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Effect of non-acoustic parameters on heterogeneous sonoporation mediated by single-pulse ultrasound and microbubbles

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ABSTRACT

Sonoporation—transient plasma membrane perforation elicited by the interaction of ultrasound waves with microbubbles—has shown great potential for drug delivery and gene therapy. However, the heterogeneity of sonoporation introduces complexities and challenges in the realization of controllable and predictable drug delivery. The aim of this investigation was to understand how non-acoustic parameters (bubble related and bubble-cell interaction parameters) affect sonoporation. Using a customized ultrasound-exposure and fluorescence-imaging platform, we observed sonoporation dynamics at the single-cell level and quantified exogenous molecular uptake levels to characterize the degree of sonoporation. Sonovue microbubbles were introduced to passively regulate microbubble-to-cell distance and number, and bubble size. 1 MHz ultrasound with 10-cycle pulse duration and 0.6 MPa peak negative pressure were applied to trigger the inertial collapse of microbubbles. Our data revealed the impact of non-acoustic parameters on the heterogeneity of sonoporation. (i) The localized collapse of relatively small bubbles (diameter, $D < 5.5$ μm) led to predictable sonoporation, the degree of which depended on the bubble-to-cell distance (d). No sonoporation was observed when $d/D > 1$, whereas reversible sonoporation occurred when $d/D < 1$. (ii) Large bubbles ($D > 5.5$ μm) exhibited translational movement over large distances, resulting in unpredictable sonoporation. Translation towards the cell surface led to variable reversible sonoporation or irreversible sonoporation, and translation away from the cell caused either no or reversible sonoporation. (iii) The number of bubbles correlated positively with the degree of sonoporation when $D < 5.5$ μm and $d/D < 1$. Localized collapse of two to three bubbles mainly resulted in reversible sonoporation, whereas irreversible sonoporation was more likely following the collapse of four or more bubbles. These findings offer useful insight into the relationship between non-acoustic parameters and the degree of sonoporation.

1. Introduction

Sonoporation refers to the process in which the interaction of low-intensity pulsed ultrasound with microbubbles transiently perforates the plasma membrane and promotes cellular uptake of local impermeable macromolecules [1–3]. Non-invasive spatiotemporal control and low cost make this physical delivery approach promising for clinical drug delivery and gene therapy [4–6].

It is generally believed that sonoporation is induced by acoustic cavitation—the oscillation and/or collapse of microbubbles driven by ultrasound [7–9]. To realize the therapeutic application of sonoporation, great effort has been focused on improving the delivery efficiency by optimizing the acoustic and non-acoustic parameters of the system [10–12]. However, in previous studies, heterogeneous effects have been observed in sonicated cells following the occurrence of acoustic cavitation. In particular, the amount of internalized exogenous molecules in sonoporated cells was found to exhibit heterogeneous characteristics, which can be classified into different subpopulations (high, low, and nominal uptake) [8,13–15]. Moreover, sonoporated cells were reported to exhibit heterogeneous and complex concomitant physiological responses, such as calcium-ion transients [16,17], calcium oscillations and waves [18], and non-unitary changes in the levels of plasma membrane potential depolarization [19]. Finally, various cellular developmental effects including proliferation inhibition

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[20], cell-cycle arrest [21], and trends in cell fate (i.e., survival, apoptosis, and necrosis) have been observed in sonoporated cells over several hours following ultrasound exposure [1,22,23]. The reason for these heterogeneous effects has yet to be identified. Moreover, for practical application, the heterogeneity of sonoporation poses more challenges in achieving predictable delivery outcome and high delivery efficiency [24]. For instance, some sonicated cells that underwent apoptosis or necrosis are not advantageous to improving the delivery efficiency, because they are essentially the side effects of sonoporation [25]. Also, previous studies have shown that trends in cell fate were correlated with the degree of sonoporation [22,26]. Therefore, it is vital to understand the key acoustic and non-acoustic parameters that are related to the heterogeneity of sonoporation.

Early studies in this area used flow cytometry to characterize heterogeneous sonoporation on a cell-to-cell basis (i.e., continuous distribution of the internalized fluorescence intensity) [11,15,22]. It was found that the degree of sonoporation could be influenced by certain acoustic parameters including the peak negative pressure (PNP), pulse repetition frequency (PRF), and duration of the applied ultrasound, as well as non-acoustic parameters, such as the bubble-to-cell ratio [3,11,13]. A positive relationship between these acoustic parameters and the pore size (i.e., degree of sonoporation) was observed using direct methods such as scanning electron microscopy (SEM) or atomic force microscopy (AFM) [27–30]. Because it is not possible to measure detailed pre-exposure microbubble parameters relative to each individual cell, neither statistical analysis nor direct observation of post-exposure samples can directly reveal the cause of heterogeneous sonoporation. To further understand the interactions between ultrasound, microbubbles, and cells, recent studies have introduced real-time imaging to spatiotemporally monitor the activities of microbubbles with respect to sonoporation [31–34]. Although real-time imaging revealed that differences in the dynamic behavior of microbubbles corresponded to differences in their effect on intracellular uptake levels and pore size [35,36], these studies emphasized the relationship between the ultrasound parameters and dynamic bubble behavior, and did not fully clarify the impact of non-acoustic parameters on the outcomes of sonoporation. Previous studies have also revealed that the extent of sonoporation can be regulated by controlling the distance between the bubble and the cell [37]; however, the influence of other non-acoustic parameters, such as the bubble size and the bubble-to-cell number, on the degree of sonoporation has yet to be fully elucidated.

In this study, we surmised that different bubble-related and bubble–cell interaction parameters would influence the degree of sonoporation at the specific acoustic pressure at which the inertial collapse of bubbles occurs. Using a customized platform for ultrasound exposure and imaging, we observed the sonoporation dynamics at the single-cell level and quantified the exogenous molecular uptake levels to evaluate the extent of sonoporation. We then statistically analyzed the relationship between the non-acoustic parameters and the degree of sonoporation.

2. Materials and methods

2.1. Experimental procedure

The experiments were performed according to the protocol illustrated in Fig. 1. The cells were first cultured in a securely sealed chamber. A membrane-impermeable fluorescence marker was then mixed into the sample chamber to track the occurrence of sonoporation. Another cell-permeable fluorescent probe was also added to evaluate the cells' viability post-exposure. Microbubbles, serving as cavitation nuclei, were finally introduced into the medium surrounding the cells. The sample chamber containing the cells, fluorescent probes, and microbubbles was mounted on an inverted fluorescence microscope, the imaging region of which was aligned with the region to be exposed to ultrasound. After the cells were subjected to a single-pulse ultrasound, the morphology and sonoporation dynamics of the cells within the region of view were recorded in real time for 10 min with a camera. Finally, the acquired bright-field and fluorescence images were statistically analyzed to clarify how the non-acoustic parameters influence sonoporation.

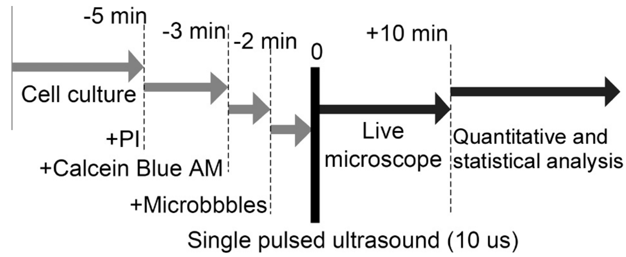

Fig. 1. Overview of the experimental procedure. The time is relative to the moment at which the ultrasound was initiated. Membrane-impermeable fluorescence marker (Propidium iodide) was used as the sonoporation tracer and Cell-permeable probe (Calcein Blue AM) was used as the fluorescence agent for the viability assays.

2.2. Cell culture

HeLa cervical cancer cells (CCL-2; ATCC, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Hyclone, Thermo Scientific Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco 10099; Invitrogen, Carlsbad, CA, USA) at 37 °C and 5% CO_2. To prepare the experimental samples, the cultured cells were washed with phosphate buffered saline (PBS), harvested by trypsinization (Gibco 25200, Invitrogen, Carlsbad, CA, USA), and seeded into Opticell chambers (155331, Thermo Scientific, USA) with 10 mL of the same culture medium. After 1 day of culture, a cell monolayer with approximately 50% confluency was obtained on one side of each chamber. Ten milliliters of Hank's balanced salt solution (HBSS) (Invitrogen 14025, NY, USA) was added to the Opticell chamber to replace the used medium. Note that the cell confluency should be less than 50% (approximately 2×10^6 cells counted by hemocytometer) to facilitate observation and measurement.

2.3. Microbubble preparation

Sonovue microbubbles (Bracco Research, Switzerland), which served as nuclei for acoustic cavitation in this study, are composed of sulfur hexafluoride gas packaged by a phospholipid monolayer shell. The diameter of these microbubbles ranges from 2 to 7 μm [38]. The non-uniform size distribution allowed us to analyze the influence of bubble size on sonoporation. Prior to their use in the experiments, the microbubbles were freshly reconstituted in 5 mL physiological saline solution. Approximately 3×10^6 bubbles were then transferred to 10 mL HBSS in the Opticell chamber and brought in close proximity to the upper cell monolayer in the sample holder through buoyancy. There was approximately one bubble positioned near each cell for 70% of cells, and there were two or more bubbles surrounding the remaining cells. The random location distribution of microbubbles enabled us to investigate the effect of the bubble-to-cell number and distance on sonoporation.

2.4. Sonoporation tracking

Propidium iodide (PI; 150 μM;) (P3566; Invitrogen) was used as a fluorescence marker to track sonoporation in the cells in the