Interaction of transient fast flows with biological cells in microfluidic confinement

Li, Fenfang

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**Interaction of Transient Fast Flows
with Biological Cells
in Microfluidic Confinement**

Fenfang Li

School of Physical and Mathematical Sciences

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Summary

In this thesis, we study the interaction of biological cells with transient fast flows mainly from bubbles in microfluidic confinement. These interactions can be utilized to stretch and porate cell membranes for biomedical application.

Deformability is an important mechanical property of cells, which has potential to serve as a label-free biomarker for various diseases. The low throughput is the main challenge for most of the current methods that measure the deformability of individual cells. In this thesis, we employ two methods of high throughput for transient stretching of cells.

The first one is utilizing the shear flow generated by a single laser-induced cavitation bubble confined within a microfluidic gap. Large initial deformations of RBCs can be achieved. The impulse-like forcing allows quantifying the cell mechanical property from a new perspective. We find a much larger area strain (around 30 %) that cells can withstand without rupture compared to only 2-3 % from the classical experiments of micropipette aspiration. Beyond this critical strain, structural membrane damage occurs with the formation of sub-nanometre sized pores, which leads to colloidal osmotic swelling of the cells to a spherical shape before their burst-like rupture.

We find this bubble method is not applicable to stretching of cancer cells. A numerical modelling reveals that the strong shear flow responsible for cell stretching is mainly located within the boundary layer close to the bubble. Only thin elastic objects located near the boundary like RBCs can be largely stretched while spherical cancer cells with a size comparable to the channel height are mainly transported by the flow, i.e. not

stretched.

Therefore we use the second method, hydrodynamic stretching, to correlate the deformability of cancer cells with their invasive potential. The results suggest that the epithelial to mesenchymal transition (EMT) might increase cellular deformability, which is responsible, at least in part, for an increased invasive potential of mesenchymal cells. Besides, we also find that the cellular deformability could be used to evaluate the effect of a chemotherapeutic drug.

Besides stretching cells, the bubble induced flow could also be used to permeabilize the cell membrane. Membrane poration is a key step for introducing foreign materials such as genes or drugs into cells. When a cavitation bubble is close to a boundary or when the gas bubbles are exposed to a shock wave, strong jetting flows occur. Once confined in a microfluidic gap, these fast and thin liquid jets are of micrometer scale and can be utilized for membrane poration of single, trapped cells. We observe localized cellular uptake from jetting gas and cavitation bubbles. For a single cavitation bubble induced microjet, we find the uptake probability is strongly dependent on the standoff distance from the bubble to the cell. The seemingly faster and thinner microjet from a single gas bubble when collapsed by a cavitation event (laser-induced shock wave and an expanding cavitation bubble) is promising for biomedical application that requires smaller and reversible pores on the cell membrane.

To optimize this microjet for cell membrane poration, we study the interaction of a single laser induced cavitation bubble with arbitrary geometry of the gas bubble(s) in a controlled manner within the microfluidic confinement. We find that a gas bubble can be collapsed with the formation of a fast liquid jet by the laser-induced shock wave. A much milder collapse of the gas bubble is also found when the cavitation bubble rebounds. When multiple gas bubbles are present, either a cascaded or a simultaneous collapse is observed, depending on their geometric arrangements. We also find strong mutual interaction between gas bubbles in close proximity, which alters their collapse

scenario. Besides, some novel phenomena are also observed from the interaction between gas bubbles and the cavitation bubble.

1 Introduction

This thesis is aimed at measuring the mechanical properties and transiently opening the plasma membrane of biological cells by using transient fast flows in microfluidic confinement.

1.1 Generating transient fast flows in microfluidics

Microfluidics has potential for large-scale automation of biology and chemistry by allowing performing many experiments in parallel while using less space and reagents [1]. However, the fluid flow in microfluidics is generally dominated by viscosity with negligible inertia. This causes challenges for various applications, such as fluid mixing and pumping in microfluidics. Recently, several methods have been demonstrated to generate transient fast flows with significant inertial effect in microfluidics, including extensional stretching flow with inertial focusing, and strong oscillating cavitation bubbles. These methods not only bring new fluid phenomena but also offer possibility toward various applications.

1.1.1 Inertial microfluidics and extensional stretching flow

The fluid flow in microfluidics is often in the regime of low Reynolds number ($Re = \rho UL/\mu$, where ρ , μ is the density and viscosity of the fluid), which defines the ratio between inertial and viscous effects. This is due to the small dimensions of the microchannel (L) and the common low operation flow rate and flow velocity (U). By neglecting the

Figure 1.1: A schematic for the inertial focusing (a) and extensional flow stretching (b). (a) Inertial focusing of particles to the channel centerline with asymmetric channel geometries (adapted from Ref. [5]). (b) Cross-slot geometry with opposing inlets and outlets for elongation of cells (adapted from Ref. [4]).

inertial, a stokes flow approximation is reached, which indicates a laminar flow in the microchannel. Yet laminar flow is not only occurring in stokes flow regime. As turbulence usually occurs at Reynolds number greater than 2,000, the flow is generally laminar and predictable below this value. However, traditionally, this middle flow regime between turbulence flow and stokes flow has not received much attention in microfluidics.

Recently, the fluid inertia in this regime is exploited in inertial microfluidics, where improved mixing, particle separation and focusing have been demonstrated [2]. Inertial microfluidics takes advantage of unique physical effects in structured microchannels and operates at high flow rates [3]. Now this emerging field is fast expanding as it allows high-throughput, simple and accurate manipulation of biological fluids for clinical diagnostics.

One of the most successful examples has been demonstrated by Gossett et al. [4]. They combined inertial focusing with cross-slot extensional flow geometry to probe single-cell deformability for large population mechanical phenotyping (hydrodynamic stretching). There, high-throughput focusing of bio-particles to the channel centerline is achieved by adopting asymmetric channel geometries [5], see Fig. 1.1 a. The lift forces from the inertial aspects of the flow and additional inertial forces provided by the asymmetric curvature lead to the migration and ordering of the flowing particles. No external manipulation or sheath flows are required for this inertial focusing approach, which simplifies the microfluidic operation system.

Using this method, cells are delivered uniformly to the center of the cross-slot geometry, which is made up of perpendicular, bisecting channels with opposing inlets and outlets, see Fig. 1.1 b. Due to this geometry, a stagnation point is formed at the center of the flow field. There the flow velocity is zero whereas the velocity gradient is large, thus an elongation flow is formed along the streamlines flowing toward the opposing outlets [6]. In this way large deformation of individual cells can be accumulated. Since the operation flow rate is very high ($500\ \mu\text{L}/\text{min}$ and more) in this hydrodynamic stretching approach, cells will pass the extensional flow region within less than $200\ \mu\text{s}$ and thus experience a transient fast stretching.

Besides biological cells, the extensional flow from the cross-slot geometry is also studied toward applications for mechanical characterization of polymer solution and microcapsules [6, 7].

1.1.2 Laser-induced cavitation bubbles in a narrow gap

Besides inertial microfluidics, another efficient method to actuate transient fast flows in microfluidics is using cavitation bubbles. These are a special type of bubbles which expand and shrink rapidly, leading to an impulsive flow dominated by inertial effects. Recently, strong oscillating cavitation bubbles have drawn increasing attention for microfluidic applications. These include enhanced microfluidic mixing [8], liquid micropump [9], microfluidic switch [10] and fluorescence activated cell sorting [11], droplet generation [12], manipulation and mechanical bending of nanotubes/nanowires [13, 14], cell lysis [15, 16], and stretching of red blood cells [17]. There the cavitation bubbles are generated either by focusing a laser pulse in liquid [18, 19], or by acoustically driven capillary waves [20]. The optical method is now widely used in microfluidics as the position and size of the cavitation bubble can be precisely controlled.

Several studies have been performed to reveal the bubble induced flow in these applications [19, 21, 22]. A single laser-induced cavitation bubble has been shown to expand

Figure 1.2: Pressure waveform of a lithotripter shock wave. Adapted from Ref. [24].

to a mainly flat and pancake shape within a narrow microfluidic gap [22]. The problem can be simplified as a planar inviscid flow by using a two-dimensional potential flow model [22]. The bubble dynamics can be described with a two-dimensional Rayleigh equation in cylindrical coordinates. However, since this potential flow model is derived for the flow in the channel center, it does not allow resolving the fluid dynamics in the boundary layers. The boundary layer flow become significant when the studied subjects are near the walls, i.e. biconcave red blood cells [17] and thin elastic objects such as nanowires [14].

Besides the generation of bubble followed by rapid expansion and collapse, a variety of other physical phenomena are also observed when focusing pulsed laser light into a liquid: dielectric breakdown with plasma generation, shock wave emission upon this breakdown, and the bubble collapse shock wave or waves [23].

1.1.3 Shockwave-gas bubble interaction

Shock waves are pressure pulses with a steep compressive shock front followed by longer duration tensile tail, see Fig. 1.2. Gas bubbles exposed to a shockwave exhibit an asymmetric collapse with the formation of a fast jet in the direction of the propagating shockwave [25, 26]. This transient fast jetting flow has been found relevant for the biomedical application in shockwave lithotripsy.

Shock wave lithotripsy (SWL) is the most common treatment for fragmentation of kidney/renal stones. It is generally thought that the following processes contribute to the stone comminution: stress waves propagating within the stone, cavitation erosion resulting from bubble collapse along the stone surface and the evolving role of intrinsic vs. extrinsic flaw distributions in the target stone [27]. In spite of the non-invasive nature, SWL can generate tissue damage as a side effect, primarily local destruction of blood vessels, bleedings, and formation of blood clots in vessels [28]. It has been suggested that cavitation is one of the primary mechanisms for this injury. The tensile (negative) pressure tail of the lithotripter pulses can lead to the formation and growth of bubbles at the focal region of the liquid environment, e.g. urine. Moreover, introducing bubble nuclei in the blood with injection of contrast agents for ultrasound imaging or fluoroscopy prior to SWL can result in a dramatic increase of the severity and extent of renal damage [29, 30]. On the other hand, elevated static pressure (overpressure) or slow pulse repetition frequency (PRT) has been shown to reduce tissue damage as they allow cavitation/gas bubbles produced from previous pulse to dissolve between lithotripsy pulses [31, 32]. Despite application of overpressure, bubbles can be stabilized within the cracked surface of the stone so that cavitation induced fragmentation of stones is maintained. All these results show clear evidence that shock wave-gas bubble interaction is responsible for both the stone comminution and the biological effect in shock wave action.

Besides, the intense jetting flow and high shear stresses from shock wave-gas bubble

interaction can be used to rupture cells for tumour therapy, e.g. histotripsy [33, 34], and temporarily open a cell membrane for gene or drug delivery both in vitro and in vivo [35, 26].

It is important to study the detailed bubble dynamics for better control of these applications. The classical experiments from Dear and Field reveal simultaneous or cascaded collapse of millimetre-sized bubbles in gelatine, depending on the geometrical arrangement [25]. Chen et al. bring this interaction into microfluidic confinement by subjecting a stable gas bubble to a laser-induced shock wave and an expanding cavitation bubble [18, 36]. Similarly, a collapse of the gas bubble with the formation of a fast jet is observed. However, their experiments are unable to continue the classic experiment in microfluidics as the experimental technique only allows one gas bubble to be generated each time. Besides, the effect of the breakdown shock wave as well as the cavitation bubble collapse shock wave on the gas bubble dynamics is not elucidated.

1.2 Stretching and membrane poration of biological cells

The transient fast flows introduced above can be used to either stretch biological cells for their mechanical properties, or temporarily create pores in the cell membrane for gene and drug delivery.

1.2.1 Deformability of biological cells

There is increasing evidence that diseased cells differ physically from healthy ones. For example, red blood cells are found to be stiffer from sickle cell anemia [37, 38] or when infected by *Plasmodium Falciparum* (malaria) [39]. A reduced stiffness has been reported for cancer cells with higher metastatic potential compared to its benign or normal phenotypes [40]. These changes of cellular mechanical property are associated with abnormalities in the cytoskeleton and nucleus structure of the diseased cells. It has been suggested that mechanical properties of cells, such as the ability to deform under a mechanical load

(deformability), could serve as a label-free biomarker for human disease [41, 42]. The advantage of using this biophysical marker is that cells can be directly assayed without time-consuming sample preparation such as laborious and costly fluorescence labelling in traditional biochemical approaches.

Growing interest has been stimulated to study the mechanical properties of cells, which leads to development of various measuring techniques. Some methods have been demonstrated to measure the overall deformability of bulk cell solution, such as light scattering [43], and microfiltration [44]. Though with high throughput, these methods neglect the heterogeneity or size variation within the cell population, which may result in misleading averages for abnormalities from a single cell or small populations. Thus it is necessary to conduct mechanical measurements on a single cell level.

A variety of such single-cell platforms are developed, including micropipette aspiration [45], atomic force microscopy (AFM) [46], micromagnetic twisting rheology [47], optical tweezers [48], optical stretchers [49] and cell transit analyser [50, 51]. Nevertheless, some drawbacks are associated with these techniques. For example, AFM allows for localized mechanical probing of a cell with precision, whereas it can also be disadvantageous as the position of the indentation influences the measured mechanical value. Similar limitations apply for micromagnetic twisting rheology and optical tweezers. Besides, these two techniques require the attachment of microbeads to the cell surface before experiment, which complicates the sample preparation and may additionally create small strains on the cells. Micropipette aspiration and optical stretcher are able to measure the whole-cell mechanical property. However, their throughput is very low (around or less than 1 cell/min). A relatively higher throughput can be achieved by cell transit analyser (up to around 1 cell/s) than other single-cell measurement techniques listed here. However, the variation of cell size and adhesiveness, particularly in heterogeneous samples, has a large impact on the transit time. Besides, the transit channels or pores are prone to clogging. In general, with these methods it is time consuming or not easy to test a large

number of cells, which, however, is required for the statistics results of measurements due to the inherent biological variation and heterogeneity.

Recently, a cavitation based technique is used to stretch red blood cells in microfluidic confinement with high throughput [17]. Many cells can be stretched simultaneously without pre-treatment by the transient fast flow (in the order of tens of microsecond) from a single laser-induced cavitation bubble. Significant statistical difference of the elastic properties has been achieved between the untreated red blood cells and those treated with stiffening or softening reagents. Particularly, this method allows stretching cells with an impulse-like forcing, which can be used to study the biophysical property of cells in a new perspective. For instance, though the mechanical property of red blood cells has been extensively studied, most of previous studies stretch the cells in a quasi-static manner or with long duration forcing. Besides, the rupture dynamics of red blood cells under excess mechanical load has not been well studied on a single cell level. In addition, this method might also be applied to probe the deformability of cancer cells.

Another high-throughput and microfluidic based approach has also been demonstrated [4]. As mentioned in section 1.1.1, single suspended cells can be uniformly delivered to the stretching region of an extensional flow with inertial focusing. The massive throughput (around 2000 cells/s) allows for rapid measurement of large cell population with improved statistic accuracy. This method has been applied to assay the deformability of malignant cells in pleural effusions and the disease state in patients with cancer can be accurately predicted [4]. In addition, this technique might also be used to study the pathophysiology of diseases such as cancer metastasis and related drug treatment.

Metastasis is the spread of cancer cells from the primary tumour to distant organ, which is the predominant cause of cancer deaths. It involves the acquisition of invasive cell phenotype in the primary tumour, invasion of these cells into surrounding matrix and blood vessels, entering and traveling through the circulation as circulating tumour cells, invading distant organ, where they adapt to the microenvironment and proliferate to a

new tumour [52]. To make the disease under control, it is important to understand the detailed mechanism for these processes such as the acquisition of invasive cell phenotype in the primary tumour.

Epithelial to mesenchymal transition (EMT) is an essential programme for embryonic development, in which polarized epithelial cells are converted into motile mesenchymal cells. However, recently it is unravelled that this important developmental programme is one of the major causes for acquisition of dedifferentiated and metastatic phenotype in the primary tumour [53, 52]. Though it has been shown that mechanical properties such as deformability can grade the metastatic potential of cancer cells [40, 54], their direct correlation with EMT has not been studied yet.

1.2.2 Cell membrane poration and delivery of foreign materials

Transferring foreign and large molecules into cells is an essential step for various biological studies and therapeutic applications such as gene therapy and drug delivery. It has been shown that modified viral vectors can delivery gene materials into cells with high efficiency [55]. However, their application is constrained as a result of possible toxicity, excited immune response and their low production.

These limitations lead to development of various nonviral methods for gene transfer, in both chemical and physical means. Chemical approaches, including cationic lipids, cationic polymers and lipid/polymer hybrids, use synthetic or natural compounds to bond with or encapsulate DNA and carry the genes for cell uptake [56]. Though most of them show high efficiency in vitro, their low efficiency in vivo transfection, possible toxicity to cells and incited immune response make the approaches less attractive for clinic application. In contrast, the physical methods are non-toxic as physical forces are utilized to create transient reversible pores in the cell membrane and allow genes or other foreign molecules to enter the cell. Due to this working principle, the physical methods are not only limited to gene transfection but also able to introduce drugs or

other reagents for cell treatment.

Electroporation and sonoporation are two major physical approaches for cell membrane permeabilization. In electroporation, an external pulsed electric field is used to create pores in the cell membrane [57]. This method has been shown to have high efficiency and is applicable to almost all cell types. Electroporation has been scaled down to microfluidic systems [58, 59] and more recently nanochannels [60] for single cell membrane poration. However, a complex fabrication process is required for these devices, such as integration of microfabricated electrodes or nanochannels. Moreover, the application of electroporation in vivo is hindered by the limited working range between the electrodes, and the requirement of surgery to treat cells deeper in the body as electrodes need to be inserted.

An alternative to electroporation is using pulsating bubbles to create transient pores in the cell membrane and facilitate the cellular uptake of foreign materials. As generally acoustic approaches such as ultrasound or shockwave are used to create these pulsating bubbles, this approach is called sonoporation [61]. Since ultrasound or shock wave can penetrate living tissues without causing damage and be focused to a specific region, this method holds good potential for noninvasive and topical gene therapy or drug delivery in vivo. However, a low delivery efficiency and a limited amount of control exist for these acoustic approaches. For example, a random distribution of pores on the cell membrane is resulted from bubbles of varying sizes and arbitrary locations when using shockwave [62, 63].

Besides the acoustic approaches, cavitation bubbles created by focused pulsed laser light are also exploited for cell membrane poration. A single laser-induced cavitation bubble has been applied to permeabilize adherent cells on a rigid substrate [64]. The easy control of the bubble size and location allow studying the effect of the standoff distance on the cellular uptake and cell viability. This optical method can be easily incorporated into microfluidic systems. Sonoporation of suspension cells in the vicinity of a single

laser-induced cavitation bubble in a microchannel has been demonstrated [65]. Moreover, Localized and directional membrane poration of single adherent cells is achieved by using a pair of laser-induced tandem microbubbles [66]. Besides, single suspension cells can be trapped by arrays of microstructures and then locally porated by the microjet from the asymmetric collapse of a single cavitation bubble near the microstructures [67]. The combination of optically generated cavitation bubbles with microfluidic confinement leads to more detailed fundamental studies about the transient bubble-cell interaction, associated fluid dynamics, and the dynamic process of cell uptake. It also holds good potential for high-throughput in vitro application.

One limitation of the optical method is the requirement for using laser-absorption dye. Without the absorption dye, high break-down laser energy need to be used, resulting in large cavitation bubbles. Under these conditions, the bubble generation is usually associated with debris of the damaged substrate, and there remains insoluble gas bubbles after collapse of the cavitation bubble, which together results in less repeatable bubble dynamics. Recently, patterned gold dots have been demonstrated to enhance laser absorption for easy alignment and consistency in bubble generation [68]. Besides, it has been reported previously that localized heating of metal surfaces immersed in water with laser can lead to the generation of vapor microbubbles [69].

1.3 Thesis outline

This thesis reports high-throughput cell stretching and localized membrane poration using transient fast flows generated in microfluidics. These are realized mainly through laser-induced cavitation bubbles. The first part of Chapter 2 (Section 2.2) investigates the threshold strains of red blood cells under an impulse-like forcing and the resultant permanent membrane poration through a single laser-induced cavitation bubble. This stretching method is applied to cancer cells in Section 2.3. This practice motivates a numerical modelling of the underlying flow field, which helps resolve the mechanisms

responsible for cell stretching in Section 2.3. It also leads to the application of inertial microfluidics and extensional stretching flow for the correlation of cellular deformability with EMT or pharmaceutical drug in Chapter 3.

The optical method for generating cavitation bubbles in Chapter 2 was improved utilizing a gold coating which lowers the energy threshold for nucleation, and therefore we can generate smaller ones and make the process more repeatable. This gold coating when exposed to a continuous wave (CW) laser assists in the generation of gas bubbles, together with a digital hologram even for arbitrary bubble patterns. Combining a pulsed and a CW laser we can now study in Chapter 4 the interaction between gas bubbles and a cavitation event in greater detail. In Chapter 5 we use again a gas bubble and a cavitation bubble to porate a single cell.

A diagram summarizing the connections between the chapters is shown in Fig. 1.3.

The novelty of Chapter 2 lies in the impulsive stretching of RBCs and the higher yield strength compared to the previous quasi-static methods. The trial for stretching cancer cells and the numerical simulation contribute to understanding the full picture, especially the boundary layer of the bubble induced flow field in the microchannel confinement. Although the extensional flow stretching method is not new, Chapter 3 is able to directly correlate the cellular deformability with EMT, which has not been studied before. The major novelty in chapter 4 and 5 is the coating of the gold nanoparticles to improve heat absorption. This allows to generate arbitrary configurations of gas bubbles in water, which is difficult in previous work. The novel experimental technique enables studying shock-wave/cavitation bubble -gas bubble interaction in a controlled manner in microfluidic channel. The resultant jet from a single gas bubble collapse is used for cell membrane poration in Chapter 5. This is different from previous technique of tandem bubbles or asymmetric bubble collapse close to a wall. The gold coating also helps avoid the commonly used laser absorbing dyes that is toxic to cells.

Figure 1.3: A schematic diagram for the thesis outline and connections between different chapters

2 Cell stretching based on cavitation bubble

2.1 Overview

It has been demonstrated in principle that a single cavitation bubble confined in a narrow gap can be used to stretch red blood cells (RBCs) and study their mechanical properties [70]. Yet the yield strength of RBCs under this impulsive stretching and the resultant cell rupture dynamics is new. Also, the flow field leading to the cell deformation is only now discussed and simulated. Furthermore, the potential application of this cavitation method to tumor cells has not yet been explored.

This chapter is organized into two parts. In the first part, we investigate the yield strength of RBCs to impulsive stretching with the cavitation method, and the induced permanent membrane poration. In the second part, we apply the cavitation method to tumor cell line RKO. A numerical simulation is also included to understand the flow field and the observed cell deformation.

2.2 Yield strength of human erythrocyte membranes to impulsive stretching

2.2.1 Introduction

Deformability of RBCs is an important mechanical property and continues to draw interest. The healthy biconcave RBCs can deform easily to traverse capillary vessels smaller than their own diameter to transport oxygen to various parts of the body [73, 74]. A degradation of this property will increase flow resistance in the microcirculation. Decreased deformability has been found for RBCs in storage [75] and in pathological conditions such as severe burns [76] and *Plasmodium Falciparum* (malaria) infected RBCs [77]. It has also been reported that mechanical properties of cells could potentially serve as a bio-indicator for human diseases [78].

So far various methods have been demonstrated to measure the mechanical properties of RBCs, among these are micropipette aspiration [79, 80], light scattering [81], flicker spectroscopy [82, 83, 84], twisting micromagnetic beads [85], atomic force microscopy [86], shear flow [87], optical tweezers [88], optical stretchers [89] and a more recent technique termed controlled cavitation rheology (CCR) [70]. The CCR method stretches a collection of RBCs using a fast and transient flow created by a single laser-induced cavitation bubble in a microchannel geometry. It has advantages over other methods in that many cells can be tested simultaneously and with an impulse like forcing. This allows one to obtain large initial deformations of the cells.

Many studies on the mechanical properties of RBC membranes have confirmed that RBC membranes can only withstand a finite strain before they rupture, as first proposed by Rand et al. [80, 79]. A classic experimental result [90] shows that the RBC membrane will rupture beyond a critical areal strain of 2-4 %. This was found in micropipette measurements of isotropic tension vs. area expansion of preswollen single human RBCs.

⁰The work of section 2.2 has been published as [71, 72]

Rapid cell lysis above a critical areal strain of $2.6 \pm 0.8\%$ was reported later with the cell poking method of osmotically swollen human erythrocytes [91]. In that method, they detect the force required to indent cells attached to a glass coverslip and use a simplified mechanical model to obtain the elastic area compressibility modulus. This agrees reasonably with the classic result from Evans et al. [90]. The work of Leverette et al. in 1972 [92] using rotational viscometer shows that beyond a threshold shear stress, extensive cell damage will occur. They summarized prior studies on hemolysis, and found a correlation in which the threshold shear stress is inversely proportional to the exposure time. The results are consistent among the various studies using RBCs of different origins and flow fields.

The present work is inspired by their result. We want to address the question of what is the maximum areal strain a RBC can sustain. Therefore we will make use of the above mentioned CCR technique where a laser induced cavitation bubble stretches cells in an impulse like fashion, e.g. within about $40\text{-}60\ \mu\text{s}$. Additionally, we supplement the CCR method with fluorescence microscopy to monitor the cell membrane integrity after the stretching.

Extensive cell membrane damage or membrane rupture beyond a critical stress or strain is due to membrane pores. It has been predicted [93] that a membrane may heal itself for sufficiently small pores (smaller than $1\text{-}10\ \text{nm}$) while membranes may not heal for larger pores. Pores in the cell membrane allow the delivery of foreign and large molecules such as genes into the cells [94, 95, 96].

Therefore, in the present work we will quantify the yield strain for RBC membrane rupture using the impulse like stretching from a single laser-induced cavitation bubble [70]. The dynamic bubble-cell interaction is recorded with high speed photography and the resultant cell membrane integrity is assessed from the efflux of fluorophores from the cell's cytoplasm into the extracellular space.

2.2.2 Materials and methods

2.2.2.1 Experimental set-up

To stretch the RBCs, we use the impulse like forcing from a laser induced cavitation bubble. The experimental technique is similar to previous studies, e.g. see [70]. Figure 2.1 depicts the experimental setup. A 532 nm, 6 ns duration laser pulse is focused with a 40 $\times$ /0.80 NA water immersion objective into the cell solution inside a microchannel. An optical breakdown at the focus of the pulsed laser beam results into a rapidly expanding bubble [97]. The bubbles created have a maximum radius of about 150-200 μm with a lifetime from 40 to 60 μs . These relatively large bubbles are needed to probe the yield strength of cells. In Ref. [70] lower laser energy was used to probe the cell rigidity without membrane rupture.

A high speed camera (Photron SA-1) is used to sequentially capture both the fast bubble dynamics and the following slower process of cell shape recovery. Therefore fast (500 frames with 10 μs time interval, 1 μs exposure time) and then slow (1999 frames with 5 ms time interval) recordings are performed. Fluorescence images of the same field of view are taken immediately after this high speed camera recording. The fluorescence images are captured with a cooled CCD camera (Sensicam QE, PCO) at 1.67 frames per second for up to 100 s. A mirror is switched to direct the light to either camera. All the timings are controlled precisely with trigger signals from a pulse delay generator (BNC model 575). During experiments we also take care that cells are only exposed to a cavitation bubble once.

2.2.2.2 Red blood cell preparations and fluorescence dye loading

Human erythrocytes were collected from a healthy donor using sodium citrate. They are washed three times with 1 $\times$ PBS (Phosphate buffered saline) containing 0.1 % BSA (Bovine serum albumin). The washed RBCs are kept at 50 % hematocrit in 1.0 mL

Figure 2.1: Experimental setup to stretch RBCs with a cavitation bubble in a microchannel. The microchannel with the cells which are preloaded with calcein is placed on the stage of an inverted microscope. A pulsed green laser (532 nm) is focused through a 40 $\times$ water immersion objective into the cell solution to create a rapidly expanding cavitation bubble. A high speed camera records the bubble dynamics and cells response. For fluorescence measurements, the green fluorescence from calcein inside the cells is excited with a mercury lamp and appropriate filter blocks (not shown here). A cooled CCD camera captures the fluorescence images. With the help of a switching mirror either the CCD or the high speed camera is illuminated.

Eppendorf tubes. Then $6\text{ }\mu\text{L}$ of these 50 % washed RBCs are diluted in $200\text{ }\mu\text{L}$ PBS. $10\text{ }\mu\text{L}$ of $100\text{ }\mu\text{M}$ calcein-AM (Biotium, USA) is added for a final concentration of $5\text{ }\mu\text{M}$. After 45 min incubation at 37°C , the cell solution is ready for use and applied immediately for experiments.

2.2.2.3 Microchannel geometry

The prepared cell solution is confined between two fastened #1 microscope cover slides with $15\text{ }\mu\text{m}$ thick flattened aluminum foils as spacers, see Ref. [70].

2.2.2.4 Image processing for maximum strain of cells

After the bubble collapses, the cells recover their shape gradually. During that time, we extract the major length L (see Fig. 2.4 *B (b)* in the later section) and the area S from the contours of the cells. This is done using image processing. The limited resolution of the camera at higher framing rates does not allow obtaining high-resolution pictures of the RBCs. Nevertheless, we can obtain rather precise contour of the cell shape using the grey level information from the pictures. The cell outline is obtained from a home built Matlab script based on the library provided by Xu and Prince [98], which is an active contour (snake) method using a gradient vector flow model. Once the contour is obtained the shape can be characterized by their center of mass, contour length, principle axes, and area. Two exemplary cells, where the contours are obtained and tracked from maximum deformation to relaxation, are shown in Fig. 2.2.

The validation for the above image analysis is shown in Fig. 2.3. The error is around 5 % to 7 % compared to that from high magnification (mag) and high numerical aperture (NA), for which the number of pixels is increased threefold. It is a systematic error because the edge of the cells is imaged with more pixels and stronger contrast. This systematic error is nevertheless eliminated in the areal strain calculation. We chose $40\times 0.80\text{ NA}$, $0.5\times$ c-mounts for the experiment because of the limited illumination

Figure 2.2: Contours from maximum deformation to relaxation of the RBCs from the home-built Matlab routine based on the snake algorithm. Two exemplary cells (left and right) are shown here. The red curve denotes the contour while the blue lines indicate the major and minor axes of the cell, respectively.

Figure 2.3: (a) Comparison of the image analyses for the same cells imaged with high magnification (mag) and high numerical aperture (NA) (60×0.90 NA, $1 \times$ c-mount, top row of the figure) and with the mag and NA for Fig. 2.2 (40×0.80 NA, $0.5 \times$ c-mounts, bottom row of the figure). The red line denotes the contour obtained from the same active contour algorithm. (b) Results of image analysis for (a). The Rel Error refers to the error of the area relative to that of the higher mag and NA.

under the high frame rate and a proper size of the view field. Higher mag and higher NA (e.g. 60×0.90 NA, $1 \times$ c-mount) will result into a lack of illumination and a small view field. In summary, we have checked the error introduced by using a rather low pixel resolution in the high-speed recordings and found that the error in measuring the total cell area is about 6 %. As only the relative change of the area is used for the areal strain we expect that the error due to a coarse resolution is less, thus below this 6 %.

Here we define the maximum linear strain γ_{lin} and areal strain γ_{area} as:

$$\gamma_{lin} = (L_{max} - L_{eq})/L_{eq}, \gamma_{area} = (S_{max} - S_{eq})/S_{eq}, \quad (2.1)$$

where L_{max} and S_{max} are the maximum length and area measured after the bubble collapses, and L_{eq} and S_{eq} when the cell has reached a constant shape.

Please note that the RBC does not deform uniformly. It is elongated along the radial direction. However, the part further from the bubble becomes thinner than the closer part. This can be clearly seen from Fig. 2.4. The rupture site of the RBC is located at this further part from the cavitation bubble, see Fig. 2.9.

2.2.2.5 Monitoring cell membrane integrity

To check thoroughly for membrane integrity, we combine the high-speed recording which covers only 10s with a slower but more sensitive imaging modality. After this 10s, the camera port is switched to the fluorescence camera (Sensicam QE) which records at 1.67frames/s for up to 100s, see the experimental set-up section.

Cells that noticeably lose their bright-field contrast or fluorescence intensity (reach the background intensity) during the total observation time of 110s are characterized as permanently porated cells. The remaining cells are characterized as viable, e.g. see Fig. 2.5 in the later section.

2.2.2.6 Statistics of cell viability as a function of maximum strain

We divide the maximum strains of all the analysed cells into several bins and count the number of viable and lysed cells within each range. To plot the percentage of viable cells for each bin the average strain is calculated and the standard deviation from the average strain is plotted as error bars.

2.2.3 Results

2.2.3.1 Bubble-cell interaction

Fig. 2.4 *A* presents an overview of the dynamics of the bubble expansion and collapse as well as the cell deformation. At time $t = -10 \mu\text{s}$ before the experiment, all the cells show the typical biconcave shape of unstretched RBCs. At $t = 0 \text{ s}$, the cavitation bubble is created. Then at time $t = 30 \mu\text{s}$, we see the dark contour of the expanding bubble and the elongated cells around it. The short exposure time of $1 \mu\text{s}$ allows us to capture the cells with little motion blur. During bubble expansion the cells are already largely stretched. At time $t = 40 \mu\text{s}$, the bubble has collapsed and the cells are stretched even further. A bubble remains visible in the center. This is different to Ref. [70] and we attribute it to the higher laser energy. This remaining gas bubble dissolves slowly due to surface tension [99]. At time $t = 40 \mu\text{s}$ the radial flow has ceased and the cells start to relax to their final shapes. At time $t = 230 \mu\text{s}$, the cells have already retracted to some extent.

Next we present the relaxation of the cell shape in greater detail and selected cells are marked with a-c ($t = 40 \mu\text{s}$) in Fig. 2.4 *A*, which are enlarged in Fig. 2.4 *B*.

Fig. 2.4 *B (b)* illustrates a cell which recovers to its original shape and still survives at the end of high-speed recording ($t = 9.87 \text{ s}$). Fig. 2.4 *B (a)* depicts the evolution of the cells which are stretched into long tethers ($t = 40 \mu\text{s}$). They complete relaxation within 115 ms ; afterward their contrast is reducing ($t = 580 \text{ ms}$) before they finally disappear ($t = 1.505 \text{ s}$) indicating cell lysis. It is noticeable that these tethered cells are stretched longer and their disappearance is faster as compared to cells without tethers. Fig. 2.4 *B (c)* shows a cell which seemingly first undergoes recovery ($t = 0\text{-}3.5 \text{ s}$) yet disappears at later time ($t = 6.5\text{-}9.5 \text{ s}$).

The obvious leakage from dye loaded red blood cells is an indicator of cell lysis. This leakage takes place on a longer timescale than the stretching and relaxation of the cells.

Figure 2.4: (A) RBCs imaged before ($-10 \mu\text{s}$), during ($30 \mu\text{s}$) and after the cavitation event ($40 \mu\text{s}$ and $230 \mu\text{s}$). Here time $t = 0$ is set as the time when the cavitation bubble is created. (B) (a)-(c) Enlarged areas demonstrating the shape recovery of the individual cells marked in (A) with a-c, respectively. The timings below indicate the elapsed time after the bubble is created. (a) The long tethered cells which disappear gradually. In (b), L is the major length which is used for calculating linear strain. Area of the cell contour is used to define the areal strain. (c) A cell which relaxes and then disappears at the end of high speed recording. The arrows in each case indicate the direction towards the bubble center. All of the scale bars denote $20 \mu\text{m}$.

Figure 2.5: Monitoring the integrity of cell membrane with fluorescence imaging. (a) and (b) The fluorescence images taken for the same cells at 25 s and 48.3 s after the bubble induced stretching, respectively. The cells marked in (a) are disappearing (denoting permanently porated cells). (c) Variation of fluorescence intensity with time for permanently porated cells and intact cells from the same experiment shown in (a). The arrows labelled with 1 and 2 point toward the permanently porated cells marked by a rectangle and a circle in (a), respectively. The three cells to their right show a mild reduction of intensity due to fluorescence bleaching (*intact cells*). Here all the timings indicate the time elapsed after the bubble has collapsed.

Figure 2.6: Change of the shape and fluorescence intensity for the individual fast leaking cells. (a) and (b) are the cells marked by 1 and 2 in Fig. 2.5 (a), respectively. (c) shows a permanently porated cell from another experiment. Here all the timings indicate the time elapsed after the bubble has collapsed.

Two exemplary frames from fluorescence microscopy are shown in Fig. 2.5 (a) and (b), which are taken 25s and 48.3s after the bubble induced stretching, respectively. It is noticeable that two of the cells lose considerably their fluorescence (labelled 1 and 2 in Fig. 2.5 (a)), while neighbouring cells seem unaffected. This strong difference in time dependent fluorescence is depicted in Fig. 2.5 (c): cells labelled 1 and 2 show a rapid decrease in intensity (within 7s from 90% to 10% and counted as permanently porated/lysed cells) while the three cells to their right (labelled as intact cells) show a mild reduction of intensity due to fluorescence bleaching (counted as viable cells).

A closer look at the leaking cells in Fig. 2.5 (a) reveals their distinct shape; cells which leak have irregular shapes and more uniform intensity distribution as compared to the intact cells. Additionally, leaking cells change shapes from irregular to spherical prior to the leakage. This behaviour is demonstrated in Fig. 2.6 where three examples of the fast leaking cells are presented. The upper two rows are cells labelled 1 and 2 from Fig. 2.5 (a) and the bottom sequence is taken from another experiment. We always find a rounding of the cells before their fast leakage of dye.

2.2.3.2 Threshold strain for RBC membrane rupture

In order to obtain a threshold strain for RBC membrane rupture from this impulsive stretching, we correlate the membrane integrity of cells with their maximum strains.

The data from 93 cells through 12 measurements has been analyzed. Among these, there are 48 viable cells and 45 non-viable cells. We obtain the statistics of viable cells (in percentage) as a function of the maximum linear/areal strain, shown in Fig. 2.7. The circle and square symbols denote the viability vs. the mean value of linear and areal strain within each strain range, respectively. It can be seen that at small strain range all cells survive. With strain increasing, the viability decreases gradually. Above a certain strain no cells remain viable, i.e. they become permanently porated. The data for the linear and area strain follow similar trends. We get a 50 % viability of 105 % for linear and 40 % for areal strain. These thresholds have been obtained through interpolation of the hyperbolic tangent functions (solid lines) which is fitted to the experimental data.

For the areal strain, some error may be involved in the neglect of a potential cell thinning during stretching. Nevertheless, we expect only a minor contribution, as the flow is essentially planar and therefore stretching should occur only in the lateral direction.

Generally speaking, the impulsive forcing of cavitation bubble results in much larger critical areal strain for permanent pore formation as compared to the classic results in micropipette aspiration which is 2-3 % [90]. This will be discussed later in Section 2.2.4.

Clarification of the areal strain by assuming a constant volume

A common challenge exists in assessing the true shape of a 3 dimensional objects such as a red blood cell from microscopic images. Only projected cell area can be obtained for the microscope. There is doubt that due to not accounting for the changes in cell thickness during the stretching we may severely overestimate our areal strain measurements [100]. Here we follow the suggestion of that comment about applying a constant volume constrain of the cell to proof our reported large strain values.

Figure 2.7: Viable cells (in percentage) as a function of the linear (*circle*) /areal (*square*) strain. The data has been collected from 93 cells through 12 measurements. The circles and squares are experimental data, demonstrating the viability vs. the mean value of each strain range with the error bars denoting standard deviation. The solid lines are fits to a tanh function.

Unfortunately, the absolute volume of the cell is not available from the microscopic pictures. Therefore, we have to assume some value to determine the thickness of the cell in its final (relaxed) state. During the stretching the thickness will change, but can now be estimated from the constant volume assumption. The cell volume is thus the measured projected area times the thickness, i.e. the cell shape is assumed as a right prism with a fixed volume of $98 \mu\text{m}^3$ [101]. Figure 2.8 compares the areal strain, S_v , from 14 cells using the constant volume assumption with the original data, S_o , which ignored a change in thickness. For moderate changes of the projected area, i.e. below about 35% the values of areal strain are similar. While for larger areal strain the method used in our paper is overestimating the stretching by roughly 40-50%. This analysis supports two main findings: very large areal strain can be achieved; and stretching of up to 30% if done sufficiently fast may not lead to cell death.

In summary we demonstrated the validity of our measurements within the experimental

Figure 2.8: Comparison of the areal strain from 14 cells using the constant volume assumption, S_v , with the original data, S_o , which neglected a change in thickness. The squares with error bars denote the calculated S_v vs. each measured S_o for living (*solid squares*) and ghost (*open squares*) cells. Here for each cell, S_o is measured 4 times by using different start points for snake algorithm under the same threshold as in Fig. 2.2

means of having only projected images of the 3-dimensional cell shape available. The important experimental finding that the area of cells can be stretched largely, here to about 30% with 50% survival probability, remains.

2.2.3.3 Leakage dynamics for cell rupture

It is interesting to note that cells do not rupture during or immediately after the impulsive stretching, but change to a spherical shape and rupture on a longer timescale. When the cell ruptures it leaks out the loaded dye to the environment. Therefore, next we will discuss the detailed process of the efflux of fluorescence from the fast leaking cells to gain some insight into the rupture process.

A time lapse sequence of the exemplary cell in Fig. 2.6 (b) is depicted in Fig. 2.9 (a). Please note that time $t = 0$ is now the time just after the leakage starts. The intensity within the cell declines gradually and seemingly homogeneously from $t = 0$ to $t = 7.2$ s. Upon closer inspection of the camera pictures we note that the intensity distribution has a dramatic change at time $t = 0$ which is hardly visible in the linearly scaled image. However, the 12-bit intensity resolution of the camera allows us to detect minute changes in the fluorescence surrounding the cell. Therefore we plot in Fig. 2.9 (b) the contour levels indicating the magnitude of fluorescence. Interestingly, at $t = -0.6$ s the background shows no fluorescent signal, while at time $t = 0$ the cell becomes embedded in a nonuniform fluorescent medium. Here a nonlinear mapping of the grey levels is used to emphasize the small changes of intensity outside the cell. From $t = 0$ to $t = 0.6$ s the fluorescence in the liquid medium increases slightly, and decays gradually from $t = 0.6$ s onwards to almost no fluorescent signal. It is noticeable that the fluorescence is inhomogeneous being stronger at the bottom left of the frames. This may be explained by a localized outflux through the plasma membrane, e.g. through single or multiple permanent pores.

Before discussing simulation results we detail the measured dynamics of the fluores-

Figure 2.9: Illustration of the detailed process for the leakage of fluorescence dye from the permanently porated cells shown in Fig. 2.5. The exemplary cell shown here is the one labelled by 2 in Fig. 2.5 (a). (a) Fluorescence images from experiment for the time from $t = -0.6$ to $t = 7.2$ s. The moment $t = 0$ is defined as the time just after the leakage starts. (b) Contour images of the experimental column in Fig. 2.9 (a) with a colour bar indicating the contour levels for intensity/concentration. Brighter colour indicates higher fluorescence intensity/concentration. The contour levels are set as 0, 0.05, 0.1, 0.3, 0.5, 0.7, 0.9, 1. (c) Contour images from the three-dimensional diffusion simulation with an effective pore diameter of $1.6 \mu\text{m}$. The same colour coding is used as in Fig. 2.9(b). (d) The relative mean fluorescence intensity/concentration as a function of time inside and surrounding the cell from the experiment (*solid squares*) and the simulation (*open circles*). The solid line is a fit of the experimental data inside the cell to an exponential decay $c = 1.028 \times \exp(-0.2278 \times t)$, where c is the relative mean concentration, t is the time (s). The pore size for simulation is of $1.6 \mu\text{m}$ diameter.

cence intensity inside and surrounding the cell: the time dependent relative mean intensity inside and outside the cell is depicted in Fig. 2.9 (d). Here the summed intensity within the cell is normalized (at $t = 0$) and plotted with squares; the emission surrounding the cell is summed over the area shown in Fig. 2.9 (b). We find the summed intensity within the cell follows an exponential decay over the timespan from $t = 0$ to $t = 7.2$ s, see the solid line. In contrast the intensity outside the cell (*squares*) is 0 before $t = 0$, reaches a maximum around $t = 0.6$ s, and then decreases gradually. The intensity may be related linearly to the dye concentration which allows us to compare the images with a diffusion model. To cater for this we have corrected all presented intensities for fluorescence bleaching, e.g. see Fig. 2.5 (c).

Besides, it is noticeable that from $t = -0.6$ s to $t = 0$ there is a rapid increase of fluorescence intensity surrounding the cell. This burst-like change is much faster than that from $t = 0$ to $t = 7.2$ s, see Fig. 2.9 (a), (b), (d). The spatial and temporal change in fluorescence intensity from $t = 0$ to $t = 7.2$ s seems to follow diffusion transport.

This transport is now tested with a simple diffusion model:

$$\partial c(\mathbf{r}, t) / \partial t = D \nabla^2 c(\mathbf{r}, t) \quad (2.2)$$

where $c(\mathbf{r}, t)$ is the dye concentration given in mol/m³ and D is the diffusion coefficient. For simplicity we assume a constant diffusion coefficient and fix it to $D = 2.2 \times 10^{-11}$ m²/s; this value is reported for calcein in PBS [102].

The diffusion transport of membrane impermeable dye calcein from the cell must occur through an opening in the plasma membrane. It has been reported that laser induced cavitation bubbles generate nanometre to micrometre sized pores [66]. Once single or multiple permanent pores in the plasma membrane are formed the calcein dye can diffuse into the extracellular medium. Therefore, the above equation is solved with a finite element solver (Comsol Multiphysics 3.4) in three dimensions where a single pore is

modelled with a variable circular opening in the spherical cell membrane.

Previously, a simple spherical diffusion model has been reported where a homogeneous leakiness of the membrane is assumed [102]. The numerical integration gives as expected a similar result. Here the finite element method allows us to include the confined geometry of the microchannel and test different pore sizes and distributions.

We study the effect of the circular pore diameter on the rate of the diffusion to compare with experiments. We understand that this approach is largely simplifying the dynamic process, i.e. the diffusion constant D may not be homogeneous in space or constant in time [103] and the pore may change location and size. The area of the simulated circular pore has to be understood as an effective area for the diffusion through multiple pores in the plasma membrane. More details about the simulation will be shown in the next section.

The simulation results with an effective pore of $1.6\mu\text{m}$ in diameter are presented in Fig. 2.9 (c) and (d) (*circles*) for comparison with the experimental data from $t = 0$ to $t = 7.2\text{s}$. Assuming that at time $t = 0$ after the pore is formed (at the lower left part of the cell), the internal concentration should follow the simulation. Indeed the good agreements within the cells both in contour images and intensity change indicate that the dye leakage during this period is driven by diffusion. The change of the concentration outside the cell follows similar trends as experiment during this time span. However, The absolute magnitudes and distributions of concentration surrounding the cell in the simulation are much smaller than seen in the experiment (from $t = 0$ to $t = 1.2\text{s}$), see Fig. 2.9 (b)-(d). This is because initially there is a rapid increase of dye outside the cell (burst-like efflux) in the experiment (from $t = -0.6\text{s}$ to $t = 0$). Therefore, the whole leakage dynamics cannot be explained with a diffusion limited process. We assume that another mechanism is responsible for the initial dye transport while diffusion dominates the later slower transport, see the discussion section.

Furthermore, it is also interesting to discuss the time-dependent inhomogeneous inten-

sity distribution for the cell leakage from Fig. 2.9. Fig. 2.10 (a) presents the summed intensity along the line in the stronger leakage direction (as indicated in the figure inset). Before the dye leakage ($t=-0.6$ s) the difference of intensity is large between inside (distance of $0-6\text{ }\mu\text{m}$) and outside the cell (distance above $6\text{ }\mu\text{m}$). From $t=0$ the intensity outside the cell increases homogeneously along the line; for later times it remains constant in space but decreases. Fig. 2.10 (b) shows the simulation result on the change of the concentration along the line connecting the centers of the cell and the pore, see the arrow in the figure inset. The simulation and experiment within the cell match very well, while stark differences in the temporal and spatial dynamics are found outside the cell.

Similar phenomena are also observed for other cells. Fig. 2.11 (a) and (b) shows fluorescence images for a few more viable and lysed cells, respectively. The viable cells still show biconcave shapes at the end of the fluorescence recording while the lysed cells all turn to spherical shapes and undergo burst-like efflux. The contours of the fluorescence intensity for the two lysed cells marked with 1 and 2 in Fig. 2.11 (b) are depicted in Fig. 2.11 (c). We can clearly see the burst-like efflux for cell 1 from $0-0.6$ s and for cell 2 from $-0.6-0$ s.

2.2.3.4 Diffusion simulation

In this three-dimensional simulation the cell is represented by a sphere and placed at the center of the liquid volume with dimensions of $60 \times 60 \times 15\text{ }\mu\text{m}^2$, i.e. representing the microfluidic channel. The pore is modelled with a variable circular opening in the sphere. At time $t = 0$ the calcein concentration is set to a constant value of 1 inside the cell and 0 outside. For simplicity we assume a constant diffusion coefficient, yet we have conducted simulations with different diffusion constants inside and outside the cell. The diffusion equation 2.2 is solved with a finite element solver (Comsol Multiphysics 3.4) for the above confines.

For a comparison of the simulation results we account for the imaging process of the

Figure 2.10: The time-dependent inhomogeneous intensity distribution. (a) The relative concentration along the arrow in the image inset from experiment at different times: -0.6 s (*cross and solid line*), 0 s (*solid line*), 0.6 s (*dashed line*), 1.8 s (*downward-pointing triangles*), 2.4 s (*dotted line*), 3.6 s (*plus signs*), 4.2 s (*dash-dot line*), 7.2 s (*circles*). The cell shown here is the same as in Fig. 2.9. All the data above has been calibrated from fluorescence bleaching. (b) Simulation result of the relative concentration along the arrow connecting the center of the cell and the pore location as shown in the figure inset. Here the timing for each line/symbol is the same as in (a).

Figure 2.11: Supplementary data on additional 6 viable and 6 lysed cells. (a) Fluorescence images of the 6 viable cells at $t=110s$ after the stretching, which is at the end of the fluorescence recording. (b) Fluorescence images of the 6 additional lysed cells just before their burst-like efflux. The timing for each cell indicates the time lapsed after the stretching. (c) Two columns (marked with 1 and 2) demonstrating the contours of time-dependent fluorescence intensity for the lysed cells numbered as 1 and 2 in (b), respectively. Please note that $t=0$ here indicates the time just after the cell leakage starts. The colour coding is the same as in Fig. 2.9.

Figure 2.12: Simulation results from the integration over the height of the microfluidic channel. (a) The relative mean concentration inside cell $\bar{c}_i(t)$ as a function of time for different pore diameters. The symbols illustrate the simulated $\bar{c}_i(t)$ for pore diameter of 0.1 μm (*point*), 0.4 μm (*cross*), 0.7 μm (*plus*), 1.1 μm (*circle*), 2.0 μm (*square*). The solid lines denote the exponential decay fittings. (b) The inverse of the characteristic time $1/\tau$ from the exponential decay fittings for all the pore sizes from simulation (*squares*), and for the experiment (*solid circles*). (c) Semi-logarithm plot of the simulation results for the 1.6 μm diameter pore, inside (*diamond*) and outside (*circle*) the cell. The solid line denotes the exponential decay fitting inside the cell.

fluorescence intensity: The images show a bottom view integrating the intensity over the height of the microfluidic channel, i.e. $15\text{ }\mu\text{m}$. We account for this in the simulations by summing the concentration over the whole channel height. The first results are presented as a relative mean concentration, $\bar{c}(t)$ which is the normalized number of dye molecules per specific volume (the volume inside and outside the cell). Thus, the initial relative mean concentration inside the cell is $\bar{c}_i(t=0) = 1$.

First we study the effect of the circular pore diameter on the rate of the diffusion. The pore diameter is varied from 0.1 to $2\text{ }\mu\text{m}$ and the concentration change inside the cell is presented in Fig. 2.12 (a). There the simulation results are plotted with symbols and fitted to an exponential function (*solid lines*):

$$\bar{c}_i(t) = ae^{-t/\tau}, \quad (2.3)$$

where a is the concentration at time $t = 0$, and τ is the characteristic time for the exponential decay. The linearity of the diffusion equation 2.2 allows us to parameterize the process with a single timescale τ . For larger pore sizes the concentration decays faster, i.e. τ decreases. Therefore, we can use this to estimate the effective pore size for the measured leakage rate. Fig. 2.12 (b) shows the measured (*solid circles*) and simulated inverse of the characteristic time (*squares*). The effective pore diameters for the measured cells shown here are around $1.4\text{--}2\text{ }\mu\text{m}$.

The results suggest that the effective pore diameter for the cell shown in Fig. 2.9 (a) is around $1.6\text{ }\mu\text{m}$. The mean concentration inside $\bar{c}_i(t)$ and outside the cell $\bar{c}_o(t)$ from the simulation result of such $1.6\text{ }\mu\text{m}$ pore is shown in Fig. 2.12 (c). The outside volume is not the full fluid domain but the volume from the measurement, see Fig. 2.9 (a). It shows that the concentration outside first increases and then decreases exponentially.

2.2.4 Discussion

During the fast event of bubble expansion and collapse in the microfluidic channel, a radial stretching of the RBCs is observed. Many of the stretched cells relax back to their original biconcave shapes, yet some of the cells form large tethers, see Fig. 2.4 *B (a)*. We explain this observation with some unspecific adhesion of the cell to glass surface or largely uneven shear stress. The latter may occur when some part of the cell is beneath the bubble, while the part just outside the bubble experiences a high shear stress.

Permanent poration or lysis of the cell is detected by ghosting and leakage of dye. All tethered cells ghost within a few seconds, and don't relax to their initial size and shape. A detailed analysis of more than 90 cells shows that more than 50 % can withstand a linear strain of 105 %, and an areal strain of 30 %. These cells neither ghost nor release the fluorescent load within 110 s. These critical strains are considerably larger than classic results from micropipette aspiration, of 2-3 % areal strain.

The much larger yield strength of cells in our experiments may be explained with the brief exposure time [92]. This short exposure time doesn't allow the cell to respond through a biochemical pathway during the stretching. It tests the purely mechanical properties of the plasma membrane and the underlying spectrin network.

Interestingly, when cells lyse, they don't rupture immediately after the cavitation exposure, but become spherical before they leak and 'disappear' gradually, see Fig. 2.4 *B (c)* (high speed recording) and Fig. 2.6 (fluorescence imaging). We find that the leakage occurs anytime between a few and tens of seconds after the stretching. Thus, pores large enough for calcein to be released can only be formed that much later. Also, the fluorescence pattern surrounding a leaking cell occurs within less than one second which is much faster than the diffusion (Fig. 2.9). This burst-like efflux together with the later slower process of diffusion may explain the observed leakage dynamics. The cell shape rounding and subsequent burst-like efflux may be the result of a pressure build up inside the cell followed by a rupturing of the membrane.

Therefore, we assume that another mechanism is responsible for the initial dye transport and suggest that *after the cavitation exposure small pores are generated and remain*. The details are presented as follows: Although the PBS solution is isotonic, the higher concentration of some of the cytoplasmic macromolecules, e.g. proteins such as hemoglobin molecules, leads to an osmotic pressure imbalance. Through pores with radii smaller than the Stokes radius of calcein ($0.6 - 0.7$ nm [104, 105]) water molecules or ions may diffuse into the cell leading to the swelling of the cell while the fluorescence dye calcein can not diffuse out. Once the cell volume exceeds the plasma membrane's capacity the cell bursts, i.e. cellular content is accelerated through the burst site. This rapid outflux is visible as a rapid increase in the calcein concentration outside the cell, see Fig. 2.9. Large pore(s) is/are formed at this burst site and then the slower diffusion process through the large pore(s) occurs. A schematic of this assumption is shown in Fig. 2.13. An effective diameter of $1.6\text{ }\mu\text{m}$ for this large pore is estimated from the diffusion model. The diffusion model only captures the transport of dye sufficiently after cell bursts and after the velocity of the fluid leaving the pore(s) is sufficiently low.

For this hypothesis the small pores must remain for a sufficient time. Long lived nanopores have been reported experimentally and theoretically for polymersomes [106, 107]. There the pores are stabilized by molecular brushes; a similar mechanism has been proposed for erythrocytes membranes where the glycocalyx may contribute to a steric stabilization of nanopores.

This mechanism of swelling and lysis is well known from electroporation of RBCs. There the electric field creates pores in the membrane and large cytoplasmic colloidal proteins cause swelling [108, pp. 54–55] which eventually leads to colloidal osmotic lysis [109]. In the present experiments only mechanical forces acting on the cell cause membrane poration. In contrast in electroporation electric field gradients lead to a complex scenario for which the exact mechanisms causing pores are still in debate [110].

Interestingly, cells which round up and burst show some irregular, non biconcave shape.

Figure 2.13: A schematic of the hypothesis for the initial dye transport

It is known that connections between the bilayer and the spectrin network, and the proteins binding the two-dimensional spectrin network are responsible for the biconcave morphology of the healthy RBCs [111]. Thus the irregular shape indicates that this underlying spectrin network has been mechanically violated, e.g. mechanical rupture of the spectrin dimer and/or anchor points of the spectrin with the plasma membrane.

Applying tension through micropipette aspiration or adhesion, e.g. Ref. [112], leads to a sustained static tension on the cells. These results typically probe the long time scale for membrane rupture. A failure model as proposed by Ref. [113] is not directly applicable to our experiments as the rupture event is not occurring during the stretching.

Nevertheless, failure models are frequently used to predict hemolysis, particularly when RBCs are exposed to high shear stress while passing through prosthetic heart valves. Ref. [114] and [115] reported on the hemolysis of RBCs from artificial heart valves, within ventricular assist devices, and blood pumps. Current computational fluid dynamics (CFD) simulations, e.g. Ref. [116], combine failure models with empirical power laws to predict the shear stress and exposure duration leading to immediate hemolysis. Our results agree with their CFD analysis that, blood cells in a rapidly fluctuating shear flow can sustain high shear stress levels for very short exposure time without hemolyzing significantly. This provides insight for the design of these medical devices.

2.3 Cavitation based rheology of cancer cells and numerical simulation for the flow field

2.3.1 Introduction

There is growing evidence that diseased state is accompanied with abnormalities in the cell cytoskeleton and nucleus structure, resulting in alterations of cellular mechanical properties [42]. For instance, increased deformability has been found for cancer cells

⁰The work in section 2.3 is submitted to Journal of Fluid Mechanics. This study is in a collaboration with my colleague Milad Mohammadzadeh, who is the main contributor to the numerical simulation.

with higher metastatic potential [40]. Most of the cancer death is caused by metastasis. Thus it is important to detect and diagnose metastasis of tumor cells for the treatment of cancer. Cellular deformability could potentially be used as a label-free biomarker for the metastatic potential of cancer cells [41, 42]. Moreover, as cells can be directly assayed, this physical biomarker can greatly reduce the time and cost for the traditional sample preparation, where laborious and expensive fluorescence labeling is often required.

In chapter 2.2, we have demonstrated large deformations of RBCs by using the controlled cavitation rheology (CCR). This high-throughput method seems promising to test the deformability of cancer cells as well.

Besides, the mechanism why red blood cells can be deformed by such flow field are not well understood yet. Modelling the fluid flow from the bubble oscillation in a narrow gap has been done to various degrees of sophistication. The problem can be simplified as a planar inviscid flow leading to a Rayleigh type equation in cylindrical coordinates [21]. This potential flow description has been extended to non-spherical bubbles in narrow gaps [117]. However, a notable deficiency of these approximations is their inability to model boundary layers, which are important when dealing with suspended objects near the walls, e.g. flat red blood cells and thin elastic objects such as nanowires [118].

Therefore, in this chapter we employ the CCR method to probe the deformability of cancer cells. In order to understand the resultant cell deformation, we conduct numerical simulations to reveal the flow pattern, especially the boundary layer generated by the bubble oscillation.

2.3.2 Materials and Methods

2.3.2.1 Cell samples preparation

Two types of cells in suspension are used in the experiments: the biconcave red blood cells (RBCs) with a diameter of 6 to $8\mu\text{m}$ and thickness of 1 to $2\mu\text{m}$, and the spherical colon carcinoma cells (RKO) with a diameter of 10 to $15\mu\text{m}$.

The following protocols are used for preparation of the cell samples. Fresh RBCs are drawn from healthy donors and washed three times with $1\times$ PBS. The cell solution is diluted $100\times$ in $1\times$ PBS containing 0.1 % bovine serum albumin and then ready for experiment. Carcinoma cell line RKO is seeded in petri dish and kept routinely in culture (37°C and 5 % CO_2). Around 90 % confluence, they are split with trypsin-EDTA and diluted $200\times$ in $1\times$ PBS containing 0.1 % bovine serum albumin. Both cell lines are applied for experiments within a few hours.

2.3.2.2 Experimental set up

The prepared cell suspensions are applied to a microchamber with a gap height of $20\mu\text{m}$. The microfluidic gap is made of two fastened microscope cover slides ($25 \times 40\text{ mm}^2$) with flattened aluminum foils as spacers. A frequency doubled Nd:YAG laser is used to focus a laser pulse (6 ns duration with a wavelength of 532 nm) into the cell solution confined within the microfluidic gap [71]. Due to a higher density, the cells settle at the lower cover slip. Nonlinear absorption of the focused and intense laser pulse in the liquid results in an optical breakdown and the formation of a plasma. Upon recombination an explosively expanding vapour bubble is created which quickly fills the gap.

The events are captured with high-speed photography from bottom view through the inverted microscope. The single bubble in the center of the channel, surrounded by cells in the liquid, shows a radial expansion and collapse from the bottom view. As a result of the fast dynamics, with velocities up to several meters per second, the surrounding cells are transported and deformed. For more details of the experimental setup, please refer to Ref. [71, 119]

2.3.3 Experimental result

To check if the method can be applied to cancer cells as well, we conduct experiments for red blood cells (RBCs) and colon cancer cell line RKO cells under the same experimental

Figure 2.14: Deformation of RBCs and RKO cells due to the cavitation bubble induced flow in a $20\text{ }\mu\text{m}$ thin gap. (a) The top image shows RBCs just before the laser-induced cavitation bubble and at the bottom are the cells just after the bubble collapses. (b) Colon cancer cells (RKO cells) exposed to a similar flow just before and after the cavitation bubble oscillation. RKO cells are much less deformed compared to RBCs (see text). In both cases, the center images are schematics of the bubble shape and cell geometry from a side view.

condition.

One example of the many experiments is shown in Fig. 2.14. Fig. 2.14 a shows a typical configuration of RBCs shortly before (*top*) and after the bubble oscillation (*bottom*) from the bottom view. RBCs near the bubble are largely stretched. However, RKO cells show very little deformation under similar flow condition, see Fig. 2.14 b. This is also observed in other repeated tests.

In the recent studies of cell stretching utilizing viscoelastic particle focusing, RBCs have an averaged elongation index of about 1.3 compared to around 0.3 for human mesenchymal stem cells (hMSCs) [120]. The elongation of RBCs from their experiments is of similar range as compared to our method. Additionally, it has been reported recently that tumor-initiating cells possess a stem-cell like deformability [121]. Therefore, we infer that RKO cells should be more rigid than RBCs. The mesenchymal cell line RKO is nevertheless quite deformable, see Fig. 3.2 and section 3.3.2.1 in the following chapter. However, with the current bubble method, their deformation can be hardly observed and analyzed. As our objective is to correlate the deformability with the metastasis of cancer

cells, we need to differentiate the rigidity of different cancer cell lines, especially between circulating tumor cells and the primary tumor cells. The result from the bubble method obviously cannot serve this purpose.

Nevertheless, we would like to understand why the bubble method cannot be applied to cancer cells. From the experiments, we didn't observe adhesion of the RKO cells to the bottom wall. Actually they are translated with the bubble induced flow. The experimental condition is similar except that the dimensions of the cells are different. RBCs are thin, bi-concave cells with a diameter of about $8\mu\text{m}$ and a thickness of less than $2\mu\text{m}$. After they are loaded inside the gap they sediment to the bottom because of their higher density than the surrounding fluid (saline buffer solution). In contrast, RKO cells have a spherical shape with a diameter of $10 - 15\mu\text{m}$ and therefore span a large part of the microfluidic gap. Thus we suspect that the flow field especially the boundary layer flow is responsible for this difference. In order to reveal the flow field caused by the bubble, we conduct numerical simulation which will be discussed in the following sections.

2.3.4 Numerical simulation

2.3.4.1 Computational domain and boundary conditions

The problem being modeled is the expansion and shrinkage of an initially spherical bubble, created by a focused, high-power laser pulse. The bubble is located at the center and between two parallel discs separated by a gap of height h . We assume axisymmetry and only model the upper quarter of the gap, i.e. we utilize symmetry to reduce computational cost, see Fig. 2.15.

The complex physical process of laser-matter interaction [122] leading to a rapidly expanding bubble is greatly simplified by starting the simulation with a bubble of finite size, filled with non-condensable gas at high pressure, similar to previous work [123, 124].

The boundary conditions as depicted in Fig. 2.15 are along $\overline{DA}$ axis of symmetry,

Figure 2.15: Sketch of the computational domain using axisymmetry and symmetry with respect to the center-plane of the gap.

between $\overline{AB}$ symmetry, at $\overline{BC}$ constant pressure p_0 , and no slip at $\overline{CD}$.

The initial pressure in the gas bubble of $R(t = 0) = 5 \mu\text{m}$ is 100 bar. The liquid is initially at atmospheric pressure $p_0 = 1$ bar. The liquid is water with a density of $\rho_l = 998.2 \text{ kg m}^{-3}$ and a dynamic viscosity of $\mu_l = 0.001 \text{ Pa}\cdot\text{s}$ while the gas viscosity is $\mu_b = 1.34 \cdot 10^{-5} \text{ Pa}\cdot\text{s}$, corresponding to water vapour. An ideal gas law for calculation of density is used for the compressible bubble content. The interfacial tension coefficient is $\gamma = 0.072 \text{ N/m}$.

2.3.4.2 Computational Method

The multiphase flow problem of the compressible gas and the incompressible liquid is modelled with volume of fluid (VOF) method accounting for interfacial tension but neglecting body forces using ANSYS Fluent 14.0 [125]. In the VOF method, a single set of momentum equations is solved for all phases, meaning that the velocity field is shared among all present phases, and the volume fraction of each phase is tracked throughout the domain. The momentum equation can be expressed as

$$\frac{\partial}{\partial t}(\rho \vec{v}) + \nabla \cdot (\rho \vec{v} \vec{v}) = -\nabla p + \nabla \cdot (\mu (\nabla \vec{v} + \nabla \vec{v}^T)) + \vec{F} \quad , \quad (2.4)$$

where ρ is the density of the phases, $\vec{v}$ is the velocity, p the pressure, and $\vec{F}$ is the surface force. The surface force is modelled as a continuum surface force (CSF) as proposed by [126]. In ANSYS Fluent, the surface curvature is calculated from local gradients in the

surface normal at the interface of phases. In solving the governing equations, the material properties, such as density, ρ , or viscosity, μ , are calculated as volume-fraction-averaged properties.

Interface tracking is done by coupling the volume of fluid with the level-set method [127, 128]. This allows for accurate interface tracking as well as mass conservation, in spite of the large density difference between the bubble content and the liquid.

An absolute convergence criteria of 10^{-6} is used for all governing equations and time step size of the simulation is 10^{-8} s. To assure the solution is mesh-independent, the simulations were conducted with two different grids, 5000 and 80000 elements, with an average of 0.4 and $0.1\mu\text{m}$ element size respectively. The difference of the solutions obtained by the two grids is negligible, therefore grid independence is indeed obtained. The results reported in this manuscript are for $0.1\mu\text{m}$ element size, while the mesh is refined for regions of high gradient, such as initial bubble-liquid interface and the vicinity of channel wall. The simulation time on a 2.1 GHz Intel Core i7 personal computer with 8 GB of RAM is approximately 24 hours for a single cycle of bubble expansion and shrinkage.

2.3.4.3 Bubble evolution and liquid velocity profile

Fig. 2.16 depicts the temporal evolution of the bubble shape over a period of $10\mu\text{s}$ in a gap of $h = 20\mu\text{m}$. The spherical bubble quickly grows into a pancake shape, forming thin liquid films at the upper and lower solid walls. Maximum bubble radius is obtained after $4\mu\text{s}$. During bubble shrinkage, the convex interface flattens and only increases curvature after $8\mu\text{s}$; that is when the internal pressure increases and dampens the collapse of the bubble. The bubble collapses to its minimum volume at $t = 10\mu\text{s}$ and rebounds afterwards (not shown here). Right to the bubble profile in Fig. 2.16 the radial velocity profile at a distance of $r = 50\mu\text{m}$ from the bubble center is shown. Initially, the liquid is at rest and develops into a plug flow with strong wall shear stress. Gradually, a more

Figure 2.16: CFD results of the bubble shape evolution every $0.5 \mu\text{s}$ together with the radial liquid velocity profile at $r = 50 \mu\text{m}$ from the center of the bubble. Please note that the reversal of the flow direction near the boundaries occurring earlier than that in the center of the gap.

parabolic profile develops. At the later expansion stage the velocity near the walls is reduced and even reversed in direction, while the velocity in the center of the gap is still outwards and positive. This flow reversal near the boundaries will be discussed in detail in the following sections. The flow reversal becomes more pronounced during bubble collapse.

Fig. 2.17 shows the projected bubble radius from Fig. 2.16 and compares it with the experimentally determined radii [129] and a 2-dimensional Rayleigh-Plesset equation, e.g. see [21]. For the chosen initial condition, i.e. radius $R(t = 0)$ and initial gas pressure, we find a good agreement between the VOF simulation and the experiment. In particular, the asymmetry of the bubble oscillation having a faster expansion than the collapse is captured in the VOF simulation. Interestingly, this asymmetry was attributed previously and in a different geometry to thermal effects [130] while the present calculations ignore thermal effects. Thus for the present case the asymmetry is a result of viscosity, i.e. the formation of boundary layers. In contrast, the inviscid Rayleigh-Plesset model also plotted in Fig. 2.17 is symmetric in time.

The late stage of bubble collapse cannot be captured with the VOF model as it as-

Figure 2.17: Comparison of experimental bubble radial dynamics [129] (*filled squares*) with the VOF solution (*filled circles*). Additionally, the solution to the 2D Rayleigh-Plesset equation starting from the maximum bubble radius is shown with a dashed line.

sumes that the liquid and bubble content are immiscible, while in experiments the laser generated bubble consists mainly of condensible vapour. Thus, our simulation predicts a milder collapse with re-expansion of the bubble. However, the experiments find a much smaller minimum bubble radius followed by fragmentation of the bubble.

2.3.4.4 Flow reversal due to adverse pressure gradient

The evolution of pressure in the channel and the liquid velocity profile at some distance from the bubble center are shown in Fig. 2.18; for clarity we have divided the results from left to right for the four different stages: early expansion, late expansion, shrinkage, and rebound. The upper frames in Fig. 2.18 show the unsteady pressure in the center of the channel from $r = 0$ to the outlet, i.e. $r = 80 \mu\text{m}$. The axial pressure gradient is negligible in comparison to the great pressure variation in the radial direction. The gas-liquid interface can be easily identified by the small pressure jump due to surface

Figure 2.18: Evolution of pressure (*top row*) and radial velocity (*bottom row*) during early (a) and late expansion (b) and early (c) and late collapse (d). The times for the pressure and velocity profiles are indicated in the upper legend of each column.

tension. The lower frames in Fig. 2.18 show the velocity profile in the liquid at a fixed distance of $r = 50 \mu\text{m}$ from the bubble center.

During the first stage, Fig. 2.18 a, $0 < t < 1.5 \mu\text{s}$, the gas pressure initially at 100 bar is accelerating the liquid outwards from zero velocity to almost 7 m/s. In consequence, the gas pressure drops within $1.5 \mu\text{s}$ below the pressure at the outlet. During this time a flat-top velocity profile develops.

The second stage, Fig. 2.18 b, corresponds to the deceleration of the flow to the maximum bubble volume at $t = 4.3 \mu\text{s}$. As the pressure in the bubble drops, an adverse pressure gradient develops ($\partial p / \partial r > 0$). This leads to detachment of the boundary layer and a reversed flow at the boundary sets in. This flow reversal is clearly visible at $t = 4 \mu\text{s}$ in the lower frame of Fig. 2.18 b. At this stage the flow profile possesses an inflection point; the liquid in the center of the channel continues to flow towards positive r while at the boundaries the flow is directed towards the bubble.

In third stage, Fig. 2.18 c, the bubble shrinks, i.e. a net flow towards the bubble sets in and eventually a purely negative velocity profile builds up. In this stage the pressure gradient is stabilizing the boundary layer and the pressure in the bubble steadily builds up.

In the last stage the bubble reaches minimum volume and rebounds, Fig. 2.18 d, $8 < t < 10 \mu\text{s}$. Similar to stage one, the internal bubble pressure is higher than the liquid pressure, but here the liquid flow is toward the bubble. Therefore, the pressure gradient once again opposes the liquid flow and leads to flow reversal near the channel walls. The flow could be described similar to stage two, but with opposite signs ($\partial p / \partial r < 0$). The liquid flow is eventually reversed at the walls in $t = 10 \mu\text{s}$, minimum bubble volume is reached, and the bubble begins to rebound.

Figure 2.19: Plot of the liquid streamlines during boundary layer separation, i.e. from $t = 3$ to $5.5 \mu\text{s}$. The radial velocity profile is plotted at $r = 50 \mu\text{m}$. Two counter rotating vortices form, move upwards and recombine.

2.3.4.5 Vorticity generation

Fig. 2.19 depicts the instantaneous streamlines during the build up and decay of the adverse pressure gradient together with a radial velocity profile at $r = 50 \mu\text{m}$, corresponding to the late stage of bubble expansion in Fig. 2.18 b. At time $t = 3.5 \mu\text{s}$ formation of vortices near the channel walls is observed. From $t = 3$ to $4.5 \mu\text{s}$ the adverse pressure gradient supports the detachment of the boundary layer, leading to a recirculating flow. Due to symmetry two vortex rings are formed. The vortices are transported towards the center of the channel. As both vortex rings migrate towards the center of the channel they merge, see Fig. 2.19, $t = 5.0$ and $5.5 \mu\text{s}$. From then on, the radial flow and the pressure gradient are aligned, stabilising the boundary layer.

2.3.5 Discussion

Utilizing a viscous model in the present study, we find that during the late expansion of a cavitation bubble in a narrow axisymmetric gap, the adverse pressure gradient leads to boundary layer separation and flow reversal in the proximity of the walls. A prominent result of the observed flow reversal and vorticity is generation of strong shear stress in the liquid close to the boundaries. The results indicate that some flows created by oscillating bubbles in narrow gaps may not be accurately described with inviscid potential flow in cylindrical symmetry. Particularly, in studying the mixing/emulsification of flows or the deformation of elastic objects, such as yeast cells [131], it is necessary to account for the vorticity generation and transport.

The numerical simulation suggests that deformable objects located within the boundary layer and close to the bubble may be stretched considerably by the shearing flow. This was indeed observed for red blood cells in the experimental studies. Since the thickness of RBCs is less than $2 \mu\text{m}$ they are located close to the bottom channel wall and are of comparable size as the vortex. After being initially translated away from the bubble center, during the late stage of expansion and the onset of shrinkage the RBCs are ex-

posed to a shearing force from the vortices near the wall. From the simulation results, neglecting the presence of cells, we obtain values of about $2 \cdot 10^6 \text{ s}^{-1}$ for the magnitude of the shear strain rate, $1/2(\partial v_r/\partial z + \partial v_z/\partial r)$, near the walls, where the RBCs are located. This can be related to a stretching of a fluid particle assuming a characteristic height and duration. Inserting $2 \mu\text{m}$ and a duration of $2 \mu\text{s}$ we obtain a length increase of $8 \mu\text{m}$, a value which is of the order of the observed stretching in experiments, e.g. [71].

To the contrary, the cells extending into the center of the channel, such as the RKO cells, will be advected with the flow. The shear stresses outside the boundary layer are considerably lower and therefore lead to weaker deformation. We find about 10% strain at the center of the channel over a thickness of $1 \mu\text{m}$. The resulting deformation of the cell may be well within the resolution limit of the imaging optics.

2.4 Conclusion

In summary, the impulsive forcing of a single laser-induced cavitation bubble is used to quantify the yield strain for RBC membrane rupture. We used a microfluidic confinement to obtain an impulse-like and sufficiently strong shear flow. The probability of cell survival is a function of the maximum strain. We find a critical areal strain of approx. 30 % where 50 % of the cells show membrane rupture. This areal strain is much larger than the classic result of 2-3 % found in micropipette aspiration [90]. Membrane poration and lysis is observed after cavitation exposure by detecting the efflux of the cytosol fluorophore calcein. We find that cells don't rupture during or immediately after the cavitation event, but turn gradually into a spherical shape before their burst-like efflux and the later slower diffusive leakage. These observations suggest that lysis of RBCs through mechanical forces is a two-step process:

First the membrane is violated through the stretching with the formation of sub-nanometre sized pores which are smaller than the Stokes radius of calcein. Second, colloidal osmotic swelling sets in until the cell bursts, which then releases the calcein

load through diffusion.

Despite the observed large deformation of RBCs, very little deformation is induced for tumor cells RKO by the cavitation method. CFD simulations reveal an adverse pressure gradient during the late expansion of the cavitation bubble in the narrow gap. This leads to boundary layer separation and flow reversal in the proximity of the walls, resulting in generation of strong shear stress in the liquid close to the boundaries. Thus only when deformable objects are located within this boundary layer and close to the bubble, they may become stretched considerably by this shearing flow. This was indeed observed in the experimental studies for RBCs and in contrast larger spherical RKO cells are mainly transported by the flow.

3 Correlation of cellular deformability with epithelial-to-mesenchymal transition and the effect of chemotherapy drug

3.1 Introduction

Invasion of cancer cells into the blood stream is an essential step for their metastatic spread from primary tumour to distant organ [52]. Recent studies suggest that the epithelial-to-mesenchymal transition (EMT) might enable metastatic dissemination [132, 133, 134]. EMT is a fundamental programme for embryonic development and differentiation of tissues/organs, where conversions of epithelial cells to mesenchymal cells occur. However, EMT can also promote tumour progression with the phenotypic changes to more migratory and invasive cells.

Biomechanical properties such as deformability have been shown to correlate with the invasion potential of cancer cells [54, 40]. However, the correlation of EMT with deformability of cancer cells has not been studied yet. Moreover, the effect of genetic

⁰The work shown in this chapter was conducted in a collaboration with Dr Igor Cima, Ms. Jess Vo Thu Hong Anh and Dr Min-Han Tan from Institute of Bioengineering and Nanotechnology, Singapore. My work is mainly on the part of microfluidic hydrodynamic stretching, e.g. design, fabrication of the device, operation of experiment, automated image processing, and data analysis. It includes cell culturing and preparation of RKO, HCT116 and PFA-treated RKO cells. All the other biological parts and the microsiege experiment were performed by the collaborators.

alterations and intracellular signalling on cell deformability has not been elucidated.

Additionally, there is growing interest in relationship between pharmacology and cellular stiffness [135, 42]. The cell deformability might serve as a label-free biomarker for testing the efficacy of pharmaceutical drugs, especially for cancer treatment.

To study cellular deformability of cancer cells, we first attempted using the method of controlled cavitation rheology (CCR). However, as mentioned in Chapter 2.3, very little cellular deformation can be induced due to the cell geometry (spherical shape in suspension).

Recently, an alternative method has been demonstrated by Dino Di Carlo's group. There, cells are delivered to the center of a microfluidic cross junction by inertial focusing and are stretched by the extensional flow [4]. Cellular deformations are recorded with a high speed camera, and data is analysed through automated image processing. The high throughput and automation of analysis allow them to rapidly link cellular deformability with various phenotypes at higher statistical significance than established methods such as atomic force microscopy. Using this method, Dino Di Carlo's group demonstrated differential deformability between native populations of leukocytes and malignant cells in pleural effusions, and accurately predicted disease state in patients with cancer and immune activation. Their work paved the way for large population mechanical phenotyping.

However, more specific biological studies such as the correlation of EMT and the effect of pharmaceutical drugs with deformability of cancer cells have not been performed with this approach. Therefore, we follow and improve this method to address the above biological issues. The first improvement is a better image quality (e.g. sharp cell edge) to resolve simultaneously the trajectory, velocity, and deformation of individual cells using our home-built image-processing algorithm (see Fig. 3.2). A second improvement is the real time cell detection using a second imaging of the cell onto a photodiode which triggers the high-speed image recording, see the materials and methods section. This

not only significantly reduces the data size and time of recording but also improves the downstream analysis, e.g. for cell separation.

Besides, we also correlate/compare the results from this method with those from the recently developed microsieve device, which has been reported for rapid isolation of circulating tumour cells from whole blood samples [136]. The hydrodynamic stretching method is capable of obtaining both cell deformability and size distribution. This can help unravel the separation mechanism for a specific cell sample when using the microsieve method, e.g. difference in size or deformability, which can in turn help choose the right pore size for the separation.

3.2 Materials and methods

3.2.1 Microfluidic device and stretching extensional flow method

We use the standard soft lithography to fabricate the polydimethylsiloxane (PDMS) microfluidic device. The homemade device is shown in Fig. 3.1 A. As the input flow rate is around several hundreds $\mu\text{L}/\text{min}$, we glue the inlet with a fast setting epoxy adhesive (Araldite) to avoid liquid leakage. The design for the above microchannel arrays is shown in Fig. 3.1 B. An enlarged schematic of the individual microchannel is shown in Fig. 3.1 C. An array of micro-filters is designed after the inlet to block cell aggregates or other debris to avoid clogging the channel. Asymmetric curvatures are used for inertial focusing of the cells towards the channel center. The cell solution comes from both directions to the stretching region in the channel center, a schematic of which is shown in Fig. 3.1 D. Cells approaching the stagnation point will experience an elongational flow and will be stretched normal to the flow direction. As the flow is symmetric, cells can reach the stretching region from below and above (see Fig. 3.1 D). Cells leave the stretching region through one of the two outlets (left and right) where they quickly recover their shape. The channel height is $26\mu\text{m}$ and the channel width over a distance of about $750\mu\text{m}$

Figure 3.1: The microfluidic device and the working principle. (A) A photograph of the fabricated microfluidic device. It contains arrays of independent microchannels for cell stretching. The width of the whole PDMS device is 50 mm. (B) The design of the microfluidic device shown in (A). The outline is of the same size as the glass substrate ($75 \times 50 \text{ mm}^2$) bonded with the PDMS channels. (C) An enlarged schematic of the individual microchannel (channel height = $26 \mu\text{m}$) as enclosed by the dashed box in (B). The channel consists of one inlet, two outlets, and a filtering region for cell aggregates and dirt. The flow comes in both directions to the central stretching region (marked by the dashed box). Cells are focused to the channel center line with the inertial focusing curvatures and get deformed at the stretching region. (D) A schematic for the stretching principle at the channel center as enclosed by the dashed box in (C).

before and after the stretching region is $67\text{ }\mu\text{m}$.

3.2.2 Microsieve method

In this method, cell solution is pumped to pass through a densely packed pore arrays within a silicon microsieve. For a specific pore size, smaller or more flexible cells can pass through the microsieve while larger or more rigid cells will be rejected. For more details about the method, please refer to Ref. [136].

3.2.3 Cell culture and preparation

3.2.3.1 HCT116, RKO and PFA treated RKO

Two kinds of human colon carcinoma cell line HCT116 and RKO are seeded separately in petri dishes with the culture medium (DMEM with 10 % (v/v) fetal bovine serum and 1 % (v/v) penicillin/streptomycin) and kept routinely in culture (37°C and 5 % CO_2). Around 90 % confluence, they are split with 0.25 % trypsin/EDTA, and then diluted in fresh culture medium at a ratio of 1:10 to 1:20 (e.g., $500\text{ }\mu\text{L}$ to 5 mL or 10 mL). The cell solution is gently transferred to a 5 mL plastic syringe (BD Bioscience) immediately before the experiment.

To stiffen the RKO cells, we use 4 % paraformaldehyde (PFA) in $1\times$ PBS (sterile filtered). PFA is a common fixative that is used to preserve cell structure. Basically, it creates covalent chemical bonds between proteins and this anchors soluble proteins to the cytoskeleton, thus, making the cells rigid. The reagent is added to the RKO cell solution (in fresh culture medium) at a final concentration of 2 %. Then the cells are incubated overnight at 4°C . After that the cells are centrifuged and resuspended in culture medium for experiments.

3.2.3.2 MCF10A wildtype and *TP53* knockout

MCF10A cells are immortalized mammary epithelial cells. Parental MCF10A cells (wildtype) are obtained from ATCC (Catalog number CRL-10317). MCF10A *TP53* knockout cells are from Sigma (Catalog number CLLS1049) and cultured using manufacturer instructions.

3.2.3.3 A549 treated with Cytochalasin D and Docetaxel

A549 cells (Adenocarcinomic Human Alveolar Basal Epithelial Cells) treated with Cytochalasin D (mycotoxins) ($10\ \mu\text{M}$, $100\ \mu\text{M}$), docetaxel (a chemotherapy drug) (5 , 10 , $50\ \text{nM}$) and untreated control cells are prepared for mechanical microfluidic hydrodynamic stretching flow. For the reagents, Cytochalasin D inhibits polymerization and induces depolymerization of actin filaments while docetaxel inhibits microtubule depolymerization and is assumed to induce cellular stiffness.

3.2.4 Experimental operation for hydrodynamic stretching

A blunt needle tip (SH23-B, SAN-EI Tech Asia Pte Ltd) is connected to the syringe loaded with cell solution and a microbore tubing (Tygon ND-100-80) with an inner diameter of $0.508\ \text{mm}$ and an outer diameter of $1.524\ \text{mm}$ is plugged to the needle tips for the syringe and the channel inlet. The channel outlets are plugged with 90 degree bend blunt needles (SH23-B-90, SAN-EI Tech Asia Pte Ltd) and connected to a waste reservoir with two tubings of equal lengths. The syringe is placed in an infusion pump (KDS200, KD Scientific) at an optimized flow rate of $500\ \mu\text{L}/\text{min}$.

The microfluidic device is placed on an inverted microscope (IX-71, Olympus) and magnified with a water immersion objective ($20\times/0.50\ \text{NA}$, Olympus). A high speed camera (Photron SA-X) is connected to the trinocular through a $0.5\times$ demagnifying C-mount. A photodiode (DET 10A/M, Thorlabs) is placed at the image plane of the microscope side port and is connected to an oscilloscope (Lecroy, wave runner 64 Xi-A).

The sensor of the photodiode is aligned with the image of the channel center (stretching region) shown in 3.1 D. When a cell passes through the stretching region, there is small fluctuation of the transmitted light intensity at the cell location. This can be detected by the photodiode and sent to the oscilloscope, which uses the signal to trigger the high speed camera for image capturing. Prior to that, the camera is set to the center mode so that it will capture images both before and after the trigger. Using this we can trigger the high-speed camera on the passing of cells and greatly reduce the file size of the image recording, i.e. this allowed us to only sample the large number of cells shown below within a reasonable time.

After the syringe is set to infuse cell solution, we wait 15-20 s for the flow field to reach a steady state and then start the image recording at a frame rate of 180,000 fps and an exposure time of 293 ns. The size of the view field is $512 \times 208 \mu\text{m}$.

3.2.5 Image processing and data analysis

We use a home-built Matlab script to process the above recorded images. To show the details of the image processing and data analysis, we illustrate an example of a single RKO cell. As shown in 3.2 A, the cell is progressively elongated when it travels to the extensional stretching region (0-105.6 μs). After that it flows toward the left outlet and the elongated shape recovers gradually (161.1-255.6 μs). To trace the cell, the current image is subtracted from an image frame without cells (background) and then the cell edge is found using thresholding, see 3.2 B (*top image*). In this way, we can roughly estimate the cell centroid through shape characterization of the detected cell edge. Thus we can get the cell trajectory in the cross channel, see 3.2 B (*middle image*). After the cell centroid is found, we can define a circular bounding box (*blue*) around the cell and then use the active contour method as mentioned in chapter 2.2 to obtain rather precise contour of the cell shape (*red*), see 3.2 B (*bottom image*). Once the cell contour is obtained, the shape can be characterized by their center of mass (a more precise centroid),

major (a) and minor (b) semi-axis.

From the above obtained cell centroids, the averaged interframe cell velocity can be calculated. As shown in the top figure of Fig. 3.2 C, the velocity decreases to a minimum when the cell travels to the center of the stretching region. When the cell leaves from the extensional stretching region to the side channel the velocity increases gradually to a constant value. The corresponding temporal evolution of the semi-axis is shown in the bottom figure of Fig. 3.2 C. Around 0-100 μs , the length of the major semi-axis progressively increases while that of the minor semi-axis decreases, leading to an elongation of the cell. Later it is the other way around, resulting in a shape recovery of the cell. The length ratio of the major to minor semi-axis (a/b) is shown in Fig. 3.2 D (*top*). It reaches a maximum value around 100 μs when the cell is around the cross channel center. Therefore, we use $(a/b)_{\text{max}}$ to define the cell deformability. Similar to previous work [4], we get statistic result of 298 RKO cells and put them into a density scatter plot (see Fig. 3.2 D (*bottom*)), where d_0 is the averaged diameter when a/b is minimum.

We omit cells which are either too large ($\geq 26 \mu\text{m}$) or too small ($\leq 10 \mu\text{m}$) for the microchannel except for the A549 case. Besides, the velocity, especially its minimum value is used as a criterion for the quality control of the inertial focusing and subsequent stretching. We reject data in which the cell is poorly focused and stays not long enough to reach a certain low velocity within the stretching flow.

3.3 Results and discussion

3.3.1 Control experiment and validation of measurements

To validate the operation and measurements of the microfluidic device, we perform a control experiment comparing the deformability of non-treated RKO cells with that of PFA treated RKO cells. The results are shown in Fig. 3.3. Differential deformability

Figure 3.2: Illustration of image processing and data analysis with exemplary RKO cells. (A) Left column: selected image sequences for the exemplary cell traveling through the extensional flow region. Right column: enlarged images for the cell in the left column. (B) Image processing: cell detection with background subtraction and thresholding (*top image*); trajectory of cell centroid across the channel (*middle image*); snake (active contour) method for extraction of cell contour, major (*a*) and minor (*b*) semi-axis (*bottom image*). Here all the numbers indicate the pixel numbers in the images. (C) Extracted data for the temporal evolution of the cell velocity (*top figure*) as well as its major (*blue*) and minor (*green*) semi-axis (*bottom figure*). (D) Temporal evolution of the ratio between the major and minor semi-axis (a/b) (*top figure*); statistic result for the deformability of 298 cells vs. their relaxation diameter d_0 using the density scatter plot (*bottom figure*). A hotter color (e.g. red color) indicates a higher data density. The deformability is defined as the length of the long axis of a deformed cell (major axis, $2a$) divided by a perpendicular shorter axis (minor axis, $2b$). Here we use the maximum value of a/b as the cell deformability while d_0 is the averaged diameter when a/b is minimum.

Figure 3.3: Control experiment for measuring the deformability of RKO and PFA treated RKO. (A) Density scatter plot for the measured deformability from the microfluidic hydrodynamic stretching. The dashed lines indicate the median deformability. (B) Averaged number of cells flowing through the microsieve per run for both non-treated (control) and PFA treated RKO with the same input number of cells.

between RKO and PFA treated RKO is observed from the microfluidic hydrodynamic stretching. The PFA treated RKO cells have a significant lower deformability, which is consistent with the fixation and stiffening effects of PFA. The result is also consistent with the microsieve measurements that fewer treated cells can pass through the microsieve than the non-treated (control) ones. Above results suggest that our measurements of the cell deformability is valid.

3.3.2 Differential deformability of various cell lines and correlation with Epithelial-to-Mesenchymal transition

3.3.2.1 HCT116 and RKO

HCT116 and RKO are two different kinds of human colon carcinoma cell lines. Their sizes are different, with the average diameter of RKO larger than that of HCT116, see Fig.3.4 A. However, when putting their cell suspensions into microsieve system, lower retrieval (higher passing through) is observed for RKO cells compared to HCT116, as

Figure 3.4: Experimental results for HCT116 and RKO passing through microsieves. (A) Statistic results for the diameter of HCT116 and RKO cell lines. Cell line diameters were extracted from images of freshly trypsinized cells in suspension using the software ImageJ. The cell images are taken from an inverted microscope (Olympus IX81) in bright field modus (B) Left: microsieves with a pore diameter of 7, 8, and 9 μm . Right: Retrieval (normalized as percentage in total input cells) of HCT116 and RKO cells from the above microsieves, respectively. (C) Percentage of both cell lines in input and after flowing through the microsieves at high flow rate of 1ml/min, and identified with fluorescent cell tracker. Cell tracker green and red are from Invitrogen, flow cytometry data are collected using FACS Calibur instrument from BD.

shown in Fig.3.4 B. Particularly, when the pore size of the microsieve increases to around $9\text{ }\mu\text{m}$ (below the averaged diameter for both cell lines), most of the RKO cells can pass through using an high flow rate of 1ml/min , while the majority of HCT116 cells are trapped on the microsieve. To further investigate this, we mix both cell lines together and pass them through a microsieve. Prior to that, the RKO cells are labeled with green fluorescent cell tracker while the HCT116 cells are marked with red fluorescent cell tracker. Results from flow cytometry reveal that RKO takes up 60% of the total cell input while HCT116 is 40%. However, in evaluating the cells which passed through the microsieve, the percentage of RKO increases to 92% while HCT116 decreases to 8%. The above results show that RKO can pass through the microsieve pores more easily than HCT116.

To check cellular deformability, both cell lines are tested for microfluidic hydrodynamic stretching. The results shown in Fig. 3.5 reveal a higher deformability of RKO cells, with a median deformability of 1.35 compared to 1.24 for HCT116 cells. That may explain why the RKO cells can pass through the microsieve more easily although they have a larger size as compared to HCT 116.

When taking a closer inspection of the cell morphology, as shown in Fig. 3.6, we can see clearly that HCT116 cells tend to stick to each other during growth which is a typical epithelial characteristic. In contrast, the RKO cells are loosely arranged as individual cells, and they spread to a larger extent on the petri-dish. It indicates that RKO is a mesenchymal cell phenotype. Epithelial and mesenchymal features for HCT116 and RKO have been reported previously [137]. The above results suggest that the mesenchymal cell phenotype correlate with higher deformability compared to the epithelial phenotype of cells from the same origin (colorectal cancer).

Figure 3.5: Experimental results for HCT116 and RKO from microfluidic hydrodynamic stretching. (A) Density scatter plot of the deformability for both cell lines. The dashed lines indicate the median deformability. (B) Statistic analysis (the boxes) of the above results for both cell lines. The line inside the boxes represents the median of the data, while the edges of the boxes are the 25th and 75th percentiles, and the ends of the whiskers denote 1.5 times the interquartile deviation (IQR). The plus symbols indicate the data not included between the whiskers.

Figure 3.6: Optical images from microscope for the cell morphology of HCT116 and RKO under the same culture condition.

Figure 3.7: Differential deformability between MCF10A wildtype (wt) and MCF10A KO (with the knockout of *TP53* gene) from the microfluidic hydrodynamic stretching. (A) Density scatter plot of the deformability for MCF10A *TP53* wt and MCF10A *TP53* KO. The dashed lines indicate the median deformability. (B) Statistic analysis of the above deformability in a similar way to Fig. 3.5. (C) Optical images for the cell morphologies under the same culture condition. The scale bar denotes 20 μm .

3.3.2.2 MCF10A wildtype and knockout of *TP53* gene

Differential deformability is also observed upon the knockout of the tumour suppressor *TP53* from the non-invasive MCF10A human breast cell line. As shown in Fig. 3.7 (A-B), larger deformability is observed for MCF10A with the knockout of *TP53* (*TP53* KO). The cell morphology is also changed from an epithelial feature (MCF10A *TP53* wt) to a mesenchymal feature (MCF10A *TP53* KO), see Fig. 3.7 (C).

The above results suggest that deletion of the tumour suppressor *TP53* gene can directly cause the MCF10A cells to increase the cellular deformability which might enhance the invasive potential of the cells. As in RKO and HCT116 cell lines, this correlates with cellular morphologies reminiscent of epithelial to mesenchymal transition. It is well known that *TP53* deletion can lead to EMT [138].

3.3.3 Correlation of cellular deformability with pharmaceutical drug treatment

Here we demonstrate the advantage of using hydrodynamic stretching over the microsieve method for probing the differential cellular deformability of A549 (Adenocarcinomic Human Alveolar Basal Epithelial Cells) cell lines of control, treated with Cytochalasin D and docetaxel (a chemotherapy drug).

Before introducing the results from the microsieve measurements, we define the microsieve cell flexibility with the input to retrieval ratio, which is the number of total input cells divided by the number of cells retrieved from the microsieve. As shown in Fig. 3.8, the input to retrieval ratios of Cytochalasin D treated cells are larger than that of the non-treated cells. Besides, the ratios increase with the concentration of Cytochalasin D used. Decreased input to retrieval ratios are observed for docetaxel treated cells. And it seems like that increasing the dosage will not lead to significant decrease of the input to retrieval ratio. Additionally, we also characterize the cell size before and after treatment, see the bottom figure in Fig. 3.8. It reveals that the cell size after treated with

Figure 3.8: Experimental results for the non-treated A549, A549 treated with Cytochalasin D and Docetaxel from microsieve measurements. Top: Input to retrieval ratio (the number of total input cells divided by the number of cells retrieved from the microsieve) for A549 control (0 μM), treated with PFA, Cytochalasin D (10, 100 μM), and docetaxel (5, 10, 50 nM). Bottom: Statistical analysis for the cell size of A549 non-treated, treated with Cytochalasin D (10, 100 μM) and docetaxel (5, 10 nM).

Cytochalasin D is similar to the non-treated ones, whereas significant increase of cell size is observed after treatment of docetaxel.

Since both the cell size and deformability will affect the input to retrieval ratio for the microsieve experiments, it is unclear that whether docetaxel can stiffen the A549 cells. Therefore, we use the microfluidic hydrodynamic stretching to compare the deformability of non-treated and treated cells. The results are shown in Fig. 3.9. The median deformability of Cytochalasin D treated A549 cells are larger than that of the non-treated cells, whereas similar deformability is observed between docetaxel treated and non-treated cells. It can also be seen that the size distribution of docetaxel treated cells is much wider than the non-treated cells and the averaged cell diameter is larger. Similarly, the deformability distribution is also larger compared to the non-treated ones, see the box-plot in Fig. 3.9.

The results from hydrodynamic stretching indicate that the decreased input to retrieval ratio for docetaxel treated cells in microsieve experiments is probably due to the increased cell size instead of decreased cell deformability. The increase of cell size is probably because cells get stuck just before mitosis in the cell cycle when treated with docetaxel. Although the drug is thought to induce cellular stiffness in the clinics by "paralyzing" the cytoskeleton, it indeed does not induce more cellular stiffness. In summary, hydrodynamic stretching and the automated analysis make it easy to discriminate between cell size and deformability for the effect of a specific cancer-treatment drug.

3.4 Conclusion and outlook

In two different experiments, we find that cells with epithelial morphology are stiffer than cells with mesenchymal morphology, supporting the hypothesis that epithelial to mesenchymal transition (EMT) is associated with increased cellular flexibility. We hypothesize that cellular deformability is responsible, at least in part, for the increased invasive property of mesenchymal cells. In our experiments, we show that loss of the tu-

Figure 3.9: Measured deformability for the non-treated A549, A549 treated with $100\ \mu\text{M}$ Cytochalasin D and $10\ \text{nM}$ Docetaxel from the microfluidic hydrodynamic stretching. Top: Density scatter plot of the deformability. The dashed lines indicate the median deformability. Bottom: Statistic analysis of the above deformability in a similar way to Fig. 3.5.

tumour suppressor *TP53* gene increases the deformability of cancer cells and is associated with morphology changes reminiscent of EMT. MCF10A cell line with deletion of the tumour suppressor *TP53* gene are significantly more flexible compared to their isogenic wildtype counterpart, indicating for the first time, that a single genetic alteration can directly impact a cellular biophysical property relevant to the invasive phenotype of cancer. Besides, we also show that cellular deformability could help evaluate the efficacy of pharmaceutical drugs for cancer treatment.

From the biological part, more work will be done in the future to test the relationship between cellular deformability and the invasive property of mesenchymal cells, e.g., invasion experiment of MCF10A *TP53* KO and wild-type. Besides, a few other cell models will be established to further test the effect of *TP53* on the cellular deformability as well as testing the alteration of cell deformability after treated with pharmaceutic drug.

From the physical side, the temporal resolution of the high speed recording allows us to study the dynamics of cell shape change under stretching flow and relate to the mechanical property of the cells in more detail. Moreover, mechanical characterization of cross-linked serum albumin microcapsules has been reported recently in similar extensional stretching flow [139]. It might inspire us for the mechanical characterization of single cancer cell in the future work. Besides, we have attempted cell separation based on their deformability in the same device but not successful. In future, we might collaborate with a numerical group for the design of a microfluidic device for deformability based cell separation.

4 Interaction of a laser-induced cavitation bubble with complex configurations of gas-bubbles within microfluidic confinement

4.1 Introduction

Shock wave-gas bubble interaction can lead to intense jetting flow and very high shear stresses. Besides the biological effect of the haemolysis of red blood cells in shock wave lithotripsy [140], it can also be applied for fragmentation of cells for tumour therapy, e.g. histotripsy [33]. Other applications include aerated explosive gels [141] in the mining industry and avoiding cavitation erosion to materials [142, 143]. However, only a limited number of results are present due to the challenges to resolve the very fast dynamics as well as small scales during the gas bubble collapse.

Early experimental studies include subjecting a stable gas bubble to a spark generated shock wave for testing the mechanisms of cavitation erosion [144], and shock wave induced collapse of arrays of planar cavities in gelatine with details for the jet formation and propagation [25]. Later Philipp et al. utilized lithotripter shock wave to interact with air bubbles (0.15 mm and 1.2 mm) stabilized below a thin plastic film [145]. The strong jets of the bubbles are shown to penetrate into the plastic film, which indicates the biological

effect in shock wave action. Similar jetting phenomena have been demonstrated to occur for smaller gas bubbles that are more relevant for biomedical application, by Ohl & Ikink [26]. In their experiment, freely rising gas bubbles of radii between $7\text{ }\mu\text{m}$ and $55\text{ }\mu\text{m}$ from electrolysis is exposed to a shock wave and a microinjection mechanism is suggested when the cells are near bubbles exposed to a shock wave.

Besides the generation from spark source and lithotripter, shock wave can also be produced from pulsed laser light induced breakdown [23]. When the energy of the focused laser light exceeds the dielectric breakdown threshold of the liquid, plasma formation and shock wave emission occurs. In the meanwhile, a cavitation bubble is generated, followed by bubble expansion and collapse. A second shock wave or pressure wave is emitted when the cavitation bubble collapses. Chen et al. [18, 36] studied the interaction of a laser induced cavitation bubble and accompanied shock wave with a stable gas bubble within a narrow gap of $10\text{ }\mu\text{m}$ between two glass slides. The gas bubble is compressed, translated, and shows a jet formation during the cavitation bubble expansion, and subsequently undergoes large expansion during cavitation bubble shrinkage. The gas bubble is compressed again and splits into two small gas bubbles after the cavitation bubble collapses. Due to the almost planar geometry, the above complex bubble dynamics is beautifully visualized. However, the effect of the accompanied shock wave on the gas bubble dynamics is not addressed, especially the first shock wave from the laser-induced breakdown. Besides, the mutual interaction between gas bubbles as demonstrated by Dear&Field [25] is not accessible as only one gas bubble is generated in their experiment.

In this chapter, we present a new method which allows preparing well controlled gas and cavitation bubbles using focused laser beams with a digital hologram and record them with high speed photography in a planar geometry. A single or multiple gas bubbles with the size of tens of micrometers are exposed to a single laser-induced cavitation bubble and accompanied shockwaves/pressure waves. First we study the effects of the accompanied shock waves/pressure waves as well as the cavitation bubble oscillation on

a single gas bubble. We extend the experiment to multiple bubbles and their mutual interaction. Here we observe that the gas bubbles can react back on the cavitation bubble dynamics. The study reveals complex scenarios, one of those is that within the liquid film near or underneath the expanding cavitation bubble secondary cavitation can be nucleated. Please note, this chapter serves as an experimental reference, i.e. to document, categorize, and discuss the observations. Numerical simulations beyond simple Rayleigh Plesset models are not undertaken but are left for future work, likely conducted by specialized groups in this area.

4.2 Materials and methods

Single or multiple micron-size gas bubble(s) are generated in water confined in a microfluidic gap by heating a gold coated substrate with a continuous wave laser beam. The position and size of the gas bubbles are defined by a digital hologram and the duration of the laser exposure, respectively. Additionally, a pulsed laser beam is focused in the liquid to generate a rapidly expanding cavitation bubble next to the gas bubble(s). The dynamics is recorded using high speed photography with frame rate of more than 2 million frames per second.

4.2.1 Microsystem preparation

A soft mask for microchannels is designed and printed. It is a simple design that only has one inlet, one outlet and a wide plain interior region ($4 \times 6 \text{ mm}^2$) for experiment, see Fig. 4.1. We use the standard soft photolithography for the microchannels fabrication. A negative photoresist (SU-8 2015, MicroChem) is spin-coated onto a pre-cleaned microscope slide ($50 \times 75 \text{ mm}^2$) at 1500 rpm to achieve a film thickness of $26 \mu\text{m}$. The substrate is first pre-baked at 95°C for 4 min, then exposed to UV light (Manual Mask Aligner MJB4, SUSS Micro-Tec) for 4 s, and subsequently post-baked at 95°C for 5 min. After that the substrate is immersed into the developer solution. After shaking the solution

Figure 4.1: Schematic of the top view of 12 microfluidic systems. The array of PDMS channels (gray part) are bonded to a microscope slide (the outer box of $75 \times 50 \text{ mm}^2$) which has been coated with a layer of gold nanoparticles (blue part). Care is taken to make sure that the gold coatings ($3.5 \times 5.5 \text{ mm}^2$) are only inside the channels. The height of the microchannel is around $26 \mu\text{m}$ and the thickness of the gold coating is around 30 nm .

mildly for 2 min, the substrate is taken out, and first rinsed further with SU-8 developer, then DI water thoroughly and air gun dried. Thus we get the patterned substrate and use it as a mold to cast microchannels in PDMS (polydimethylsiloxane). The base and curing agent for PDMS (Sylgard 184 Silicone Elastomer Kit, Dow Corning) is mixed at a weight ratio of 10:1, degassed and then poured onto the mold. The PDMS layer is cured after 4 h at 60°C . Then it is peeled off from the mold, followed with inlet and outlet punched and surface thoroughly cleaned for bonding process.

Inspired by the work of Fang Yuan et al. [68] where tandem bubbles are generated by laser absorption through a pair of gold dots, we simply coat the glass substrate with a layer of gold nanoparticles to improve the laser absorption. The strong absorption

is likely due to surface plasmonic resonance of gold nanoparticles and its photothermal properties. For a discussion of the mechanism we refer to Ref. [146].

A pre-cleaned microscope slide is covered with a designed mask so that only uncovered regions will be coated with gold nanoparticles. The slide is applied for sputtering (JFC-1600, Auto fine coater, JEOL) at 10 mA for 150 s to achieve a coating thickness of around 30 nm. The topography of the coated gold layer is shown in Fig. 4.2. Then the slide is released from the mask, bonded with the PDMS layer using a plasma cleaner (Expanded Plasma Cleaner PDC-002, Harrick Plasma). During the bonding process, care is taken to make sure that the gold coated parts are inside the channel interiors. A schematic of the top view of the bonded microsystem is shown in Fig. 4.1.

4.2.2 Experimental setup

The experimental setup consists of two laser sources, a spatial light modulator (SLM), a few optical components, an inverted microscope, and a high speed camera to generate and image the bubbles, see Fig. 4.3.

The continuous wave (CW) laser (laser Quantum, excel, 2 W) is used to generate complex configurations of gas bubbles. The emission power can be adjusted continuously with the laser controller (MPC6000). The reflective SLM (model LC-R-2500, Holoeye Photonics AG, Berlin, Germany) is used for phase modulation of the laser light, which depends on the polarization state of the incident beam. Therefore, the CW laser beam (532 nm) is expanded by a telescope (L1 and L2) to fill the active area of the SLM before it is adjusted by a half wave plate to optimize the phase modulation. The hologram for the desired pattern is calculated with the commercial software (Holoeye software version 2.2) on a computer and combined with a lens phase before sent to the SLM. The lens phase is used to separate the depth of focus of the ineffective zero-order undiffracted portion from that of the diffracted beam. A polarizer is used subsequently to decrease the unwanted higher order diffraction portion of the beam. After that the laser beam

Figure 4.2: Topography of the gold nanoparticles coated on the glass substrate from atomic force microscopy. (a) 3D topography of a $5 \times 5 \mu\text{m}^2$ gold coating layer. (b) 2D topographical image of the same region. (c) The height measurement curve along the line in (b)

is collimated by the lens L3 ($f = 250$ mm) before projecting the desired pattern at the focal plane of the microscope objective (water immersion, $20\times$, $NA = 0.50$, Olympus). For more details of the SLM setup, please refer to Ref. [147].

The microchannel is placed on the translation stage of an inverted microscope (IX-71, Olympus), see the insert of Fig. 4.3. The PDMS channel with a height of $26\text{ }\mu\text{m}$ is filled with DI water. The CW laser beam is focused on the layer of gold nanoparticles inside the channel. The gold coating absorbs the energy of the CW laser light. Therefore, at each laser focus, the substrate and the liquid nearby is heated and a gas bubble is nucleated which grows gradually. By changing the hologram pattern, different configurations of gas bubbles can be achieved. The size of the gas bubbles can be adjusted with the input CW laser power, and the duration of the laser beam. A mechanical shutter (SH1/M, Thorlabs) is used to control the exposure duration of the CW laser beam. The setup allows a very accurate positioning of the gas bubbles through the hologram pattern.

A second laser source, the frequency doubled Nd:YAG laser (Orion, New Wave Research, Fremont, CA) gives a laser pulse (532 nm , 6 ns) to generate single cavitation bubble in the microchannel. Similarly, the laser beam is expanded with a telescope (L4 and L5) to fill the back aperture of the microscope objective. A 70% - 30% mirror is used to reflect 70% of the CW laser light and transmit 30% of the pulse laser light into the same microscope platform. The gold coating has good absorption of the pulsed laser light and allows reliable generation of cavitation bubbles [68]. The pulsed laser energy can be adjusted directly on the panel of the laser pump as well as with the delay time between the pump and the Q-switch.

During a typical run of the experiment, we first shine with the CW laser light for a certain power and duration onto the gold layer to create gas bubbles of the desired size. Then just before switching off the CW laser light, we trigger the pulse laser to generate an explosive cavitation bubble near the gas bubbles. The resulting complex bubble dynamics is recorded with a high speed camera (Photron SA-Z, Japan) at up to 2.1 million frames

per second. The timing is controlled precisely with a pulse delay generator (Model 575, Berkeley Nucleonics Corporation, San Rafael, CA, USA), i.e., switching on/off for the CW laser light, and triggering the pulse laser and the high speed camera.

4.3 Results and discussion

4.3.1 Pressure waves emitted from single cavitation event

In order to understand the interaction between the cavitation bubble and the gas bubbles, it is necessary to measure the pressure waves generated from the laser-induced cavitation event. Therefore, using the same experimental setup, we put a drop of DI water on top of a gold coated substrate (see Fig. 4.1), shoot a laser pulse and place a hydrophone (ONDA Model HNR-1000, serial No.1640) next to the laser focus. The hydrophone detects the pressure wave emitted from the laser-induced cavitation event and converts the pressure signal to a voltage. We also use a photodiode (DET 10A/M, Thorlabs) to detect the precise timing of the pulse laser light before it enters the microscope. Dynamics of the laser-induced cavitation bubble is recorded with the high speed camera. An oscilloscope (Lecroy, Wave Runner 64 Xi-A, 600 MHz, 10 Giga samples per second) is used to monitor the signals, i.e., the voltage signal from the hydrophone, the laser signal from the photodiode, the trigger signal from the delay generator to the high speed camera and the exposure sequence of the high speed camera. One example of the many measurements taken is illustrated in Fig. 4.4.

Fig. 4.4 A presents the measured signals from the oscilloscope while Fig. 4.4 B demonstrates the corresponding bubble dynamics recorded with the high speed camera. The camera is triggered (blue curve) and starts recording before the arrival of the pulse laser light (black line). During the second exposure (the second peak of the green curve) time, the laser light arrives and an intense plasma is recorded (see the red arrow on the left). A pressure peak is detected with the hydrophone around $1.5\ \mu\text{s}$ after the arrival of the pulse

Figure 4.3: Experimental setup: The continuous wave (CW) laser beam at 532 nm is controlled by a spatial light modulator (SLM) and a few other optical components to generate complex configurations of laser foci inside a microchannel which is filled with DI water. A layer of gold nanoparticles on the substrate absorbs the focused CW laser light. A gas bubble is formed at each laser focus and grows gradually with the CW laser light. Just before switching off the CW laser light, a single laser pulse of 532 nm, 6 ns duration is focused into the liquid to generate single cavitation bubble next to the gas bubbles. A high speed camera is used to record the fast dynamics of the cavitation - gas bubble interaction. The timing is precisely controlled by a delay generator for the high speed camera, the pulse laser, and the mechanical shutter of the CW laser. Insert is the side view of the microsystem which is placed on the translation stage of an inverted microscope.

Figure 4.4: Measurement of the pressure waves from single laser-induced cavitation event: (A) Oscilloscope recordings: pressure signal received by the hydrophone (red curve), pulse laser light detected by the photodiode (black line), trigger signal to the high speed camera (blue curve) and exposure sequence of the high speed camera (green curve). (B) The corresponding cavitation bubble dynamics recorded with the high speed camera at 300,000fps and 0.29 μs exposure time. The red arrows correlate the exposure timing of the camera with the recorded images. The consecutive frame number is marked in each image frame. The scale bar denotes 100 μm .

laser light. This time delay is the traveling time of the acoustic wave from the laser spot to the hydrophone. Then a cavitation bubble is generated (frame 3), expands (frame 3-8) and collapses (frame 9-13) rapidly within around $40\mu\text{s}$. At frame 14, the bubble starts to rebound. After the cavitation collapses (frame 14), we find another pressure peak detected by the hydrophone. This pressure wave is induced by the rebounding of the cavitation bubble (between frame 13 and 14) and it takes some time to reach the hydrophone. There are also some low-amplitude signals picked up by the hydrophone around $5\text{-}10\mu\text{s}$ and $25\text{-}45\mu\text{s}$, which is likely due to the reflection of the pressure wave from the glass edge.

The hydrophone has a sensitivity of 50 mV/bar . Therefore the amplitude of the initial acoustic transient is about 1.6 bar . The distance from the laser focus to the hydrophone can be estimated as $d \approx 2.3\text{ mm}$ assuming a speed of sound of 1500 m/s in water. It is known that the strength of the shock wave decreases with its traveling distance: $P(r) \propto A/r$, where r is the traveling distance, $P(r)$ is the pressure of the shock wave at distance r , A is a constant. Therefore the pressure amplitude at $100\mu\text{m}$ away from the laser focus can be estimated as $P_1(r = 100\mu\text{m}) = P_1(d) \times d/r \approx 36.8\text{ bar}$. This distance of $100\mu\text{m}$ is a typical distance used later in the experiments.

A few more measurements are performed for cavitation bubble with R_{max} between $64\text{-}314\mu\text{m}$ and we obtain $7.6\text{-}65\text{ bar}$ for $P_1(r = 100\mu\text{m})$, as shown in Fig. 4.5 (*circles*). The measurements also indicate that the amplitude of the pressure wave increases with the maximum bubble radius, see the solid line.

Though the cavitation bubble measured here is not confined in the microchannel, it helps us to understand the temporal behavior of the shock waves or pressure waves from the laser induced breakdown and the cavitation bubble collapse. By using this method we can also estimate the pressure exerted on the gas bubbles nearby.

Figure 4.5: Pressure amplitude at $100 \mu\text{m}$ away from the laser focus $P(r = 100 \mu\text{m})$ for cavitation bubbles of different maximum radius R_{max} . The *circles* depict the calculated value from the measurements. The *solid line* shows a linear fit of the experimental data. All the bubble dynamics are recorded at 300,000 fps and $0.29 \mu\text{s}$ exposure time. The signal monitoring is the same as described in Fig. 4.4.

4.3.2 Cavitation interaction with single gas bubble

In order to reveal the fast process of the bubble dynamics, we first use the highest frame rate available in spite of a compromise at the spatial resolution, as shown in Fig. 4.6. Fig. 4.6 (a) shows the bottom view for the interaction of a single cavitation bubble with one gas bubble at an interframe time of $0.476 \mu\text{s}$ (2100,000 frames per second (fps)). The initial distance between the centers of the two bubbles is $108 \mu\text{m}$. At frame 1, we see the nearly hemi-spherical shape of the gas bubble before the arrival of the pulse laser light. An intense plasma emission is observed when the pulsed laser is focused in frame 2. Then a cavitation bubble is expanding, and the gas bubble is translated to the left while being compressed. A jet traveling from the right to the left is formed during this process, see frame 4. In the later dynamics, the gas bubble expands (frame 5-6) before it is compressed again (frame 7-9). Subsequently the gas bubble expands again when the cavitation bubble starts to collapse (frame 11-23). After the cavitation bubble

collapses and rebounds, compression and the subsequent cusp shape of the gas bubble is observed (frame 24-36). We can also see that the tip of the liquid jet breaks up resulting in a small droplet trapped inside the gas bubble between frame 5 and frame 6.

The experimental configuration in Fig. 4.6 (b) is similar but with a larger size of the cavitation bubble, as listed in Fig. 4.6 (c). Thus a stronger and faster compression and jetting of the gas bubble is observed (frame 3), followed by an expansion from frame 4 to frame 5. Subsequently, it is notable that the gas bubble is compressed again to a large extent during the cavitation bubble expansion (frame 7-9) before it expands in the later process. Additionally, when the gas bubble is pushed away during the cavitation expansion, a small gas pocket is left behind at the original site. It grows and finally merges with the gas bubble when the latter are attracted back by the cavitation shrinkage.

In order to see more clearly the effect of the cavitation bubble dynamics on the size and position of the gas bubble, we plot the radius of the cavitation bubble (*diamond*) together with the diameter of the gas bubble (*circle*) and the distance d between them (in the bottom) as a function of time in Fig. 4.7. The time 0 here corresponds to the first frame in Fig. 4.6. The case in Fig. 4.7 a and Fig. 4.7 b corresponds to that in Fig. 4.6 a and Fig. 4.6 b, respectively. In both cases, the gas bubble is compressed to a minimum size soon after the generation of the cavitation bubble. Before the cavitation bubble expands to its maximum size, the gas bubble is compressed for a second time, which is notable in Fig. 4.7 b while much milder in Fig. 4.7 a. This might be due to a stronger pressure wave exerted on the gas bubble as the cavitation bubble is bigger in Fig. 4.7 b. After the cavitation bubble collapses, a sharp compression of the gas bubble can be clearly seen in Fig. 4.7 a. This compression is also observed in Fig. 4.7 b. However, it is much slower compared to Fig. 4.7 a. From the bottom curves of Fig. 4.7, we can see that the gas bubble is pushed away during the expansion of the cavitation bubble; then attracted back during the shrinkage of the cavitation bubble; and again translated away after the gas bubble is collapsed for the third time. Similarly, the final repulsion of the gas bubble

Figure 4.6: Interaction of a single cavitation bubble (*right*) with one gas bubble (*left*) at the highest frame rate of 2100,000fps and an exposure time of $0.16\mu\text{s}$. The frame number is indicated on the upper left part of each frame with an interframe time of $0.476\mu\text{s}$. (a), (b) are the recorded bubble dynamics for cavitation bubble of different sizes. (c) shows a schematic of the experimental configuration and a list of the parameters for the above cases. Both the scale bars denote $50\mu\text{m}$.

Figure 4.7: The time dependent size of the gas bubble and the distance d from the gas bubble to the cavitation bubble as a result of the cavitation bubble dynamics. a, b corresponds to the case in Fig. 4.6a and Fig. 4.6b, respectively. The top figure shows the temporal variation of the radius of the cavitation bubble (*diamond*) and the diameter of the gas bubble (*circle*). The bottom curve shows the distance d vs. time. The time 0 here corresponds to the first frame in Fig. 4.6. The dotted line indicates the moment when the cavitation bubble reaches its maximum size.

is much weaker in Fig. 4.7 b compared to in Fig. 4.7 a. We hypothesize that in the case of Fig. 4.7 b, the left behind gas pocket might affect the later dynamics of the gas bubble, i.e., reduce or increase the volume of the gas bubble when the gas pocket is left behind or merged again, and hindering the pressure wave from the cavitation bubble to the gas bubble.

The first compression and jetting of the gas bubble is likely due to the pressure wave emitted from the laser induced breakdown while the final compression and cusp shape might be a result of the emitted pressure wave when the cavitation bubble rebounds. These two pressure transients are also detected for the laser-induced cavitation bubble on a glass plate, as mentioned in the previous section. However, the mechanism for the second compression of the gas bubble during the expansion of the cavitation bubble is unknown yet. We hypothesize that the microfluidic confinement and a resonant oscillation of the gas bubble with the cavitation bubble might contribute to this second compression. The immediate expansion after compression of the gas bubble might be due to the tensile tail of the pressure wave, see Fig. 4.4 A. During the shrinkage of the cavitation bubble, the increasing velocity of the induced flow leads to a declining pressure in the liquid nearby, as according to Bernoulli's principle. Therefore, the gas bubble keeps expanding during the collapse phase of the cavitation bubble.

To get better spatial resolution of the bubble dynamics, we lower the frame rate to 1200,000 fps and two similar cases recorded with only the cavitation bubble size different are shown in Fig. 4.8. Similarly, in Fig. 4.8 a, the gas bubble is compressed and jetting ($0.83\text{-}2.49\mu\text{s}$) after the pulse laser light arrives. Subsequently the gas bubble expands during the late expansion and the collapse of the cavitation bubble ($3.32\text{-}10.79\mu\text{s}$). After the cavitation bubble rebounds, the proximal parts of the gas bubble to the cavitation bubble are compressed and two sub-cusp shapes are formed ($11.62\text{-}16.60\mu\text{s}$), which is also observed in the previous work (Figure 6 of Ref. [25]). A smaller cavitation bubble is shown in Fig. 4.8 b. Similar phenomena are observed. As a result of the weaker pressure

wave from this smaller cavitation bubble, the impacts on the gas bubble are milder compared to the case in Fig. 4.8 a. Additionally, it is noticeable that in both cases the cavitation bubble deforms and jets away from the gas bubble during its collapse .

4.3.3 Cascaded collapse of two/three gas bubbles

The experimental setup allows us to generate complex configurations of gas bubbles. Depending on their geometric arrangement, either simultaneous or cascaded collapses of the gas bubbles are observed when they are exposed to the laser induced cavitation event. In this section we will introduce the cascaded collapse of two or three gas bubbles arranged along a line together with the cavitation bubble. For convenience, we name the gas bubble in the closest proximity to the cavitation bubble as b1, the next one b2, and so on.

4.3.3.1 Two gas bubbles of different size ratios

We vary the sizes of the two gas bubbles to investigate the effect of their size ratios. The case for two gas bubbles of similar sizes (b1 and b2) is shown in Fig. 4.9 (a) with the cavitation bubble being to their right. Bubble b1 is compressed and jets in the right-to-left direction from $t=0$ to $1.388\ \mu\text{s}$. Then it expands until the cavitation bubble collapses at $t=11.798\ \mu\text{s}$. After this, the cavitation bubble rebounds, and bubble b1 is compressed again and deforms into a cusp shaped bubble, between $t=12.492$ and $16.656\ \mu\text{s}$). Bubble b2 is hardly affected until bubble b1 rebounds from its first compression. Then bubble b2 translates laterally and is compressed, between $t=2.776$ and $5.552\ \mu\text{s}$. After that b2 rebounds and expands until bubble b1 is compressed again to a cusp shape in $16.656\ \mu\text{s}$. Then we see another compression and bubble b2 develops a cusp.

Next cases with two gas bubbles of different size are investigated. To confirm the cascaded collapse, we compare the experiments with a single gas bubble. Experiments where b1 is larger than b2 are shown in Fig. 4.10.

(c)

Unit (μm)	Diameter (gas bubble)	Rmax (cavitation bubble)	Distance between them
exposure time: 0.16 μs			
(a) 1200kfps	21	56	104
(b) 1200kfps	19	44	102

Figure 4.8: Interaction of single gas bubble with cavitation bubble of different size: (a), (b) shows the high speed images for the bubble dynamics with the cavitation bubble size of 56 μm and 44 μm , respectively. (c) is a list of the frame rates, exposure time for the above recordings, and the initial diameter of the gas bubble, the maximum radius of the cavitation bubble, the initial distance between the cavitation bubble and the gas bubble centers. The standard deviation for the size measurement is $\pm 2 \mu\text{m}$.

Figure 4.9: Interaction between one cavitation bubble and two gas bubbles of similar sizes. (a) Bubble dynamics recorded by the high speed camera with $0.694 \mu\text{s}$ interframe time and $0.16 \mu\text{s}$ exposure time. (b) A schematic of the geometric arrangement for the cavitation bubble and gas bubbles (b1 and b2), and a list of the parameters including the maximum radius R_{max} of the cavitation bubble, the initial radii of the gas bubbles R_1 , R_2 , and the initial distance between the cavitation bubble and each gas bubble d_1 , d_2 .

Figure 4.10: Interaction between a single cavitation bubble and two gas bubbles with b_1 larger than b_2 , as shown in the left columns of (a) and (b). The right columns are the corresponding control cases with only one gas bubble. The size of each frame is $350 \times 80 \mu\text{m}^2$. The interframe time is $2.22 \mu\text{s}$ with an exposure time of $0.29 \mu\text{s}$. The table on the bottom shows the parameters for each case.

In the left column of Fig. 4.10 (a), two gas bubbles with a size ratio $R1/R2 = 1.57$ are exposed to a laser-induced cavitation bubble being to their left. At frame 3, we see that b1 collapses with a jet away from the cavitation bubble. Bubble b2 expands slightly with its central part compressed, while in the control case on the right column a cusp shape is formed. When b1 rebounds in frame 4, b2 collapses seemingly with a jet away from b1. Judging from the motion blurring the collapse of b2 seems slightly stronger than in the control case. After that b1 expands until the cavitation bubble rebounds. Then a second pressure wave starts to collapse b1 (frame 9-10), during which b2 is attracted toward b1 and expands. Also bubble b1 moves towards the cavitation bubble. This rapid translational motion might excite a surface wave which first focuses into a cusp on the right hand side of bubble b1 (frame 9 of Fig. 4.10 (a)). The cusp continues to move through the bubble and develops into a blob on the left hand side of bubble b1, see frames 11-12. Simultaneously bubble b1 is compressed by the pressure wave emitted from the cavitation bubble collapsing between frames 8 and 9.

In the control case, after the cavitation bubble rebounds, there is not much change in the size of b2. In addition, it is noticeable that during the collapse, the cavitation bubble deforms to a larger extent and collapses more asymmetrically (with a jet) away from the two gas bubbles when compared to the control case.

Similar phenomena are observed in Fig. 4.10 (b). Due to a larger size of the cavitation bubble, a stronger jetting of b1 is observed, which causes the breakup of b1 to the main part and a tiny gas pocket in frame 5. The surface wave induced by the motion of b1 is stronger and now leads to a splitting of the bubble between frames 13 and 14 of Fig. 4.10.

The second case with different sized bubbles is shown in Fig. 4.11, where b1 is smaller than b2. For the dynamics of b2, there is not much difference between the two gas bubbles case and the control case when the standoff distance is large (e.g. $d2/R_{max} \approx 2.6$), see Fig. 4.11 (a). Although the cavitation bubble is also deformed and collapses with a jet away from the gas bubble in the control case, its deformation and the jetting is slightly

Figure 4.11: Interaction between a single cavitation bubble and two gas bubbles with b_1 smaller than b_2 , as shown in the left columns of (a) and (b). The left columns of (a) and (b): two gas bubbles with $R_1 < R_2$ in a horizontal row with a cavitation bubble; The right columns are the corresponding control cases. The size of each frame is $350 \times 80 \mu\text{m}^2$ in (a) and $430 \times 80 \mu\text{m}^2$ in (b), respectively. The table on the bottom shows the parameters for each case.

weaker than for the two gas bubbles case, see Fig. 4.11 a control. It seems like that b1 have already experienced compression and jetting before its expansion in frame 3 on the left column. Additionally, it is compressed for a second time during the expansion of the cavitation bubble in frame 4. This is also observed in Fig. 4.11 (b), where the standoff distance is smaller ($d2/R_{max} \approx 1.8$).

In Fig. 4.11 (b), in frame 3, bubble b1 is already in the expansion phase after its first collapse and jetting, and we can also see the motion-blurred image of b2, which combines the compression and jetting of b2 during the short exposure time of 290 ns. Here it is clearly obvious that the jet of bubble b2 is faster when bubble b1 is present. After that b2 expands due to the collapse of b1 in frame 4, then shrinks after bubble b1 rebounds (frame 5), and subsequently expands again together with b1 during the collapse of the cavitation bubble, see frame 6-12 in Fig. 4.11 b. After the cavitation bubble collapses, the rebound pressure wave compresses bubble b1 again (left column, frame 13-14). Subsequently bubble b1 rebounds, and collapses bubble b2, which has a penetrating jet from the left to the right while in the control case (right column) not much impact is observed on the gas bubble b2.

4.3.3.2 Three gas bubbles

Quite similar events are observed when three gas bubbles along a line are exposed to a laser-induced cavitation event. We have selected a few cases together with a schematic of the geometric arrangement in Fig. 4.12.

Figure 4.12 A and B show three bubbles of similar size, while in Fig. 4.12 C and D the bubble b3 (the most distant bubble from the cavitation bubble) is smaller than the other two bubbles, i.e. b2 and b1. Let us start with the equal sized bubbles shown in Fig. 4.12 A. In frame 3, bubble b1 already rebounds from its collapse while Bubble b2 is collapsed and then rebounds at frame 4. Bubble b3 becomes compressed between frame 3 and 4. Interestingly, during the cavitation expansion, b1 collapses a second time

Figure 4.12: A horizontal array of three gas bubbles exposed to a laser-induced cavitation event. A, B shows three gas bubbles of similar sizes. The gas bubbles in A are smaller than in B while the cavitation bubble size is larger. In C and D, b1 and b2 have similar sizes while b3 is about half the size. A schematic of the experimental geometry is shown below the images, with a table listing all the parameters. The distance from each gas bubble to the cavitation bubble is similar for all the cases. The interframe time for all the images is $0.992 \mu\text{s}$ with an exposure time of $0.16 \mu\text{s}$.

around frame 6. It seems that this collapse induces a “chain of reactions” for b2 and b3 as the rebound of one gas bubble causes the collapse in the outward direction. After the cavitation bubble rebounds in frame 20, b1 collapses again and may trigger another “chain of reactions” for bubbles b2 and b3. Yet, this time the dynamics is slower and leads to lesser deformations of bubbles b2 and b3.

A very similar case is shown in Fig. 4.12 B; here the gas bubbles are just larger than in Fig. 4.12 A. Again, a cascaded collapse is observed after the laser-induced breakdown and the rebound of the cavitation bubble. The first and second cascaded collapse is slower compared to the case in Fig. 4.12 A, which might be due to the smaller cavitation bubble as well as the larger size of the gas bubbles.

Three gas bubbles of different sizes are shown in Fig. 4.12 C and D, where b1 and b2 have similar sizes while b3 is smaller. Similar phenomena are observed for both cases as in Fig. 4.12 B. As the cavitation bubble is larger in Fig. 4.12 C, the collapses of the gas bubbles are stronger than in Fig. 4.12 D. Comparing Fig. 4.12 B with Fig. 4.12 D, the parameters are similar except that b3 is smaller in the latter case. The collapse of b3 in Fig. 4.12 D is stronger and faster than in Fig. 4.12 B, which indicates that smaller gas bubbles are easier to be collapsed.

In summary, mild and rather pronounced cascaded collapses are observed when two or three gas bubbles with the appropriate size ratio and position are exposed to a laser induced cavitation event of sufficient strength. When two/three gas bubbles are present, the cavitation bubble deforms and collapses asymmetrically with a jet away from the gas bubbles. Strong interaction between the cavitation bubble and the gas bubbles as well as mutual interaction between the gas bubbles are observed. Besides, we find that only when b1 is small (compared to the cavitation bubble and the other gas bubble(s)) a second compression of b1 can be observed which occurs during the expansion of the cavitation bubble.

4.3.4 Simultaneous collapse of multiple gas bubbles on pentagons and hexagons

Another obvious arrangement is to place the gas bubbles at equal distance from the cavitation bubble. A few examples are shown in Fig. 4.13. In Fig. 4.13 (a), five gas bubbles of similar sizes are created on the corners of a pentagon with the cavitation bubble being generated approximately at their geometrical center (frame 2-3). In these experiments with more than 3 bubbles it is more difficult to control their size as good as before. Although each of the gas bubbles collapses with a jet away from the cavitation bubble (frame 3-4), one bubble has a closer distance to the cavitation bubble due to its larger size (1 o'clock position). The interaction is stronger leading to a larger/faster jet (frame 3 and 4 of Fig. 4.13 b). Later in frame 4 the cavitation bubble deforms inward to a larger extent on its proximal side to this gas bubble. All the gas bubbles expand during the collapse of the cavitation bubble (frame 4-6) until the latter rebounds. Then we see another concerted collapse of the gas bubbles in frame 7, which is much milder as compared to the first collapse.

A larger cavitation bubble is created in Fig. 4.13 (b), where the gas bubbles are of different sizes. In frame 3, we see a stronger collapse for each of the gas bubbles with a fast penetrating jet. This fast liquid jet even breaks up and extrudes out a portion of the right top gas bubble (frame 4). Due to the larger sizes of gas bubbles at the 1 o'clock position and 8 o'clock position, stronger interaction with the cavitation bubble is observed. The cavitation bubble is partially squeezed on these two sides until it collapses with a jet away from these bigger gas bubbles (frame 4-6). After the cavitation bubble rebounds, all the gas bubbles are compressed slightly (frame 7).

To obtain more details about the bubble dynamics, i.e., capturing the bubble shapes at different timings, we shift the exposure sequence of the high speed camera with respect to the arrival timing of the pulsed laser light, as shown in Fig. 4.13 (c). As a result, we see in frame 2 a small expanding cavitation bubble instead of the laser-induced plasma.

The gas bubbles around it are slightly compressed. Similar phenomena as the above cases are observed later. It is noticeable that frame 4 captures a pentagonal shape of the cavitation bubble during the early collapse state, which is due to its strong interaction with the gas bubbles proximal to each side of the pentagon. At last an example with six gas bubbles oriented on a hexagon is shown in Fig. 4.13 (d). The dynamics is very similar to the three previous cases.

4.3.5 Change of the jet orientation for neighboring bubbles

In the above sections, the gas bubbles are rather widely spaced from each other while surrounding the central cavitation bubble. The jetting direction of each gas bubble is always along the direction from the cavitation bubble center to the center of the gas bubble. We expect that bubbles sufficiently close to each other may affect each other's jetting directions. Therefore we position the gas bubbles at equal distance from a cavitation bubble; see Fig. 4.14, where three gas bubbles are closely arranged on an arc surrounding the cavitation bubble.

In Fig. 4.14 A1, the central gas bubble is larger than the side ones. They jet away from the cavitation bubble at $5.56 \mu\text{s}$. It is noticeable that the jetting directions of the gas bubbles on the two sides deviate from the radial direction, but is toward the central gas bubble, which still remains to jet in radial direction. Then the gas bubbles expand until the cavitation bubble collapses with a jet away from them in $13.90 \mu\text{s}$. Subsequently, the gas bubbles are compressed due to the rebound of the cavitation bubble. Interestingly, the side gas bubbles are attracted toward the central one during this compression. In Fig. 4.14 A2, the same geometric arrangement is applied but the gas bubbles are smaller. A similar phenomena is found. Yet, for the gas bubbles on the two sides, not only the change of their jetting direction but also the attraction of them towards the central gas bubble is weaker as compared to the case in Fig. 4.14 A1.

Figure. 4.14 B1 and B2 explore the effect of the distance between the gas bubbles. In

Figure 4.13: Simultaneous collapse of 5 (a-c) or 6 gas bubbles (d) in a ring surrounding a laser-induced cavitation bubble. The interframe time is $2.78 \mu\text{s}$ for (a), (b),(d) with an exposure time of 248 ns. In (c), the interframe time is $3.33 \mu\text{s}$ with an exposure time of 293 ns. All the scale bars denote $50 \mu\text{m}$.

the latter figure the distance between the gas bubbles are larger. Please note that the image scaling for these two cases are different, see the scale bars. All other parameters are similar. In Fig. 4.14 B2, the bubbles jet radially while in Fig. 4.14 B1, where the bubbles are closer, the jet is deflected towards the central larger bubble.

Next we explore the effect of the size of the central gas bubble. Figure. 4.14 C1 and C2 reveal that when the central gas bubble is smaller than the side ones, the mutual interaction is strongly reduced. By comparing this case with Fig. 4.14 B2, where all the parameters are similar except that the central gas bubble is larger than the side ones, the two cases here hardly show any change of the jetting directions or any obvious attraction of the side gas bubbles toward the central one.

In summary, only when the gas bubbles are close enough with the central gas bubble larger than the side ones, can we see a noticeable change of the jetting orientation. Then some attraction of the side gas bubbles toward the central one is observed, too. With a larger size of the gas bubbles and a closer distance between them, stronger mutual interaction is observed.

4.3.6 Oscillation of small gas bubble after cavitation event

After the impact from a laser-induced cavitation bubble and accompanied pressure waves, oscillations of small gas bubbles are observed. One example is shown in Fig. 4.15 a, where a single gas bubble is exposed to a large cavitation bubble. During the rapid expansion of the cavitation bubble, the gas bubble is pushed away and leaves behind a small gas pocket which seems like pinned at the original site. Then the cavitation bubble expands over the gas pocket and the low pressure inside the cavitation bubble makes the gas pocket expand. Later the cavitation bubble retracts back and leaves behind the gas pocket, which now becomes a small gas bubble. Subsequently this small gas bubble is oscillating at some high frequency.

To study this oscillation in greater detail, 60 consecutive images for this small gas

Figure 4.14: Mutual interaction between gas bubbles when they are close. Image sequences A1-C2 illustrate examples of three close gas bubbles with similar distances to a cavitation bubble. The interframe time is $2.78\mu\text{s}$ with an exposure time of 293 ns . A schematic of the geometric arrangement is shown on the left bottom. The parameters for each case are listed in the table on the bottom: r_1, r_2, r_3 is the radius of the gas bubble b1, b2, b3; d_1, d_2, d_3 is the distance from the gas bubble center to the cavitation bubble center; d_{12}, d_{23} is the distance between gas bubbles.

bubble following the last frame of Fig. 4.15 a are listed in Fig. 4.15 b. From the change of the bubble shape, we estimate 8 cycles of oscillation between frame 5 and frame 51. Thus one period time of oscillation is estimated as $T = 4.79 \mu\text{s}$. Therefore the frequency is around 208.7 kHz while the frame rate for the recording is 1200 kHz . Since the period of bubble oscillation is not exact a multiple of the interframe time, the recorded bubble shapes vary slightly for each cycle.

In order to see the temporal evolution of the bubble shape with more details, we sort the above images according to their phase of oscillation, as shown in Fig. 4.15 c. Starting from 0.61π where the gas bubble looks round, the gray value of its interior increases gradually. From 0.87π onwards, there appears a small but brighter point in the center of the gas bubble. Then around 1.1π , the bubble interior becomes uniformly bright. Interestingly, from 1.2π to 1.4π the central part of the gas bubble becomes darker while the contrast for the edge decreases. Subsequently, we see a narrow oval shape of the gas bubble ($1.5\pi - 1.7\pi$), which then expands gradually ($1.8\pi - 2\pi(0\pi) - 0.17\pi$) and finally evolves to a circular shape again ($0.26\pi - 0.52\pi$). Please note that all the images shown here are from bottom view.

The classical work from Crum [148] helps us to understand the above shape oscillation. In his work, a millimeter sized vapor-air bubble near a solid boundary is driven near its resonance frequency. The large pulsations lead to the development of surface modes and jetting as shown in Fig. 4.15 d. On the left shows the well-known “Crum’s jet” already within the bubble and moving to the boundary below. The image sequence on the right shows the bubble oscillation and generation of cusp shaped bubbles pointing towards and away from the boundary in a side view. In our experiment, the recorded bubble oscillation is from the bottom view. Nevertheless, by considering the refraction of the illumination light from water to gas at the bubble wall, we can estimate the curvature of the bubble from the recorded gray value.

The variation in gray levels is consistent with the sketch of the shape of the gas bubble

proposed in Fig. 4.15 e. It shows the cross section of the bubble shape, which is along the x axis of the bottom view image in Fig. 4.15 c. The first schematic image shows the convex bubble shape from 0 to 0.7π , where a small bottom part of the gas bubble is pinned at the surface. Then a concave shape appears on the top central part of the bubble as a jet is developing from inside the bubble to the substrate around 0.87π . According to Crum's work, the other top surface surrounding the jet becomes flat when this jet proceeds. Therefore we see a bright bubble interior with an even brighter central point at 1π . Afterwards, the jet vanishes and the bubble restores to a flat shape on the top surface around 1.1π . This explains why we see a uniformly bright bubble interior in Fig. 4.15 c. Subsequently, the bubble protrudes from the top surface and forms another convex shape, leading to a dark part in the center, as seen from 1.2 - 1.3π in Fig. 4.15 c. From 1.4π to 1.6π the bubble deforms further to the middle and upper directions while the other dimension perpendicular to this cross section expands (indicated by the dashed line). Finally a narrow and elongated shape is formed. Thus we see a dark and narrow ellipse from the bottom view. After that the bubble deforms gradually to the quasi-hemispherical shape around 0.61π then another cycle of the oscillation starts.

From the recorded images of the small oscillating bubble, we can extract the bubble contour through image processing using a home-built routine in Matlab. From this we obtain the projected bubble area, and major and minor semi-axis for each image frame. One example is shown in Fig. 4.16 A for the temporal evolution of the projected bubble area. Here we set the time $t=0$ as the moment just after the cavitation bubble rebounds, corresponding to the frame marked with an asterisk in Fig. 4.15 a. In Fig. 4.16 A, we can see that the small gas bubble oscillates and expands at the beginning and before it enters a more stable mode of oscillation. After around $170\mu s$, this stable oscillation stops abruptly as the projected area hardly changes any more while the position of the bubble centroid keeps the same, see Fig. 4.15 a and b. We suppose that originally a small bottom part of the gas bubble is tethered or pinned on the substrate resulting in a

Figure 4.15: Oscillation of a small pinned gas bubble after the cavitation event. (a) Consecutive images for one gas bubble exposed to a large cavitation event, recorded by the high speed camera with an interframe time of $0.83 \mu\text{s}$ and an exposure time of $0.16 \mu\text{s}$. The size for each frame is $256 \times 64 \mu\text{m}^2$. The images are ordered in columns from top to down and then from left to right. (b) Following the last frame of (a), a consecutive cropped image sequence of the small gas bubble pointed in (a) is listed with the same interframe time. (c) Re-arrangement of (b) in the order of calculated phase. The number on the top of each image indicates the oscillation phase in radian for the gas bubble. The “pi” symbol denotes π . There is slight difference between the same phase values shown here. (a)-(c) are all from top view. d) The image on the left is the photograph of jet formation from the side view from Crum [149]; the image sequence on the right shows the side view of the bubble oscillation and liquid jet production [148]. (e) Proposed shape oscillation for the gas bubble in (c) from a side view.

Figure 4.16: Temporal variation of the projected area for the oscillating gas bubble in Fig. 4.15 (a) and extraction of the oscillating frequency using Fast Fourier Transform analysis. (A) Time evolution of the projected area of the gas bubble from bottom view, from after collapse of the cavitation bubble (the frame marked by the star in Fig. 4.15 (a)) until the oscillation of the gas bubble totally stops. (B) (i), (ii) is enlarged oscillation curve from earlier and later time during the gas bubble oscillation (as indicated by the double arrows for the time spans in (A)), respectively. (C) (i), (ii) shows the frequency domain for (B) (i), (ii) after Fast Fourier Transform, respectively. The highest peak in (i) and (ii) depicts the dominant oscillation frequency, which is 221 kHz and 211 kHz , respectively.

thin liquid film between the gas bubble and the substrate, as shown in Fig. 4.15 e. After a certain period of bubble oscillation in both vertical (z) and horizontal planar (x-y) direction, this liquid film finally ruptures leading to the attachment of the whole bottom part of the gas bubble to the substrate. Then the gas bubble is fixed.

We can obtain the oscillation frequency of the gas bubble from Fig. 4.16 A. Furthermore, to check if there is any obvious damping effect before the oscillation stops, we conduct a Fourier transform for both the earlier and the later times in Fig. 4.16 B (i) and (ii), respectively. The corresponding result of the frequency domains is shown in Fig. 4.16 C (i) and (ii). We can see that the dominant oscillation frequency for the earlier and later time is $221k$ Hz and $211k$ Hz, respectively. This indicates a small damping effect for the oscillation.

We can also estimate the natural frequency of the gas bubble. After the gas bubble stops oscillation it keeps a constant area of $142 \times 10^{-12} \text{ m}^2$. The bubble radius in equilibrium is thus around $6.72 \mu\text{m}$. Therefore the natural or Minnaert frequency [150] is $f_0 = 3 \text{ m/s} \div (6.72 \mu\text{m}) = 446k \text{ Hz}$. Our measured frequency of the gas bubble is different to this fundamental resonance. It is reasonable as the bubble is not oscillating at its volume mode, but at some surface mode.

4.3.7 Activation of cavitation nuclei from cavitation bubble expansion close to surfaces

4.3.7.1 Nucleation from gaseous remains

When the standoff distance from a gas bubble to a single cavitation bubble is below 1 ($d/R_{max} < 1$), the cavitation bubble will expand over the original site of the gas bubble. For this condition, some new phenomena are observed. For simplicity, we will first discuss the cases of one cavitation bubble with one gas bubble, as shown in Fig. 4.17.

Figure. 4.17 (a-c) depict cases where the gas bubbles are of similar size but located at different standoff distance to the cavitation bubble, i.e. the maximum radius of the

Figure 4.17: Gas bubble dynamics after the passage of an expanding cavitation bubble (*right*) over a single gas bubble (*left*). The geometric arrangement is the same as in Fig. 4.6 and the corresponding parameters are listed at the left bottom of the figure. a-c show three comparative cases, where all the other parameters are similar except the cavitation bubble size is increasing (from left to right). Note, the CW laser light is still on during the recording. (d) is a control case with similar geometric parameters but the CW laser light is already off before the cavitation expansion starts. All the images are taken at 300,000 fps with 293 ns exposure time. The scale bars denote $50 \mu\text{m}$.

cavitation bubble is increasing from left to right in Fig. 4.17. The gas bubble is always formed before the cavitation bubble is nucleated. In Fig. 4.17 (a), the gas bubble is pushed during the rapid expansion of the laser-induced cavitation bubble ($3.33\text{-}6.66\ \mu\text{s}$). The overall gas bubble dynamics is resembling the one previously described, see section 4.3.2. However, it is interesting that there are some tiny gas pockets left behind at the original site of the gas bubble. After the cavitation bubble expands over these gas pockets, they grow into a larger gas bubble ($9.99\ \mu\text{s}$). For clarity, we call this kind of gas bubble a surface trapped bubble (STB). Subsequently, the cavitation bubble starts to shrink, and we see the retracting wall of the cavitation bubble moving across the STB at $13.32\ \mu\text{s}$. Afterwards the cavitation bubble wall seems to be attached with and dragged by this STB while the other part of the wall continues to move inward ($16.65\ \mu\text{s}$). At $t=19.98\ \mu\text{s}$ the STB disappears, probably due to the merging with the cavitation bubble. The cavitation bubble keeps this protrusion part on the left side before it condenses back to the liquid ($19.98\text{-}23.31\ \mu\text{s}$).

Similarly, in Fig. 4.17 (b), the STB is also formed when the cavitation bubble expands over the original site of the gas bubble. Due to a larger size of the cavitation bubble, the STB remains longer underneath the cavitation bubble and grows to a larger size ($6.66\text{-}19.98\ \mu\text{s}$). After that the STB also attaches to and deforms the cavitation bubble wall when the latter moves across the former ($19.98\text{-}23.31\ \mu\text{s}$). It can be clearly seen that subsequently the STB merges with the cavitation bubble, which forms a protrusion part on its left side ($26.64\ \mu\text{s}$). Similar phenomena are also observed in Fig. 4.17 (c). As a result of an even smaller standoff distance, the original gas bubble is pushed out of the view field and a larger STB is formed underneath the cavitation bubble. Interestingly, there is another tiny gas bubble left behind (on the left side of the original gas bubble site) due to the violent interaction between the cavitation bubble and the original gas bubble. This tiny gas dot also expands underneath the cavitation bubble and displays similar dynamics as the original STB ($6.66\text{-}23.31\ \mu\text{s}$).

The continuous wave (CW) laser light is still on during the above process. To check if the formation and expansion of the STB is affected by the CW laser light, we perform a control experiment where the CW laser light is already off before the generation of the cavitation bubble, as shown in Fig. 4.17 (d). At $t=0$, the vapor inside the gas bubble already condenses slightly as a result of the cooling which sets in quickly. The dynamics of the STB in Fig. 4.17 (d) where the light is off and in Fig. 4.17 (c) where the light is on are very similar. This indicates that the formation and expansion of the STB is not a thermally driven process.

What else could cause the nucleation of this secondary cavitation event? It is likely that gas pockets are left behind when the original gas bubble is pushed away by the fast expansion of the cavitation bubble. This might be due to the surface roughness at the site of the original gas bubble. As we use a sputtering method to prepare the gold coated substrate, the surface is not perfectly smooth. After the impulsive pushing away of the original gas bubble, some small gas pockets remain trapped/pinned at the original site. During the expansion of the cavitation bubble ($6.66\text{-}9.99\text{ }\mu\text{s}$), the pressure within its interior drops continuously. The pressure of the liquid film underneath the cavitation bubble should be very similar to that inside the bubble, due to the boundary layer approximation and the continuity of the pressure at the flat free interface. As a result the trapped gas experiences a pressure drop of about 1 bar and forms the STB. Afterwards, the cavitation bubble begins to retract back with an accelerating velocity. The bubble wall is approaching the STB before moving across it. Therefore, during this process, the velocity of the liquid film where the STB locates is accelerated, leading to a decrease of the local pressure according to Bernoulli's principle. This might explain the continuous growing of the STB during the shrinkage of the cavitation bubble. These two mechanisms of pressure drop might also be applicable to the expansion of any tiny gas component left in the liquid film underneath the cavitation bubble, as in the case of Fig. 4.17 (c).

Besides, it is noticeable that the edge of the STB changes brightness and shape after $6.66 \mu\text{s}$ in Fig. 4.17 (b-d). The small gas pockets as well as the STB in Fig. 4.17 (a) are darker in color and remains. The difference is that the STB in Fig. 4.17 (a) is closer to the rim of the cavitation bubble wall, i.e. the lubricating film between the cavitation bubble and the glass surface is likely thicker.

The gas bubble illuminated from behind diffracts light, i.e. it is a phase object. The change from a dark to a bright object can be explained by a change of curvature of the diffractive element, which we explain in Fig. 4.18. When the cavitation bubble (blue) expands in the microchannel, thin liquid films (yellow) are formed between the bubble wall and the channel wall. Initially, inside this liquid film, the trapped gas pockets are small and grow to form a small STB (black, which is smaller than the thickness of the liquid film. It looks dark at the edge due to its convex cap (a negative lens), as shown in Fig. 4.18 (a). Afterwards, the liquid film becomes thinner due to the further expansion of the cavitation bubble. In the meanwhile, the STB also grows to a larger size. These lead to the thinning of the liquid film between the STB and the cavitation bubble. Finally this thin liquid film ruptures, resulting in the contact of the STB top surface with the bottom wall of the cavitation bubble, as shown in Fig. 4.18 (b). During further expansion, the STB merges with the cavitation bubble, as shown in Fig. 4.18 (c). After they merge together, a concave shape is formed near the outer boundary of their contact region. The center where the trapped gas is located is more flat and hardly diffracts light. The white ring surrounding the center is likely formed by the varying curvature of the gas interface connecting with the cavitation bubble. As for Fig. 4.17 (a), where the standoff distance is larger we expect a thicker STB film. Therefore, the STB is not able to merge (drain the liquid film) with the cavitation bubble and keeps its convex shape, i.e. it remains as a dark object in high-speed images.

The above phenomena of STB are also observed for the cases of multiple gas bubbles. Here we show the cases of 6 surrounding gas bubbles run over by the cavitation bubble

Figure 4.18: A schematic on the dynamics of the liquid film rupture between the cavitation bubble and the STB: (a) The STB (black) is initially smaller than the thickness of the liquid film (yellow) underneath the cavitation bubble (blue); (b) The STB grows to touch the bottom surface of the cavitation bubble; (c) The STB merges with the cavitation bubble.

nucleated in their geometrical center. One example is shown in Fig. 4.19. At $t=5\mu\text{s}$, the plasma created by the pulsed laser light is visible and is surrounded by 6 gas bubbles. At $t=10\mu\text{s}$, these gas bubbles are jetting and pushed away during the cavitation bubble expansion. The original sites of the gas bubbles are now underneath the cavitation bubble and STBs are formed there. These STBs grow gradually to larger sizes ($15\mu\text{s}$ - $35\mu\text{s}$). It is also noticeable that the cavitation bubble wall is strongly deformed at the proximal side to the original gas bubbles, e.g., protrusions form at $10\mu\text{s}$, and indentations form between $15\mu\text{s}$ to $30\mu\text{s}$. At $t=35\mu\text{s}$, the bubble wall of the shrinking cavitation bubble is moving across the STBs. Besides, the bubbles formed underneath the cavitation bubble center also grow up, contact with and finally merge with the STBs ($10\mu\text{s}$ - $45\mu\text{s}$). Similar to the one gas bubble case, these STBs also drag, deform and finally merge with the cavitation bubble (40 - $50\mu\text{s}$). The formation of gas/vapor bubbles in the liquid film right underneath the cavitation bubble center is observed here as well as in Fig. 4.17 (d). We speculate that the strong heating from the high-intensity pulsed laser light used could lead to film boiling in its neighborhood.

Another example with six surrounding bubbles is shown in Fig. 4.20, where the stand-off distance is larger and more details for the bubble dynamics are revealed due to a shorter interframe time of $2.78\mu\text{s}$. At $t=5.56\mu\text{s}$, the gas bubbles are pushed away by the expanding cavitation bubble, leaving behind small gas pockets at the original sites. At $t=8.34\mu\text{s}$, the cavitation bubble wall reaches the small STBs which are formed from the gas pockets. Subsequently, the STBs grow to larger sizes and deform the cavitation bubble wall locally inward ($11.12\mu\text{s}$ - $13.90\mu\text{s}$). Besides, it looks like that the STBs now attach to one part of the cavitation bubble wall. At the moment it is not clear whether it is the top part or the bottom part of the cavitation bubble. Afterwards, the cavitation bubble starts to shrink. Its attached part is dragged by the growing STBs while the other part moves faster inward ($16.68\mu\text{s}$ - $25.02\mu\text{s}$). At $t=27.80\mu\text{s}$, the part, which moves faster, merges with the bubble formed underneath the cavitation bubble

Figure 4.19: Expansion of a cavitation bubble over the original sites of 6 surrounding gas bubbles. The interframe time is $5\mu s$ with an exposure time of 248 ns for each frame. The scale bar in the first frame denotes $100\mu m$.

center, indicating that it is the bottom part of the cavitation bubble. The top wall of the cavitation bubble slowly moves across the STBs ($19.46\mu s - 27.80\mu s$). The latter also move inward with the former, leaving some gas remains at the original sites. Interestingly, these gas remains connect the STBs with the original gas bubbles that are attracted back by the inward flow from the cavitation bubble collapse ($27.80\mu s - 33.36\mu s$). Finally, these connections or ties break up due to the fast collapse and condensation of the cavitation bubble to the surrounding medium ($36.14\mu s - 38.92\mu s$).

As mentioned previously, the formation and growth of STBs are due to the left behind gas pockets and reduction of the pressure from the pulsation of the cavitation bubble. To check for an nucleation threshold of STBs, we alter the CW laser energy at each laser focus so that some spots gas bubbles appear while other spots not, e.g. as shown in

Figure 4.20: Expansion of a cavitation bubble to the original sites of 6 surrounding gas bubbles. The interframe time is $2.78\mu\text{s}$ with an exposure time of 248 ns for each frame. The scale bar in the first frame denotes $100\mu\text{m}$.

Fig. 4.21. At $t=0$, there are 6 CW laser foci among which two are forming stable gas bubbles while at the other 4 focal spots the laser energy is below the threshold. We keep the CW laser light on. At $5\ \mu\text{s}$, the plasma from the pulsed laser light is observed; a cavitation bubble is generated and rapidly expands over the original sites of the CW laser foci. The 2 gas bubbles are pushed away from the original sites and STBs are formed there. Interestingly, at other laser focal spots small bubbles are also formed and grow gradually ($10\ \mu\text{s}$ - $35\ \mu\text{s}$). Similarly, the original 2 gas bubbles deform the cavitation bubble wall locally inward ($15\text{-}20\ \mu\text{s}$) and gradually merge with the cavitation bubble, after which surface instabilities (Faraday waves) are observed at those sites ($25\ \mu\text{s}$ - $35\ \mu\text{s}$). Later the bubble dynamics is similar as in the previous cases. The formation of bubbles at the sites where the CW laser energy is lower is facilitated by the previously described low pressure effects from the cavitation bubble event. The CW laser may have created cavitation nuclei, yet did not exceed the threshold for bubbles expanding to visible size. This only occurs once the pressure drops. The nature of the nuclei is not understood, it could be regions of higher gas saturation or nanoscopic gas bubbles.

4.3.7.2 Nucleation from continuous wave laser light outside the liquid film

Now we want to check if the above nucleation/growth of bubbles can still occur when the cavitation bubble does not expand over the CW laser foci. To do this, we control the size and position of the cavitation bubble and we also adjust the CW laser energy so that at some focal spots gas bubbles are generated while at other spots the energy is just below the threshold. Examples of such experiments are shown in Fig. 4.22.

In Fig. 4.22 a, there are two laser foci. At the left focus appears a gas bubble while at the right focus no gas bubble is formed. Immediately after the cavitation bubble is generated and starts expansion, a tiny gas or vapor dot appears at the right laser focus ($2.22\ \mu\text{s}$). Then at $4.44\ \mu\text{s}$, it grows to a larger size and forms a tear shaped bubble toward the cavitation bubble while the left gas bubble is jetting away from the cavitation bubble.

Figure 4.21: Expansion of a cavitation bubble over the original sites of 2 surrounding gas bubbles, and 4 focal spots of CW laser light where the laser energy is below the threshold for generating gas bubbles. The interframe time is $5\mu\text{s}$ with an exposure time of 248ns . The scale bar in the first frame denotes $100\mu\text{m}$.

During the shrinkage of the cavitation bubble, it is attracted toward the former and keeps expanding ($6.66\text{-}13.32\ \mu\text{s}$). Finally, when the cavitation bubble rebounds, it jets away and condenses back into the liquid. In contrast, the jetting of the gas bubble on the left side is much slower, and there is still large volume of gas remaining ($15.54\text{-}19.98\ \mu\text{s}$).

Similar phenomena are observed in Fig. 4.22 b, where the two laser foci are close to each other. The jetting direction of the gas bubble on the bottom is not affected by the newly induced bubble ($5\ \mu\text{s}$). A more interesting case is shown in Fig. 4.22 c. There are 4 CW laser foci and only two gas bubbles are formed on the left bottom of the view field. When the cavitation bubble is formed and starts to expand, two tiny gas or vapor dots appear at the other two CW laser focal spots ($2.78\ \mu\text{s}$). Then they grow to larger sizes during the cavitation bubble expansion while the original two gas bubbles are jetting away from the cavitation bubble ($5.56\ \mu\text{s}$). They keep expanding during the shrinkage of the cavitation bubble ($8.33\text{-}13.89\ \mu\text{s}$). It is interesting that after the cavitation bubble rebounds, they deform gradually and finally collapse toward each other, leaving behind only some tiny gas amount ($16.67\text{-}27.78\ \mu\text{s}$).

Here the bubble formation at the CW laser spots with lower energy is probably also due to the lowering of the pressure from the expanding cavitation bubble. However, the newly formed bubbles have different bubble dynamics compared to the originally generated gas bubble(s). They do not jet together with the originally generated gas bubbles during the cavitation bubble expansion. Thus they must have been formed after the passage of the shock front. Besides, they jet toward each other during collapse and condense almost completely back to the surrounding medium after collapse. All these suggest that they are low pressure induced cavitation bubbles.

4.4 Conclusions and outlook

We have demonstrated an experimental technique that allows preparing well controlled gas and cavitation bubbles. A continuous wave laser controlled by a digital hologram

Figure 4.22: Cavitation bubble(s) formed at CW laser focal spot(s) where the laser energy is below the threshold for generating gas bubbles. Pulsed laser-induced plasma and cavitation bubble are shown in the second frame of each case. The timing is indicated on each image frame with scale bars denoting 50 μm . The exposure time of the camera for (a-b) and (c) is 293 ns and 248 ns, respectively.

in combination with a pulsed laser enables the study of complex cavitation-gas bubble interaction.

Shock waves or pressure waves are generated from the pulsed laser induced breakdown and the cavitation rebound. The first shock wave or pressure wave causes an asymmetric collapse of the gas bubble(s) with the formation of a fast jet away from the cavitation bubble. The rebound shock wave or pressure wave leads to another collapse of the gas bubble(s). By varying the size of the cavitation bubble, size of the gas bubble(s) as well as the distance between both, different jetting patterns are observed during the collapse. Depending on the geometric arrangement of an array of gas bubbles, either cascaded or simultaneous collapse of the gas bubbles is observed. Strong influence of the gas bubbles on the cavitation bubble dynamics is also found, e.g. the cavitation bubble deforms on the proximal surface and collapses away from the gas bubbles. Besides, the mutual interaction between the gas bubbles is observed to alter their collapse scenario, i.e. the jet orientation of gas bubbles in close proximity is deviated from the usual direction.

Besides this new observation for jet orientation, a few other interesting phenomena are also found. First, in addition to the two collapses induced by the shockwave or pressure waves, in some cases another collapse of the gas bubble(s) is observed during the expansion of the cavitation bubble. The exact mechanism for this collapse is unknown yet. It might be due to the impulsive flow from the cavitation bubble expansion, or a resonant response of small gas bubble with the cavitation bubble oscillation, or simply the effect of the elasticity of the PDMS layer. Further investigations will be conducted to test these possibilities. Second, the fast expansion of the cavitation bubble leaves a liquid film on the substrate and causes a pressure drop inside or in close proximity to the cavitation bubble. When the cavitation bubble expands over or across the original site(s) of the gas bubble(s), expansion of left-behind gas pocket(s) is observed. The thin film may drain and rupture. Moreover, these low-pressure zones can nucleate cavitation bubbles at the CW laser foci which do not grow "regular" gas bubbles. Third, pronounced oscillations of

left-behind gas bubble are observed after the main cavitation bubble has collapsed. The shape oscillation suggests that jets are formed very similar to that reported by Crum [148] through parametric amplification of the surface modes.

To understand these complex interactions between the cavitation event and the gas bubble(s), numerical modelling is strongly needed. The difficulties are that compressible effects in 3-dimensional geometry have to be resolved. For some of the reported experiments even viscous effects can not be neglected. As a starting point we compare the fast jetting of neighboring bubbles, see section 4.3.5. Dr. Stephen James Shaw (Xi'an Jiaotong Liverpool University) has kindly run first tests of his complex 3D code and the result are reported in Fig. 4.23. When two gas bubbles in close proximity are exposed to a cavitation event (Fig. 4.23 a), their jet orientations (as marked by the yellow arrows) deviate from the radial direction (blue arrows) that is usually observed for a single gas bubble (Fig. 4.23 b). Figure 4.23 c shows the numerical modeling about 3D impact of a planar shock wave (traveling from the bottom to the top) on two close gas bubbles in water. The simulation results agree well with our experimental observations.

Although the gas bubbles from the simulation are of millimeter size, the numerical method looks very promising to resolve the underlying mechanism for the experimental results. The numerical modelling is currently on the way accounting for micron-size gas bubbles for different experimental parameters. These runs take several weeks and months thus will be reported in our future work.

Partially similar to the classical experiments from Dear&Field on millimetre-size 2D bubbles in gelatine, our technique allows the easy generation of arbitrary gas bubble geometries on a scale relevant for biology. We have already applied the induced jets from single cavitation bubble with or without a gas bubble for membrane poration of biological cells, which will be discussed in the following chapter. Yet, more complex studies looking for the effects of the presence of gas bubbles on the rupture of red blood cells could be envisioned. These may help to understand and optimize histotripsy [33], cavitation

Figure 4.23: Change of jet orientation when two gas bubbles in close proximity are exposed to a cavitation event. (a) Experimental results showing that the jet orientation is changed from the usual radial direction (indicated by the blue arrows) to the direction marked by the yellow arrows. (b) Control experiment for one gas bubble exposed to a cavitation event with similar geometric parameters as in (a). The gas bubble is jetting along the radial direction, as marked by the arrow. (c) Numerical simulation for the 3D impact of a planar shock wave of initial strength 200 MPa on two gas bubbles in water initially 2.5 bubble radii apart (x-z plane). The color map indicates the pressure distribution around the gas bubbles.

enhanced HIFU [151] and hemorrhages observed in shock wave lithotripsy [140].

5 Poration of single suspension cells with bubbles in microfluidic confinement

5.1 Introduction

Shock wave-gas bubble interaction may lead to intense jetting flows and high shear stresses. A microinjection mechanism has been suggested for shock wave permeabilization and transfection of biological cells. For instance, when biological cells are near micron-size gas bubbles exposed to a shock wave, they can be temporarily permeabilized with the induced microjet [26]. In contrast, it has been shown that shockwave-gas bubble interaction is responsible for the haemolysis of red blood cells in shock wave lithotripsy [140] and fragmentation of cells for tumour therapy in histotripsy [33]. To promote these beneficial applications while prevent the adverse biological effect, it is important to study the detailed interaction between jetting bubbles and cells, especially on

Figure 5.1: Fast jetting of a gas bubble (*right*) exposed to a cavitation event (*left*). The motion blurred image in the second frame shows the traveling of the jet across the entire gas bubble ($31\ \mu\text{m}$) within a short exposure time of 247 ns. The averaged jet velocity is thus more than 125 m/s.

the single cell level. However, so far only a few experiments have been demonstrated to study this interaction [66, 152].

In Chapter 4, we have demonstrated the generation of gas bubbles with radii of 10-20 μm , which is of the scale relevant in cell biology. Furthermore, fast jetting of the gas bubbles when exposed to a cavitation event has been observed. For example, in the simplest case of a single gas bubble, the impulsive acceleration of the liquid causes an asymmetric collapse of the gas bubble with the formation of a fast jet (with the averaged velocity more than 125 m/s) away from the cavitation bubble, as shown in Fig. 5.1. Therefore, the technique may serve as an experimental method to learn more about the transient gas bubble-cell interaction where the gas bubble is collapsed by the accompanied shock waves from a laser-induced cavitation bubble. The induced microjet might be utilized for localized membrane poration of biological cells.

Sonoporation of suspension cells or single trapped cells with laser induced cavitation bubbles in a microfluidic confinement has been demonstrated previously [65, 66, 152]. The position and size of the laser induced cavitation bubble can be precisely controlled. However, all these techniques require the use of dyes, e.g., trypan blue, for linear absorption of the laser light to generate smaller bubbles. Generally, laser absorption dyes are toxic to biologic cells, which limits their application for laser induced cavitation bubble toward cell transfection and rheology [153]. Recently, it has been shown that vapour microbubbles can be generated via laser-induced localized heating of gold nanoparticles immersed in water [68]. In Chapter 4, we have shown that cavitation bubbles of desired sizes can be generated using plasmonic absorption through gold nanoparticles coated on the glass substrate. Besides, gas bubbles can also be formed by heating the gold nanoparticles using a continuous-wave laser beam. Most importantly, it is known that the gold nanoparticles are biocompatible [154]. Therefore, in this chapter, we will apply gold-nanoparticles coated surfaces for cell membrane poration instead of using laser absorption dyes. Additionally, to keep cells from being flushed away by the bubbles induced

Figure 5.2: A schematic of the gold coated microchannel and cell trapping structures. *Left:* A single PDMS microchannel (*green*) bonded with a glass substrate (*gray*), which is coated with a layer of gold nanoparticle (*brown*). The gold coating layer is within the interior of the PDMS channel. *Right:* Enlarged schematic for arrays of cell trapping structures inside the microchannel. Inset is a single trapping structure. The right image is adapted from Ref.[152].

flows, we adopt the trapping structures from Ref. [152] to immobilize single cells.

Therefore, the present chapter consists of two parts. In the first part, we use the gold coated surfaces to extend the previous work of Li Zhenguo et al. [152] since their work is compromised with the use of cellular toxic dye trypan blue. There, the microjet from a single cavitation bubble near a boundary is utilized for the membrane poration of single trapped cells. In the second part, the microjet from a single gas bubble exposed to a cavitation event is utilized to interact with single trapped cells.

5.2 Materials and methods

5.2.1 Microchannel design

The microfluidic chip used here is a combination of the design in Fig. 4.1 of Chapter 4 with that reported in Ref. [152], see Fig. 5.2. A PDMS layer with arrays of cell trapping structures is bonded with a glass substrate coated with a layer of gold nanoparticles. Care is taken to make sure that the gold coating is inside the channel interior. The

details for the fabrication of the device are the same as in Chapter 4.

5.2.2 Cell culture and preparation

Carcinoma cell lines HeLa and MCF7 are seeded separately in petri dishes with the culture medium (DMEM with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin) and kept routinely in culture (37°C and 5% CO₂). Around 90% confluence, the cells are split, diluted in fresh culture medium, and then washed three times with 1× PBS to get rid of serum and antibiotics. Propidium iodide (PI) dye is added to the cell solution immediately before experiment. Subsequently, the cell solution is passed through a filter with 25 μm pore size to get rid of cell aggregates or undesired debris. Then the cell solution is gently withdrawn to a 1 mL syringe and pumped slowly to the microchannel, which is pre-washed with 1× PBS. Care is taken to avoid introducing gas bubbles for the above process.

MCF7 cells are used for the cell poration experiment with a single cavitation bubble, with a PI working solution of 15 μM while HeLa is applied for one cavitation - one gas bubble experiment with a PI working solution of 30 μM . As PI is toxic to cells after long time, we change the microchannel and cell solution every 1-2 hrs.

5.2.3 Experimental setup

5.2.3.1 Setup utilizing a single cavitation bubble

Similar to the previous setup used for investigating the yield strength of RBCs in Chapter 2.2, we focus a pulsed laser light (532 nm wavelength and 6 ns duration) to generate a single cavitation bubble near a trapped cell, as shown in Fig. 5.3. The fast bubble dynamics and cell response are recorded with a high speed camera at 300k-480k frames per second, after which the imaging system is switched immediately to the fluorescence microscopy. To avoid fluorescence bleaching, a home-built mechanical shutter is utilized to synchronize the fluorescence exciting light with the exposure time of the cooled CCD

camera, which takes an image every 3 s or 6 s. The total time for fluorescence monitoring is 5-10 min. A pulse delay generator controls the above timing for triggering the pulsed laser, high speed camera, cooled CCD camera and the mechanical shutter. For more details of the equipment, please refer to the experimental setup in Chapter 2.2.

5.2.3.2 Setup utilizing one cavitation bubble - one gas bubble

The experimental setup is similar to that utilizing a single cavitation bubble. The only difference is that we add a CW laser to create a gas bubble before the pulsed laser-induced cavitation bubble. The setup and control for the CW laser light is similar to that in Chapter 4. A schematic for the setup and the working principle is shown in Fig. 5.4.

5.3 Results and discussion

5.3.1 Single cell membrane poration by a single cavitation bubble induced microjet

We utilize the microjet of a single cavitation bubble collapsing near the cell trapping structure for single cell membrane poration. We alter the bubble size and distance to the cell to check the effect of the standoff distance γ on the cell uptake, as shown in Fig. 5.5. When the standoff distance is small ($\gamma = 0.22$), as shown in the left column of Fig. 5.5 (A), the cavitation bubble squeezes the cell into the triangular trap during the bubble expansion (0-6.66 μ s). During the bubble shrinkage, the cell volume first restores (9.99 μ s) and later is compressed again by the microjet from the bubble collapse (13.32 μ s, the microjet is not visible but can be deduced from the splitting of the bubble). After the bubble collapses, the cell gradually recovers its shape (16.65-29.97 μ s). The fluorescence microscopy shows that the cell starts to uptake PI dye after around 87 s (*right column*). At 297 s, the entire cell is stained by the fluorescence dye.

Figure 5.3: Experimental setup for single cell membrane poration by the microjet of a single cavitation bubble. (a) The setup is similar to that used for investigating the yield strength of red blood cells in Chapter 2.2. The differences are that we use green exciting light for the PI dye and get red fluorescence emission. Besides, to avoid fluorescence bleaching, we installed a mechanical shutter to synchronize the fluorescence exciting light with the exposure time of the CCD camera. (b) A schematic shows the jetting of a cavitation bubble toward a single trapped cell in the microchannel. The standoff distance from the bubble to the cell is defined as $\gamma = d/R_{max}$, where d is the distance from the bubble center to the cell frontier and R_{max} is the maximum radius of the cavitation bubble.

Figure 5.4: Experimental setup for single cell membrane poration by the microjet of a single gas bubble exposed to a cavitation event. (a) A schematic of the side view: first, a CW laser light is focused to the gold coated microchannel to generate a single gas bubble (B1) near a trapped cell (*red*), then a pulsed laser light is used to create a cavitation bubble (B2). (b) Bottom view: the emitted shock wave from the laser induced breakdown makes the gas bubble (B1) jetting toward the trapped cell. Other settings, i.e., image recording, fluorescence monitoring, timing control, are similar as Fig. 5.3.

Another case with a larger standoff distance ($\gamma = 0.65$) is shown in Fig. 5.5 (B). At $3.33\ \mu\text{s}$, the trapped cell is compressed by the expanding bubble. Then the cell is elongated during the bubble shrinkage and at $6.66\ \mu\text{s}$ we can see clearly that the cavitation bubble jets toward the elongated cell, which leads to a compression of the cell again. Subsequently, the cell gradually recovers to its original shape ($9.99\text{-}283.05\ \mu\text{s}$). The fluorescence images on the right column reveal that a spherical vesicle appears around the impact site of the microjet. It grows gradually to a size of around $5\ \mu\text{m}$. This is a characteristic of cell membrane resealing after mechanical damage without Ca^{2+} in the surrounding medium ($1\times\text{PBS}$) [66]. It indicates that around this standoff distance, the bubble induced microjet is capable of transiently opening the cell membrane. Smaller molecules than PI dye might be able to transfect to the cells at similar experimental condition.

At even larger standoff distance ($\gamma = 1.16$, see Fig. 5.5 (C)), similar cell deformation is observed during the bubble expansion and shrinkage ($0\text{-}6.66\ \mu\text{s}$). However, the microjet produced from the bubble collapse is not able to directly impact on the cell surface, see the remaining two small gas bubbles at $9.99\ \mu\text{s}$. The fluorescence microscopy shows that no uptake is observed even after 10 min. This is because the jet losses most of its strength traveling through the intervening liquid when at a large standoff distance.

To quantify the uptake process, we get the temporal evolution of the mean fluorescence intensity inside the cell shown in Fig. 5.5 (A). The result is shown in Fig. 5.6 (a). It indicates an exponential increase of the fluorescence dye inside the cell, which is typically a diffusion limited process, see the diffusion simulation in Chapter 2.2. It is also noticeable that the cell volume increases after the cavitation event. Therefore, we plot the projected cell area (normalized) as a function of time, as shown in Fig. 5.6 (b). The projected area increases more than 50 % within 5 min. The increasing trend is similar to a previous study [152]. The progressive enlargement of the cell volume is due to osmotic swelling, as the cell cannot reseal the membrane disruption site and extracellular liquid is driven

Figure 5.5: Three exemplary cases for a single cavitation bubble induced microjet toward single cell membrane poration. (A) The left column illustrate the bright field (BF) images for the bubble-cell interaction while the right column shows the fluorescence images (Fluor) of the cell after the cavitation bubble impact. The case shown in (B) is similar to (A) but with a larger standoff distance γ . (C) Another case with an even larger γ . The upper image sequence shows the BF for bubble-cell interaction, and the bottom image is the fluorescence recording of the cell at 10min after the cavitation bubble. The exposure time is 1 μs for all the BF images and 0.5 s for all the fluorescence images. The value of γ is 0.22, 0.65 and 1.16 for the case shown in (A),(B),(C), respectively. For better image quality, a minimized illumination light is combined to the fluorescence microscopy. Here the contrast for the fluorescence images is adjusted for better visualization (not to scale).

Figure 5.6: (a) Temporal evolution of the mean fluorescence intensity inside the cell shown in Fig. 5.5 A. (b) Increasing of the projected area (normalized) with time for the cell shown in Fig. 5.5 A. (c) Statistic of 13 cells for the uptake with respect to the standoff distance.

into the cell by the osmotic pressure imbalance. We also summarize all the studied cases with the standoff distance, see Fig. 5.6 (c). It indicates that most of the cells will show uptake when $\gamma < 0.4$, while at larger standoff distance the uptake probability is very low. Although only a few experiments have been conducted, we reveal the importance of the influence of the standoff distance on the cell uptake.

To reveal more details on the bubble-cell interaction, we now used a higher frame rate ($480k$ fps) with a compromise on the size of the field of view, see Fig. 5.7 (A). At $t=0$, the trapped cell has an almost spherical shape. Then it is pushed by the expanding cavitation bubble to squeeze inside the trap ($2.08\text{-}4.16\mu\text{s}$). Later when the bubble shrinks ($6.24\text{-}8.32\mu\text{s}$), the cell retracts from the trap. At $10.4\mu\text{s}$ the bubble collapses with a microjet toward the cell. After that the cell is strongly deformed ($12.5\mu\text{s}$), with a thinning part at the impact region as indicated by the arrow. Later the cell gradually restores to its original shape ($18.7\mu\text{s}\text{-}10.4\text{ ms}$), with a small bulge where the jet has impacted/squeezed

Figure 5.7: One exemplary case with more details for the bubble-cell interaction and demonstrating localized cell uptake. (A) Bright field image sequences for the bubble-cell interaction and subsequent cell shape recovery. The timing for each frame is indicated at the bottom left corner with an exposure time of 293 ns. The yellow arrows point to the impact region of the microjet. (B) Fluorescence images showing the cell uptake after the cavitation bubble event. The exposure time is 0.3 s for each frame. The arrow indicates the pin-like start point for the uptake.

the cell. This characteristic location indicated with an arrow in Fig. 5.7 A at $t = 10.4 ms$ can also be identified in the sequence taken with the fluorescence camera, see the same arrow in Fig. 5.7 B. The uptake of PI into the cell starts exactly at this jet-impact region and progresses from there into the cytoplasm of the cell, i.e. from $t = 48 s$ to $t = 264 s$.

5.3.2 Single cell membrane poration from gas bubble-cavitation bubble interaction

As shown in Chapter 4 the interaction of cavitation bubble close to a stable gas bubble may lead to a fast microjet. Here we demonstrate its use for cell membrane poration. Results from one experiment are illustrated in Fig. 5.8. In the left column of Fig. 5.8 (A), a gas bubble is formed near a trapped cell at $t = 0$. Then a cavitation bubble is generated on the left side of the gas bubble. At $t = 2.08 \mu s$, we can see clearly the expanding cavitation bubble and the jetting gas bubble toward the cell. The arrow points to the jet-impact region on the cell membrane. Later at $t = 4.16 \mu s$, due to the wall effect of the trapping structure, the cavitation bubble collapses asymmetrically toward the right

direction. Thus it deforms toward and connects with the expanding gas bubble, squeezing together the cell partially into the trapping structure. From $t=6.24-8.32\ \mu\text{s}$, the cavitation bubble merges with the gas bubble on the right side while a liquid jet forms toward the trapping structures on its left side. It is also noticeable that two small remaining gas bubbles are left behind during this asymmetrical collapse of the cavitation bubble (on the left side of the image frame). However, due to the large standoff distance of the cavitation bubble to the trapping structure (similar to the case in Fig. 5.5 (C)), this second jet vanishes before it can impact on the cell ($t=8.32-12.48\ \mu\text{s}$). At $t=4.97\ \text{ms}$, the cell has restored its shape to some extent and the gas bubble also shrinks due to diffusion. The arrow labels the jet-impact region on the cell membrane. The right column shows the fluorescence images. From $t=24$ to $t=36\ \text{s}$, we can still see the dissolving gas bubble on the left side. The cell shows no obvious uptake until $114\ \text{s}$, after which progressive uptake is observed ($144\ \text{s}$ to $20\ \text{min}$).

A similar case with a smaller gas bubble is illustrated in Fig. 5.8 (B). Here a slightly lower frame rate ($300\ \text{kfps}$) is used for larger view field to get more details for the cavitation bubble dynamics. It seems like that after the fast jet of the gas bubble, the major part of the cavitation bubble evolves to jet toward the right direction while the other part deforms and jets away from the gas bubble, leaving two small un-condensable gas bubbles behind. The inset image show cell uptake after $20\ \text{min}$ (*bottom*) compared to the fluorescence image before the bubble (*top*).

5.4 Conclusion and outlook

Uptake can be induced by single bubble oscillations near a trapped suspension cell. Using the gold coating we can generate sufficiently small bubbles to create a microjet able to pierce the cell membrane. Therefore, we do not have to deal with potentially toxic dyes to absorb the laser light [67]. The important parameter controlling uptake is the normalized distance of the bubble to the cell or trapping structure.

Figure 5.8: Preliminary results utilizing the microjet from one cavitation bubble-one gas bubble interaction for single cell membrane poration. (A) *Left column*: bright field images for the bubble-cell interaction. *Right column*: fluorescence images showing the cell uptake after the bubbles. (B) Another similar case with a smaller gas bubble. The main image sequence shows the BF recording for the bubble-cell interaction while the inset images indicate the fluorescence uptake of the cell before and after the bubbles. For both cases, the timing for each frame is indicated on the upper right corner. The exposure time for the BF images is 293 ns while for the fluorescence images is 0.3 s.

A similar approach but using two bubbles and adherent cells has been shown by the research group of Prof. Pei Zhong [66, 68]. They utilize two pulsed lasers to generate very fine jets, while the cells are attached to the substrate. Using one CW laser and one pulsed laser as compared to two pulsed lasers has the advantage of reducing some complexity in the setup. Still more work is needed to understand if stable gas bubbles could offer other advantages. It seems like that the microjet from the gas bubble is faster and thinner compared to that from a cavitation bubble near the trapping structures. It might create smaller and reversible pores on the cell membrane, which is more suitable for localized cell membrane poration. However, the bubble dynamics adds complexity as compared to the single cavitation bubble method. As shown in Fig. 5.8, some part of the cavitation bubble may move dramatically toward and finally merge with the gas bubble, and then extrude together to further squeeze the cell into the trapping structure. This might be a result of the wall effect (from the cell trapping structures) on the cavitation bubble. The shock wave from the pulsed laser induced breakdown is occurring much earlier than the cavitation bubble collapse. Thus we first see the jet of the gas bubble due to the shock wave and only later the translation and jet of the cavitation bubble toward the wall, during which the cavitation bubble merges with the gas bubble before they move together to the structure.

Further work is needed to reveal more details for the bubble dynamics of this method, especially the cavitation bubble evolution. Besides, we need to conduct parameter studies to optimize the microjet from the gas bubble as well as minimizing the wall effects. There may be a possibility to use gas bubbles rather than the trapping structure to fix the cell in place, thus to reduce the complexity of the current setup with on demand traps made of gas bubbles.

6 Conclusion and Outlook

6.1 Conclusions

This thesis deals with the utilization of transient fast flows for measurement of mechanical properties and membrane poration of biological cells in microfluidic confinement. The general theme of this work is the generation and interaction of transient fast flows, particularly through laser-induced cavitation bubble in a narrow microfluidic gap.

The rapid expansion and collapse (within tens of microsecond) of a single laser-induced cavitation bubble causes an impulse-like and strong shear flow in the microfluidic confinement. This provides an opportunity to quantify the yield strength of RBCs under impulsive stretching in Chapter 2.2. In agreement with the previous prediction [92], with a very short exposure time, RBCs can withstand a much larger area strain (approx. 30 %) compared to the classical result from micropipette aspiration, which is only 2-3 % [90]. Beyond this critical strain, excessive cell damage occurs resulting in cell lysis. However, instead of being lysed directly during the cavitation event, the cells evolve to a spherical shape before their burst-like efflux and subsequent diffusive leakage. This helps to shed light on the rupture dynamics of RBCs under mechanical load: Subnanometer sized pores are formed due to excessive stretching of the cell membrane; this results in a colloidal osmotic swelling of the cell to a spherical shape, which finally bursts and ruptures.

Though large deformations of RBCs can be achieved using this method, we find in Chapter 2.3 that cancer cells are hardly stretched under similar experimental condition. The thin biconcave RBCs sediment to the bottom wall of the gap, whereas the spherical

cancer cells RKO extend into the center of the channel. This geometrical difference indicates that the boundary layer flow might be responsible for the stretching results. This motivates a numerical simulation to resolve the full picture of the bubble induced flow. We find an adverse pressure gradient during the late expansion of the cavitation bubble, which leads to a boundary layer separation, flow reversal and vorticity generation close to the boundaries. Strong shear stress is thus generated within the boundary layer and close to the bubble. Outside the boundary layer the shear stresses are considerably lower and very small deformation can be induced. This explains why RBCs can be stretched considerably while the cancer cells are mainly transported by the flow.

In order to probe the deformability of cancer cells and correlate with their invasive potential, another method called hydrodynamic stretching is utilized in Chapter 3. There cells are delivered with inertial focusing to experience a transient extensional stretching flow. We find RKO cells with mesenchymal morphology are more flexible than HCT116 cells with epithelial morphology while both cell lines are from the same origin. Moreover, the deletion of the tumour suppressor *TP53* gene in MCF10A cells results in a significant increase of the cellular deformability as well as morphology change from epithelial to mesenchymal feature. These two different cases suggest that epithelial to mesenchymal transition (EMT) is associated with increased cellular deformability, which might be responsible for the increased invasive potential of mesenchymal cells. We think that this is the first time that a single genetic change is found to be capable of directly altering a cellular biophysical property relevant to the invasive phenotype of cancer. Additionally, the correlation of cellular deformability with the effect of chemotherapy drug (docetaxel) is demonstrated. This kind of correlation helps to discriminate between cell size and deformability for the effect of a specific cancer-treatment drug.

Besides, strong jetting flows will occur when gas bubbles are in the proximity of a single laser-induced cavitation bubble. This transient fast jetting flow in microfluidic confinement is studied in detail in Chapter 4. Gas bubble(s) can be collapsed by the shock

waves / pressure waves from both the pulsed laser-induced breakdown and the cavitation bubble rebound. A fast liquid jet away from the cavitation bubble is formed during the impact of the first shock wave / pressure wave on the gas bubble. The experimental technique utilizing a coated layer of gold nanoparticles on the microchannel substrate allows generating smaller cavitation bubble and complex configurations of gas bubbles. Depending on the geometric arrangement, either cascaded or simultaneous collapse of the gas bubbles is observed. Moreover, when gas bubbles get closer, their mutual interaction becomes pronounced, i.e. the jet orientation of gas bubbles in close proximity deviates from the usual radial direction. Additionally, some novel phenomena from the interaction between gas bubbles and the cavitation event are also presented in Chapter 4, i.e., an extra/additional collapse of the gas bubbles during the cavitation bubble expansion, activation of bubble nuclei when the cavitation bubble expands over or close to them, and pronounced oscillations of surface trapped bubble (STB) following the cavitation event.

The gold nanoparticles coated on the glass substrate in Chapter 4 are cell friendly and allow controlled bubble generation. By using this gold coating we are able to generate a sufficiently small cavitation bubble close to a trapped suspension cell, where the bubble induced microjet is utilized to pierce the cell membrane and induce cellular uptake in chapter 6. We find that the cellular uptake is strongly dependent on the standoff distance from the cavitation bubble to the trapped cell. In this way we improve the previous experiment of Li Zhenguo et al. [67] by avoiding the use of potentially toxic dyes to absorb the laser light. Besides, the microjet from a single gas bubble when collapsed by a cavitation event as studied in Chapter 4 is also exploited in chapter 6 for cell membrane poration. Both methods have shown capability of inducing localized cell membrane poration and molecular uptake. Moreover, compared to the case of a single cavitation bubble near the trapping structures, the microjet from the gas bubble seems faster and thinner, which is promising for generating smaller and reversible pores on the

cell membrane toward the application of localized gene or drug delivery.

In summary, this thesis work demonstrates the potential of utilizing transient fast flows for biomedical application in microfluidic confinement, such as probing the mechanical properties of cells and permeabilization of cell membrane for molecular uptake. The generation of various transient fast flows and their interaction with biological cells are elaborated.

6.2 Future work

Following are some future work for this thesis:

To confirm the simulation results in Chapter 2.3, experimental work will or should be conducted in future to directly visualize the flow field of the boundary layer by using particle image velocimetry (PIV).

For the cancer cell stretching in Chapter 3, further work can be done for both biological and the fluid mechanic parts. To correlate the cellular deformability with the invasive potential of mesenchymal cells, invasion experiments can be performed such as on MCF10A *TP53* KO cells and its wild-type. Besides, to further check the effect of *TP53* gene and pharmaceutical drug on the cellular deformability, a few more cell models should be tested. The dynamic cell shape change as recorded by the high speed camera may allow the mechanical characterization of single cancer cell with more details in the future. Furthermore, the deformability cytometry used in this chapter might be combined with a separation system for deformability based cell sorting. We might collaborate with a numerical group for the design of this system.

Numerical modelling is strongly needed for understanding the complex interactions between the gas bubbles and the laser-induced cavitation event in Chapter 4. Currently a numerical simulation is on the way modelling the deviation of the fast jetting of micro-size gas bubbles in close proximity. Besides, experimental studies could be envisioned to investigate the effects of the presence of gas bubbles on the rupture of RBCs, as it may

help to understand and optimize histotripsy [33], cavitation enhanced HIFU [151] and avoid hemorrhages observed in shock wave lithotripsy [140].

For the experiments of cell membrane poration in chapter 6, in both methods more cells need to be studied to obtain statistically relevant results. Moreover, although the microjet from a single gas bubble is thinner and faster, the presence of the cell trapping structure results in complexity to the bubble dynamics, especially for the cavitation bubble. Therefore, parameter studies are needed to optimize the impact of the microjet from the gas bubble and in the meanwhile minimize the wall effect on the cavitation bubble. In addition, other approaches may be explored to immobilize the cell without using the trapping structure, i.e. using gas bubbles to fix the cell in place , thus to reduce the complexity of the current setup.

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