Hypersonic Poration of Membranes: From Triggered Release and Encapsulation to Drug Delivery

Yao Lu

**HYPERSONIC PORATION OF MEMBRANES:
FROM TRIGGERED RELEASE AND
ENCAPSULATION TO DRUG DELIVERY**

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DISSERTATION

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This thesis is dedicated to my family

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Chapter 1

General Introduction

In the past decades, membrane-disruption methods have been proposed as an important physical approach to address a subset of functions, specifically nucleic acid delivery to the certain primary cells.^[1, 2] However, the delivery of any cargo to any cell type is still a big challenge for intracellular delivery.^[3, 4] Recently, numerous studies have been focusing on the precision membrane disruption at the micro and nanoscale aiming to surmount current delivery limitations.^[5, 6] With improved precision, these physical methods can be applied to accurately control the membrane permeability of single cells and target specific sites on the cell membrane.^[7, 8] The delivery efficiency can be brought to high throughput for diverse cells with the incorporation of microfluidics and microsystem techniques.^[9, 10]

In this thesis, hypersonic poration is introduced as a new physical method to precisely control membrane permeability for the applications of controlled release and encapsulation, and enhanced drug delivery. Bulk acoustic wave (BAW) resonators of gigahertz (GHz) frequency have been fabricated using microelectromechanical system (MEMS) technologies to generate GHz ultrasound (i.e. hypersound). The mechanism of hypersonic poration has been analyzed step by step using a variety of model systems, from the supported lipid bilayer (SLB), to giant unilamellar vesicles (GUVs), polymer-shelled vesicles (PSVs), and cancer cells. These experiments have provided a deep insight into the formation of hypersonic nanopores from planar lipid membranes to complex cell systems. This innovative poration method has the potential to be applied for intracellular delivery and other biomedical applications.

Chapter 2 gives an overview of the precision membrane-disruption methods at the micro/nanoscale for intracellular delivery. Recent developments of these physical poration methods, including electroporation, optothermal poration, nanoinjection, microfluidic squeezing, sonoporation and hypersonic poration, are presented in detail. The selected examples focus on developing high precision membrane disruption in combination with advanced techniques in nanotechnology, microfluidics, laboratory-on-chip (LOC), and

microsystems. The mechanism and merit of each approach are discussed, aiming to highlight new ways to deliver any cargo to any cell type.

In Chapter 3, the fabrication of an integrated chip involving a bulk acoustic wave (BAW) resonator and a gold electrode is presented to study hypersonic poration on a supported lipid bilayer (SLB). The high-frequency BAW resonator of 1.6 GHz has been used to generate hypersound, while the gold electrode has been used as the extended gate of a field effect transistor (EFGET) to facilitate electrical measurements of the lipid membrane. With this integrated platform, the electric behavior of hypersonic nanopores is analyzed to provide comprehensive information on the mechanism of hypersonic poration.

In Chapter 4, the giant unilamellar vesicle (GUV) is introduced as a cell model to study the effects of hypersonic poration on lipid membranes. GUVs with a diameter of 15-20 μm have been immobilized on a SLB via the biotin-streptavidin affinity pair. The deformation of GUVs induced by hypersound has been analyzed using laser scanning confocal fluorescence microscopy (CLSM), while the size of hypersonic pores has been estimated by loading fluorescent polystyrene (PS) beads with different diameters into GUVs. This study suggests the potential of hypersonic poration in applications of cell manipulation.

In Chapter 5, a study is presented on the application of hypersound to a supramolecular system for controlled encapsulation and release. Supramolecular polymer-shelled vesicles (PSVs), either suspended in solution or immobilized on a surface, have been constructed to understand the effects of hypersonic poration and to achieve controlled loading and releasing of fluorescent dyes. The studies of hypersound from lipid vesicles to supramolecular vesicles extend the application of such physical poration effects.

Chapter 6 describes the use of hypersound to enhance the intracellular delivery of polymer-wrapped mesoporous silica nanoparticles (PMSNs) loaded with Doxorubicin (Dox). The cellular uptake and distribution of Dox-loaded PMSNs have been analyzed by fluorescence measurements. The cell viability has been compared with both Dox-loaded PMSNs and free Dox molecules under the stimulation of hypersound. The mechanism of hypersound-enhanced cellular uptake has been studied further through inhibitor experiments. This study provides a better understanding of hypersonic poration on enhanced intracellular delivery.

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Chapter 2

Precision Membrane-Disruption Methods at the Micro/nanoscale for Intracellular Delivery

Intracellular delivery of materials, such as drugs, genes, dyes, and nanoparticles, has become a critical component of cell-based therapies and genome-editing applications. Limitations of current techniques continue to motivate the development of systems that can deliver a broad variety of cargos to diverse cell types. In this chapter, we reviewed recent advances of micro and nano-scale membrane-disruption methods that create specific exogenous stimuli, such as electrical, optothermal, mechanical, and acoustic. Techniques of nanotechnology, microfluidics, lab-on-chip and microsystems were combined with these methods to enable intracellular delivery with spatial and temporal precision, high throughput, and minimal cell perturbation. The challenges and opportunities of these intracellular delivery technologies have been addressed.

2.1 Introduction

Efficient and targeted intracellular delivery of exogenous molecules and compounds is a critical issue in biological research and therapeutic applications. To surmount the biological barrier of the cell membrane and to direct the site-specific internalization, various chemical and physical methods have been developed. These methods can be categorized into carrier-based^[1, 2] and membrane-disruption-based approaches.^[3, 4] Carrier-based methods use viral vectors^[5-7] or chemically synthesized carriers (liposomes,^[8-10] polymersomes,^[11-13] dendrimers,^[14-16] and nanoparticles,^[17-19] etc.) to package cargos and release them through endocytotic pathways. However, challenges such as immune response, safety and complexity of preparation are concerns for introducing foreign agents.^[20] Furthermore, the delivery efficiency is limited by numerous obstacles^[21] and is generally cell-type dependent.^[22]

As an attractive candidate for more universal delivery systems, membrane-disruption modalities have been developed to enhance the cellular uptake of “naked” cargo (e.g., DNA, RNA, proteins, etc.), aiming to deliver almost any target molecule to any cell type. These methods can be regarded to induce permeabilization or direct penetration of cell membranes in response to physical stimuli, such as electric field,^[23-25] heat,^[26, 27] and mechanical forces.^[28-31] By creating transient pores in the cell membrane, the membrane-disruption approaches allow the passage of submicrometer materials into cells, in a manner that is less dependent on cargo properties. However, conventional membrane disruption shows significant limitations in translocation efficiency, cell viability and transfection uniformity. For example, the adverse side effects, including pH changes and significant joule heating, in bulk electroporation can induce a high rate of cell death,^[32, 33] while the requirement of bubble agents in sonoporation makes it difficult to control the delivery efficiency.^[34, 35]

To overcome such challenges, conventional membrane-disruption methods have been improved by advances in the fields of nanotechnology, microfluidics, lab-on-chip (LOC) devices, and other microsystems. Nanofabrication techniques facilitate the micro and nano-scale membrane disruption, concentrating precise perturbing effects to the subcellular scale.^[36, 37] The combination of microfluidic-based chips achieves advantages of mimicking the cellular microenvironment, which largely reduces the consumption of reagents and directly couples to downstream analytical chemistry platforms.^[38] The precision membrane-

disruption methods at the micro and nano-scale have provided many unique advantages, including negligible cell damage and good dosage control capabilities with single-cell resolution. Therefore, these methods promise to enable more biomedical applications of intracellular delivery.

In this review, we give an overview of the main micro/nanoscale membrane-disruption techniques for intracellular delivery and their mechanisms of function, as well as cover several examples of studies on membrane disruption, including electroporation, thermal poration and mechanical poration. Two major types of mechanical poration, including solid-contact methods (i.e., nanoinjection and microfluidic squeezing) and fluid-shear methods (i.e., sonoporation and hypersonic poration) are discussed in combination with the potentially transformative techniques of nanotechnology, microfluidics, LOC, and microsystems. In particular, the method of hypersonic poration, based on gigahertz ultrasound (i.e., hypersound), is an innovative way to enhance the efficiency of intracellular delivery, as will be discussed in detail in the following chapters.

2.2 Membrane disruption at the micro/nanoscale

2.2.1 Electroporation

As a leading physical delivery method, electroporation has been widely used in gene transfer with high-throughput to introduce diverse (bio)molecules to millions of cells.^[39] In a bulk electroporation system, cells are suspended in solution and dispersed between two parallel plates (Figure 2.1a).^[40, 41] Once a series of intense electrical pulses are applied to the plates, a transmembrane potential is generated across the cell membrane and the lipid molecules within the membrane are rearranged to form small openings. It is found that the initially hydrophobic openings induced by the lateral fluctuation of lipid molecules are soon transferred to hydrophilic pores, with the transmembrane potential reaching a critical value (Figure 2.1b).^[42, 43] The accumulation of hydrophilic pores in the membrane is the reason for the changes of membrane permeability in electroporation.^[44] Despite multiple practical advantages of bulk electroporation, like well-established protocols and user-friendliness, its high working voltage (more than 1000 V) induces a high rate of cell death, which continues to be a limitation for real life therapeutic applications.^[45]

Figure 2.1. (a) Schematic picture of (bulk) electroporation in a cuvette-based system. The gap between the electrodes is larger than the size of the cells by several orders of magnitude. Reproduced with permission.^[45] Copyright 2016, The Royal Society of Chemistry. (b) Schematic illustration of two basic pore types formed during the electroporation process, (i) a hydrophobic and (ii) a hydrophilic pore. Here, r indicates the specific radius of intermediate hydrophobic pores that are transformed into hydrophilic ones. Reproduced with permission.^[46] Copyright 2011, Springer.

To realize effective electroporation at significantly lower voltages compared to bulk electroporation, microscale electroporation has been developed based on two prototypes, namely microfluidic and microelectrode electroporation.^[45] In a microfluidic electroporation system, an electric field is applied as the cells flow through a pair of electrodes in a microfluidic channel. The key factor to achieve successful delivery is the precise synchronization between the flow rate and the implementation of the electric field. Some studies have addressed this issue by focusing the electric field on the narrow section of a microfluidic channel where only single cells can flow through.^[47–52] As shown in Figure 2.2a, electroporation in microfluidic droplets was demonstrated to deliver genes into cells at a relatively low voltage (less than 7 V).^[53] The electroporation occurred when the cell-containing droplets (in oil) flowed through a pair of microelectrodes at a constant voltage, which facilitated gene delivery at high throughput. To improve the transfection efficiency, a vortex-based electroporation system (Figure 2.2b) was developed to induce hydrodynamic forces from vortex motion and rotation, which enabled uniform permeabilization of the cell membrane.^[54] Due to advances in microfabrication techniques, the microelectrodes in microfluidic systems can be easily configured in different manners and shapes, which varies the distribution of the electrical field aiming to improve the performance of microscale electroporation.

Figure 2.2. Strategies for microfluidic electroporation. (a) Schematic of the droplet-based microfluidic electroporation device. Inset images illustrate the processing of the droplets at different sections of the device. Cell-containing droplets rapidly flow through the two microelectrodes on the substrate (each electrode was 25 μm wide, and the distance between the two electrodes was 20 μm). Reproduced with permission.^[53] Copyright 2009, American Chemical Society. (b) A vortex-assisted microfluidic device with a spiral electroporation section for gene delivery. The device had two wide sections (3 cm long and 500 μm wide) and one narrow section (4.768 mm long and 35 μm wide). The depth of the channel was 75 μm . Reproduced with permission.^[54] Copyright 2010, The Royal Society of Chemistry.

In the microelectrode electroporation system, a single or an array of microelectrodes is manufactured at dimensions comparable to the cell size to generate localized electric field to a single cell, so that the field intensity at a lower voltage can be sufficient enough for reversible membrane disruption. Figure 2.3a shows a microfluidic chip with individual lateral cell trapping sites to selectively and locally induce electropores on single cells.^[55] Such design can focus the electric field thus achieve dielectric breakdown of the cell membrane at rather low applied voltages. In particular, the deformation of cells can be visualized at a single cell level in this microelectrode electroporation system.^[56] Subsequently, a device was reported that can independently electroporate multiple cells with multi-microholes in between two parallel channels (Figure 2.3b).^[57] Cells were trapped in the microholes by generating a pressure difference between the parallel channels, which essentially concentrated the electric field at the trapped cells to achieve localized membrane disruption. With such configuration, a biased voltage of less than 4 V gave successful transfection of cells with a transfection efficiency of 75% and a cell viability of almost 100%.

Figure 2.3. Strategies for microelectrode electroporation. (a) Schematic of the chip for single-cell electroporation. An electrode was connected to one of the main channels and the other electrode was connected to the small channel where the cell was trapped. Reproduced with permission.^[55] Copyright 2005, The Royal Society of Chemistry. (b) A microfluidic device for single cell electroporation and gene transfection. (i) Artistic 3D impression of trapped cells. (ii) Microfluidic chip layout, zoom-in of trapped single cells. Reproduced with permission.^[57] Copyright 2008, The Royal Society of Chemistry.

2

To precisely and quantitatively control the delivered amount of substances for intracellular delivery, microscale electroporation systems were further miniaturized to the nanoscale.^[45] In a nanoscale electroporation system, the porating electric field can be focused on a nanosized section of the cell membrane, which greatly increases the electric potential while minimizing the damages to the cells.^[58, 59] The first nanoscale electroporation system was realized with a combined structure composed of one nanochannel and two microchannels (Figure 2.4a).^[60] The cell to be transfected was positioned in one microchannel using optical tweezers, and the transfection agent was located in the second microchannel. This technique created a single pinpoint hole in the cell membrane rather than numerous small pores induced by conventional electroporation, which could deliver well-defined amounts of a variety of transfection agents into living cells. To increase the scalability of nanoscale electroporation, a three-dimensional (3D) electroporation system was developed (Figure 2.4b).^[61] In this system, 3D nanochannel (300–600 nm in diameter, 10 μm long) arrays were fabricated to transfect numerous cells in a controlled manner. The transfection efficiency was improved to approximately 90%. Before the effective implementation of nanochannel-based electroporation, several approaches, including micro-capture structures, dielectrophoresis and magnetic tweezers, were normally applied to achieve precise positioning of cells,^[62–64] which is important for the localized membrane disruption.

Figure 2.4. Strategies for nanoscale electroporation. (a) Schematic of a two-dimensional (2D) nano-channel electroporation. A single cell was precisely positioned against the nanochannel outlet via the optical tweezer and stimulated by the electric field. The nano-channel was 90 nm in diameter and 3 mm long. Reproduced with permission.^[60] Copyright 2011, Nature Publishing Group. (b) A three-dimensional nanochannel electroporation (3D NEP) platform for high-throughput cell transfection. (i) The cross-sectional schematic of the system, which consists of a 3D NEP chip, a support platform, two polydimethylsiloxane (PDMS) spacers, and a bottom electrode. (ii) The 3D model showing all components stacked on a substrate and bonded with two clamps. (iii) Scanning electron microscopy (SEM) image of a cell loaded onto the nano-channel side of the 3D NEP chip ready for electroporation. Reproduced with permission.^[61] Copyright 2016, The Royal Society of Chemistry.

2.2.2 Optothermal poration

Normally, bulk thermal stimulation is capable of perturbing lipid membranes to induce membrane disruption and to deliver small molecules into cells.^[65, 66] However, the detrimental effects from high temperature on cells preclude its widespread applications. Thus, more precise approaches have been proposed using absorbent nanoparticles as nucleation sites to confine the intense heating to a localized area.^[67, 68] An example is shown in Figure 2.5a, presenting a bio-photonic laser-assisted surgery tool (BLAST) fabricated to achieve massively parallel delivery of large cargo into mammalian cells.^[69] The platform comprised an array of transmembrane holes, the side walls of which were coated with crescent-shaped titanium (Ti) films aiming to harvest laser energy. The rapid and pulsed laser triggered the vibration of bubbles, thus disrupting the cell membrane and inducing partial membrane disruption. With this localized optothermal poration method, cargos such as plasmid DNA, enzymes, antibodies and even large-sized bacteria can be efficiently internalized into cells.

To realize targeted delivery to a single cell, a photothermal nanoblade made of a Ti-coated microcapillary pipette was fabricated and placed on top of a mammalian cell (Figure 2.5b).^[70] The laser-induced bubble cavitation can generate high-speed and localized fluidic flows that disrupted the cell membrane, which significantly enhanced the delivery of molecules into the cell cytosol. With this method, quantum dots (QDs), which are normally difficult to be internalized into cells, were efficiently delivered into cells via the localized and intense membrane disruption.

Figure 2.5. Strategies for localized optothermal poration. (a) Schematic of the BLAST large-cargo delivery platform. The platform contained an array of transmembrane holes patterned on a 1.5- μm -thick silicon dioxide (SiO_2) film. Crescent-shaped Ti films were asymmetrically coated on the side walls of these holes to harvest laser pulse energy. After membrane opening, an external pressure source was applied to deform the bottom flexible PDMS storage chamber to push cargos into the cytosol of cells via these transient membrane pores. Reproduced with permission.^[69] Copyright 2015, Nature Publishing Group. (b) Schematic of ultrafast membrane disruption induced by a photothermal nanoblade for cargo delivery into cells. A Ti thin film was coated on the outside of a glass micropipet. Upon excitation by a nanosecond laser pulse, the Ti heated rapidly, along with a thin surrounding aqueous layer through heat conduction. Reproduced with permission.^[70] Copyright 2011, American Chemical Society.

2.2.3 Mechanical poration

2.2.3.1 Nanoinjection

To overcome the limited accuracy of microinjection,^[71, 72] nanoinjection has been developed as a more precise method for intracellular delivery.^[73, 74] Advances in modern nanofabrication technology has enabled such mechanical poration method with improved precision. The generation of nanometer features, capable of penetrating the cell membrane and providing access to the cytosol, has facilitated the direct injection of targeted materials through a hollow nanoneedle or nanostraw.^[75, 76] As shown in Figure 2.6a, a cell culture

platform of templated “nanostraws” that can pierce the cell membrane was demonstrated as a permanent fluidic pipeline for cytosolic delivery.^[77] In this case, sequential delivery was carried out without the need to continually rupture the cell membrane. The intracellular delivery of molecules ranging from ions to 5000-basepair DNA constructs was possible with sub-minute temporal resolution within an easy-to-use sample well platform. In addition, this method opened the way for active and reproducible delivery of a wide variety of species into cells without endocytosis.

Moreover, as a high-aspect-ratio nanostructure, nanowires were also employed as a nanoneedle for nanoinjection.^[78, 79] As shown in Figure 2.6b, surface-modified vertical silicon nanowires were used for the spatially localized delivery of biomolecules into the primary mammalian cells.^[80] The vertical silicon nanowires penetrated the cell membrane and subsequently released the surface-bound molecules directly into the cell cytosol, thus allowing the efficient delivery of biomolecules into cells without any chemical modification or viral packaging. This modality enabled the introduction of a broad range of biological effectors (i.e. DNA, RNA, peptides, proteins and small molecules) into almost any cell type.

Figure 2.6. Strategies for membrane disruption with direct injection through nanometer structures. (a) Nanostraws for direct fluidic intracellular delivery. (i) Schematic of a typical device used to deliver biomolecules into cells via nanostraw-mediated delivery. (ii) SEM image of nanostraw membranes. Reproduced with permission.^[77] Copyright 2011, American Chemical Society. (b) Silicon nanowires (Si NWs) as a generalized platform for delivering a wide range of biological effectors. (i) Schematic of cells (pink) on Si NWs (green) at early and late stages of penetration, respectively. (ii) SEM image of vertical Si NWs fabricated by reactive ion etching. Reproduced with permission.^[80] Copyright 2010, National Academy of Sciences.

2.2.3.2 Microfluidic squeezing

Microfluidic squeezing involves the rapid deformation of cells as they pass through the constriction of the size of approx. half to one-third of a cell's diameter in a microfluidic channel.^[81, 82] Intracellular delivery of a variety of cargos including proteins, nucleic acids, QDs, carbon nanotubes and other nanomaterials, has been demonstrated with this membrane-disruption method.^[83, 84] A major advantage of microfluidic squeezing is the simplicity of the device, with no moving parts or need for an external power supply. The energy for membrane disruption comes from the flow through a static structure.^[3] Moreover, this approach has shown the high-throughput ability to handle over a million cells per second. To ensure the delivery efficiency for different cell types, microfluidic channels of various constriction geometries have been designed to address cells with different sizes. Figure 2.7 shows a vector-free microfluidic platform for the cytosolic delivery of macromolecules.^[85] Transient membrane disruption has been produced on cells as they were mechanically deformed at the constricted positions, which facilitated the passive diffusion of materials into the cell cytosol.

Figure 2.7. Schematic of membrane disruption by microfluidic squeezing. The rapid deformation of a cell occurred as it passed through a microfluidic constriction, generating transient membrane holes. TEM image of the parallel channel with blue cells included for illustration. Reproduced with permission.^[85] Copyright 2013, National Academy of Sciences.

Cell squeezing has been combined with microscale electroporation within a microfluidic channel to enhance nuclear delivery.^[86] As shown in Figure 2.8, cells passing through the microfluidic constructions were mechanically deformed to cause transient disruption of the cell membrane, which allowed a subsequent electric field to further disrupt the nuclear

envelop and to drive DNA molecules into both the cytoplasm and nucleus. The results indicated that the delivery of DNA and messenger RNA was significantly dependent on the electric field, while protein delivery was more dependent on the mechanical disruption. Thus, the combined mechanical and electrical membrane disruption was capable of co-delivering nucleic acids and proteins for both nuclear and cytosolic delivery.

Figure 2.8. High-throughput nuclear delivery via a combined mechanical and electrical membrane-disruption method. (a) Schematic illustration of the working principle. (i) Cell membranes were mechanically disrupted as cells passed through the microfluidic constriction. (ii) The electric pulses reversibly disrupted the nuclear envelope and drove DNA into the cytoplasm and nucleus. Purple dashed lines indicate the applied electric field. (b) Magnification of a set of identical microfluidic constrictions etched into a silicon wafer (left) and a set of microelectrodes deposited on a Pyrex wafer (right). (c) Optical image of the microfluidic device by bonding the silicon and Pyrex wafers together. The scale bar represents 1 mm. Reproduced with permission.^[86] Copyright 2017, Nature Publishing Group.

2.2.3.3 Localized sonoporation

Sonoporation has been developed as an important permeabilization approach since its introduction in the 1980s.^[87] Microbubble interaction with cells under the stimulation of ultrasound is the key step in a sonoporation process, in which the cell membrane is acoustically disrupted to open temporary pores for intracellular delivery. These microbubbles are gas-filled structures stabilized by a lipid, protein or polymer shell, which can also be used

as ultrasound contrast agents.^[88, 89] The process of alternately growing and shrinking of microbubbles is referred as cavitation, which can be divided into stable cavitation (Figure 2.9a) and inertial cavitation (Figure 2.9b and c).^[90-92] For stable cavitation at lower ultrasound intensities, microbubbles expand and contract in an oscillatory manner, creating local acoustic streaming that may result in transient openings of the cell membrane. In contrast, inertial cavitation at higher ultrasound intensities may lead to microbubbles oscillating with increasing amplitudes and then bursting with sufficient energy to permeabilize the cell membrane.

Figure 2.9. Mechanisms of microbubble cavitation. (a) Stable cavitation: microbubbles can oscillate around their resonant size while generating acoustic streaming that exerts shear stresses on cells. Initial cavitation has two effects: (b) sudden collapse of microbubbles leads to shock waves that are capable of disrupting the cell membrane, (c) the collapsing microbubbles near a membrane surface also experience non-uniformities in their surroundings, which results in a high-velocity microjet to penetrate into the cell membrane. Reproduced with permission.^[90] Copyright 2012, Future Science Group.

Although such mechanism has been clearly demonstrated and accepted by most researchers, reproducibly controlling sonoporation at the micro and nanos-scale has proven difficult which is due to fact that the random and violent cavitations are heterogeneous, and some cells undergo excessive damages while others remain unaffected.^[93] Thus, targeted cavitation was proposed to precisely control the position of cavitation bubbles and locally generate shear forces at a specific distance to the target cell.^[94, 95] Figure 2.10a shows a microfluidic device that simultaneously applied microscale electroporation and sonoporation to the cells flowing through the microchannel.^[96] In this case, the two different fields (electric field and ultrasound) in perpendicular directions allowed the formation of transient pores along two axes of the cell membrane at reduced poration intensities, hence maximizing the delivery efficiency while minimizing the cell death. To further achieve targeted sonoporation on a single cell, a surface acoustic wave (SAW) resonator of 24 MHz was used to control the

position of a microbubble cluster relative to the target cell (Figure 2.10b).^[97] Both spatial manipulation and acoustic excitation of microbubbles were controlled with this microfluidic SAW-based device, and the precisely positioned microbubbles could induce localized cavitation of cells, which achieved a high delivery efficiency while maintaining cell viability. Subsequently, a microfluidic chip involving an array of cell trapping structures was presented for the single-cell sonoporation at high throughput (Figure 2.10c).^[37] The asymmetrical growth and collapse of the cavitation bubbles near the trapped cells induced microjetting to deform individual cells in suspension, which allowed for a fast, repeatable, and localized rupture of the cell membranes.

Figure 2.10. Strategies for localized sonoporation. (a) Schematic of a microfluidic electro-sonoporation device. Cells passing through the microfluidic channel made of two opposing 3D microelectrodes were exposed to an electric field generated between the microelectrode pair. An ultrasonic transducer placed on top of the channel coupled ultrasound to cells. Reproduced with permission.^[96] Copyright 2013, The Royal Society of Chemistry. (b) Schematic of targeted cavitation with a SAW-based microfluidic device. Microbubbles were positioned toward the targeted cell to achieve localized sonoporation. Reproduced with permission.^[97] Copyright 2014, AIP Publishing. (c) Schematic of a microfluidic chip with an array of cell trapping structures. A cavitation bubble was created at a stand-off distance to the trapped cell. Then, the bubble expanded to its maximum size and collapsed asymmetrically caused by the solid boundary condition of the trapping structure. Thereby, microjetting was induced to deform the cells. Reproduced with permission.^[37] Copyright 2013, The Royal Society of Chemistry.

2.2.3.4 Hypersonic poration

Recently, a new type of cell poration method, namely hypersonic poration which uses GHz ultrasound was developed to enhance intracellular delivery, especially nuclear uptake.^[98] Different from sonoporation, transient nanopores can be directly created by hypersonic without any assistance of bubble agents. Here, hypersonic is referred to as the acoustic wave above GHz frequency, which is tens to hundreds times higher than conventional ultrasonic waves.^[99, 100] Since the wavelength of the GHz hypersonic is at the submicron scale, it

strongly couples with cells to exert both normal and shear stresses on the cell membrane, which directly induces transient nanopores in the membrane. As shown in Figure 2.11, hypersound was generated by a microfabricated bulk acoustic wave (BAW) resonator of 1.6 GHz frequency.^[98] Free doxorubicin (Dox) molecules have been efficiently delivered into HeLa cells through transient nanopores induced by hypersound during the membrane-disruption process. It was found that the pore formation was temporary and reversible, which ensured high cell viability. Combined with conventional sonoporation, this system offered a clean and efficient method to enhance cellular uptake of different sized molecules without requirements of any additional chemicals. The action region and the delivery amount can be well controlled by the input power and duration of hypersound, providing potentials to realize the localized delivery with accurate amounts of therapeutic agents. Since hypersonic poration requires no additional chemicals, complicated preparation steps and does not induce any immune response, it can be extensively applied to drug and gene delivery.

Figure 2.11. Schematic of the hypersound-assisted intracellular delivery system. (a) The hypersonic wave was generated by a microfabricated BAW resonator. It propagated in the liquid and interacted with HeLa cells. (b) Explanation of interactions between hypersound and cells. The hypersound induced artificial hypersonic nanopores in cell membrane, allowing foreign molecules to access into the cell. 2D finite element modelling (FEM) simulations of (c) ultrasonic wave of 30 MHz and (d) hypersonic wave of 1.6 GHz. The color patterns outside the hemisphere represent the distributions of the acoustic pressure in water and the color patterns on the cell represent the stress on the cell membrane. The simulation results show that both the normal and the shear stress induced by the hypersound are strongly enhanced compared with the conventional ultrasound. (e) SEM images of cell membranes after the treatment of hypersound for 10 min. The bottom image was the enlarged image of the top one. Reproduced with permission.^[98] Copyright 2017, Wiley-VCH.

2.3 Conclusions and outlook

Over the past decades, membrane-disruption methods have been successfully developed for intracellular delivery. In this chapter, we have focused on the recent trends in high-precision membrane-disruption methods applied for drug and gene delivery. These physical approaches have advantages in taking several salient features of the micro and nano-scale: (i) able to accommodate diverse cells, (ii) independent of delivery materials, (iii) compatible with intracellular targeting strategies, and (iv) high cell viability after the process. Some representative examples of intracellular delivery were discussed in detail, which can help to understand the mechanism of membrane disruption at the micro and nano-scale. In addition, advances in nanotechnology, microfluidics, lab-on-chip (LOC), and microsystems have been combined with these poration methods. By concentrating precision membrane-perturbing effects to the cellular or subcellular scale, the potential exists to address applications that are underserved by current techniques.

For further advances, high delivery efficiency should be combined with scalability, tunable throughput, low cost and user-friendliness. An ideal delivery system would be able to deliver any cargos to any type of cells at desired sites, which can be accomplished with a combination of targeted and stimuli-responsive carriers. In such case, the membrane disruption must be sufficient to introduce the intended cargo, yet the cell must be capable of repairing itself without permanent damage. The high-precision membrane-disruption techniques have great potential for accelerating the progress in studies of drug and gene delivery, which can be further applied to clinical therapeutic applications.

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Chapter 3

Real-time Detection of Hypersonic Poration of Supported Lipid Bilayers

As a new type of membrane-disruption method, hypersonic poration has been introduced to improve the efficiency of drug and gene delivery for biomedical applications. In this chapter, an integrated microchip, composed of a bulk acoustic wave (BAW) resonator and a gold electrode as the extended gate of a field effect transistor (EGFET), was fabricated to study the effects of hypersonic poration on a supported lipid bilayer (SLB). The high-frequency BAW resonator was used to generate hypersound, while the EGFET facilitated the conductivity measurements of the SLB assembled on top of the device. The real-time detection revealed that hypersound could induce transient nanopores in the membrane, which acted as the equivalent of ion channels and changed the membrane permeability of the SLB. Further characterization confirmed the reversibility and controllability of the hypersonic nanopores, which provided insight into the mechanism of hypersonic poration and thereby contributed to the development of an approach to control the membrane permeability in real time.

3.1 Introduction

Physical methods based on the principle of membrane disruption, such as thermal poration,^[1-3] optoporation,^[4-6] electroporation^[7-9] 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 the physical interaction with cells, which improves the permeability of the cell membrane without any further damage. 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, 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] Thus, an acoustic wave of gigahertz (GHz) frequency and (sub)micrometer wavelength, which is defined as hypersound,^[33, 34] has been proposed to affect membrane permeability. 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.^[35] 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.^[36]

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 chapter, hypersonic poration of a supported lipid bilayer (SLB) was studied. A microfabricated bulk acoustic wave (BAW) resonator with a frequency of 1.6 GHz has been used to generate hypersound with a submicron wavelength. On the same microchip, a gold electrode has been integrated and connected with the extended gate electrode of a field effect transistor (EGFET) to monitor the currents through the SLB induced by hypersound. The ion-channel effects of hypersonic nanopores were measured and analyzed with salt solutions containing different cations. 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.

3.2 Results and discussion

3.2.1 System design and working principle

In this work, a micro-electromechanical system (MEMS) microchip, integrated with a bulk acoustic wave (BAW) resonator and a gold electrode, was fabricated to generate and monitor the hypersound effects on a supported lipid bilayer (SLB), that is assembled on top of the device, in real time (Figure 3.1a). Scanning electron microscopy (SEM) images of the BAW resonator are shown in Figure 3.1b. The typical structure of the BAW resonator consists of a piezoelectric film of aluminium nitride (AlN) and two electrodes (top and bottom) of molybdenum (Mo).^[37] By coupling the vertical electric field through a specific piezoelectric coefficient, the BAW resonator vibrates in a longitudinal mode and generates hypersound of gigahertz (GHz) frequency.^[38] Here, the quarter-wavelength Bragg reflector composed of Mo and silicon dioxide (SiO₂) thin films was deposited under the resonance structure to avoid the dissipation of energy into the silicon substrate.^[39] The polygon shape of the resonator was designed to enhance the main-mode vibration while minimizing the parasitic effect.^[35]

The working principle of the integrated system is illustrated in Figure 3.1c. An artificial 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 BAW resonator and the gold electrode. Once activated, the vibrating interface of the resonator drove the motion of the SLB that was placed on top of the resonator and induced a propagation of hypersound within the lipid membrane, from the vibrated area of 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 by the FET.^[40] Since a SLB is highly resistive and impermeable to hydrated ions, the electric potential of the underlying gold electrode can remain constant in a pure electrolyte solution by the isolation caused by the intact SLB. However, when this insulating SLB is stimulated with hypersound, the continuous motion of the lipid membrane may induce a change of the membrane structure. Once membrane defects occur during this process, the SLB becomes permeable to ions, thus inducing membrane conductivity which can be detected by the electrical measurements from the back-end FET.

Figure 3.1. A microfabricated chip integrating a high-frequency BAW resonator (1.6 GHz) with a gold electrode. (a) Top view of the integrated microchip. (b) SEM images of the polygon-shaped BAW resonator. The BAW resonator is a film-stacked structure with a piezoelectric layer of AlN sandwiched between two Mo electrodes. (c) Schematic illustration and working principle of the integrated system. The SLB was coated on the surface of the microchip, covering both the BAW resonator and the gold electrode. The gold electrode is connected with the gate electrode of the FET (called an extended-gate FET, EGFET) and is used as the front-end sensing electrode for electrical measurements.

The lipid bilayer was fabricated using the Langmuir-Blodgett (LB) method.^[41] Figure 3.2a shows the surface pressure–area (π -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 $\text{K}_3\text{Fe}(\text{CN})_6$ as a redox probe to confirm the integrity of the artificial SLB. As shown in Figure 3.2b, 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.

Figure 3.2. Characterization of the SLB made of POPG and assembled on the MEMS-fabricated BAW/EGFET device. (a) π -A compression isotherm of POPG at the air-water interface at room temperature. (b) CV responses of 1 mM $\text{K}_3\text{Fe}(\text{CN})_6$ in 1 M KCl at the gold electrode of the integrated microchip (black: bare electrode without SLB, blue: electrode coated with the SLB).

3.2.2 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 hypersonic. As shown in Figure 3.3a, the current curve instantaneously increased when turning on the hypersonic and was held at a constant value during the sustained stimulation. The current recovered to its original value when switching off the hypersonic. These results suggest that the applied hypersonic facilitated the SLB to become conductive by changing its membrane permeability, which can be attributed to hypersonic poration. As illustrated schematically in Figure 3.3c, some pores were created in the SLB by the stimulation of hypersonic, allowing the translocation of ions across the

membrane and inducing an immediate increase of the ion current. The concomitant recovery of the much lower base current by switching off the hypersound indicates the reversibility of the pore formation, which can be explained by the flexibility of the lipid membrane and molecular diffusion of the lipid molecules. The acoustic pressure induced by hypersound disrupts the order of the lipid membrane with mechanical effects, but the defects occurred in the membrane structure can be healed during the re-assembly process. Interestingly, the transmembrane current induced by hypersound is similar to the stochastic “gating” behavior of biological ion channels.^[42] Instead of modulating the ion-channel currents with reversibly binding blocker molecules, the current through the SLB can be mediated by transient pores from hypersound.

Subsequently, the ion current was modulated with hypersound of different input powers as illustrated in the current-time trace (Figure 3.3a). The first step of the ion current was in response to hypersound of 3.2 mW; thereafter the current value was increased step by step with the increase of input power, suggesting the generation of more transient nanopores with hypersound of higher input power. The magnitude of the current through the transient pores was analyzed as a function of input power and fitted by an exponential equation (Figure 3.3b). The fitted curve shows a gradual transition in the response to hypersound. 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.

Figure 3.3. (a) Real-time detection of ion current through the SLB on the BAW/EGFET device by alternately switching on (green arrows) and off (orange arrows) the hypersound (the input powers were successively 3.2, 10, 20, 32, 50, 100, 160, 250, 320 and 500 mW). The buffer solution was PBS (0.1 M, pH 7.4). (b) Data points (markers) and exponential fitting (line) of the ion current as a function of input power. (c) Schematic illustration of the formation and recovery of transient pores in the SLB induced by hypersound.

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 Figure 3.4a. To ensure the applied hypersound was in the linear range, the value of the input power was limited to 250 mW. It was found that the current curves all increased with hypersound of higher input powers, and importantly, the currents were different with solutions containing different salts. It is likely that the increased current responses for CaCl₂ and FeCl₃ compared with KCl can be attributed to two reasons. Since each electrolyte solution contains the same concentration of metal ions (i.e. 2 mM K⁺, Ca²⁺ and Fe³⁺), the valence of these ions plays the main role in determining the current flow induced by the translocation of cations, which agrees with the phenomena that the current is almost proportional to the valence of metal ions. On the other hand, the different concentrations of the chloride anion in these solutions of KCl, CaCl₂ and FeCl₃ can be

another reason for the different current responses. These results further confirm the ion channel-like behavior of hypersonic nanopores. Similar results have been reported previously for pores formed in the cell membrane by electroporation.^[43] This study supports the prediction that currents through a lipid bilayer can be generated by increasing the membrane conductance due to the formation of pore structures in a pulsed electric field. In the present study, the current mediated by hypersound can also be considered as an analogous channel current, which is generated from the translocation of ions through transient nanopores in the SLB. The ion mobility across the pore structures can be affected by both the charge and concentration of ions, which may altogether determine the magnitude of hypersonic current.

In Figure 3.4b, 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 Figure 3.3b (below 250 mW). It is therefore likely that the value of the current through the transient pores in the SLB can be quantitatively controlled by adjusting the intensity of hypersound, which can be consequently used to adjust the permeability of lipid membrane to different degrees.

Figure 3.4. (a) Real-time recording of the ion current through the SLB on the BAW/EGFET device by alternately switching on (purple arrows) and off (orange arrows) the hypersound (the input powers were successively 3.2, 10, 20, 32, 50, 100, 160, 250 mW). The electrolyte solutions were respectively KCl, CaCl_2 and FeCl_3 of the same concentration (2 mM in pure water). (b) Data points (markers) and linear fitting (lines) of the ion current as a function of input power.

3.2.3 Characterization of hypersonic poration

To evaluate the formation of hypersonic pores at the lipid membrane, cyclic voltammograms of 1 mM $\text{Fe}(\text{CN})_6^{3-}$ in 1 M KCl were recorded with the SLB-coated gold

electrode in the presence and absence of hypersound. As shown in Figure 3.5a, the redox response increased with switching on the hypersound (500 mW) in real time, indicating electron transfer to the gold electrode, which was induced by the exposure of electrode upon immediate pore formation. Once the last CV cycle was ended, another CV cycle was started again with switching off the hypersound, and the duration of this hypersonic treatment was less than 1 min. As shown in the figure, the redox peaks were reduced immediately and overlapped with the CV curve obtained in the absence of hypersound, indicating that the oxidation and reduction of ions was reduced to the base level at the shielded electrode. This data confirms that the hypersound-induced pore formation is fast and reversible, and the SLB can restore its integrity after the stimulation by hypersound.

The morphology of the SLB was analyzed by atomic force microscopy (AFM) before and after the stimulation by hypersound. All the measurements were conducted in liquid phase using tapping mode. Since the platform of AFM is not compatible with the hypersound setup, it cannot provide real-time information of morphological changes of SLB. Therefore, hypersound 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 hypersound until the redox peaks did not reduce any more after switching off the hypersound. As shown in Figure 3.5b, 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.^[43] The morphology of SLB was first detected after a short treatment of hypersound (250 mW, 5 min), where no obvious difference was observed on the membrane surface (Figure 3.5c), indicating that hypersound of lower intensity and duration does not damage the structure of SLB irreversibly. In contrast, when the applied hypersound was increased to 500 mW for 30 min, the morphology of the membrane was changed (Figure 3.5d). The average height increased from 13.7 ± 0.6 nm (Figure 3.5b) to 16.8 ± 1.0 nm, and clear defects were observable on the membrane surface (Figure 3.5d). These results confirm the effects of hypersound on the changing of the membrane structure of SLBs.^[44]

Figure 3.5. Characterization of hypersonic poration at a SLB. (a) Real-time CV responses of 1 mM $\text{K}_3\text{Fe}(\text{CN})_6$ in 1 M KCl at SLB by switching on (500 mW, <1 min) and off the hypersound. The black curve was obtained with the original SLB before any treatment of hypersound. The green curve was obtained by simultaneously switching on the hypersound. After the duration of a CV cycle, hypersound was immediately switched off and another CV cycle (blue) was recorded. (b) AFM images of SLB (b) before any treatment of hypersound. (c) after treated with hypersound of 250 mW for 5 min and (d) then treated with hypersound of 500 mW for 30 min.

To assess the structural changes of the membrane, laser scanning microscopy (LSM), which is compatible with the hypersound setup, was used for studying the hypersonic pores at the SLB in a real-time fashion. The duration of hypersound for all the microscopy measurements was less than 1 min during the scanning process. Figure 3.6a shows the morphology of the SLB coated on top of the activated BAW resonator in a confocal image. Here, the color and contrast were governed by the membrane structure at varied reflectivity levels. Once activated, wave-like patterns were observed on the surface of the SLB, centering at the resonator and radiating towards the outside, which indicates the propagation of hypersound within the bilayer structure. As the zoom-in image shows, the lipid membrane

was patterned by the propagation of hypersound, which induced morphological deformation of the membrane.

Subsequently, the morphology 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 (Figure 3.6b), 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 (Figure 3.6c, 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 propagation of hypersound.

Figure 3.6. Real-time LSM images of a SLB on a BAW/EGFET device in the absence or presence of the stimulation by hypersound (500 mW). (a) Morphology of SLB on top of the activated BAW resonator. The sizes of the 2D map and the 3D zoom-in image are respectively $450 \times 550 \mu\text{m}^2$ and $100 \times 100 \mu\text{m}^2$. Morphology of SLB on top of the gold electrode before (b) and after (c) the stimulation by hypersound. Hypersound 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 $220 \times 220 \mu\text{m}^2$ and $2 \times 2 \mu\text{m}^2$.

3.3 Conclusions

In this chapter, the behavior of a supported lipid bilayer (SLB) stimulated by the hypersound of gigahertz frequency was detected by an integrated microchip composed of a bulk acoustic wave (BAW) resonator and a gold electrode, which facilitates the real-time electrical detection of hypersonic poration. The “gating”-shaped current induced by hypersound reveals that some pores were created on the SLB with the propagation of hypersound, which can be regarded similar to 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 rate and magnitude of hypersonic pores can be controlled in a real-time manner by correspondingly adjusting the duration and input power of hypersound. The simultaneous effects of ion valence and concentration on the formation of hypersound-induced current confirms the behavior of hypersonic nanopores on lipid membranes as the ion channel. Furthermore, characterization experiments (cyclic voltammetry, Atomic force microscopy and laser scanning microscopy) were conducted to evaluate the properties of hypersonic nanopores on the SLB. 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.

3.4 Acknowledgements

MEMS&NEMS group (Tianjin University) is acknowledged for the microfabrication of the integrated chip composed of a bulk acoustic wave (BAW) resonator and a gold electrode. Menglun Zhang is thanked for the circuit design of the BAW resonator.

3.5 Materials and methods

3.5.1 General

All chemicals including phosphate-buffered saline (PBS), potassium chloride (KCl), calcium chloride (CaCl_2), iron (III) chloride (FeCl_3) and potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$) 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. $\text{K}_3\text{Fe}(\text{CN})_6$ was dissolved in 1 M KCl solution to obtain 1 mM solution for the cyclic voltammetry tests.

3.5.2 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 $0.5 \text{ mg} \cdot \text{mL}^{-1}$. The supported lipid bilayer (SLB) was fabricated and coated on the integrated chip by the Langmuir-Blodgett (LB) method using a Langmuir trough (Kibron, MicroTrough XL). In brief, 50 μL of the POPG solution was spread onto the surface of pre-cleaned Milli-Q water and then compressed at a speed of $10 \text{ mm} \cdot \text{min}^{-1}$ until the targeted surface pressure of $25 \text{ mN} \cdot \text{m}^{-1}$ 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 sub-phase at a constant speed of $1 \text{ mm} \cdot \text{min}^{-1}$ to form a single lipid layer and then down into the LB trough again to form the SLB.

3.5.3 Fabrication of the integrated microchip

The microfabrication process was according to a previously published article (Figure 3.7).^[40] Before fabrication of the resonance part of a bulk acoustic wave (BAW) 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. The top layer of 600 nm Mo was used as the bottom electrode followed by the sputtering of highly c-axis oriented aluminum nitride (AlN) film (1 μm) as a piezoelectric layer and another layer of Mo (600 nm) as the top electrode. Finally, 300 nm Au was employed as the gold electrode, which was used as the extended gate electrode of a field effect transistor (EGFET).

Figure 3.7. Microfabrication process flow.

3.5.4 Setup for the real-time detection of hypersonic poration

The integrated chip was mounted onto a two-channel evaluation board with the wire bonding technique. Sinusoidal signals of 1.6 GHz were generated by a signal generator (Agilent, N5181A), amplified by a power amplifier (Mini-Circuits, ZHL-5W-422), and sent to the BAW resonator, which then transduced the electrical supply to mechanical vibrations and generated hypersound. The gold electrode was connected to a commercially available back-end field effect transistor (FET, ALD110800A, Advanced Linear Devices). The back-end transistor was an n-channel FET with a zero-volt threshold voltage necessary to avoid the destruction of lipid membranes at any positive or negative voltage. A miniature Ag/AgCl reference electrode (World Precision Instruments, Inc.) was used to bias the transistor to the desired working point. FET characteristics was confirmed off-line using a Keithley 2636 and 2400 Source Measure Unit (SMU) before real-time measurement at a constant bias voltage of 0.3V to ensure the FET working in the linearly amplified zone.

3.5.5 Characterization

Cyclic voltammetry (CV)

The CV results were obtained using a CHI 660E (Huachen, Shanghai) potentiometer. During the CV test, a time-dependent potential signal (from -0.2 mV to 0.7 mV) was applied to the working electrode, and the current that flowed between the working and counter electrodes was recorded. The counter electrode used in this work was a platinum wire, and the reference electrode a Ag/AgCl electrode.

Atomic force microscopy (AFM)

The AFM analysis was performed using a Bruker ICON AFM. The Bruker's Sharp Nitride Lever (SNL-10) probe was used for high-performance imaging with tapping mode in fluid. The cantilever was oscillated below resonance, which resulted in a continuous series of force-distance curves. The varying degrees of force constants and resonance frequencies of the probe provided enough versatility to cover the lipid membranes.

Laser scanning microscopy (LSM)

The LEXT OLS4000 (Olympus) laser scanning microscope was used to analyze the 3D morphology of the SLB with or without the stimulation of hypersound. All the microscopy images were obtained in confocal mode, which was excited by a 405 nm semiconductor laser. The area was scanned with an ultra-fast acquisition function to reach a rate of 16 frames per second (fps). A dedicated 50x objective lens (NA 0.95) was used to observe the membrane surface with minimum aberration. The open platform of the LSM makes it possible to place the integrated chip on the microscope stage and enables the real-time detection of the membrane morphology.

3.6 References

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Chapter 4

Hypersound-induced Deformation of and Encapsulation by Giant Unilamellar Vesicles

The ability of hypersound to induce acoustic pressure in a liquid and exert shear stress on a membrane surface is at the root of hypersonic poration. In this chapter, giant unilamellar vesicles (GUVs) were prepared and immobilized on a supported lipid bilayer (SLB) to study the hypersonic poration effect. Real-time microscopy measurements revealed that the deformability of GUVs can be controlled with hypersound of different input powers. Moreover, the deformation changed with the distance between the GUVs and the BAW device. The size of the hypersonic pores induced by the deformation of GUVs was analyzed by loading polystyrene (PS) beads of different sizes into the GUVs. The results described here provide a deeper understanding of hypersonic poration, which can be further applied in cell manipulation and cell membrane permeation.

4.1 Introduction

As an important physical method for enhancing transportation of external molecules like proteins,^[1-3] drugs^[4-6] and genetic materials^[7-9] into cells, acoustic approaches have triggered a lot research interest due to their non-invasiveness and biocompatibility. Numerous studies have been performed to understand the mechanism of pore formation in the acoustic field, among which the deformation of cells via acoustically actuated microbubbles was considered as one of the important factors for transient membrane disruption.^[3, 10-12]

In microbubble-based poration processes,^[13-16] the oscillation of the air-liquid interface on the microbubbles perturbs the liquid nearby, which thereby results in localized acoustic streaming in liquid.^[17-23] When cells are placed in the acoustic flow, hydrodynamic forces will be exerted on the cell membrane, which then stretch and deform the cells. To further understand the working mechanism of such deformability, artificial cell models^[24-26] are usually applied. Among these models, giant unilamellar vesicles (GUVs) have gained special interest since their size is comparable with cells, while their spherical shape facilitates the characterization of the deformability with microscopic techniques.^[27-30] It was found that the mechanical forces generated from the acoustic streaming can deform the elastic vesicles resulting in pore structures on the membrane.^[31, 32] Here, the collapse of microbubbles to concentrate forces, called cavitation, is an essential mechanism of sonoporation.

Recent studies prove that gigahertz ultrasound (i.e. hypersound) can also induce hypersonic poration effects on membrane structures, which has been verified with both theoretical simulations and experimental cell experiments.^[33] It was found that the acoustic streaming generated from hypersonic waves can induce acoustic pressure in liquid and exert shear stress on the surface of the membrane, which mechanically changes the permeability of the cell membrane by creating transient nanopores. This formation of hypersonic nanopores was also confirmed for the planar supported lipid membrane as described in Chapter 3. Since the acoustic streaming in the high-frequency acoustic field of hypersound is induced by the propagation of microscale acoustic waves instead of the vibration of microbubbles, the triggered deformation of cells and GUVs by hypersound should be

different from the acoustically actuated microbubbles, but this has not yet been studied in detail.

In this chapter, hypersound generated from a BAW resonator of 2.5 GHz frequency was applied to trigger the deformation of giant unilamellar vesicles (GUV) that were immobilized on a supported lipid bilayer (SLB). Confocal laser scanning microscopy (CLSM) was applied to monitor the real-time deformation of GUVs. Different powers were applied to the resonator, and the distance between the GUVs and the resonator was varied to further understand the functioning of the hypersound process. To better understand the mechanism of hypersonic deformation, a 3D finite element model (FEM) was employed to simulate the deformation of GUVs in a force analysis. Moreover, the hypersound-driven encapsulation of nanoparticles of different sizes by GUVs was studied, which in turn supports the formation of hypersonic pores and verifies the size of these pore structures. These studies help to understand hypersonic deformation and membrane poration processes, and provide insight into the mechanical effects of hypersound.

4.2 Results and discussion

4.2.1 GUVs immobilized on a supported lipid bilayer

In this work, giant unilamellar vesicles (GUVs) were immobilized on a supported lipid bilayer (SLB) as a cell model to study the deformation of vesicles stimulated by hypersound. As shown in Figure 4.1a, a BAW resonator of 2.5 GHz frequency was placed into a reservoir to apply hypersound. GUVs were adhered to the SLB on the bottom glass substrate of the reservoir. The distance between the GUVs and the hypersonic source was defined as the vertical distance between the bottom substrate and the resonator.

As shown in Figure 4.1b, both the GUVs and the SLB were made from lipid mixtures of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), Texas Red-functionalized 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (Texas Red-DHPE), and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(cap biotinyl) (Biotinyl Cap-PE). After the deposition of the biotinylated SLB, streptavidin (SAv) was anchored onto the membrane surface, which was followed by the adhesion of biotinylated GUVs via biotin-SAv affinity

pairs. Here, Texas Red-DHPE was used as one of the lipid components to facilitate the fluorescence imaging of the immobilized GUVs.

Figure 4.1. (a) System for the detection of hypersound-induced deformation of immobilized GUVs. The BAW resonator was mounted on a long rectangle-shaped evaluation board, which can be inserted into the reservoir at a variable depth. The distance between the immobilized GUVs and the mounted resonator was controlled by the inserted position of the evaluation board. The GUV-SLB system was kept in sucrose buffer (100 mM, 20 mM Tris, pH 7.4) to keep the GUVs stable. (b) Schematic picture of the biotin-SAv binding motif used for the adhesion of GUVs to the SLB. Both the GUVs and the SLB are composed of DOPC, Texas Red-DHPE and Biotinyl Cap-PE.

Before the adhesion of GUVs to the SLB, the mobility of the lipid membranes was assessed through qualitative fluorescence recovery after photobleaching (FRAP) experiments. As shown in Figure 4.2a, the Texas Red-labeled SLB, which was fabricated using the vesicle fusion method,^[34, 35] emitted uniform red fluorescence before the bleaching step. After exposure to high-intensity excitation at a wavelength of 595 nm for 10 min, a clearly defined dark patch was observed on the SLB, indicating an almost complete bleaching of the Texas Red dye. However, the red fluorescence increasingly recovered over the course of 20 min after the laser was shut off, suggesting that the lipid molecules in the membrane can move in and out of the bleached area through lateral diffusion. This confirms the proper formation of the SLB, which therefore provides a flexible interface for the following adhesion of GUVs and reduces the risk of vesicle rupture that can occur on a rigid surface.

The fluorescence images obtained from different focal planes for the same GUV are shown in Figure 4.2b. Here, Alexa Fluor 488-labeled SAv was used to facilitate the visual imaging of the adhesion interface. As the figure shows, GUVs with diameters of 15 to 20 μm stayed intact upon adhesion to the SLB (image i). By gradually decreasing the focal plane close to the binding surface (image ii), the contact area between the adhered GUV and SLB

became apparent as a small dark patch (image iii), which overlapped with the areas of green fluorescence emitted from the Alexa Fluor 488-labeled SAV (image iv). It is likely that the adhesion of GUVs led to concentration of the anchored SAV molecules at the SLB to support the immobilization of vesicles, which consequently induced a local high fluorescence intensity at the binding site.

Figure 4.2. (a) Fluorescence images of the Texas Red-labeled SLB before bleaching (left), 10 min later after exposure to high-intensity laser with the excitation wavelength of 595 nm (middle), and 20 min after the excitation laser was shut down (right). (b) Fluorescence images of immobilized GUVs obtained at different focal planes. Image i to iii correspond to the GUVs emitted from Texas Red fluorescence. Image iv correspond to the SAV emitted from Alexa Fluor 488 fluorescence. The adhesion of GUVs to the SLB was controlled by inter-membrane affinity pairs between SAV and biotin moieties on both GUV and SLB. The scale bars represent 20 μm .

To investigate the morphology of immobilized GUVs, 3D reconstructions of a GUV loaded with Fluo-4 dye were composed from the confocal z-stacks (Figure 4.3b); the corresponding 2D cross-sectional images are shown in Figure 4.3a. As the Texas Red (lipid) channel shows, the adhering GUV was shaped as a truncated sphere on the surface of the SLB. The green fluorescence emitted from Fluo-4 was clearly defined as a sphere and coincided with the shape of the lipid shell, confirming the integrity of the immobilized GUV. These results enable us to distinguish easily between adhering and non-adhering vesicles, which can be beneficial to observe dynamic responses of the GUVs under the application of hypersound.

Figure 4.3. (a) Confocal 2D cross-sectional images and (b) 3D reconstructions of an immobilized GUV on the SLB. The confocal z-stacks were obtained by scanning the GUV from bottom to top with distance steps of 0.3 μm . The scale bars represent 10 μm .

4.2.2 Hypersound-induced deformation of GUVs

Due to the micrometer size of GUVs, their response to hypersound can be monitored in situ using confocal laser scanning microscopy (CLSM). The real-time deformation of a GUV, which was induced by hypersound of different input powers, was recorded and is shown in Figure 4.4a. In this process, the stimulation of hypersound was applied at 200 ms and switched off at 800 ms. As a control, the GUV without any hypersonic treatment was first tracked for the same duration. With the input power of hypersound increasing from 100 mW to 500 mW, the deformation of the GUV became observable and progressively increased, which suggests that the shape of the GUV was varied depending on the input power of hypersound.

To analyze the deformability by hypersound quantitatively, the outline (dashed line in Figure 4.4a) of the GUV at each frame was extracted by an image processing program, from which the long and short axes (a and b in Figure 4.4a) were estimated, and the aspect ratio (a/b) of the GUV was derived thereof. Figure 4.4b shows the change of the aspect ratio for a GUV treated with hypersound at different input powers. Once hypersound was applied, the aspect ratio immediately increased and maintained at a higher value during the stimulation, while it returned to its initial value upon switching off the hypersound. This result indicates that the deformation of a GUV induced by hypersound was instant and reversible. By increasing the input power of hypersound, the aspect ratio of the GUV was also increased corresponding to a larger degree of deformation. Subsequently, the projected area of the vesicle was also monitored as a result of the deformation of the GUV (Figure 4.4c), which shows a similar trend as the aspect ratio (Figure 4.4b). The deformability in terms of both aspect ratio and projected area can be attributed to elastic and reversible stretching of the membrane of a GUV induced by the mechanical effects of hypersound.

Figure 4.4. (a) Time sequences of CLSM images showing the deformation of a GUV upon hypersound of different input powers (0, 100, 300 and 500 mW). The distance between the GUV and the resonator was fixed at 100 μm in each measurement. The scale bars indicate 10 μm. Changes of the (b) aspect ratio (as marked in Figure 4.4a, aspect ratio = a/b) and (c) projected area of the GUV under the stimulation of hypersound.

Notably, both the aspect ratio and the projected area of the GUV at a hypersonic power of 500 mW did not completely recover to their original values upon turning off the power. To explain this irreversible deformation, further experiments were performed with hypersound of high input power. As shown in Figure 4.5a, hypersound of 500 mW was continuously applied to two adjacent GUVs for 600 ms, during which the relative distance between the two vesicles was gradually reduced and finally reached a minimum value that

was stable after switching off the hypersound. The corresponding binding areas of the two GUVs with the SLB are shown in Figure 4.5b. Compared to the original state (image i), the binding areas were significantly increased after the stimulation by hypersound (image ii), which is likely due to the compression of the GUVs onto the SLB. This assumption was confirmed by the 3D reconstructions of a GUV from the confocal z-stacks (Figure 4.5c), which shows that the height of the GUV was decreased after treatment with hypersound of 500 mW for 600 ms. These results suggest that the contact area between the GUV and the SLB was also changed during the deformation with hypersound, which may enhance the binding of biotin receptors on the GUV with the anchored SA_v on the SLB and thereby increase the number of bound affinity pairs in the contact area. By this enhanced interaction, the hypersound-treated GUV cannot totally recover to its original shape.

Figure 4.5. (a) Time sequence of CLSM images showing the deformation of two adjacent GUVs stimulated by hypersound of 500 mW during 600 ms. The distance between the two GUVs and the hypersonic source was fixed at 100 μm . (b) The binding areas between the GUVs and the SLB before (i) and after (ii) the stimulation by hypersound (500 mW, 600 ms). (c) 3D reconstructions of an immobilized GUV before (i) and after (ii) the stimulation by hypersound (500 mW, 600 ms). The scale bars indicate 10 μm .

In principle, the intensity of hypersound will be attenuated with energy dissipation in solution during its propagation process.^[33] To evaluate the effects of hypersound as a function of distance (referred to as the marked distance in Figure 4.1a), the deformation of a GUV was monitored with hypersound of the same input power (300 mW) at different distances (Figure 4.6a). The extracted aspect ratio (Figure 4.6b) and projected area (Figure 4.6c) of the GUV confirm the changes of deformation at different distances. In Figure 4.6c, the recorded area of GUV treated with hypersound at 300 μm was larger than that at the other distances,

which is likely due to the spreading out of the GUV upon prolonged observation. Interestingly, with the distance between the GUV and the hypersonic source increasing from 100 to 300 μm , the degree of deformation became weaker and almost reached zero at 300 μm , as witnessed from the images, the aspect ratio as well as the area shown in Figure 4.6. Surprisingly, the deformation of the GUV increased when the distance was increased from 50 to 100 μm . These results indicate that the generation of hypersound has a limited effective range, with an optimum distance of approx. 100 μm .

Figure 4.6. (a) Time sequence of CLSM images showing the deformation of GUV with hypersound at different distances (50, 100, 200 and 300 μm). The input power of hypersound was fixed at 300 mW in each measurement. The scale bars indicate 10 μm . Corresponding changes of the (b) aspect ratio and (c) projected area of the GUV upon the stimulation by hypersound (300 mW).

4.2.3 FEM simulations of hypersound-induced vesicle deformation

To understand the mechanism of deformation induced by hypersound, a 3D finite element model (FEM) was employed for theoretical simulations. In the model, hypersound was generated by a BAW resonator (2.5 GHz) that was localized above the GUV. The GUV, which was surrounded by water, was substituted by an elastic sphere with a diameter of 20 μm at coordinates of (45 μm , -45 μm) relative to the resonator center. The vertical distance between the resonator and the GUV was fixed at 100 μm . Figure 4.7a shows the patterns of acoustic streaming induced by hypersound, where four micro-vortices were observed around the resonator. A zoom-in image of the GUV in the acoustic field is shown in Figure 4.7b. The simulated color in the water medium represents the distribution of acoustic pressure generated by the acoustic streaming, and the color represents the shear stress exerted on the GUV induced by the acoustic pressure. The figure clearly shows that the GUV deformed under the action of shear stress. The nonuniform distribution resulting from the shear force that acted in the direction of the acoustic streaming led to the deformation of the GUV.

Figure 4.7. Working mechanism of hypersonic deformation of an immobilized GUV as modeled by FEM simulations. (a) Simulation results of the acoustic streaming patterns distributed around the GUV. (b) Zoom-in image of panel (a) showing the nonuniform flow field near the vesicle and the shear force at the vesicle. The right color bar indicates the magnitude of the streaming velocity from min (blue) to max (red), while the left color bar indicates the magnitude of the shear stress from min (red) to max (pink).

Subsequently, the acoustic field around the GUV was calculated, and the hydrodynamic traction was integrated over the surface of the GUV to calculate the shear stress exerted on the vesicle. Due to the nonuniform acoustic pressure, the shear stress experienced by the

GUV was different for the vesicles at different distances relative to the vibrating resonator. The simulation results with hypersound of different input powers and different distances are summarized in Table 4.1 and the corresponding 3D plot is shown in Figure 4.8. For the results obtained at each distance, the shear stress increased by increasing the input power of hypersound from 100 to 500 mW, which was in accordance with changes of deformation in Figure 4.4. However, for GUVs that were treated with hypersound of the same input power but at different distances, the shear stress always reached its maximum value at 100 μm and decreased at lower (50 μm) or higher distances (200 and 300 μm). It is likely that the shear stress on the GUV is proportional to the input power of hypersound, which is not fully proportional with distance. These results confirm that the deformation of the GUV can be controlled by tuning the input power of hypersound as well as the distance of the GUV relative to the resonator.

Table 4.1. Shear stresses on the surface of a GUV induced by hypersound of different input powers (100, 300 and 500 mW) at different distances (50, 100, 200 and 300 μm) to the resonator.

Distance (μm)	Power (mW)	Shear stress on GUV (pN)
50	100	0.226
50	300	0.818
50	500	1.590
100	100	0.380
100	300	1.260
100	500	2.320
200	100	0.243
200	300	0.739
200	500	1.250
300	100	0.138
300	300	0.411
300	500	0.679

Figure 4.8. 3D plots of shear stress as a function of input power and distance according to the data in Table 4.1.

4.2.4 Hypersound-induced encapsulation by GUVs

Since hypersound has been studied in previous research to enhance the delivery of drug molecules by creating transient nanopores on the cell membrane, we hypothesize that the effect of deformation induced by hypersound is an important factor to trigger the pore formation on membrane structures. To evaluate this assumption, hypersound was applied to study the encapsulation of positively charged polystyrene (PS) beads into negatively charged GUVs at different input powers. The duration of stimulation for all the measurements was 10 min. Here, the encapsulation efficiencies of PS beads of 50 nm (blue fluorescence), 100 nm (orange fluorescence) and 200 nm (red fluorescence) were investigated and compared. To avoid the fluorescence interference by PS beads with orange/red fluorescence, Top Fluor-labeled GUVs that emitted green fluorescence were used instead of red-emitting Texas Red-labeled GUVs.

As shown in Figure 4.9a, by switching on the stimulation of hypersound, the blue fluorescence, emitted from 50 nm PS beads, was observed inside the GUV, and its fluorescence intensity gradually increased with hypersound of higher input power. This suggests that the PS beads of 50 nm were successfully taken up by GUVs upon applying hypersound, which in turn verifies the formation of transient nanopores induced by the rapid mechanical deformation of the GUVs. To evaluate the pore size relative to the deformation of the vesicles, PS beads of 100 and 200 nm were also used in encapsulation experiments

into GUVs using hypersound of different input powers. It was found that the orange fluorescence, emitted from the PS beads of 100 nm, was only observed inside the GUV when hypersound of 300 and 500 mW was used (Figure 4.9b). The absence of orange fluorescence under hypersound of 100 mW indicates that the PS beads of 100 nm cannot be loaded into GUVs, which is likely due to an insufficiently large pore size induced by hypersound of 100 mW necessary for the penetration of the 100 nm PS beads. Furthermore, the red fluorescence, emitted from the PS beads of 200 nm, was only visible around but not inside the GUV even with hypersound of 500 mW (Figure 4.9c). It indicates that the PS beads of 200 nm were too large to be loaded inside GUVs with hypersound of 500 mW.

These results reveal that the encapsulation of PS beads is dependent on the input power of hypersound, which we assume is also related to the deformation of GUVs. Apparently, only when the deformation of GUVs was strong enough to open nanopores of sufficient size that allows the uptake of PS beads, the encapsulation can be achieved. These results suggest a method to control the efficiency and selectivity of encapsulation via the pore size on the membrane by adjusting the input power of hypersound.

Figure 4.9. CLSM images of Top Fluor-labeled GUVs encapsulated with positively charged PS beads of (a) 50 nm (blue fluorescence), (b) 100 nm (orange fluorescence) and (c) 200 nm (red fluorescence) without (control) or with (100, 300, 500 mW) the stimulation by hypersound at a fixed distance of 100 μm for 10 min. The scale bars indicate 10 μm .

4.3 Conclusions

In this chapter, the deformation of giant unilamellar vesicles (GUVs) induced by hypersound of 2.5 GHz frequency was monitored in real time and analyzed with theoretical simulations. Both the aspect ratio and projected area of GUVs were quantitatively studied to reveal the deformation mediated by hypersound of different input powers. These results confirm that the shear stress generated from the acoustic streaming can stretch the lipid membrane and reversibly deform the shape of GUVs. Since GUVs were flexibly immobilized on the surface of a supported lipid bilayer (SLB), the binding area between the bound GUV and the SLB was also affected upon the application of hypersound, which in turn verifies the compression of GUVs by the acoustic pressure. These studies clearly show the mechanical effects of hypersonic deformation, which can be potentially used as a new tool for biophysical analyses and further development of controlled drug delivery methods.

4.4 Acknowledgements

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4.5 Materials and methods

4.5.1 General

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(cap biotinyl), sodium salt (Biotinyl-Cap-PE), and 1-palmitoyl-2-(dipyrrometheneboron difluoride)undecanoyl-*sn*-glycero-3-phosphoethanolamine (Top Fluor-PE) were purchased from Avanti Polar Lipids and dissolved in chloroform. Texas Red-modified 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (Texas Red-DHPE), non-fluorescent and Alexa Fluor 488-labeled streptavidin (SAv) were purchased from Thermo Fisher Scientific Inc. SAv were dissolved in 0.1 M PBS buffer (0.1 M sodium dihydrogen phosphate and 0.15 M sodium chloride, pH 7.4) at a concentration of 20 $\mu\text{g mL}^{-1}$. Amine-

modified polystyrene (PS) beads (2.5 wt.% in aqueous solution) with different fluorescence markers were purchased from Sigma Aldrich and diluted 10 times with Milli-Q water before use.

4.5.2 Preparation of the supported lipid bilayer

In this work, lipid mixtures of DOPC, Texas Red-DHPE and Biotinyl-cap-PE (molar ratio is 99.85:0.05:0.1) were used to form the supported lipid bilayer (SLB) using the vesicle fusion method. First, small unilamellar vesicles (SUVs) were prepared using the hydration and extrusion method.^[36] In brief, a chloroform solution of DOPC (10 mg mL⁻¹, 99.85 μ L), Texas Red-DHPE (0.2 mg mL⁻¹, 7.07 μ L) and Biotinyl-cap-PE (0.2 mg mL⁻¹, 8.78 μ L) was injected into a glass vial and evaporated under a gentle flow of nitrogen gas until the film of lipid was visible on the wall. Then, the vial was put into a vacuum desiccator for more than 1 h to dry the lipids. Thereafter, the dry lipid mixtures were hydrated with 1 mL Milli-Q water and vortexed until all lipids were removed from the vial. The obtained lipid solution was then extruded 11 times using a Mini-Extruder kit (Avanti Polar Lipids) equipped with a polycarbonate membrane of 100 nm pores (Whatman) to form SUVs. In this work, the supported lipid bilayer (SLB) was deposited in the wells of a 96-well plate with a glass bottom. Before the formation of the SLB, 400 μ L sodium hydroxide (NaOH, 2 M) solution was added to glass substrate for 1 h to form a hydrophilic surface. Afterwards, the wells were rinsed with Milli-Q water three times and incubated with 200 μ L SUV solution for 30 min at room temperature. A defect-free SLB was then formed by the rupture of SUVs onto the hydrophilic glass substrate. Excess lipids were removed from the well by rinsing with Milli-Q water three times.

4.5.3 Preparation of giant unilamellar vesicles

Two types of giant unilamellar vesicles (GUVs) were prepared using an electrical method.^[37] One was composed of DOPC, Texas Red-DHPE and Biotinyl-cap-PE with a molar ratio of 99.85:0.05:0.1. The other one was composed of DOPC, Top Fluor-PE and Biotinyl-cap-PE with a molar ratio of 99.7:0.2:0.1. The mixed lipid solutions were dropped on a titanium oxide-coated glass slide and evaporated with a flow of nitrogen gas to create a uniform lipid film. The lipid-coated slide was dried in a vacuum desiccator for 1 h. Then, the

dried lipid-coated slide and a clean non-lipid-coated slide were put together to form a capacitor cell. The conductive sides of both slides were faced inward and were fixed with a clamp to form a chamber. The chamber was filled with 200 mM sucrose (Sigma-Aldrich) solution in Tris buffer (20 mM) and sealed with plastic paraffin film. Electro-formation was then carried out using a function generator. A 10 Hz sinusoidal potential with a 1 V peak-to-peak amplitude was applied across the chamber for 2 h, after which the frequency was reduced to 2 Hz for 1 h. The GUVs were extracted from the chamber using a pipette and stored in a vial (Eppendorf) in the dark.

4.5.4 Immobilization of giant unilamellar vesicles

Before the adhesion of GUVs, the SLB-coated substrate was incubated with SAv/ Alexa Fluor 488 SAv (200 μL , 20 $\mu\text{g mL}^{-1}$) for 20 min and then rinsed consecutively with PBS and sucrose buffer, each for three times. After that, 50 μL GUV solutions were added to the well and incubated for another 30 min. Then, the well was gently rinsed with sucrose buffer three times to remove excess GUVs.

4.5.5 Fluorescence measurements

Confocal laser scanning microscopy (CLSM, Nikon A1) and fluorescence microscopy (Olympus, BX53) were used to observe immobilized GUVs and the SLB. The Texas Red-labeled GUVs were examined by CLSM and fluorescence microscopy with an excitation wavelength (λ_{ex}) of 595 nm, while the Top Fluor-labeled GUVs were measured with λ_{ex} of 495 nm.

4.6 References

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Chapter 5

Hypersound-Controlled Release and Uptake of Cargo by Polymer-Shelled Vesicles

Polymeric nanocarriers are used increasingly in controlled release to achieve complex drug-release patterns in response to a specific stimulus. In this chapter, a miniaturized high-frequency acoustic resonator was applied to generate hypersound of gigahertz (GHz) frequency for the controlled release and uptake of cargo from/to polymer-shelled vesicles (PSVs). PSVs either suspended in solution or immobilized on a surface were addressed by this hypersound-controlled release method, and the release rates increased linearly with hypersound of longer duration and higher input power. Encapsulation studies revealed that the uptake can be well controlled by adjusting the exposure time of hypersound at different input powers. PSVs showed structural integrity after treatment of hypersound, verifying that the mechanical stress induced by hypersound does not destroy the assembled supramolecular structures of PSVs. This hypersound-controlled release and encapsulation system may become beneficial to the applications of controlled drug delivery.

5.1 Introduction

The controllable release of drug molecules from nanocarriers is an important aim in the field of drug delivery. Numerous studies have focused on developing novel drug carriers based on supramolecular self-assembly methods to realize controlled drug release, where both the release and encapsulation process can be controlled quantitatively.^[1-6]

Vesicles are well-known, promising systems for the encapsulation and delivery of biologically active substances. A broad spectrum of compounds can be easily loaded into these micro/nano scale containers, and many strategies exist to control their properties.^[7-9] Polymer-shelled vesicles (PSVs) are highly stable and effective for the encapsulation of small hydrophilic molecules.^[10] Considering the controlled release of cargo for biomedical applications, stimulus-responsive vehicles capable of reacting to a specific trigger are highly required. For example, redox-responsive PSVs comprising a cyclodextrin vesicle core and a reductively cleavable polymer shell have been synthesized for intracellular delivery.^[11] Some thermosensitive cross-linked polymer vesicles have been used to create a temperature-controlled release system.^[12, 13]

A variety of stimuli have been employed to accomplish the triggered release of cargo from vesicles, including internal conditions such as pH,^[14-16] and redox state^[17-19] as well as external triggers such as light,^[20-22] temperature,^[23-25] ultrasound^[26-28] and magnetic field.^[29-31] However, these methods are limited by either chemical properties of the vesicles or specifications of the operating environment. For practical applications, it is desired to develop methods without limitations imposed by the chemical properties of the system or the external environment. Physical stimuli provided by micro/nano scale devices are thought to be potential candidates to realize controlled release owing to the small device size and high precision.^[32] Micro-electromechanical systems (MEMS) such as microneedles^[33-35] and micro-pumps^[36, 37] have been developed for controlled release.

According to chapter 3 and 4, hypersound of gigahertz frequencies can directly interact with cell membranes and contribute to the deformation of the vesicle membrane.^[38] The mechanical stress from the membrane deformation can induce the transient formation of nanopores, allowing the translocation of substances from/into vesicles and even cells.

In this chapter, miniaturized high-frequency acoustic resonator has been applied to generate hypersound (1.6 GHz) to quantitatively control the release and encapsulation of cargo by polymer-shelled vesicles (PSVs). The hypersound-controlled release and encapsulation of cargo from/into PSVs, employing vesicles both suspended in solution and immobilized on a surface, are demonstrated here. The release and encapsulation kinetics have been assessed as a function of duration and input power of the hypersound, in order to assess the function of triggered release.

5.2 Results and discussion

5.2.1 Release of cargo from PSVs suspended in solution

Figure 5.1a shows the system of hypersound-controlled release from PSVs suspended in solution. Two chambers were sealed in stack and separated by a filter membrane as a dialysis system. An acoustic resonator of 1.6 GHz^[39] was placed at the bottom to generate hypersound in order to induce a swift micro-vortex and to trigger the release of cargo from vesicles in the lower chamber. Here, PSVs were used as a highly stable model carrier to study the hypersound-controlled release behavior in solution. As shown in Figure 5.1b, PSVs were created from cyclodextrin vesicles, onto which adamantyl-functionalized poly(acrylic acid) (Ad-PAA) was attached by host-guest recognition, followed by cross-linking of the carboxylic acid groups on the vesicle surface.^[6] Carboxyfluorescein (CF) dye was encapsulated into the PSVs as a model compound to allow quantification by fluorescence measurements. Once treated with hypersound, the permeability of PSVs should be changed by creating (transient) pores in the polymer-cyclodextrin vesicle shell, triggering the release of the CF dye from the PSVs. The released amount is evaluated from the fluorescence intensity of the liberated CF dye in the upper chamber.

Figure 5.1. Hypersound-controlled release of cargo from PSVs in solution. (a) Chamber-based system for studying dye release from PSVs in solution: A filter membrane (cut-off 12–15 kDa) sandwiched between two PDMS chambers to keep the PSV-encapsulated CF dye in the lower chamber, while liberated CF dye can pass the membrane. (b) Schematic representation of PSV based on cyclodextrin vesicles, decorated with Ad-PAA and covalently cross-linked using a bis-amine. The permeability of PSVs are changed with hypersonic poration effects.

Hypersound at different durations and input powers was applied to the dye-encapsulated PSVs to investigate the controlled release rate in solution. Figure 5.2a–c shows the fluorescence spectra of CF in the upper chamber after treating with hypersound of 100, 300 and 500 mW, respectively. At each power, the fluorescence intensity gradually increased as the duration of hypersound was extended, suggesting that more CF dye was released from PSVs upon prolonged hypersound treatment. This implies that the released amount can be controlled by the exposure time of hypersound. As a control experiment, the solution in the upper chamber was also studied by fluorescence spectroscopy in analogous manner (marked as control group in Figure 5.2a–c), which showed no significant fluorescence intensity when no hypersound was applied.

To gain further insight into the release kinetics in solution, peak fluorescence intensities in Figure 5.2a–c were extracted and investigated as a function of time. As shown in Figure 5.2d, the fluorescence intensity of released CF increased with the shift of hypersound from 100 mW to 500 mW at the same duration, suggesting a more rapid release with hypersound of higher power. The power dependence is attributed to the micro-vortex created in the solution as induced by hypersound.^[38, 39] Higher input power increases the velocity of the microfluidic flow, thus enhancing the shear stress executed on the PSV surface and speeding up the release in solution. In addition, the release curves show a linear trend in the initial

period of time (initial 10-20 min) and thereafter gradually level off to a plateau, indicating a concentration balance of released CF between both chambers. The initial slopes of the linearly fitted sections of the curves are regarded as the release rates under hypersound of different input powers, which shows a linear dependence as a function of power (Figure 5.2e).

Figure 5.2. Fluorescence detection of hypersound-controlled dye release from PSVs in solution. Fluorescence spectra of released CF dye triggered by hypersound of different input powers: (a) 100 mW, (b) 300 mW, and (c) 500 mW, or without applying hypersound (control) as a function of time. In the control experiment, the fluorescence intensity of the solution in the upper chamber is detected after waiting for 20 min after dye-encapsulated PSVs were put in the lower chamber. (d) Fluorescence intensity as a function of time and input power, and linear fits of the initial parts of these curves. (e) Initial release rate (determined from the linear fits in d) as a function of hypersound input power.

To assess any structural changes of PSVs upon hypersound-controlled release, dynamic light scattering (DLS) and transmission electron microscopy (TEM) measurements were performed. As shown in Figure 5.3a, no obvious changes occurred in the size distribution of PSVs upon hypersonic treatment, indicating that the PSVs maintained their original size and stayed intact under the hypersonic stress. This observation is supported by the TEM images (Figure 5.3b). Before any hypersonic treatment (image i) in Figure 5.3b, the PSVs appeared as circular objects as typically observed for polymer-stabilized vesicles,^[40] and no apparent difference was observed after treatment of hypersound (image ii) in Figure 5.3b). These results suggest that the mechanical stress induced by hypersound does not destroy the structure of the PSVs and that the permeability of membrane is reversible. We attribute the

hypersound-controlled release to hypersonic poration resulting in an enhanced permeability of the highly dynamic, host-guest-stabilized polymer-cyclodextrin vesicle layer.^[41]

Figure 5.3. Characterization of PSVs: (a) Size distributions of PSVs before and after hypersound according to DLS. (b) TEM images of PSVs before i) and after ii) treatment with hypersound (500 mW for 20 min). The scale bar is 100 nm.

5.2.2 Release of cargo from PSVs immobilized on a surface

As demonstrated above, hypersound can be used as an effective source to trigger the release of dyes from PSVs suspended in solution. However, for some applications, it can be beneficial to anchor carriers onto substrates to realize controlled release. For this purpose PSVs can be conjugated with biotin to specifically immobilize them on streptavidin (SAv)-coated surfaces via biotin-SAv recognition applying a recently published procedure.^[10] Figure 5.4a shows the system for hypersound-controlled release of dyes from PSVs immobilized on a surface. A 600- μm -high chamber was mounted on an acoustic resonator with a glass slide covering on top to form a sealed compartment. As shown in Figure 5.4b, the glass slide was functionalized with biotin-functionalized poly-L-lysine (PLL-*g*-OEG-biotin) and streptavidin (SAv), and PSVs functionalized with biotin terminal groups were subsequently immobilized on this surface via biotin-SAv affinity pairs. When studying the release, biotinylated PSVs encapsulated with CF were immobilized on the glass slide and immersed in HBS buffer. The released amount of dye was determined from fluorescence measurements of the solution in the chamber as well as from fluorescence microscopy images of the PSVs on the glass slide.

Figure 5.4. (a) Chamber-based system for hypersound-controlled dye release and encapsulation from/into PSVs immobilized on a surface. Hypersound is generated by the acoustic resonator at bottom. It propagates through the solution and interacts with PSVs immobilized on the cover glass. The vertical distance between the acoustic resonator and the PSV areas (i.e. the height of the PDMS chamber) is 600 μm . (b) Sketch of the biotin-streptavidin binding motif used for the immobilization of biotinylated PSVs.

To study hypersound-controlled release of cargo from PSVs immobilized on a surface, the fluorescence intensity of released CF in the sealed chamber was measured. The fluorescence spectra of CF released from PSVs triggered by hypersound of 100, 300 and 500 mW are respectively shown in Figure 5.5a-c. Similar to the release of cargo from PSVs suspended in solution, the fluorescence intensity of CF for immobilized PSV also increased when the duration of hypersound was extended, showing a faster release of CF at higher input power of hypersound. No significant amount of CF was released from the biotinylated PSVs when no hypersound was applied (marked as control in Figure 5.5a-c). These results indicate that the release of cargo from PSVs immobilized on a surface can also be well controlled by adjusting the duration and input power of the applied hypersound.

The release kinetics on a surface was monitored by calculating the fluorescence intensity of released CF as a function of time upon hypersound treatment at different input powers. As shown in Figure 5.5d, all three release curves show a linear trend, and the fitted slopes of the curves are regarded as the release rates. Note that the release profiles on a surface did not reach a plateau at 15 min, meaning that the final release was not complete, while for the release in solution triggered by hypersound of 500 mW, a clear plateau was observed in the same period. As shown in Figure 5.5e, the fitted release rates were found to be linearly dependent on the input power. The main driving force to trigger the release of cargo from

PSVs immobilized on a surface is the acoustic shear force induced by the high-speed microvortex at the membrane-liquid interface.^[38] The kinetics of this hypersound-controlled release on a surface shows a similar behavior to the mechanism of release in solution. Therefore, we expect that also here hypersonic poration causes the release.

Figure 5.5. Fluorescence detection of dye release from PSVs immobilized on a surface. Fluorescence spectra of released CF triggered by hypersound of different input powers: (a) 100 mW, (b) 300 mW and (c) 500 mW. (d) Fluorescence intensity as a function of time, and linear fits (release rate) at different input powers. (e) Release rate as a function of hypersound input power.

For visual monitoring of hypersound-controlled release on a surface, a cover glass substrate was patterned with PLL-g-OEG-biotin using the micromolding in capillaries (MIMIC) technique,^[42] before SAV and CF-loaded PSVs were immobilized. PSVs loaded with 100 μ M of CF were used to prevent self-quenching of the dye and to allow visualization by fluorescence microscopy. Figure 5.6a shows the line-patterned fluorescence microscopy images of immobilized, CF-encapsulated PSVs with and without stimulation by hypersound. Compared with the controls (no hypersound applied), the stimulation of hypersound led to an obvious decrease of fluorescence intensities in the patterned areas, indicating the successful release of CF from PSVs on the substrate. With the input power of hypersound increasing from 100 mW to 500 mW, lower fluorescence intensities were observed from the patterned PSV areas, confirming the stronger release of CF dye under hypersound of higher input power. Note that all fluorescence images in Figure 5.6a were taken at the substrate area straight above the acoustic resonator. To quantitatively calculate the changes of fluorescence

intensity induced by hypersound of different input powers, line profiles of the patterned fluorescent PSVs are shown in Figure 5.6b-d. Figure 5.6e shows the power-dependent fluorescence changes as a function of time, illustrating the power-dependent release process on a surface.

Figure 5.6. (a) Fluorescence imaging of line-patterned (by MIMIC), immobilized, CF-loaded PSV before and after stimulation of hypersound. The scale bars are 10 μm . Changes of fluorescence intensity under hypersound at input powers of (b) 100 mW (c) 300 mW and (d) 500 mW. (e) Time-dependent changes of fluorescence intensity under hypersound of different input powers.

To verify that PSVs stay immobilized on the surface during treatment of hypersound, PSVs covalently labeled with rhodamine B were used for the fluorescence measurements. As shown in Figure 5.7a, the fluorescence intensities of rhodamine B labeled PSVs did not change after the stimulation by hypersound (500 mW, 20 min), suggesting that the PSV are not detached from the surface and the covalently labeled dye cannot be released by hypersound.

Figure 5.7. (a) Fluorescence microscopy images of rhodamine-B labeled PSVs with (500 mW for 15 min) and without stimulation of hypersound. The scale bar is 10 μm . (b) Line profiles of fluorescence intensities extracted from immobilized PSVs.

5.2.3 Encapsulation of cargo into PSVs immobilized on a surface

In the case of encapsulation, empty biotinylated PSVs were used and immobilized on SAV-coated substrates (similar as shown in Figure 5.4b), and the samples were immersed into a concentrated 5 mM CF solution. The encapsulation of the CF dye triggered by hypersound was studied by fluorescence microscopy imaging of the PSVs on the cover glass. Figure 5.8a shows the fluorescence microscopy images of immobilized PSVs with and without stimulation by hypersound. PSVs treated with hypersound of higher input powers and longer durations exhibited a green color of higher intensity, indicating that a larger amount of CF was encapsulated into the vesicles. Figure 5.8b-d shows the line profiles of fluorescence intensities obtained from PSVs triggered by hypersound in CF dye solutions. The encapsulation kinetics is illustrated in Figure 5.8e. The encapsulation yields increased with the loading time, and the encapsulation rates increased with input power. These results demonstrate that hypersound can not only promote the release of a dye from pre-loaded PSVs, but also enhances the uptake of fluorescent molecules into empty vesicles. Additionally, it confirms that, in the release experiments with the substrate-immobilized

PSVs described above, detachment of the vesicles is not an issue, but that instead the dye is released from the vesicles while the vesicles remain intact and immobilized.

Figure 5.8. Fluorescence imaging of PSVs after loading with CF under hypersound of different input powers and duration time. The scale bar is 10 μm . Line profiles of fluorescence intensity under hypersound of (b) 100 mW (c) 300 mW and (d) 500 mW. (e) Time-dependent changes of fluorescence intensity under hypersound of different input powers.

5.3 Conclusions

In this work, hypersound was used as a physical stimulus to control the release and encapsulation of cargo from polymer-shelled vesicles (PSVs) either suspended in solution or immobilized on a surface. The hypersound-controlled release and encapsulation kinetics were analyzed with hypersound of different durations and input powers. All kinetic data confirm a power dependence, thus providing possibility to precisely control the extent of release or encapsulation by setting the conditions of hypersound. The hypersound can exhibit reversible and instant-responsive membrane permeability of PSVs, providing an “on-off” switch to quantitatively control the release and encapsulation of cargo in real time. In particular, alteration of duration and input power of hypersound can modulate the cumulative release/encapsulation of cargo from/into PSVs on demand. This method can be further used to change the permeability of the supramolecular structure by controlling the assembly or disassembly of the supramolecules, creating the possibility of applying hypersound in controlled release of self-assemble materials. The constant release rate at a given input power can be further used for pulsatile stimuli-responsive release instead of sustained release in controlled drug delivery.

5.4 Acknowledgments

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5.5 Materials and methods

5.5.1 General

All chemicals were purchased from Sigma Aldrich, VWR or TCI and used without further purification. 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffered

saline (HBS, 20 mM HEPES, 150 mM NaCl, pH 7.4) was prepared using ultrapure water and used as the buffer solution throughout the work in this chapter. Adamantane-terminated poly(acrylic acid) (Ad-PAA),^[6] amphiphilic β -cyclodextrin,^[43,44] rhodamine B labeled amphiphilic β -cyclodextrin,^[45] and biotin-NH₂^[10] were prepared according to previously described procedures.

5.5.2 Preparation of polymer-shelled vesicles (PSVs)

Polymer-shelled vesicles (PSV) were prepared according to a previously described method.^[10] Briefly, a 2 mM stock solution of amphiphilic β -cyclodextrin in chloroform was added in a round bottom flask and evaporated under a stream of argon to obtain a thin film. Residual solvent was removed under vacuum before the film was hydrated by addition of buffer or carboxyfluorescein (CF) dye solution and stirred for 2 h. The solution was sonicated for 15 s and repetitively passed through a polycarbonate membrane with 100 nm pore size in a Liposofast manual extruder (AVESTIN) to yield cyclodextrin vesicles (CDVs). To stabilize CDVs with a polymer shell, 25.0 μ M Ad-PAA was added to a buffered solution of CDVs (100 μ M amphiphilic β -cyclodextrin), and this mixture was gently stirred for 30 min to obtain polymer-decorated vesicles. For crosslinking of the polymer shell, 9.00 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) was added. After 20 min, 600 μ M 2,2'-(ethylenedioxy)bis(ethyleneamine) and optionally, for the biotin-conjugated PSVs, 30 μ M biotin-NH₂ was added, and the colloid was slowly stirred overnight. Purification of PSVs was performed as described below.

For release from PSVs in solution, PSVs were prepared in HBS buffer containing 5 mM CF. Before the release experiment PSVs were separated from excess CF dye and byproducts by size exclusion chromatography (Sephadex G-50 Superfine) with HBS as the eluent.

For DLS and TEM experiments, PSVs were prepared in HBS buffer and purified by dialysis (Spectra/Por regenerated cellulose (RC) dialysis membranes, MWCO 6–8 kDa, Spectrum Laboratories) against HBS (3 $\times$ buffer exchange within 48 h).

For the release of cargo from PSVs immobilized on a surface, PSVs conjugated with biotin-NH₂ were prepared in HBS buffer with either 5 mM CF for fluorescence spectroscopic investigations, or with 100 μ M CF for fluorescence microscopic measurements. Before immobilization aiming for fluorescence spectroscopic investigations, PSVs were separated

from excess CF dye and byproducts by size exclusion chromatography (Sephadex G-50 Superfine) with HBS as the eluent. PSVs for fluorescence microscopic experiments were purified by dialysis (Spectra/Por regenerated cellulose (RC) dialysis membranes, MWCO 6–8 kDa, Spectrum Laboratories) against a 100 μ M solution of CF in HBS (3 $\times$ buffer exchange within 48 h).

Rhodamine B-labelled PSVs conjugated with biotin-NH₂ were prepared from CDVs containing 5 mol% rhodamine B-labelled amphiphilic cyclodextrin in HBS. PSVs were purified by dialysis (Spectra/Por regenerated cellulose (RC) dialysis membranes, MWCO 6–8 kDa, Spectrum Laboratories) against HBS (3 $\times$ buffer exchange within 48 h).

For encapsulation of cargo into PSVs immobilized on a surface, PSVs conjugated with biotin-NH₂ were prepared in HBS and purified by dialysis (Spectra/Por regenerated cellulose (RC) dialysis membranes, MWCO 6–8 kDa, Spectrum Laboratories) against HBS (3 $\times$ buffer exchange within 48 h).

5.5.3 Characterization of PSVs

Dynamic light scattering (DLS)

DLS was performed using a Nanotracer instrument (Anaspec), and data were processed with the Microtrac FLEX Operating Software. All the measurements were performed in HBS buffer using 1 mL centrifuge tubes. The average of five measurements was used as the size distribution of PSVs for each experiment.

Transmission electron microscopy (TEM)

5 μ L of the PSV sample was incubated on a carbon coated copper grid (glow-discharged in an oxygen plasma) for 2 min and gently blotted with filter paper. The sample was stained with 5 μ L of 0.5 % (w/w) aqueous phosphotungstic acid for 1 min and again gently blotted with filter paper. TEM measurements were performed at the Institute of Materials Physics, Münster, Germany, using a Zeiss 200 FE electron microscope with Schottky emitter and energy Ω filter operating at 200 kV. The microscope was equipped with a CCD camera Gatan USC 4000, and pictures were processed using ImageJ version 1.51n.

5.5.4 Immobilization of PSVs

Preparation of glass substrates

After sonication in acetone, ethanol and ultrapure water (each for 10 min), the pre-cleaned substrates of cover glass were immersed in a freshly prepared piranha solution (concentrated $\text{H}_2\text{SO}_4/\text{H}_2\text{O}_2$ (30%) = 3/1 (v/v)) for 30 min. Then the substrates were extensively washed with ultrapure water and dried in a stream of nitrogen.

Preparation of PDMS stamps

PDMS stamps were prepared by casting a 10:1 mixture of poly(dimethyl siloxane) and curing agent (Sylgard 184, Dow Corning) on a line-patterned silicon master. The silicon master with 10 μm wide line features separated by 10 μm spacing was fabricated by photolithography. The PDMS mixture was cured at 80 $^\circ\text{C}$ overnight. The cured stamp was peeled off from the master at the curing temperature and cut into square pieces.

Patterning PSV by micromolding in capillaries (MIMIC)

The patterned PDMS stamp was oxidized in oxygen plasma for 30 s to form hydrophilic interface and quickly placed in contact with the cleaned glass substrate, so that the grooves in PDMS form capillaries between the surface and the stamp. Poly-L-lysine PLL-g-OEG-biotin (200 μL , 0.1 $\text{mg}\cdot\text{mL}^{-1}$, HBS) was pipetted and dropped at the open end of the stamp. The solution spontaneously filled the channels under the capillary pressure. After 20 min, the stamp was gently removed, and the glass substrate was repeatedly washed with HBS (three times). Afterwards, the glass substrate was incubated with streptavidin (200 μL , 30 $\mu\text{g}\cdot\text{mL}^{-1}$, HBS) for 15 min and rinsed with HBS again (three times). After that, the patterned glass substrate was incubated with biotinylated PSVs for 5 min and rinsed with HBS (three times) to remove excess biotinylated PSV.

5.5.5 Device fabrication

Acoustic resonator

The acoustic resonator was fabricated using standard microfabrication according to a previous published process.^[38] A typical sandwiched structure that comprising two

molybdenum (Mo) layers as electrodes (top and bottom electrode) and a piezoelectric layer of aluminum nitride (AlN) in the middle was deposited on stacked reflector substrates to generate hypersound. In this chapter, the frequency of the acoustic resonator is 1.6 GHz, which was obtained by a network analyzer (Agilent E5061B).

Chamber-based release/encapsulation system

For PSVs release system in solution, PDMS with thickness of 1.6 cm was cut into square pieces and punched a hole in the center (diameter: 1.5 cm) to form chambers (volume: 150 μL). A PDMS chamber was first sealed on top of the acoustic resonator and filled with CF-encapsulated PSVs, then a dialysis membrane (MWCO 12–15 kDa, Spectrum Laboratories) was covered on top the chamber, followed by overlay of another chamber with the same size. Before adding hypersound, the upper chamber was filled with HBS buffer, which was then taken out for fluorescence measurements.

For PSVs release or encapsulation system on a surface, a square piece of PDMS of 600 μm was punch a hole in the center (diameter: 1.5 cm) and sealed on top of the acoustic resonator. After filling with HBS (or CF dye solution, 50 μL), a cover glass that immobilized with biotinylated PSVs was then covered on top of the chamber, aligning the center of the acoustic device. Sinusoidal signals of 1.6 GHz were generated by a signal generator (Mini-Circuits, D500), amplified by a power amplifier (Mini-Circuits, ZHL-5W-422), and sent to the device, which then transduced the electrical supply to mechanical vibrations in solution. The electric input can be conveniently switched on and off using a program control, thus the hypersound duration is well controlled.

5.5.6 Dialysis experiments

In the dialysis experiments for cargo release from PSVs in solution, fluorescence spectra of CF were measured immediately or upon waiting for 20 and 40 min after hypersound was turned off to ensure that the released CF dye in the lower chamber can pass through the filter membrane and equilibrate before fluorescence quantification. As shown in Figure 5.9, the fluorescence intensity detected at 20 min is higher than right after applying hypersound, while the peak intensity at 40 min is similar to that at 20 min, indicating that the released CF

dye has reached equilibrium within 20 min after hypersound is turned off. Therefore, all other measurements were done while waiting for 20 min after ending hypersound.

Figure 5.9. Fluorescence spectra of CF in the upper chamber measured immediately or upon waiting for 20 and 40 min after hypersound was turned off.

5.5.7 Fluorescence measurements

Fluorescence spectrometer

The emission spectra of CF (excitation at $\lambda = 488$ nm) and Rhodamine B (excitation at $\lambda = 550$ nm) were recorded using a fluorescence spectrometer (Perkin Elmer). For control experiments, HBS buffer in the upper PDMS chamber was detected without applying hypersound in the lower chamber, however, the fluorescence measurement still started after 20 min to keep in accordance with the conditions of hypersound-triggered experiments.

Fluorescence microscopy

Fluorescent images were taken using an inverted research microscope (Olympus, IX71) equipped with a mercury burner (U-RFL-T) as a light source and a digital camera (Olympus, DP70) for image acquisition. Green excitation light ($510 \text{ nm} \leq \lambda \leq 550 \text{ nm}$) and red emission light ($\lambda > 590 \text{ nm}$) was filtered using a filter cube (U-MWG Olympus). To avoid the photo-bleaching of CF, new areas were selected after each shot. The focusing time before taking the micrographs was 20 s and the magnification times is 10 in all cases.

5.6 References

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Chapter 6

Hypersound-Enhanced Drug Delivery with Mesoporous Silica Nanoparticles

The intracellular delivery efficiency of drug-loaded nanocarriers is often limited by biological barriers arising from the cell membrane and the cell interior. In this chapter, hypersound was applied to enhance the cellular uptake of polymer-wrapped mesoporous silica nanoparticles (PMSNs) loaded with Doxorubicin (Dox). Both fluorescence and cell viability measurements revealed that the delivery efficiency of Dox-loaded PMSNs was significantly improved by hypersound. The delivered amount was controlled by adjusting the input power and duration of hypersound. Furthermore, it was found that Dox-loaded PMSNs were internalized into cells by direct penetration instead of a traditional endocytotic pathway. These results confirm the mechanism of hypersonic poration for cellular uptake, which reversibly changes the membrane permeability by creating transient nanopores at the cell membrane. This method enables the drug-loaded carriers to overcome biological barriers during intracellular delivery, which improves the delivery efficiency and provides a versatile way for biomedical applications in drug delivery.

6.1 Introduction

The efficiency of intracellular delivery system is an important issue in therapeutic applications.^[1] Existing methods are mainly focused on addressing drug-loaded nanocarriers into cells and releasing the drugs while suppressing limitations from biological barriers.^[2] Chemically synthesized nanocarriers have been developed to surmount the restrictions of delivery from both the cell membrane and the cell interior environment, including polymeric micelles,^[3, 4] liposomes,^[5, 6] dendrimers,^[7, 8] nanoparticles,^[9, 10] and nanogels.^[11, 12] Among these systems, mesoporous silica nanoparticles (MSNs) have triggered a lot research interest,^[13] since their well-defined pores can hold numerous drug molecules for a variety of different applications. In particular, polymer-wrapped MSNs (PMSNs) have been developed by capping the pores that encapsulated with drugs via disulfide cross-linkable polymers to achieve high loading efficiency.^[14] It has been reported that the drug molecules loaded in the PMSNs can be released by the degradation of the polymer shell in the intracellular reducing microenvironment, which consequentially induces cell death.^[15]

In a carrier-mediated delivery system, efficient drug release relies on the ability of drug carriers to enter into the cell interior and release the loaded drug molecules to the cytoplasm. However, in most cases, drug carriers are internalized through an endocytotic pathway,^[16, 17] which makes them trapped in endo-lysosomal vesicles and prevents the further release of loaded drugs into the cytoplasm. In order to enhance the endosomal escape during intracellular delivery, different strategies have been developed such as pH-controlled release^[18, 19] and the “proton sponge” effect.^[20, 21] Physical stimuli have been proposed as well, such as thermal,^[22, 23] light,^[24-26] ultrasound,^[27-29] and electric field.^[30-32] These physical methods are based on the principle of membrane disruption to change the permeability of the cell membrane in order to realize direct translocation of drug carriers into cells.^[32, 33]

According to the results reported in Chapters 3-5, hypersound generated by a high-frequency bulk acoustic wave (BAW) resonator can induce transient nanopores on the supported lipid bilayer (SLB) and membranes of synthesized vesicles. This poration method can also change the membrane permeability and result in the delivery of exogenous substances.

In this chapter, hypersound was applied to enhance the intracellular delivery of PMSNs loaded with Doxorubicin (Dox). A chamber-based system was fabricated to facilitate the *in-vitro* delivery of Dox-loaded PMSNs into HeLa cells under the stimulation of hypersound. The cellular uptake and distribution of Dox-loaded PMSNs was measured and analyzed by fluorescence microscopy. Cell viability was studied to evaluate the effects of hypersonic poration with both Dox-loaded PMSNs and free Dox molecules. The mechanism of hypersound-mediated cellular uptake was also studied through experiments with inhibitors that affect the traditional endocytotic pathways. This study improves our understanding of hypersonic poration on cellular uptake and confirms the potential of hypersound as a non-invasive modality to enhance the efficiency of intracellular delivery.

6.2 Results and discussion

6.2.1 Intracellular delivery of Dox-load PMSNs

The setup for an intracellular delivery system triggered by hypersound is shown in Figure 6.1a. A polydimethylsiloxane (PDMS) chamber was sealed on a bulk acoustic wave (BAW) resonator^[34] and filled with cell culture medium containing drug carriers. HeLa cells were seeded on a glass slide, which was placed on the top of the chamber and aligned above the center of the BAW resonator. When the resonator is activated, it vibrates and creates hypersound in the cell culture medium, triggering the delivery of drug carriers into the HeLa cells.

In this work, polymer-wrapped mesoporous silica nanoparticles (PMSNs, MCM-41 structure, diameter 160 nm) loaded with Doxorubicin (Dox) were used as a model drug carrier for the evaluation of the intracellular delivery triggered by hypersound.^[35] As shown in Figure 6.1b, Dox molecules are encapsulated into the pores of MSN and non-covalently capped with copolymers containing 2-pyridyl disulfide hydrochloride (PDS) and poly(ethylene glycol) (PEG). Here, PEG is used to provide water solubility and to prevent nonspecific interactions with bio-macromolecules. The PDS groups are used to facilitate wrapping of the nanoparticles through multiple weak electrostatic interactions. Subsequently, the copolymers are crosslinked by disulfide exchange to obtain stable polymer networks to create the shell that traps the Dox molecules. After entering into cells, the Dox molecules

loaded within the PMSNs are released by the degradation of the polymer shell in the intracellular reducing microenvironment. A transmission electron microscope (TEM) image of Dox-loaded PMSNs is shown in Figure 6.1c. Dynamic light scattering (DLS) and zeta-potential tests were used to check their size (192.1 nm) and surface charge (-9.97 mV). These results show that the synthesized Dox-loaded PMSNs are uniform and stable in aqueous suspension.

Figure 6.1. (a) Schematic setup for the hypersound-triggered intracellular delivery of Dox-loaded PMSNs into cells. A PDMS chamber (height: 600 μm , diameter: 6 mm) is sealed on top of an acoustic resonator and filled with the cell culture medium that contains Dox-loaded PMSNs. HeLa cells are cultured on the glass slide that is placed on top of the PDMS chamber. (b) Cartoon of the Dox-loaded PMSNs and chemical structure of the wrapping polymer. (c) TEM image of the Dox-loaded PMSNs.

The cellular uptake of Dox-loaded PMSNs under the stimulation of hypersound is evaluated using confocal laser scanning microscopy (CLSM). As shown in Figure 6.2, the red fluorescence emitted by Dox molecules was distributed in the cells after exposure to hypersound of 250 mW for 10 min, which indicates that a detectable quantity of Dox-loaded PMSNs was internalized into the cells within this short time. The Dox fluorescence increased when the input power was increased to 500 mW, suggesting that more Dox-loaded PMSNs were delivered into cells and this hypersound-triggered process was power-dependent. In a

control experiment, HeLa cells were incubated with the same amount of Dox-loaded PMSNs without any hypersonic treatment for 10 min. The fluorescence intensity of Dox in this case was much lower compared to that in the presence of hypersound.

Furthermore, the cell nuclei were selectively stained with 4',6-diamidino-2-phenylindole (DAPI) to show the distribution of Dox-loaded PMSNs within the cells. As the merged images in the right column show, for cells exposed to hypersound of 250 mW for 10 min, the Dox fluorescence concentrated around the DAPI-stained areas without any overlap, indicating that the Dox-loaded PMSNs remained in the cytoplasm after uptake into cells by the hypersonic treatment. As the input power was increased to 500 mW (duration was still 10 min), the localized Dox fluorescence was enhanced in the border of the nuclei and a small fraction of red fluorescence overlapped with the DAPI signal, suggesting that Dox entered into the nucleus with the hypersound of higher input power. Since the polymer shell of Dox-loaded PMSNs can be dissolved due to the reducing conditions in the cell interior,^[35] the red fluorescence inside the nucleus can be from either the Dox-loaded PMSNs or the Dox molecules released from them.

Figure 6.2. Confocal laser scanning microscopy (CLSM) images of the intracellular uptake and distribution of Dox-loaded PMSNs in HeLa cells. For the hypersound-treated groups, HeLa cells were incubated with Dox-loaded PMSNs and exposed to hypersound of 250 or 500 mW for 10 min. For the control group, HeLa cells were incubated with Dox-loaded PMSNs without any hypersonic treatment for 10 min. The concentration of Dox-loaded PMSNs (Dox loading: 24 wt.%) in 0.1 M phosphate-buffered saline (PBS, pH 7.4) was $10 \mu\text{g mL}^{-1}$.

To assess the intracellular delivery efficiency of Dox-loaded PMSNs under the stimulation of hypersound, the cell viability of HeLa cells was tested using the Presto Blue assay. As shown in Figure 6.3a, after incubation with Dox-loaded PMSNs for 10 min, the cell viability slightly decreased compared with the cells incubated with cell culture medium (blank group). However, the percentage of dead cells was increased to 45% after exposure to hypersound of 250 mW for 10 min, and an even higher cell apoptosis was observed with an input power of 500 mW for 10 min (58%). These results indicate that Dox-loaded PMSNs were rapidly internalized into the cells with the treatment of hypersound, which further confirms that hypersound can enhance the intracellular delivery of nanoparticles and induce the apoptosis of cancer cells. As a control, the cells were exposed to hypersound of 500 mW for 10 min in the absence of Dox-loaded PMSNs. The identical cell viability compared to the blank group suggests that the hypersound itself is nontoxic for use as a stimulus to promote drug delivery. The nontoxic nature of the PMSN carriers has also been verified in a previously published result.^[35] All-in-all, these results show the synergistic and specific effect of the combined use of Dox-loaded PMSNs and hypersound.

Figure 6.3. (a) Comparison of the cell viability between Dox-loaded PMSNs and the free Dox molecules. Blank: cells were incubated with cell culture medium without any hypersonic treatment. Control: cells were exposed to hypersound of 600 mW for 20 min. Dox-loaded PMSN group: cells were incubated with Dox-loaded PMSNs without or with the treatment of hypersound. Free Dox molecules group: cells were incubated with free Dox molecules without or with the treatment of hypersound. The input power of hypersound was 250, 500 and 600 mW; treatment time was 10 and 20 min. The concentration Dox-loaded PMSNs (Dox loading: 24%) was $10 \mu\text{g mL}^{-1}$ (i.e. the concentration of Dox encapsulated within the MSN carriers was $2.4 \mu\text{g mL}^{-1}$). The concentration of free Dox molecules was $2 \mu\text{g mL}^{-1}$. Data present mean $\pm$ SD ($n = 6$). $*P < 0.05$; $**P < 0.01$, analyzed by student t-test. The * indications above the green columns refer to the comparison between groups treated with free Dox molecules and the blank group. (b) Fluorescence microscopy images for the distributions of Dox-loaded PMSNs and the free Dox molecules after treatment with hypersound of 600 mW for 20 min. The scale bars are 25 μm .

Drug-loaded carriers are widely used in the chemotherapeutics of cancers since drug molecules that freely diffuse and distribute throughout the blood circulation will result in

undesirable side effects and limit achievement of proper doses required for efficacious responses.^[36] However, the conventional delivery efficiency of carrier-formulated drugs is limited due to biological barriers resulted from the carrier effect.^[37] Here, the cell viability was analyzed with both Dox-loaded PMSNs and free Dox molecules to compare the delivery efficiency with hypersound of different intensities. As shown in Figure 6.3a, free Dox molecules presented a similar trend of reduced cell viability as the Dox-loaded PMSNs upon treatment with hypersound, indicating that both free drug molecules and drug-loaded carriers can be successfully internalized into cells with hypersound and induce apoptosis. Note that upon hypersound of 250 and 500 mW for 10 min, free Dox molecules showed a higher toxicity compared with Dox-loaded PMSNs, which can be attributed to the carrier effect of the PMSNs. After entering into cells, Dox-loaded PMSNs first open the polymer shell with the assistance of the reducing intracellular environment, then release the trapped Dox molecules to kill the cancer cells. In contrast, free Dox molecules can directly interact with the cancer cells after entering the cell interior. Interestingly, when the input power of hypersound was increased to 600 mW for 20 min, no significant difference was observed between Dox-loaded PMSNs and free Dox molecules, which suggests that the delivery efficiency of drug-loaded carriers and free drug molecules achieved the same level. It is likely that the Dox molecules encapsulated within PMSNs are also released from the carriers upon treatment of hypersound and the intracellular delivery of drug-loaded carriers can be as efficient as the free drug molecules with hypersound of sufficient intensity. The equal efficiency achieved by the carrier is a very important issue for the intracellular delivery of carrier-formulated drugs *in vivo*. In addition, carriers also have proven advantages over the use of free drug molecules by solubilizing therapeutic cargos, substantially prolonging the circulation lifetimes of drugs and targeting the cancer cells with specific ligands.^[37]

To understand the mechanism of the same efficiency between the carrier-formulated drugs and free drug molecules under hypersound of 600 mW for 20 min, the distributions of Dox-loaded PMSNs and free Dox molecules inside the HeLa cells were confirmed using fluorescence microscopy. As shown in Figure 6.3b, after treatment with hypersound, the red fluorescence emitted either from Dox-loaded PMSNs or free Dox molecules was homogeneously distributed within the cells and totally overlapped with the DAPI-stained nuclei. This suggests that even with drug carriers, the loaded drug molecules can be

efficiently delivered to the cell nucleus with the assistance of hypersound. According to a previous study, the accumulation of drug molecules within the cell nucleus creates the opportunity for the drug to interact with nuclear DNA and induce cell apoptosis, which is a major approach for most anticancer drugs to achieve nucleotropic ability.^[38] These results further support the similar delivery efficiency of Dox-loaded PMSNs as free Dox molecules, which is realized with hypersound by releasing the loaded drug molecules without restrictions of cytoplasmic trafficking and breaking the barrier of the nuclear envelope.

6.2.2 Uptake mechanism of hypersound-induced delivery

Further experiments were conducted to verify that the Dox-loaded PMSNs were internalized into the cells under the stimulation of hypersound instead of the Dox molecules released from the nanocarrier before uptake. Here, crosslinked, layer-by-layer assembled mesoporous silica nanoparticles (CLM-MSNs) were used for the intracellular delivery triggered by hypersound.^[39] As shown in Figure 6.4a, raspberry-type MSNs were wrapped with two oppositely charged copolymers. Positively charged poly(2-(dimethylamino)ethyl methacrylate-*co*-2-(pyridyl disulde)ethyl methacrylate) (PDMAEM-*co*-PPDEM) and negatively charged poly(methacrylate-*co*-2-mercaptoethyl methacrylate) (PMA-*co*-PMEM) were used to form the layer-by-layer (LbL) films. The crosslinking in the polyelectrolyte films was achieved by thiol exchange between the pyridyl disulfide in PDMAEM-*co*-PPDEM and the thiol in PMA-*co*-PMEM.^[40] Then the crosslinked film was labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI) and 3,3'-dioctadecyloxacarbocyanine (DiO), which was covalently functionalized with the polyelectrolyte films and cannot be removed by hypersound. The TEM image of the CLM-MSNs is shown in Figure 6.4b. DLS and zeta-potential tests were used to check their size (264.2 nm) and surface charge (-2.24 mV).

Figure 6.4. (a) Schematic of the crosslinked layer-by-layer (LbL) systems of PDMAEM-co-PPDEM and PMA-co-PMEM (x : 0.33; y : 0.67). (b) TEM image of the CLM-MSNs.

As shown in Figure 6.5, after exposure to hypersound of 500 mW for 10 min, both fluorescence emitted from DiI and DiO was observed within the cells, whereas the fluorescence intensity of the cells incubated with dye-labeled CLM-MSNs in the absence of hypersound for 10 min was much lower, which confirms the hypersound-triggered uptake of CLM-MSNs into cells and evidence the possibility of cellular uptake of nanoparticles with a diameter of 100-200 nm with hypersound. We assume that the same mechanism applies to the Dox-loaded PMSNs, i.e., uptake of intact, Dox-loaded carriers into the cells, after which release of the Dox occurs.

Figure 6.5. Fluorescence microscopy images of intracellular uptake and distributions of dye-labeled CLM-MSN in HeLa cells. For the hypersound treated groups, HeLa Cells were incubated with CLM-MSNs and exposed to the hypersound of 500 mW for 10 min. For the control group, HeLa cells were incubated with CLM-MSNs without any hypersonic treatment for 10 min. The concentration of CLM-MSNs (Dye loading capability: DiI 0.9 wt.%, DiO 0.8 wt.%) is $10 \mu\text{g mL}^{-1}$. The scale bar is $25 \mu\text{m}$.

To further understand the mechanism of hypersound-triggered drug delivery, the intracellular uptake pathway of Dox-loaded PMSNs under the stimulation of hypersound was analyzed. Two inhibitors (permethylated- β -cyclodextrin (m- β CD) and sucrose)^[41] were used to identify first the particular uptake pathway of Dox-loaded PMSNs into cells in the absence of hypersound treatment. The internalization of Dox-loaded PMSNs was monitored by fluorescence measurements after the pretreatment with inhibitors for 30 min. In Figure 6.6a, the fluorescence intensity of Dox randomly increased with m- β CD at higher concentration, suggesting that m- β CD, an inhibitor of caveolin-dependent endocytosis,^[42] did not reduce the uptake of Dox-loaded PMSNs. In contrast, sucrose greatly prevented the uptake of Dox-loaded PMSNs at increased concentration, which indicates that the primary pathway of uptake of Dox-loaded PMSNs is by clathrin-mediated endocytosis.

To evaluate the intracellular pathway under the stimulation of hypersound, cells pretreated with inhibitors were incubated with Dox-loaded PMSNs with and without the hypersound treatments. As shown in Figure 6.6c, in the control group, cells pretreated with m- β CD (80 mM) emitted nearly equal red fluorescence as the cells without inhibitor, while the cells pretreated with sucrose (1800 mM) almost presented no red fluorescence. This result confirms the inhibiting effect of sucrose on the uptake of Dox-loaded PMSNs in the absence of hypersound, which is in accordance with the results shown in Figure 6.6a. However, cells exposed to hypersound of 500 mW for 10 min emitted a much stronger red fluorescence compared with the control group, regardless whether they were pretreated with inhibitors or not, suggesting that the uptake of Dox-loaded PMSNs induced by hypersound was not inhibited by the sucrose. It is therefore likely that the hypersound-mediated pathway of Dox-loaded PMSNs is not by endocytosis. This behavior is attributed to the hypersonic poration effect.^[34] As illustrated in Figure 6.6b, we envisage that the cell membrane is disrupted by the shear stress from the hypersound and some transient nanopores occur, which increases the membrane permeability and allows the translocation of Dox-loaded PMSNs into the cells. We therefore presume that by the direct penetration under the stimulation of hypersound, trapping of Dox-loaded PMSNs into endosomes is avoided, and the loaded drug molecules are released directly into the cytoplasm.

Figure 6.6. (a) Fluorescence intensity of HeLa cells pretreated with inhibitors (m-βCD or sucrose) for 30 min and then incubated with Dox-loaded PMSNs for 20 min. The applied concentrations of m-βCD were: 10, 20, 40, 60 and 80 mM (saturated). The concentrations of sucrose were: 225, 450, 900, 1350 and 1800 mM (saturated). (b) Schematic illustration of the hypersound-mediated direct penetration of Dox-loaded PMSNs in HeLa cells with or without the pretreatment with inhibitors. The control group: cells with or without pretreatment with inhibitor were incubated with Dox-loaded MSNs ($10 \mu\text{g mL}^{-1}$). The hypersound group: cells with or without pretreatment with inhibitor were exposed to hypersound of 500 mW for 10 min.

6.3 Conclusions

In this chapter, hypersound was used to enhance the intracellular delivery of polymer-wrapped mesoporous silica nanoparticles (PMSNs) loaded with Doxorubicin (Dox). The delivery efficiency of Dox-loaded PMSNs was enhanced by hypersound at increasing input powers and durations. Most likely, hypersound creates transient nanopores at the cell membranes, through which Dox-loaded PMSNs can directly penetrate to reach the cytoplasm and release the loaded Dox molecules to the cell nucleus. Compared with the traditional

endocytosis of nanoscale drug carriers, the hypersound-mediated penetration of Dox-loaded PMSNs overcomes the biological barriers of endosomes, which improves the efficiency of intracellular delivery. In principle, this membrane-disruption based strategy is not limited to one single kind of drug carrier or drug, and it can be further applied to different nanocontainers encasing multiple drug combinations for therapeutic applications in a variety of disease states.

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6.5 Materials and methods

6.5.1 General

Chemicals for the synthesis of nanoparticles were purchased from Sigma Aldrich or TCI and used without further purification. Phosphate-buffered saline (PBS) solution was composed of 0.1 M sodium dihydrogen phosphate and 0.15 M sodium chloride. The pH was adjusted to 7.4 with sodium hydroxide. Dulbecco's modified eagle medium (DMEM), 1,1'-Diocadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI) and 3,3'-diocadecyloxacarbocyanine (DiO) were purchased from Thermo Fisher Scientific Inc. Doxorubicin hydrochloride (Dox) was purchased from Ontario Chemicals Inc.

6.5.2 Preparation of Dox-loaded PMSNs

Preparation of MSNs

Mesoporous silica nanoparticles (MSNs) were synthesized according to a previously published result.^[35] In brief, NaOH solution (2 M, 3.5 mL) was mixed with distilled (DI) water (480 mL). Hexadecyltrimethylammonium bromide (CTAB) (1.02 g) was added to the solution with stirring and heating at 80 °C. After CTAB was completely dissolved, tetraethyl

orthosilicate (TEOS) (4.67 g) was added dropwise slowly and stirred for 2 h at 80 °C. The resulting precipitate was filtered off and washed twice with DI water, and then dried at 60 °C overnight. Finally, the dried sample was calcined at 550 °C for 5 h in air.

Preparation of Dox-loaded MSNs

MSNs (10 mg) were added to ultrapure water (1 mL) and sonicated to disperse the MSNs until a uniform colloidal solution was observed. An amount of 5 mg Dox was dissolved in this solution and allowed to stir for 3 h. Then, the dispersion was centrifuged to collect the Dox-loaded MSNs and the supernatant was used to calculate the drug loading content. In order to remove the Dox from the exterior surface of the MSNs, the drug-loaded MSNs were washed twice with ultrapure water and collected by centrifugation.

Preparation of PDS-PEG copolymer

A mixture of 2-cyano-2-propyl benzodithioate (RAFT reagent, 9.44 μ L, 0.05 mmol), PDSEMA (1 g, 3.92 mmol), poly (ethylene glycol) methacrylate (931 mg, 0.98 mmol) and AIBN (2.4 mg, 0.0146 mmol) was dissolved in THF and then degassed by argon purge. The reaction vessel was sealed and then placed in a pre-heated oil bath at 70 °C for 24 h. In order to remove unreactive monomers and purify the polymer, the resultant mixture was precipitated in diethyl ether, which yields the random copolymer.

Preparation of Dox-loaded PMSNs

The polymer solution of PDS-PEG was added to Dox-loaded MSNs and stirred for 3 h at room temperature. Then dithiothreitol (DTT) (60 mol % against PDS) was added to the mixture and stirred for 3h to allow for in-situ crosslinking. The polymer cross-linked PMSNs were collected by centrifugation. The unabsorbed and unreacted DTT were removed by washing with DI water by centrifugation and re-dispersion cycles.

6.5.3 Preparation of CLM-MSNs

First, raspberry-type MSNs (rMSNs) were synthesized using soft-templating method.^[43] A mixture of cetyl-trimethylammonium bromide (CTAB), triethanolamine and distilled water were stirred for a period of 1 h at 80 °C. And tetraethyl-orthosilicate (TEOS) was

quickly added into the surfactant solution to this mixture. Then, rMSNs were alternately immersed in the phosphate buffer (0.1 M, pH 7.4) of PDMAEM-co-PPDEM (1 mg mL⁻¹) and PMA-co-PMEM (1 mg mL⁻¹) for 5 min for each step. rMSNs were washed with the 0.15 M NaCl solution after each step. Finally, the layer-by-layer film was crosslinked the thiol-exchange reaction and functionalized the fluorescent dyes by placing them respectively in the solution of DiI (1 mg mL⁻¹) and DiO (1 mg mL⁻¹).

6.5.4 Characterization of Dox-loaded PMSNs and CLM-MSNs

Transmission electron microscope (TEM)

TEM analysis was performed using JEOL-2100 microscope at an accelerating voltage of 200 kV. To prepare the sample for TEM, a droplet of Dox-loaded PMSNs/CLM-MSNs was casted on a 200 mesh copper grid for 2 min, and then gently wiped with a sterile paper.

Dynamic light scattering (DLS) and zeta-potential measurements

DLS and zeta-potential measurements were carried out simultaneously using Malvern ZS 90 Zetasizer instrument. 0.1% suspensions of Dox-loaded PMSNs/CLM-MSNs were prepared in MilliQ water and these suspensions were sonicated for 5 min before the analysis.

6.5.5 Setup of intracellular delivery system

A polydimethylsiloxane (PDMS) chamber (thickness: 600 μm) with a diameter of 6 mm was sealed on top of a BAW resonator (1.6 GHz, as the same one described in Chapter 5). After filling cell culture medium containing Dox-loaded PMSNs (typical 50 μL), a glass slide that seeded with approximately 1×10^4 HeLa cells was covered on top of the PDMS chamber, aligning above the center of the BAW resonator. Sinusoidal signal of 1.6 GHz was generated by a signal generator (Agilent, N5181A) and amplified by a power amplifier (Mini-Circuits, ZHL-5W-422), which then transduced the electrical signal to mechanical vibrations of resonator and generated hypersound. The input power of hypersound was conveniently controlled by adjusting the power of the signal generator.

6.5.6 Cell viability analysis

In vitro cytotoxicity was evaluated by the Presto Blue assay (Thermo Fisher Scientific Inc.). HeLa cells were first seeded into a 96-well plate at a density of 1.0×10^4 cells per well in 100 μL DMEM and incubated at 37 °C in 5% CO_2 atmosphere for 24 h. Afterwards, the culture medium was replaced with 10 mg mL^{-1} Dox-loaded PMSNs (Dox loading density 24wt.%) or 2 $\mu\text{g mL}^{-1}$ Dox molecules that dissolved in DMEM and treated with hypersound of different input powers. HeLa cells that incubated with Dox-loaded PMSNs/Dox but without any treatment of hypersound were used as a negative control. HeLa cells that that incubated with DMEM and treated with hypersound were used as a positive control. After incubation, the Presto Blue stock solution (5 mg mL^{-1} , 10 μL) was added to each well and incubated for another 20 min. The absorbance was monitored using a microplate reader (Thermal Scientific, Multiskan GO) at the excitation wavelength of 540 nm. The efficiency was assayed in terms of the percentage of cell viability compared to untreated blank control cells.

6.5.7 Fluorescence measurements

Confocal laser scanning microscope (CLSM, Nikon A1) and fluorescence microscope (Olympus, BX53) were used to evaluate the intracellular uptake and distribution of Dox-loaded PMSNs or free Dox molecules. After each hypersonic treatment, the HeLa cells were washed with PBS buffer three times to remove the redundant nanoparticles or drug molecules. The HeLa cells were then fixed with 4% paraformaldehyde aqueous solution for 10 min at room temperature and rinsed with PBS buffer again. Afterwards, the HeLa cells were stained with DAPI at room temperature for 5 min and rinsed with PBS buffer twice. The prepared slides of fixed samples were examined by CLSM and fluorescence microscope at excitation wavelength (E_x) of 485 and 403 nm, emission wavelength of (E_m) 550 and 450 nm respectively for Dox and DAPI. To quantify the cellular uptake Dox-loaded PMSNs with inhibitors, the fluorescence intensity of HeLa cells was measured by a fluorescence spectrophotometer (Thermal Scientific, Varioksan LUX).

6.6 References

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Summary

This thesis contributes to the continuing studies of biological materials, such as cells and tissues, using micro/nano-physical methods. In this work, hypersonic poration has been applied to induce reversible membrane disruption in different assembled systems, including the supported lipid bilayer (SLB), giant unilamellar vesicles (GUVs), polymer-shelled vesicles (PSVs), and cells. The application of this physical poration method can be directed toward two aspects: controlled release/encapsulation and triggered intracellular delivery. For carriers employed in drug delivery, like liposomes, polymersomes and micelles, the sustained release can be controlled by transient disruption of the carrier membrane, which in principle allows for tailored release profiles with excellent spatial, temporal and dosage control. On the other hand, when hypersonic poration is applied to a cell system, it can be used to control the permeability of cell membranes by inducing transient nanopores, which may enhance the process of intracellular delivery.

Chapter 1 provides a general introduction to this thesis.

Chapter 2 reviews recent progress of membrane-disruption methods for intracellular delivery. Special attention was given to methods at the micro and nanoscale, which maintain numerous advantages such as improved precision, high throughput and delivering diverse types of cargos to target cells, in combination with advances in nanotechnology, microfluidics, laboratory-on-chip (LOC), and microsystems.

Chapter 3 has studied the mechanism of hypersonic poration with a planar lipid membrane. The behavior of a supported lipid bilayer (SLB) under the stimulation of hypersound was analyzed by the real-time electrical measurements of hypersonic poration. The “gating”-shaped current changes revealed the formation of transient nanopores, which were regarded as analogous to ion channels in the membrane. These instantaneous pore structures created a possibility to control the membrane permeability by hypersound of different input powers and durations, which can be further applied in controlled release and intracellular delivery.

Chapter 4 has described the effects of hypersound on a vesicular system. It was found that hypersound induced the reversible deformation of giant unilamellar vesicles (GUVs)

within milliseconds. Changes of aspect ratio and projected area were monitored in real time with microscopy techniques and quantitatively analyzed to reveal the degree of deformation with hypersound of different intensities. This study confirmed the mechanism of hypersonic poration: the shear stress generated by acoustic streaming affects the lipid membrane to induce nanopores and change the membrane permeability.

Chapter 5 has described the use of hypersound to control the release and encapsulation of cargo from polymer-shelled vesicles (PSVs), either suspended in solution or immobilized on a surface. The kinetics of release and encapsulation were analyzed to reveal the dependence on the hypersonic power. The mechanism of reversible membrane disruption was further verified with these hypersound-controlled processes, which created the possibility to realize pulsatile stimuli-responsive release.

Chapter 6 has shown the capability of hypersound to enhance the delivery of drug-loaded carriers into cancer cells. The work revealed that mesoporous silica nanoparticles of approx. 100 nm could be quickly internalized into cells through direct penetration under the stimulation of hypersound, whereas larger particles were not taken up. Both fluorescence and cell viability measurements confirmed that the delivered amounts could be quantitatively controlled through the input power and duration of hypersound. This study shows the potential of hypersound to increase the delivery efficiency by surmounting the biological barriers in cellular environments. In principle, this membrane-disruption method is not limited to one single kind of drug carrier or drug, and can be further applied to different nanocontainers encasing multiple drug combinations for therapeutic applications.

Collectively, this thesis has introduced a new physical poration method to non-invasively change the permeability of membranes. By carefully inducing shear stress by acoustic pressure, transient nanopores can be created in the membrane to obtain reversible membrane disruption. However, the request for effective delivery to some patient-derived cells, such as immune cells, neurons and stem cells, is still difficult to realize. Despite the barriers that remain, the membrane disruption induced by hypersound can be potentially applied to controlled release and intracellular drug delivery in combination with other advanced techniques such as microfluidics and micro/nanotechnology. To this end, hypersonic poration can be further developed to achieve high spatial and temporal precision,

which promises to become a valuable way to deliver various cargos to diverse cells on demand.

Samenvatting

Dit proefschrift draagt bij aan de voortdurende studie van biologische materialen, zoals cellen en weefsels, met behulp van micro/nano-fysische methoden. In dit onderzoek is hypersonic poration toegepast om een reversibele verstoring teweeg te brengen van membranen in verschillende geassembleerde systemen, waaronder de lipid bilayer op een oppervlak (supported lipid bilayer, SLB), grote unilamellaire vesicles (giant unilamellar vesicles, GUVs), polymeer-bedekte vesicles (polymer-shelled vesicles, PSVs) en cellen. De toepassing van deze fysische poration-methode kan op twee aspecten gericht worden: geregleerde afgifte/inkapseling en prikkelgestuurde intracellulaire afgifte. Voor dragers die gebruikt worden in medicijnafgifte, zoals liposomen, polymersomen en micellen, kan de afgifte geregeld worden door tijdelijke verstoring van het membraan van de drager, hetgeen in principe toegespitste afgifteprofielen toelaat met uitstekende controle in tijd, ruimte en dosering. Bovendien kan hypersonic poration in systemen met cellen gebruikt worden om de permeabiliteit van celmembranen te regelen door tijdelijke nanoporiën te induceren, hetgeen het proces van intracellulaire afgifte kan bevorderen.

Bij elkaar genomen heeft dit proefschrift een nieuwe fysische poratiemethode geïntroduceerd om niet-invasief de permeabiliteit van membranen te veranderen. Door voorzichtig parallelle krachten aan te brengen door middel van akoestische druk, kunnen tijdelijke nanoporiën in het membraan gemaakt worden om voor reversibele membraanverstoring te zorgen. Echter, de vraag naar effectieve afgifte aan sommige patiënt-verkregen cellen, zoals immuuncellen, neuronen en stamcellen, is nog steeds moeilijk te realiseren. Ondanks de resterende barrières, kan membraanverstoring door hypergeluid mogelijkwerwijs toegepast gaan worden in geregleerde en intracellulaire afgifte van medicijnen in combinatie met andere geavanceerde technieken zoals microfluidica en micro/nanotechnologie. Met dit doel kan hypersonic poration verder ontwikkeld worden om hoge precisie te bereiken in tijd en ruimte, hetgeen een waardevolle manier belooft te worden om verscheidene ladingen in diverse cellen op aanvraag te bezorgen. Verdere details staan beschreven in de Engelse samenvatting (summary).

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Yao Lu

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About the author

Yao Lu was born on April 7th 1989 in Shaanxi, China. She obtained her Bachelor degree of Electrical Engineering from the College of Precision Instrument and Opto-Electronics Engineering at Tianjin University in 2012. In the same year, she became a PhD candidate under the supervision Prof. Hao Zhang in the MEMS&NEMS lab at Tianjin University. Her first project was to study acoustic resonators, filters and duplexers for a radio frequency microelectromechanical system (RFMEMS). Then she continued to study the applications of acoustic resonators as sensors and transducers in biochemistry under the supervision of Prof. Xuexin Duan, in the Mbios group at the State Key Laboratory of Precision Measuring Technology & Instruments. In 2016, she started the subject of controlled release and encapsulation of vesicles as an exchange PhD student under the supervision of Prof. Jurriaan Huskens, in the Molecular Nanofabrication (MnF) group at the University of Twente. The collaborative research work between Tianjin University and University of Twente is described in this thesis.

List of publications

1. Z. Zhang, **Y. Lu**, W. Pang, D. Zhang, H. Zhang, “A High Performance C-band FBAR Filter. *IEEE Microwave Conference Proceedings (APMC)*, **2013**, 923-926.
2. **Y. Lu**, Y. Chang, N. Tang, H. Qu, J. Liu, W. Pang, H. Zhang, D. Zhang, X. Duan, “Detection of Volatile Organic Compounds Using Microfabricated Resonator Array Functionalized with Supramolecular Monolayers.” *ACS Appl. Mater. Inter.* **2015**, 7, 17893.
3. **Y. Lu**, Y. Chang, N. Tang, H. Qu, W. Pang, D. Zhang, H. Zhang, X. Duan, “Concentration-independent Fingerprint Library of Volatile Organic Compounds Based on Gas-surface Interactions by Self-assembled Monolayer Functionalized Film Bulk Acoustic Resonator Arrays.” *IEEE SENSORS*, **2015**, 1-4.
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5. Z. Zhang, Y. Wang, H. Zhang, Z. Tang, W. Liu, **Y. Lu**, Z. Wang, H. Yang, W. Pang, H. Zhang, D. Zhang, X. Duan, “Hypersonic Poration: A New Versatile Cell Poration Method to Enhance Cellular Uptake Using a Piezoelectric Nano-Electromechanical Device.” *Small* **2017**, 13, 1602962.
6. Q. Zhang, Y. Liu, J. Lai, S. Qi, C. An, **Y. Lu**, X. Duan, W. Pang, D. Zhang, D. Sun, J. Chen, “Liquid Phase Mass Production of Air-stable Black Phosphorus or Phospholipids Nanocomposite with Ultralow Tunneling Barrier.” *2D Mater.* **2018**, 5, 2.

Manuscripts in preparation

1. **Y. Lu**, H. Zhang, Z. Wang, S. Jeong, M. Jo, M. Park, X. Duan, “Real-time Detection of Nanoparticles Interaction with Lipid Membranes Using an Integrated Acoustical and Electrical Multi-mode Biosensor.” *submitted*.

2. Hypersound-Controlled Release and Uptake of Cargo by Vesicles, *in preparation*.
3. Real-time Detection of Hypersonic Poration of Supported Lipid Bilayers, *in preparation*.
4. Hypersound-Enhanced Drug Delivery with Mesoporous Silica Nanoparticles, *in preparation*.

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