Probing the Bioeffects of Cavitation at the Single-Cell Level

by

Fang Yuan

Department of Mechanical Engineering and Materials Science
Duke University

Date: _____

Approved: _____

Pei Zhong, Supervisor

Chuan-Hua Chen

Franklin Hadley Cocks

Fan Yuan

Dissertation submitted in partial fulfillment of the requirements for
the degree of Doctor of Philosophy in the Department of
Mechanical Engineering and Materials Science in
the Graduate School of
Duke University

2013

ABSTRACT

Probing the Bioeffects of Cavitation at the Single-Cell Level

by

Fang Yuan

Department of Mechanical Engineering and Materials Science
Duke University

Date: _____

Approved: _____

Pei Zhong, Supervisor

Chuan-Hua Chen

Franklin Hadley Cocks

Fan Yuan

An abstract of a dissertation submitted in partial fulfillment of the requirements for
the degree of Doctor of Philosophy in the Department of
Mechanical Engineering and Materials Science in
the Graduate School of
Duke University

2013

Copyright by
Fang Yuan
2013

Abstract

The primary goal of this dissertation research is to develop an experimental system and associated techniques that can be used to investigate the bioeffects produced by cavitation bubbles at the single cell level. Such information has been lacking due to the randomness and complexity in cavitation inception and subsequent bubble-bubble interaction generated by an acoustic field typically used in therapeutic ultrasound applications. Connection between cavitation activities and bioeffects produced in cells nearby presents another challenge that has not been resolved satisfactorily. In this work, we developed a laser-based system for generating tandem bubbles with a maximum diameter about 50 μm (i.e., on the scale of a single cell) in a microfluidic channel of 25 μm in height and 800 μm in width. We further developed techniques for micropatterning of individual gold dots (15 nm thick and 6 μm in diameter) used for bubble generation, which are precisely aligned at various stand-off distances (SD) from individual islands (32 x 32 μm^2) coated with fibronectin used for cell adhesion. The dynamics of tandem bubble interaction with resultant jet formation, microstreaming and vortex flow in the microfluidic channel were captured by high-speed imaging and particle image velocimetry (PIV). The deformation of the target cell was recorded by high-speed imaging as well (using a second camera) immediately after the tandem bubble interaction and assessment of membrane strain was aided with 2 μm sized polystyrene beads attached to the cell membrane. Membrane poration was characterized by uptake of fluorescent propidium iodide (PI) into the target

cell, from which the normalized maximum pore size was estimated. Using this experimental system, we have observed the complete process of bubble-bubble interaction with resultant jetting flow, cell deformation, and localized pinpoint membrane rupture with progressive diffusion of macromolecules into the target cell. Furthermore, we observed a clear SD dependence in the treatment outcome produced by the tandem bubbles. At short SD of 10 μm , all treated cells underwent necrosis with high yet unsaturated level of PI uptake, indicating that the cell could not reseal the poration site. At intermediate SD of 20 ~ 30 μm , 58% to 80% of the cells were observed to have repairable membrane poration with low to medium but saturated level of PI uptake. At long SD of 40 μm , no detectable PI uptake was observed, corresponding to no membrane compromise. Within the repairable membrane poration group, the sub-population of cells that eventually survived without apoptosis increased from about 9% at SD of 20 μm with strong adhesion to about 70% at SD of 30 μm with no adhesion at the leading edge facing the jetting flow. The maximum PI uptake, pore size, and membrane strain estimated could vary by more than an order of magnitude, which is similar to the magnitude of variations in pore size (0.2 ~ 2 μm) produced by tandem bubbles observed by SEM. The large principal strain ($> 500\%$) with associated high strain-rate ($> 10^6 \cdot \text{s}^{-1}$) produced by the tandem bubbles provide a unique tool to examine the bioeffects of cavitation at the single cell level with potentially a diverse range of applications to be explored.

Contents

Abstract	iv
List of tables	ix
List of figures	x
Acknowledgements	xiii
1. Introduction	1
1.1 Cavitation and its biomedical implications	1
1.2 Interaction of cavitation bubbles with cells	3
1.3 Bubble dynamics and cell rheology	6
1.4 Cell mechanics and cell mechanotransduction	11
1.5 Cell micropatterning in microfluidics	13
1.6 Objectives	15
2. Pulsating tandem bubbles for localized and directional single cell membrane poration	17
2.1 Introduction	17
2.2 Experimental setup	18
2.3 High-speed image and μ PIV	19
2.4 Cell culture and experimental design	20
2.5 Bubble-bubble and microjet-cell interaction	21
2.6 Flow field around the pulsating microbubble(s)	23
2.7 Cell membrane poration	25
2.8 Localized and directional membrane poration	28
2.9 Discussion and summary	30

3. Micropatterning for controlling the generation of cavitation bubbles and precise culture of individual cells in microfluidics devices	32
3.1 Intorduction.....	32
3.2 Materials and methods	35
3.2.1 Design of hybrid patterns in a microfluidic channel.....	35
3.2.2 Patterning of micron-sized gold dots in microfluidic channels	36
3.2.3 Fabrication of hybrid patterns in microfluidic channels	38
3.2.4 Cell seeding into the patterned microfluidic channel	41
3.3 Results and discussion	42
3.3.1 Single bubble from gold dot	42
3.3.2 Single cell patterning in microchannels.....	43
3.4 Summary	46
4. Dynamics of tandem bubble interaction in a microfluidic channel	47
4.1 Introduction.....	47
4.2 Theoretical background	49
4.3 Materials and methods	50
4.3.1 Laser-induced microbubble(s) in the microfluidic channel	50
4.3.2 High speed imaging and flow visulization.....	52
4.4 Results and discussion	53
4.4.1 Dynamics of tandem bubble – the effects of phase delay.....	53
4.4.1.1 General features of tandem bubble oscillation.....	53
4.4.1.2 Effects of phase delay on tandem bubble interaction	57
4.4.1.3 Temporal development of the tandem bubble oscillation.....	59
4.4.1.4 Jet velocity	61

4.4.2 Characterization of tandem bubble interaction and jet formation in the preserce of single cells nearby	62
4.5 Summary	64
5. Biological and mechanical responses of single cells to cavitation bubbles – effects of standoff distance and adhesion pattern	65
5.1 Intorduction.....	65
5.2 Materials and methods	66
5.2.1 Experimental system and sample preparation.....	66
5.2.2 Cell treatment and image acquisition.....	68
5.2.3 Analysis of PI uptake and pore size estimation	70
5.2.4 Analysis of cell membrane deforamtion, strain and strech ratio estimation....	72
5.2.5 Data analysis and statistics.....	74
5.3 Results and discussion	74
5.3.1 PI uptake	74
5.3.1.1 Kinematics and general features of PI uptake in the target cell.....	74
5.3.1.2 Assessment of pore size variation, apoptosis and phenotype	78
5.3.4 Cell membrane deformation, strech ratio and strain calculations.....	82
5.4 Summary	93
6. Future work.....	95
References.....	98
Biography.....	109

List of Tables

Table 5.1: Three different outcomes in membrane poration and PI uptake following tandem bubble-cell interaction at various standoff distances (SD) in group “H” and group “I”	77
Table 5.2: The fate of treated cells with PI uptake	81
Table 5.3: Summary of strain calculation results from 6 cases with different SD s and adhesion patterns.....	93

List of Figures

Figure 1.1 A spherical bubble in an infinite field of liquid	6
Figure 1.2 Decomposition of flow field.....	9
Figure 1.3 Cell deformation due to extensional flow.....	10
Figure 2.1 Experimental setup – tandem bubble.....	19
Figure 2.2 Dynamics of tandem bubble and bubble-cell interaction.....	22
Figure 2.3 Modeling results of the oscillation of tandem bubble (bottom row) compared with the experimental results from high speed imaging (top row).....	23
Figure 2.4 Fluid motion.....	24
Figure 2.5 Deformation of the imaginary material lines in the flow field around tandem bubble.....	25
Figure 2.6 Treatment of two individual Rat mammary carcinoma cells at SD = 20 mm by the tandem bubble.....	26
Figure 2.7 Trypan blue uptake.....	27
Figure 2.8 Membrane poration.....	29
Figure 3.1 A schematic diagrams of the microfluidic chip design layout.....	35
Figure 3.2 Schematic diagrams showing the protocol for fabrication of microfluidic chip with gold dot patterns	36
Figure 3.3 A schematic diagram of the fabrication procedures used for micropatterning on the surface of glass substrate.....	39
Figure 3.4 A schematic diagram of the procedures involved in microchannel construction and cell seeding.....	40
Figure 3.5 Dynamics of single bubble oscillation produced by laser absorption from a single gold dot patterned on the glass bottom of a microfluidic channel.....	42

Figure 3.6 Single cell patterning in proximity to a pair of gold dots.....	44
Figure 3.7 Cell seeding in the microchannel with patterned adhesive regions.....	45
Figure 4.1 A schematic diagram of the experimental setup for laser-generated tandem bubble in a microfluidic channel.....	51
Figure 4.2 Laser generated tandem bubble oscillation and jet formation produced inside the PDMS-glass microchannel.....	54
Figure 4.3 Dynamics of laser generated tandem bubble oscillation and jet formation produced inside PDMS-glass microchannel.....	58
Figure 4.4 Temporal development of the top and bottom poles of the tandem bubble....	60
Figure 4.5 Relation between jet velocity and time delay between generation of the first and second bubbles.....	61
Figure 4.6 Dynamics of tandem bubble interaction at different <i>SDs</i> in two adhesion groups “H” and “I”.....	62
Figure 4.7 Jet speed produced by tandem bubble interactions with the presence of a single cell at different <i>SDs</i> from B_1 in two adhesion groups “H” and “I”.....	63
Figure 5.1 Timeline of imaging acquisition before and after tandem bubble-cell interaction.....	69
Figure 5.2 General observation of cell morphology change and PI uptake after bubble-cell interaction	75
Figure 5.3 Time course of PI uptake in the target cell after tandem bubble interaction at different standoff distances (<i>SD</i>).....	76
Figure 5.4 Intracellular PI amount at saturation level	78
Figure 5.5 Normalized maximum pore size of individual cells	79
Figure 5.6 Time course of phenotype changes of survival and apoptotic cells after tandem bubble treatment	80

Figure 5.7 Schematic diagrams illustrating tandem bubble-cell interaction and associated flow field produced in a microfluidic channel.....	83
Figure 5.8 Deformation of a HeLa cell grown on a “I” pattern with beads attached on the membrane at a SD of 30 μm	84
Figure 5.9 Regional difference in membrane displacement of a HeLa cell on “H” pattern and SD = 30 μm	84
Figure 5.10 The stretch ratio of 4 line segments defined between adjacent two beads attached on the cell membrane (see Fig. 5.9).....	85
Figure 5.11 Deformation of the leading edge of a HeLa cell on “I” pattern and SD of 20 μm	86
Figure 5.12 Time history of the displacement trajectories of 5 beads near the leading edge of a target cell in Fig. 5.11 along the jet direction.....	87
Figure 5.13 Deformation of two HeLa cells on “I” pattern in response to the jetting flow produced by the tandem bubbles at SD = 20 μm (top row) and SD = 40 μm (bottom row).....	88
Figure 5.14 Comparison of membrane deformation of individual cells grown on “H” vs. “I” adhesive patterns.....	89
Figure 5.15 Characteristic time course of the principal strains and projected normal strain along (ε_x) or perpendicular (ε_y) to the jet direction near the leading edge of the target cell (see Fig. 5.14).....	90
Figure 5.16 Illustration of different bi-axial strain configurations produced the jetting flow from tandem bubbles in the proximal region of a cell.....	91
Figure 5.17 Examples of deformation of cells grown on “H” and “I” patterns produced by tandem bubbles at different standard off distances.....	92

Acknowledgements

I am truly indebted to many people for their help and support during my time as a Ph.D. student. Foremost, I would like to thank my advisor, Dr. Pei Zhong, for providing incredible help through my graduate studies (experiments, presentations, and publications) and especially during this dissertation work. I am very grateful for the opportunity to work in his group so I have learned to become a better researcher. I am also grateful to my dissertation committee members, Drs. Chuan-Hua Chen, F. Hadley Cocks, Fan Yuan and Stefan Zauscher for their insightful critiques and valuable advice on my project.

I would like to thank Dr. Georgy Sankin who has worked with me in the lab for most of my Ph.D. program. His innovative ideas and enthusiasm for scientific research have been extremely motivating to me. I am also grateful to Xiaoyi Huang, Jaclyn Lautz, Dianna Liu, Chen Yang, Ying Zhang and Joseph Kleinhenz, who have spent time and helped me on the tandem bubble project. I also want to thank Drs. Neal Simmons and Nathan Smith. It was a great pleasure working with you in the same group.

I would also like to thank several staff members at Duke and outside for giving me tremendous technical support during my research. They are Jay Dalton from SMIF, Yasheng Gao from LMCF, and Bill Faught from Zeiss.

I would especially like to thank my parents, Aisheng Yuan and Jiayun Li, far away in China for their patience and love through the past eight years.

1. Introduction

1.1 Cavitation and its biomedical implications

Cavitation is the formation and subsequent dynamics of gas or vapor bubbles in a liquid, induced either by acoustically or hydrodynamically produced tension, or by local energy deposition from laser or high-energy particles [1]. Acoustic and hydrodynamic cavitations are efficient in producing physical changes in a bulk solution, while optic and particle cavitations are typically related to localized individual bubble dynamics [2].

Hydrodynamic cavitation has been exploited in the past decade, as a new technique for large-scale microbial cell disruption and extraction of intracellular components. Compared to conventional cell disintegrators, such as high-speed agitator bead mills and high-pressure homogenizers, the disruption efficiency per unit energy can be increased by 10-20 folds using hydrodynamic-driven cavitation reactors [3]. Enzyme and protein can be selectively released [4], depending on their location in the cell, periplasm or cytoplasm [5].

Ultrafast (picosecond and femtosecond) laser has been applied to remove organelles (mitochondria and lysosomes) or to cut sub-cellular structures (actin filaments, microtubules and mitotic spindles) in living cells [6]. Laser-assisted photoporation has been developed for contact-free, non-disruptive, and targeted transfection of individual cells using CW [7] or pulsed [8] lasers. With pulse repetition frequency (PRF) in the kHz range, cavitation bubbles are thermoelastically generated to enable nano-dissection or

even membrane perforation. In comparison, at a PRF in the MHz range, nano-dissection is mainly produced by the accumulative effects of chemicals breaking integrity of macro-biomolecules. Because the mechanical disruption induced by inertial cavitation bubbles can be highly violent, the energy required for nanosurgery with kHz pulse is normally only 10 percent of that with MHz pulses [9].

Acoustic cavitation generated by ultrasound (US) provides a unique non-invasive tool in a diverse range of therapeutic applications, including shock wave lithotripsy [10], high-intensity focused ultrasound (HIFU) [11], and ultrasound-mediated drug, gene and siRNA delivery [12]. Ultrasound can be used for acoustic detection, and also for producing desirable changes in biological structures and processes [13]. Shock wave induced cavitation can damage cells but may also alter cell membrane integrity without causing cell death. The latter phenomenon has been used to deliver plasmid DNA into cells, leading to transgene expression and augment chemotherapeutic effects when applied concomitantly with a suitable drug [14]. Ultrasound has evolved rapidly from a diagnostic modality into a promising tool for non-invasive therapy and drug delivery [15]. High intensity focused ultrasound has been used for tissue destruction (ablation) [16, 17], blood clot dissolution (thrombolysis) [18]) and vascular occlusion (acoustic hemostasis) [19], as well as non-invasive opening of blood-brain-barrier (BBB) [20]. Further development of HIFU therapy entails shortening treatment time, minimizing undesirable prefocal damage [11, 21] and real-time monitoring tissue damage [22]. At moderate intensity, ultrasound assisted by ultrasound contrast agents (UCAs) has been shown to

temporarily permeabilize the cell membrane and drive the uptake of DNA, proteins, and drugs from extracellular environment [23, 24]. Therapeutic agents can be concentrated in high-capacity drug carriers made of liposomes and polymers. Ultrasound can be used to activate lipid- or protein-based vesicles that encapsulate both gas and drug inside at the site of interest [25, 26]. With therapeutic agents directly delivered to a specific region of cells or tissue, systemic toxicity can be minimized. Acoustic cavitation can also facilitate, via mechanical or thermal means, drug transport across impermeable interfaces to reach the target tissue, across blood-brain-barrier, and in solid tumors and blood clots [18, 27].

1.2 Interaction of cavitation bubbles with cells

Acoustic cavitation can be categorized into non-inertial or inertial cavitation, depending on the amplitude and frequency of the ultrasound exposure. In non-inertial cavitation, a bubble is driven into oscillation with relatively small change in size. The membrane permeability of a cell in close proximity can be altered by shear stresses associated with microstreaming produced by oscillating bubbles [28]. In contrast, an inertial cavitation bubble expands more than 3 times of its initial size and then implodes violently [29]. The presence of a rigid boundary nearby can cause the inertial bubble to collapse asymmetrically, leading to the formation of a high-speed liquid jet directed towards the boundary. Jet formation from asymmetric bubble collapse can concentrate momentum and energy into a small area, often strong enough to lyse the target cell. Moreover, when an inertial bubble collapses symmetrically at a large distant from a

boundary, extremely high temperature and pressure can be produced during the final stage of the bubble collapse, rendering the formation of sonochemically induced free radicals which are cytotoxic [30]. *In vitro* studies have demonstrated that cavitation can produce, in addition to cell lysis, multiple sublethal cellular effects, including changes in i) cell morphology; ii) membrane permeability; iii) cell proliferation; iv) genetic DNA damage or mutation.

Although acoustic cavitation is known to be strongly associated with cellular bioeffects [31], the exact mechanism by which cavitation bubbles perturb the cell membrane and induce subsequent cellular responses remains illusive. Despite that cavitation-induced shear stresses and microstreaming have been suggested as a primary mechanism for cell membrane permeabilization [32, 33], how cells respond to the high strain-rate loading produced by cavitation bubbles is largely unknown. The complexity of cavitation-induced flow field, non-uniform bubble size, and bubble-bubble interaction and jet formation in acoustic cavitation make it difficult to achieve controllable sonoporation outcome [34].

The capability to investigate cavitation-induced bioeffects at the single-cell level may help improve the efficacy and safety in modern biomedical applications of therapeutic ultrasound. Two critical processes involved in designing a cell-bubble interaction model are: *i*) to bring bubble(s) and cell(s) close to each other for interaction; *ii*) to control the characteristics of the bubble oscillation and therefore the resultant bioeffects on the cell(s). For example, individual xenopus oocytes, as a single cell model, have been used

to assess the variation of transmembrane ion flow via voltage-clamp technique during US exposure [35]. More sophisticated experimental systems are required to achieve a similar spatiotemporal specificity on the cellular events if only individual micron-sized biological cells are targeted by cavitation activities [36]. Individual UCA was manipulated by buoyancy or optical tweezers to place on the surface of a target cell, and bubble oscillation was activated by an incident ultrasound wave. Depending on the cavitation intensity, the target cell membrane could either be permanently ruptured by a strong liquid jet [37] from inertial collapse of the cavitation bubble or reversibly perforated by microstreaming associated with non-inertial cavitation [38]. However, the response of UCAs to ultrasound exposure showed significant variations due to shell non-uniformity and mechanical rigidity, which could also be influenced by the chemical composition of the shell [39]. Therefore, a statistically significant correlation between bubble dynamics and resultant biological effects is often difficult to establish.

In contrast to acoustic cavitation, optical cavitation produced by a focused laser pulse is highly reproducible with the resultant bubble dynamics much more predictable. Laser induced cavitation has been produced in microfluidic devices to generate single bubble events in a confined quasi-2D space [40]. The microfluidic channel, constructed using glass or PDMS, allows light transmission for optical imaging and manipulation. Microfluidic systems also enable cell confinement and monitoring of cell deformation and growth over time [41]. For example, microfluidic devices have been used to monitor cell culture [42] and to analyze gene expression activities [43]. Overall, microfluidics

technique provides an attractive platform for investigating bubble-cell interaction and the resultant bioeffects produced by cavitation bubbles at the single cell level.

1.3 Bubble dynamics and cell rheology

Fig. 1.1 A spherical bubble in an infinite field of liquid

Generation of cavitation bubbles typically involves a phase change from liquid state to vapor/ gas state, fostered either by underpressure or superheating. Sir Lord Rayleigh first analyzed an isolated spherical bubble of radius $R(t)$ (t is time) in an incompressible liquid of density ρ_L under driven pressure $p_\infty(t)$. Assuming spherical symmetry throughout the bubble oscillation, dynamics of a single bubble in 3-D without nearby boundaries can be described by the Rayleigh-Plesset (R-P) equation [44]:

$$\frac{p_B(t) - p_\infty(t)}{\rho_L} = R \frac{d^2 R}{dt^2} + \frac{3}{2} \left(\frac{dR}{dt} \right)^2 + \frac{4\nu_L}{R} \frac{dR}{dt} + \frac{2S}{\rho_L R} \quad (1.1)$$

Here, $p_b(t)$ is the gas pressure inside the bubble, ν_L viscosity of the surrounding fluid, and S surface tension. The L.H.S. of (1.1) presents the pressure difference across the bubble wall. On the R.H.S of (1.1), the first two terms are related to inertial force, and the third and fourth terms account for the effects of viscous stress and surface tension, respectively.

Several different types of cavitation-based applications in microfluidic systems have been explored, including mixing [45], micropump [46], switching [47], and cell lysis [48]. Among all these applications, one unique feature worth noting is that inertial bubble oscillation can carry surrounding flow to the regime of high Reynolds number within a several hundred μs , which is usually difficult to acquire in conventional microfluidic devices. Dynamics of single cylindrical bubble in this quasi-2D geometry can be described by the 2-D R-P equation [49]:

$$\left(\frac{d^2 R}{dt^2} + \left(\frac{dR}{dt} \right)^2 \right) \log \frac{R}{R_\infty} + \frac{1}{2} \left(\frac{dR}{dt} \right)^2 = \frac{p_\infty}{\rho_L} \quad (1.2)$$

where R_∞ is the distance at which fluid velocity drops to zero. Accounting for only fluid inertia, viscous term and surface tension term are ruled out compared to 3-D governing equation [50]. Justification of this change is made by examining Reynolds number (Re , Eq. 1.3) and characteristic diffusion time of the viscous boundary (τ_{visc} , Eq. 1.4):

$$Re = \frac{2R_{\max}^2}{t_c \cdot \nu_L} \quad (1.3)$$

$$\tau_{visc} = \frac{H^2}{4 \times 1.72^2 \nu_L} \quad (1.4)$$

Here, ν_L is liquid kinematic viscosity, R_{max} the maximum bubble radius, t_c bubble collapse time, and H channel height. Stokes flow can be ruled out if $Re \gg 1$; cylindrical bubble shape and 2-D potential flow hold true if τ_{visc} (e.g., $\sim 53 \mu s$ for $H = 25 \mu m$) is longer than t_c (e.g., $\sim 10 \mu s$ for $R_{max} = 25 \mu m$).

Two bubbles oscillating in-phase in a quasi-2D configuration has been studied recently [51], assuming 2-D potential flow and cylindrical bubble shape. By adapting Lagrangian

$$\left(\ddot{R}_1 R_1 + \dot{R}_1^2\right) \log \frac{R_1}{R_\infty} + \frac{\dot{R}_1^2}{2} = \frac{P_\infty}{\rho_L} - \frac{U_1^2}{2} - \left(\ddot{R}_2 R_2 + \dot{R}_2^2\right) \log \frac{r_{12}}{R_\infty} \quad (1.5)$$

formalism [52], Laplace equation was derived similar to the 2-D R-P equation but with two additional terms on the R.H.S. of (1.5). On the R.H.S of (1.5), the second term represents dynamic pressure reduction from bubble translation, and the third term accounts for the effect of the imposed pressure from a neighboring oscillating bubble. Because of the simplification in spherical bubble geometry and the neglect of thermal and mass transfer across the bubble wall, this model is limited to later stage of bubble expansion (cylindrical shape already developed) and the earlier stage of the 1st bubble collapse (without strong elongation or jet formation).

Bubble oscillation can be modeled using boundary element method (BEM) [90, 97]. Based on the assumption for an irrotational and incompressible flow, a velocity potential ϕ can be introduced that satisfies the Laplace's equation:

$$\nabla^2 \phi = 0 \quad (1.6)$$

Eq. 1.6 can be solved with a boundary integral method using Green's function G , which

can be assumed to be harmonic as in the form:

$$G = -\frac{1}{|x - y|} \quad (1.7)$$

Where x is a point in the integration domain, and y is a point on the boundary surface S . With this simplification, the velocity potential can be expressed by:

$$\Omega\phi(x) = \int_S n_y \cdot [\phi(y)\nabla G(x, y) - G(x, y)\nabla\phi(y)] dS \quad (1.8)$$

where Ω is the solid angle at x , and n_y is the local normal unit vector of an elementary surface dS . Eq. 1.8 indicates that if the velocity potential ϕ and its normal derivatives are known on the boundary surface S of a domain V , where ϕ satisfies the Laplace equation, then ϕ can be determined anywhere in V by integration over the boundary surface. This BEM has been widely used to simulate bubble-bubble interaction due to its computational efficiency [90, 97, 99].

Figure 1.2 Decomposition of flow field [48]

Following the Cauchy–Stokes decomposition theorem, an arbitrary instantaneous state of flow motion may be resolved at each point into a uniform translation, a dilatation along three mutually perpendicular axes, and a rigid rotation of these axes. The flow field

around an oscillating bubbles can therefore be decomposed into 3 components (Fig. 1.2), i.e. translational flow, extensional flow and rotational flow [53]. Only extensional flow can deform a suspended cell [54], which can even be ruptured if the areal strain on the cell membrane exceeds 2-3% [55]. An adherent cell may deform when exposed to extensional, translational or rotational flow because of the anchorage of cell onto supporting substrate. Cell poration may be triggered because of the stretch along the flow direction or the velocity gradient across the flow direction.

Cell deformation induced by flow can be inertial-/viscous- dominant (Fig. 1.3)

Figure 1.3 Cell deformation due to extensional flow

determined by the relative magnitude of fluid inertial and cell membrane tension [53]. If cell membrane is very weak compared to fluid inertial, cell deformation can be described directly by flow kinematics (jet impact); otherwise cell deformation is mainly caused by tangential viscous stresses exerted by fluid on non-slip cell wall boundary (microstreaming). Cell deformation from hydrodynamic flow induced by bubble oscillation may involve both mechanisms considering dissipation of fluid inertial with time, and entire cell is initially deformed by fluid inertial followed by only viscous stress on cell membrane.

Previous studies have been shown improved efficiency in cell lysis by modifying the shock wave front from a typical lithotripter pulse [56]. Cell lysis was increased one fold by re-focusing the shock wave front. Analogous to this idea, the energy from a single bubble collapse can also be focused by a second bubble oscillating anti-phase with respect to the first bubble. By increasing the velocity gradient along the cell membrane, cell deformation may be enhanced by two pulsating bubbles with comparable size to a cell.

1.4 Cell mechanics and cell mechanotransduction

Cells are highly complex multiscale systems composed by components with distinct mechanical, chemical and biological properties [57]. The primary cell mass comes from membrane and cytoskeleton, which together maintain cell integrity and cell shape. Cells can respond and adapt to external mechanical stimuli, accompanied by changes in morphology and signaling pathways that ultimately direct the function and fate of the cell. In this process, the cytoskeleton plays a central role transferring the stimuli-related structural deformation across the entire cell body, through actin microfilaments, microtubules and intermediate filaments [58]. Cell response to external mechanical stimuli starts with the *in situ* physical membrane deformation, and thereafter the strain is transmitted through the cytoskeleton elements to distal sites. The stimuli at different sites may then simultaneously convert to chemical activities followed by the downstream activation of transcription factors and upregulation of protein expression [59, 60].

Original efforts on theoretically modeling cell mechanics were based on the continuity assumption at a cellular length scale, so a constitutive model can be used to describe phenomenologically the cell properties. With this assumption, the cytoskeleton can be modeled as a simple viscoelastic continuum, porous gel and soft glassy material. A classical example is the application of linear viscoelastic constitutive model to estimate the Young's modulus based on micropipette aspiration of leukocytes. In general, these models can work properly as long as the scale of the interested region is significantly larger than its microstructural characteristics by several orders of magnitude. The subcellular structures, however, are spatially heterogeneous with different micro- to nano-compartments. For example, the fibrous and other discrete cytoskeleton elements contribute differently in cell mechanotransduction, and the external stimuli is likely to be redistributed or integrated at the subcellular level. This type of decentralization nature is normally included in discrete micromechanics models [61].

The mechanical property of cytoskeleton has been characterized previously under quasi-static loading conditions, but the results often vary significantly depending on the experimental conditions. Without the intervention of an external force, the cytoskeleton structures can be studied by monitoring the Brownian motion of inherent or introduced particles, in which fluorescence correlation spectroscopy and dynamic light scattering are often employed. The cell rheology can also be probed by applying external forces, such as micropipette aspiration, microplates, optical traps, magnetic traps and atomic force microscope [62]. All these methods, however, cannot mimic the high strain-rate loading

produced by cavitation bubbles. We therefore need to develop new tools that can be used to study cavitation bubble(s)–cell interaction and the associated cell mechanics relevant to therapeutic ultrasound applications.

1.5 Cell micropatterning in microfluidics

Cell micropatterning allows precise spatial control of the location and geometry of single cells on a patterned substrate surface [63]. Previous studies have shown that cell morphogenesis and functions depend on the *in situ* characteristics of the cell occupied region, such as topography [64], chemistry [65], wettability [66], roughness [67] and surface charge [68]. All these microenvironmental cues contribute to the localized cell adhesion machinery, which can further influence cell architecture and polarity, and regulate cell growth and differentiation [69]. Cell micropatterning on a surface can be achieved by either chemical or physical means. Chemically, cells can be selectively attached to bio-adhesive regions surrounded by non-adhesive background [70]. Physically, cells can be confined within topographically or structurally distinguishable spaces [64]. By manipulating the localized surface property, cell micropatterning techniques can be used to investigate how cells sense changes and function in a diverse range of microenvironments [71]. The engineered substrate can be extremely valuable in mimicking the *in vivo* tissue structure that cannot be easily implemented in a conventional *in vitro* cell culture system. Besides the fundamental cell biology studies

[72], cell micropatterning has also been exploited in the field of cell-based sensing [73], tissue engineering [74], and drug screening [75].

Chemical micropatterning defines region-specific cell adhesiveness, preparing surface for cell adhesion with exclusive spatial resolution at single protein/receptor molecular level [76]. Cell-substrate adhesion can be enhanced by coating the surface with poly-lysine, extracellular matrix proteins (*e.g.* fibronectin, laminin, and collagen), or RGD (Arginine-Glycine-Aspartic amino acid) containing synthetic polymers. To prevent cells from growing beyond adhesive regions, non-fouling (non-adhesive) molecular systems have been widely used, such as bovine serum albumin, phospholipids, ethylene glycol derived polymers, and hydrogels (*e.g.* agarose, PVA, PAA). A variety of methods have been developed to successfully produce micropatterns: photo-(*e-beam*, x-ray) lithography [77], micro-contact printing [78], stencil-assisted patterning [79], laser/UV etching [80], microfluidic patterning [81], UV-induced photochemical patterning [82]. Recent efforts on surface micropatterning have been made to fabricate physico-chemically dynamic/switchable surfaces [83], whose bio-adhesiveness *in situ* can be changed under stimulus of temperature [84], electric potential [85] or light [86]. These kinds of surfaces have been applied for co-culturing multiple types of cells [87] and exploring dynamic cell behaviors [88].

Integration of micropatterned substrate surfaces into microfluidic-based cell culture systems has opened up new opportunities to perform single cell analysis because of

easier handling of individual cells, precise control of microenvironments for cell cultures, and faster detection of cell behavior with smaller reagent volume. Although two-dimensional (2-D) cell cultures on patterned substrate surfaces enclosed in microfluidic systems do not mimic *in vivo* 3-D physiological environments, they remain to be widely used research approaches because of its simplicity and low operation cost. All patterned cells sitting on the same focal plane can be monitored in real-time using conventional microscopy; transport of fluids and soluble factors can be regulated in precisely defined spatiotemporal patterns; targeted cells can be easily presented with controllable and reproducible external stimulus. These advantages facilitate high speed manipulation and detection of a number of cells for highthroughput screening. Integration and automation are two ultimate goals for the development of cell-base microsystem under the framework of so-called ‘micro-total-analysis-system’ (μ TAS), where all typical operation units (e.g. growth, treatment, selection, lysis, separation and analysis) are incorporated into a single robust microdevice [89].

1.6 Objectives

Recent mechanistic studies on the bioeffects of cavitation bubble have shown the ability to establish or isolate an observation window, preferably at single cell level, to monitor the response of individual target cells with high spatiotemporal precision [36, 37]. Membrane poration is recognized as one of the major processes responsible for

majority of the biological consequences. However, the complete process starting from the bubble oscillation, followed by bubble-cell interaction, cell deformation, pore formation, molecules uptake, pore resealing, and finally phenotype development has not been successfully investigated in sequence. In this dissertation study, we will establish a new experimental system to monitor and reproduce all the events ever happened in order to make a close connection between them. In chapter 2, we demonstrate the tandem bubble technique for localized and directional cell membrane poration which provides a unique approach to apply precisely mechanical stresses on individual biological cells. In chapter 3, we introduce a method for fabricating paired gold dots in a microfluidic channel where tandem bubbles can be initiated in a biocompatible microenvironment that is also used for patterned cell culture, treatment and analysis. In chapter 4, we evaluate the dynamics of tandem bubble interaction in the microfluidic channel under different phases without the presence of a cell nearby. We also examine tandem bubble interaction with anti-phase in the presence of a cell nearby. In chapter 5, we investigate the bioeffects produced by the tandem bubbles on single cells grown on different adhesion patterns and at various stand-off distances. The characteristics of membrane poration, membrane deformation and phenotype of the cell after the treatment are evaluated and compared. Finally in chapter 6, future work is recommended.

2. Pulsating Tandem Bubbles for Localized and Directional Single Cell Membrane Poration

2.1 Introduction

Cavitation, the formation and dynamics of vapor/gas bubbles in aqueous medium, plays a pivotal role in a diverse range of biomedical and engineering applications [12, 90]. An oscillating bubble can release energy upon symmetric collapse in the form of heat, light and shockwave [91] or high-speed liquid jet due to asymmetric collapse either near a boundary [90] or under the influence of an impinging shock wave [92]. In contrast to damaging ship propellers and hydraulic structures [93], the power of cavitation has often been harnessed in biomedical applications for the treatment of cancers by high-intensity focused ultrasound, non-invasive disintegration of kidney stones by shock wave lithotripsy, and ultrasound-mediated drug and gene delivery [12]. However, understanding the dynamic bubble-cell interaction and its causal relation with the biological consequences still remains to be a significant challenge. Only a few studies have been performed to investigate bubble-cell interaction under well-controlled experimental conditions, using either a small air bubble [32, 94] or ultrasound contrast agents as stabilized cavitation nuclei [37] in an acoustic field, or a laser-generated millimeter-size bubble to interact with adherent or suspension cells [41]. Overall, these previous studies are limited in resolving the transient bubble-cell interaction, associated fluid dynamics, and membrane poration by the cavitating bubble, as well as subsequent transport of macromolecule into the cell.

In this chapter, we introduce a method for generating individual pairs of tandem bubbles in close proximity that leads to alternating formation of microjets in opposite directions. The transient bubble-bubble and microjet-cell interaction, as well as the resultant microstreaming and vortex formation are characterized by high-speed imaging and micro-Particle Image Velocimetry (μ PIV). Using this method, localized and directional membrane poration in single biological cells can be produced in an orientation and proximity dependent manner.

2.2 Experimental setup

A Zeiss Axio Observer inverted microscope was configured to incorporate two laser beams for tandem bubble generation and high-speed cameras for imaging of bubble dynamics, bubble-cell interaction, and μ PIV of fluid motion (Fig. 2.1a). Two Q-switched Nd:YAG lasers of 5 ns pulse duration (MiniLase I (LASER1) and Solo PIV (LASER2), New Wave Research) with wavelength of 1064 nm and 532 nm, respectively, were transmitted through a 63X long working distance objective. The incident angle of LASER1 was slightly tilted so that the foci of the two laser beams in the image plane of the objective were separated by a distance of $\Delta = 40 \mu\text{m}$. Each laser beam was preferentially absorbed by the fluid medium containing 0.4% Trypan blue in a microfluidic channel (25 μm in height, Fig. 2.1b), generating a single bubble via optical breakdown [41, 91, 95]. By controlling the release of individual laser pulses using a

Figure 2.1 Experimental setup. (a) tandem bubbles are generated by two pulsed Nd:YAG lasers, which are separated by $\Delta = 40 \mu\text{m}$ in the focal plane of a 63 $\times$ objective. (b) Microfluidic exposure chamber with 25 μm in height, 10 mm $\times$ 25 mm in channel width and length.

digital delay generator (BNC 555, Berkeley Nucleonics Corporation), tandem bubbles with different oscillation phases can be produced.

2.3 High-speed imaging and μPIV

Two 45°-dichroic mirrors (900dcsp and z532dcrb, Chroma Technology Corp.) were used for reflection of the laser beams and simultaneous transmission of illumination light for high-speed imaging. A fiber-optic coupled Xenon flash lamp (ML-1000, Dyna-Lite) was used to provide backlight illumination, partitioned into an 80/20 intensity ratio between two high-speed cameras (Imacon 200, DRS Hadland and Phantom v7.3, Vision

Research). The Imacon 200 was operated at 1 million frames /second, 50 ns exposure time, and 14 frames per sequence to capture the dynamics of bubble-bubble-cell interaction. The Phantom camera was used to record the subsequent uptake and progressive diffusion of the Trypan blue into the targeted cell within 1 minute. Moreover, a Shimadzu HPV-1 camera operated at 1 million frames/second, 250 ns exposure time, and 100 frames per sequence was used for μ PIV. For flow visualization, polystyrene beads of 2 μ m in diameter (R0200, Duke Scientific Corp) were added in the medium as tracers at 1×10^9 beads/ml concentration. The acquired images were processed using DaVis 7 software (LaVision, GmbH) for determining the velocity field around pulsating bubble(s).

2.4 Cell culture and experimental design

Rat mammary carcinoma (R3230Ac) cells maintained routinely in cell culture (37⁰C) were trypsinized and re-seeded in a 60-mm diameter Petri dish at 10^5 cells/ml concentration one day before the experiment. When cell growth reached about 10% confluence, the culture medium was replaced by saline solution containing 0.4% Trypan blue (T8154, Sigma-Aldrich) at room temperature ($\sim 20^0$ C). Subsequently, two 25- μ m platinum wires (A-M Systems, Inc.) were placed in parallel in the Petri dish to form a channel of ~ 10 mm wide and 25 mm long. A 1.0-mm thick glass plate was then placed on top of the platinum wires to form a microfluidic exposure chamber (see Fig. 2.1b).

Trypan blue enhances laser absorption to facilitate optical breakdown while providing concomitantly a biomarker for membrane poration.

Adherent cells of spindle shape were selected and randomly assigned to one of the seven groups. Cells in groups G_1 to G_3 were treated at different orientations (0^0 and 90^0) with respect to tandem microbubble axis while maintaining the same stand-off distance (SD) of 10 μm . Cells in groups G_4 to G_6 were treated at SD = 20, 30, and 40 μm , respectively, while maintaining the same orientation (0^0 , as used in case G_1). In group G_0 (control), cells were treated by a single bubble (i.e., B_1) produced at SD = 10 μm .

2.5 Bubble-bubble and microjet-cell interaction

Similar to shockwave-bubble interaction [95, 96], the most effective energy transfer is produced when the collapse time of the bubble matches with the duration of external driving force. By releasing the two laser pulses in tandem with a 4 μs interpulse delay, a first bubble (B_1) can be generated (at time = 0 μs) that expands to a maximum size of about 50 μm before a second bubble (B_2) of similar size is produced (Fig. 2.2a, 4 μs). Consequently, the collapse of B_1 is coupled with the simultaneous expansion of B_2 , leading to asymmetric deformation of the bubbles with microjets formation (Fig. 2.2a, 6 μs) under the influence of secondary Bjerknes forces [97]. Thereafter, B_2 begins to collapse with its elongated portion in contact with B_1 contracting rapidly, producing a second microjet moving away from B_1 (Fig. 2.2a, 8 ~ 12 μs). This alternating

Figure 2.2 Dynamics of tandem bubble and bubble-cell interaction. (a) Dynamics of tandem bubble (generated by both lasers with a 4 μs interpulse delay) and their interaction with an adherent cell (pointed to by arrow) near the first bubble (B₁). (b) Dynamics of single bubble (generated by LASER1) and its interaction with an adherent cell (pointed to by arrow). The asymmetric deformation with resultant formation of alternating microjets in opposite directions along the tandem bubble axis is not observed during the symmetric expansion and collapse of the laser-generated single bubble of comparable size

formation of microjets moving in opposite directions along the axis of the tandem bubble is not observed during the symmetric expansion and collapse of a single bubble of comparable size produced by one laser (Fig. 2.2b). Similar bubble dynamics have been observed in an unconstrained fluid medium using spark-generated vapor bubbles of millimeter size [98].

The dynamics of tandem bubble interaction in the experiment have been simulated using the BEM module of 3DYNAPS[®] [99] and the results are shown in Fig. 2.3. It is observed from the pressure contours that the first bubble acts virtually like a free surface locally when grown close to its maximum size. The first bubble is forced to collapse due to the local high pressure generated by the rapid expansion of the second bubble. At this moment, a fast reentrant jet is developed, which draws in liquid and elongates the second bubble. The reentrant jet eventually touches down the opposite side of the bubble. The collapse of the second bubble then triggers the formation of a re-entrant jet in the second

Figure 2.3 Modeling results of the oscillation of tandem bubble (bottom row) compared with the experimental results from high speed imaging (top row). Top row: bubble-bubble-cell interaction extracted from Fig. 2.2a (4-9 μ s); Bottom row: simulated tandem bubble with color scheme for pressure field mapping (the color in blue to red is corresponding to pressure from -2×10^4 Pa to 9×10^4 Pa).

bubble in the opposite direction to the first jet [99].

2.6 Flow field around the pulsating single or tandem bubble(s)

Following the 1st optical breakdown, the fluid is initially pushed outward from the center of B_1 before reversing the direction of flow motion during subsequent collapse after the maximum bubble expansion. In contrast, following the 2nd optical breakdown, the initial symmetric expansion of B_2 is quickly distorted with its proximal end being sucked in by the collapsing B_1 , leading to the formation of the first microjet (J_1 in Fig. 2.4a, 6 μ s). The acceleration of J_1 produces a microstreaming impulse with a maximum flow velocity about 10 m/s near the jet tip (5 to 6 μ s). Upon maximum expansion, the

elongated B_2 collapses asymmetrically and generates the 2nd microjet (J_2) that moves away from B_1 (7 to 11 μs). As a consequence of the alternating microjet formation, two pairs of vortices are generated around B_1 and B_2 , one rotating clockwise and the other counter-clockwise (Fig. 2.4b), reaching a maximum vorticity of $350,000 \text{ s}^{-1}$ at 6 μs .

Based on the μPIV results, the flow field around the tandem bubble can be reconstructed. Figure 2.5 shows four imaginary material lines originally located at a SD

Figure 2.4 Fluid motion. (a) High-speed imaging of bubble dynamics and micro-particle image velocimetry (μPIV) using $10 \times 10 \text{ }\mu\text{m}$ interrogation windows with 25% overlap indicating the flow direction around individual oscillating microbubbles. Flow tracers (i.e., $2 \text{ }\mu\text{m}$ polystyrene beads) were used to facilitate visualization of the velocity field. (b) Streamline display of three μPIV images, demonstrating vortex formation in the flow field, with one pair of vortices rotating clockwise and the other counterclockwise within $22 \text{ }\mu\text{s}$ following the generation of the tandem bubble.

Figure 2.5 Deformation of the imaginary material lines in the flow field around tandem bubble. Four material lines are located 10, 20, 30, and 40 mm away from B_1 .

of 10, 20, 30 and 40 μm (from left to right) in parallel from B_1 and their deformation 30 μs thereafter. It can be seen that at short SD of 10 and 20 μm , the central portion of the material lines along the axis of the tandem bubble has been pushed forward faster than its counterpart at long SD of 30 and 40 μm . Off-axis, the material lines move backwards presumably by the vortex flow. This directional and proximity dependent displacement of the fluid particles around the tandem bubble suggests that the deformation of a target cell nearby could also depend on SD and orientation.

2.7 Cell membrane poration

Because of the unique flow pattern produced by the pulsating tandem bubble, we have examined its influence on the morphology and membrane permeability of individual adherent cells (Rat mammary carcinoma) placed nearby. Following initial pilot tests, we

Figure 2.6 Treatment of two individual Rat mammary carcinoma cells at $SD = 20\ \mu\text{m}$ by the tandem bubble, resulting in different levels of trypan blue uptake.

soon realized that the heterogeneity in cell shape or morphology may significantly influence the outcome of membrane poration. In particular, we observed that cells grown in spindle-like shape with their membrane stretched primarily in one direction are more vulnerable to the impact of the tandem bubbles with resultant membrane poration and Trypan blue uptake than cells that stretch out in all possible directions (Fig. 2.6). In subsequent experiments, we determined to focus on the spindle-like shape cells only.

Figure 2.7 Trypan blue uptake. (a) Cell deformation produced by the microjet and vortex flow initiated from the pulsating tandem bubbles. Representative images of individual cells (circled) before, at 10 and 65 μ s, and 1 min after the interaction with either single (case G_0) or tandem (case G_2 or G_3) bubble. (b) Probability of Trypan blue uptake in 1 min after bubble-cell interaction: effect of orientation (G_1 , impacted by J_1 ; G_2 , impacted by J_2 ; and G_3 , parallel to J_1). The stand-off distance between the proximal cell surface and bubble center is 10 μ m. (c) Probability of Trypan blue uptake in 1 min after bubble-cell interaction: effect of stand-off distance. Individual cells are located on the axis of the tandem bubble, with a bubble-cell stand-off distance varying from 10 μ m to 40 μ m, at an interbubble separation distance $\Delta = 40$ μ m. Error bars indicate intervals $\pm 1/\sqrt{N}$, where N ($= 15$ to 27) is the number of cells treated in each group. Two-tailed test of proportions was used for statistical analysis for comparing results between different groups.

As shown in Fig. 2.7a, a cell placed on the axis of B_1 and B_2 with a SD of 10 μ m from the proximal surface of B_2 (case G_2) can be deformed significantly (see Fig. 2.7a for an example of case G_1) while its counterpart in the symmetric flow field of a single bubble is only strained mildly (case G_0). The microjet-flow-cell interactions along the tandem bubble axis causes a micron-scale bending and shear deformation of the cell (65 μ s in Fig. 2.7a for case G_2) that leads to localized membrane disruption and uptake of Trypan blue within 1 minute. In comparison, when a cell is placed at the same SD but rotated by 90°

from the axis of B_1 and B_2 (Fig. 2.7a, case G_3) or subjected to the symmetric expansion and collapse of a single bubble (case G_0), much milder strain with no concomitant Trypan blue uptake is observed. Also, no appreciable membrane poration is produced when the cell is located either off the tandem bubble axis with a SD greater than $10\text{ }\mu\text{m}$ or on the axis with a SD greater than $40\text{ }\mu\text{m}$. Quantitatively, the membrane poration produced by the tandem bubble shows a clear dependence on orientation (Fig. 2.7b) and stand-off distance (Fig. 2.7c). Statistically significant ($p<0.05$) increase in Trypan blue uptake is observed in G_1 and G_4 compared to G_0 with maximum probability for Trypan blue uptake produced at $SD = 20\text{ }\mu\text{m}$ along the tandem bubble axis.

2.8 Localized and directional membrane poration

The membrane disruption is highly localized. Using time-lapse imaging (Fig. 2.8a), we can observe a pinpoint entrance of the Trypan blue from the cell surface proximal to the incident jet in 6 s. The dye gradually diffuses and spreads out in the cytosol towards the distal end in 24 s, with subsequent directional entrance and staining of the cell nuclei by 42 s. Using our setup, the cell can be quickly fixed by a perfusion flow of 2.5% glutaraldehyde, started in about 10 seconds after the tandem bubble-cell interaction. Subsequently, cell location and jet impact direction are registered by laser-produced markings at the bottom of the Petri dish (Fig. 2.8b). Scanning electron microscopy of the treated cell reveals highly localized membrane disruption with single or dual pore formation at the site of membrane surface facing the microjet impact. The pore size

Figure 2.8 Membrane poration. (a) Time-lapse images showing pinpoint entrance of Trypan blue into the cell from the site of microjet impact (pointed to by arrow) with subsequent progressive diffusion in the cytosol and staining of the nuclei. (b) Laser-generated marking dots (circled) on the bottom surface (~ 1.0 mm thick polystyrene) of the Petri dish, which are used to register the treated cell, site and direction (distal to “T” marking) of the microjet impact. (c) SEM images of the porated cell shown in (b), demonstrating the formation of a $200\ \text{nm}$ pore (pointed by arrow) at the site of microjet impact. (d) SEM images of another porated cell, showing the formation of two adjacent pores (pointed to by arrow) of about $1 \sim 2\ \mu\text{m}$ in individual pore size. Spherical vesicles of less than $1\ \mu\text{m}$ in size were observed near the disruption site, which is characteristic of cell membrane resealing after mechanical damage.

varies from $200\ \text{nm}$ to $2\ \mu\text{m}$ (Fig. 2.8c and 2.8d). Because the cell is treated in Trypan blue solution (with no Ca^{2+} content), active repair of the pore is likely to be significantly compromised with less than $1\ \mu\text{m}$ spherical vesicles observed at the disruption site (see Fig. 2.8d) [100, 101]. Therefore, the observed pore size may represent an upper limit in membrane disruption produced by the tandem bubbles under physiological conditions.

2.9 Discussion and summary

A new method is developed to produce localized and directional membrane poration on single cells with high precision and reproducibility. The pore size can be varied from nano- to micro-meter range, limited only by the ability of the cell to reseal the disruption site [15]. Because jet diameter is usually about one-tens of the maximum bubble size, the potential energy accumulated by a single bubble with comparable size to a cell (i.e., $\sim 20 \mu\text{m}$) is limited and insufficient to produce measurable membrane disruption when collapsing near a boundary [102]. By confining the bubble generation in a quasi-2D thin layer, and furthermore, by transferring and focusing energy through tandem bubble interaction, we have demonstrated the feasibility for generating pinpoint mechanical forces for highly localized membrane poration.

Microjetting induced flow shear stress and resultant bending (and stretching) of the cell body may contribute to the observed pore formation. The flow-induced shear stress (τ) near the microfluidic channel (height = $2h = 25 \mu\text{m}$) surface can be estimated by $\tau = \mu(V_j/\eta)$ where μ is dynamic viscosity of the medium and V_j is jet speed. In our setup, the maximum shear stress is estimated to be about 1 kPa, which is within the range of previously reported critical shear stress of 0.1 kPa for cell detachment [102] and water-hammer pressure of 3 kPa for jet impact induced membrane rupture [37].

Individual cells placed on the axis of the tandem bubble (see Fig. 2.2a, Fig. 2.4a, and Fig. 2.7a) show different degrees of bending deformation produced by jet impact and

resultant microstreaming and vortex flow. The central portion of the cell directly under jet impact is detached, while one or both distal ends of the cell remain attached, leading to a significant stretching of the cell membrane. The stretch ratios of material lines located at different orientations and *SDs* from the tandem microbubble (data not shown), estimated based on μ PIV images, show a good correlation with the membrane poration.

It is envisioned that the technique developed in this work can be easily integrated in microfluidics and thus possessing great potential in high-throughput biosynthesis and bioengineering applications, and for mechanistic investigation of cavitation-induced bioeffects.

3. Micropatterning for Controlling the Generation of Cavitation Bubbles and Precise Culture of Individual Cells in Microfluidics Devices

3.1 Introduction

In Chapter 2, we developed a new experimental method and system for producing tandem bubbles by ns pulsed lasers and demonstrated the feasibility of using tandem bubble-produced jetting flow to create directional and controlled membrane poration at the single-cell level. Similarly, tandem bubble approaches have been used for characterizing material properties of nanotubes [103], and for cell sorting [104]. In all these previous studies, cavitation bubbles were produced by laser-induced rapid heating of fluids containing color dyes. In some cases, the dye (such as Trypan blue) was used both to enhance laser absorption and also served conveniently as a biomarker for cell membrane permeabilization [105, 106]. However, because most of the dyes are either cytotoxic or non-biocompatible, their presence in the fluid medium could severely limit the utility of these methods in biomedical and biotechnological applications. In this chapter, we aim to make a translation from the open experimental system (in Petri dish) used in Chapter 2 to a closed and high-throughput experimental system constructed in a microfluidic device. Specifically, we want to replace trypan blue with patterned micron-sized gold dots (25 nm thick) decorated on the glass substrate of a microfluidic channel for precise generation of tandem bubbles, and isolated individual areas (32 x 32 μm^2) covered with fibronectin on the same glass surface for cell culture to control the

location, shape and adhesion pattern of individual cells to be treated by tandem bubbles.

The random spreading of anchorage-dependent cells has been related to the variation of traction force that cells exert on the substrate in previous studies [78]. This variation is transmitted to cytoskeleton and further regulates cell phenotype along different pathways [107]. The uncertainty in cell phenotype caused by the variations in cell spreading pattern presents a significant challenge in deciphering or comparing the outcomes even after the same treatment. For example, we have noticed in Chapter 2 that spindle-shaped cells tend to be porated more easily than fully spread ones. Standardization of the cell shape has been shown to provide a more homogeneous microenvironment in cell cultures [69], which may benefit the analysis of the biological and mechanical responses of otherwise similar individual cells exposed to a particular cavitation event. To determine the effect of cell traction on treatment outcomes, we will design a square-shaped cell adhesion pattern with two anchorage edges connected in the middle and two free standing edges, namely “H” and “I” patterns (Fig. 3.1).

Surface patterning, based on surface chemistry and material science, has been used as a versatile strategy for controlling the spatial placement and spreading geometry of individual cells on a surface [70]. Cellular response and development, such as proliferation, migration, differentiation, and apoptosis of individual cells, can be guided by surface cues through intracellular signaling pathways [78]. Engineered surfaces with controlled chemistry [108], topography [109] and stiffness [110] have provided new insights into cell-surface interaction. Cellular patterns have been employed in cell-based

biosensors for diagnostics and in cell arrays for drug discovery [111]. Because of the advantages in precise geometry control and high-throughput potential, patterned surfaces can be used to create a uniform biochemical and biomechanical background or microenvironment for the growth and treatment of individual cells in a microfluidic device.

In the past decade, a variety of engineering approaches have been developed for cell patterning [112]. They all share the same design principle, i.e., to combine microfabrication with biochemical functionalization to produce biological cues in spatially controlled regions while the background is rendered non-adhesive (or non-fouling) by chemical modifications. In addition, a microfluidic system will further allow us to control precisely the microenvironment surrounding the cell, facilitated by the convenience in medium exchange in a microfluidic channel [113]. For example, individual cells can be tagged or stained before and after the tandem bubble treatment for downstream analysis. During the experiment, the culture medium can be adjusted to deliver chemical cues to the target cells. In this chapter, we will develop a microfluidic chip with hybrid micropatterns on the same glass substrate for both bubble generation and cell growth. To promote single cell attachment and spreading, individual “H” or “I” shaped cell adhesive areas ($32 \times 32 \mu\text{m}$ in size, $8 \mu\text{m}$ in line width) will be accurately produced and aligned to individual pairs of gold dots, while the remaining surface of the glass substrate will be passivated with Polylysine grafted Polyethylene (PLL-g-PEG).

3.2 Materials and methods

3.2.1 Design of hybrid patterns in a microfluidic channel

Design of the microfluidic chip with specific pattern layouts inside each channel were carried out using AutoCAD (Autodesk Inc., San Rafael). As shown in Fig. 3.1, three

Figure 3.1 A schematic diagram of the microfluidic chip design layout. Each channel is made of PDMS with a glass bottom. Channel dimension is 25 μm in height and 800 μm in width. Individual cells are cultured in either the “H” or “I” patterned area, which is separated from a pair of gold dots at various standoff distances (SD) from 10 to 40 μm . The interbubble distance (Δ) is 40 μm .

channels of 25 mm in nominal length were constructed in parallel with inlet and outlet ports connected to both ends of the channels to facilitate injection and exchange of different fluid media (e.g., for cell culture and bioassays) during the experiment. Each channel has a dimension of 800 μm by 25 μm in width and height, and contains 10 units with varied standoff distance (SD), ranging from 10 to 40 μm , between the tandem bubble and the leading edge of the target cell. Inside each unit, 30 sets with either “H” or

“T” shaped regions are designed for cell adhesion. A pair of gold dots with a fixed interbubble distance ($\Delta = 40 \mu\text{m}$) will be placed nearby for tandem bubble generation. Numerical markers are integrated into the channel design, so that the location of individual cells can be registered, and also easily tracked during and after the experiment. In total, 800 sets are assembled in one chip so that multiple test sites can be created within each channel. Such a chip device provides a high-throughput platform for assessing tandem bubble-cell interaction under a wide variety of experimental conditions.

3.2.2 Patterning of micron-sized gold dots in microfluidic channels

Figure 3.2 Schematic diagrams showing the protocols for (a) patterning of micron-sized gold dots on a glass surface using metal lift-off technique and (b) fabrication of PDMS-glass microfluidic chips. (c) A photograph of the integrated microfluidic chip with individual channels filled with a blue solution. Blunt needles are connected to the ports punctured on the PDMS.

It has been shown previously that vapor microbubbles can be generated via laser-induced localized heating of metal surfaces immersed in water [114] or nano-particle dispersed tissue mimicking materials [115]. To produce tandem bubbles

with precision in nucleation sites and desirable spatial distribution, a glass surface was treated by coating it with an array of paired gold dots having a separation distance of 40 μm using metal lift-off technique [116, 117]. Briefly, as shown in Fig. 3.2a, a thin layer of photoresist (NFR 015, JSA Micro.) was first spin-coated on a 1-mm thick microscopic glass slide (#48300, VWR International). A chrome master photomask (Photo Sciences) with designed patterns was then used to perform photolithography on the coated layer of photoresist using a lithography mask aligner (MA6/BA6, Karl Suss). An array of paired gold dots of 6 μm in diameter and 15 nm thick was deposited on the glass surface using an electron beam evaporator (PVD 75, Kurt Lesker) preceded by a 2-nm thick chrome underlayer deposition in order to enhance the adhesion between the glass surface and the gold dots. The 6- μm diameter gold dots were selected to ensure sufficient area for laser absorption while providing a small enough target ($<$ the focal spot of the laser beam) for easy alignment and consistency in bubble generation.

Based on the Drude free-electron theory [118], the attenuation coefficient (a) of an incident laser pulse at near-infrared wavelengths by a metal nanolayer can be estimated by:

$$\ln(1 - \alpha) \propto -n_e a \lambda^3 \quad (3.1)$$

where n_e is the electron density in the metal, λ is the laser wavelength, and a is the metal thickness. This leads to an estimated maximum absorption about 70% by the gold nanolayer at 1064 nm wavelength. It is worth noting that while a thicker layer of gold deposition on the glass surface will increase laser absorption, it will also generate more

insoluble gas and metal debris during plasma formation, making the resultant bubble dynamics less reproducible.

The glass substrate patterned with gold dots was bound with a polydimethyl-siloxane (PDMS, which has an elastic modulus of 1.8 MPa [119]) block containing several microchannels (Fig. 3.2b). The PDMS (Sylgard 184, Dow Corning) microchannels were produced from a silicon master mold (Stanford Microfluidic Foundry) using soft lithography technique [120]. Fluid access ports (~ 1 mm in diameter) were punctured at both ends of the PDMS microchannel. The surfaces of the glass substrate and the PDMS slab (~ 2 mm thick) were hydrophilized in an oxygen plasma asher (K-1050X, Emitech), aligned using position pins, and brought into conformal contact. The integrated chip was then baked on a hot plate to strengthen the bonding at the glass-PDMS interface. For visualization, the channels in the microfluidic chip were filled with dye containing water (Fig. 3.2c).

3.2.3 Fabrication of hybrid patterns in microfluidic channels

For cell adhesion, separate sets of “H” or “I” patterns of $32 \times 32 \mu\text{m}$ in size, which define the individual adhesive regions, were produced by molecular assembly pattern lift-off (MAPL) method [121]. Started from the glass substrate with patterns of gold dots, a 2- μm thick layer of positive photoresist (S1813, positive, Shipley, MA) was spin-coated on the glass surface. A chrome master photomask (with alignment marks) was aligned to the gold patterns using a lithography mask aligner (MJB3, Suss MicroTec). After UV illumination and subsequent development, “H” (or “I”) shaped photoresist patterns were

produced next to each pair of gold dots (Fig. 3.3). PLL-g-PEG (0.5 mg/ml) solution was

Figure 3.3 A schematic diagram of the fabrication procedures used for micro-patterning on the surface of glass substrate

prepared in HEPES buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH adjusted to 7.4 with 0.1M NaOH solution), and a 200 μl solution drop was placed on a paraffin film. Glass slide with patterned gold dots on the surface facing down was plated onto the liquid drop, leading to an anti-biofouling layer formed electrostatically on the surface in 45 min [112]. The sample was then soaked in N-Methyl-2-pyrrolidone (NMP) with ultrasonic agitation to lift-off photoresist. Consequently, the gold patterned glass surface was covered with PLL-g-PEG except for the individual “H” (or “I”) areas next to each pair of gold dots.

The glass substrate covered with a PLL-g-PEG chemical monolayer is vulnerable to plasma exposure, so the patterned region needs to be protected during plasma assembly

of the microfluidic chip. As shown in Fig. 3.4, a PDMS slab used as the protective cover

Figure 3.4 A schematic diagram of the procedures involved in microchannel construction and cell seeding

during plasma etching and a PDMS housing for microchannel construction were prepared from a silicon mold. Using gold patterns as a reference, the PDMS slab (800 μm wide groove structure) was placed in conformal contact with the glass slide to protect the patterning regions. Reactive oxygen plasma (100 W, 500 mTorr, 60 s) was applied to remove PLL-g-PEG from the area with no PDMS protection. A second PDMS slab with pre-prepared fluid access ports was treated by reactive ion etching (RIE, 25 W, 500 mTorr, 25 s), and then aligned to the patterned glass and brought in contact under a stereoscope. After binding, the microchannel was first primed with PBS for 30 min at 1 $\mu\text{l}/\text{min}$, followed by 30 min perfusion treatment of fibronectin solution (25 $\mu\text{g}/\text{ml}$ in PBS, 1 $\mu\text{l}/\text{min}$) to fill the “H” (or “T”) regions. To confirm the specific regions for fibronectin

deposition, fibronectin was bioconjugated with amine reactive dye (Dylight 488, Thermo Scientific) and the location of fibronectin can be visualized under fluorescence microscope. Thereafter the fibronectin solution was flushed out by PBS injection at a flow rate of 30 $\mu\text{l}/\text{min}$ for 10 min to remove any residual fibronectin on the glass substrate outside the patterned areas. After washing, the microchannel was then filled with PBS during storage before cell seeding.

3.2.4 Cell seeding into the patterned microfluidic channel

HeLa cells were maintained in DMEM supplemented with 10% FBS and 1% antibiotic/ antimycotic solution. After the patterned regions were coated with fibronectin, cells were trypsinized from the cell culture flask (or dish), resuspended in culture medium ($\sim 5 \times 10^6 / \text{ml}$) and introduced into the channel through a 20 mm TYGON[®] plastic tubing with 0.02" ID. Once the channels were filled with cell suspension, the outlet of the channel was clamped, and the chip was placed in an incubator for 30 min to allow the cells to initiate adhesion onto the "H" or "I" regions. The chip was then perfused with cell culture medium at a flow rate of $\sim 30 \mu\text{l}/\text{min}$ to flush out non-attached cells, and then placed in the incubator for 1.5 hours during which the culture medium was slowly perfused into the channel at a low flow rate of $\sim 0.5 \mu\text{l}/\text{min}$ to maintain sufficient nutrient supply.

3.3 Results and Discussion

3.3.1 Single bubble from gold dot

The generation of a cavitation bubble via laser-irradiation of a 6 μm gold dot on a glass surface is highly localized and reproducible. A representative high-speed imaging

Figure 3.5 Dynamics of a single bubble oscillation (i.e., expansion and collapse) produced by laser absorption from a single gold dot patterned on the glass bottom of a microfluidic channel (the brighter interior in the collapse phase compared to the expansion phase may suggest a transition from an initial 3D hemispherical expansion to a two-dimensional cylindrical collapse of the bubble). The insets in the upper-left and upper-right corners of the plot show a gold dot before and after a single cavitation event, respectively, demonstrating thermal ablation and cavitation damage to the gold layer

sequence of laser-induced single bubble oscillation in the microfluidic channel is shown in Figure 3.5. Based on the acquired images, bubble diameter vs. time plot was obtained from 12 individual measurements using different gold dots. The strong absorption of the

incident laser pulse by the gold dot leads to the instantaneous creation of a plasma (at $t = 0$ s), which expands rapidly to form a vapor bubble of a maximum projected diameter (in the x-y plane) of 50 μm in 2 μs . The rapid initial increase in the projected bubble diameter may be related to the creation of a hemispherical bubble around the gold dot, which is gradually transformed, upon further expansion, into a cylindrical bubble constrained by the top and bottom surfaces of the microfluidic channel. Thereafter, the bubble starts to collapse, reaching a minimum size in about 3.7 μs , followed by a slight rebound. Overall, the collapse time of the bubble (T_c) in the PDMS-glass microchannel is 5.7 μs . In comparison, using the same experimental setup T_c of a bubble with a 50 μm maximum diameter in a glass-glass microchannel of 25 μm height was found to be 8.8 μs . The difference is likely attributed to the compliance of the PDMS boundary, which is known to shorten the collapse time of a bubble in close proximity [122]; in contrast, a rigid boundary (i.e., glass) can lengthen the collapse of a similar bubble [90, 122].

3.3.2 Single cell patterning in microchannels

Figure 3.6 shows a portion of the fabricated micropatterns of gold dots (for laser bubble generation) aligned in regular SD with the “H” regions (for cell adhesion). The photoresist patterns could be initially visualized in red fluorescent imaging. After MAPL process, the photoresist patterns were removed and then replaced by fibronectin in the microchannel. The remaining part of the glass surface was electrostatically covered with a layer of PLL-g-PEG, which serves as a non-biofouling background. This is confirmed by the strong green fluorescence emitted from Dylight-488 tagged fibronectin, limited

only to the “H” regions. Once cells were introduced into the channel, their adhesion and spreading could only be supported within the area covered by the fibronectin. Similar patterns could be seen in other parts of the microchannel with different *SDs* or adhesion patterns (*i.e.* “I”).

Figure 3.6 Single cell patterning in proximity to a pair of gold dots. MAPL chemistry treatment starts from the photoresist patterns in alignment to pairs of gold dots on the glass substrate. The individual regions initially occupied by the photoresist were finally grown with single cells

The patterned regions were successfully protected through oxygen plasma exposure before its irreversible bonding with the PDMS housing. Figure 3.7 shows the completed microfluidic chip with tubing connected to the inlet and outlet through metal pins (stainless steel, 0.025” OD × 0.017” ID, 0.5” long). It was observed the suspended HeLa

cells injected into the microchannel quickly attached to the “H” or “I” regions within 30 min. Following a 90-min culture in the incubator, most of the attached cells were fully spread out owing to the fibronectin coating. In total, 30% of the adhesive regions (“H” or “I”) were typically and successfully grown with single cells, providing more than 200 individual cells that can be selected for the tandem bubble treatment.

Figure 3.7 Cell seeding in the microchannel with patterned adhesive regions. The microfluidic chip was made from plasma-bonded PDMS and a patterned glass substrate. The microchannel was filled with blue dye for visualization. About 30% of the cell adhesive regions were occupied by individual HeLa cells. The numerical numbers in the peripheral area of the microchannel were used to track the location of the target cells throughout the tandem bubble-cell interaction experiment.

3.4 Summary

In this chapter, we have developed a microfluidic chip with hybrid patterns fabricated on the surface of glass substrate. Cavitation bubble can be generated in the microfluidic channel via laser absorption by patterned micron-sized gold dots. The dynamics of single bubble oscillation produced in such a microchannel are highly reproducible. Single adherent cells with desirable shape were also successfully introduced onto the microfluidic chip and placed in close proximity to individual pairs of gold dots. This integrated microfluidic system design provides a versatile platform for single cell treatment by tandem bubbles under a wide variety of test conditions, with the resultant cell response readily available for analysis through combination with high-speed imaging and sequential microscopy that have been set up in our experimental system. Therefore, this microfluidic system may provide a versatile and high-throughput platform for exploring the mechanism of cavitation bubble induced bioeffects at the single cell level, as well as a diverse range of biomedical and biotechnology applications.

4. Dynamics of Tandem Bubble Interaction in a Microfluidic Channel

4.1 Introduction

The interaction of cavitation bubbles in a fluid medium or near a boundary occurs frequently in a wide range of engineering and biomedical applications [12, 90, 123]. Bubble-bubble interaction with resultant violent collapse of the bubble and subsequent shock wave emission or high-speed liquid jet formation have been associated with erosion damage on solid surfaces in hydraulic machinery [93] and certain adverse effects on cells and tissue exposed to diagnostic and therapeutic ultrasound [124]. However, the power of cavitation has also been harnessed in biomedical applications to improve the disintegration of kidney stones in shock wave lithotripsy [125], to facilitate targeted drug and gene delivery [12, 95], and to enhance the efficacy of high-intensity focused ultrasound in cancer therapy [11].

Several groups have investigated the dynamics of bubble-bubble interaction using laser-based experimental systems with high spatial and temporal precision in bubble generation. Lauterborn and Hentschel showed examples of the interaction of two bubbles of similar size in water, demonstrating mutual attraction of the bubbles with re-entrant jet formation toward each other [126]. Blake and colleagues examined the interaction of two different-sized bubbles in water near a solid boundary [127]. Their results demonstrate that the mutual interaction between two bubbles in close proximity is as important as the presence of a rigid boundary in determining the overall behavior of the bubbles (i.e.,

migration, asymmetric deformation, jet formation and bubble splitting). Sato and Tomita studied non-spherical motion of two laser-generated bubbles near a solid boundary [128]. By adjusting the time delay (and thus the phase difference) between the two bubbles, they were able to control the direction of jet formation either toward or away from the boundary. Within an optimal range of phase delay, they were able to increase the jet speed produced by the tandem bubble interaction compared to its counterpart from a single bubble of similar size near the boundary.

The general features of bubble-bubble interaction observed experimentally have been captured by model stimulations based on potential flow and boundary element method (BEM) [127, 128]. Similarities with a single bubble oscillation either near a rigid or soft boundary were noted [90]. The BEM approach has also been used to describe the dynamic bubble interaction in a multi-bubble cloud [97]. Moreover, Ilinskii and colleagues have investigated bubble-bubble interaction in an acoustic field based on Lagrangian and Hamiltonian mechanics, demonstrating coupled pulsation with translational motions [52]. Higher order surface disturbances of the bubble can also be introduced to model jet formation [129].

In recent years, there has been a growing interest in tandem bubble interaction in microfluidic devices [130-132], as exemplified in Chapter 2 by the demonstration of directional and localized membrane poration on single cells [105]. In Chapter 3, we further refined the experimental system using microfabrication techniques to improve the reproducibility in bubble generation and precision control in tandem bubble-cell

interaction. In this chapter, we will first summarize the theoretical background for the mutual interaction between two oscillating bubbles. We will then describe the experimental methods for high-speed imaging of bubble dynamics and flow visualization via particle imaging velocimetry (PIV). This will be followed by the results of laser-generated tandem bubbles in the microfluidic channel with particular emphasis on the effects of phase delay on the dynamics of tandem bubble interaction and jet formation without the presence of a cell nearby. Finally, we will present the results on the dynamic interactions of tandem bubbles with the presence of a target cell grown on “H” and “I” adhesion patterns and at various standoff distances.

4.2 Theoretical background

The mutual interaction between two oscillating bubbles in water without nearby boundaries can be described by the secondary Bjerknes forces [133, 134]. In the simplest case, the pressure field (p_I) produced by a spherically oscillating bubble (referred as bubble 1 or B₁) is given by:

$$p_I = \frac{\rho}{r} \frac{d}{dt} (R_I^2 \dot{R}_I) \quad (4.1)$$

Where ρ is the density of water, r is the radial coordinate, R_I is the time-varying radius of B₁, and the dot denotes time derivative. A second bubble (B₂) located at an inter-bubble distance Δ from B₁ will experience a time-varying radiation force (F_{I2}) produced by p_I given by:

$$F_{12} = -V_2 \nabla p_1 \quad (4.2)$$

where V_2 is the volume of B_2 . Integrating Eq. 4.2 over a period of the volume oscillation of the bubble, one can determine the secondary Bjerknes force by:

$$F_B = \langle F_{12} \rangle = -\frac{\rho}{4\pi\Delta^2} \langle \dot{V}_1 \dot{V}_2 \rangle e_r \quad (4.3)$$

where $\langle \rangle$ denotes time average, V_I is the volume of B_I , and e_r is the unit vector along the radial direction. It has been shown that the secondary Bjerknes forces of two spherically oscillating bubbles are symmetric: $\langle F_{12} \rangle = -\langle F_{21} \rangle$ [134].

The most significant implication of Eq. 4.3 is that when two bubbles oscillate in phase, they attract each other. In contrast, when oscillating out of phase, they repel each other.

4.3 Materials and methods

4.3.1 Laser-induced microbubble(s) in the microfluidic channel

The fabricated microfluidic chip (see Chapter 3) was placed on the stage of an experimental system constructed from an inverted microscope (Axio Observer D1, Zeiss) described previously [105]. Two Q-switched Nd:YAG lasers of 1064 nm wavelength and 5 ns pulse duration [MiniLase I (Laser 1) and Orion (Laser 2), New Wave Research] were focused through a 63 $\times$ objective (LD Plan Neofluar, Zeiss) and projected on a pair of gold dots (Fig. 4.1). Alignment of the laser foci with the gold dots was facilitated by a rotating slide holder (H22ROTS, Prior Scientific) in combination with the x-y translational stage of the microscope. Bubble dynamics and the flow field around the

pulsating bubble(s) can be visualized through the same objective by high-speed cameras connected to the microscope. In this study, the output power of each laser was adjusted to be $\sim 10 \mu\text{J}$ so that the maximum bubble diameter produced from individual gold dots is $50.0 \pm 0.9 \mu\text{m}$ (mean $\pm$ standard deviation, $n = 12$).

Figure 4.1 A schematic diagram of the experimental setup for laser-generated tandem bubble in a microfluidic channel (inter-bubble distance $\Delta = 40 \mu\text{m}$). The mid-point between the pair of gold dots is chosen as the origin “O” of a Cartesian x - y - z coordinate system, where the positive direction of the y axis is pointed from B_2 ($y = -20 \mu\text{m}$) to B_1 ($y = +20 \mu\text{m}$) and the z -axis is from glass ($z < 0$) to PDMS ($z > 25 \mu\text{m}$)

The non-dimensional standoff distance (γ) between a pair of equal-sized tandem bubble can be defined by the ratio of the inter-bubble distance (Δ) divided by the maximum diameter of the individual bubbles ($2R_{\text{max}}$) [98]:

$$\gamma = \frac{\Delta}{2R_{\text{max}}} \quad (4.4)$$

In this study, γ was kept constant at 0.8, corresponding to a strong mutual interaction between the tandem bubbles due to the secondary Bjerknes force. However, the phase delay between the oscillations of the two bubbles can be adjusted using a digital delay generator (BNC 555, Berkeley Nucleonics) that controls the timing of the trigger signals to the two lasers. The time delay τ between individual laser pulses was monitored by a photodiode (DET210, Thorlabs) connected to an oscilloscope (500 MHz WaveRunner 6050A, LeCroy).

4.3.2 High speed imaging and flow visualization

The dynamics of laser-generated tandem bubble oscillation in the microfluidic channel were captured using a high-speed camera (Imacon 200, DRS Hadland) operated at 50 ns exposure time and a framing rate of 1 ~ 10 million frames/second. To examine the dynamics of tandem bubble with a target cell nearby, the microchannel was seeded with HeLa cells (see §3.2.4) before high speed imaging. Backlight illumination was provided by a fiber-optic coupled Xenon flash lamp with 200 μ s pulse duration (ML1000, Dyna-Lite). To facilitate the visualization of flow motion generated by the bubble oscillation via PIV, 2 μ m polystyrene beads (R0200, Duke Scientific) were added as neutral buoyancy tracers in the fluid medium at a concentration of 1×10^9 beads/ml. The trajectories of the tracers were recorded by a second high-speed camera (Phantom V7.3, Vision Research) operated at 1 μ s exposure time and 20,000 frames/second. The acquired image sequences (at 1.5 pixel/ μ m resolution) were processed offline by DaVis7 software (LaVision, GmbH) using a 16×16 pixel interrogation window with 50% overlap to

determine the velocity and vorticity fields averaged over every 50 μs after the tandem bubble interaction.

4.4 Results and discussion

4.4.1 Dynamics of tandem bubble – the effects of phase delay

4.4.1.1 General features of tandem bubble oscillation

In our experiment, two equal-sized laser-generated bubbles ($R_{max} = 25 \mu\text{m}$) are created in close proximity to each other ($\Delta = 40 \mu\text{m}$ or $\gamma = 0.8$). Therefore, strong interaction due to the secondary Bjerknes force between the two bubbles is observed, leading to asymmetric bubble deformation and axial jet formation (Fig. 4.2). In particular, we focus on the effects of the time delay (τ , varying from 0 and T_c) between the generation of the two bubbles on their dynamic interaction and subsequent jet and vortex formation in the surrounding fluid.

The general features of tandem bubble oscillation are investigated by high-speed photography at a framing rate of 1 million frames/second. As shown in Fig. 4.2a (1st row), when the two bubbles are generated simultaneously (*i.e.*, in-phase), both B_1 and B_2 start to expand concurrently after the plasma formation. The proximal walls of the two bubbles quickly approach each other, become stagnated and flattened along the mid-plane between the two bubbles, while their distal walls continue to expand outward for about 3 μs . Upon maximum expansion, the tandem bubble begins to collapse in unison toward the flattened proximal walls in the mid-plane. Because of their larger

Figure 4.2 (a) Laser-generated tandem bubble oscillation and jet formation produced inside the PDMS–glass microchannel at an inter-bubble time delay of 0, 1, 2, 3, 4, 5 μs (from top to bottom). High-speed imaging sequences were taken with a framing rate of 1×10^6 frames/s; (b) jet formation from in-phase tandem bubble oscillation; (c) jet formation from out-of-phase tandem bubble oscillation; and vorticity field averaged over $t = 100\text{--}150 \mu\text{s}$ after (d) in-phase and (e) out-of-phase tandem bubble oscillation.

curvatures, the sections of the distal walls along the axis of the tandem bubble collapse much more rapidly than the rest of the bubble surface (see $t = 5 \sim 7 \mu\text{s}$), leading to the formation of two axial jets moving toward each other (see Fig. 4.2b). The collision of the two axial jets in the mid-plane breaks up the tandem bubble into two pairs of small daughter tandem bubbles that move away from each other along the x-axis.

The dynamics of in-phase tandem bubble interaction is analogous to a single bubble oscillation near a rigid boundary (that serves as a mirror of the single bubble), with the

centroids of the bubbles and/or axial jets migrating toward each other (or toward the boundary) [51, 90]. This observation is supported by the progressive thinning of the gap between the proximal walls of the tandem bubble, not only during the initial expansion but also in the collapse phase when the mutual attraction associated with the secondary Bjerknes force increases significantly with decreased inter-bubble distance and much more rapidly changed bubble volume (see Eq. 4.3). Furthermore, the collapse time of the tandem bubble is lengthened ($T_c = 7.5 \mu\text{s}$) compared to its counterpart of a single bubble ($T_c = 5.7 \mu\text{s}$). This observation is consistent with the findings of previous studies [51, 90, 135].

In contrast, when two equal-sized bubbles are generated at different times within a single oscillation period, the characteristics of the tandem bubble interaction change dramatically. As shown in Fig. 4.2a, the original symmetric geometry (in the projected x-y plane) in B_1 oscillation is disrupted immediately after the generation of B_2 (with a time delay τ varying from 1 to 5 μs in the 2nd to 6th rows), whose radial oscillation, in turn, is also severely distorted. In general, the two bubbles are found to oscillate mostly out of phase and therefore, repel each other by the secondary Bjerknes force. This mutual interaction leads to the formation of axial jets moving away from each other (see for example Fig. 4.2c), and the breakdown of B_1 into several smaller daughter bubbles drifting along the axis of the tandem bubble (or y-axis). Details of the interaction and characteristics of the jet formation depend on the phase delay between the two bubbles, which, for example, can provide a means to optimize the tandem bubble interaction for

maximizing the resultant jet velocity.

In particular at $\tau = 2 \mu\text{s}$ (the 3rd row in Fig. 4.2a), B_1 has just reached its maximum expansion and is ready to collapse when B_2 is produced. Therefore, the two bubbles oscillate completely out of phase (i.e., anti-phase), resulting in the collapse of B_1 being greatly accelerated by the concomitant expansion of B_2 with a significantly shortened T_c of $3.7 \mu\text{s}$.

The dynamics of anti-phase tandem bubble interaction is analogous to a single bubble oscillation near a free surface (that serves as a deformable boundary of constant pressure), with the centroids of the bubbles and/or axial jets migrating away from each other (or the free surface) [136]. Our observation of the tandem bubble interaction in a narrow fluid gap (a quasi-2D configuration) is similar to the general features of tandem bubble interacting in free field (a 3D configuration) [128]. The primary difference is in the geometry of the bubble (cylindrical vs. spherical) and associated dependency of the strength of the Bjerknes attraction ($1/d$ vs. $1/d^2$) [51, 137].

The flow field around the tandem bubble during $t = 100 \sim 150 \mu\text{s}$ has been characterized by PIV, which reveals a pattern of streamlines that is consistent with the deformation of the bubbles, jet formation, and subsequent displacement of the bubble remnants. Furthermore, the PIV results shown in Fig. 4.2d and Fig. 4.2e demonstrate the formation of two pair of vortices around the center of the tandem bubble (i.e., the origin of the x-y-z coordinate system O). The two pairs of vortices associated with the tandem bubble oscillating in-phase tend to stay close to each other with the vortices

rotating counter-clockwise in quadrants I and III, yet clockwise in quadrants II and IV (Fig. 4.2d). In contrast, the two pairs of vortices generated by the anti-phase oscillating tandem bubble rotate in the opposite direction of their in-phase counterpart and gradually drift outward away from each other (Fig. 4.2e). It has been observed that these long-lasting vortices can sustain the movement of bubble remnants for more than 150 μs . During $t = 100$ and $150 \mu\text{s}$, the magnitude of the maximum vorticity produced by the anti-phase tandem bubble oscillation is $2.8 \times 10^3 \cdot \text{s}^{-1}$, compared to $1.8 \times 10^3 \cdot \text{s}^{-1}$ produced by the in-phase tandem bubble oscillation. These findings support the potential utility of tandem bubbles in various biological and biotechnology applications, such as single cell membrane poration [105] and directional displacement of micro-particles and suspended cells in a microfluidic channel [104].

4.4.1.2 Effects of phase delay on tandem bubble interaction

The effects of the phase delay on the dynamics of tandem bubble interaction and jet formation are examined in detail at a framing rate up to 10 million frames/second (Fig. 4.3). Several interesting features can be noticed. First, the rapid expansion and subsequent elongation of B_2 toward B_1 as a result of their mutual interaction causes an asymmetric deformation of the proximal walls of the tandem bubble, leading to the formation of a first liquid jet (J_1) inside the collapsing B_1 (see for example, $t = 1.3$ to $1.6 \mu\text{s}$ in Fig. 4.3a). The proximal walls of the two bubbles appear to be separated by a thin layer of liquid even when J_1 is piercing through the interior of B_1 (see for example, $t = 2.3$ to $2.6 \mu\text{s}$ in Fig. 4.3b). This observation is consistent with the results of potential

flow modeling of tandem bubble interaction in 3D, which also suggest the formation of a

Figure 4.3 Dynamics of laser-generated tandem bubble oscillation and jet formation produced inside the PDMS–glass microchannel at an inter-bubble time delay of (a) 1 μ s, (b) 2 μ s, (c) 3 μ s, and (d) 4 μ s. High-speed imaging sequences were taken with a framing rate up to 10×10^6 frames/s.

high pressure region between the asymmetrically deformed proximal bubble walls that drives the development of J_1 [128]. Second, B_1 breaks up into several small daughter bubbles upon collapsing into a minimum volume shortly before or after J_1 pierces through the distal wall of B_1 . Subsequently, the remnants of B_1 are carried away from the center of the tandem bubble along the y-axis by the resultant vortex flow (see also Fig. 4.2e). Third, T_c of B_1 is found to vary significantly with the phase delay between the two bubbles. The minimum in T_c for B_1 (i.e., 3.8 μ s) is produced at an inter-bubble time delay of $\tau = 2 \mu$ s, which, interestingly, also correlates to the largest elongation of B_2 produced (see $t = 3.0 \mu$ s in Fig. 4.3b; the elongation is measured by the ratio of the maximum

bubble diameter along the y-axis over its counterpart along the x-axis). Fourth, upon maximum expansion B_2 starts to collapse with its highly elongated proximal wall contracting rapidly backward, forming a second jet (J_2) piercing through the interior of B_2 toward its distal wall (see for example, $t = 4.3$ to $6.5 \mu\text{s}$ in Fig. 4.3c). The push-and-pull action of the tandem bubble (more specifically of B_2 , as shown in Fig 4.3) has been coined the ‘catapult’ effect [138].

4.4.1.3 Temporal development of the tandem bubble oscillation

The dynamics of tandem bubble oscillation can be further illustrated by the temporal variations of its boundaries along the y-axis. Based on the data shown in Fig. 4.3, the trajectories of the top and bottom locations of the bubble boundaries as well as the tip locations of the jets over time are plotted. For the in-phase oscillation (Fig. 4.4a), the proximal walls of the bubbles move toward each other and stagnate in the mid-plane while the distal walls expand outwards more extensively before collapsing in unison back toward the mid-plane (see also the 1st row in Fig. 4.2a). The oscillations of the two bubbles are merely mirror images of each other, as well as the two axial jets, which move faster than the collapse of the remaining portions of the distal walls of the bubbles. In contrast, the expansion and collapse of the two bubbles in the anti-phase oscillation (Fig. 4.4b) are significantly different from each other in terms of bubble collapse time and maximum axial elongation. For example, the extrusion of the proximal surface of B_2 is clearly coupled with the advance of the tip of J_1 inside the collapsing B_1 . However, the advance of the tip of J_2 inside the collapsing B_2 is largely de-coupled with the rebound

Figure 4.4 Temporal development of the top and bottom poles of the tandem bubble along the y axis (see the coordinates in Fig. 2): (a) in-phase oscillation, (b) anti-phase oscillation. Solid lines: B₁ top and B₁ bottom; dashed lines: B₂ top and B₂ bottom; dotted lines: B₁ jet tip and B₂ jet tip

(or re-expansion) of B₁ because of its rapid break up due to surface instability.

Consequently, the speed of J_2 in general is found to be significantly slower than J_1 (Fig. 4.5). Furthermore, the diagram illustrates clearly the lengthening or shortening in collapse time when the tandem bubble oscillates either in-phase or out-of-phase, respectively.

4.4.1.4 Jet velocity

Figure 4.5 Relation between jet velocity and time delay between the generation of the first and second bubbles. J_1 and J_2 indicate the jets from the collapse of the first and second bubbles, respectively. The jet speed is averaged within 500 ns after the initiation of the collapse of each bubble, and the results are obtained from six individual measurements.

Figure 4.5 shows the dependency of jet velocity on the time delay (τ) between the generations of the two bubbles. The speed of J_1 was measured by averaging the tip displacement on the proximal wall of B_1 over the first 500 ns starting from the initial expansion of B_2 . The results show that the maximal speed of J_1 (95 ± 7 m/s) is reached at

$\tau = 2 \mu\text{s}$ for the anti-phase bubble oscillation, which is likely correlated with the most efficient energy transfer from B_2 to B_1 [132]. The speed of J_2 was measured by averaging the tip displacement on the proximal wall of B_2 over the first 500 ns from the initial collapse of B_2 . The speed of J_2 was found to increase progressively from $\tau = 1$ to $4 \mu\text{s}$.

4.4.2 Characteristics of tandem bubble interaction and jet formation in the presence of single cells nearby

Figure 4.6 Dynamics of tandem bubble interaction at different SDs in two adhesion groups “H” and “I”.

Fig. 4.6 shows representative image sequences of the dynamic interaction of tandem bubbles at different SDs (10 to 40 μm) in two adhesion groups (“H” and “I”). Only

results from experiments with the maximum bubble size within the range $50 \pm 1 \mu\text{m}$ were selected for analysis. At $SD = 10 \mu\text{m}$ (e.g., H-10 and I-10), the expansion of B_1 was found to be disturbed slightly in the axial direction by the target cell (see top boundary of B_1 at $3 \mu\text{s}$). Under these experimental conditions, the radius of the bubble at its maximum expansion ($25 \mu\text{m}$ in the projected image plane) already exceeded the SD , and therefore the leading edge of B_1 along the axial direction of the tandem bubble could be in touch with the target cell. In contrast, when the SD was equal to or greater than $20 \mu\text{m}$ the interaction of B_1 with the target cell during bubble expansion was hardly noticed. The first jet J_1 (shown in Fig. 4.6), developed from B_2 towards B_1 , was observed to reach the distal end of B_1 between 3.75 and $4 \mu\text{s}$. Because J_1 is primarily responsible for creating the mechanical stress and resultant membrane deformation on the target cell, we calculated the average speed of J_1 during the collapse period of B_1 in different cases

Figure 4.7 Jet speed (J_1) produced by tandem bubble interactions with the presence of a single cell at different SD s from B_1 in two adhesion groups “H” and “I”.

based on the acquired high-speed images. The results, shown in Fig. 4.7, reveal that the existence of the target cell nearby does not affect significantly the evolution of J_1 during

the collapse of B₁. An average speed of ~50 m/s was obtained in all cases. In a separate experiment, the “H” or “I” regions were not seeded with cells, and the bubble dynamics was captured in the cell culture medium with the same acquisition sequence. The jet speed was calculated to be 50 ± 5 m/s similar to all the cases with cells nearby.

4.5 Summary

In this chapter, we investigated the dynamics of tandem bubble interactions in a microfluidic channel developed for single cell treatment under well-controlled experimental conditions. In close proximity ($\gamma = 0.8$), the coupled oscillation of the tandem bubble produces asymmetric deformation of the bubble walls, leading to axial jet formation either toward or away from each other depending on the phase relationship between the two bubbles. The rotational direction and strength of the resultant vortices around the tandem bubble, as well as the axial jet speed can also be adjusted and controlled by changing the phase delay. Based on the jet speed measurement, the development of jetting flow was found to be consistent and minimally influenced by the presence of a single adherent cell nearby. Considering that the jetting flow is presumably and primarily responsible for the bioeffects induced in the target cell, this experimental system provides a reliable source of high strain-rate loadings that can be applied to a localized area of a target cell to assess the mechanical and biological responses of a single cell to cavitation exposure.

5. Biological and Mechanical Responses of Single Cells to Tandem Bubbles – Effects of Standoff Distance and Adhesion Pattern

5.1 Introduction

Previous studies on cavitation-induced bioeffects have been focused on specific and often individual processes in the bubble-cell interaction, such as bubble dynamics [37] or macromolecular uptake in the target cell(s) after cavitation exposure [36]. However, there is a disconnect between bubble dynamics and the resultant cell response produced by the stresses imposed by the bubble oscillation. Most importantly, the intermediate process of cell deformation that relates critically cavitation-induced stress and strain on the cell membrane to different types of cell response has not been analyzed. Although techniques exist for analyzing cell deformation under quasi-static loading conditions [139], they have not been applied to quantify the rapid deformation and recovery of cell membrane under the high strain-rate loadings produced by cavitation bubbles. Furthermore, the lack of an appropriate constitutive model for biological cells makes it difficult to carry out in-depth analyses of the stresses induced and transmitted through the target cell during cavitation exposure. In light of this background, we will make a preliminary effort in this chapter to characterize the dynamic processes of cell deformation produced by tandem bubbles, using 2 μm sized polystyrene beads attached to the cell membrane. In combination with high-speed imaging of bubble dynamics and sequential microscopy of PI uptake, as well as extended cell culture for assessing the long-term bioeffects in the

targeted cell after the tandem bubble treatment, we hope to gain more insights into the mechanism of cavitation-induced bioeffects at the single-cell level.

5.2 Materials and methods

5.2.1 Experimental system and sample preparation

A microfluidic chip with patterned areas on the glass substrate for cell adhesion and tandem bubble generation, as described in Chapter 3, was seeded with HeLa cells at a concentration of 5×10^6 cell/ml using a flow rate of 30 $\mu\text{L}/\text{min}$. The flow was then stopped and the chip was maintained in a cell culture incubator (NU-8500, NuAire) for 30 min to allow the cells to initiate adhesion to the substrate. The chip was subsequently perfused with cell culture medium at the abovementioned flow rate to flush out non-attached cells, followed by incubation for 1.5 hours with a slow perfusion of the culture medium into the channel at a low flow rate of ~ 0.5 $\mu\text{L}/\text{min}$ to maintain sufficient nutrient supply. As shown in Fig. 3.7, the two inlets of the microfluidic chip were connected through 0.5'' long and 0.025''/0.017'' in outer/inner diameter metal pins (New England Small Tube) and 20 mm long and 0.02'' inner diameter TYGON[®] tubing (#AAQ04103, Saint-gobain Ppl) to individual 1 ml syringes (#309628, BD) mounted on two syringe pumps (Nanojet, Chemyx). In general, PBS buffer was maintained in syringe #1 and used as a flushing agent when the medium in the microchannel needed to be replaced. In comparison, DMEM cell culture medium was loaded initially in syringe #2 to supply nutrients to individual cells that had spread out and adhered to the patterned

“H” or “I” areas in the microfluidic channels. Thereafter, the microfluidic chip assembly together with the two syringes was transported onto the microscope stage. The pure DMEM medium in syringe #2 was replaced by 100 µg/ml PI solution (in DMEM), which was slowly perfused into the channels at a flow rate of 0.5 ml/min so that the adherent cells would not be detached by flow-induced shear stresses as confirmed by microscopy. To avoid introducing any air bubbles into the microfluidic channels during this procedure, the following protocol was employed for medium exchange. First, the metal pin together with connected tubing was unplugged from inlet #2 of the microfluidic chip. Second, syringe #1 was used to pump PBS buffer into the channels at a flow rate of 5 µl/min until a liquid droplet was formed on top of the inlet #2. Third, syringe #2 was switched to PI solution and injected through the tubing to form a liquid droplet at the tip of the metal pin. At this moment, it was safe to plug the metal pin back into the port of inlet #2. The two droplets would merge together when brought into contact at the connection site, so entrapment of air bubbles could be prevented. After tandem bubble treatment, a similar procedure was performed to replace PI solution in syringe #2 with pure DMEM cell culture medium. During the tandem bubble treatment, the microfluidic channels were perfused continuously with the PI solution at a constant flow rate of 0.5 µl/min. Cells grown in the patterned areas showed no detectable signs of degradation within the 2-hour experiment, during which a total of 40 cells could be treated together with acquisition of pre- and post-treatment images. After the experiment, the PI solution in the microfluidic channels was replaced by a combination of FITC-Annexin V (20× dilution from stock

solution) and PI (100 $\mu\text{g/ml}$) staining solution in PBS for assessment of apoptosis and cell viability. The tandem bubble treated cells were incubated in the Annexin V/PI staining solution for 15 min before fluorescent imaging. After all the target cells were imaged, the Annexin V/PI staining solution in the microfluidic channels was replaced with normal DMEM cell culture medium, and the microfluidic chip was placed back in the cell culture incubator. Cell culture medium was maintained at a flow rate of 0.2 $\mu\text{l/min}$ with the syringe pump for overnight culture. Next day, the microfluidic chip was transported on the microscope stage and the cell culture medium was replaced by the PI staining buffer solution as previously described. The target cells were imaged again after 15 min incubation.

5.2.2 Cell treatment and image acquisition

The microfluidic chip, prepared as described above, was placed on the stage of an inverted microscope (Axio Observer D1, Zeiss). Two Q-switched Nd:YAG lasers of 1064 nm wavelength and 5 ns pulse duration [MiniLase I (Laser 1) and Orion (Laser 2), New Wave Research] were focused through a 63 $\times$ objective (LD Plan Neofluar, Zeiss) and projected on a pair of gold dots with the aid of the microscope stage. Laser 1 was triggered first, generating bubble 1 (B_1) and Laser 2 was triggered in 3 μs thereafter to generate bubble 2 (B_2). Therefore the expansion and collapse of the two bubbles were out of phase, leading to the formation of a liquid jet (J_1) during the collapse of B_1 towards the target cell (see Fig. 4.6).

Since PI was mixed in the culture medium, it could be used as a fluorescent marker for

molecular uptake if membrane poration occurred as a result of the tandem bubble treatment. For viability test, PI combined with Annexin V in PBS was perfused through the microchannels in 2 hours following the beginning of the tandem bubble treatment. The timeline for a typical treatment is summarized in Fig. 5.1.

For imaging acquisition, the original intracellular PI intensity and geometry of the cell before the tandem bubble treatment were recorded by the Zeiss camera using red fluorescence (FL) and bright field (BF) imaging, respectively. Zeiss AxioVision software was used to program and control the shutter on/off switch for the light source and the filter cube positions in the turret. A trigger signal was captured by a photodetector placed between light source and microscope condenser when the shutter for bright field luminescence was opened. This trigger signal was received by the delay generator, and used as a time reference to control the release of the trigger signals to lasers and cameras

Figure 5.1 Timeline of imaging acquisition before and after tandem bubble-cell interaction.

sent out by the multichannel delay generator with programmed delays (see Fig. 5.1).

The dynamics of tandem bubble interaction were captured by the Imacon camera with 1

μs interframe time (IFT) and 50 ns exposure time at gain 8 in a 14 μs duration following the trigger of the first pulsed laser. In some experiments, the IFT was shortened to 150 ns in order to record clearly the characteristics of jet formation and progression. Immediately after the tandem bubble-cell interaction, the deformation of the target cell was recorded for 1 ms using the phantom camera at 12 μs IFT and 1 μs exposure time. Thereafter, the Zeiss camera, operated at 2 to 10 s IFT, was used to capture the dynamic process of PI diffusion from the poration site into the target cell for the time period from 2 to 120 s. Finally, Annexin V and PI staining performed at 2 and 24 hours after the tandem bubble treatment were also imaged with the Zeiss camera.

5.2.3 Analysis of PI uptake and pore size estimation

For the cells in which the intracellular PI intensity reached a saturation level within 2 minutes after the tandem bubble treatment, a quasisteady-state diffusion model [36] was adapted to estimate the maximum pore size initially formed on the cell membrane. It was assumed that the PI molecules presented in the extracellular solution would diffuse across the cell membrane rapidly during the transient pore opening and resealing period, and then bound to intracellular nucleic acids to form PI-DNA or PI-RNA complexes that emit fluorescence upon excitation. After the pore resealed, the PI-DNA or PI-RNA complexes further diffused within the cytoplasm, leading to spatiotemporal changes in intracellular PI intensity that could be quantified experimentally. Detailed description of the quasisteady-state diffusion model can be found in a recent paper published by Cheri Deng's group at University of Michigan [36]. Briefly, the time dependent pore size is

described by an exponential function:

$$r_a(t) = r_0 e^{-\beta t} \quad (5.1)$$

where r_0 is the maximum pore radius and β is a pore resealing time constant. With the assumption that the pore resealing is slower than PI diffusion across the cell membrane, the PI transport into the cell is considered quasisteady-state, and the overall amount of PI entering the cell is a time integral of the PI influx at the poration site. With these assumptions, the time dependent intracellular PI fluorescence intensity change (ΔPI) can be determined by:

$$\Delta PI(t) = \alpha \pi D C_0 r_0 (1 - e^{-\beta t}) / \beta \quad (5.2)$$

where α is a coefficient that relates to the amount of PI molecules with PI-RNA fluorescence intensity measured by the experimental imaging system, D is the diffusion coefficient of PI inside the cell, and C_0 is the PI concentration in the surrounding medium. With α , D and C_0 all being constant, the maximum pore size (r_0) is proportional to the product of the time constant β and the maximum PI fluorescence intensity change ($\Delta PI_{\max}$).

Because we haven't determined the exact values for α and D in our experimental system, the maximum pore size cannot be calculated for individual cases. Instead, we calculated the normalized maximum pore size (Eq. 5.3), based on the PI measurement from each cell divided by the average maximum pore size estimated in the I-20 group where the tandem bubble-induced PI uptake (and thus the corresponding pore size) is minimal.

$$\overline{\Delta PI_{\max}} = \Delta PI_{\max} / \langle \Delta PI_{\max} \rangle_{I-20} \quad (5.3)$$

5.2.4 Analysis of cell membrane deformation, strain and stretch ratio estimation

Cell is a 3D object with irregular shape and complicated intracellular structures, and deforms non-homogeneously [62]. However, for the adherent cells that spread out on the “H” and “I” patterned areas in this study, the lateral dimension of the cell (i.e., 32 μm) is significantly larger than its height (i.e., < 8 μm). Therefore finite plane strain can be assumed when calculating the strain in a small portion of the cell membrane, as done previously in studies of analyzing neutrophils migration [140] and bi-axial stretch of cultured vascular smooth muscle cells on a deformable substrate membrane [141]. To characterize the cell membrane deformation in response to tandem bubble-induced jetting flow, we traced the displacements of polystyrene beads that were chemically bound to the cell membrane. The polystyrene beads (2 μm in diameter) with carboxyl functional group on the surface were conjugated with RGD peptide (Peptide-2000) first to facilitate their binding to the integrins on the cell membrane [142].

A triangulation scheme, which defines a small but finite area of interest, was adapted to analyze the local membrane strain. Specifically, a triad of beads were identified from the high-speed image sequences captured by the Phantom camera and tracked throughout the tandem bubble-cell interaction for up to 1 ms. The beads coordinates over time were recorded from the time-lapse images, and strain calculation was carried out in a custom code written in Matlab (The MathWorks) following the approach originally described by

Fung [143] and subsequently adapted by others [140]:

$$\Delta s^2 - \Delta s_0^2 = 2 \varepsilon_{ij} \Delta a_i \Delta a_j \quad (5.3)$$

where Δs_0 and Δs are the distance between two beads before and after stress, ε_{ij} is the strain tensor, Δa_i and Δa_j are the distance between the two beads along axis i and j in a orthogonal coordinate system (such as the one used the original image frame). At least three beads are required to calculate three strain components ε_{11} , ε_{12} , ε_{22} in this orthogonal coordinate system. Thereafter, two principal strain components ε_k ($k = 1, 2$) in orthogonal directions $v_1^{(k)}$, $v_2^{(k)}$ can be computed by solving the following eigenvalue problem:

$$(\varepsilon_{ij} - \varepsilon_k \delta_{ij}) v_i^{(k)} = 0 \quad (5.4)$$

where δ_{ij} is the Kronecker symbol.

To facilitate data interpretation, the calculated principal strain ε_1 and ε_2 were also transformed to strains along (or in the x-axis) and perpendicular (or in the y-axis) to the jetting flow direction based on the Mohr's circle analysis for plane strains as follows:

$$\varepsilon_x = \frac{\varepsilon_1 + \varepsilon_2}{2} + \left(\frac{\varepsilon_1 - \varepsilon_2}{2} \right) \cos 2\theta \quad (5.5)$$

$$\varepsilon_y = \frac{\varepsilon_1 + \varepsilon_2}{2} - \left(\frac{\varepsilon_1 - \varepsilon_2}{2} \right) \cos 2\theta \quad (5.6)$$

$$\frac{\gamma_{xy}}{2} = - \left(\frac{\varepsilon_1 - \varepsilon_2}{2} \right) \sin 2\theta \quad (5.7)$$

where ε_x and ε_y are the normal strains along the x- and y-axis, and γ_{xy} is the shear strain in the x-y plane, and θ is the angle between ε_1 and ε_x .

In some cases, a pair of beads with close proximity can be identified on the cell membrane. The stretch ratio ε of the line element between the pair of beads can be calculated based on the normalized length change ($L \rightarrow L'$) as follows:

$$\varepsilon = (L' - L) / L \quad (5.8)$$

5.2.5 Data analysis and statistics

Data collection was carried out from cells adhered to either the “H” or “I” patterns in the microfluidic channels. For cells in each adhesion pattern (referred to as group “H” or group “I” henceforth), different *SDs* were evaluated. Under a specific test configuration (i.e., fixed *SD* and adhesion pattern), the experiments were repeated for 14 ~ 17 times to facilitate statistical analysis of the results. Student t-test was used in each adhesion pattern when comparing the results between two different *SDs*. Statistical analysis of the results between the two adhesion pattern groups was performed using two-way ANOVA analysis with VassarStats online calculator.

5.3 Results and discussion

5.3.1 PI uptake

5.3.1.1 Kinematics and general features of PI uptake in the target cell

Three different types of acute responses were observed from individual cells treated by the tandem bubbles at different *SDs* in group “H” (Fig. 5.2). The target cell could be killed instantly when the *SD* was small (i.e., 10 μm), which led to necrosis. As shown in the left column of Fig. 5.2, cell membrane clearly lost its integrity and cell nucleus was

heavily stained with PI. This result suggests the jet-induced mechanical stress on the cell

Figure 5.2 General observation of cell morphology change and PI uptake after tandem bubble-cell interaction. Colocalization of PI and Hoechst (blue fluorescence for live cell nuclear staining) at the nucleus site indicates cell death.

membrane had exceeded the irreparable damage threshold. When the *SD* was large (*i.e.*, 40 μm), the PI uptake in the target cell was negative (see images in the right column Fig. 5.2), indicating that the cell membrane integrity was not compromised by the tandem bubble interaction. Most interestingly, when the *SD* was in the middle range (*i.e.*, 20 to 30 μm), a detectable level of PI uptake was often observed while the target cell still maintained its integrity after interaction with the tandem bubbles. Cells in group “I”

showed a similar trend with a clear SD dependency in PI uptake.

Figure 5.3 Time course of PI uptake in the target cell after tandem bubble

Depending on the SD , the time-lapse fluorescent images of PI uptake showed three characteristic appearances (Fig. 5.3). At SD of 10 μm , PI started to diffuse from the poration site at the proximal edge of the cell facing the jetting flow into the cytosol of the target cell, and gradually accumulated at the nucleus site. The average intensity of the PI inside the target cell, however, wouldn't reach a plateau within the 2-minute observation window. This result indicates that the membrane poration is severe and cannot be

repaired by the target cell, which will eventually lead to necrosis of the cell [144]. In comparison, at $SD = 20$ or $30 \mu\text{m}$, PI started to diffuse into the cytosol of the target cell from the proximal edge without a clear built-up in the nucleus site. The PI intensity inside the target cell also increased less rapidly and reached a plateau level within 30 seconds, indicating a reseal of the poration site [144]. Thereafter, the cell may survive. At $SD = 40 \mu\text{m}$, no detectable PI uptake was observed in the target cell and the intracellular PI intensity remained at a level comparable to the pre-treatment background value.

Based on the ΔPI vs. time plots, we therefore further divided the outcome for all treated cells in group “H” (or “I”) into 3 categories: necrosis, uptake and no uptake. The

Group “H”				Group “I”			
SD	Necrosis	Uptake	No uptake	SD	Necrosis	Uptake	No uptake
10 μm	100%	0	0	10 μm	100%	0	0
20 μm	42%	58%	0	20 μm	13%	80%	7%
30 μm	0	73%	27%	30 μm	0	80%	20%
40 μm	0	0	100%	40 μm	0	0	100%

Table 5.1 Three different outcomes in membrane poration and PI uptake following tandem bubble-cell interaction at various standoff distances (SD) in group “H” and group “I”.

percentage of each category under different treatment conditions is summarized in Table 5.1. It was observed that at $SD = 10 \mu\text{m}$ all cells were killed instantly while no membrane compromise was produced at $SD = 40 \mu\text{m}$, in dependent of the adhesion patterns. In comparison, when SD was in the range of 20 to $30 \mu\text{m}$, significant number of cells

showed appreciable amount of PI uptake while reaching a saturation level within 2 min. Overall, there is shift in the percentage of cells undergoing necrosis to repairable membrane poration to no membrane poration after the tandem bubble treatment as the SD increases from 10 μm to 40 μm . Also, cells in group “I” appear to have a higher percentage in repairable membrane poration than their counterparts in group “H”.

5.3.1.2 Assessment of pore size variation, apoptosis and phenotype

Figure 5.4 Intracellular PI amount at saturation level

The repairable membrane poration observed at SD = 20 ~ 30 μm has potential utility in targeted gene delivery to selected individual cells, in which the porated cell must survive in order to allow for gene expression in several hours to one day after the treatment. We therefore repeated the experiments in the “uptake” regime for both “H” and “I” groups to determine whether there are significant differences in the amount of PI uptake at the saturation level among different groups and in the estimated pore size based

on a quasisteady state diffusion model described in section 5.2.3 [36]. In addition, Annexin V staining was applied 2 hours after the tandem bubble treatment to detect early stage apoptosis while phenotype of the treated cells were evaluated following extended culture over 24 hours after the tandem bubble treatment.

Figure 5.5 Normalized maximum pore size of individual cells

The PI uptake at the saturation level is shown in Fig. 5.4. Although PI level varied significantly at in each group at a specific SD , the averaged PI level at $SD = 20 \mu\text{m}$ in either group was found to be significantly higher than their counterparts at $SD = 30 \mu\text{m}$. Between the two adhesion patterns, cells in group “H” showed significantly higher PI uptake than those in group “I” orientation. Moreover, the normalized maximum pore size

Figure 5.6 Time course of phenotype changes of survival and apoptotic cells after tandem bubble treatment

for each cell was calculated, and the results are shown in logarithmic scale in Fig. 5.5. Overall, the calculated maximum pore size spans over a range of two orders of magnitude. Even within each group, the difference in maximum pore size can change by an order of magnitude at different *SDs*. This result may reflect the high degree of

heterogeneity in biological cells from the same population [57]. It may also be caused by the significant differences in the cytoskeleton structure of individual cells and therefore in their response to similar external mechanical stresses. The averaged maximum pore size, however, shows a similar trend as in the PI uptake (Fig. 5.4), and significant differences were observed in terms of their dependence SD and adhesion patterns.

Following prolonged cell culture, we observed two typical responses of the cells (survival and apoptotic), revealed by their phenotype and viability/apoptosis analysis. As shown in Fig. 5.6, an example of survival Cell #1 was found to spread out with limited and discrete Annexin-V staining at 2 hours after the tandem bubble treatment, and subsequently divided into two daughter cells within 24 hours. In contrast, apoptotic Cell #2 started to round up with dispersed Annexin-V staining at 2 hours after the treatment, and disintegrated into small pieces within 24 hours. In total, 14 ~17 cells were tracked for each case, and the percentage of cells in either the survival or apoptotic group are summarized in Table 5.2. The cell survival rate at $SD = 20 \mu\text{m}$ in both “H” and “I”

SD	Group “H”		Group “I”	
	Survival	Apoptotic	Survival	Apoptotic
20 μm	15%	85%	47%	53%
30 μm	53%	47%	87%	13%

Table 5.2 The fate of treated cells with PI uptake

groups is lower than their counterparts at $SD = 30 \mu\text{m}$. For a given SD , less cells survived

in the “H” group than in the “I” group. Although the percentage of cells with saturated PI uptake is not statistically different between SD of 20 and 30 μm in the “H” and “I” groups (see Table 5.1), the cells in these group will presumably recover differently, leading to significant differences in their survival and apoptosis outcome. These differences in the subpopulations appear to be associated with the variations in the PI saturation level and the maximum pore size (see Fig. 5.4 & 5.5). In summary, cells with higher PI uptake are likely to be associated with larger pore size, which, although beneficial for macromolecular delivery, also increases the risk for apoptosis.

5.3.4 Cell membrane deformation, stretch ratio and strain calculations

We hypothesize that the differences in PI uptake, maximum pore size and cell phenotype are related to the mechanical strains induced on the cell membrane by the jetting flow produced by tandem bubbles (Fig. 5.7). In principle, cell membrane strain can be estimated using micron-sized beads attached to the cell surface. In this preliminary study, we aim to provide some evidence of support for such an approach, which has not been utilized in characterizing cell membrane deformation produced by the high strain-rate loading associated with cavitation bubbles.

A typical example of the cell membrane deformation in response to the jetting flow produced by the tandem bubbles is shown in Fig. 5.8. Following the passage of the jetting flow (at about $t = 0$ s), the cell membrane was observed to deform significantly with the leading edge of the cell being shear-stretched inwards along the axis of the jetting flow. The largest deformation of the cell membrane was generally observed in

Figure 5.7 Schematic diagrams illustrating tandem bubble-cell interaction and associated flow field produced in a microfluidic channel: a) Side view, b) top view, c) x-y coordinate system established for membrane strain calculation

the second or third image frame (*i.e.*, within $14 \mu\text{s}$), which was then followed by a longer period ($> 500 \mu\text{s}$) during which the deformed cell gradually recovered back to its initial shape. During this rapid deformation and recovery period, all the beads were found to remain firmly attached to the cell membrane. This general observation from numerous cells under a variety of treatment conditions suggests that the binding between the beads

Figure 5.8 Deformation of a HeLa cell grown on a “I” pattern with beads attached on the membrane at a SD of 30 μm (i.e., “I-30”). The original boundary of the target cell is sketched with red lines on each frame.

and cell membrane was sufficiently strong to overcome the mechanical shear force imposed by the jetting flow. Therefore the beads can be used reasonably as surface markers to assess membrane deformation and to calculate resultant strains as appropriate. The time in each image frame is in reference to the instance when the first bubble was generated.

Figure 5.9 Regional difference in membrane displacement of a HeLa cell on “H” pattern and SD = 30 μm (i.e., “H-30”) along the direction of jetting flow produced by the tandem bubble. Five beads in a row along the jet direction, indicated by the yellow arrow, are marked with red crosses and tracked during cell membrane deformation. The original boundary of the target cell is sketched with black lines on each frame.

Because the distribution of the beads on the cell membrane is random and difficult to control, we have selected 12 examples from a total of 72 cells treated in this series of experiments for further analysis using the following criteria:

- 1) the beads remained clearly focused on the image plane in frames where coordinates were recorded;

2) the beads were not constrained in their motion by other neighboring beads;

Several interesting observations can be made based on these selected cases. In Fig. 5.9, five beads from a HeLa cell on “H” pattern and SD = 30 μm (i.e., “H-30”) located approximately in a row along the jet direction were chosen to calculate the stretch ratio of the line segments between them. Following the tandem bubble interaction, the line segment in the proximal region (between bead#1 and #2) was elongated, while the line segment in the central region (between bead#2 and #3) showed minimal deformation,

Figure 5.10 The stretch ratio of 4 line segments defined between adjacent two beads attached on the cell membrane (see Fig. 5.9). Each curve (from top to bottom) corresponds to the time-history of the stretch ratio of a line segment with its midpoint at 11.4, 15.7, 21.2 and 26.6 μm , respectively, from the leading edge of the target cell.

and yet the line segments in the distal region of the cell were compressed (Fig. 5.10). These regional differences in membrane deformation are apparent in most of the cells analyzed, which is a reflection of the localized stress produced by the tandem bubble as well as the heterogeneous strain configuration across the cell membrane. The pinpoint rupture and directional diffusion of Trypan blue (Fig. 2.8a) and PI (Fig. 5.3) into the target cell, together with the SEM evidence of the membrane damage after the tandem bubble treatment (Fig. 2.8c&d) all suggest that high mechanical stress and strain are likely produced near the leading edge of the target cell. In the following, we will focus on the strains induced in the proximal region of the target cell and examine their dependency on *SD* and adhesion pattern (“H” vs. “I”).

Figure 5.11 Deformation of the leading edge of a HeLa cell on “I” pattern and *SD* of 20 μm (i.e., “I-20”). The jet direction is indicated by the yellow arrow. The original boundary of the target cell is sketched with solid black lines while the deformed leading edge of the cell boundary is sketched with dashed black curves. The displacement of 5 beads (marked with red crosses) near the leading edge are tracked during cell membrane deformation caused by the tandem bubble.

A second example is a HeLa cell grown on “I” pattern with *SD* = 20 μm (i.e., “I-20”). Five beads near the leading edge, which were initially located almost in a line perpendicular to the jet direction, were tracked (Fig. 5.11). The displacement of the beads together with the leading edge showed a clear bending and stretch deformation along the orthogonal direction of the jetting flow. The trajectories of a material line connected

through the five beads at different time points after the tandem bubble interaction are shown in Fig. 5.12. It can be seen clearly that the maximum deformation (bending +

Figure 5.12 Time history of the displacement trajectories of 5 beads near the leading edge of a target cell in Fig. 5.11 along the jet direction.

stretch) was produced at $t = 12 \mu s$, followed by a slow and gradual recovery of the material line towards its original position at $t = -12 \mu s$. Moreover, beads (#2, 3 & 4) located closer to the x-axis were displaced more along the jet direction than beads (#1&5) located off-axis. The line elements between beads #2&3 and #3&4 were elongated during the process of cell deformation, indicating that the central portion on the leading edge of the cell was stretched under tension perpendicular to the jet direction. It is also important to note that the beads near the jetting axis (i.e., #2, 3 and 4) show a radial outward displacement pattern, suggesting a locally impulse stress loading at the center of

the leading edge. In contrast, beads near the peripheral edges of the cell where the membrane is anchored on the substrate in the “I” pattern (i.e., #1 and 5) show a contraction toward the central axis, indicating again the tension produced in the central portion of the stretch leading edge in the direction perpendicular to the jetting flow.

Figure 5.13 Deformation of two HeLa cells on “I” pattern in response to the jetting flow produced by the tandem bubbles at $SD = 20 \mu\text{m}$ (top row) and $SD = 40 \mu\text{m}$ (bottom row). The dotted lines highlight the bending deformation of the leading edge of the target cell, which is significantly larger at $SD = 20 \mu\text{m}$.

The dependency of cell deformation on SD can be observed clearly from cells grown on “I” pattern, based on the extent of bending and stretch of the leading edge after the tandem bubble interaction. As shown in Fig. 5.13, the leading edge of a cell at $SD = 20 \mu\text{m}$ was clearly stretched more significantly than a cell at $SD = 40 \mu\text{m}$. Within $500 \mu\text{s}$, the cell at $SD = 40 \mu\text{m}$ already recovered to its original status, while the cell at $SD = 20 \mu\text{m}$ still showed some residual strain indicated by the dotted line.

The differences in membrane deformation of cells grown on “H” and “I” patterns, especially near their leading edges can be seen in Fig. 5.14. Depending on the adhesion strength to the substrate, the leading edge of the target cell in response to the jetting flow

Figure 5.14 Comparison of membrane deformation of individual cells grown on “H” vs. “I” adhesive patterns. The cell on the “H” pattern (top row) has its leading edge anchored on the substrate during cell membrane deformation. In comparison, the cell on the “I” pattern (bottom row) has its leading edge in free standing without binding to the substrate. Therefore, the leading edge (as sketched in dotted line) shows significant bending deformation in response to the jetting flow produced by the tandem bubble. A triad of beads, marked with red crosses on the membrane of each cell, is identified for strain calculation.

may remain anchored, such as in the cell grown on “H” pattern, or significantly displaced and stretched, such as in the cell grown on “I” pattern. We have further identified three beads in the proximal region of each cell and applied the triangulation scheme described in §5.2.4 to calculate the principal strains and the projected normal strains in the x-y coordinate system (Fig. 5.7). The results are shown in Fig. 5.15. In both cases, the magnitude of the principal strains (Fig. 5.15a and 5.15b) were observed to increase rapidly following the impact of the jetting flow, reaching their respective peak values in 12 μ s. Thereafter, the magnitudes of the principal strains were reduced gradually, approaching the baseline levels after 500 μ s. This result is consistent with the observations from the high-speed images (Fig. 5.14). For the cell grown on “H” pattern, the maximum and minimum principal strains are both positive, indicating a bi-axial tensile strain configuration (see top row in Fig. 5.16). In contrast, for the cell grown on

Figure 5.15 Characteristic time course of the principal strains and projected normal strain along (ϵ_x) or perpendicular (ϵ_y) to the jet direction near the leading edge of the target cell (see Fig. 5.14). “H” cell (Fig. 5.14 top) - a) Principal strains; c) ϵ_x and ϵ_y . “I” cell (Fig. 5.14 bottom) - b) Principal strains; d) ϵ_x and ϵ_y .

“I” pattern, the maximum principal strain is positive while the minimal principal strain is negative, indicating a hybrid bi-axial compressive-tensile strain configuration (see bottom row in Fig. 5.16). The projected normal strains along (or x-axis) and perpendicular (or y-axis) to the jet direction (Fig. 5.15c and 5.15d) show a time course and sign pattern similar to the principal strains. In particular, the proximal region near the leading edge of the cell grown on “H” pattern was found to reach a maximal tensile strain of 520% and 230% in the x- and y-axis, respectively. This finding

Figure 5.16 Illustration of different bi-axial strain configurations produced the jetting flow from tandem bubbles in the proximal region of a cell grown on (a) “H” and (top row) (b) “I” patterns (bottom row).

corresponds to a maximal strain rate of $7 \times 10^6 \cdot \text{s}^{-1}$. The difference in the strain configuration produced at the leading edge of a cell grown on “H” vs. “I” pattern is presumably associated with the different propensity in membrane poration seen in the PI uptake (Fig. 5.4). These exceedingly high strains at such a high strain rate are most likely responsible for the large pore formation and unsaturated PI uptake in target cells located in close proximity to the tandem bubbles.

Finally, pairs of image frames extracted before and $12 \mu\text{s}$ after the tandem bubble-cell interaction from six representative cases of different *SDs* and adhesion patterns are shown in Fig. 5.17. A triad formed by three beads (highlighted with red crosses) on each

cell membrane was selected and used for strain calculation. The results summarized in Table 5.3 show that in both “H” and “I” groups there is a *SD* dependency in principal

Figure 5.17 Examples of deformation of cells grown on “H” and “I” patterns produced by tandem bubbles at different standard off distances. A triad formed by three beads in the proximal region of the cell membrane facing the jetting flow is used to estimate the regional plane strain.

strains, with higher values at smaller *SD*. This *SD* dependency can be related to the aforementioned tandem bubble induced bioeffects on the target cell. Specifically, higher principal strains may presumably lead to large pore formation, high level of PI uptake, and increased risk of post treatment apoptosis. The differences in the bi-axial strain configuration and magnitude of the maximum principal strains are also noticed between the “H” and “I” group at a given *SD*. Since cell membrane rupture is more likely to be induced under bi-axial tensile strain than a hybrid compressive and tensile strain [143], cells in the “H” group would be injured more severely compared to those in the “I” group at the same *SD*. The area stretch ratio calculation also suggests that the cell membrane is under tensile stress in “H” group and under compressive stress in “I” group. Specifically,

at $SD = 20$ and $30\ \mu\text{m}$ when transient pore is preferably produced on the cell membrane (see Table 5.1), the membrane is stretched over 50% for “H” group. This value is

Adhesion pattern	SD (μm)	Normal strain			Principal Strain		Area stretch ratio	Principal direction ($^{\circ}$)
		Along jet	Ortho jet	Shear	Max	Min		
H	20	4.64	2.87	0.3	5.19	2.31	19.5	-29
H	30	0.38	0.1	0.12	0.53	-0.05	0.5	20
H	40	0.27	0.12	-0.3	0.48	-0.16	0.2	35
I	20	-0.31	0.26	-0.07	0.27	-0.31	-0.1	-7
I	30	-0.25	-0.03	-0.13	0.03	-0.31	-0.3	-25
I	40	-0.27	0.00	-0.03	0.00	-0.27	-0.3	-6

Table 5.3 Summary of strain calculation results from 6 cases with different SDs and adhesion patterns.

significantly higher than the threshold ($\sim 3\%$) to cause membrane rupture under quasi-static tension. Interestingly, up to 50% of the porated cells are capable to fully recover and function normally given enough time (see Table 5.2). This result indicates that the cells may respond themselves differently under high strain-rate loading, and the strain threshold for repairable or permanent cell damage could be potentially much higher compared to the corresponding values for the cells under quasi-static stress loading.

5.4 Summary

With spatially defined hybrid gold and fibronectin patterns created on the bottom

surface of the microchannel, we have demonstrated the feasibility of an experimental system for single cell treatment with high throughput potential. We have investigated PI uptake, membrane strain and phenotype changes of the targeted cells at different standoff distances in two different adhesion patterns with either an anchorage edge (in “H”) or a free standing edge (in “I”) facing the jet impact direction. In short SD of 10 μm , all cells will be killed or undergoing necrosis while in long SD of 40 μm , all cells will survive with no membrane compromise, independent of the adhesion pattern. In the medium SD of 20 ~ 30 μm , 58 ~ 80% of the treated cells show repairable membrane poration. The maximum pore size in the repairable poration range can vary up to two orders of magnitude, depending on SD and adhesion strength. Strong adhesion leads to bi-axial tensile strains on the membrane, large pore size and high uptake of PI, but increased risk for apoptosis. In contrast, low adhesion leads to hybrid compressive and tensile strain on the cell membrane, small pore size and lower PI uptake, but higher survival rate. Cell may sustain more than one order of magnitude higher stress under higher strain-rate loading produced by tandem bubble-induced jetting flow than under quasi-static loading. Future work will focus on the completion of the membrane strain characterization with the current experimental system, and exploration of potential applications of this technology for the delivery of therapeutic agents to individual cells, or to guide the differentiation pathway of non-mature cells.

6. Future Work

In this dissertation, we have performed initial proof-of-principle study and follow up transition to a microfluidic based system that was used subsequently to assess the bioeffects produced by tandem bubbles of consistent dynamics and jetting formation on single cells grown on adhesion patterns of defined size and geometry. Beyond these initial efforts and the promising preliminary results, several critical issues warrant further investigations in the near future.

First, the dynamics of tandem bubble interaction need to be further investigated both experimentally and through numerical modeling. The characteristics of jet formation in the microfluidic channel, especially its orientation with respect to channel walls and target cell need to be determined. The transient flow field induced by the tandem bubble needs to be characterized at higher framing rates using μ PIV so that the mechanical stress field imposed by the jetting flow on the target cell membrane during the early stage of the tandem bubble-cell interaction (and pore formation) can be described.

Second, the transport of extracellular macromolecule through the poration site needs to be further analyzed through both numerical modeling and cleverly designed experiments. The effects of convective transport on the macromolecular delivery should be considered.

Third, the changes in cytoskeleton and extracellular matrix binding of the target cell with substrate in response to tandem bubble-induced jetting flow should be investigated.

This study will be critical for pinpointing the location and possibly the size of the pores formed on the cell membrane.

In terms of practical issues, we have demonstrated in Chapter 4 that small pore size is accompanied by low PI uptake, but it has the benefit of reduced risk for apoptosis and permanent cell damage. To improve the efficiency of macromolecular delivery while maintaining a high survival rate of the target cell by tandem bubbles, new treatment strategies need to be explored. Considering that tandem bubble-cell interaction is directional and localized (Chapter 2), it is possible that the target cell can be treated multiple times in sequence from several different directions. This strategy can be readily implemented by a more sophisticated pattern design of the microfluidic channel and an upgrade in the positioning control of the experimental system. Conceptually, tandem bubble treatment should be performed under a condition that will lead to sufficient membrane poration without causing apoptosis or permanent damage in the target cell. Under such a condition, multiple small pores may be created sequentially in different regions of the target cell by tandem bubbles, and thus improving the overall efficiency and quantity of macromolecular delivery. Moreover, the pore opening and resealing is mediated by the wound healing processes in the target cell after the tandem bubble treatment. The intracellular machinery for injury repair is driven largely by the influx of Ca^{2+} from the poration site. Therefore, by adjusting the Ca^{2+} concentration and other chemical cues in the medium, one may be able to modulate the dynamics of pore opening and closure for improved macromolecular delivery.

Furthermore, this dissertation is mainly focused on mechanistic studies of tandem bubble-cell interaction. Therefore, only HeLa cells were used in the bioeffect studies in microfluidic channels with various patterns due to time constraint. However, with the establishment of such a new and versatile technology, we are in a unique position to explore applications in a variety of cells with high translational or clinical relevance. For example, we may employ tandem bubbles to apply localized and precisely controlled shear stresses to stem cells in order to guide their differentiation and phenotype with potential applications in regenerative medicine. We may also use tandem bubbles to study the response of neurons to cavitation-induced shear stresses, which may help to understand the mechanisms of traumatic brain injury by blast waves at cellular level.

Finally, tandem bubble technique described in this dissertation is based on two inertial cavitation bubbles of similar size but with different phase of oscillation. The mutual interaction between the two bubbles is intense and the resultant directional jet is violent, with a strain rate induced on the membrane of a target cell in close proximity exceeding $10^4 \cdot \text{s}^{-1}$. The dynamic response of biological cells under such high strain-rate loading needs to be further investigated in relation to cytoskeleton re-arrangement and extracellular matrix binding and detachment before the mechanism of tandem bubble-induced pore formation can be clearly elucidated.

References

1. Bjorno, L., *Cavitation and Inhomogeneities in Underwater Acoustics - Gottingen, Frg, 9-11 July 1979*. Ultrasonics, 1980. 18(2): p. 94-94.
2. Gogate, P.R. and A.M. Kabadi, *A review of applications of cavitation in biochemical engineering/biotechnology*. Biochemical Engineering Journal, 2009. 44(1): p. 60-72.
3. Shirgaonkar, I.Z., R.R. Lothe, and A.B. Pandit, *Comments on the mechanism of microbial cell disruption in high-pressure and high-speed devices*. Biotechnology Progress, 1998. 14(4): p. 657-660.
4. Balasundaram, B. and S.T.L. Harrison, *Study of physical and biological factors involved in the disruption of E. coli by hydrodynamic cavitation*. Biotechnology Progress, 2006. 22(3): p. 907-913.
5. Balasundaram, B. and A.B. Pandit, *Significance of location of enzymes on their release during microbial cell disruption*. Biotechnology and Bioengineering, 2001. 75(5): p. 607-614.
6. Ben-Yakar, A. and F. Bourgeois, *Ultrafast laser nanosurgery in microfluidics for genome-wide screenings*. Current Opinion in Biotechnology, 2009. 20(1): p. 100-105.
7. Paterson, L., et al., *Photoporation and cell transfection using a violet diode laser*. Optics Express, 2005. 13(2): p. 595-600.
8. Tirlapur, U.K. and K. Konig, *Cell biology - Targeted transfection by femtosecond laser*. Nature, 2002. 418(6895): p. 290-291.
9. Vogel, A., et al., *Mechanisms of femtosecond laser nanosurgery of cells and tissues*. Applied Physics B-Lasers and Optics, 2005. 81(8): p. 1015-1047.
10. Zhu, S.L., et al., *The role of stress waves and cavitation in stone comminution in shock wave lithotripsy*. Ultrasound in Medicine and Biology, 2002. 28(5): p. 661-671.
11. Kennedy, J.E., *High-intensity focused ultrasound in the treatment of solid tumours*. Nature Reviews Cancer, 2005. 5(4): p. 321-327.
12. Mitragotri, S., *Innovation - Healing sound: the use of ultrasound in drug delivery and other therapeutic applications*. Nature Reviews Drug Discovery, 2005. 4(3): p. 255-260.
13. Nyborg, W.L., *Biological effects of ultrasound: Development of safety guidelines. Part II: General review*. Ultrasound in Medicine and Biology, 2001. 27(3): p. 301-333.

14. Ferrara, K., R. Pollard, and M. Borden, *Ultrasound microbubble contrast agents: Fundamentals and application to gene and drug delivery*, in *Annual Review of Biomedical Engineering*, 2007. p. 415-447.
15. Coussios, C.C. and R.A. Roy, *Applications of acoustics and cavitation to noninvasive therapy and drug delivery*. *Annual Review of Fluid Mechanics*, 2008. 40: p. 395-420.
16. Leslie, T.A. and J.E. Kennedy, *High intensity focused ultrasound in the treatment of abdominal and gynaecological diseases*. *International Journal of Hyperthermia*, 2007. 23(2): p. 173-182.
17. Roberts, W.W., et al., *Pulsed cavitation ultrasound: A noninvasive technology for controlled tissue ablation (histotripsy) in the rabbit kidney*. *Journal of Urology*, 2006. 175(2): p. 734-738.
18. Datta, S., et al., *Correlation of cavitation with ultrasound enhancement of thrombolysis*. *Ultrasound in Medicine and Biology*, 2006. 32(8): p. 1257-1267.
19. Vaezy, S. and V. Zderic, *Hemorrhage control using high intensity focused ultrasound*. *International Journal of Hyperthermia*, 2007. 23(2): p. 203-211.
20. Hynynen, K., et al., *Non-invasive opening of BBB by focused ultrasound*, in *Brain Edema XII*. 2003. p. 555-558.
21. Ter Haar, G. and C. Coussios, *High intensity focused ultrasound: Physical principles and devices*. *International Journal of Hyperthermia*, 2007. 23(2): p. 89-104.
22. Rivens, I., et al., *Treatment monitoring and thermometry for therapeutic focused ultrasound*. *International Journal of Hyperthermia*, 2007. 23(2): p. 121-139.
23. Zarnitsyn, V.G. and M.R. Prausnitz, *Physical parameters influencing optimization of ultrasound-mediated DNA transfection*. *Ultrasound in Medicine and Biology*, 2004. 30(4): p. 527-538.
24. van Wamel, A., et al., *Controlled drug delivery with ultrasound and gas microbubbles*. *Journal of Controlled Release*, 2005. 101(1-3): p. 389-391.
25. Allen, T.M. and P.R. Cullis, *Drug delivery systems: Entering the mainstream*. *Science*, 2004. 303(5665): p. 1818-1822.
26. Arora, M. and C.D. Ohl, *Bubble assisted drug delivery*. *Journal of Controlled Release*, 2005. 101(1-3): p. 363-365.
27. Jang, S.H., et al., *Drug delivery and transport to solid tumors*. *Pharmaceutical Research*, 2003. 20(9): p. 1337-1350.

28. Wu, J. and W.L. Nyborg, *Ultrasound, cavitation bubbles and their interaction with cells*. Advanced Drug Delivery Reviews, 2008. 60(10): p. 1103-1116.
29. Church, C.C., *Spontaneous homogeneous nucleation, inertial cavitation and the safety of diagnostic ultrasound*. Ultrasound in Medicine and Biology, 2002. 28(10): p. 1349-1364.
30. Miller, M.W., D.L. Miller, and A.A. Brayman, *A review of in vitro bioeffects of inertial ultrasonic cavitation from a mechanistic perspective*. Ultrasound in Medicine and Biology, 1996. 22(9): p. 1131-1154.
31. Hallow, D.M., et al., *Measurement and correlation of acoustic cavitation with cellular bioeffects*. Ultrasound in Medicine and Biology, 2006. 32(7): p. 1111-1122.
32. Marmottant, P. and S. Hilgenfeldt, *Controlled vesicle deformation and lysis by single oscillating bubbles*. Nature, 2003. 423(6936): p. 153-156.
33. Ohl, C.D., et al., *Sonoporation from jetting cavitation bubbles*. Biophysical Journal, 2006. 91(11): p. 4285-4295.
34. Kinoshita, M. and K. Hynynen, *Key factors that affect sonoporation efficiency in in vitro settings: The importance of standing wave in sonoporation*. Biochemical and Biophysical Research Communications, 2007. 359(4): p. 860-865.
35. Zhou, Y., J.M. Cui, and C.X. Deng, *Dynamics of sonoporation correlated with acoustic cavitation activities*. Biophysical Journal, 2008. 94(7): p. L51-L53.
36. Fan, Z.Z., et al., *Spatiotemporally controlled single cell sonoporation*. Proceedings of the National Academy of Sciences of the United States of America, 2012. 109(41): p. 16486-16491.
37. Prentice, P., et al., *Membrane disruption by optically controlled microbubble cavitation*. Nature Physics, 2005. 1(2): p. 107-110.
38. van Wamel, A., et al., *Vibrating microbubbles poking individual cells: Drug transfer into cells via sonoporation*. Journal of Controlled Release, 2006. 112(2): p. 149-155.
39. Takeuchi, S., T. Sato, and N. Kawashima, *Nonlinear response of microbubbles coated with surfactant membrane developed as ultrasound contrast agent - experimental study and numerical calculations*. Colloids and Surfaces B-Biointerfaces, 2002. 24(3-4): p. 207-216.
40. Zwaan, E., et al., *Controlled cavitation in microfluidic systems*. Physical Review Letters, 2007. 98(25).
41. Le Gac, S., et al., *Sonoporation of suspension cells with a single cavitation bubble in a*

- microfluidic confinement*. Lab on a Chip, 2007. 7: p. 1666-1672.
42. Gomez-Sjoberg, R., et al., *Versatile, fully automated, microfluidic cell culture system*. Analytical Chemistry, 2007. 79: p. 8557-8563.
 43. Zhong, J.F., et al., *A microfluidic processor for gene expression profiling of single human embryonic stem cells*. Lab on a Chip, 2008. 8(1): p. 68-74.
 44. Plesset, M.S. and A. Prosperetti, *Bubble Dynamics and Cavitation*. Annual Review of Fluid Mechanics, 1977. 9: p. 145-85.
 45. Hellman, A.N., et al., *Laser-induced mixing in microfluidic channels*. Analytical Chemistry, 2007. 79(12): p. 4484-4492.
 46. Dijkink, R. and C.D. Ohl, *Laser-induced cavitation based micropump*. Lab on a Chip, 2008. 8(10): p. 1676-1681.
 47. Wu, T.H., et al., *Pulsed laser triggered high speed microfluidic switch*. Applied Physics Letters, 2008. 93(14).
 48. Quinto-Su, P.A., et al., *Examination of laser microbeam cell lysis in a PDMS microfluidic channel using time-resolved imaging*. Lab on a Chip, 2008. 8(3): p. 408-414.
 49. Lohse, D., et al., *Impact on soft sand: Void collapse and jet formation*. Physical Review Letters, 2004. 93(19).
 50. Zwaan, E., et al., *Controlled cavitation in microfluidic systems*. Physical Review Letters, 2007. 98(25).
 51. Quinto-Su, P.A. and C.D. Ohl, *Interaction between two laser-induced cavitation bubbles in a quasi-two-dimensional geometry*. Journal of Fluid Mechanics, 2009. 633: p. 425-435.
 52. Ilinskii, Y.A., M.F. Hamilton, and E.A. Zabolotskaya, *Bubble interaction dynamics in Lagrangian and Hamiltonian mechanics*. Journal of the Acoustical Society of America, 2007. 121(2): p. 786-795.
 53. Lokhandwalla, M. and B. Sturtevant, *Mechanical haemolysis in shock wave lithotripsy (SWL): I. Analysis of cell deformation due to SWL flow-fields*. Physics in Medicine and Biology, 2001. 46(2): p. 413-437.
 54. Carstensen, E.L., et al., *Lysis of Erythrocytes by Exposure to Cw Ultrasound*. Ultrasound in Medicine and Biology, 1993. 19(2): p. 147-165.
 55. Evans, E.A., R. Waugh, and L. Melnik, *Elastic Area Compressibility Modulus of Red-Cell*

- Membrane*. Biophysical Journal, 1976. 16(6): p. 585-595.
56. Lokhandwalla, M., et al., *Mechanical haemolysis in shock wave lithotripsy (SWL): II. In vitro cell lysis due to shear*. Physics in Medicine and Biology, 2001. 46(4): p. 1245-1264.
 57. Mofrad, M.R.K. and R.D. Kamm, eds. *Cellular Mechanotransduction: Diverse Perspectives from Molecules to Tissues*. 2009, Cambridge University Press.
 58. Helmke, B.P., A.B. Rosen, and P.F. Davies, *Mapping mechanical strain of an endogenous cytoskeletal network in living endothelial cells*. Biophysical Journal, 2003. 84(4): p. 2691-2699.
 59. D'Addario, M., et al., *Cytoprotection against mechanical forces delivered through beta(1) integrins requires induction of filamin A*. Journal of Biological Chemistry, 2001. 276(34): p. 31969-31977.
 60. Mayer, M.P., et al., *Multistep mechanism of substrate binding determines chaperone activity of Hsp70*. Nature Structural Biology, 2000. 7(7): p. 586-593.
 61. Hatami-Marbini, H. and C. Picu, *Modeling the Mechanics of Semiflexible Biopolymer Networks: Non-affine Deformation and Presence of Long-range Correlations*, in *Advances in Soft Matter Mechanics*. 2012, Springer Berlin Heidelberg. p. 119-145.
 62. Mofrad, M.R.K., *Rheology of the Cytoskeleton*, in *Annual Review of Fluid Mechanics*. 2009. p. 433-453.
 63. Nakanishi, J., et al., *Recent advances in cell micropatterning techniques for bioanalytical and biomedical sciences*. Analytical Sciences, 2008. 24(1): p. 67-72.
 64. Flemming, R.G., et al., *Effects of synthetic micro- and nano-structured surfaces on cell behavior*. Biomaterials, 1999. 20(6): p. 573-588.
 65. Scotchford, C.A., et al., *Growth of human osteoblast-like cells on alkanethiol on gold self-assembled monolayers: The effect of surface chemistry*. Journal of Biomedical Materials Research, 1998. 41(3): p. 431-442.
 66. Tan, J.L., et al., *Simple approach to micropattern cells on common culture substrates by tuning substrate wettability*. Tissue Engineering, 2004. 10(5-6): p. 865-872.
 67. Jiang, X.Y., et al., *Controlling mammalian cell spreading and cytoskeletal arrangement with conveniently fabricated continuous wavy features on poly(dimethylsiloxane)*. Langmuir, 2002. 18(8): p. 3273-3280.
 68. Webb, K., V. Hlady, and P.A. Tresco, *Relative importance of surface wettability and charged functional groups on NIH 3T3 fibroblast attachment, spreading, and cytoskeletal organization*.

- Journal of Biomedical Materials Research, 1998. 41(3): p. 422-430.
69. Thery, M., *Micropatterning as a tool to decipher cell morphogenesis and functions*. Journal of Cell Science, 2010. 123(24): p. 4201-4213.
 70. Falconnet, D., et al., *Surface engineering approaches to micropattern surfaces for cell-based assays*. Biomaterials, 2006. 27(16): p. 3044-3063.
 71. Lim, J.Y. and H.J. Donahue, *Cell sensing and response to micro- and nanostructured surfaces produced by chemical and topographic patterning*. Tissue Engineering, 2007. 13(8): p. 1879-1891.
 72. Mrksich, M., *What can surface chemistry do for cell biology?* Current Opinion in Chemical Biology, 2002. 6(6): p. 794-797.
 73. Pancrazio, J.J., et al., *Development and application of cell-based biosensors*. Annals of Biomedical Engineering, 1999. 27(6): p. 697-711.
 74. Hasirci, V. and H. Kenar, *Novel surface patterning approaches for tissue engineering and their effect on cell behavior*. Nanomedicine, 2006. 1(1): p. 73-89.
 75. Bhadriraju, K. and C.S. Chen, *Engineering cellular microenvironments to improve cell-based drug testing*. Drug Discovery Today, 2002. 7(11): p. 612-620.
 76. Arnold, M., et al., *Activation of integrin function by nanopatterned adhesive interfaces*. Chemphyschem, 2004. 5(3): p. 383-388.
 77. Veiseh, M., et al., *Guided cell patterning on gold-silicon dioxide substrates by surface molecular engineering*. Biomaterials, 2004. 25(16): p. 3315-3324.
 78. Chen, C.S., et al., *Micropatterned surfaces for control of cell shape, position, and function*. Biotechnology Progress, 1998. 14(3): p. 356-363.
 79. Folch, A., et al., *Microfabricated elastomeric stencils for micropatterning cell cultures*. Journal of Biomedical Materials Research, 2000. 52(2): p. 346-353.
 80. Doyle, A.D., et al., *One-dimensional topography underlies three-dimensional fibrillar cell migration*. Journal of Cell Biology, 2009. 184(4): p. 481-490.
 81. Delamarche, E., et al., *Patterned delivery of immunoglobulins to surfaces using microfluidic networks*. Science, 1997. 276(5313): p. 779-781.
 82. Herbert, C.B., et al., *Micropatterning gradients and controlling surface densities of photoactivatable biomolecules on self-assembled monolayers of oligo(ethylene glycol)*

- alkanethiolates*. Chemistry & Biology, 1997. 4(10): p. 731-737.
83. Liu, Y., et al., *Controlled switchable surface*. Chemistry-a European Journal, 2005. 11(9): p. 2622-2631.
 84. Yamato, M., et al., *Thermally responsive polymer-grafted surfaces facilitate patterned cell seeding and co-culture*. Biomaterials, 2002. 23(2): p. 561-567.
 85. Yeo, W.S., M.N. Yousaf, and M. Mrksich, *Dynamic interfaces between cells and surfaces: Electroactive substrates that sequentially release and attach cells*. Journal of the American Chemical Society, 2003. 125(49): p. 14994-14995.
 86. Dillmore, W.S., M.N. Yousaf, and M. Mrksich, *A photochemical method for patterning the immobilization of ligands and cells to self-assembled monolayers*. Langmuir, 2004. 20(17): p. 7223-7231.
 87. Kikuchi, Y., et al., *Arraying Heterotypic Single Cells on Photoactivatable Cell-Culturing Substrates*. Langmuir, 2008. 24(22): p. 13084-13095.
 88. Nakanishi, J., et al., *Photoactivation of a substrate for cell adhesion under standard fluorescence microscopes*. Journal of the American Chemical Society, 2004. 126(50): p. 16314-16315.
 89. El-Ali, J., P.K. Sorger, and K.F. Jensen, *Cells on chips*. Nature, 2006. 442(7101): p. 403-411.
 90. Blake, J.R. and D.C. Gibson, *Cavitation Bubbles near Boundaries*. Annual Review of Fluid Mechanics, 1987. 19: p. 99-123.
 91. Ohl, C.D., et al., *Bubble dynamics, shock waves and sonoluminescence*. Philosophical Transactions of the Royal Society of London Series a-Mathematical Physical and Engineering Sciences, 1999. 357(1751): p. 269-294.
 92. Dear, J.P., J.E. Field, and A.J. Walton, *Gas-Compression and Jet Formation in Cavities Collapsed by a Shock-Wave*. Nature, 1988. 332(6164): p. 505-508.
 93. Arndt, R.E.A., *Cavitation in Fluid Machinery and Hydraulic Structures*. Annual Review of Fluid Mechanics, 1981. 13: p. 273-328.
 94. Rooney, J.A., *Hemolysis near an Ultrasonically Pulsating Gas Bubble*. Science, 1970. 169(3948): p. 869-&.
 95. Sankin, G.N. and P. Zhong, *Interaction between shock wave and single inertial bubbles near an elastic boundary*. Physical Review E, 2006. 74(4).

96. Sankin, G.N., et al., *Shock wave interaction with laser-generated single bubbles*. Physical Review Letters, 2005. 95(3).
97. Chahine, G.L. and R. Duraiswami, *DYNAMIC INTERACTIONS IN A MULTIBUBBLE CLOUD*. Journal of Fluids Engineering-Transactions of the Asme, 1992. 114(4): p. 680-686.
98. Khoo, B.C., et al., *Multiple Spark-Generated Bubble Interactions*. Modern Physics Letters B, 2009. 23(3): p. 229-232.
99. Hsiao, C.T., et al., *Modelling single- and tandem-bubble dynamics between two parallel plates for biomedical applications*. Journal of Fluid Mechanics, 2013. 716: p. 137-170.
100. Zhou, Y., et al., *Effects of extracellular calcium on cell membrane resealing in sonoporation*. Journal of Controlled Release, 2008. 126(1): p. 34-43.
101. McNeil, P.L. and R.A. Steinhardt, *Plasma membrane disruption: Repair, prevention, adaptation*. Annual Review of Cell and Developmental Biology, 2003. 19: p. 697-731.
102. Ohl, C.D. and B. Wolfrum, *Detachment and sonoporation of adherent HeLa-cells by shock wave-induced cavitation*. Biochimica Et Biophysica Acta-General Subjects, 2003. 1624: p. 131-138.
103. Quinto-Su, P.A., et al., *Manipulation and Microrheology of Carbon Nanotubes with Laser-Induced Cavitation Bubbles*. Physical Review Letters, 2010. 104(1).
104. Lautz, J., et al., *Displacement of particles in microfluidics by laser-generated tandem bubbles*. Applied Physics Letters, 2010. 97(18).
105. Sankin, G.N., F. Yuan, and P. Zhong, *Pulsating Tandem Microbubble for Localized and Directional Single-Cell Membrane Poration*. Physical Review Letters, 2010. 105(7).
106. Le Gac, S., et al., *Sonoporation of suspension cells with a single cavitation bubble in a microfluidic confinement*. Lab on a Chip, 2007. 7(12): p. 1666-1672.
107. Wang, N., et al., *Micropatterning tractional forces in living cells*. Cell Motility and the Cytoskeleton, 2002. 52(2): p. 97-106.
108. Castner, D.G. and B.D. Ratner, *Biomedical surface science: Foundations to frontiers*. Surface Science, 2002. 500(1-3): p. 28-60.
109. Craighead, H.G., C.D. James, and A.M.P. Turner, *Chemical and topographical patterning for directed cell attachment*. Current Opinion in Solid State & Materials Science, 2001. 5(2-3): p. 177-184.

110. Wong, J.Y., J.B. Leach, and X.Q. Brown, *Balance of chemistry, topography, and mechanics at the cell-biomaterial interface: Issues and challenges for assessing the role of substrate mechanics on cell response*. Surface Science, 2004. 570(1-2): p. 119-133.
111. Bretagnol, F., et al., *Surface functionalization and pPatterning techniques to design interfaces for biomedical and biosensor applications*. Plasma Processes and Polymers, 2006. 3(6-7): p. 443-455.
112. Fink, J., et al., *Comparative study and improvement of current cell micro-patterning techniques*. Lab on a Chip, 2007. 7(6): p. 672-680.
113. Velte-Casquillas, G., et al., *Microfluidic tools for cell biological research*. Nano Today. 5(1): p. 28-47.
114. Yang, G.W., *Laser ablation in liquids: Applications in the synthesis of nanocrystals*. Progress in Materials Science, 2007. 52(4): p. 648-698.
115. Farny, C.H., et al., *Nucleating cavitation from laser-illuminated nano-particles*. Acoustics Research Letters Online-Arlo, 2005. 6(3): p. 138-143.
116. Minter, J., et al., *Advanced metal lift-off process using electron beam flood exposure of single layer photoresist*. Microlithography 1999: Advances in Resist Technology and Processing Xvi, Pts 1 and 2, 1999. 3678: p. 1074-1082.
117. Schoning, M.J., et al., *Amperometric PDMS/glass capillary electrophoresis-based biosensor microchip for catechol and dopamine detection*. Sensors and Actuators B-Chemical, 2005. 108(1-2): p. 688-694.
118. Johnson, P.B. and R.W. Christy, *OPTICAL-CONSTANTS OF NOBLE-METALS*. Physical Review B, 1972. 6(12): p. 4370-4379.
119. Schneider, F., et al., *Mechanical properties of silicones for MEMS*. Journal of Micromechanics and Microengineering, 2008. 18(6).
120. Duffy, D.C., et al., *Rapid prototyping of microfluidic switches in poly(dimethyl siloxane) and their actuation by electro-osmotic flow*. Journal of Micromechanics and Microengineering, 1999. 9(3): p. 211-217.
121. Falconnet, D., et al., *A combined photolithographic and molecular-assembly approach to produce functional micropatterns for applications in the biosciences*. Advanced Functional Materials, 2004. 14(8): p. 749-756.
122. Brujan, E.A., et al., *Dynamics of laser-induced cavitation bubbles near elastic boundaries: influence of the elastic modulus*. Journal of Fluid Mechanics, 2001. 433: p. 283-314.

123. Pishchalnikov, Y.A., et al., *Cavitation bubble cluster activity in the breakage of kidney stones by lithotripter shockwaves*. Journal of Endourology, 2003. 17(7): p. 435-446.
124. Miller, D.L., *A Review of the Ultrasonic Bioeffects of Microsonation, Gas-Body Activation, and Related Cavitation-Like Phenomena*. Ultrasound in Medicine and Biology, 1987. 13(8): p. 443-470.
125. Xi, X.F. and P. Zhong, *Improvement of stone fragmentation during shock-wave lithotripsy using a combined EH/PEAA shock-wave generator - In vitro experiments*. Ultrasound in Medicine and Biology, 2000. 26(3): p. 457-467.
126. Lauterborn, W. and W. Hentschel, *CAVITATION BUBBLE DYNAMICS STUDIED BY HIGH-SPEED PHOTOGRAPHY AND HOLOGRAPHY. I*. Ultrasonics, 1985. 23(6): p. 260-268.
127. Blake, J.R., et al., *Interaction of 2 Cavitation Bubbles with a Rigid Boundary*. Journal of Fluid Mechanics, 1993. 255: p. 707-721.
128. Sato, K. and Y. Tomita, *Non-spherical Motion of Two Cavitation Bubbles Produced with/without Time Delay near a Boundary*. in *Third International Symposium on Cavitation*. 1998. Grenoble, France.
129. Kurihara, E., et al., *Model for the dynamics of two interacting axisymmetric spherical bubbles undergoing small shape oscillations*. Journal of the Acoustical Society of America, 2011. 130(5): p. 3357-3369.
130. Chen, Y.H. and I. Lin, *Dynamics of impacting a bubble by another pulsed-laser-induced bubble: Jetting, fragmentation, and entanglement*. Physical Review E, 2008. 77(2): p. 026304.
131. Chen, Y.H., H.Y. Chu, and L. I., *Interaction and fragmentation of pulsed laser induced microbubbles in a narrow gap*. Physical Review Letters, 2006. 96(3).
132. Chang, C.M., et al., *Efficient transfer and concentration of energy between explosive dual bubbles via time-delayed interactions*. Microfluidics and Nanofluidics, 2010. 9(2-3): p. 329-340.
133. Lauterborn, W. and T. Kurz, *Physics of bubble oscillations*. Reports on Progress in Physics, 2010. 73(10).
134. Mettin, R., et al., *Bjerknes forces between small cavitation bubbles in a strong acoustic field*. Physical Review E, 1997. 56(3): p. 2924-2931.
135. Lauterborn, W., *CAVITATION BUBBLE DYNAMICS - NEW TOOLS FOR AN INTRICATE PROBLEM*. Applied Scientific Research, 1982. 38: p. 165-178.

136. Blake, J.R., B.B. Taib, and G. Doherty, *TRANSIENT CAVITIES NEAR BOUNDARIES .2. FREE-SURFACE*. Journal of Fluid Mechanics, 1987. 181: p. 197-212.
137. Chahine, G.L., *Experimental and Asymptotic Study of Non-Spherical Bubble Collapse*. Applied Scientific Research, 1982. 38: p. 187-197.
138. Fong, S.W., et al., *Interactions of multiple spark-generated bubbles with phase differences*. Experiments in Fluids, 2009. 46(4): p. 705-724.
139. Bao, G. and S. Suresh, *Cell and molecular mechanics of biological materials*. Nature Materials, 2003. 2(11): p. 715-725.
140. Barbee, K.A., E.J. Macarak, and L.E. Thibault, *Strain-Measurements in Cultured Vascular Smooth-Muscle Cells Subjected to Mechanical Deformation*. Annals of Biomedical Engineering, 1994. 22(1): p. 14-22.
141. Gilchrist, C.L., et al., *Measurement of intracellular strain on deformable substrates with texture correlation*. Journal of Biomechanics, 2007. 40(4): p. 786-794.
142. Poh, Y.-C. and N. Wang, *The use of Optical Magnetic Twisting Cytometry and Flourescence Resonance Energy Transfer to quantify force-induced protein dissociation in the nucleus of a living cell*. 2012.
143. Fung, Y.C., *Foundations of Solid Mechanics*. International Series in Dynamics. 1965: Prentice Hall.
144. Fan, Z., et al., *Intracellular delivery and calcium transients generated in sonoporation facilitated by microbubbles*. Journal of Controlled Release, 2010. 142(1): p. 31-39.

Biography

Name Fang Yuan
Birth Date 09/22/1981
Birth Place Dongtai, Jiangsu, P.R.China
Education Duke University, Durham, NC
Ph.D. candidate in MEMS (2005-2013)

University of Science and Technology of China
M.S. in Polymer Science and Engineering (2002-2005)

University of Science and Technology of China
B.S. in Polymer Chemistry and Physics (1998-2002)

Refereed Journal Publications

G.N. Sankin, **F. Yuan**, P. Zhong: Pulsating Tandem Microbubble for Localized and Directional Single-Cell Membrane Poration, Physical Review Letters 105 (7), 78101 (2010)

J. Lautz, G.N. Sankin, **F. Yuan**, P. Zhong: Displacement of particles in microfluidics by laser-generated tandem bubbles, Applied Physics Letters 97 (18), 183701-3 (2010)

F. Yuan, G.N. Sankin, P. Zhong: Dynamics of tandem bubble interaction in a microfluidic channel, The Journal of the Acoustical Society of America 129, 2587 (2011)

X. Huang, **F. Yuan**, M. Liang, et al.: M-HIFU Inhibits Tumor Growth, Suppresses STAT3 Activity and Enhances Tumor Specific Immunity in a Transplant Tumor Model of Prostate Cancer, PLOS ONE 7(7): e41632 (2012)

C.T. Hsiao, J.K. Choi, S. Singh, G.L. Chahine, T.A. Hay, Y.A. Ilinskii, E.A. Zabolotskaya, M.F. Hamilton, G.N. Sankin, **F. Yuan**, P. Zhong: Modeling Single and Tandem Bubble Dynamics between Two Parallel Plates for Biomedical Applications, Journal of Fluid Mechanics 137 – 170, 716 (2013)

Awards

DOD Predoctoral Training Fellowship (2008)

Honorarium 3rd Place Poster Award, MEMS Retreat, Duke University (2009)