THE ROLE OF CELL MEMBRANE STRAIN IN SONOPORATION CHARACTERISED BY MICROFLUIDIC-BASED SINGLE-CELL ANALYSIS

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ABSTRACT

In the present study we have investigated the sonoporation dynamics in a single cell using a novel microfluidic-based approach. Our methodology has successfully addressed the biophysical mechanisms underlying US-induced cell membrane sonoporation by performing in situ measurement of localised cell membrane deformation, and simultaneous quantification of both intracellular calcium concentration ($[Ca^{2+}]_i$) and transmembrane transfer of extracellular membrane-impermeable probes. We have highlighted novel aspects of microbubble-cluster dynamics combined with localised cell membrane strain, which could be responsible for membrane permeabilisation and transmembrane pore formation correlated with the transduction of intracellular biochemical signals (i.e. $[Ca^{2+}]_i$ influx) as a result of microbubble-cell interaction.

KEYWORDS: Sonoporation, Microfluidics, Microbubble-cluster, Membrane deformation, Biochemical signals

INTRODUCTION

Cell membrane sonoporation using Ultrasound-activated Contrast Agent (UCA) offers a promising strategy for intracellular drug and gene delivery [1]. Ultrasound-driven bubble oscillations (or bubble cavitation) nearby cells have been observed to alter cell membrane permeability [2] and induce the formation of transient membrane pores (sonoporation) through which cell exchanges molecules such as DNAs, siRNAs and pharmaceutical drugs with the extracellular fluid [3], both in vitro and in vivo. Although several possible mechanisms have been reported for the enhanced uptake of extracellular molecules, the sonoporation dynamics at the cellular level and its underlying microscale cell membrane mechanisms remain widely elusive and only few in vitro studies have focused on ultrasound-induced cell response [4]. Furthermore, efficiency of drug/gene delivery and sonoporation-associated side effects have mainly been studied through post-sonication analyses due to the lack of methods for real-time monitoring of cell-membrane sonoporation.

THEORY

In the present study we have investigated the bio-physical mechanisms of sonoporation dynamics and the intracellular change in $[Ca^{2+}]_i$ within a microconfined fluidic environment. The effect of oscillating microbubble-clusters (MCs) exposed to Ultrasonic Standing Wave (USW) was investigated at a single-cell level and showed distinct characteristics with respect to single-bubble induced membrane response. In situ single-cell analysis combined with modeling of the stress field generated by MCs in the microfluidic device have non-intuitively predicted that cell membrane has responded to the mechanical stimuli originating from UCA stable cavitation and transduced it into localised membrane deformation dynamics (i.e. cyclic strain and plastic deformation). Membrane deformation has further been associated with increased cell membrane permeability and uptake of fluorescent propidium iodide (PI) dye localized within microdomains in the cellular membrane.

EXPERIMENTAL

Single-cell analysis was performed within a glass-PDMS hybrid microfluidic device equipped with a piezoelectric transducer (PZT) acoustically coupled to the glass surface. The PDMS microfluidic device (channel width: 1mm, channel height: 50μm) was fabricated with Polydimethylsiloxane (PDMS) polymer using a standard Soft Lithography technique [5]. The PDMS layer containing a linear microchannel was then plasma bonded with piranha-cleaned glass slide. PZT was attached to the glass surface and the microfluidic device was mounted on the stage of a confocal fluorescent microscope to allow monitoring and quantitation of cell-bubble interaction. USW (resonant frequency: 2.14 MHz, peak-to-peak voltage: 10V) was generated within the microdevice for < 10 minutes.

Human cervical cancer (HeLa) cells were selected as a biological model and cultured following standardized protocols. HeLa cells (concentration: 10^6 cells/mL) were seeded and grown in the microfluidic device in Minimal Essential Medium (MEM, HyClone) supplemented with 10% heat-inactivated fetal calf serum (FBS, HyClone) and 100 U/mL penicillin and 0.1 mg/mL streptomycin (GIBCO) in a humidified incubator at 37°C and 5% CO_2. Membrane deformation studies were performed by using fibronectin-coated fluorescent polystyrene beads (0.5µm diameter, Molecular Probes), linked to the cell membrane via integrins [6]. Optison© contrast agent ($5x10^6$ microbubbles/mL, General Healthcare) was suspended in Phosphate Buffer Saline (PBS) and injected into the microdevice. When exposed to USW, oscillating microbubbles were observed

to form clusters that came into direct contact with the cell membrane (Figure 1). UCA clusters induced localized fluid flow perturbations (in close proximity to the cell) that were visualized by in house micro-Particle Image Velocimetry (μPIV) methods. This resulted in the displacement of membrane-linked beads which was quantified using a Multiple Particle Tracking (MPT) algorithm (Figure 1) [6]. Furthermore, sonoporation dynamics was quantified through the uptake of extra-cellular propidium iodide (PI, Molecular Probes), while $[Ca^{2+}]_i$ dynamics was evaluated using Fluo-3-AM fluorescent probe (Molecular Probes).

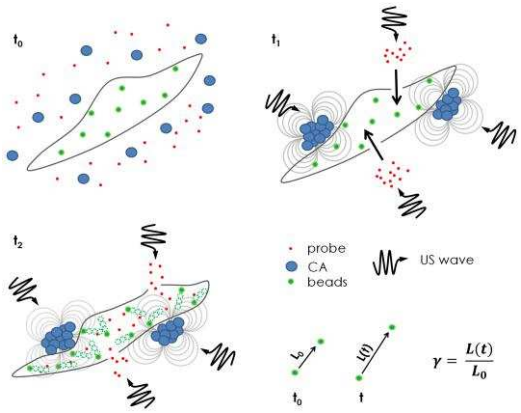


Figure 1: Schematic representation of the experimental approach. At $t=t_0$ (initial condition) no US are applied. Propidium iodide (PI probe) and contrast agent (CA) are homogeneously dispersed within phosphate buffer saline (PBS). At $t=t_1$ (intermediate condition) US is applied at a resonant frequency (f_R) of 2.14MHz and a peak-to-peak voltage of 10V. CA clustering is observed in proximity to the cell membrane. Clusters oscillation generates localized microstreaming that induces cell deformation. At $t=t_2$ (final condition) cell membrane is deformed and PI diffuses into the intra-cellular compartment through the generated membrane pores. *Membrane strain (γ) is calculated by tracking the displacement of membrane*-linked fluorescent microbeads.

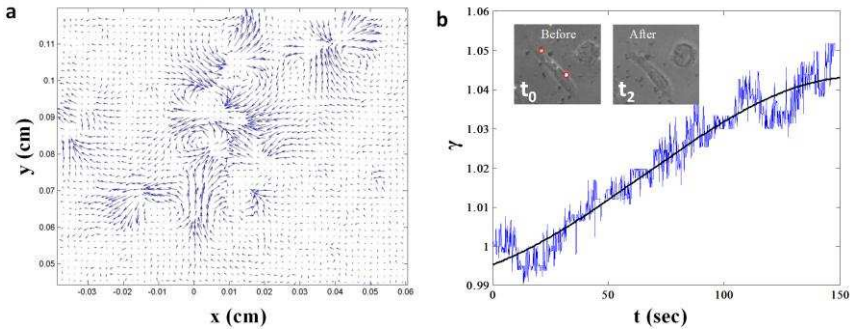


Figure 2: (a) Representative vectorial field around UCA clusters, measured using micro-PIV. (b) Representative trend of membrane strain measured using MPT method. The black line corresponds to the interpolation trend.

RESULTS AND DISCUSSION

Microbubbles oscillate under the influence of USW which creates three dimensional (3D) streaming within the flow field. This in turn creates local vortices around the cluster as shown in Figure 2a. When living cells are in the vicinity of the oscillating MC, perturbations are generated by large spatial and temporal gradients of fluid shear-stress associated with microstreaming. This induces the deformation of cell membrane, which is tracked using the aforementioned MPT method (Figure 2b). Cell membrane deformation (i.e. membrane strain) was observed to vary with the distance from the UCA cluster. However a generalized behaviour was detected, characterised by short-term instantaneous fluctuations and a long-term sinusoidal-like trend originating from residual cell membrane plastic deformation (Figure 3). In sharp contrast to the homogenous uptake of PI in dead cells, we observe uptake of PI in localised microdomains (inset, Figure 4a). Normalised PI fluorescence intensity is observed to increase exponentially with time, although after 4 mins of the exposure to the USW. In this respect, it may be inferred that membrane sonoporation is caused by long-term cyclic strain (i.e. fatigue condition), after several cycles of stretching and relaxing of the membrane revealed through the deformation measurements (Figure 3). The localized nature of PI up-

take could thus be addressed to a spatially-heterogeneous membrane response that originates from membrane structural heterogeneity and the distance from the oscillating MC. In contrast, $[Ca^{2+}]_i$ showed a sudden exponential-like reduction (Figure 4b) that may be induced by short-term cell membrane deformation, which results in instantaneous transient formation of small pores independent of the role of other membrane bound ion-channels.

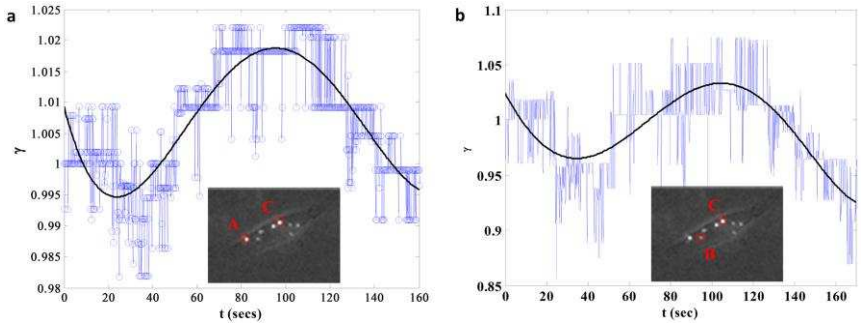


Figure 3: Cell membrane strain measured by particle tracking of bead A (a) and bead B (b), in respect to bead C.

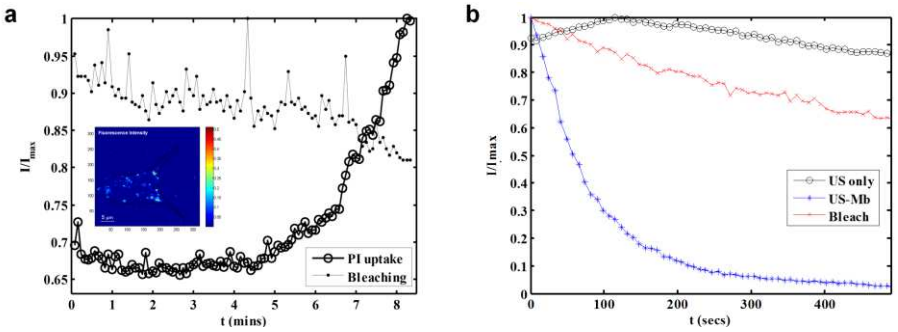


Figure 4: (a) Intra-cellular fluorescence intensity (I, measure of PI concentration) as a function of time (t). Fluorescence is normalized with respect to the maximum value (I_{max}). Bleaching trend is reported. A colour-map of the intracellular fluorescence at t=8min is shown (inset). (b) Intra-cellular fluorescence intensity (I, measure of Calcium concentration) as a function of time. Fluorescence is normalized in respect to the maximum value (I_{max}). Bleaching (red), US-only (black) and US+contrast agent (blue) trends are reported.

CONCLUSION

Our study revealed unique insights into the biophysical mechanisms regulating cell membrane sonoporation within microfluidic confinement. Experimental observations suggested that sonoporation dynamics is primarily caused by cell membrane deformations, and that different temporal scales can be detected regarding cell membrane response to stable microstreaming of microbubble-clusters. Spatio-temporal membrane deformation induced increase in cell membrane permeability and uptake of membrane-impermeable probes in the form of localised microdomains in the cell membrane. This mechano-responsive behaviour of the cell is transduced into biochemical signal by triggering intracellular calcium flux as a result of sonoporation. The microfluidic based single-cell analysis approach provides high degree of similitude with the in vivo response condition of cells, with insights into the non-trivial mechanisms underlying membrane sonoporation under microconfinement.

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