from left to right). The scale bar is 10 μm .

3.6.3 Calcein uptake kinetics

In this irreversible sonoporation process, after 100 s, the calcein fluorescence inside the GUV reaches equilibrium with the surrounding buffer, resulting in the indistinguishability of the GUV in the calcein fluorescence image (Figure 25b). The corresponding kinetic curve (Figure 26) confirms that inside the GUV, the calcein fluorescence gradually increases from 0 to 100 s and then levels off at a maximum normalized intensity, equivalent to the fluorescence of the surrounding buffer. Unlike resealable sonoporation, where calcein uptake is limited by membrane resealing, the calcein concentration gradient in this case results in unrestricted molecular diffusion to complete equilibrium.

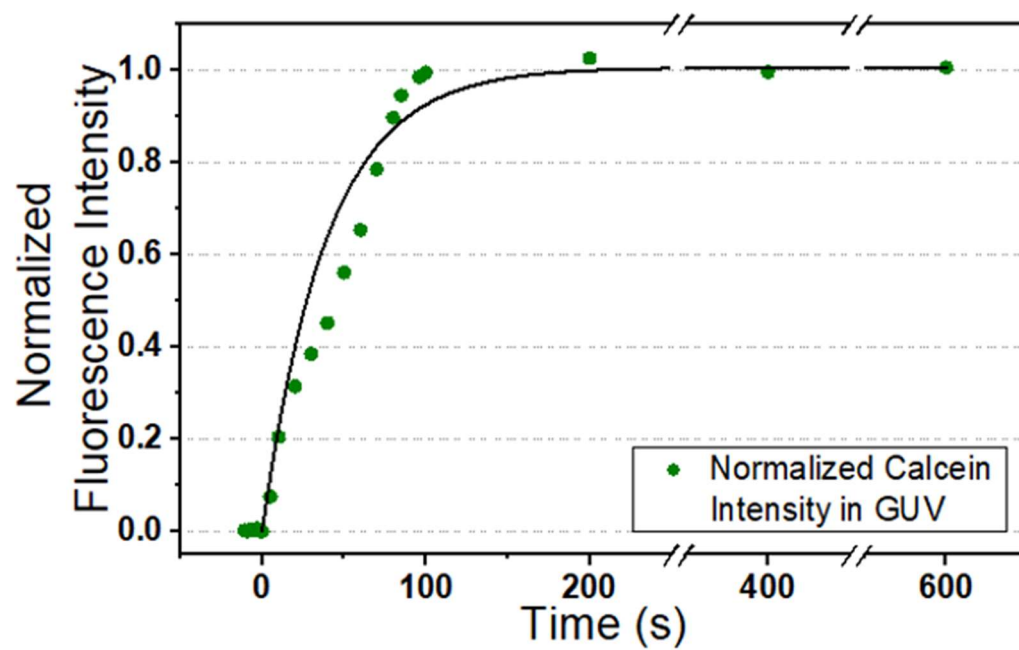


Fig 27 Normalized fluorescence intensity of Calcein inside the GUV after pulsed ultrasound.

Chapter 4 Interpretations and Significance of Study

Findings

4.1 Summary of contributions

This study is the first to directly investigate the role of phospholipid bilayers in the resealing of sonoporation sites, specifically demonstrating that membrane tension actively contributes to the membrane reseal process. While previous studies have confirmed that cell membranes can reseal after sonoporation, they did not establish whether membrane tension plays a direct role in this process. Our work fills this gap by showing that the physical forces within the lipid bilayer, particularly membrane tension, are sufficient to drive resealing at sonoporation sites, independent of intracellular repair mechanisms. Using GUVs as a simplified membrane model, we provide the first direct evidence that membrane-driven self-reseal occurs purely through biophysical forces, without requiring cellular processes. From a methodological perspective, this is also the first study to design and use GUVs to investigate the biophysics of sonoporation, marking a significant departure from previous work that primarily relied on live cells. GUVs offer a well-controlled, cell-organelle-free environment, allowing us to isolate the effects of membrane tension and lipid dynamics in pore resealing. This experimental approach provides a new framework for studying sonoporation mechanics, as it enables systematic modifications of lipid composition, cholesterol content, and other membrane components to explore their impact on repair efficiency.

Furthermore, our findings align with prior studies showing that larger microbubbles ($>8\ \mu\text{m}$) create pores that are less likely to reseal, reinforcing the concept that larger pore sizes require greater membrane tension for successful closure. However, unlike earlier research that attributed irreversible sonoporation primarily to intracellular factors, our study identifies membrane tension itself as a key determinant of whether resealing can occur. This novel insight has direct implications for optimizing ultrasound-mediated drug delivery strategies, as it suggests that tuning microbubble size and membrane composition could actively enhance membrane recovery dynamics. By establishing that membrane tension is a direct and essential driver of sonoporation repair, this work not only fills a critical knowledge gap in the field but also provides a foundation for future studies exploring how physical forces regulate membrane integrity in biomedical applications.

4.2 Significance of study findings

4.2.1 Preparation of GUV and cationic MB

In this study, we optimized the preparation of GUVs by designing a custom-built electroformation chamber, allowing for high-yield and high-quality vesicle production. Unlike conventional electroformation methods, which often result in polydisperse vesicle populations with variable membrane properties, our approach employed a gradual voltage and frequency modulation strategy, which improved membrane uniformity and structural integrity. Additionally, we introduced a filtration step to precisely control GUV size distribution, ensuring that only vesicles within a defined size range were selected for experimentation. This enhancement not only standardized the experimental conditions but also ensured reproducibility in studying sonoporation-induced

membrane dynamics. To facilitate MB attachment to GUV membranes, we modified the MB shell composition to generate cationic MBs, introducing positively charged lipids into the lipid monolayer. This modification significantly enhanced the electrostatic interaction between MBs and the negatively charged GUV membranes, leading to a spontaneous and stable MB-GUV attachment. Unlike conventional MB formulations, which may distribute randomly in solution or form aggregates, this tailored interaction enabled precise single-MB attachment to individual GUVs, ensuring a controlled and localized sonoporation process.

The combination of optimized GUV preparation, cationic MB engineering, and electrostatically driven attachment established a highly stable and reproducible model system for studying sonoporation. This system allowed for single-site MB-mediated ultrasound exposure, ensuring that sonoporation events were both consistent and well-characterized.

4.2.2 Build an ultrasound exposure system combining fluorescence microscope and high-speed camera

In this study, we developed an integrated ultrasound exposure system that combines fluorescence microscopy and high-speed imaging, enabling real-time visualization of sonoporation events with high temporal and spatial resolution. This system was specifically designed to capture membrane perforation, MB cavitation dynamics, and post-sonoporation resealing processes with exceptional clarity. While optimized for GUV-based sonoporation studies, this setup is highly versatile and can be readily applied to a broad range of sonoporation experiments, including those involving live cells, lipid bilayers, and microfluidic platforms, allowing for detailed characterization of ultrasound-induced biophysical effects in various experimental contexts. To ensure precise ultrasound delivery, we designed a custom waveguide system that effectively channels ultrasound energy to the focal plane of the microscope. This waveguide configuration ensures that ultrasound exposure remains well-aligned with the imaging field, preventing off-axis distortions or energy dispersion that could otherwise introduce inconsistencies in experimental results. One of the key advantages of this waveguide system is its adaptability—it can be easily integrated with standard microscope stages and sample holders without requiring additional modifications to existing imaging setups. This plug-and-play compatibility eliminates the need for customized hardware adaptations, making it a universally applicable tool for sonoporation studies across different experimental models. By combining high-speed imaging, fluorescence microscopy, and a precisely controlled ultrasound exposure system, our setup provides real-time insights into sonoporation dynamics. The flexibility and modularity of the system ensure that it is not limited to GUV studies but can be widely implemented in various biomedical and biophysical applications, making it a powerful platform for advancing ultrasound-mediated drug delivery, gene therapy, and membrane biophysics research.

4.2.3 Membrane investigation protocol during sonoporation based on GUV model

Based on the ultrasound microscopy system described in the previous section, we developed a novel experimental protocol and platform for in vitro sonoporation studies that provides a standardized and reproducible framework for studying membrane dynamics under ultrasound exposure. Unlike

traditional cell-based sonoporation studies, where intracellular processes can obscure direct membrane responses, our approach leverages GUVs as a controlled cell-mimicking model that enables accurate characterization of pure biophysical membrane responses to MB-mediated sonoporation.

This study is the first systematic use of GUVs as an experimental model for membrane dynamics during sonoporation, allowing for high-resolution visualization of membrane perforation, resealing, and lipid reorganization without interference from cellular repair mechanisms. By integrating fluorescence microscopy, high-speed imaging, and a precisely guided ultrasound delivery system, this protocol enables direct observation of membrane deformation, transient pore formation, molecular uptake kinetics, and resealing kinetics. The ability to track individual sonoporation events under well-defined experimental conditions establishes a new experimental model (GUVs) and protocol for probing membrane behavior under ultrasound exposure.

4.2.4 Demonstrate GUV membrane self-resealing after sonoporation

This study successfully demonstrates that GUV membranes are capable of self-resealing after sonoporation, providing direct experimental evidence that membrane tension plays a critical role in the repair process. By systematically tracking membrane dynamics in real time using fluorescence microscopy and high-speed imaging, we observed that small pores created by MB-mediated sonoporation could spontaneously close without the involvement of intracellular repair mechanisms. These findings establish that the restoration of membrane integrity is primarily driven by lipid bilayer self-organization, where phospholipids rapidly migrate toward the pore edge to facilitate closure. The ability of GUVs to undergo self-resealing supports the hypothesis that membrane tension acts as a key driving force in the reseal process. Unlike live cells, which rely on complex biological pathways such as cytoskeletal remodeling and vesicle trafficking for membrane repair, GUVs provide a minimal yet highly controlled system to isolate the purely biophysical contributions to membrane reseal. The observed resealing behavior aligns with theoretical predictions that local increases in membrane tension at the pore boundary enhance lipid mobility, accelerating the resealing process. By directly demonstrating membrane self-resealing, this study provides quantitative validation of lipid bilayer reseal mechanisms in sonoporation, bridging a crucial knowledge gap in ultrasound-mediated drug delivery and membrane biophysics.

4.3 Limitations of the proposed method

4.3.1 Sonoporation based on polydispersed microbubbles

One of the primary limitations of the proposed method is the use of polydispersed microbubbles (MBs), which introduces variability in the sonoporation process due to differences in MB size distribution. In this study, MBs were not monodisperse but rather exhibited a range of diameters, meaning that the cavitation behavior, ultrasound energy absorption, and resulting membrane interactions varied significantly between different MBs. This heterogeneity affects the consistency and reproducibility of sonoporation events, as MBs of different sizes generate different levels of mechanical stress, leading to variations in pore size, membrane deformation, and resealing kinetics across experiments. The polydispersity of MBs also impacts on the efficacy and predictability of

membrane resealing, as demonstrated in our results, where smaller MBs tend to produce resealable pores, while larger MBs generate irreparable disruptions. This variation makes it challenging to precisely control the sonoporation outcome, particularly when aiming to study specific membrane repair mechanisms. Additionally, MBs of different sizes have different resonance frequencies, meaning that the ultrasound parameters optimized for one MB size may not be equally effective for all MBs in the sample, further complicating the standardization of the sonoporation process.

Furthermore, the random distribution of MB sizes makes it difficult to ensure that each GUV is exposed to an MB with a controlled cavitation response. This lack of uniformity limits the ability to draw quantitative conclusions about sonoporation-induced membrane dynamics, as the observed effects are influenced by the inherent size variability of the MB population rather than being purely a function of the experimental parameters. Moving forward, achieving a more controlled MB size distribution, such as through microfluidic-based MB synthesis or size fractionation techniques, would enhance the reliability and precision of GUV-based sonoporation studies.

4.3.2 Sonoporation observation based on inverted fluorescence microscope

A key limitation of the proposed method is that the inverted fluorescence microscope used in this study only captures a two-dimensional (2D) projection of the sample, whereas both GUVs and MBs are inherently three-dimensional (3D) structures. As a result, the imaging system provides a flattened view of membrane dynamics, which limits the ability to accurately assess the full spatial characteristics of MB-GUV interactions, pore formation, and membrane resealing during sonoporation. Because the inverted fluorescence microscope collects data from a single focal plane, it does not provide depth-resolved information on membrane deformation, MB positioning, or cavitation effects occurring outside of the focal plane. This means that crucial details, such as the exact spatial orientation of the MB relative to the GUV membrane or whether pore formation extends beyond the imaged cross-section, may not be fully captured. Additionally, since the entire GUV is not always within the same focal plane, membrane regions at different depths may appear out of focus, further reducing the accuracy of quantifying pore dimensions and resealing kinetics.

4.3.3 Quantification of GUVs membrane tension

While this study successfully demonstrated that membrane structure and tension contribute to the resealing process after sonoporation, a key limitation is the lack of direct quantification of membrane tension. The experimental findings provide strong evidence that membrane tension plays a critical role in driving lipid migration and pore closure, but the exact magnitude of tension changes during and after sonoporation remains undetermined. In the current setup, membrane resealing dynamics were observed qualitatively through fluorescence imaging, showing that smaller MB-induced pores tend to close, whereas larger MBs generate irreversible disruptions. However, without quantitative tension measurements, it is not possible to determine the precise threshold at which a membrane can self-reseal, or the critical tension required for lipid reorganization. This limitation prevents a more detailed analysis of how specific physical parameters, such as lipid composition, membrane elasticity, or cholesterol content, influence membrane self-reseal.

4.4 Future directions

4.4.1 Sonoporation investigation based on modified GUV

A promising future direction for this research is to modify the lipid composition of GUVs to better simulate the diverse biophysical properties of natural cell membranes and to investigate how different lipid environments influence membrane resealing after sonoporation. The current study utilized simple phospholipid bilayers, which provided a well-controlled system for isolating the purely biophysical aspects of membrane repair. However, cell membranes are far more complex, containing cholesterol, membrane proteins, and specialized lipid domains, all of which play crucial roles in membrane permeability, fluidity, and mechanical stability. One approach to advanced sonoporation studies is to introduce membrane-associated proteins into GUVs, such as transmembrane receptors, cytoskeletal anchors, or lipid-binding proteins, to evaluate their effects on pore formation and resealing dynamics. Additionally, modifying the cholesterol content of GUVs could provide insights into how membrane stiffness and fluidity influence the efficiency of sonoporation and the probability of successful resealing. Given that cholesterol-rich membranes are more rigid, increasing or decreasing cholesterol levels in GUVs could mimic the mechanical properties of different cell types, such as the highly flexible membranes of cancer cells or the structurally rigid plasma membranes of neurons. By systematically altering the lipid composition, protein incorporation, and cholesterol content, future research can explore how biologically relevant membrane variations impact sonoporation efficiency, membrane reseal, and drug uptake kinetics.

4.4.2 Integration of optical trapping for controlled MB-GUV interaction

A potential future direction for sonoporation research is to integrate optical trapping techniques to precisely control MB-GUV interactions prior to ultrasound exposure. In the current experimental setup, MBs spontaneously attach to GUVs via electrostatic interactions, resulting in random positioning and orientation of MBs on the GUV membrane. While this approach effectively promotes sonoporation, the lack of spatial control over MB placement results in variations in pore formation locations, cavitation dynamics, and membrane deformation patterns.

By incorporating optical tweezers or microfluidic device-based trapping systems, MBs can be precisely positioned and manipulated relative to the GUV surface prior to ultrasound exposure. This level of control will allow researchers to systematically study the effects of MB positioning on sonoporation efficiency, pore morphology, and membrane reseal dynamics. Furthermore, controlled MB placement can be used to comparatively study sonoporation effects at different membrane regions, such as polar versus equatorial attachment or localized versus distributed MB aggregation.

4.5 Research summary

In this study, we investigated the membrane dynamics and self-sealing behavior of phospholipid bilayers during sonoporation using GUVs as a model system. The primary goal was to determine whether membrane structure and tension are sufficient to drive resealing after sonoporation independently of intracellular membrane reseal mechanisms. To achieve this goal, we first optimized the preparation of GUVs by designing a custom electroformation chamber and

implementing a stepwise voltage and frequency modulation strategy to ensure high yield, high-quality vesicles with controlled size distribution. Furthermore, we engineered cationic MBs to promote spontaneous electrostatic attachment to the GUV membrane, enabling a stable and reproducible sonoporation platform. Next, we developed an integrated ultrasound exposure system that combines fluorescence microscopy and high-speed imaging to allow real-time observation of MB-induced membrane perforation and resealing processes. Finally, we demonstrated that the GUV membrane can autonomously reseal after sonoporation, confirming that membrane tension plays a key role in driving the reseal process, with smaller MB-induced pores closing successfully while damage produced by larger MBs remains unsealed. This study is the first direct experimental validation that membrane tension alone is sufficient to restore bilayer integrity after sonoporation, filling a critical knowledge gap in ultrasound-mediated drug and gene delivery research. Furthermore, it establishes a novel methodological framework for studying sonoporation-induced membrane dynamics, providing a controllable, reproducible, and scalable system for studying the biophysical mechanisms of membrane repair. For future studies, modifying the lipid composition of GUVs by incorporating membrane-associated proteins or altering cholesterol content could help mimic different mechanical properties of biological membranes, allowing for a more comprehensive analysis of how different cell types respond to sonoporation. Furthermore, integrated optical trapping techniques allow for precise control of MB-GUV interactions, reducing experimental variability and providing a deeper understanding of spatially dependent membrane dynamics. These advances will help improve efficiency, reproducibility, and therapeutic potential of ultrasound-mediated drug delivery systems.

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