A NOVEL DESIGN OF MICRO-SIZE TRANSDUCER BASED SINGLE CELL BIO-CHIP

A Dissertation in Engineering Science and Mechanics

by

An Cheng

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The dissertation of An Cheng was reviewed and approved* by the following:

Jian Xu  
Assistant Professor of Engineering Science and Mechanics  
Dissertation Advisor  
Chair of Committee

Jerzy Ruzyllo  
Professor of Electrical Engineering and Materials Science and Engineering

Michael T. Lanagan  
Associate Professor of Engineering Science and Mechanics  
Associate Director of Material Research Institute

Mark W. Horn  
Associate Professor of Engineering Science and Mechanics

Judith A. Todd  
P. B. Breneman Department Head Chair  
Head of the Department of Engineering Science and Mechanics

*Signatures are on file in the Graduate School
ABSTRACT

Cell membranes pose one of the greatest barriers to the delivery of drugs, proteins, DNA and other molecules into cells and tissues. The ability of ultrasound to transiently disrupt these barriers in living cells could provide an important tool, especially for delivery of macromolecular drugs or other compounds requiring access to the cytosol. The relatively large size of ultrasound-created disruptions indicates that this method could be used to deliver a broad range of compounds, such as drugs, proteins, DNA and RNA, etc.

The advance of micro-fabrication techniques enabled the development of novel bio-chip being able to catch, and study the structure, function and characteristic of cells in single cell level, which are concerned with our understanding of the nature and ourselves.

This thesis is aimed towards development of a novel micro-sized ultrasound transducer based bio-chip. Besides being able to catch the single cells, it can generate a confined and low-power ultrasound wave to facilitate the cell intake of small particles, especially for drug molecules. The innovative device fabrication process is addressed in detail. Quantum dots are adopted as the testing agent to conduct test on living cell. The cell intake of quantum dots of both natural diffusion and ultrasound facilitation processes are compared and quantified. A series of ultrasound conditions are tested. It was approved that by controlling the size of the transducer, power and frequency of the ultrasound wave the transducer emits, acoustic wave can be precisely targeted on each individual single cell, therefore to facilitate the cell intake of small particles without noticeable impact on other neighbor cells.
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Chapter 1

INTRODUCTION

1.1 Research Objectives

The cell membrane poses one of the greatest barriers to the delivery of drugs, proteins, DNAs and other molecules into cells and tissues. The ability of ultrasound to transiently disrupt these barriers in living cells could provide an important tool, especially for delivery of macromolecular drugs or other compounds that requires access to the cytosol. The relatively large size of ultrasound-created disruptions indicates that this method could be used to deliver a broad range of compounds, such as drugs, proteins, DNAs and RNAs.

The advance of micro-fabrication techniques enables the development of novel devices that are able to catch and trap cells at single cell level. This in turn boosts the growing interest in single cell study that used to be impractical due to the difficulties in trapping single cell as well as relatively high cost to fabricate the micro-size feature devices used for cell trapping.

Although the single-cell based bio-chip is getting a lot of research attention and starting to show promising potential in fields ranging from drug testing, drug screening, cell sorting, fundamental cell-biology study to environment monitoring, one major problem that hinders the development of single cell-based bio-chips is the relatively low
efficiency in cell uptake. The cell membrane acts as the barrier between cell and cell media and thus prevents external testing agents, (drug molecules, proteins, bio-agents, DNA, RNA) from entering the cell. Therefore, a slow or weak cell response seems to be unavoidable.

This thesis is aimed towards development of a novel micro-sized ultrasound transducer based bio-chip that can be utilized in single cell study. The idea is to combine the advanced micro-fabrication techniques with the knowledge of ultrasound enhanced drug molecule delivery into the cell in order to solve the biggest obstacle, i.e., low efficiency in uptake of the testing molecule, which single cell based bio-sensors are facing. We hope that by controlling the physical sizes of our micro-size transducers and the acoustic power and frequency of the ultrasound wave they emit, acoustic energy can be precisely targeted on each or several individual single cells without any noticeable impact on other neighbor cells, as well as causing any cell-to-cell interaction.

1.3 Outline of Thesis

Chapter 2 consists of a systematic literature review on the application of ultrasound technology in biology and bio-medical study; it traces the history of ultrasound application in cell study and new developments and the evolution in recent years. The tremendous significance of single cell study and the potential of a single cell based bio-chip are also addressed in chapter 2. At the end of chapter 2, an innovative design of micro-sized transducer based single cell bio-chip is proposed. Chapter 3 introduces the unique process approach in the fabrication of the micro-size transducer based bio-chip, detail study from material selection to key processing techniques including, plasma etching, lithography, lapping, etc are addressed. Chapter 4 describes
the characterization of the fabricated micro-sized transducer and its acoustic field measurement. Quantum dots were utilized as the cell labeling agent. The cell culture, experiment design and realization of cell testing using quantum dots are addressed in detail. Chapter 5 summarizes the entire work, and provides important direction and improvement needed to be done for future work.
Chapter 2

ULTRASOUND’S APPLICATION IN DRUG DELIVERY AND THE DESIGN OF AN INNOVATIVE MICRO-SIZE TRANSDUCER BASED BIO-CHIP

2.1 Introduction of Ultrasound

Sound is a form of energy generated from a vibrating object. Sound energy is a mechanical energy propagates by physical movement of the molecules and particles in its propagating media. Sound travels in the form of a wave, a coordinated disturbance movement at a fixed speed of media. As the sound source vibrates, the pressure wave travels. The frequency of the sound wave is determined by the number of the oscillations per second made by the sound source. In physics, the term "ultrasound" applies to all acoustic energy with a frequency above human hearing (20,000 Hz or 20 kHz) [1].

2.2 Ultrasound’s Application in Bio-science and Medication

Over the past several decades, ultrasound has become increasingly important in the practice of medicine. The application of ultrasound to medical diagnosis imaging has achieved continuous development and growth over several decades. It is based on the theory that when acoustic wave encounter an object, a static or dynamic acoustic radiation force is generated on the object (echo). By detecting and analyzing the reflected wave, 2-D or 3-D imaging of the object can be generated. Improvement in technology has
been followed by widespread acceptance and use of ultrasound in medical diagnosis. Applications have progressed from simple measurement of anatomical dimensions. For diagnostic applications, ultrasound (Sonography 1-20 MHz) is one of the most frequently employed clinical imaging modalities. For tissue and body imaging, and has become indispensable in the disciplines of cardiology, obstetrics, and gynecology [2].

Ultrasound is also widely used in therapeutic applications to bring heat or agitation into the body. Therefore, much higher energies are used in diagnostic ultrasound. In many cases, the range of frequencies used are also very different. Ultrasound sources may be used to generate regional heating in biological tissue, e.g. in occupational therapy, physical therapy and cancer treatment. Focused ultrasound may be used to generate highly localized heating to treat cysts and tumors (benign or malignant). These procedures generally use lower frequencies than medical diagnostic ultrasound (from 250 kHz to 2000 kHz), but significantly higher energies [2], [3].

Another physiological effect of low-intensity ultrasound has been discovered and is playing an ever increasing role in bio-medical field. It is the ultrasound facilitation on the delivery of therapeutic agents including drug molecules, genetic material, proteins, and chemotherapeutic agents. The primary advantage of ultrasound is that it is a physical, rather than a chemical approach, the enhancement is likely to be broadly applicable to a variety of drugs and cell types [28]. Traditional drug delivery methods, such as injections and oral medications, are often unsuitable for proteins, DNA, and other biotherapeutic compound. Drug delivery methods need improvement to increase drug and gene efficacy by enhancing intracellular delivery and to increase drug safety by targeting specific cells or organs to reduce side effects. Enhanced and targeted delivery methods are both limited
by insufficient or uncontrolled transport across cell membranes. To address these delivery problems, ultrasound has been used to increase plasma membrane permeability and thereby loading living cells with drugs, proteins and genes. [23]

It was found that the pressure wave of ultrasound traveling in the cell media can produce forces that permeabilize cell membranes to accelerate the agent molecule diffusion process and disrupt the vesicle that carry agent molecules [4], [5].

Until now, the real mechanism of ultrasound facilitation on drug delivery has not been fully understood, cavitations has been reported to possibly play an important role. Cavitation is the formation and activity of gas-filled bubbles in medium exposed to ultrasound [6], [7]. As the pressure wave passes through the media, gas bubbles will start to oscillate. The circulating flow created by bubble oscillation can generate shear forces associated with the oscillating amplitude which are capable of opening red cells [8] and synthetic vesicles such as liposome [9]. Furthermore, the collapse of the cavitation can generate super high pressure up to 1.6 MPa [9], [13] in very small range, the associated shear force can transiently open or rupture the cell membrane.

It was reported that ultrasonication and cavitation can be involved in drug delivery by several mechanism. First mechanism is from the oscillatory motion of the fluid driven by ultrasound. The oscillating fluid increase the effective diffusivity of drug molecules, whether they were free or bound to carrier, will be increased [10]. This enhanced transport can occur within blood, cells, and extracellular fluids [8], [9].

Perturbation of the drug carrier is another impact of ultrasound toward drug delivery. The shear force in ultrasonic filed can help to shear the drug form the carrier
polymer backbone [11], [12], and then release the drug molecule. In the case of drug carried inside the liposome vesicle, when the shear stress generated in ultrasonic filed exceeds the strength of vesicle, it will rupture and release the interior content [11].

The third contribution of ultrasound to drug delivery relates to the stresses that pressure wave applied on cells and tissues, resulting in cell permeabilization and capillary rupture. Cells in an environment with cavitation events are subject to shear force due to microstreaming, sonic jets, and shock waves. It is possible that a small jet of liquid would shoot into the cells directly at sonic speed if a large semi rigid cell is adjacent to a small cavitating bubble which has an asymmetric bubble collapse [14]. This kind of activities can probably rupture the cell membrane. Likewise a collapse of a microbubble near a capillary or blood vessel wall will cause the liquid jet to shoot right into the wall. Such a collapse may be the source of the large amount of extravasation that is caused in tissue exposed to ultrasound in the presence of microbubble contrast agents [15], [17]. Figure 2.1 illustrates various modes by which drug delivery can be enhanced by ultrasound.
2.3. Major Methods for Cell Invitro Drug (Molecule) Delivery Using Ultrasound Facilitation

2.3.1 Laser-Induced Shock Waves

In a number of laser-induced stress wave experiments, it was shown that cell can...
be loaded with different types of molecules that were present in the extracellular medium such as dyes, drugs and genes, this suggest the possibility of laser generated stress wave to deliver molecules (e.g., genes and drugs) into cell for medical applications[16]. S. Lee, and T. Anderson [18] generated stress wave by ablation of 127 um thick polyimide film with an ArF excimer laser. The pressure waves generated in the polyimide propagate ~0.3 mm through the media to the mouse breast sarcoma cell line, EMT-6. The stress wave is about 100 ns duration with a rise time of 10 ns. The magnitude of stress wave measured at the position of the cells was 300±70 bar. During 80 seconds of the application of stress wave, they observed an average 10% decrease of the fluorescence intensity of membrane impermeable calcein for each individual cell indicating the efflux of calcein molecule from cell into the media. The efflux stopped after 80 seconds indicating the membrane opening is a transient process associated with the ultrasonic pressure wave. Figure 2.2 illustrate the experimental set up for laser induced shock wave.

Fig. 2.2 Experimental arrangement of laser induced shock wave to facilitate molecule delivery into cell
In another similar experiment arrangement, D. J. Mcauliffe, S. Lee [19] approved that laser induced stress wave can induce a transient permeabilization of the plasma membrane. The opened channel in cell membrane closes in ~10 seconds. They focused ArF excimer laser source (193 nm, 200 mJ) on polyimide to generated stress wave in medium containing human peripheral blood mononuclear cells. The repetition rate of wave is 0.1 Hz, with 10 nm second peak time and 80 seconds exposure duration. The peak pressure was measured at 650 bar. It was found out that after 2-3 stress waves, up to 10% tritiated thymidine (a viable stain which has very slow diffusion rate through cell membrane. It is normally used to investigate and quantify the uptake of the molecules by cells) uptake was achieved. More importantly, they also approved that the cell damage is relatively low for stress wave exposure. The viability of the cell culture was >93% in the experiments. Furthermore, the size distribution of cell cultures exposed to stress wave showed no discernible difference from that of the controls.

S. R. Visuri and N. Heredia [20] demonstrated successful cell transient permeation by using high-frequency optically produced low-energy stress wave (opto-acoustic ultrasound). 14-fold improvement in fluorescence dye uptake was achieved by using the opto-acoustic technique. In their experiment set up, a diode-pumped 532 nm Nd:YAG laser was chosen. The laser emission was coupled to a 50 um diameter silica optical fiber and delivered to the absorbing cell media. Pulsed energy exiting the optical fiber ranged form 100 to 350 uJ. The resulting optical power delivered ranges from 150-300 mW with sufficiently short duration produce (10-200 MHz). Acoustic wave has very low duty cycle (0.1%).
Multiple cell lines were investigated including: MES-SA (human uterine sarcoma), 769P (human renal carcinoma) and NCTC (mouse fibroblast). Approximately 50% of opto-acoustic treated cells incorporated dye whereas control cells incorporated only 6% of cells. Measurement from hydrophone showed that the cells experienced ~10 bar of pressure magnitude. Cell viability test two hours after treatment shows 96% of cell are still alive.

However, there are some drawbacks of laser induced ultrasound, Beside shock wave, some ancillary effect such as bubble formation, ultraviolet (UV) radiation and formation of radicals can bring negative effects on the cells being tested, especially UV radiation which are fatal to cells.
2.3.2 Transducer Induced Shock Waves

Besides laser, another common approach to generate ultrasound to permeabilize the membrane of cells is transducer induced acoustic wave. Shock waves with amplitude in the range of 10-1000 bar have been shown to induce membrane disruption [29], [30].

W. J. Greenleaf and M. E. [21] Bolander used Human chondrocytes cell (cell line CD4 C20-A4) and lipsome to test the acoustically induced transfection on cells. In their experiment set up, Figure 2.4, two 35mm-diameter air-backed ultrasound transducers were placed 3 mm under the bottom of the of a six-well culture plates. Continuous-wave (CW) ultrasound of 1.0 MHz frequency was delivered through the bottom of six-well plates with an average peak pressure amplitude of up to 0.41 MPa for 20 seconds. It was found that transfection efficiency increased linearly with ultrasound exposure, (Figure 2.5). At 0.41 Mpa, transfection rate reach 43%, four times of 20 seconds exposure at 0.20 Mpa.

Fig. 2.4 A 35-mm-diameter 1.0 MHz transducer was placed below each of two wells of a six-well culture plate. [21]
M. Pong [22] investigated the changes in membrane permeation (leakage mimicking drug release) in vitro during exposure to ultrasound applied at two frequency ranges: conventional therapeutic ultrasound range (1 MHz and 1.6 MHz), and low (20 kHz) frequency range. Phospholipids vesicles were used as controllable biological membrane models. Egg phosphatidylcholine vesicles were prepared with sizes ranging from 100 nm to 1 μm. Leakage was quantified in terms of temporal fluorescence intensity changes observed during carefully controlled ultrasound ON/OFF time intervals. With the peak-to-peak pressure change from 0.03-0.94 MPa, observed acoustic power intensity ranges from 0.13 to 46.90 W/cm². All the three frequencies have impact on the membrane leakage. It was found that the 20 kHz seems to generate more leakage than that observed at 1 and 1.6 MHz. Also, decreasing the membrane curvature by increasing vesicle diameter can effectively enhance the leakage caused by the same acoustic wave. Figure 2.6 shows the experiment setup.

Fig. 2.5 Transfection rates of living cells after ultrasound exposure as a function of average peak pressure of the 20 s burst of 1.0 MHz ultrasound [21].
In the study performed by J. Sundaram [28], he examined cell viability and cellular uptake of calcein using 3T3 mouse cell suspension as a model system. Experiment set up is similar with figure 2.4. The transducer faced down to the cell meida, and cell is suspended in the medium instead of sitting on the bottom (figure 2.7). Cell suspension medium were exposed to varying acoustic energy doses at four different frequencies in the low frequency regime (20 – 100 kHz). At all frequencies, fluorescence dye uptake by cell increase corresponding to the increase of energy density. However, at each frequency, cell viability, decreased with increasing energy dose. The energy density at which viability drops below 50% is: 10 J/cm², 45 J/cm², 40 J/cm², and 60 J/cm², respectively at 20 kHz, 57 kHz, 76 kHz, and 93 kHz.

Fig. 2.6 Experiment setup used for ultrasound application to cell suspension set up in [22]
In research work reported in [23] [24] [26] [27], researcher went even further. Besides testing the extensive intracellular uptake for molecules induced by ultrasound, they also investigate the detail ultrasound mechanism and what ultrasonic wave does to cell to facilitate intracellular uptake (e.g. its detail impact on cell membrane structure). Transport can persist for >1 min after sonication. DU 145 prostate-cancer cells were used and cells were sonicated using 20 acoustic pulses at 24 kHz with pressure amplitude at 7 atm. All the cells were sonicated for 240 seconds in 15 second intervals. Flow cytometry analysis of sonicated cells showed 10% to 40% increase in fluorescence which is an indicative of molecular uptake. Fluorescence molecules used in the experiment span a range of sizes (0.6 to 2,000 kDa). Figure 2.8 showed that all of the molecules were transported into the cytosol of living cells when present in the media during sonication. However, smaller molecule appears in the nucleus at high concentration. Further SEM imaging showed localized effect to the cell plasma membrane, including micro-sized
changes in surface morphology and cell’s self repairing process on the broken plasma membrane continuity after sonication (Figure 2.9).

Fig 2.8 Intracellular delivery by ultrasound. (a) Confocal micrographs showing a nonsonicated DU 145 cell exposed to calcein (A1) and sonicated cells exhibiting uptake of calcein (A2), bovine serum albumin (A3), 150 (A4), 500 (A5) and 2000 kDa (A6) dextrans. Scale bars are 1 um [23].

Fig. 2.9 Ultrastructural analysis of ultrasound’s effect on plasma membrane. (a) Evidence of structural changes in plasma membrane due to ultrasound: SEM of (a1) nonsonicated DU 145 cell and (a2,a3) sonicated cell, shown at two levels of magnification, with a region lacking characteristic membrane surface topography and revealing exposed cytoskeleton; and (b) evidence of repairing wounds: SEM of (b1) nonsonicated cell and (b2,b3) sonicated cell, [23]
Table 2-1 summarizes the key experimental parameters, measuring result and experiment outcome from previous researches of using ultrasound wave to transiently enhance the cell membrane permeation, therefore increase the efficiency of cell’s uptake of drug molecule, gene molecule, medical agents, etc. It is noticed from the table that the key factors vary greatly from different experiments. Frequency ranges from 20 KHz to 200 MHz; pressure amplitude ranges from 300 kPa to 400 bars and ultrasound activation time ranges from 10 seconds to 20 minutes.

Table 2-1

<table>
<thead>
<tr>
<th>Ref</th>
<th>Stress wave source</th>
<th>Application</th>
<th>Frequency</th>
<th>Duty cycle</th>
<th>Exposure time</th>
<th>Working mode</th>
<th>Power density</th>
<th>Pressure amplitude</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>[37]</td>
<td>Transducer</td>
<td>Transdermal drug delivery</td>
<td>19.6-93.4 kHz</td>
<td>100%</td>
<td>15 minutes</td>
<td>continuous</td>
<td>0.2 W/cm²-2.7 W/cm²</td>
<td>n/a</td>
<td>Skin conductivity enhanced up to 200% @ 1.08 W/cm²</td>
</tr>
<tr>
<td>[38]</td>
<td>Transducer</td>
<td>Transfection, DNA delivery</td>
<td>2 MHz</td>
<td>10%</td>
<td>3×10 seconds</td>
<td>Pulse (10kHz repetition)</td>
<td>80 W/cm²</td>
<td>n/a</td>
<td>Transfection rate 7.53%</td>
</tr>
<tr>
<td>[22]</td>
<td>Transducer</td>
<td>Drug delivery</td>
<td>20 kHz</td>
<td>100%</td>
<td>10 min with 10 min interval</td>
<td>continuous</td>
<td>0.13 W/cm²</td>
<td>0.06 MPa</td>
<td>30% drug leakage</td>
</tr>
<tr>
<td>[22]</td>
<td>Transducer</td>
<td>Drug delivery</td>
<td>1.0 MHz</td>
<td>40%</td>
<td>10 min with 10 min interval</td>
<td>continuous</td>
<td>3.0 W/cm²</td>
<td>0.47 MPa</td>
<td>~12% drug leakage</td>
</tr>
<tr>
<td>[22]</td>
<td>Transducer</td>
<td>Drug delivery</td>
<td>1.63 MHz</td>
<td>40%</td>
<td>10 min with 10 min interval</td>
<td>continuous</td>
<td>46.9 W/cm²</td>
<td>1.88 MPa</td>
<td>~12% drug leakage</td>
</tr>
<tr>
<td>[23]</td>
<td>Transducer</td>
<td>Drug delivery</td>
<td>2.4 MHz</td>
<td>10%</td>
<td>10 Hz</td>
<td>Pulse</td>
<td>n/a</td>
<td>7 bar</td>
<td>10-80% increase of molecular uptake</td>
</tr>
<tr>
<td>[30]</td>
<td>Transducer</td>
<td>Transdermal insulin delivery</td>
<td>20 kHz</td>
<td>20%</td>
<td>200s</td>
<td>Pulse</td>
<td>0.5-0.9 W/cm²</td>
<td>n/a</td>
<td>Insulin delivery increase 50%</td>
</tr>
<tr>
<td>[19]</td>
<td>Laser 193nm</td>
<td>Drug delivery</td>
<td>0.1 Hz</td>
<td>n/a</td>
<td>15 min</td>
<td>Pulse</td>
<td>2000 mW</td>
<td>380 ± 40 mW</td>
<td>10% of cell uptake reached</td>
</tr>
<tr>
<td>[20]</td>
<td>Laser, 532nm</td>
<td>Drug delivery</td>
<td>10-200 MHz</td>
<td>1.25-10.20 MHz</td>
<td>Pulse</td>
<td>150-300 mW</td>
<td>14 fold improvement in cell uptake</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[20]</td>
<td>Transducer</td>
<td>Gene transfection</td>
<td>1.0 MHz</td>
<td>n/a</td>
<td>20× up to 4 times</td>
<td>Pulse</td>
<td>10 W/cm²</td>
<td>0.15-0.41 MPA</td>
<td>Up 50% gene transfection @ 0.41 MPA</td>
</tr>
<tr>
<td>[39]</td>
<td>Transducer</td>
<td>Gene transfection</td>
<td>1.0 MHz</td>
<td>n/a</td>
<td>60 seconds</td>
<td>Pulse</td>
<td>0.4 W/cm²</td>
<td>n/a</td>
<td>15% increase on DNA transfection</td>
</tr>
<tr>
<td>[40]</td>
<td>Transducer</td>
<td>Drug delivery</td>
<td>M Hz</td>
<td>50-100%</td>
<td>20 minutes</td>
<td>Pulse</td>
<td>0.4 W/cm²</td>
<td>n/a</td>
<td>2-6 fold increase in drug uptake by cell</td>
</tr>
<tr>
<td>[41]</td>
<td>Transducer</td>
<td>Gene Transfection</td>
<td>1.0 MHz</td>
<td>50-100%</td>
<td>20-60 sec</td>
<td>Pulse</td>
<td>n/a</td>
<td>319-411 kPa</td>
<td>2-4% transfection rate compared to 0.34% without ultrasound</td>
</tr>
</tbody>
</table>
2.4 Importance of Single Cell Study in Cell Screening and Drug Testing

Cell-based biosensors incorporate cells as sensing elements that convert changes in immediate environment to signals for processing. Cell-based biosensors can possibly enable people to monitor the extracellular action potential of the single living cell under stimulants. Nowadays, more and more scientist have been interested in the study of structure, function and characteristic of living cells, which are concerned with our understanding of the nature and ourselves. Cells are equipped with a host of receptors that can convert chemical signals into electrical signal and light signals. Cells function as versatile biosensors in a variety of applications. By using living cells as sensitive element, cell-based biosensors are able to response to many biological analytes in a physiologically relevant manner. It carries potential in providing rapid, sensitive and low cost measurement technology to detect functional information.

Major approaches for transduction of cellular signals include measures of cell metabolism, impedance, intracellular potentials, and extracellular potentials. All those approaches, network of excitable cells cultured on microelectrode arrays, are uniquely poised to provide rapid and functionally effective cell-based biosensor [35]. So cell based biosensor can be useful for a wide range of application ranging from pharmaceutical screening to environmental monitoring [34].

The integrated biological analysis systems have received an increased attention in the areas of point-of-care (POC) diagnostics, food pathogen screening, environmental monitoring and biomedical research for drug discovery by providing automated and potentially portable solutions to a wide range of fluid-base solutions. One of the key issues in developing these systems is simple and high-throughput cell separation [32].
As the smallest sustainable unit of life-isolated biological cells in cell cultures, single cells are good experimental model system for examining complex biological processes and functions in a controlled and straightforward manner [31]. Current diagnostic techniques or pharmacological studies requires parallel analysis of millions of cells, which is not only costly but also time consuming for sample preparation. Therefore a cell-based system for efficient and parallel analysis of drug candidates is very attractive for reducing the complexity and cost. In the last two decades, there has been numerous interest and effort on cell trap chips to trap and align cells. Among those chips, dielectrophoresis (DEP) chip is the most popular methods. It uses dielectrophoresis phenomena to trap and align cells on planar electrodes on the chip surface. DEP is a physical phenomenon whereby neutral particles, in response to a spatially non-uniform electric field $E$, experience a net force directed toward locations with increasing or decreasing field intensity according to the physical properties of the particle and medium. An device demo is shown in Figure 2.10 [33]. Owing to the progress and development people have made in the field of micro/nano fabrication, the size of planar electrodes on DEP chip can be made to micrometer level which matches the size of the single cell, therefore make the single-cell resolution achievable. Chuang and Y. Lee [33] further improve the cell trapping technique of traditional DEP chips have by fabricating 3D concave microstructures of single-cell-based DEP chip to and successfully used it to study the apoptosis of U937 & A431 cells. Device structure is shown in figure 2.11 and cell trapping result is shown in figure 2.12.
Fig 2.10: Traditional DEP chip with planar electrode pad to trap cell. The optical micrographs of living cell U937 immersed in RPMI/Sucrose (1:10), (a) suspension in the medium before DEP; (b) After DEP. [33]

Fig 2.11 SEM pictures 3D concave microstructures single-cell-based DEP chip by excimer laser machining (a) The diameter of microstructure is about 30 µm; (b) The 10 by 10 array on SU-8. [33]
Due to the increasing interest in single cell study, especially with the techniques developed to allow researchers to catch and separate each single cell, single cell based bio-sensors are being explored and used in much wider fields. Majority of those applications requires a single cell to uptake small particles such as drug molecules, DNAs,
RNAs, proteins, or dye agents for cell labeling research and to monitor the subsequent cell response. However, until now, the slow transfer of those testing particle into single cells through cell membrane still hinder the research potential of single cell based bio-sensor. Therefore, a device which is capable of using low power and confined ultrasound wave to focus on individual cell and transiently open the cell membrane to facilitate the cell uptake on testing particle will be very valuable for the fundamental and basic studies single-cell and single cell related bio-sensor development.

In this dissertation, we proposed to develop a micro-sized transducer based bio-sensor. Instead of using a large transducer to deliver ultrasound wave into cell media, which impacts a huge population of cells at one time, our device will position a micro-size single element transducer underneath each single cell trapped on the cell trapping pad to generate low power and space confined ultrasound wave targeting on one or several individual cells. This chip design could dramatically shorten the time for single cell on-chip inspection, and carry potential for efficient, site-specific, acoustic induced particle intake by single cell, which could be applied to variety applications. Figure 2.13 shows the structure of our proposed micro-sized based single cell bio-chip
Fig 2.13 Structure of proposed micro-sized based single cell bio-chip, in which there is a single micro-sized transducer underneath each single cell to generate low power and space confined ultrasound wave to facilitate the particle intake of each individual cell.
References


Chapter 3

DEVICE FABRICATION

3.1 Introduction of Piezoelectric Materials

Piezoelectric materials are the basic materials for making transducers. The piezoelectric effect describes the reversible relationship between a mechanical stress and an electrical voltage in solids. An applied mechanical stress will generate a voltage and an applied voltage will change the shape of the solid by a small amount. Figure 3.1 illustrates the piezoelectric effect. This is the electromechanical quality of piezoelectric materials which has led to their application in transducers which converts electrical energy to mechanical energy and vice versa.

![Piezoelectric effect](image)

Fig 3.1 Piezoelectric effect [1]
Since the discovery of ferroelectricity of polycrystalline ceramic (BaTiO$_3$) during the early 1940s, a succession of new piezoelectric materials have been discovered. Such as Pb(Zr$_{1-x}$Ti$_x$)O$_3$ (PZT) and Pb(B’B’’)O$_3$, (B’=Zn, Mg, In, Yb and Sc et al, B’’=Nb, Mo, Ta and W) in the form of polycrystalline and single crystal [3]. After the discovery of PZT, numerous new materials such as Pb(Zn$_{1/3}$Nb$_{2/3}$)O$_3$-PbTiO$_3$ (PZN-PT), Pb(Mg$_{1/3}$Nb$_{2/3}$)O$_3$ – PbTiO$_3$ (PMN – PT), etc are also found and investigated [4]. Most piezoelectric materials in the market are either polycrystalline or single crystal. Single crystal piezoelectric materials have considerably higher piezoelectric coefficients and electromechanical coupling factors than polycrystalline materials, and as a result they are being used in fabrication of ultrasound transducers with unprecedented bandwidth (>100%) and sensitivity, especially for HF transducer fabrication. Table 1 shows properties of a few important piezoelectric materials used in high frequency transducer designs. Single crystals have been widely used for certain applications since 1990s [6], [7].

High quality piezoelectric single crystals such as lithium niobate (LiNbO$_3$), Pb(Zn$_{1/3}$Nb$_{2/3}$)O$_3$-PbTiO$_3$ (PZNT), Pb(Mg$_{1/3}$Nb$_{2/3}$)O$_3$-PbTiO$_3$ (PMNT) and polycrystalline material such as lead titanate (PbTiO$_3$) have been investigated since 1950s [3]. The single crystals’piezoelectric strain remains nearly hysteresis free up to levels of ~0.5 to 0.6% depending on the crystal composition, which is desired for many piezoelectric actuations. Single crystals such as Pb(Mg$_{1/3}$Nb$_{2/3}$)O$_3$-PbTiO$_3$ (PMN-PT), exhibit large increases in strain over conventional piezoelectric materials due to the ability to orient the crystals along a preferred high strain crystallographic direction [8].
Lastly, compared to the polycrystalline material with grain size in micrometer level, the single crystal with no grain boundary would be more suitable for the micro-level dimension of a transducer to be used in the single cell bio-chip. Single crystal materials also have proven to be able to retain their bulk material properties better at higher frequencies than their polycrystalline counterparts [5]. All the unprecedented characteristic of single crystal piezoelectric material and its wide availability from a manufacturer make PMN-PT single crystal the ideal material for making the micro-sized transducer in our bio-chip.

Table 3-1 Properties of a few important piezoelectric materials used in high frequency transducer designs. [5]

<table>
<thead>
<tr>
<th>Property</th>
<th>PVDF</th>
<th>PZT–5H</th>
<th>PbTiO₃</th>
<th>PMN–PT Crystal (33%PT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$d_{33}$ ($10^{-12}$ C/N)</td>
<td>–33</td>
<td>593</td>
<td>60</td>
<td>5,500–6,500</td>
</tr>
<tr>
<td>$k_t$</td>
<td>0.12–0.15</td>
<td>0.51</td>
<td>0.49</td>
<td>0.58</td>
</tr>
<tr>
<td>$k_{33}$</td>
<td>–</td>
<td>0.75</td>
<td>0.51</td>
<td>0.94</td>
</tr>
<tr>
<td>$\varepsilon_{33}^S/\varepsilon_0$</td>
<td>5–13</td>
<td>1,470</td>
<td>180</td>
<td>680–800</td>
</tr>
<tr>
<td>$c$ (m/s)</td>
<td>2,200</td>
<td>4,580</td>
<td>5,200</td>
<td>4,610</td>
</tr>
<tr>
<td>$\rho$ (kg/m³)</td>
<td>1,780</td>
<td>7,500</td>
<td>7,660</td>
<td>8,060</td>
</tr>
<tr>
<td>Curie temp. (°C)</td>
<td>100</td>
<td>200</td>
<td>260</td>
<td>130</td>
</tr>
</tbody>
</table>

$\varepsilon_{33}^S/\varepsilon_0$ is the relative clamped dielectric permittivity and $\rho$ and $c$ denote density and plate mode sound velocity, respectively. These values are approximate and will vary with the manufacturing and/or testing methodology used.
3.2 Current Fabrication Method for HF Transducers

The traditional method for fabricating small size transducers includes dicing, molding, etc. [9], [10]. Either a piezoelectric thin film, polycrystalline, or a single crystal substrate is cut by a dicing saw with thin blade. Figure 3.2 shows a standard process flow of a PZT-Polymer composite transducer array by using the dicing technique. A dicing saw is used to cut the bulk material into individual posts, and then epoxy is filled into the saw kerfs. After the epoxy is cured, the substrate is removed by lapping and grinding. However, the ultimate goal of this single cell-biochip is to conduct study on each individual cell trapped on the electrode, this requires that the size of designed micro-size transducer under each cell trapping electrode must be at the same size scale as the single cell (down to 5-20 um) to be studied. This posts a difficult challenge for dicing method. Besides, >2:1 aspect ratios for thickness mode operation makes the designed transducer pillar extremely fragile for the traditional dicing technique which can easily cause pillar breakage and chipping.

![Diagram](image_url)

Fig 3.2 Conventional dice and fill PZT-Polymer composite transducer fabrication method.
Photolithography based micro-fabrication has several advantages, compared to dicing technique: submicron machining precision, batch fabrication, and a low-stress mechanical environment for fragile and fine structures. For single crystal piezoelectrics, both wet etching and dry etching could be used to fabricate transducers. Wet etching (HF, HCl) [11] is usually a cheap process. Good adhesion between the photoresist and the single crystal substrate is required, and the etching solution should not attack the photoresist. But for ultrasound transducers, particularly those made from single crystals, the most important requirement of any micro-fabrication process is the ability to make features with aspect ratios greater than 2:1. This is required so that the transducer resonates in a pure mode that yields the maximum electromechanical coupling coefficient. High aspect ratio also dampens lateral modes; both effects are needed to achieve a broad bandwidth and high sensitivity. Dry etching is preferable because, unlike wet etching which is isotropic, dry etching can be anisotropic.

Plasma dry etching has been shown to be an effective method for machining high aspect ratio, anisotropic features in materials that do not exhibit a high degree of wet etching anisotropy. Dry etching is also potentially less damaging than mechanical micromachining methods. Deep RIE etching of PZT ceramic has been investigated by a number of researchers. Either photoresist or metal masks have been used to define the etching pattern. The etchant chemistries used include SF$_6$; SF$_6$/N$_2$/Ar; HBr/Ar; CF$_4$; Cl$_2$/CF$_4$, for example [12] [13] [14] [15]. Table 3-2 presents the etching chemistry, etch rate and sidewall profile characteristics. Etching depths over 100 microns have been reported [12]. Unfortunately, for all the etch result, the sidewall angles are less than 80° (Figure 3.3, 3.4 and 3.5). In order for our designed micro-sized, single element,
transducer to achieve better lateral working efficiency, a higher aspect ratio and more vertical sidewall etching is desirable. This will also ease the difficulties for later lithography processes such as electrode and connection line patterning. To the authors’ knowledge, no plasma dry etching work has been reported on single crystal PMN-PT piezoelectric material. In this thesis, a new method of plasma-etching of PMN-PT single crystal material was developed to facilitate the fabrication of single cell bio-chip.

**Table 3-2. Summary of key characteristic of PZT plasma etching**

<table>
<thead>
<tr>
<th>Etch Chemistry</th>
<th>Etch Rate</th>
<th>Mask material</th>
<th>Etch Depth</th>
<th>Side wall Angle</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl₂/Ar, C₂F₆/Ar, Cl₂/C₂F₆/Ar, HBr/Ar</td>
<td>900~1300 A/min</td>
<td>Photoresist</td>
<td>0.25 μm</td>
<td>50~80°</td>
<td>[15]</td>
</tr>
<tr>
<td>CF₄/O₂, CF₄/N₂</td>
<td>~ 60 nm/min</td>
<td>Photoresist, Pt</td>
<td>-</td>
<td>-</td>
<td>[13]</td>
</tr>
<tr>
<td>Cl₂/CF₄</td>
<td>~90 nm/min</td>
<td>Photoresist, Pt</td>
<td>-</td>
<td>-</td>
<td>[14]</td>
</tr>
<tr>
<td>SF₆</td>
<td>300 nm/min</td>
<td>Ni</td>
<td>70 μm</td>
<td>~75°</td>
<td>[13]</td>
</tr>
<tr>
<td>SF₆</td>
<td>~120 nm/min</td>
<td>Photoresist</td>
<td>2 μm</td>
<td>85°~90°</td>
<td>[12]</td>
</tr>
<tr>
<td>SF₆, SF₆/N₂, SF₆/Ar</td>
<td>~160 nm/min</td>
<td>Ni</td>
<td>&gt;100 μm</td>
<td>~72°</td>
<td>[12]</td>
</tr>
</tbody>
</table>
Fig. 3.3. SEM images of PZT bulk material etched in ICP chamber with SF$_6$[13].

Fig. 3.4. SEM images of PZT bulk material etched in RIE chamber with SF$_6$[12].

Fig. 3.5 SEM images of PZT films etched in ICP chamber with HBr/Ar, chemistry [15].
3.3 Design of Device Fabrication Process

One important step for plasma etching is to choose an appropriate etch mask. Because it has a melting point of 1455 °C, boiling point of 2730 °C, and strong resistance to chemical corrosion and physical bombardment, Ni has become a common plasma etch mask material and is being widely used in plasma etch [20], [21]. Nickel chloride, an etch product produced in chlorine based plasma etch, has high melting point of 1001 °C, which makes it hard to be removed. Therefore it prevents further Ni etching. All these characteristics make Ni an ideal etch mask for our fabrication which employs chlorine chemistries. Below is the detail of each step of the fabrication process step. Figure 3.6 illustrates the whole process flow.

1. Evaporate thin (200 nm) Ni film on a polished PMN-PT wafer surface to be used as a seed layer for subsequent Ni electroplating.
2. Use photolithography to define the transducer areas.
3. Grow thick Ni etch mask by electroplating using photoresist (from step 2).
4. Remove photoresist, leave Ni mask and start plasma etching to etch through the seed layer, then form vertical transducer pillars with high aspect ratio.
5. Fill epoxy between the etched the pillars, then lap the epoxy thickness down until the top surface of etched pillars are exposed.
6. Flip sample over, mount lapped side on glass substrate using silver epoxy (public electrode), then lap down the PMN-PT substrate until all substrate material is removed, so that all pillars are disconnected and embedded in the epoxy.
7. Put on top electrode and connection line on top of exposed PMN-PT pillars using photolithography.
8. Deposit parylene film (acoustic matching layer + device encapsulation)

9. Deposit cell trapping electrodes on parylene thin film above each transducer.

Fig 3.6 Designed process flow for micro-sized transducer based bio-chip
3.3.1 Photolithography:

Since photoresist was used as the mold in our process design for growing Ni mask by electroplating, it is required to have at least the same thickness as the desired Ni mask. SPR series i-line photoresist from “Megaposit” is designed to achieve 1-10 um film thickness in a single coat with good uniformity. So they were used to meet the different thickness requirements in our tests. Contact aligner was used to pattern the photoresist. To avoid the interfering reflection of the light reflected from the bottom of surface to degrade exposed image, an anti-reflection coating layer (Barli) is put down below the photoresist to absorb the light which passes through the Photoresist. Figure 3.7 shows the SEM image of an exposed photoresist mold for Ni mask growing. By optimizing the photolithography parameters, a clear and smooth profile is achieved.

![Fig. 3.7 SEM images of exposed photoresist with clear and smooth profile.](image)
3.3.2 Etching Mask Growth

Due to the low etch rate of PMN-PT and the relatively low selectivity of Ni over PMN-PT, a thick Nickel layer becomes necessary in order to be able to protect material underneath until the desired etch depth is achieved. To deposit the Nickel mask, an e-gun evaporation technique was originally chosen due to its advantage to deposit pure, high quality metal films. However, evaporation deposited Nickel showed significant tensile stress [22]; thin film start to peel off once the thickness exceeded 2500 Å with smallest feature size of 25 um, which is far from the desired Nickel etch mask thickness.

Because of serious stress issue of evaporation deposited Ni, electroplating becomes more reasonable approach. Electroplating is a widely used technique for depositing metal thin films. However, electroplated metal film quality can vary a lot depending on the electroplating conditions including: pH value, current density, bath temperature, deposition mode (AC, DC, pulse), etc. By carefully controlling these parameters, the Nickel layer internal stress can be minimized [25]. Another important Ni film property for our plasma etching is the film density. Denser films can provide a stronger etch resistance [25] and therefore increase the etch selectivity.

Research found that pulsed plating leads to smaller metal grain size and less porosity due to a higher deposition potential [22]. The higher frequency of the pulse also leads to a smaller internal stress in the resulting deposit. Also, the deposited metal film hardness decreases with the increasing current density [26]. Plasma etching experiments were conducted to test the etch resistance of Ni thin film deposited under different conditions. The parameters for etching in the ICP chamber were kept the same (800 W coil power, 200 V bias, 100 sccm Cl₂ flow, 200 mTorr). Table 3-3 showes the test results
for Ni thin film deposited by high frequency pulse plating. Note that the film made with the smallest current density delivered the best etching resistance of 15.2 nm/min. Figure 3.8 is a SEM image showing two electroplated Ni layer deposited on one sample in serial sequence, the first Ni layer was deposited with high frequency (2.5 MHz) pulse plating current density at 10 mA/dm². The second Ni layer was deposited at DC current with 35 mA/dm². It can be seen that the Ni layer formed from large DC current has much bigger grain size.

Table 3-3: Summary of Nickel etch rate with different electroplating condition

<table>
<thead>
<tr>
<th>Mode</th>
<th>Pulse</th>
<th>DC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Current density</strong> (Frequency)</td>
<td>10 mA/dm²</td>
<td>10 mA/dm²</td>
</tr>
<tr>
<td></td>
<td>(2.5 MHz)</td>
<td>(200 Hz)</td>
</tr>
<tr>
<td><strong>Etch rate</strong></td>
<td>15.2 nm/min</td>
<td>19.7 nm/min</td>
</tr>
</tbody>
</table>

Fig. 3.8 Cross-sectional SEM image of two different Ni layer: bottom layer was deposited by 2 MHz pulse plating with current density of 10 mA/dm²; top layer was deposited by DC plating with current density of 35 mA/dm²
Finally, after finding the optimum experimental condition of both photolithography part and Ni electroplating part, a condense Ni etch mask with small grain size and straight sidewall was formed. Figure 3.9 A and B show the FESEM image of growing Ni in the photoresist mold and the Ni mask profile after removing the photoresist by solvent and oxygen plasma. The Ni mask size is around 25 μm.

Fig. 3.9 Condense Ni film was achieved by optimized photolithography and electroplating. (A) Ni growing in photoresist mold. (B) Ni mask after removing photoresist, the sample is 30 degree tilt, so real height is two times SEM measured height.
3.3.3 Plasma Etching Study

3.3.3.1 Overview of Plasma Etching Technique:

In the 1960's, plasma processing was first introduced. This technology is referred to as dry etching. Comparing with wet etching, dry etching is more effective in producing controllable etch profiles, selectivity to masks, and etch uniformity. Today, plasma dry etch techniques have found a wide application in semiconductor manufacturing, microelectronics, lasers, light emitting device fabrication, microelectromechanical systems (MEMS), and micromachining material, etc. Among all the plasma dry etch methods, reactive ion etching (RIE) is the most common technique and was the only production dry etching process in the production industry until 1980’s. Dry etch schemes are more attractive for device fabrication due to the anisotropic profiles that can be produced along with critical dimensional control, increased etch uniformity, and reproducibility \[27\].

RIE System:

As the most common dry etch technique, RIE is based on capacitively coupled plasma. During the etching, only one power source is employed. The applied RF chuck power induces a dc self-bias on the chuck, which can accelerate ions. One of the biggest drawbacks of traditional RIE systems is that the physical impact of the ions on the samples can cause and accumulate damage on the device due to the significantly accelerated ion energy \[28\]. Also, RIE technique requires relatively high pressure to stabilize the plasma due to its low ionization efficiency. RIE’s typical operating pressures are 50-200 mTorr.
The etch uniformity of etched samples is somewhat related to the chamber pressure. However, low pressure processing is desirable to obtain better uniformity over the sample surface. An other limitation to RIE is the relatively low ion flux (≤10⁹ cm⁻³) which limits the initial bond-breaking that must precede the formation of the etch product. A high ion flux, if possible, can assist desorption of these products once they have formed, more quickly leading to faster etch rates.

**New High Density Plasma Etching Techniques**

Because of these limitations of most RIE systems, new plasma etching techniques have been developed, such as reactive ion beam etching (RIBE), magnetically enhanced reactive ion etching (MERIE), electron cyclotron resonance (ECR) RIE, and inductively coupled plasma (ICP) etching. Due to the ability to offer higher ion density (>10¹¹ cm⁻³) and near independent control of ion energy and ion density, ECR and ICP are becoming more commonplace in research and industry. ICP and ECR operate as simple RIE reactors if the respective high density plasma source is not applied. The major difference between ECR and ICP is the way they generate a high density plasma. In an ECR chamber, microwaves are introduced into the chamber resonance cavity through a wave guide producing a dynamic electric field which dissociates the process gas and generates the electrons and ions of the plasma that diffuse to the wafer surface. High-density plasmas can be formed at low pressure with low plasma potentials and ion energies. In ICP, the plasma is formed in a dielectric vessel encircled by an inductive coil into which RF power is applied. A strong magnetic field is induced in the center of the chamber, which generates high-intensity plasma due to the circular region of the electric field that
exists concentrically to the coil. At low pressure, the plasma diffuses from the generation region and drifts to the substrate at relatively low energy [29]. Thus, ICP is expected to produce low damage while achieving a high etch rate. Anisotropic profiles are obtained by superimposing a RF bias on the sample so that ion energy can be controlled independently. Figure 3.10 shows the different chamber schematics of major plasma etchers, such as RIE, ECR and ICP.

Fig. 3.10 Schematic of major plasma etching system (a) RIE, (b) ECR, (c) ICP. [29].
3.3.3.2 Design of Experiment

Although both ICP and ECR reactors provide another process parameter to control ions, which is a more advanced process concept than the RIE-only approach, ICP etcher was chosen in our etch study due to its availability issue. An ICP-RIE plasma etcher was utilized to conduct an etching study. The etch chamber was designed for 6” wafer processing and has a substrate backside Helium cooling configuration. The 6” Silicon carrier wafer is clamped to the cathode by a ceramic ring. Process gas flow is precisely controlled by electronic mass flow controllers. The chamber was evacuated using turbo and rotary pump to maintain the base pressure of less than 1x10^{-4} Torr. The process pressure is controlled with a throttle valve and measured by a baratron gauge. The Ar/Cl\textsubscript{2} high-density plasma discharge is generated by applying a 2MHz RF power to the single turn inductive coil wound around the chamber ceramic bell jar with maximum output of 2500 W. The plasma diffuses from top of the chamber and drifts to the substrate surface. The substrate cathode stage is biased with 13.56 MHz RF power to control the energy of incident ion from the plasma with the maximum bias to 300 V. Besides the capability to operate at lower pressure (less than 10 m Torr), its two plasma power input configuration (coil power for generating high density plasma, RF power to create DC bias) provides us almost independent control of ion density and ion energy. All of these features enable the possibility of etching a vertical and high aspect ratio single-element transducer pillar on PMN-PT single crystal piezoelectric material. These advanced features also give us with more flexibility in later process optimization

In this research, as discussed in the previous section, ICP-RIE was selected as the dry etching method to fabricate the micro-size transducer. The PMN-PT single crystal
plates were prepared as wafers and lapped on both sides and polished on one, then embedded in a 2.5” diameter PZT wafer to avoid the difficulties in micro-fabrication process for small samples. Figure 3.11 shows 1.5 cm diameter single crystal PMN-PT embedded in a 2.5” PZT ceramic wafer. After putting the Ni mask on the single crystal surface, the whole sample was attached on a 6” Si carrier wafer using “REVALPHA” thermal release tape from “NITTO DENKO, Inc”. The "REVALPHA" is a unique adhesive tape that adheres tightly at room temperature and can easily be peeled off just by heating. It helps the heat dissipation from the sample by creating an uniform thermal conduction between backside of the sample and the carrier wafer. So a better temperature control can be gained by only adjusting the setting of the Helium backside cooling. The samples were etched using varying conditions in gas flow, pressure, gas composition, ICP source power, and substrate bias.

Fig. 3.11 PMN-PT single crystal embedded in a 2.5” PZT ceramic wafer

At the beginning, we focus on studying the etch rate which is the most important outputs. One of the problems in studying and optimizing the etching process is the large
numbers of process variables and the complex interaction between them. It typically requires an impractically large number of process runs to come up with the optimized process to obtain the desired etch rate and. The application of scientific process optimization methods such as Design of Experiment (DOE) can traditionally reduce the numbers of runs required [30]. Factorial design is a powerful tool which indicates possible values of optimum process parameters thereby enabling rapid determination of process parameter for optimum results [31] [32].

To study the effect of different process parameters on the etch rate, a design of experiment (DOE) methodology was applied. A standard 24 full factorial design was used which enabled the study of combined effects of the process parameters on a response. Table 3-4 shows the raw data of the 16 runs. The four process parameters which are important for the etching were identified to be ICP power (200-2000 W), dc bias (-50- -280 V), chamber pressure (5-150 mTorr), Ar percentage in total gas mixture (10-75%). The response output considered was the etch rate of PMN-PT. To unambiguously validate the conclusions from DOE, the orders of the runs was completely randomized. Before each run, the etch chamber was completely cleaned and conditioned. Data analysis and regression model generation were done by statistical software “JMP”. We use regression analysis to find the direction of maximum variance in the data by least square fitting it to the four independent process parameters that were varied during the experiments.
Table 3-4. Raw data for DOE study of plasma etching

<table>
<thead>
<tr>
<th>Level</th>
<th>Coil power A (w)</th>
<th>dc bias B (V)</th>
<th>pressure C (m Torr)</th>
<th>Ar fraction D %</th>
<th>Etch Rate (nm/min)</th>
</tr>
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<tbody>
<tr>
<td>Low (-)</td>
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<td>-50</td>
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<td>-280</td>
<td>150</td>
<td>75</td>
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<table>
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<tr>
<th>Run</th>
<th>Coil power A (w)</th>
<th>dc bias B (V)</th>
<th>pressure C (m Torr)</th>
<th>Ar fraction D %</th>
<th>Etch Rate (nm/min)</th>
</tr>
</thead>
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</table>
RSquare shows >93% of total variation can be explained by this model.

F-test result indicates the model is significant.

All effects are significant.

Non-significant effects.

Significant effects.

Fig. 3.12. JMP analysis result showing the significant factor and summary of fit of the regression model generated.
Figure 3.12 shows the result of JMP analysis based on Table 3’s experiment design and run result. All parameters including coil power, DC bias, pressure and Ar fraction play significant role in determining etch rate. Besides, among all the between-factors interactions, coil power*DC bias has also has significant impact on the etch rate. The JMP generated regression model Summary of fit is 93.19% shows that the regression model is a perfect fit for etch rate prediction and > 90% of variation can be explained by this regression model. Figure 3.13 shows impact on etch rate of each individual parameter and Figure 3.14 shows the match response etch rate by predicted Plot.

**JMP generated regression model:**

\[
\text{Etch Rate} = 131.375 + 70.5E_{\text{coil power}} + 32E_{\text{DC Bias}} + 27.5E_{\text{Pressure}} - 16.875E_{\text{Ar Fraction}} + 22.875E_{\text{Coil Power}}E_{\text{DC Bias}}
\]

Fig. 3.13. JMP analysis result showing impact on etch rate of each individual parameter.
After DOE analysis using JMP, we found the most significant process parameters that contribute to the etch rate. We generated a regression model with satisfied prediction precision to quickly find out the best setting to achieve fast etch rate. However, Ni etch mask selectivity is also an important factor we need to consider besides etch rate. Low selectivity will not only make the fast etch rate meaningless, but also will damage the etched device profile due to the mask corrosion. Also, result from DOE is based on statistical analysis. In order to be able to achieve a stable and robust process and be ready for further adjustments on process requirement or optimization, a further understanding of the real etching mechanism of PMN-PT single crystal etch in Cl₂/Ar ICP plasma is still necessary. Therefore, some experiments for the analysis of the input of each individual process parameter on etch rate, and etch mask selectivity were conducted.

Fig. 3.14 JMP analysis result showing response etch rate actual by predicted plot.
3.3.3.3 Gas Ratio

In our study, instead of pure Cl₂ based etching, Cl₂/Ar gas mixture was used to conduct the dry etching in ICP chamber. The motivation of choosing Ar as the additive gas is to help stabilize the plasma and enhance the physical and chemical etching mechanism [33]. As a high mass atom, Ar can help the sputtering process, that reduces the etch resistance of the surface caused by etch product re-deposition. Ar⁺ ions play a key role in both the initial bond-breaking in the materials, and in etch product desorption by ion enhancement. It helps in enhancing surface physical bombardment to obtain a smooth sidewall [34]. In our study, we found the Ar and Cl₂ ratio dramatically affect etch rate and the sidewall roughness. Etch rate of both PMN-PT and Ni increased as the Ar is added into the pure Cl₂. Hence it proves that the combination of chemical and physical etching mechanism will promote the etch rate. The PMN-PT etch rate increased almost 60% with 20% Ar in gas mixture. However, as Ar percentage approaching 40%, the etch rate of both materials started to drop and selectivity over Ni mask also decreases showing a reactant limited etch mechanism. As the physical bombardment started to dominate, insufficient etching flux contribute to the decreased etch rate. The AFM measurement also showed bigger surface roughness. Figure 3.15 shows the etch rate of PMNPT , Ni and the selectivity as function %Ar in the Cl₂/Ar gas mixture.
3.3.3.4 Pressure Effect:
The etch rate was also measured as a function of pressure. Pressures lower than 10 mTorr were not investigated due to the difficulty in maintaining a stable pressure. An increase in pressure will decrease the mean-free-path of electrons, causing a higher frequency of collision for electrons and ions, and thus create more reactive species during the collisions [35]. The initial increase in etch rate from 10 to 100 mTorr suggests a reactant limited etch regime for the baseline etch as a higher pressure yields more reactive Cl species, when the pressure is increased above 150 mTorr, the etch rate started to drop. It is because of the increase in the collision frequency, which results in the smaller

Fig.3.15. Etch rate of PMN-PT, Ni and selectivity as a function of % Ar in Ar/Cl\textsubscript{2} mixture in ICP etching: Total gas flow= 35 sccm, coil power=500w, dc bias=120V, pressure=25 mTorr
mean-free-path, that in turn lowers the kinetic energy of the ions. This is an indicator of a sputtering desorption limited etch mechanism. Figure 3.16 shows the different etch results from etchs of 10 mTorr and 200 mtorr. It is worth noticing that the selectivity over Ni mask also increases as the pressure gets higher. It once again shows Ni’s stronger resistance to chemical element rather than physical element in plasma etching. The coil power, DC bias and gas flow were set to 500w, 120V, 20 sccm Cl₂ and 15 sccm Ar respectively. Increase in pressure will decrease the mean-free-path of electrons, which causes a higher frequency of collision among electrons and ions, and then creates more reactive species during the collision. The etching mechanism then shifts more toward a chemical regime. As the pressure keep increasing from 150 mTorr, the etch rate started to drop. As explained before, at higher pressure, the mean-free-path decreases, which cause the electrons and ions to gain less energy, then hinder impact of the physical bombardment in plasma etch. The increase in ion collision at high pressure causes the randomization of the reactive species moving direction, an effect, which results in the isotropic-like sidewall. Furthermore, the surface roughness becomes worse and it might be due to the enhanced formation of involatile etching products with the increase of process pressure.
3.3.3.5 Coil Power Effect:

Coil power plays an important role for controlling the etch rate. As the ICP coil power is increased, plasma density is increased by generating more reactive species (ion and neutral), then normally leads to higher etch rate [36]. However, higher density reduces the mean free paths and the ion energy may be decreased due to the higher collision frequency [37]. In the experiment, the ICP power is increased from 200 W to 2000 W, due to the higher concentration of the reactive species, there will be an increase in the bond breaking and sputter desorption. It was observed that the increase of ICP coil power had a huge impact on etch rate. PMN-PT etch rate increased more than 300% when coil power increase from 200W to 1500 W. Increasing the coil power also increases the etch
rate at the expense of the etch selectivity. It was noticed that the Ni mask in high coil power etch is much rougher than the one in low coil power etch. The increased surface temperature in higher density plasma might also contribute to this effect. Figure 3.17 shows the different etch results from coil power varies from 200 to 1500 W.

![Graph showing etch rate of PMN-PT, Ni and selectivity as a function of coil power in ICP etching: Cl₂=20 sccm, Ar=15 sccm, dc bias=120 V, pressure=25 mTorr]

3.3.3.6 DC Bias Effect

DC bias also play an important role in controlling the etch rate. Higher ion energy results in higher etch rates and also helps remove the etch products. Ionic species, which gained more kinetic energy from the higher dc bias, accelerate in a directional manner towards the sample. This also enhances the sputtering effect on the sidewall surface to remove the etch product and etched mask material deposited on the sidewall [38].
As shown on Figure 3.18. In our study, the dc bias was changed from 50 V to 250 V. The etch rate peak at around 200 V and stop increasing as the dc bias keep increase. Initially, as ion energy increases, the etch rate will increase due to the improved sputter desorption efficiency of the etch products from the surface. In addition, higher ion energy improves the ability to break surface bonds with essentially increase the reactivity of the surface and reaction kinetics of etch gases. However, as the ion energy continues to increase, it is possible to reach a state where the ions sputter the reactants from the surface before they can react, so the etch rate saturate. However, higher bias values reduce the etch selectivity between the masking material and the substrate.

Fig.3.18. Etch rate of PMN-PT, Ni and selectivity as a function of dc bias in ICP etching: Cl₂=20 sccm, Ar=15 sccm, Coil power=500w, pressure=25 mTorr.
3.3.4 Real Etching Result

The systematic etch study and optimization we conducted above guided us to a successful ICP etch process that fulfilled the requirement of deep PMN-PT etching, fast etch rate, acceptable mask selectivity and high aspect ratio anisotropic etched profiles. The optimized values for bias voltage, ICP coil power, chamber pressure, and balanced Ar/Cl₂ gas mixture were -100V bias voltage, 1500 W coil power, 25 mTorr chamber pressure and a mixed gas flow of 10 sccm Cl₂ and 15 sccm of Ar. The roughness of the facet as viewed in a FESEM was less than 40 nm. The sidewall angle with respect to the normal surface was less than 2°. Figure 3.19 A shows SEM images of etched features of 3x3 matrix of transducers with different designed size: 25, 50, 75, 100 µm were etched in four different quadrants. Figure 3.19 B shows detail of the etched 25 µm square area transducer matrix. Sample was tilted by 30 degrees in SEM, so the real etched depth will be 2 times the depth of SEM measured, i.e. >120 µm.

Fig. 3.19 SEM image of etched micro size transducer, (A) 3x3 matrix of transducer of with 25, 50, 75, 100 µm square area in each quadrant (B) 3x3 matrix of 25 µm square area with etched depth up to 2x60 µm=120 µm.
Figure 3.20 A shows the SEM images of etched 3x3 matrix of transducer with 75 um square area, Figure 3.20 B shows more detail of a single element of this 3x3 matrix. The etch depth was approximately $2 \times 64 = 128$ um with sharp edge and high verticality.

Fig.3.20 (A) SEM of etched 3x3 matrix of transducers with 75 um square area (B) single element transducer with 75 um square area, etch depth reached $2 \times 64 = 128$ um.
3.3.5 Epoxy Filling and Wafer Lapping:

After etching, the device was placed on the bottom of a mold made of Teflon. Then “EPO-Tech 301” epoxy was poured into the mold shown in Figure 3.21 A. After 24 hours, epoxy was fully cured and the whole epoxy wafer was taken out the Teflon mold shown in Figure 3.21 B. The PMN-PT substrate was embedded in the epoxy wafer with etched pillars facing down. Figure 3.22 showed the following lapping process on epoxy wafer to removed etched left PMN-PT substrate. Figure 3.22 A shows the substrate being left from etching start to be lapped away from the middle and Figure 3.22 B shows the status that the substrate was almost removed. Once the whole substrate was removed, all the etched pillars will no longer be connected and become single elements embedded in an epoxy wafer, they are then ready for later process to put down Au electrode and connection lines, which drive the each individual transducer.

![Fig. 3.21 (A) Epoxy was filled into the space between etched pillars, A Teflon mold was used to hold the epoxy until it cures (B) the epoxy wafer was taken out of the mold, with PMN-PT substrate embedded in it, (etched pillars facing down).](image)
In order to ensure the quality of transducer electrode contact and improve the device final top surface topography, a flat and smooth transducer top surface and single crystal, epoxy interface is desired. This makes surface polishing after the lapping process necessary. A two step polishing was used in our device fabrication. The 3 um Al₂O₃ lapping powder was adopted first for rough polishing and then 0.1 um 3M super fine polishing slurry was used as the final finishing process. Figure 3.23 compare the SEM surface condition of transducer after Al₂O₃ power and after fine slurry polishing. Profilometer results indicated the final surface roughness less than 1500 Å.

Fig 3.22 Lapping process: (A) shows the left etched substrate start to be removed from the middle, (B) The substrate was almost removed, only single element pillar left in the epoxy wafer.
3.3.6 Electrode and Connection Lines Patterning

We have investigated Cl$_2$/Ar based PMN-PT single crystal material dry etching using ICP-RIE. Relationship between each rate, selectivity, and sidewall roughness were examined as a function of process parameters, including source power, bias power, pressure, and Ar fraction. The optimization of etch parameters is essential to obtain a acceptable etch rate and an anisotropic etch profile. We have approved that high etch rate and anisotropic profile could be achieved by precisely controlling the etch conditions. After the lapping and polishing, the device is ready for lithography and metal evaporation.
for final electrode and connection line patterning. Figure 3.24 shows 500x200 um contact pad on the edge of devices for external connection. Transducer devices are in the middle area (B). In each quadrant, there are three 3x3 transducer arrays. The transducer size varies between each quadrant with 25, 50, 75, 100 um of one side width.

Fig. 3.24 The final device after the electrode and connection line patterning: 500x200 um contact pads were placed on the edge of devices for external connection. Transducer devices are in the middle area (B). Three 3x3 transducer matrix is located in each quadrant. Transducer size varies in each quadrant with 25, 50, 75, 100 um.
Reference


[27] From on line source: www.triontech.com


Chapter 4
DEVICE TESTING ON A LIVING CELL

4.1 Transducer testing and characterization

Before living cell testing is conducted on our micro-size transducer devices, acoustic measurements were conducted to evaluate the pressure amplitude generated by each single element transducer. Figure 4.1 shows the experimental set up. The device is put into the water in a scanning water tank. A hydrophone is attached to a positioning device, which moves it about in the ultrasound beam; the hydrophone signal (Voltage) is recorded for various positions in the beam. By multiplying the recorded signal by the amplitude coefficient calibrated for that hydrophone, the pressure amplitude can be calculated as a function of position.

![Experimental set up of acoustic field measurement for micro-sized transducers.](image)

Fig 4.1 Experimental set up of acoustic field measurement for micro-sized transducers.
After recording the pressure amplitude, the acoustic power intensity can be further calculated from the equation [12]

\[ I = \frac{P^2}{2\rho c} \]  

(4-1)

Where \( \rho \) is the density of the medium (which in this case is \( 10^3 \) kg/m\(^3\)) and \( c \) is the speed of sound in water, which is 1500 m/s in our test. In our experiment, the hydrophone was scanned on a 1cm x 1cm square area centered on the tested transducer. As cells will be grown directly on the chip surface instead of being suspended in the solution, the knowledge of acoustic power density at the chip surface will be very useful in evaluating the ultrasound impact on a cell. So the hydrophone was desired to be placed as close to transducer as possible. However, the extreme small size (75 um diameter) and special structure of the hydrophone make it very fragile and easy to be broken. In our test, the closest distance between hydrophone and chip surface was around 5 mm (roughly estimated by visual measurement through the scanning tank). Transducers of three different sizes, 25, 50, 75 um were tested. All the devices were driven by the same signals; a 30 MHz continuous wave signal with 50 mV output from function generator; the signal from function generator was amplified by a power amplifier with 10 db amplification. The reason to choose a relatively high frequency (30 MHz) as a starting frequency is to increase the length of near field (NFL) acoustic field from micro-size transducer and also to reduce the ultrasound beam divergence so that we can confine the ultrasound impact on the single cell level instead of the population level. Figure 4.2 shows the two zones in the acoustic field generated from an unfocused single-element transducer.
remains well collimated in the near field. The region beyond near field is far field, where the beam becomes smooth and diverges. The NFL and far field divergence angle $\theta$ is calculated by Equations 4-2 and 4-3 [12].

$$NFL = \frac{D^2}{4\lambda} \quad (4-2)$$

$$\sin \theta = \frac{1.2\lambda}{d} \quad (4-3)$$

Where $\lambda$ is the acoustic wave length and D is the transducer diameter in Eq. 4.2, In Eq. 4.3, $\lambda$ is ultrasound wave length which is determined by $\lambda = \frac{c}{f}$, and $f$ is the ultrasound frequency. From equation 4-2 and 4-3, we can tell that the higher the frequency, the smaller the wave length, therefore, the longer the NFL, the smaller the divergence angle $\theta$. (For 30 MHz ultrasound in water with $c=1500$ m/s, $\lambda =0.05$ mm), we roughly use $d=70$ um to represent our transducer with 50 um square area, then to calculate $\theta$ using equation 4-3. The calculated $\theta$ and NFL are roughly $60^\circ$ and 13 um separately. As our parylene coating between cell and transducer is about 15 um, 30 MHz is the smallest frequency that can bring us relatively confined acoustic field which is desired for our later living cell testing.

![Fig. 4.2 Near field and far field of acoustic field from single element unfocused transducer.](image)
By using the hydrophone scanning results and equation 4-1, we calculated the acoustic power density across the whole 1cm x 1cm scanning field to be 0.2 ±5mW/cm² for 25, 50, 75um transducers. We believe the reason we have such an even power density distribution across the whole area is because the distance between hydrophone and device surface (5mm) is much larger than the NFL (15 um), and that the acoustic signal the hydrophone picked up is highly diverged.

To further characterize the transducer, the impedance and phase spectrum of transducers were scanned by an impedance network analyzer. Figure 4.3 (A, B, C, D) shows the results of the scan. The resonance frequency was found to be 61, 57, 54 and 50 MHz for 100, 75, 50 and 25 um square area transducers respectively. This matches the theory that the higher the aspect ratio (diameter/thickness), the lower resonance frequency will be [13]. It is worth noticing that the impedance spectrum of 25 um transducer in Figure 4.3D is more spread up than other three spectrums which deserve further investigation.

Fig 4.3 (A) Impedance and phase spectrum of transducer with 100 um square area transducer
Fig 4.3 (B) Impedance and phase spectrum of transducer with 75 um square area transducer

Fig 4.3 (C) Impedance and phase spectrum of transducer with 50 um square area transducer
Fig 4.3 (D) Impedance and phase spectrum of transducer with 25 um square area transducer
4.2 Cell culturing and preparation

In order to conduct the cell testing on our single cell bio-chip to prove the concept and feasibility, living cells were cultured on the top surface of our device. The Melanoma LU1205 cell line was chosen for this task. Malignant melanoma is an aggressive skin cancer with no current viable therapy. Melanoma is one of the fastest growing cancers in the developing world with the incidence having tripled in the last three decades. Chemotherapy, immunotherapy and vaccines have all produced very limited benefits especially since the responses are typically short-lived, with no significant effect on overall survivals [1]. The fast growth rate, good morphology, well established proliferation, relative simple to maintain, and robustness to less friendly growing environments [2] make the LU1205 melanoma cancer cell line a perfect candidate for our device testing.

4.2.1 Cell Thawing

A vial of LU1205 melanoma cancer cell line was taken out from the freezer at -80 °C, then thawed quickly in a 37°C water bath for 2 minutes until the medium became liquid. The cells were then transferred into a 100 ml Petri dish with cell media, the media contained 90% Dulbecco’s modified Eagle medium (DMEM)+10% fetal bovine serum (FBS). The whole transfer process takes place in a sterile hood with an air curtain. The total solution volume is 10 ml (9 ml medium+1 ml cell from the frozen vial). The Petri dish was then placed in the incubator with a constant temperature of 37°C and 5% CO₂ environment for 5 hours for cells to recover and settle on the surface.
4.2.2 Cell Passing and Culturing

After 6 hours of leaving the cells in the incubator, the LU1205 were ready for passing and culturing on the surface of micro-sized transducer chip. 30 ml of cell culture medium (DMEM+10% FBS) was warmed up to 37°C and centrifuged. After washing the cells two times with 5 ml DPBS (without Ca and Mg), then 1 ml trypsin was placed into the dish for 1 minute to detach the cell from the bottom of the Petri dish. After cells begin to round-up and detach, most of the trypsin was aspirated. Then cell culture media was poured into the Petri dish. Before passing the cells onto the device surface using a pipette, the medium was gently washed and agitated to fully detach and break the clumps of the cells. Then, the cells suspended in the media were transferred to the glass container with our micro-sized transducer bio-chip sitting at the bottom. Finally, the glass container was put back into the incubator for further cell growth. LU1205 melanoma cells were allowed to grow for 36 hours on the device surface until the cells were 50-70% confluent.

To determine the cell confluence, 5 nl sample is taken from the cell suspension in each Petri by syringe, then the sample is injected into the cell counting chamber which has counting grid. To compare the number of cell count in the grid with the well established reference number of cell count of 100% confluence, the living cell confluence of each Petri dish can be determined. Figure 4.4 shows LU1205 melanoma cancer cells in Petri dish after 24 hours culturing, where the cells reached about 50% confluent.
Fig. 4.4 Melanoma LU1205 cancer cell line after 24 hours culturing in cell culture medium (DMEM+10% FBS) in incubator with 37°C and 5% CO₂ flow, reached ~50% confluence.

Fig. 4.5 Melanoma LU1205 cancer cells grow on the top coating, parylene C’s surface of the micro-sized transducer bio-chip after 24 hours culturing. (A) Low magnification optical microscope image of device surface with gold electrodes and connections lines. (B) High magnification showing melanoma cells growing on top of a 50 um square electrode with parylene C film between them.
Figure 4.5 shows the optical microscope image of the growth condition of Melanoma LU1205 cancer cells on a parylene C thin film coated on the very top of the micro-sized transducer bio-chip, after 24 hours of cell culturing. Figure 4.5 A shows a low magnification optical microscope image of device surface with gold electrodes and connections lines, Figure 4.5 B shows healthy melanoma cells on a 50 um square area electrode coated with parylene C film. The micro-sized transducer is located underneath the electrode which will be driven to generate an acoustic wave and to create impact on cell bio-mechanism, so possible drug delivery facilitation can be observed.

4.3 Quantum Dot Labeling

For initial drug delivery testing on the living cell, using a real drug molecule would bring a lot of challenges from drug tracking perspective. It would also be difficult to quantify cell intake and viability. Agents which can be tracked by fluorescence emitting light can overcome these difficulties and make the cell tracking and observation much easier. The most popular dye agents widely used in cell study are organic based, which in general get bleached and lose fluorescence quickly. Quantum dots are a new labeling agent group just beginning to be used in cell studies in recent years. It has a similar size as most drug molecules and it is stable inside cell without causing noticeable change in cell characteristic [3]. All these characteristics make QDs the perfect candidate for living cell testing on our bio-chip.

Quantum dots (QDs) are a colloidal semiconductor nanocrystals with typical size range from 5 to 30 nm. QDs continuous absorption spectra and narrow, size-dependent
fluorescent emission make them ideally suited for multiplexed fluorescence detection, while their improved brightness and photostability permit long-term, dynamic imaging application. QDs have shown a great deal of promise in fluorescence imaging applications since their first reported use as biological probes in 1998 [4]. In the past 10 years, QDs have been tested as “smart” targets for diagnostic or therapeutic purposes in the areas such as cancer tumor detection and drug delivery [5]. Their unique photophysical properties make QDs an alternative to organic dyes and fluorescence proteins to label and track cells both invitro and invivo. Traditional organic dyes readily photobleach and lose fluorescence, therefore, are only useful for short-term cell labeling. QDs are photostable and maintain fluorescence intensity in a cell culture for prolonged time [10]. Some noteworthy imaging applications of QDs have utilized small molecules [5] and antibody [6], [7]. Other biomedical benefits in incorporating QDs as a major read-out component of cell based cell-signal platform have been demonstrated by Michael. R. Warnement et al. [7], [8]. Besides long term photostability, these nanoparticles are resistant to chemical and metabolic degration. They have also been proved to be nontoxic to the cell and did not trigger any noticable change to cell behavior and property [9].

All these properties of QDs make them the perfect candidates of for testing the effect our micro-sized transducer based bio-chip on cell membrane permeation of. The QDs particles used in our experiment are made of CdSe with an outside shell of ZnS. The CdSe/ZnS QDs were coated with an amine terminated polymer to make the QDs surface hydrophilic and more soluble to water based cell media. Figure 4.6 shows the 3D
structure of CdSe and CdSe/ZnS core-shell quantum dots. The colloidal quantum dots were readily ingested by cells.

Since QDs’ narrow emission spectrum and efficient energy conversion are very critical to the accuracy of the experimental output, both absorption and emission spectrum of CdSe/ZnS QDs were characterized before being placed in the cell media. Figure 4.7 A shows the photoluminescence emission at 613 nm wave length with a FWHM at only 20 nm. This indicates a narrow size distribution and means that majority of QDs taken in by cells will emit light by single narrow emission. Therefore, the reading of fluorescence intensity can precisely predict the quantity of QDs being taken by cells. Figure 4.7 B shows the QDs first absorption peak is 608 nm. The small stokes shift (difference between the PL and the 1st absorption peak) shows a dominant band edge emission indicating the high quality semiconductor structure of the CdSe/ZnS QDs we used.

4.3.1 Fluorescence Detection

To prepare the QDs for the labeling experiment, melanoma cancer cells were cultured in 20 ml Petri dishes for 48 hours until 50% of confluence is reached. 0.05 ml of 8 uM CdSe/ZnS core-shell QDs in DI water solution were filtered by an acrodisc syringe filter that has 0.2 um HT Tuffryn membrane. There are two purposes for this filtering procedure. First is to sterilize the QDs solution and filter out most bacteria and second is to prevent the congregation of QDs particles before they are evenly placed and diffuse into the cell culturing media. The QDs were diluted by cell culture media to 100 nM and poured into three Petri dishes. Then all three Petri dishes were put back to incubator for 2 hrs, 6 hrs and 12 hrs respectively. After being taken out of the incubator, each Petri dish was washed three times by new cell culturing media to wash away all the background
QDs that were still suspended in the cell culture medium and any QDs that were possibility adsorbed on the cell membrane surface. For all three samples with different QDs diffusion time, both bright field (cell image) and fluorescence (QDs image) microscope images of cells on same location were taken and overlapped together for QDs uptake comparison. Figure 4.8 shows the QD uptake comparison for different diffusion periods of 2 hrs, 6 hrs and 12 hrs. From the intensity of the lamination, it clearly shows that the uptake of: 12 hrs (D) > 6 hrs (C) > 2 hrs (B). Figure 4.8 (A) shows the fluorescence image without washing the media. The whole background is full of red, and no cell can be identified, so cell media wash is a necessary step before fluorescence imaging.

Fig. 4.8 QDs uptake comparison of different doses. (A) Fluorescence image before media wash, showing whole red background. (B) QDs uptake of cell for 2hrs. (C) QDs uptake by cell for 6 hrs. (D) QDs uptake of by cells for 12 hrs.
Because the fluorescence images showed in Figure 4.8 were generated by overlapping two images (bright field for cell, fluorescence for QDs) together, there is a possibility that the quantum dots were just sticking to the melanoma cancer cell surface without getting inside the cell due to some cell membrane biochemical property. To clear this concern, 3D confocal microscope image was taken by “Olympus FluoView 1000” triple laser confocal microscope. Figure 4.9 show the 3D Z-axis confocal image of QDs uptake by melanoma cancer cell. The QDs were evenly diffused into the cell media with 100 nM concentration 24 hours before the image was taken. The cells were washed three times before confocal microscope observation. No red fluorescence emission was noticed in the background. Besides topside (A) and bottom side (F) of the cell, all other four Z-levels have clear QDs emissions from different location inside the cells confirming that those QDs were inside the cell. Also, the majority of QDs emission was located inside the cell rather than edge area, this showed that those QDs sticking on cell membrane are negligible. Figure 4.10 shows the 3D images of QDs distribution inside cells generated by confocal microscope.
Fig. 4.9. Confocal microscope image of QDs’ distribution in different Z-axis levels inside the cell, 2 um increment in Z-axis between each image from A to F. (A) is the bottom of the cell. (F) is the top of the cell.
Flow cytometry was used to further quantify the uptake of QDs into the cell as a function of time. Six more cell samples were prepared. For all the samples, the cell concentration was counted and relative adjustment was made to maintain the cell concentrations at \(~0.5\times10^6\)/ml. The QDs were evenly diffused into the culturing media of every sample except one control sample. After 1 hr, 3 hrs, 6 hrs, 12 hrs and 24 hrs, cells were washed, detached, agitated and suspended in the new media. Then sample was taken and put into a flow cytometer (Guava PCA-96 System\textsuperscript{”} for fluorescence intensity analysis). PCA-96 system shines a 532 nm green laser into the cell suspending media and uses two detectors to detect excited emission from QDs inside the cells. So the higher the concentration of QDs inside the media, the higher the intensity reading will be. Table 4-1 shows the flow cytometer reading of control samples (sample from 1 hr, 3 hrs, 6 hrs, 12 hrs and 24 hrs QDs intake). “\% Total” represents the relative fluorescence intensity.
Figure 4.11 is Histogram based on flow cytometer fluorescence intensity measurement result from table 4-1. It clear shows that at the same initial QDs concentration of 100 nM in cell media, the longer the incubation time of cells in QDs solution, the more is the intake of QDs by the cell. Figure 4.12 shows 2-D image of the fluorescence particle counting of different samples in Table 4-1.

<table>
<thead>
<tr>
<th>Table 4-1</th>
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<tbody>
<tr>
<td><strong>Control</strong></td>
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<tr>
<td>X-Mean</td>
</tr>
<tr>
<td>683.7</td>
</tr>
<tr>
<td>684.0</td>
</tr>
<tr>
<td><strong>3hrs</strong></td>
</tr>
<tr>
<td>X-Mean</td>
</tr>
<tr>
<td>1219.8</td>
</tr>
<tr>
<td>1211.5</td>
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<tr>
<td><strong>12hrs</strong></td>
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<tr>
<td>X-Mean</td>
</tr>
<tr>
<td>1124.1</td>
</tr>
<tr>
<td>1099.7</td>
</tr>
</tbody>
</table>

Fig. 4.11 Histogram of flow cytometer fluorescence intensity measurement result from table 4-1.
Fig. 4.12. 2-D image of fluorescence particle counting of different samples in flow cytometer. The number of fluorescence particle (dark dots) increase as the incubation time of cell in QDs solution increase from 1 hr to 24 hrs (QD dose are kept same as 100 nM, control sample does not have any QDs in cell media).
4.4 Living Cell Testing on Bio-chip

4.4.1 Experiment Set up

Since we have approved that the CdSe/ZnS quantum dots can be uptaken by the Melanoma LU1205 cancer cell, and the amount of uptake is time dependent, the next step will be using our micro-sized transducer based bio-chip to test it on LU1205. This will verify whether the acoustic wave generated by our transducer can really facilitate the cell intake in shorter time compared with the natural QDs diffusion experiment we conducted. Figure 4.13 and Figure 4.14 are a diagram and a real picture of the experiment set up of the living cell testing on our micro-sized transducer based bio-chip. An HP function generator generates a continuous wave with 30 MHz. The high frequency signal was amplified by power amplifier, and then delivered to each individual transducer. A fluorescence microscope was used to detect fluorescence intensity from a single Melanoma cancer cell after the application of the transducer-induced stress wave.

![Diagram of experimental setup of the cell testing on micro-sized transducer based bio-chip.](image)

Fig. 4.13 Diagram of experimental setup of the cell testing on micro-sized transducer based bio-chip.
In our experiment, each single element transducer was driven for 3 minutes at 30 MHz, with amplified acoustic power varying from 50 mW to 500 mW.

4.4.2 Cell Permeability Observation:

Determination of cell permeability was made via fluorescent light microscopy. After the acoustic treatment on each single transducer element, the QDs solution was removed from the Petri-dish, and then cells were thoroughly washed three times with new cell culturing media to remove the background QD fluorescence in the media. Cells cultured around the micro-size transducer were observed under both optical microscope and fluorescence microscope. Cells were analyzed within 1 hour after treatment. Fluorescence activity was quantified by capturing an image with a CCD camera and integrating fluorescence intensity over a defined area. Comparisons between treated and
non-treated areas were made in Figure 4.15 and Figure 4.16. Figure 4.15 shows the fluorescence image (bright spot) after three 75 um transducers (red circle) in a 3x3 transducer array were driven by 80 mW electrical signal for 3 minutes (red circles indicate the transducers that were activated). It was clear that there were a large number of QDs in the cells located above the acoustically activated transducer elements compared to those where transducers underneath were not activated. Figure 4.16 shows the QDs fluorescence intensity after two 25 um transducers in the area were driven by 40 mW electrical signal for 3 minutes (red circle indicate the transducers that were activated). The cell uptake again is obvious, and compared to the QDs uptake in Figure 4.15, both fluorescence intensity and QDs emission areas are smaller. This is probably due to the smaller ultrasound acoustic power. Figure 4.17 shows the fluorescence intensity in areas where two 75 um transducers were driven by 500 mW electrical signal for 3 minutes. QDs emission is all over the microscope viewing field indicating majority of cells in a much bigger area received the impact from the emitted ultrasound wave.

Fig. 4.15 Cell’s QDs uptake comparison between areas with and without ultrasound, three transducers were driven with 80 mW electrical signal for 3 minutes.
Fig. 4.16 Cell’s QDs uptake comparison between areas with and without ultrasound, two 25 um transducers were driven with 40 mW electrical signal for 3 minutes.

Fig. 4.17 Cell’s QDs uptake, after two 75 um transducers were driven with 500 mW electrical signal for 3 minutes.
4.4.3 Cell Viability Testing

Cell viability testing after the application of ultrasound by the micro-sized transducers was carried out with Trypan Blue exclusion assay that is well known in the bio-field. Post-ultrasound, the Trypan Blue exclusion assay was added into the cell media. Then, the treated cells were incubated for 2 hours. After that, cells were taken out and lifted by cell-lifting agent, Trypsin. The lifted cells were extracted from Petri-dish and injected into cytometer for living cell counting (dead cell will be dyed blue by Trypan blue agent). The viability assays demonstrated minimal effects of acoustic ultrasound on short term viability. A small fraction (<10%) were considered dead.

4.4.4 Experiment for Further Improvement on Testing Result

Although we observed the fact that ultrasound wave emitted by micro-sized transducers can facilitate the cell intake of QDs in just three minutes (which is significantly faster than natural diffusion process (2-24 hrs)), the impact area of cells was not confined enough. Cells on surrounding areas of transducers which were activated also had noticeable QDs intake (from the fluorescence images). Since the initial testing results indicated that the acoustic power might be the key factor that affects the cells intake of QDs, we decided to lower the acoustic power. In the meanwhile, the transducer activation period was also shortened to further reduce the ultrasound impact. In the new experiments, a batch of fresh Melanoma LU1205 cancer cells were passed and cultured on the devices surface. After the QDs were added into the cell media solution, we drove
each individual transducer at 20 mW acoustic power for 30 seconds. Unlike last experiment in which we only drove two or three transducers from the 3x3 matrix group due to the limited knowledge of ultrasound impact, in this experiment, we drove all the 9 individual 25 um transducers. With all the posted ultrasound procedure being kept same, the testing result was shown in figure 4.18. In figure 4.18 (A), no QDs uptake was found on areas above one group of transducers without activations. In figure 4.18 (B), cell’s QDs uptake was clearly observed and well confined on top of each transducer element after all 9 transducers were separately driven by 20 mW power signal for 30 seconds. Compared to the last experiment, it clearly shows that the area of cells being impacted by ultrasound is more confined on top of each activated transducer. It further proves that the acoustic power and transducer driving time play an important role in controlling the area on which cells were impacted by ultrasound.
Fig. 4.18 (A) No QDs uptake was found in areas without transducer activations; (B) Cell’s QDs uptake was well confined on top of each transducer element after all 9 transducers were separately driven by 20 mW power signal for 30 seconds.
4.4.5 Result Discussion:

The increase in the total fluorescence intensity in melanoma cancer cells we observed (when we conducted the QDs intake vs. time) experiment is because of a net movement of the QDs particles from cell media through the cell membrane and into the cell cytoplasm. The kinetics of this molecular movement can be described by a diffusion process from high concentration to low concentration. However, this is a relatively slow process because the cell membrane acts like a barrier between cell media and cytoplasm. In the living cell testing on our micro-sized transducer based bio-chip, tremendous QD uptake was observed after only 3 minutes of ultrasound. This verifies that the ultrasound waves generated by our micro-sized transducer have the capability seen in traditional transducers (cm diameter size level) adopted in previous research, they can facilitate the intake of molecules into cells by transiently increasing the cell membrane permeability. It has also been shown that the acoustic power (pressure amplitude) generated by our micro-sized transducers has key impact on the uptake of QDs uptake from both the amount of QDs in the cells and the size of area on which cells were impacted by the ultrasound waves. With a modified experimental setup (30 MHz frequency, 10 mW power input and 30 seconds driving period) on the 25 um square shape transducer, we successfully further confined the size of ultrasound impacted area to the same of the size of the transducer surface.
References


Chapter 5

CONCLUSIONS AND FUTURE WORK

5.1 Conclusions

In this dissertation, we introduced fabrication and testing of single element micro-sized transducer based multi-function bio-chip for single cell studies that includes cell screening, new drug molecule testing, protein, ligment delivery and gene transfection facilitation. To our knowledge, this is the first time this kind of concept has been realized at a device level and a promising initial experimental result on cell testing has been accomplished. The complete bio-chip system is based on a full analysis, design and fabrication approach.

A systematic plasma dry etching study of Cl\(_2\) based plasma etching on single crystal piezoelectric material PMN-PT in ICP chamber was performed. DOE based statistical analysis was used to identify the effect of each individual process parameter and their interactions on the PMN-PT etch rate. A regression model was applied based on the statistical analysis which provides >80% accuracy in etch rate predication, and gives us a guide line in controlling the etch rate. A more detailed study on the relationship between the etch selectivity and the etch rate of Ni mask, and each individual process parameter including process pressure, plasma power, DC bias, Cl\(_2)/Ar\) ratio, was also conducted to better understand the etching mechanism. This etching study laid a solid groundwork for future device fabrication.
The fabricated micro-sized single crystal transducer elements were characterized to make sure that they have the frequency of interest. The acoustic field measurement was also conducted to estimate the power generated by each single element.

The feasibility test of using Quantum dots (QDs) to track and label melanoma LU1205 cancer cell was carried out and confirmed. The QDs uptake as a function of time was quantified. A living cell test on bio-chip was performed and showed promising results. It was found that uptake of QDs in the cells is significantly affected by the acoustic waves being generated by the micro-size transducers. Instead of having an impact on cell (cell permeation) in a macro level, QDs uptaking from the cell only happened in small area surrounding micro-sized transducer after getting an impact by acoustic wave. For the first time, we gained the ability to manipulate the cell membrane permeability in single-cell level. This achievement certainly promises tremendous new opportunities for single cell study including cell mechanism analysis, cell sorting, drug screening, sorting, especially in facilitating the fast chemical molecule (drug) delivery. Since the whole experiment can be performed right on the devices, it reduces the possibility of contamination compared to most other methods in cell analysis in vitro. This form of transfection could also be used to introduce foreign DNA into plant cells. Therefore, this approach can possibly carry single cell study to a new level.

5.2 Future Work

1. More than 80% of the cells appear to be viable after the application of acoustic wave generated by micro-size transducer. The cell viability test was performed in one hour
after ultrasound treatment, therefore there is a possibility that the bacteria in the un-
sterile environment during fluorescence imaging experiment may start to disturb
cell’s viability, which in turn, may give us mistaken impression that it was our
ultrasonic wave that killed cells. A detail and systematic study on cell damage and
long term viability needs to be performed to further. A better understanding of cell
damage or injury caused by pressure wave generated by our micro-size single element
is still needed. A transmission electron microscopy (TEM) will be a good approach to
study the detailed cell structural alternation after ultrasound exposure.

2. Quantum dots used in our cell testing possess an average dimension of only ~10 nm.
   Therefore, those QDs can be uptaken by the cells easily, organic and inorganic
   molecules with different dimensions should also be used to further verify the cell
   uptake by using our devices.

3. It was shown that the cells which were and close to the area of micro-sized transducer
   have obvious quantum dots intake compare to cells that were farther away from the
   transducer. Therefore the latter didn’t get impact or get less impact by acoustic wave.
   However, in order for this device to become applicable for single cell study, cell
   trapping structure (physical confining, surface chemistry modification, micro-
   patterned polymer, etc) is needed to be integrated to the existing device structure.

4. There are only two traditional methods to generate acoustic wave to facilitate drug
   molecule delivery into cells, which are laser induced acoustic wave and transducer
   induced wave. The acoustic waves generated in these approaches are delivered to cell
   media first, and then the waves propagate and reach to a population of cells. The
smallest transducer they used is above 1 cm diameter. However, in our device, the transducer is really in the micro scale level, this theoretically limit its power output level. Instead of looking at the acoustic impact at macro level with a large population of cell, we are interested in manipulating single cell at micro level. Since this is the first time this kind of device has been accomplished, there is no result from previous studies that can be used as references. A better understanding of the cell permeation mechanism induced by such a small transducer is mandatory. No available hydrophone can be used to detect the acoustic field so close to the device surface (concern of breaking the hydrophone), a systematic mathematical model and calculation is need to estimate the actual acoustic power from measured data.

5. Based on the results from previous studies on facilitating drug molecule permeation through cell membrane by using ultrasound, drug molecule permeation is both energy and frequency dependent. In our cell on device testing, only 30 MHz frequency was tested, with 50 mW to 500 mW delivered power. The impact of a wider frequency (2k-100 MHz) range with different power intensity on cells must be tested. Transducers with different sizes also carry some interest. A good RC impedance matching net work must be introduced between transducer and driving circuit to prevent the generation of reflective power from transducer, thereby increasing the power delivery efficiency.

6. In the device’s electrical connection configuration, each transducer is connected to a individual connecter that is connected to power amplifier. This configuration can only allow only one single element transducer to be driven at one time. This is enough for
feasibility test and proof of concept. However, not only is this setup time consuming, but it also requires a lot of labor work when switching between transducers. A redesigned electronic connection structure to have all the transducers being connected and controlled simultaneously by an high frequency multiplexer will fix this problem.

7. Due to the complicated micro-fabrication processes such as photolithography, deep plasma etch, thin film deposition, and lapping & polishing, the fabrication cost will be relatively high. Optimization of process to increase the device robustness and to reduce the cost is necessary in order to make the device practical and ready for researcher in bio-medical field.
Appendix

Nontechnical Abstract

The cell is the structural and functional unit of all living organisms. It is the smallest unit of an organism that is classified as living, and is often called the building block of life. Therefore, studying the structure, function and characteristic of cells concerned with our understanding of the nature and ourselves. However, cell membranes pose one of the greatest barriers to the delivery of small particles such as drug molecules, proteins, DNA and other testing agents into the cells which are important to cell study.

Ultrasound is a sound pressure with a frequency greater than the upper limit of human hearing (20000 hertz). The production of ultrasound is used in many different fields, from RADAR detection, ultrasound cleaning, to noninvasive diagnosis and medical imaging. In recent years, a new application of ultrasound in biological field was developed. It is to use ultrasound to facilitate the cell intake of drug molecules and other small particles. This is due to the fact that lower ultrasound energy can temporarily open the cell membrane without causing unrepairable damage. This dramatically increases the efficiency of cell intake of drug molecules and other compounds requiring access to the cytosol. The relatively large size of ultrasound-created disruptions indicates that this method could be to deliver a broad range of compounds, such as drugs, proteins, DNA and RNA. However, all the studies of this small particle delivery facilitation using ultrasound were done only at large cell population level.

The advance of micro-fabrication techniques enables the development of novel
device being able to catch and trap cells in single cell level, this trigger and boost the
growing interest in single cell study which used to be impractical due to the difficulties in
trapping single cell and relatively high cost to fabricate the micro-size feature device used
for cell trapping.

Although the single-cell based bio-chip starts to show their promising potential in
fields from drug testing, drug screening, cell sorting, fundamental biology study to
environment monitoring. One big problem that prevents the further development of single
cell-based bio-chip is that the relatively low efficiency in cell intake.

This thesis is aimed towards development of a novel micro-sized ultrasound
transducer based bio-chip. Besides being able to catch the single cells, it can generate a
confined and low-power ultrasound field to facilitate the cell intake of small particles,
especially for drug molecules. The innovative device fabrication process is addressed in
detail. Quantum dots are adopted as the testing agent to conduct test on living cell. The
cell intake of quantum dots of both natural diffusion and ultrasound facilitation processes
are compared and quantified. A series of ultrasound conditions are tested. It was
approved that by controlling the size of the transducer, power and frequency of the
ultrasound wave the transducer emits, acoustic wave can be precisely targeted on each
individual single cell, therefore to facilitate the cell intake of small particles without
noticeable impact on other neighbor cells.
VITA

An Cheng

EDUCATION

• **M. Eng.** in Mechanical Engineering, Department of Mechanical Engineering, Tianjin University, September 1997-Jan 2000.
• **B. Eng.** in Mechanical Engineering, Department of Mechanical Engineering, Tianjin University, September 1993-July 1997.

Working Experience

• **Process engineer** in 8” semiconductor wafer fab (MOS17), January 2000-August 2003.