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Experimental study on cell self-sealing during sonoporation

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ABSTRACT

Reparable sonoporation of human breast cancer cells was achieved during exposure to moderate ultrasound (spatial peak acoustic pressure, p_{sp}=0.25 MPa, 1 MHz tone-bursts, 20 cycles per tone-burst at pulse repetition frequency of 10 kHz) up to 40 s assisted by the presence of encapsulated microbubbles (EMBs). It was demonstrated that shear stress generated by oscillating EMBs at the cell membranes introduced small transient pores in cell membranes by which cells were able to uptake some extracellular fluid and meanwhile triggered the repairing process through self-sealing during sonoporation. It was also indicated by post-sonoporation analysis using the fluorescent microscopy, scanning electron microscopy, and the Bradford assay which determined the protein content in cell supernatant that the self-sealing might be established by lysosomal-associated membrane protein, LAMP-1, fusing with the plasma membrane under the stressful condition in sonoporation.

1. Introduction

It has been reported [1–10] that oscillating encapsulated microbubbles (EMBs) excited by moderate ultrasound (US) could make cell membranes of nearby cells temporarily 'open' letting macromolecules be delivered into cells. The process usually is a transient phenomenon; the cells can reseal themselves and still keep their vitality after sonication. This technique is called reparable sonoporation [11]. Reparable sonoporation has the potential to be a non-viral transfection tool to deliver DNA or drug safely and efficiently into a cell [12,13], because it has a unique niche such as site specificity (US can be easily focused into a desired volume) and ease of manipulating parameters of US for applications *in vivo*. Several groups of researchers [14–18,19] have tried to understand the possible mechanisms of the reparable sonoporation. Their results have suggested that the possible candidates may include (1) EMBs excited by US promote endocytosis, a process by which a cell uptakes some of its extracellular fluid including material dissolved or suspended in it through its endogenous cellular machinery, e.g., surface receptor sites such as caveoli; (2) shear stress produced by the interaction among cells and oscillating EMBs generates transient, nanometer size pores in plasma membranes through which macromolecules may get inside of cells [17,20].

It is known that when cell membranes suffer emergent disruption, 'resealing' process may be triggered. 'Resealing' is a complex and dynamic cell adaption process which is needed for cell survival [21–23]. It has been reported that cell membrane disruption may trigger Ca^{2+} entering the disruption area and cause vesicles to present in cytoplasm underlying the disruption site to fuse rapidly with one another and also with the adjacent plasma membrane [21,24]. Lysosomes, membrane-bound organelles, have been recognized to play an important role in repairing of plasma membrane 'wounds' [21]. It was also found that LAMP-1, a lysosomal-associated membrane protein, normally is not presented at the plasma membrane surface, became ubiquitously exposed on the cell surface [21,22] when lysosomes were triggered to fuse with the plasma membrane under stressful condition. Up to the authors' knowledge, there is no publication which has addressed the question whether the similar process occurs during the reparable sonoporation; i.e., the shear stress generated by the oscillating EMBs at the cell membrane surfaces would trigger the similar resealing process to make cells repair themselves. To answer this question by using convincing experimental results would also help to find an optimum acoustic pressure amplitude range achieving best delivery-efficiency and minimize possible side-effects in future clinical applications such as the targeting drug delivery.

The main goal of this study is to further understand the mechanisms of the reparable sonoporation. It is hypothesized that protein content in a plasma membrane of a cell which experiences reparable sonoporation caused by nearby oscillating EMBs under US excitation would increase as a consequence of the self-resealing action described above. Furthermore, it is hypothesized that there should be an optimum acoustic pressure amplitude range *in situ* that would generate most effective reparable sonoporation, below which no sonoporation effect would be observed and above which the disruption generated by sonoporation would be too severe to be repaired.

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2. Materials and methods

2.1. The preparation of NBD-labeled microbubbles

1, 2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy (polyethylene glycol)-2000] (ammonium salt) (DPPE-PEG2k) and 25-{*N*-[(7-nitro-2-1, 3-benzoxadiazol-4-yl)-methyl] amino}-27-norcholesterol (25-NBD-cholesterol) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). The L-α-phosphatidylcholine(PC, lyophilized powder) was purchased from Sigma-Aldrich, Inc. N_2 and all other reagents were analytical grade and were used as received.

PC/DPPE-PEG2k/25-NBD-cholesterol (90:5:5, molar ratio) dissolved in chloroform were added to the round bottom flask. Chloroform was removed under a vacuum evaporation until the thin film formed. A phosphate buffer solution (PBS, pH=7.4±0.1) was added to the dried lipids thin films to create a lipid concentration of 1 mg/ml. Then the lipid suspension was well mixed above the phase transition temperature of the lipids (60 °C) to form a milky solution of multi-lamellar liposomes with 25-NBD-cholesterol incorporated into the lipid membrane. Then the multi-lamellar liposomes suspension was continuously sonicated at 100 W with a probe while constant purging using a steady (4 ml/min) stream of N_2 gas for 5 min to form the microbubbbles. After centrifugal separation at 1500 rpm for 5 min (Eppendorf centrifuge 5804R, Brinkmann Instruments, Wesbury, NY, USA), the diameter of microbubbles was measured using Mastersizer 2000 (Malvern, England). Microbubbles for immunofluorescence experiment, the composition is PC/DPPE-PEG2k/cholesterol (90:5:5, molar ratio).

2.2. Cell culture and pretreatment

MCF7 (a human breast cancer cell line) cells, were cultured as monolayers in RPMI1640 media and 10% fetal bovine serum (FBS). They were grown in a humidified 5% CO_2 atmosphere at 37 °C. For US exposure experiments, exponentially growing cells were harvested and resuspended in fresh RPMI 1640 media with 10% FBS. The concentration of the cells was diluted to a concentration of ~10^6 cells/ml and mixed with the NBD-labeled microbubbles solution of the same concentration. For each trial, 1 ml cells suspension and 0.5 ml microbubbles solution were placed into a plastic tube of 15 mm diameter and 75 mm length (Kimble, Owens-Illinois, Toledo, OH).

2.3. Ultrasound apparatus and the ultrasound exposure

The ultrasound exposure system was shown in Fig. 1. An arbitrary waveform generator (Agilent 33250A, USA) was used to produce a sinusoidal radio frequency signal; it contained repeated 1 MHz tone-bursts, 20 cycles per tone-burst at a pulse repetition frequency (PRF) of 10 kHz. It was then amplified by a 50 dB broadband RF power amplifier (ENI 2100L, Rochester, NY, USA), and used to drive a self-made focusing transducer of radius 9.2 cm. The central frequency of the transducer is 1 MHz and the focal distance was 8 cm. The plastic test tube of 15 mm diameter and 75 mm length filled with cell and microbubble suspension capped by a rubber stopper (a rubber stopper was used as an sound absorber to minimize a standing-wave effect; there was no air between the cap and suspension) was rotated at 60 rpm by a DC motor throughout the exposure period; the rotation helped to mix microbubbles with cells evenly.

The transducer and test tube were immersed and mounted in a glass tank filled with de-ionized and degassed water. The test tube was aligned axially with the transducer in such a way that the center of the cell-suspension in a test tube was situated at an 8 cm distance from the surface of the source transducer.

A calibrated needle broadband hydrophone (TNU0001A, NTR, Seattle, WA, USA) with an active diameter of 0.6 mm and an upper frequency limit of 20 MHz and a low-noise 30 dB preamplifier (HPA30, NTR, Seattle, WA, USA) were used to measure the acoustic pressure *in situ*. The calibration of hydrophone was done using the combination of the beam scan-integration technique and the acoustic power measurement using the acoustic irradiation force [25]. The attenuation of the wall of test tubes was found by measuring the ultrasound amplitude with/without placing the test tube *in situ* and before the hydrophone using a short US tone-burst. The *in situ* spatial peak-pressure amplitude after attenuation correction, p_{sp}, changed with applied voltage to the power amplifier. In the experiment, output of the function generator was adjusted ranging from 400 mV to 1000 mV with a 200 mV increment, corresponding to 400 mV, 600 mV, 800 mV and 1000 mV voltage, the respective acoustic pressures amplitudes, p_{sp}, were 0.19, 0.25, 0.38, 0.48 MPa as measured by using the calibrated hydrophone. The total exposure time was 40 s.

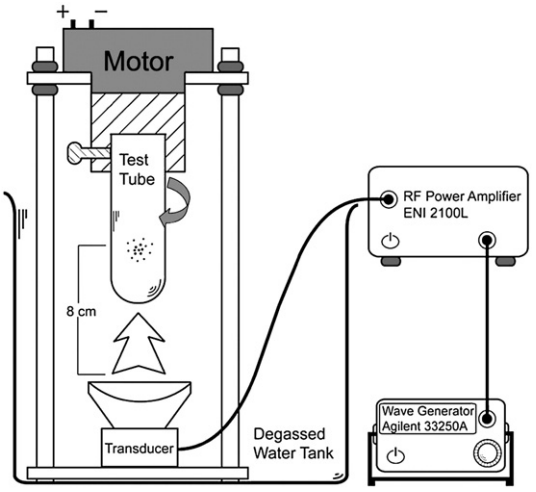

Fig. 1. The schematic diagram of ultrasound exposure apparatus. A focusing transducer (radius=9.2 mm and focal length=8 mm) of 1 MHz was used.

2.4. Characterization of cell morphology after the ultrasound exposure

2.4.1. Fluorescent microscopy

The optical observations and imaging were achieved by using the epifluorescent mode (excitation wavelength 440 nm, fluorescence wavelength 530 nm) of a microscope equipped with a digital Coolsnap MP3.3 camera (Axioskop 200, Carl Zeiss, Germany). The samples were imaged in real time immediately after sonication.

2.4.2. Scanning electron microscopy (SEM)

To observe the effects of ultrasound exposure on cell membranes, MCF7 breast cancer cells for each case were imaged using the scanning electron microscopy (SEM) at the different magnification. After ultrasound exposure, each sample was fixed with 2.5% glutaraldehyde solution for 1 h at −4 °C and then washed twice in 0.1M phosphate buffered saline (PBS, pH=7.2±0.1). Alcohol dehydration was followed in 33%, 50%, 66%, 80%, 90% and 100% ethanol for 20 min respectively, each stage being repeated twice. Then critical point drying was performed using Critical Point Driers (Emitech K850X, UK), after which the samples were gold sputter-coated for 5 min at 125 mA in an argon atmosphere with the approximately 50 nm coating (Emitech K550X Sputter Coating Systems, England). A field emission scanning electron microscope (SEM, FEI Sirion-200, USA) was used with a gun acceleration voltage of 20.0 kV and a working distance of 8 mm. The secondary electron detector was used to image the samples condition.

2.4.3. Flow cytometry assay

After the US exposure, the cells were separated from NBD-labeled microbubbles in suspension by centrifugation (1000 rpm, 8 min, Eppendorf centrifuge 5804R, Brinkmann Instruments, Wesbury, NY,