CELL MEMBRANE DYNAMICS DURING SONOPORATION INVESTIGATED BY MICROFLUIDIC SINGLE-CELL ANALYSIS

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INTRODUCTION

Cell membrane sonoporation using stabilized gas bubbles (ultrasound-activated Contrast Agents, CAs) represents a promising strategy for intracellular delivery of bioactive molecules [1]. Ultrasound-driven bubble oscillations nearby cells have been observed to increase membrane permeability *via* formation of transient membrane pores (sonoporation) through which cell can exchange molecules such as DNAs, siRNAs and pharmaceutical agents with the extracellular milieu [2]. Although several potential mechanisms have been reported for ultrasound-induced membrane poration [4], the sonoporation dynamics at the cellular level and its underlying microscale mechanisms remain widely elusive and only few *in vitro* studies have focused on ultrasound-induced cell response [5]. Furthermore, sonoporation-associated bioeffects have been widely studied through post-sonication analyses, which impede to capture the on-time cell membrane dynamics. In the present study we have investigated the bio-physical mechanisms associated with cell sonoporation in the presence of stabilised Microbubble Clusters (MCs) under exposure to an Ultrasonic Standing Wave (USW) field. *In situ* microfluidic-based single-cell analysis was employed in order to quantify the dynamics of cell membrane strain induced by USW application, and the consequent uptake of extracellular molecules (i.e. fluorescent probes, ions).

MATERIALS AND METHODS

Single-cell analysis was performed within a glass-PDMS *hybrid* microfluidic device equipped with a piezoelectric transducer (PZT) acoustically coupled to the glass surface (Fig.1a). A straight microchannel (width: 1mm, height: 50μm) was fabricated within a Polydimethylsiloxane (PDMS) layer, using standard Soft Lithography technique, and then plasma bonded with a 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 for single-cell analyses. USW (resonant frequency: 2.14 MHz, peak-to-peak voltage: 10V) was generated within the microdevice for < 10 minutes.

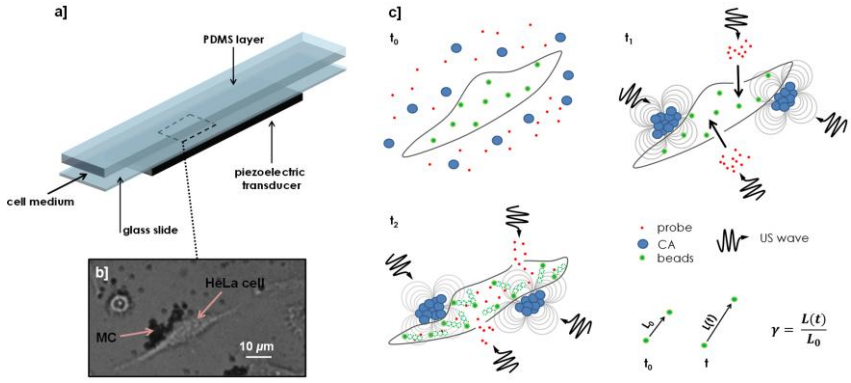

Figure 1: a) Schematic depiction of the microfluidic device. b) Microscope image showing MC-cell interaction during USW application. c) Schematic depiction of the problem under investigation. At $t=t_0$ (initial condition) no US is applied. Propidium iodide (PI probe) and contrast agent (CA) are homogeneously 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 intracellular compartment through the generated membrane pores. Membrane strain (γ) is calculated by tracking the displacement of membrane-linked fluorescent microbeads.

Human cervical cancer (HeLa) cells were selected as a biological model and cultured following standardized protocols. Cells were seeded and grown within the microfluidic device 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 [6]. Optison© contrast agent (3.0-4.5μm diameter, GE) was suspended in Phosphate Buffered Saline (PBS) and injected into the microdevice. When exposed to USW, oscillating microbubbles were observed to form *clusters* under the effect of secondary radiation forces. MCs came into direct contact with the cell membrane (Fig. 1b-c) and induced localized fluid flow perturbations (microstreaming) that were visualized by micro-Particle Image

Velocimetry (μPIV) methods (Fig.2a). This resulted in the displacement of membrane-linked beads which was quantified using a Multiple Particle Tracking (MPT) algorithm (Figure 1c) [6]. Furthermore, membrane permeabilisation dynamics was quantified through the uptake of extracellular FITC-dextran (70kDa) or Propidium Iodide (PI).

RESULTS AND DISCUSSION

Fluid streaming generated by MC oscillation in close proximity to the cell was visualized by μPIV (Fig.2a). The enhanced spatial velocity gradients associated with the onset of microstreaming (Fig.2a) caused a heterogeneous spatial distribution of fluid shear stress on the cell membrane, which resulted in spatially heterogeneous membrane deformation (Fig.2b-c). As a result, the uptake of probe molecules (i.e. FITC-dextran 70kDa in Fig.3b, and PI in Fig.3c) was observed to happen only through specific microdomains of the cell membrane, rather than a homogeneous membrane permeabilisation. In addition, the uptake of probe molecules was observed to increase significantly only after few minutes from the onset of USW (Fig.4c), which is in contrast with other drug delivery techniques (*i.e.* inertial cavitation, shock waves, fluid jetting). It is likely that such sonoporation dynamics is correlated with the time evolution of membrane strain. Notably, membrane strain displayed a unique trend characterised by short-term instantaneous fluctuations (blue line, Fig.2b-c) and a long-term sinusoidal-like trend (red line, Fig.2b-c), which likely originates from the competitive action of MC-induced fluid shear stress and cytoskeletal retraction forces. In this respect it may be inferred that the observed long-term PI uptake is caused by a *fatigue* mechanism, after several cycles of membrane stretching and relaxing; which will likely depend on the spatial distribution of fluid shear stress and on the local membrane mechanical properties (heterogeneous PI uptake). Despite the uniqueness of these observations, further investigations are currently undergoing in our laboratories in order to gain a deeper understanding of the underlying physical mechanisms.

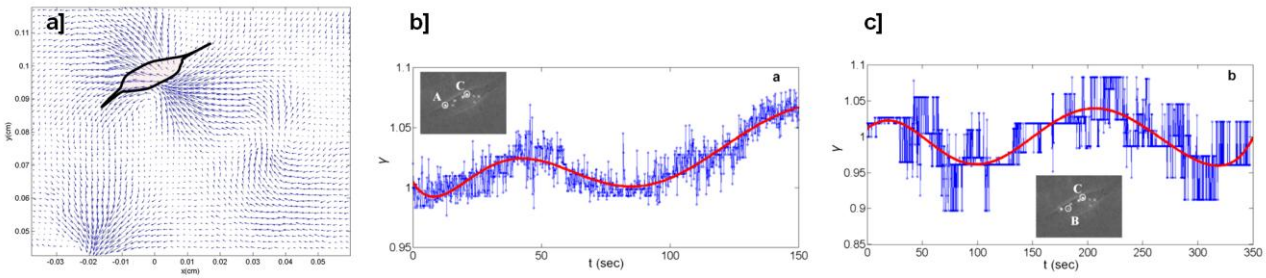

Figure 2: a) Velocity vector field nearby a single-cell. b-c) Cell membrane strain measured by tracking of bead A (b) and bead B (c), in respect to bead C (reference).

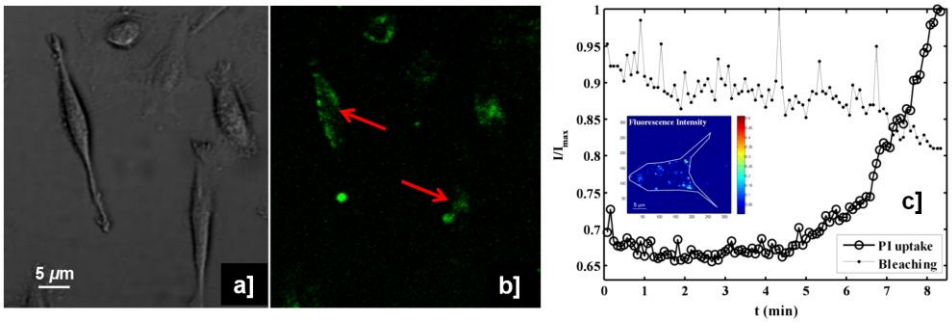

Figure 4: (a) Bright field microscope image of adhering HeLa cells within the microdevice, and corresponding fluorescent image showing the uptake of FITC-dextran 70kDa after sonoporation (red arrows, b). c) Intracellular fluorescence intensity (I, measure of PI concentration) as a function of time (t). Fluorescence is normalized with respect to the maximum value (I_{max}). Bleaching trend is reported. A colourmap of the intracellular fluorescence, at t=8min, is shown (inset).

References

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