Hypersonic poration of supported lipid bilayers†

Yao Lu, a,b Jurriaan Huskens, b,* Wei Pang a and Xuexin Duan a,b,*

Hypersound (ultrasound of gigahertz (GHz) frequency) has been recently introduced as a new type of membrane-disruption method for cells, vesicles and supported lipid bilayers (SLBs), with the potential to improve the efficacy of drug and gene delivery for biomedical applications. Here, we fabricated an integrated microchip, composed of a nano-electromechanical system (NEMS) resonator and a gold electrode as the extended gate of a field effect transistor (EGFET), to study the effects of hypersonic poration on an SLB in real time. The current recordings revealed that hypersound enabled ion conduction through the SLB by inducing transient nanopores in the membrane, which act as the equivalent of ion channels and show gating behavior. The mechanism of pore formation was studied by cyclic voltammetry (CV), atomic force microscopy (AFM) and laser scanning microscopy (LSM), which support the causality between hypersonic-triggered deformation and the reversible membrane disruption of the SLB. This finding contributes to the development of an approach to reversibly control membrane permeability by hypersound.

Introduction

Physical methods based on the principle of membrane disruption, such as thermal poration,1–3 optoporation,4–6 electroporation7–9 and sonoporation,10–12 have been widely adopted to improve the efficiency of intracellular delivery. The key to the enhanced uptake of extracellular substances lies in their physical interaction with the cells, which can improve the permeability of the cell membrane without any further damage to assist the entry of foreign molecules. Among these techniques, sonoporation induced by ultrasound has gained much attention in various drug delivery and therapeutic applications.13–16

In a conventional sonoporation process, the most significant effect of ultrasound involves the nucleation, growth and oscillation of microbubbles, a phenomenon referred to as cavitation.17–19 Cavitation includes either the rapid collapse of microbubbles (inertial cavitation) or the sustained oscillatory motion of microbubbles (stable cavitation), both of which can induce strong mechanical effects on the cell membrane.20–22 The collapse of microbubbles generates shock waves with an extensive amplitude that disturbs the cell membrane, whereas the stable oscillation of microbubbles can induce acoustic pressure in the liquid and exert shear stress on the membrane.23–25 The degree of membrane permeability upon sonoporation mainly depends on the frequency and duration of the applied ultrasound.26–28 Since ultrasound spans a frequency of roughly 15 kHz to 10 MHz in liquid, with an associated acoustic wavelength of 10 to 0.01 cm, no direct coupling of the acoustic field with the cell membrane can be detected at a molecular level,29 which restricts the direct formation of pores at the cell membrane and creates a strong dependence on microbubble agents.30,31

In principle, the increase of the ultrasonic frequency will accelerate the oscillation of cavitation bubbles as well as enhance the acoustic pressure.32,33 Thus, an acoustic wave of gigahertz (GHz) frequency and (sub)micrometer wavelength, which is defined as hypersound,34,35 has been proposed to affect membrane permeability.36 It has been reported that hypersound can be applied to enhance the delivery of drug molecules into cancer cells by creating transient nanopores in the cell membrane, which showed no cytotoxicity.37 The mechanical stress on the membrane surface is significantly enhanced by hypersonic poration compared with the conventional ultrasonic treatment. Furthermore, hypersound has also been applied in a layer-by-layer (LbL) system to control the disassembly of supramolecular membrane structures.38 Despite the strong potential of hypersound in both drug delivery and controlled release, the mechanism of hypersonic poration is still not fully understood.

In this work, hypersonic poration was studied by analyzing the acoustic behavior of a supported lipid bilayer (SLB). A high-frequency nanofabricated resonator of 1.6 GHz was used to generate hypersound with a submicron wavelength. On the same microchip, a gold electrode has been integrated and
connected with an extended gate electrode of a field effect transistor (EGFET)\(^\text{19,40}^\) to monitor the currents through the SLB induced by hypersonic vibration. The ion-channel effects of hypersonic poration were measured and analyzed with salt solutions containing cations with different valences. Characterization by cyclic voltammetry (CV), atomic force microscopy (AFM), and laser scanning microscopy (LSM) was performed to feature the properties of hypersonic nanopores. This study thus aims to provide a better understanding of the mechanism of hypersonic poration and to create an approach capable of changing and monitoring membrane permeability in real time.

### Experimental section

#### Materials

All chemicals including phosphate-buffered saline (PBS), potassium chloride (KCl), calcium chloride (CaCl\(_2\)), iron(III) chloride (FeCl\(_3\)), and potassium ferricyanide (K\(_3\)Fe(CN)\(_6\)) were purchased from Sigma Aldrich. PBS was dissolved in ultrapure water to obtain the 0.1 M buffer solution (including 0.1 M sodium dihydrogen phosphate and 0.15 M sodium chloride, pH 7.4). The metal ion chlorides were dissolved in pure water to obtain 2 mM electrolyte solutions. K\(_3\)Fe(CN)\(_6\) was dissolved in 1 M KCl solution to obtain a 1 mM solution for the cyclic voltammetry tests.

#### Fabrication of the supported lipid bilayer

The lipid 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-1'-glycerol (POPG) was purchased from AVANTI and dissolved in chloroform with a typical concentration of 0.5 mg mL\(^{-1}\). The supported lipid bilayer (SLB) was fabricated and coated on the integrated chip by the Langmuir–Blodgett (LB) method\(^\text{41,42}^\) using a Langmuir trough (Kibron, MicroTrough XL). In brief, 50 \(\mu\)L of the POPG solution was spread onto the surface of pre-cleaned Milli-Q water and then compressed at a speed of 10 mm min\(^{-1}\) until the targeted surface pressure of 25 mN m\(^{-1}\) was reached. Afterwards, the integrated chip, which was previously oxidized in air plasma for 5 min to create a hydrophilic interface, was vertically pulled up from the water subphase at a constant speed of 1 mm min\(^{-1}\) to form a single lipid layer and then down into the LB trough again to form the SLB.

#### Membrane characterization

The SLB coating on the integrated chip was characterized by cyclic voltammetry (CV, CHI 660E, Huachen), fluorescence microscopy (Olympus IXplore Pro), atomic force microscopy (AFM, Veeco, Nano Scope III) in tapping mode and laser scanning microscopy (LSM, Olympus LEXT OLS4000).

#### Fabrication of the integrated microchip

The integrated device was fabricated according to a previously published process (see ESI,† Fig. S1).\(^\text{43}^\) Before the fabrication of the resonator, a Bragg reflector was mounted on the silicon wafer by alternately depositing three pairs of silicon dioxide (SiO\(_2\)) and molybdenum (Mo) thin films.\(^\text{44}^\) The first layer of the Bragg reflector was used as the bottom electrode followed by the sputtering of highly c-axis oriented aluminum nitride (AlN) film as a piezoelectric layer and another layer of Mo as the top electrode.\(^\text{45}^\) Finally, 300 nm Au was deposited and employed as the extended gate electrode of a field effect transistor (EGFET).

### Results and discussion

#### Working principle

The microchip integrated with an acoustic nano-electromechanical system (NEMS) resonator and a gold electrode is shown in Fig. 1a. The polygonal shape of the NEMS resonator was designed to enhance its main-mode vibration while minimizing the parasitic effect.\(^\text{47}^\) By coupling the vertical electric field through a specific piezoelectric coefficient, the resonator vibrates in a longitudinal mode and generates hypersonic gigahertz (GHz) frequency.\(^\text{47}^\) A quarter-wavelength Bragg reflector was placed under the resonance structure to avoid the dissipation of energy into the silicon substrate.\(^\text{48}^\)

The working principle of this integrated microchip is illustrated in Fig. 1b. An artificial supported lipid bilayer (SLB) made of 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-1'-glycerol (POPG) was deposited on the surface of the microchip, covering both the resonator and the gold electrode. POPG was used as the component of the SLB to mimic the negatively charged nature of cell membranes and because it shows good flexibility at room temperature which facilitates membrane deformation and poration. Once activated, the vibrating interface of the resonator drives the motion of the SLB deposited on top of the resonator and induces a propagation of hypersonic wave within the lipid membrane, from the vibrated area of the resonator to the gold electrode. The embedded gold electrode was connected with the gate electrode of a back-end field effect transistor (FET) and was used as the charge-sensitive interface for current measurements.\(^\text{43}^\) Since an SLB is highly resistive and impermeable to hydrated ions,\(^\text{49}^\) the electrical potential of the underlying gold electrode can remain constant in a pure electrolyte solution by the isolation from the intact SLB. However, when hypersonic...
is applied, the transduction of the motion of the resonator to and through the SLB may deform the membrane structure. Once membrane defects occur during this process (as shown in the zoom-in image), the SLB becomes permeable to ions, thus inducing membrane conductivity that can be detected by electrical measurements from the back-end FET.

**Characterization of the supported lipid bilayer**

Fig. 2a shows the surface pressure–area ($\pi$–$A$) compression isotherm of POPG. The target surface pressure for the formation of SLB was fixed at 25 mN m$^{-1}$ to ensure the compactness of the membrane. Cyclic voltammetry (CV) tests were conducted using $K_3$Fe(CN)$_6$ as a redox probe to confirm the integrity of the artificial SLB. As shown in Fig. 2b, the formation of the SLB on the gold electrode significantly reduced the measured faradaic current compared with the uncovered electrode, suggesting that an insulating SLB was successfully placed on top of the device with the LB method.

**Real-time detection of hypersonic poration**

The real-time electrical response of the SLB was recorded by periodically switching on and off the stimulation of hypersound.
As shown in Fig. 3a, the current curve exhibited an instantaneous increase when turning on the hypersound and maintained a constant value during sustained stimulation (see also Videos S1–S3 in ESI†), indicating that the applied hypersound caused conductivity through the SLB, ascribed to induced permeability to the ions present in the buffer solution. The current curve recovered readily to its original value upon switching off the hypersound, indicating the reversibility of the induced membrane permeability. As the patterns of the SLBs in Videos S1–S3 (ESI†) show, it is likely that the lipid membrane coated on the microchip was mechanically disrupted by deforming the SLB through the propagation of hypersound. In this acoustical disruption process that occurs by coupling with the acoustic waves at a sub-micron length scale, some transient nanopores are generated to allow for the ion transfer across the SLB. Since hypersound can form strong turbulent flow inside fluids, the ion transfer can also be facilitated by the accompanying acoustic streaming effects. Due to the flexibility of the lipid membrane and the molecular diffusion of the lipid molecules therein, the membrane defects can then be healed in a re-assembly process by turning off the hypersound. Interestingly, the transmembrane current induced by hypersound resembles the stochastic “gating” behavior of biological ion channels. Instead of modulating the ion-channel currents with reversibly binding blocker molecules, the current through the SLB can be mediated by the formation and closure of the transient pores formed by hypersound.

Subsequently, the ion current was modulated with hypersound of different input powers as shown in the current–time trace (Fig. 3a). The first step of the ion current was in response to hypersound of 3.2 mW; thereafter the current values increased step by step with the increase of input power. It is likely that more and/or longer lasting transient nanopores were generated with hypersound of higher power, and this high acoustic intensity also enhanced the ion transfer by accelerating the velocity of acoustic streaming. The magnitude of the current through the transient pores was plotted as a function of input power, which shows a response to hypersound that levels off at high powers (Fig. 3b).

In the low power range (less than 250 mW), the current almost increased linearly with the input power, while at higher input power, the current increased much more slowly and presented a trend of saturation, which is likely due to limitation of the number of hypersonic pores within the restricted area of the SLB. In the absence of an SLB, a significantly higher current was observed (Fig. S5, ESI†), and changing the KCl concentration from 2–100 mM in the presence of an SLB showed little variation of the hypersound-induced current (Fig. S6, ESI†). These results support that the limited formation of hypersonic pores is the main factor for the current saturation observed in Fig. 3b.

To understand how GHertz hypersound was generated and its waves propagated along the surface of the NEMS resonator, a 2D finite element model (FEM) of the single resonator was employed (Fig. S3, ESI†). It shows that the surface of the resonator deformed to generate hypersound, the magnitude of which is around several nanometers. We assume that the nanoscale deformation of the resonator drives the deformation of the SLB coating on its surface. By increasing the input power of hypersound, the surface of the resonator deformed more strongly (illustrated in Table S1, ESI†), which is in accordance with the increased currents in Fig. 3.

To further investigate the mechanism of hypersonic poration, ion currents through the SLB were measured in solutions containing salts with cations of different valences. The current curves probed in solutions of KCl, CaCl2 and FeCl3 of the same concentration (2 mM in pure water) are shown in Fig. 4a. Similar measurements were also performed in solutions of NaCl and MgCl2 (Fig. S7, ESI†), and these results resemble those of KCl and CaCl2, respectively. To ensure the applied hypersound was in the linear range, the value of the input power was limited to 250 mW. All current curves were found to increase with hypersound of higher input powers, and importantly, the currents were different for solutions containing different salts. In Fig. 4b, the fit results of currents generated from solutions containing the different salts all showed a linear trend as a function of input power, which is in accordance with the results presented in Fig. 3b (below 250 mW).
These results support therefore that the value of the current through the transient nanopores in the SLB can be quantitatively controlled by adjusting the intensity of hypersonic sound, which can be consequently used to adjust the premeability of the lipid membrane.

The increased current responses for CaCl₂ and FeCl₃ compared with KCl can possibly be attributed to (i) increased ionic strength, (ii) increased concentration of chloride, and/or (iii) a direct effect of the valence of the cation. Because the negative surface charge of POPG is screened by mobile counterions with positive charge, the overall ionic current through the SLB is mainly induced by the translocation of cations through the pore-like structures. Therefore, the non-linear current trend for different cations can probably be related to a complex and combined effect of ionic strength and specific cation properties regarding their valence and possibly to mobilities affected by acoustic streaming. However, more data would be needed to discriminate between these possibilities.

Characterization of hypersonic poration

To evaluate the formation of hypersonic pores at the lipid membrane, cyclic voltammograms using the ferro/ferricyanide redox couple were recorded with the SLB-coated gold electrode in the presence and absence of hypersonic sound. As shown in Fig. 5a, the redox response increased with switching on the hypersonic sound (500 mW) in real time, indicating a transport of the redox couple to the gold electrode coupled to electron transfer, which was induced by the exposure of the electrode upon pore formation. Once the last CV cycle was ended, another CV cycle was recorded upon switching off the hypersonic sound, and the duration of this hypersonic treatment was less than 1 min. As shown in Fig. 5a, the redox peaks were reduced immediately and overlapped with the CV curve obtained in the absence of hypersonic sound, indicating that the oxidation and reduction of ions was reduced to the base level at the shielded electrode. This data confirms that the hypersonic-induced pore formation is fast and reversible, and the integrity of the SLB is restored upon ending the stimulation by hypersonic sound.

The morphology of the SLB was analyzed by atomic force microscopy (AFM) before and after the stimulation by hypersonic sound. All the measurements were conducted in liquid phase using tapping mode. Since the platform of AFM is not compatible with the hypersonic setup, it cannot provide real-time information of morphological changes occurring at the SLB during hypersonic operation. Therefore, hypersonic sound was applied to the SLB for a prolonged period of time until the transient nanopores transformed into permanent defects, which can then be detected with AFM. The transition from reversible pores to irreversible damage was determined by CV by gradually increasing the duration of hypersonic sound until the redox peaks did not diminish any more after switching off the hypersonic sound (Fig. 5b). As shown in Fig. 5b, the original surface of the SLB was essentially flat and featureless. Note that the detected height of the lipid bilayer was slightly higher (approx. 10 nm) than the normal value (4–5 nm) obtained from dry lipid membranes, which can be attributed to the effects of liquid in tapping mode. The morphology of the SLB was first detected after a short treatment of hypersonic sound (250 mW, 5 min), where no obvious difference was observed on the membrane surface (Fig. 5c), indicating that hypersonic sound of lower intensity and duration does not damage the structure of the SLB irreversibly. In contrast, when the applied hypersonic sound was increased to 500 mW for 30 min, the morphology of the membrane was changed (Fig. 5d). The average height increased from 13.7 ± 0.6 nm (Fig. 5b) to 16.8 ± 1.0 nm, and clear defects were observable on the membrane surface (Fig. 5d). These results confirm the existence of morphological effects of hypersonic sound on the SLBs.

To assess the structural changes of the membrane, laser scanning microscopy (LSM), which is compatible with the hypersonic sound setup, was used for studying the hypersonic pores at the SLB in a real-time fashion. The duration of hypersonic sound for all the microscopy measurements was less than 1 min during the scanning process. One intact SLB made of POPG was deposited on the integrated device, covering both the NEMS resonator and the embedded gold electrode to guarantee the membrane continuity from the resonator to the gold electrode. The confocal image of Fig. 6a shows the morphology of the section of the SLB...
Fig. 5 Characterization of hypersonic poration at a SLB. (a) Real-time CV responses of 1 mM K₃Fe(CN)₆ in 1 M KCl at SLB by switching on (500 mW, <1 min) and off the hypersonde. The black curve was obtained with the original SLB before any treatment of hypersonde. The green curve was obtained by simultaneously switching on the hypersonde. After the duration of a CV cycle, hypersonde was immediately switched off and another CV cycle (blue) was recorded. (b) AFM images of SLB before any treatment of hypersonde. (c) AFM images after treated with hypersonde of 250 mW for 5 min and (d) then treated with hypersonde of 500 mW for 30 min.

Fig. 6 Real-time LSM images of a SLB made of POPG on the integrated device in the absence or presence of the stimulation by hypersonde (500 mW). (a) Morphology of the SLB on top of the activated NEMS resonator (the surface layer is AlN). The sizes of the 2D map and the 3D zoom-in image are respectively 450 × 550 μm² and 100 × 100 μm². Morphology of SLB on top of the gold electrode before (b) and after (c) the stimulation by hypersonde. Hypersonde was on only during scan (b), and each scan took <1 min. The sizes of the 2D maps and the 3D zoom-in images are respectively 220 × 220 μm² and 2 × 2 μm².
coated on top of the activated NEMS resonator. Here, the color and contrast are governed by the deformation of the membrane structure induced by hypersound. Upon turning on the hypersound, wave-like patterns were observed on the surface of the SLB (which can be observed as well by the fluorescent SLB in Videos S1–S3, ESI†), centered at the resonator and radiating outward, which indicates the propagation of hypersound within the bilayer structure. As the zoom-in image in Fig. 6a shows, the morphology of the lipid membrane was affected by the propagation of hypersound.

Subsequently, the morphology of the section of the SLB on top of the gold electrode was analyzed to evaluate the membrane structure affected by the propagation of hypersound. For comparison, the surface of the SLB was first imaged without any stimulation of hypersound (Fig. 6b), which indicates a uniform and featureless surface without defects. However, upon switching on the hypersound (500 mW, <1 min), some sub-micrometer features were immediately generated on the SLB at the same position (Fig. 6c, see zoom-in image). Although the exact sizes of the pores cannot be accurately determined because of the limitation by optical resolution of the confocal microscope, these results confirm the formation of pore structures induced by the deformation of the SLB with hypersound propagating within the membrane.

Conclusions

In this work, the behavior of a supported lipid bilayer (SLB) stimulated by hypersound of gigahertz frequency was characterized by a microchip integrating a nano-electromechanical system (NEMS) resonator and a gold electrode, which facilitates the real-time electrical detection of hypersonic poration. The “gating”-shaped current induced by hypersound revealed that some pores were created in the SLB with the propagation of hypersound, which resembles the behavior of ion channels at lipid membranes. It was found that the formation of these pore structures was instantaneous (at least sub-second, as we did not probe the dynamics any faster) in response to hypersound, indicating that the number and/or lifetime of hypersonic pores can be controlled in a real-time manner by correspondingly adjusting the duration and input power of hypersound. The effects of ion valence and/or concentration on the formation of hypersound-induced current confirms the behavior of hypersonic nanopores in lipid membranes as the ion channel. Furthermore, characterization experiments (cyclic voltammetry, atomic force microscopy and laser scanning microscopy) showed that during the propagation of hypersound within the SLB, the membrane was laterally deformed, which supports the formation of transient pore-like defects. The generation of switchable and reversible nanopores enables the active control of membrane permeability, which can be further applied in controlled release and drug delivery systems.

Conflicts of interest

The authors declare no competing financial interest.
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