Sonoporation with single bubbles in microfluidic systems

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Abstract. When bubbles oscillate close to cells in suspension they can cause viable or permanent poration of the cell membrane. This method named as sonoporation offers potentials for novel therapeutic applications in medicine i.e., cell killing or actively induced drug uptake. Until now, the details of bubble-to-cell interactions are not clarified especially due to a lack of experimental methods. Here we show that sonoporation of cells in suspension is made possible and can be studied in details when using a microfluidic system together with laser-induced cavitation bubbles.

Keywords: cavitation, lab-on-a-chip, sonoporation, suspension cells
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INTRODUCTION

When cells are exposed together with bubbles to ultrasound they can loose their membrane integrity. This occurrence is commonly termed *sonoporation* which can either last for a short duration (repairable sonoporation) or lead to cell death (permanent sonoporation). A common method to probe the integrity of the cell membrane consists of loading the cells with appropriate fluorescence marker such as Calcein AM; upon cell membrane poration or damage the dye leaks out of the cell causing a change in the fluorescence pattern and intensity.

Until now, it was not possible to probe cavitation bubble-induced leakage of individual cells in suspension because of the difficulties involved in tracking cells as the fast bubble-induced flows result in cell motion [1,2]. Also fixing the cell in space - for example with optical tweezers - is ruled out because of the high forces needed and motions may be biaised. The new approach presented here consists of investigating bubble-to-cell interactions not in the bulk but in a confined microfluidic environment, e.g. a microfluidic chamber so as to trap the cells in a given location. Additionally, only a single pulsation of the bubble is studied which allows for correlating cell response to fluid dynamics only.

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EXPERIMENTAL APPROACH

The experimental procedure is separated into the following steps. First cells are loaded with a fluorescent dye, rinsed and subsequently injected into a microfluidic system together with a growth medium supplemented with a light absorbing dye, and a single bubble of variable size and at variable distance from the cells is created in the microfluidic network

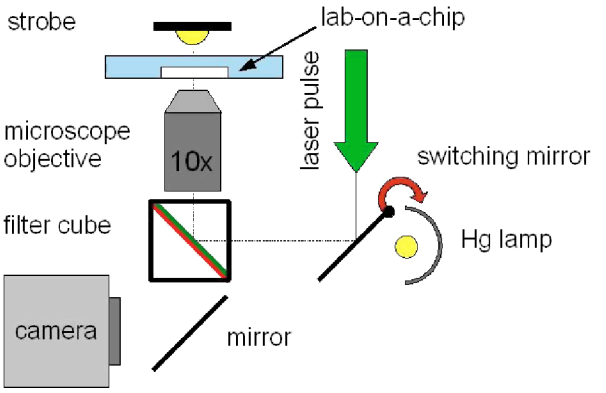

FIGURE 1. A lab-on-a-chip system filled with cells and a light absorbing dye is mounted on a 5-axis translation stage. A bubble is generated in the microchamber with a short pulse from an intense green laser (Solo PIV, New Wave, CA, USA) focused with a 10x microscope objective. A sensitive camera (Sensicam QE, PCO, Weilheim, Germany) records the bubble oscillation and the cell displacement with a time interval of approximately 10μs. Illumination is achieved using a strobe lamp. Before and after the fast dynamics fluorescent pictures of the cells are taken by directing light with a switchable mirror from a mercury lamp through an appropriate filter block onto the dye-loaded cells.

A sketch of the experimental setup for the generation of laser-induced bubbles and the recording of the fluorescence images is depicted in Fig. 1. By focusing an intense pulse from a frequency doubled Nd:YAG laser (λ=532nm, 6ns pulse duration) into a microchip filled with cells and an absorbing dye (see below) single bubbles are generated. For laser energies between 10 and 100μJ at the focus (10x magnification, N.A. 0.25) a transient cavitation bubble is created with a lifetime of 10 to 30μs. An even further increase of the laser energy leads to the formation of a stable gas bubble which dissolves slowly by the action of surface tension pressure.

Lab-on-a chip devices are fabricated using a polymer-based material, PDMS (PolyDiMethylSiloxane) using conventional molding procedure. A silicon mold is first fabricated in a clean-room environment using photolithography and dry-etching techniques (Bosch process). A mixture of a PDMS pre-polymer and a curing agent (10:1) (Sylgard 184, Dow Corning) is subsequently poured on the resulting mold and cured for 2 hours at 60°C. After curing, the PDMS slab is removed from the mold and reservoirs are punched using a blunt needle. Finally, chips are bonded to a microscope glass slide or a polypropylene foil after plasma-based activation of the surface(s) [3].

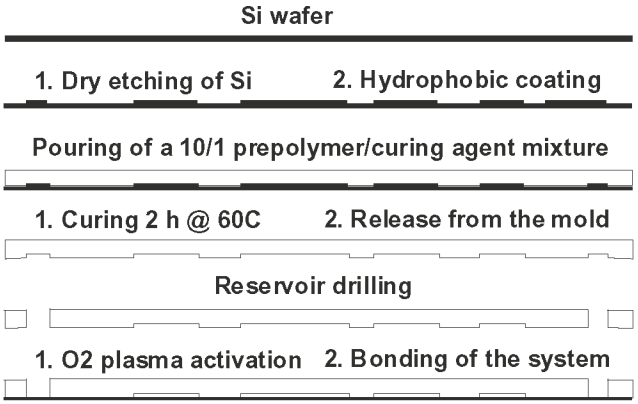

FIGURE 2. Flow-chart illustrating the fabrication of PDMS microfluidic systems using a silicon mold

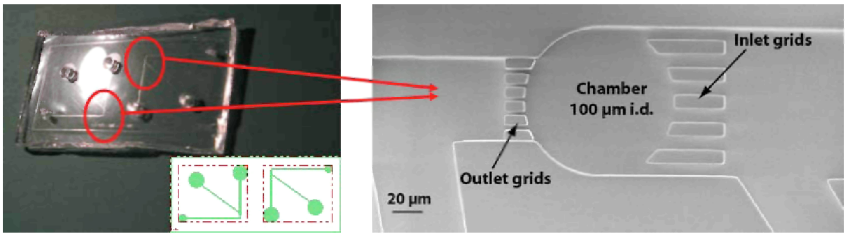

FIGURE 3. Picture and layout of a PDMS microchip of 0.8 cm×1.8 cm (right); details on a chamber of 100 µm diameter (SEM picture, left).

The layout of the used microchips includes a chamber for cell confinement and inlet and outlet microchannels. Microchambers are of 100-160 µm diameter and 27 µm height, and are surrounded by grids (see Fig. 3) so as to create a confined environment and thereby to prevent cells from escaping once they are in the chamber.

Microchips are treated with appropriate cell-friendly coating before their use. HL 60 cells (human leukemia cell line) are loaded with Caclein AM dye, washed in fresh RPMI+ medium and re-suspended in a trypan blue-supplemented solution before their introduction in the microchips. Calcein AM is used to probe the integrity of the cell membrane; fluorescence occurs inside cell cytoplasm after the hydrolysis of the acetoxymethyl ester moiety by intracellular enzymes to give Calcein. Cell membrane damage results in a leakage of the dye, and a loss of fluorescence inside the cell and an increase of the fluorescence background in the surrounding liquid. Trypan blue is added in the medium for two purposes. It is first used as a stain for probing cell viability as it accumulates in dead cells which subsequently get a dark bluish color. Secondly, trypan blue absorbs the green light generated by the laser, helping thereby to generate cavitation bubbles.

EXPERIMENTAL RESULT

Bubble-to-cell interaction in a chamber

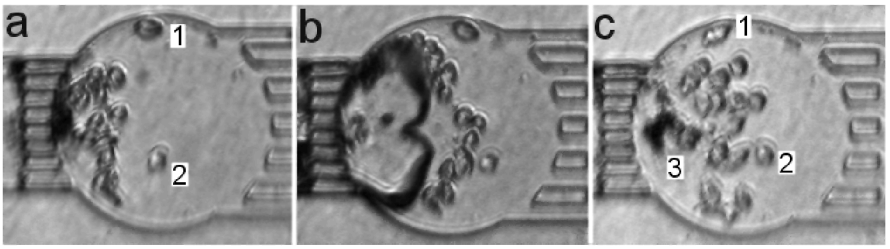

FIGURE 4. (a) The circular chamber in PDMS with a diameter of 160μm and a height of 27μm contains 19 viable HL 60 cells. Due to the fine structured grids cells are collected in the chamber. Frame (b) depicts the very fast bubble oscillation collapsing with a jet towards the left grid structure. 10 minutes later (c) the cells which are labeled as **1** and **2** have been only slightly displaced and show no release of Calcein through their membrane. The dark stained cells close to label **3** are dead cells, presumably the same as the ones close to the left grid in frame (a).

The *microchamber* in which fluid is flushed from the right is surrounded by two series of grids; the left more dense one (see Fig. 4) stops the cells, whereas the right grid allows their passage but prevents their later escaping. Thereby, cells become accumulated and can be exposed to a single cavitation bubble pulsation. A sequence of light microscopy pictures are displayed in Fig. 4. Approximately 4 HL 60 cells in the reservoir are already stained as dead (trypan blue uptake) while 19 remain viable. Figure 4b (8μsec after the bubble generation) depicts the bubble covering around a third of the chamber while collapsing. This creates a jet flow towards the left grid pattern. During bubble activity all cells in the reservoir reveal clear displacement. Figure 4c depicts the cells 60s after the bubble treatment. The cells closer to the bubble reveal larger displacement than the cells located further away, e.g. the cell labeled as **1** in Fig. 4 comes to rest with little net movement.

The release of Calcein from the cells is demonstrated in the sequence Fig. 5a and b. By comparing the fluorescence intensity of both frames it is evident that the signal has dropped considerably for the cells being initially close to the bubble. Cells further away from the bubble (labeled as **1** and **2** in Fig. 5a and b) are hardly affected; their fluorescence remains constants within 3% indicating that no bleaching occurred.

Additionally, weak background fluorescence is found originating from the fluid in the reservoir. The fluorescence intensity from the reservoir before and after bubble activity is depicted in Fig. 5c as a function of the distance. The origin is indicated with the cross in Fig. 5c and is measured towards the right. Before bubble activity the background fluorescence is approximately constant about its mean value (open symbol in Fig. 5c).

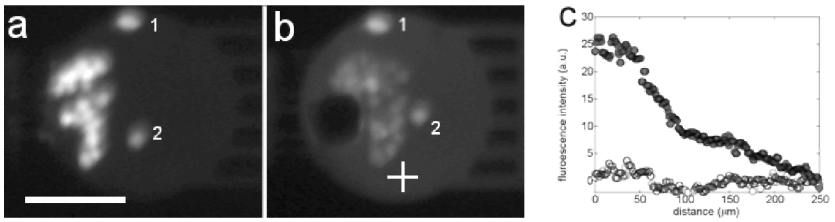

FIGURE 5. Fluorescence stained cells (Calcein) in a microfluidic chamber **(a)** before and **(b)** 85s after the cavitation bubble event. The mean intensity values of cells marked 1 and 2 are 313 and 179 in a), respectively and are affected by less than 3% by the bubble pulsation resulting into a mean intensity value of 305 and 182 for 1 and 2 in b), respectively. However, for the cells closer to the bubble a strong change in the fluorescence signals is found, e.g. the maximum intensity level drops by a factor of 2.5 from 893 to 356. The scale bar denotes 100 µm. **(c)** Fluorescence intensity in the chamber before (open symbols) and after the bubble pulsation as a function of the distance (cross in Fig. 2b).

However, after the bubble pulsation (closed symbols in Fig. 5c) a clear increase of the fluorescence signal from the liquid is found which decreases over a distance of 250µm function to the initial value.

Effect of bubble-to-cell distance

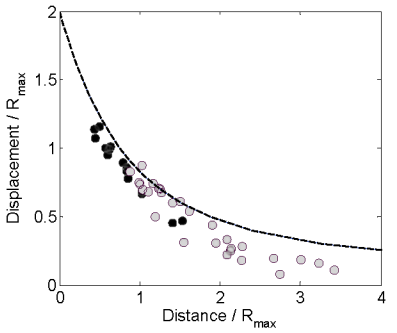

Figure 6. The viability of the cells is presented as a scatter-plot with respect to the initial distances from the bubble center and path traveled by the cells. Both parameters are normalized by the maximum bubble radius, R_{max}. At short distances, largest cell displacement and highest percentage of cell killing (black ○) is found. Cells being at distances $1.5 \times R_{max}$ and beyond survive a single bubble oscillation (bright ○). The dashed line shows the theoretical displacement of a fluid particle in an axisymmetric geometry.

Here we examine the influence of the cell-to-bubble distance value on the threshold for permanent poration of the cell membrane. Therefore, the experiments are conducted in a 2d-quasi free liquid where the boundaries are at least 0.2 mm from the bubble center. This geometry leads to disc-like bubble dynamics with no jet formation, in contrast to the dynamics found in the chambers (see Fig. 4b).

Resuts are summarized in Figure 6. Cells initially close to the bubble center, $d<R_{max,}$ are killed after a single bubble oscillation, whereas cells being at distances $d>1.5\times R_{max}$ survive the bubble treatment. Assuming that the fluid dynamics is limited to 2 dimensions the velocity drops with $1/r$, where r is the distance from the bubble center. Thus, we can derive the length of the traveled path, p, of a fluid particle starting at a distance d from the bubble wall as $p = 2\sqrt{d^2 + R_{\max}^2} - 2d$. This maximum displacement line is plotted in Fig. 6 as a dashed line. Within the measurement errors, all cells travel less than this distance presumably due to the fluid drag.

The limited amount of data available doesn't allow for deriving firm conclusions yet. Nevertheless, Fig. 6 suggests that above a certain distance from the bubble this cell type survives a single cavitation event. This so called 'killing radius' is roughly estimated to be in the order of the maximum bubble radius. Still we have to keep in mind that this finding is based on only very preliminary data and more measurements are necessary to support this hypothesis.

CONCLUSIONS

The main findings are:

- Viable and permanent poration of cells in the close vicinity of a cavitation bubble is found already from a single oscillation cycle.
- Cells at further distance (above approx. one bubble radius) are moving but are not permanently sonoporated.
- After the bubble pulsation the cells leak dye which is measurable in the surrounding liquid.

The presented approach of sonoporation in a microfluidic environment opens not only novel experimental possibilities to understand in greater details bubble-to-cell interactions but might also allow for controlling cell membrane poration by adjusting the fluid dynamics parameters (bubble size, distance from the cell, and the type of bubble dynamics) with respect to the treated cells.

ACKNOWLEDGMENTS

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